Copper Active Sites in Biology


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1. Introduction

Based on its generally accessible I/II redox couple and bioavailability, copper plays a wide variety of roles in nature that mostly involve electron transfer (ET), O₂ binding, activation and reduction, NO₂⁻ and N₂O reduction and substrate activation. Copper sites that perform ET are the mononuclear blue Cu site that has a highly covalent CuII-S(Cys) bond and the binuclear Cuₐ site that has a Cu₂S(Cys)₂ core with a Cu-Cu bond that keeps the site delocalized (Cu(1.5)₂) in its oxidized state. In contrast to inorganic Cu complexes, these metalloprotein sites transfer electrons rapidly often over long distances, as has been previously reviewed.¹⁻⁴ Blue Cu and Cuₐ sites will only be considered here in their relation to intramolecular ET in multi-center enzymes. The focus of this review is on the Cu enzymes (Figure 1). Many are involved in O₂ activation and reduction, which has mostly been thought to involve at least two electrons to overcome spin forbiddenness and the low potential of the one electron reduction to superoxide (Figure 2).⁵,⁶ Since the Cu(III) redox state has not been observed in biology, this requires either more than one Cu center or one copper and an additional redox active organic cofactor. The latter is formed in a biogenesis reaction of a residue (Tyr) that is also Cu catalyzed in the first turnover of the protein. Recently, however, there have been a number of enzymes suggested to utilize one Cu to activate O₂ by 1e⁻ reduction to form a Cu(II)-O₂⁻ intermediate (an innersphere redox process) and it is important to understand the active site requirements to drive this reaction. The oxidases that catalyze the 4e⁻ reduction of O₂ to H₂O are unique in that they effectively perform this reaction in one step indicating that the free energy barrier for the second two-electron reduction of the peroxide product of the first two-electron step is very low. In nature this requires either a trinuclear Cu cluster (in the multicopper oxidases) or a Cu/Tyr/Heme Fe cluster (in the cytochrome oxidases). The former accomplishes this with almost no overpotential maximizing its ability to oxidize substrates and its utility in biofuel cells, while the latter class of enzymes uses the excess energy to pump protons for ATP synthesis. In bacterial denitrification, a mononuclear Cu center catalyzes the 1e⁻ reduction of nitrite to NO while a unique μ₄S₂-Cu₄ cluster catalyzes the reduction of N₂O to N₂ and H₂O, a 2e⁻ process yet requiring 4Cu’s. Finally there are now several classes of enzymes that utilize an oxidized Cu(II) center to activate a covalently bound substrate to react with O₂.
This review presents in depth discussions of all these classes of Cu enzymes and the correlations within and among these classes. For each class we review our present understanding of the enzymology, kinetics, geometric structures, electronic structures and the reaction mechanisms these have elucidated. While the emphasis here is on the enzymology, model studies have significantly contributed to our understanding of O\textsubscript{2} activation by a number of Cu enzymes and are included in appropriate subsections of this review. In general we will consider how the covalency of a Cu(II)–substrate bond can activate the substrate for its spin forbidden reaction with O\textsubscript{2}, how in binuclear Cu enzymes the exchange coupling between Cu’s overcomes the spin forbiddenness of O\textsubscript{2} binding and controls electron transfer to O\textsubscript{2} to direct catalysis either to perform two e\textsuperscript{−} electrophilic aromatic substitution or 1e\textsuperscript{−} H-atom abstraction, the type of oxygen intermediate that is required for H-atom abstraction from the strong C-H bond of methane (104 kcal/mol) and how the trinuclear Cu cluster and the Cu/Tyr/Heme Fe cluster achieve their very low barriers for the reductive cleavage of the O-O bond.

Much of the insight available into these mechanisms in Cu biochemistry has come from the application of a wide range of spectroscopies and the correlation of spectroscopic results to electronic structure calculations. Thus we start with a tutorial on the different spectroscopic methods utilized to study mononuclear and multinuclear Cu enzymes and their correlations to different levels of electronic structure calculations.

2. Electronic Structure and Spectroscopy

2.1 Cu(I) sites

Cuprous complexes have a closed shell, 3d\textsuperscript{10}, configuration and thus must be studied by methods involving either electron ionization or transitions into higher energy unoccupied valence orbitals. The former methods include photoelectron spectroscopy where a valence or core electron is ionized with a photon of known energy and the electron flux at a given kinetic energy is directly recorded\textsuperscript{7} (not useful for metalloproteins due to the approximately 10 Å escape depth of the emitted electron) and K-\textbeta emission where a core 1s electron is ionized and photon emission to fill the core hole is measured (see section 2.1.2). The latter method is X-ray absorption spectroscopy (XAS) at the Cu(I) K-edge where a 1s electron is excited into the Cu 4p levels. Since s→p transitions are electric dipole allowed, these produce intense absorption features that are sensitive to the Cu(I) ligand environment. XAS is commonly performed using synchrotron radiation emitted by electrons moving in an orbit at relativistic speeds. This has a continuous range of energies, providing wavelengths in the UV, soft X-ray, and hard X-ray regions and is polarized in the plane of the ring. Third generation synchrotron sources produce extremely intense beams (many orders of magnitude higher than conventional X-ray tubes, which only produce discrete energies). This has made it possible to perform XAS on samples as dilute as ~0.1 mM.

2.1.1 Cu(I) K-edge X-Ray Absorption Spectroscopy (XAS)—The Cu 1s→4p transition at approximately 8984 eV constitutes the lowest energy pre-edge region of the Cu(I) K-edge with reasonable intensity. From spectroscopic studies on structurally defined model complexes these transitions are sensitive to the coordination environment of the Cu(I) center.\textsuperscript{8} As shown in (Figure 3 left) a linear two coordinate (2C) ligand environment raises
the energy of the Cu 4p\textsubscript{z} orbital resulting in a low energy 1s→4p\textsubscript{x,y} set of transitions at 8984 eV. Addition of a third ligand splits this degeneracy resulting in the double peaked spectrum in (Figure 3 green). Thus both 2C and 3C Cu(I) sites have a low energy feature at 8984 eV but the intensity in this lower energy feature is greatly decreased in 3C sites. For a four coordinate approximately tetrahedral Cu(I) site, all three 4p levels interact with the ligand field (LF) and are destabilized to higher energy. Thus a feature at −8984 eV in Cu K-edge XAS is characteristic of reduced Cu, its shape defines the coordination environment at the Cu(I) site and its intensity can be used to quantify the amount of reduced copper in a metalloprotein sample.

2.1.2 K-\textbf{\textbeta} emission—X-ray emission spectroscopy at the K-edge involves using the synchrotron radiation to ionize a Cu 1s core electron and measuring the photon emission associated with an electron filling the hole. The K-\textalpha region between 8030–8050 eV involves a 2p→1s transition and is the most intense emission observed (dipole allowed) but it is not very sensitive to the chemical environment of the Cu. Alternatively, K-\textbeta emission is sensitive to oxidation state, spin state, ligand character, and coordination number. The K-\textbeta region (Figure 4 left) consists of the main peaks (K-\textbeta\textsubscript{1,3} and K-\textbeta') between 8900–8910 eV involving a Cu 3p→1s transition and valence peaks (K-\textbeta'' and K-\textbeta\textsubscript{2,5}) between 8960–8990 eV involving valence/ligand→1s transitions. Figure 4 right shows the K-\textbeta region of a 3d\textsuperscript{10} transition metal, Zn(II) (qualitatively similar to Cu(I) but at higher energy). The K-\textbeta' peak is missing in the spectra of d\textsuperscript{10} systems but is present for Cu(II) complexes at ~10 eV lower energy than the K-\textbeta\textsubscript{1,3} emission and with less intensity.

The K-\textbeta\textsubscript{1,3} is the second strongest feature observed in an emission spectrum and is roughly \(1/8\textsuperscript{th}\) as intense as the K-\textalpha peak. It is an electric dipole allowed 3p→1s transition where the 1,3 refers to the spin-orbit splitting of the 3p, which is not resolved in the lineshape. In contrast to Cu(I) complexes, for Cu(II) complexes the hole in the 3d will exchange couple with the hole in the 3p resulting in the K-\textbeta feature when both holes have the same spin.\textsuperscript{9} The final state with opposite spins in the 3p and 3d orbitals gives rise to the main K-\textbeta\textsubscript{1,3} peak. In Cu(II) there can also be a ligand to metal charge transfer (LMCT) process that occurs due to the 3d relaxation associated with the increase in \(Z_{\text{eff}}\) caused by the 1s core hole, resulting in the transfer of a ligand electron in to the 3d orbital. This new \(1s\textsuperscript{d}\textsuperscript{10}\textsuperscript{L}\) excited state is lower in energy than the \(1s\textsuperscript{d}\textsuperscript{9}\textsuperscript{L}\) excited state (the 1s and \(L\) are holes in the orbitals indicated). The 3p→1s transition associated with this final state can also lead to a K-\textbeta' feature, whereas the 3p→1s transition associated with the \(1s\textsuperscript{d}\textsuperscript{9}\) state leads to the main K-\textbeta\textsubscript{1,3} peak. In Cu(II) complexes, weaker final states can also arise on the lower energy side of the K-\textbeta\textsubscript{1,3} feature due to d electron excitations into the \(d\textsubscript{c2y2}\) hole (in the ground state). K-\textbeta valence peaks lie higher in energy at 8960–8990 eV (Figure 5) than the main \(\textbeta\textsubscript{1,3}\) peaks at 8900–8910 eV and are ~100 times weaker than the main peaks. These transitions involve electrons from valence/ligand orbitals filling the metal 1s hole. The K-\textbeta valence region is divided into the K-\textbeta'' (the so called crossover peak) and the K-\textbeta\textsubscript{2,5} lines. The crossover peak is less intense and ~10–20 eV lower in energy than the K-\textbeta\textsubscript{2,5} peak. The nomenclature of the K-\textbeta region originated from the spectroscopy of high Z materials, such as uranium. K-\textbeta\textsubscript{2,5} was originally assigned to 4p→1s transitions and K-\textbeta\textsubscript{5} to 3d→1s transitions. This nomenclature has been transferred to transition metals where an understanding of the dominant
interactions and transitions in this energy region has made the nomenclature lose its original
physical meaning. The K-β₂,5 transitions are now known to originate from ligand np states
(where n=2, 3, etc. depending on ligand type) mixing with metal p character giving these
metal p→s electric dipole intensity. The valence 3d→1s quadrupole allowed transitions are
also in the same energy range but carry very little intensity. The K-β" peak at 8955–8970 eV
are assigned as ligand ns (n=2, 3, etc.) to metal 1s transitions. The intensity of these
transitions is also thought to originate from mixing of metal p character into the ligand ns
orbital. The energies of these peaks correspond mainly to the ligand ns binding energies.
The absolute and relative energies of the K-β" peak shifts to lower energies relative to the K-
β₂,5 peak with increase in ligand Z_eff (from C to F) as shown for Zn complexes in Figure
6A. The intensity of these transitions depends on the number of ligands and the metal-
ligand distance, thus providing complementary information to EXAFS (extended X-ray
absorption fine structure). The K-β" crossover peaks are also sensitive to different
protonation states and can be used to distinguish H₂O from a OH⁻ ligation as shown again
for Zn complexes in Figure 6B. The energy positions of the crossover peak can thus give
valuable information about the ligand environment.

2.2 Mononuclear Cu(II) sites

2.2.1 Ligand Field Theory (LFT)—While there is now much interest in Density
Functional Theory (DFT) in modern physical-inorganic chemistry (see section 2.5) it must
be emphasized that LFT still provides the most accurate correlation to spectroscopic data
involving transitions of electrons between d-orbitals. In an octahedral LF, the five 3d
orbitals split into a three fold degenerate t²g and a two fold degenerate e⁸ set at higher
energy. This splitting is given by 10Dq, the spectroscopic parameter of LFT (D relates to the
ligand electrostatic charge distribution and q the radial integral over the d-orbitals). Cu(II)
has a 3d⁹ configuration that results in a ²Eg ground state Figure 7A. This is orbitally
degenerate leading to a Jahn-Teller distortion that lowers the symmetry of the complex,
splits the orbital degeneracy and lowers the total energy of the system. Generally this leads
to a tetragonal distortion, i.e. elongation along z and contraction in the x,y plane, and a
limiting square planar structure. A key feature of LFT is that the energy splitting of the d-
orbitals is sensitive to the ligand environment at the metal center. For the square planar limit
this leads to the splitting pattern of Figure 7A center, where the highest energy half occupied
orbital is dₓ²-y² as its lobes are oriented along the ligand-metal bonds and thus has the
largest repulsion (i.e. antibonding) interaction with the ligands. A structural coordinate that
has been important in Cu(II) (bio)inorganic chemistry is the distortion from square planar to
a D₂d distorted tetrahedral structure. This involves raising two trans ligands above and two
trans ligands below the molecular plane. Note that in the tetrahedral limit (Figure 7A right)
the complex would have a ²T₂ ground state which is again degenerate and unstable to a
Jahn-Teller distortion to the D₂d structure. In the D₂d distorted tetrahedral site the ground
state is still the half occupied dₓ²-y² orbital (often referred to as dₓ²-y² when the coordinate
system is oriented such that the x,y axes are along the perpendicular C₂'s of the D₂d
structure). Note from Figure 7A that the energy splitting of the d orbitals in the D₂d structure
is much reduced relative to that of the square planar structure. This reflects the fact that
10Dq of an approximately tetrahedral structure is ~4/9 the 10Dq of an octahedral (or related
square planar) structure. We finally note that in square planar structures the dₓ² level is often
lower in energy than predicted by LFT. This reflects the fact that the metal 4s mixes with the 3d_{2z} level in planar structures, an interaction not directly included in LFT but present in molecular orbital theory (section 2.5).

A second coordinate in Cu(II) (bio)inorganic chemistry should also be considered. Five coordinate (5C) sites can be described as located along the square pyramidal to trigonal bipyramidal coordinate in Figure 7B. This idealized C_{4v} to D_{3h} correlation can be achieved either through a C_{2v} distortion (top; i.e. the Berry pseudo-rotation) or a C_{3v} distortion (Figure 7B bottom), the latter being relevant to square planar associative ligand displacement reactions where a ligand binds axially and rotates into the plane to displace an equatorial ligand. Note that the square pyramidal structure has a half occupied d_{x^2-y^2} orbital and similar d orbital splittings as in the square planar structure but the fifth, axial ligand shifts the Cu out of the equatorial plane. This increases the LF along the z axis (and decreases the d_{2z/4s} mixing) and decreases the LF in the equatorial plane. The net effect is that d_{2z} is no longer the lowest energy level in the square pyramidal structure. Distortion to a trigonal bipyramidal structure changes the d orbital splitting and results in a new half occupied d_{z^2} ground state. Note that this involves a change of coordinate system where the C_{3z} axis is now perpendicular to the C_{4z} axis of the square pyramidal structure. (In the original coordinate system the ground state is 3/4 d_{x^2-y^2} + 1/4 d_{z^2}). These ligand field energy splittings of the d-orbitals determine the ground state and low lying LF excited state spectral features of Cu(II) sites.

2.2.2 Cu(II) Ground States

2.2.2.1 Electron Paramagnetic Resonance (EPR) g and A^{M} values: The unpaired electron in the half occupied d orbital has S_{TOT} = 1/2, M_{S} = ±1/2, thus a doublet ground state. This M_{S} = ±1/2 spin degeneracy splits in a magnetic field (H) by g\beta H (the Zeeman effect, \beta is the Bohr magneton that converts an angular momentum into a magnetic moment), leading to the EPR spectrum that gives the g value characteristic of the ground state.

In the EPR experiment (Figure 8) a fixed energy microwave source is used (X band ~ 9GHz) and the magnetic field is varied until the resonance condition, g\beta H = h\nu (the microwave energy), is satisfied and microwave photons are absorbed. Thus for a known source energy, the magnetic field at which the microwaves are absorbed gives the experimental g value (g = h\nu/\beta H = 0.71448h\nu(MHz)/H(Gauss)). The g values of a Cu(II) complex are generally anisotropic, i.e. dependent on the orientation of the z axis of the complex relative to the applied magnetic field. A frozen solution of a Cu(II) complex will have all orientations relative to the fixed magnetic field direction, and the microwave absorption will spread from H_{1} (Figure 9 top) for complexes with H parallel z to H_{2} (for H perpendicular z). Thus the EPR absorption signal can spread over hundreds of Gauss with intensity increasing towards H_{2} due to the greater number of molecules with z perpendicular to H. The EPR signal is modulated to increase sensitivity leading to a first derivative spectrum (Figure 9 middle) with features at the turning points, i.e. a peak at the magnetic field associated with g_{∥} (i.e. the z axis of the complex parallel to H) and an intense derivative shaped feature at g_{⊥} (i.e. the g value for the Zeeman splitting of the ground doublet with the z axis of the complex perpendicular to the magnetic field). Finally, Cu has a nuclear spin (I) of 3/2 which couples

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to the electron spin to produce a hyperfine splitting of the spectrum into 2I+1 = 4 hyperfine lines split by the metal hyperfine coupling constant A\textsuperscript{M}, which can also be anisotropic (A\textsuperscript{M∥} for the z axis of the complex parallel to the magnetic field, A\textsuperscript{M⊥} for the z axis perpendicular to the field). From Figure 9 bottom |A\textsuperscript{M∥}| > |A\textsuperscript{M⊥}| and the latter is often not resolved in the EPR spectrum. Note that only the magnitude of A\textsuperscript{M} is measured in the EPR spectrum; generally A\textsuperscript{M∥} is negative due to the physical origin of the hyperfine coupling (see below). Thus we experimentally derive g\textsubscript{i} and |A\textsuperscript{Cu,i} (i=x, y, z) of a metal site by EPR spectroscopy and interpret these experimental parameters in terms of LFT.

In contrast to organic radicals, metal complexes often have g values that deviate greatly from the free electron value of 2.0023. This derives from the fact that transition metal ions have large spin orbit coupling (SOC) constants; for Cu(II) λ\textsubscript{3d} = −830 cm\textsuperscript{−1} while for N and O radicals this value is approximately −70 cm\textsuperscript{−1}. This large SOC mixes the LF excited states described in section 2.2.1 into the ground state and leads to the large deviations of the g values from 2.00 observed experimentally. The expression describing the SO contribution to the g values is\textsuperscript{15}:

\[ g\textsubscript{i} = 2 - 2\lambda k^2 \sum_{n \neq 0} \frac{\langle \psi\textsubscript{0} | L_i | \psi\textsubscript{n} \rangle \langle \psi\textsubscript{n} | L_i | \psi\textsubscript{0} \rangle}{E_n - E_0} \]

where \psi\textsubscript{0} is the ground state, \psi\textsubscript{n}’s the excited states at LF energies, E\textsubscript{n}−E\textsubscript{0} above the ground state (section 2.2.1) and the values for the effects of the orbital angular momentum operator L\textsubscript{i} (i = x, y, z directions of the molecule relative to the magnetic field) on electrons in the d-orbitals are given in table 1. The k\textsuperscript{2} in equation 1 are the Steven’s orbital reduction factors\textsuperscript{16} that account for the fact that the d-orbitals have ligand character due to covalent bonding and this decreases the magnitude of the orbital angular momentum (and SOC) over electrons in the d-orbitals. Thus k\textsuperscript{2} < 1 due to covalency and can be calculated from the coefficients of molecular orbitals as described in reference 17. Note from equation 1, the observed g values directly reflect the nature of the ground state thus ligand environment. For a d\textsubscript{x2−y2/dxy} ground state (Figure 7B left) g∥ > g⊥ > 2.00 while for a d\textsubscript{z2} ground state (Figure 7B right) g⊥ > g∥ = 2.00.

The metal hyperfine couplings A\textsuperscript{M,i} (i=x, y, z) are also dependent on the nature of the ground state and its covalency. Those for a d\textsubscript{x2−y2/dxy} ground state are given in equation 2.\textsuperscript{18,19}

\[ A\textsubscript{∥} = \beta_1^2 \left( A_{\text{Fermi}} + A_{\text{spin–dipole}} \right) + P d \left[ \frac{3}{2} (g\textsubscript{∥} - 2.00) + \frac{1}{2} (g\textsubscript{⊥} - 2.00) \right] \]
\[ A\textsubscript{⊥} = (A_x, A_y) = \beta_1^2 \left( A_{\text{Fermi}} - \frac{1}{2} A_{\text{spin–dipole}} \right) + P d \left[ \frac{3}{2} (g\textsubscript{∥} - 2.00) + \frac{1}{2} (g\textsubscript{⊥} - 2.00) \right] \]

The first term in equation 2 is the Fermi Contact interaction of the electron spin with the nuclear spin at the nucleus. As only s orbitals can have electron spin density at the nucleus, for electrons in d-orbitals this involves their spin polarization of electron pairs in the ns, n = 1,2,3 orbitals such that there is a net negative spin density at the nucleus. The Fermi Contact term is isotropic, thus contributes equally in all directions. The second, spin dipolar, term involves the dipole interaction of the electron spin, averaged over the shape of the half
occupied d orbital, with the nucleus spin on the metal. For an electron spin in a d_{x^2-y^2} orbital, the A_{∥} spin dipolar term is also negative and large. It is purely anisotropic thus sums to zero over the three molecular directions, (i.e. A_{⊥} = -1/2A_{∥}). The third term involves the magnetic dipole interaction of the orbital angular momentum, obtained from SOC to LF excited states as described above, with the Cu nuclear spin and thus can be estimated from the g value deviations from 2.00. Finally as the electron spin is delocalized onto the ligands due to covalency, its interaction with the nuclear spin on the Cu decreases. In equation 2 this is given by \( \beta^2 \), the amount of metal character in the half occupied d_{x^2-y^2}/d_{xy} orbital which decreases from 1.0 as the covalency increases, and the fact that experimental g values are used in equation 2 which have \( k^2 < 1 \) in equation 1 to account for covalency.

### 2.2.2.2 Superhyperfine coupling:
As covalency delocalizes the electron spin onto the ligands it can couple to the ligand nuclear spin \( I_L \) and further split each hyperfine line in Figure 9 bottom into 2I_{L}+1 components. This superhyperfine splitting \( A_{L}^{i} \) is anisotropic and a direct measure of the amount of that ligand valence orbital contained in the half occupied “d” orbital, i.e. the covalency of this bond. As shown in Figure 10 the covalent mixing \( (\beta^2)^2 \) of ligand i character into a half occupied d orbital delocalizes the electron spin into a hybrid valence orbital on that ligand, where the hybrid has \( n_s \) and \( \sqrt{1 - n^2} \) ligand orbital character. The s character gives an isotropic Fermi contact contribution to the superhyperfine coupling of the nuclear spin \( I_L \) to the electron spin, while the ligand p character in the hybrid produces an anisotropic spin-dipolar contribution. The net effect of these considerations gives the superhyperfine coupling in equation 3,

\[
A_{∥}^{ii}(\text{covalency}) = \left( \beta_{2}^{Li} \right)^2 \left( n^2 A_{iso}^{Li} + (1-n^2) A_{aniso}^{Li} \right) \quad A_{∥}^{ii}(\text{covalency}) = \left( \beta_{2}^{Li} \right)^2 \left( n^2 A_{iso}^{Li} - \frac{1}{2} (1-n^2) A_{aniso}^{Li} \right)
\]

where the values for the Fermi contact and spin dipolar interactions of one electron spin in a biologically relevant ligand valence orbital with the nuclear spin on that ligand is given in table 2.\(^{20}\) Thus by experimentally measuring the superhyperfine coupling constants \( A_{L}^{i} \) (i = x,y,z) one can obtain the covalency of that ligand \( (\beta^2)^2 \) and its hybridization \( (n^2) \) involved in bonding to the metal ion.

This superhyperfine coupling is generally very small and often not resolvable in the standard EPR experiment at X-band. However it can be obtained by double resonance and pulsed EPR experiments. In electron-nuclear double resonance (ENDOR) the EPR signal is used to probe NMR transitions on directly coordinated ligand nuclei.\(^{21-23}\) Thus in addition to the electron Zeeman effect, the ENDOR spectrum also involves the nuclear Zeeman effect \( (g_N \beta_N H) \), the ligand hyperfine coupling to the electron spin and a quadrupole term if the ligand nuclear spin is greater than or equal to 1. For protons, where the nuclear Zeeman is large relative to the hyperfine coupling, as shown in Figure 11 left, two ENDOR peaks are observed centered at the proton Larmor frequency (~14MHz in X band) split by the hyperfine coupling; while for other nuclei (e.g. N) where the nuclear Zeeman is small, transitions are centered at A/2 and split by twice the nuclear Zeeman effect (~1MHz for N at X band) with a small additional splitting due to the quadrupole coupling (2P in Figure 11 right). Note that the ENDOR spectrum can be taken at different fields associated with...
different EPR g values, thus providing the anisotropic components of the ligand superhyperfine coupling. Note also that the z axis of the superhyperfine is often not along the molecular z axis but rather the z axis of the ligand p orbital involved in covalent bonding to the half occupied d orbital in Figure 10.

Finally for very small superhyperfine couplings (as, for example, the non-coordinated N of a Histidine ligand), electron spin echo envelope modulation (ESSEM) spectroscopy is used.24–29 An intense microwave pulse sequence is applied to obtain an electron spin echo whose decay is modulated by weak electron spin coupling to remote nuclear spins. The Fourier Transformation (FT) of the modulation in Figure 12 top produces an ENDOR like spectrum in the 0–5 MHz region in Figure 12 bottom. As shown in Figure 12 bottom for the remote N of a His ligand to a Cu(II) site, two dominant features are observed in the FT of the ESEEM spectrum (ν− and ν+). For the remote N of a His ligand, the hyperfine coupling is small enough that it just cancels the nuclear Zeeman splitting and the resultant signal observed directly reflects the quadrupole splitting of the remote N nuclear spin (I_N = 1) caused by the electric field gradient of its chemical environment (equation. 4),

\[ e^2Qq = \frac{2(ν_+ + ν_-)}{3} \eta = 3\frac{(ν_+ - ν_-)}{(ν_+ + ν_-)} \quad [4] \]

where \( e^2Qq \) is the quadrupole coupling constant (Q the nuclear quadrupole moment, q the electric field gradient) and \( \eta \) is the asymmetry parameter (i.e. for x ≠ y). Thus ESSEM spectroscopy can provide chemical insight into weak interactions such as H-bonding or deprotonation of a coordinated His ligand.

### 2.2.3 Ligand Field Excited States—
Near IR/Visible photons excite d electrons into the half occupied d orbital in the LF energy level diagrams in section 2.2.1 and Figure 7. These transitions thus directly probe the ligand environment around the Cu(II) site. However these \( d \rightarrow d \) or LF transitions are parity forbidden, therefore weak in the absorption spectrum. This intensity is given by the oscillator strength, f, associated with the electric dipole transition moment between the ground and excited state (g and e, respectively in equation 5)

\[ f \propto \langle \psi_g | r | \psi_e \rangle^2 \quad r = x, y, z \quad [5] \]

LF transitions generally have molar extinction coefficients \( \varepsilon < 100 \text{ M}^{-1} \text{ cm}^{-1} \) and can be difficult to observe in metalloprotein absorption spectra. Alternatively due to different selection rules, \( d \rightarrow d \) transitions can be intense in Circular Dichroism (CD) and Low Temperature Magnetic Circular Dichroism (MCD) spectra. The intensity in a CD spectrum is given by the rotational strength, R, which is proportional to the projection of the electric dipole transition moment between the ground and excited state into the magnetic dipole moment \( (M_i, i = x, y, z) \) for this transition (equation 6).

\[ R \propto \langle \psi_g | r | \psi_e \rangle \cdot \langle \psi_e | M_i \psi_g \rangle \quad [6] \]
Comparing equations 5 and 6, transitions that are intense in the CD spectrum relative to the corresponding absorption spectrum are generally magnetic dipole allowed (and have a nonzero projection into the electric dipole transition moment which can only occur in a chiral environment). The magnetic dipole operator corresponds to a rotation of an electron from one orbital to another, thus d→d transitions are generally magnetic dipole allowed and relatively intense in CD spectra.

The “C-term” selection rule for low temperature MCD intensity of paramagnetic systems is given by equation 7.\textsuperscript{31–35}

$$\Delta \epsilon_{L-R} \alpha g_z \langle \psi_y | \gamma | \psi_x \rangle \langle \psi_z | \gamma | \psi_y \rangle + g_y \langle \psi_y | \gamma | \psi_x \rangle \langle \psi_x | \gamma | \psi_y \rangle + g_z \langle \psi_x | \gamma | \psi_y \rangle \langle \psi_y | \gamma | \psi_x \rangle$$ \textsuperscript{[7]}

Thus, two perpendicular electric dipole transition moments are required for a transition to a given excited state to absorb circular polarized light in a longitudinal magnetic field. Active sites in Cu(II) proteins are generally of low symmetry thus all electronic states are nondegenerate and electronic transitions are uni-directional. Low temperature MCD intensity thus requires SOC between excited states that are perpendicular in polarization. As indicated above, the SOC of a transition metal ion is much larger than that of a ligand. Therefore bands that are intense in the MCD relative to the corresponding absorption spectrum (known as the C/D ratio) are metal centered LF transitions.\textsuperscript{36} As a rule for Cu(II) sites, LF transitions have C/D > 0.1. It should be noted that SOC over all excited states leads to the sum rule where the MCD intensity in left and right circular polarization tend to be equal and sum to zero over spin orbit coupled excited states.\textsuperscript{37} Deviations from the sum rule (i.e. dominant intensity in one circular polarization) reflects spin orbit coupling of a low lying LF excited state into the ground state.

### 2.2.4 Charge Transfer Excited States

At higher energy than the LF transitions are the LMCT transitions. These involve excitation of electrons from filled ligand valence orbitals into the d hole on the Cu(II). Thus these transitions involve a large change in electron density. These are electric dipole allowed and can be intense in the absorption spectrum and provide a direct probe of the ligand valence orbitals involved in bonding. In bioinorganic chemistry we are mostly interested in donor bonding interactions. All ligands will have a lone pair available for σ donor bonding to the metal, and those that have additional electron pairs available can also π donor bond to the metal center. Since σ donor bonding is stronger than π donor bonding due to better directional overlap, the σ valence orbital on the ligand will be at more negative energy than the π donor orbitals (Figure 13, $H_{M-L}$ is the resonance integral of the molecular Hamiltonian over the metal and ligand valence orbitals and thus proportional to their overlap). The intensity of a charge transfer (CT) transition is proportional to the overlap of the donor and acceptor orbitals involved in the CT excitation. Thus from Figure 13 the π CT will be at lower energy and relatively weak in absorption, while the σ CT transition will produce a higher energy, intense feature in the absorption spectrum. The stronger the donor bonding interaction of the ligand with the metal the more intense the CT transition.
From molecular orbital theory bonding is dominated by ligand and metal valence orbitals that are close in energy and have good overlap. Thus ligands that produce low energy intense CT absorption features form highly covalent bonds with the metal center. In copper bioinorganic chemistry these are generally the oxygen intermediates, where as described in section 3 their highly covalent bonding activates selective reactivity in biological function. Other highly covalent L-M bonds in Cu biochemistry (i.e. active sites that exhibit low energy, intense CT absorption features) are the sulfide bridge in nitrous oxide reductase (N\textsubscript{2}OR) (Section 5.0), the cysteine crosslinked phenolate ligand radical in Galactose Oxidase (Section 3.4), the phenolate ligand in pre-processed amine oxidase and the thiolate ligand in blue Cu, Cu\textsubscript{A} and in preprocessed Cu(II) Galactose Oxidase.

In Resonance Raman (rR) spectroscopy a Laser is tuned into an intense absorption band (i.e. a CT transition) and vibrations associated with the chromophore (i.e. the active site in the protein and the specific L-M bond associated with the charge transfer) become greatly enhanced in Raman scattering intensity as illustrated from the data in Figure 14. In Cu bioinorganic chemistry this generally involves an “Albright” A-term intensity enhancement mechanism. A-term rR intensity reflects excited state distortions. The CT transition of ligand L involves excitation of an electron from a L-M bonding donor orbital mostly on the ligand into an L-M antibonding acceptor orbital mostly on the metal. Therefore the L-M bond associated with the CT transition probed by rR spectroscopy will elongate and its associated L-M stretching vibration will be greatly resonance enhanced. Other active site L-M bonds can also be enhanced (but with less intensity) as the Cu d acceptor orbital is generally antibonding to all equatorial ligands. Other intraligand and bending modes may also be observed in rR spectroscopy dependent on how the bonding changes in the CT excited state being probed by rR relative to the ground state.

2.2.5. XAS K-edge—An open shell Cu K-edge typically consists of two distinct spectroscopic features—the pre-edge and the shakedown as shown in Figure 15. The lowest energy pre-edge transition is the 1s→3d transition that can be used to distinguish between Cu(II) and Cu(III) higher redox states. The Cu(II) pre-edge is around 8979 eV (Figure 15 inset). Cu(III) has a $Z_{\text{eff}}$ and contracted ligand field that results in a higher pre-edge energy of approximately 8981 eV (Figure 15 inset). The edge is also shifted to higher energies in Cu(III) compared to Cu(II). Thus the pre-edge position can be used to differentiate between Cu(II)$_2$ side-on peroxo and Cu(III)$_2$ bis-$\mu$-oxo complexes and quantitate the amount of each component present. The pre-edge transition: The K-pre-edge 1s→3d transition has low intensity because it is electric dipole forbidden. In centrosymmetric complexes, such as in D$_{4h}$ [CuCl$_4$]$^{2-}$ that have no 3d/4p mixing, the pre-edge transition (at 8979 eV) gains intensity mostly through the electric quadrupole operator (Figure 16 left black). Blue Cu on the other hand has a tetrahedrally distorted site, and a higher pre-edge intensity owing to some 4p mixing into the 3d orbital (Figure 16 left inset red). This 4p mixing into the 3d orbital while small has a substantial impact on the intensity of the preedge because an electric dipole transition is ~2 orders of magnitude stronger than an electric quadrupole transition in this energy region. From polarized single crystal XAS the origin of the pre-edge intensity in blue Cu has been
shown to be from Cu 4px,4py mixing into the 3d_x2-y2 ground state as seen in the xy-polarized K-edge (Figure 16 right).\textsuperscript{\textit{43}} No pre-edge intensity is observed in the z-polarized spectrum showing that the 4pz orbital does not mix into the 3d_x2-y2 ground state in blue Cu. Thus, the pre-edge intensity in an important spectroscopic probe of 3d-4p mixing that is allowed by ligand field theory.

The 3d/4p mixing is indirect and occurs by overlap of both the 3d and 4p metal orbitals with the same ligand based valence orbital.\textsuperscript{\textit{44}} This mechanism of mixing can be described using the configuration interaction (CI) model given below (equation 8):

\[
\begin{array}{ccc}
<\text{ligand}3p|&<\text{metal}3d|&<\text{metal}4p| \\
<\text{ligand}3p|&0-E&T_I&T_2 \\
<\text{metal}3d|&T_I&\Delta_1-E&0 \\
<\text{metal}4p|&T_2&0&\Delta_2-E \\
\end{array}
\]

The \(T_1\) and \(T_2\) terms give the bonding interaction between the ligand p and metal d and between ligand p and metal p orbitals, respectively. \(\Delta_1\) and \(\Delta_2\) are the energy difference between the ligand 3p and metal 3d configuration and between ligand 3p and metal 4p configuration, respectively. Solving this determinant shows that there is some metal p mixing into the ground state d wavefunction as both overlap the same ligand p orbital. The K-pre-edge intensity thus provides a direct probe of the metal 3d-4p mixing and an indirect probe of the metal-ligand bonding.

\textbf{2.2.5.2 Shakedown transition:} The K-edges of Cu(II) and Cu(III) systems commonly contain a transition between 8986–8988 eV that is described as a shakedown transition (Figure 15). In systems with more covalent interactions with the ligands, this feature appears to the lower end of this range. The shakedown transition is a two electron process that results from a 1s\(\rightarrow\)4p electric dipole-allowed transition. The 1s core hole created results in relaxation of the Cu valence orbitals to lower binding energies. For Cu this relaxation of the 3d orbitals is large enough to cause a transfer of a second electron from the ligand valence to the metal 3d orbital (an LMCT process). The sudden approximation formalism can be used to quantify the intensity of this shakedown (1s\(\rightarrow\)4p + LMCT) feature as a percentage of the total 1s\(\rightarrow\)4p transition intensity.\textsuperscript{\textit{43}} The main-to-shakedown peak intensity ratio \(I_{m}/I_s\) is given by equation 9:

\[
I_{m}/I_s=\tan^2(\theta'-\theta)\quad [9]
\]

with \(\tan 2\theta' = 2T/(\Delta-Q)\) and \(\tan 2\theta = 2T/\Delta\), where \(T = \langle \Psi (3d^9)|H|\Psi(3d^{10}L)\rangle\) is the interaction matrix element between the configurations contributing to the ground state wave functions, and \(\Delta\) is the energy difference between the two configurations in the ground state as shown in Figure 17A. \(Q\) accounts for the increase in the effective nuclear charge (Ze\textsubscript{eff}) felt by the 3d\textsuperscript{9} configuration upon creation of the \(\xi 3d^9\) configuration (where \(\xi\) denotes a core orbital hole) (Figure 17B). Thus, the change in wavefunction \(\theta'-\theta\) upon creation of the 1s core hole determines the main-to-shakedown peak intensity ratio. Using this configuration interaction formalism, the shakedown transition intensity in a D\textsubscript{2d} [CuCl\textsubscript{4}]\textsuperscript{2-} system was calculated to be 59% of the total 1s\(\rightarrow\)4p transition intensity, similar to that obtained from fitting the z-polarized XAS spectrum (~60%) (Figure 18).
2.2.6. XAS L-edge—The Cu L-edge spectrum results from a metal 2p→3d electric dipole-allowed transition and consists of the L_{3} (2p_{3/2}→3d) and L_{2} (2p_{1/2}→3d) edges separated by ~20 eV due to 2p spin-orbit coupling. The L_{3} to L_{2} intensity ratio is ~2:1 due to the 4:2 degeneracy of the 2p_{3/2} and 2p_{1/2} states. For typical Cu(II) complexes, the L_{3}-edge occurs at ~930 eV, whereas the L_{2}-edge occurs at ~950 eV and is ~1.5 times broader from an additional Coster-Kronig auger decay channel of this excited state. For Cu(III) complexes, the L_{3}- and the L_{2}-edges are ~1.5–3 eV higher compared to those observed for Cu(II). The 2p→4s transition is Δl = −1 and is thus, ~20–30 times lower in intensity than the Δl = +1 (p→d) transition. Multiplet and shakeup effects, which usually lead to a redistribution of the intensity of the L_{3} and L_{2} peaks do not contribute to the L-edge spectra of 3d^{9} complexes.

The total area under the L_{3}- and L_{2}-peaks reflects the extent of metal-ligand covalency. As the unoccupied Cu 3d orbital mixes with filled ligand orbitals, the intensity of the L-edge transition decreases because of the metal localized nature of the Cu 2p→ψ^{*}_{-LUMO} transition. The ground state wavefunction (ψ^{*}_{-LUMO}) of a d^{9} Cu complex can be expressed as equation 10:

\[ \psi^{*}_{-LUMO} = (1 - \beta^2)^{1/2} \phi(Cu(3d)) - \beta \phi(Ligands(np)) \]  

where, 1 – β² and β² correspond to the Cu 3d and ligand np character, respectively. By comparing the total area under the L-edge spectra to that of D_{4h} [CuCl_{4}]^{2−} (a system that has been studied by a wide energy range of spectroscopies, and found to have 61 ± 4% Cu character in the ψ^{*}_{-LUMO}), we can get a quantitative estimate of the amount of Cu character in the ground state wavefunction of other Cu complexes. An important example of the use of L-edge intensity to quantify covalency is for the blue Cu site in Figure 19. Its L_{3}-edge has 0.67 times the intensity of square planar CuCl_{4}^{2−}. D_{4h} [CuCl_{4}]^{2−} has 61% Cu d_{x2-y2} character in its ground state. Thus, the blue Cu site in plastocyanin has only 41% Cu d character reflecting a highly covalent ground state wavefunction, which is important in its function of long-range electron transfer.

2.2.7. XMCD—X-ray magnetic circular dichroism (XMCD) combines L-edge XAS with MCD. The XMCD signal is the difference in the L-edge spectra obtained with right and left circularly polarized light in the presence of an external magnetic field. The low temperature (<1 K) XMCD signal at the L-edge of plastocyanin (S = 1/2 mononuclear blue Cu center) is shown in Figure 20A. The XMCD intensity at the L_{3} edge is seen to be negative. It is interesting to compare the negative XMCD L_{3} intensity for the mononuclear blue Cu site with the signal for the Cu in [(F_{8}TPP)Fe^{III}-O-Cu^{II}(TMPA)](ClO_{4}) which is positive (Figure 20B). The binuclear complex (shown in Figure 20 C) has an Fe(III) (S = 5/2) antiferromagnetically coupled to the Cu(II) (S = 1/2) to produce an S = 2 ground-state. This S = 2 spin vector aligns with the magnetic field and thus produces a field of opposite sign at the Cu due to the Cu(II) S = 1/2 antiferromagnetic coupling to the high spin S = 5/2 Fe(III). The opposite sign of the XMCD signal confirms the antiferromagnetic coupling of the Cu to the Fe and thus a powerful use of XMCD lies in studying ferro- and antiferromagnetic coupling of Cu to other paramagnetic centers.
2.3 Binuclear Copper Sites

2.3.1 Two Copper(II): Magnetic Coupling

2.3.1.1 Ground state Zero Field Splitting (ZFS) and Antiferromagnetic coupling: If two Cu(II), each with S=1/2, are closer than ~ 6 Å apart their electron spins will dipole-dipole couple. This produces an S=1 triplet EPR signal that has characteristic spectral features (Figure 21). First there is a half field (or $\Delta M_S = 2$) transition at ~1500 Gauss (twice the $g$ value) in X-band. This however must be distinguished from “junk” Fe(III) generally present in biological systems, and the $\Delta M_S = 2$ transition is often only observed at very low temperature. Further the $g \approx 2.0$ ($\Delta M_S = 1$) region will broaden or split into two dominant spectral features (called fine structure). The splitting between these spectral features derives from the Zero Field Splitting (ZFS) of the triplet. As shown in Figure 21 the magnetic field splitting between these transitions (or better from simulation of the spectrum) gives the ZFS parameter $D$ which in turn gives the distance between the two Cu(II) centers ($r$ in equation 11).

$$D = \frac{-3}{2} \frac{g^2 \beta^2}{r^3}$$ \text{[11]}

If the two Cu(II)’s have a bridging ligand this can provide a superexchange pathway for antiferromagnetic coupling between the two S=1/2’s to produce an $S_{TOT} = 0$ ground state. The origin of this coupling is illustrated in Figure 22A left. The half occupied d orbital on Cu$_A$ overlaps a filled bridging ligand valence orbital. The electron pair in this ligand orbital polarizes such that one electron spin dominantly pairs with the spin on Cu$_A$ and the second electron on the ligand polarizes towards the remote side of the filled ligand orbital. If Cu$_B$(II) overlaps the same ligand orbital its electron pairs with this remote polarized spin and the net effect is the antiferromagnetic coupling of the electron spins on the two Cu(II) though the bridging ligand superexchange pathway. Note from Figure 22A right that if the two Cu(II) overlap orthogonal orbitals on the bridging ligand, the two electron exchange interaction between the second electron in each of the two orthogonal ligand orbitals will lead to a parallel, ferromagnetic alignment of the electron spins on the two Cu(II) with an $S_{TOT} = 1$ ground state. The molecular orbital version of this valence bond description is shown in Figure 22B. From group theory one takes symmetric and antisymmetric combinations of the half occupied d orbitals on the two coppers and allows for their bonding interaction with the valence orbital on the bridging ligand. From Figure 22B, only the antisymmetric combination ($\varphi_2$) can have a non-zero overlap with the occupied $p_z$ orbital on the bridging ligand. Thus, $\varphi_1$ and $\varphi_2$ split in energy and this leads to the spin paired antiferromagnetic ground state.

This coupling of the spins on the two Cu(II) is described by the Heisenberg-Dirac-Van Vleck Hamiltonian (equation 12)

$$H = -2J S_A \cdot S_B E_{S_{TOT}} = - J [S_{TOT}(S_{TOT}+1) - S_A(S_A+1) - S_B(S_B+1)]$$ \text{[12]}

which leads to an energy dependence on the total spin, $S_{TOT} = S_A + S_B$, $S_A + S_B$ $-1$,...,$S_A - S_B = 1$, 0 for 2 Cu(II) with $S = \frac{1}{2}$. Thus, antiferromagnetic coupling ($J$ negative)
leads to an \( S_{\text{TOT}} = 0 \) ground state with the \( S_{\text{TOT}} = 1 \) at \( 2J \), where \( J \) quantifies the exchange coupling. An antiferromagnetic coupled Cu(II) dimer has an \( S = 0 \) ground state with no EPR signal but can show a contribution to EPR, MCD and magnetic susceptibility if the \( S_{\text{TOT}} = 1 \) level becomes thermally populated.

In an exchange coupled dimer there is an additional contribution to the ZFS of the triplet considered above (\( D \) in Figure 21). This is the pseudo-dipolar or anisotropic exchange contribution.\(^{50-52}\) This derives from the second order SOC of a LF excited state into the ground state of one Cu and the exchange interaction of this component with the ground state of the second Cu(II). Since SOC is also responsible for the \( g \) values deviating from 2.00, the anisotropic exchange contribution to the ZFS parameter \( D \), Figure 21, for an exchange coupled Cu(II) dimer can be related to the ground state \( g \) values as given in equation 13, for a \( d_{x^2-y^2} \) ground state:

\[
D_{x^2-y^2}^{d_{x^2-y^2}} = -\frac{1}{16} (g_\| - 2)^2 J_{x^2-y^2,xy} + \frac{1}{4} (g_\perp - 2)^2 J_{x^2-y^2,xy} \tag{13}
\]

The problem with using this expression is that in addition to the ground state \( g \) values which are obtained by EPR, it requires values from the magnetic exchange interaction between the ground state on one Cu and the ligand field excited state on the second Cu(II) (\( J_{x^2-y^2,xy} \) and \( J_{x^2-y^2,xz,yz} \)). These ground/excited state exchange couplings can, in principle, be observed in LF excited states. However, this has only been investigated in one case (see ref 53).

Alternatively, if the sign and magnitude of \( D \) is measured experimentally, the electron spin dipolar contribution to \( D \) is subtracted off using equation 11, and the first term in equation 13 is assumed to dominate, then \( J_{x^2-y^2,xy} \) can be obtained from the EPR experiment.

**2.3.1.2 Excited States:** Exchange coupling in bridged binuclear Cu(II) dimers can affect both the LF and CT excited states. The effect on the \( d \rightarrow d \) transitions is to split each into four states where the splitting can provide the different ground to excited state and excited to excited state exchange pathways associated with the bridging ligand. In practice this requires very detailed spectroscopy on single crystals to resolve these effects (see ref 53) and generally will not produce significant features in the LF region of low resolution spectra of binuclear Cu(II) model complexes or protein sites. Alternatively, the CT transitions of a bridging ligand can show large deviations relative to the CT transitions on Cu(II) monomers (these are the “dimer bands” in the absorption spectra of binuclear model complexes), and these deviations directly probe the superexchange pathway for the ground state antiferromagnetic coupling.\(^54\)

As shown in Figure 23, starting from the AF coupled ground state discussed above, the CT transition of a bridging ligand to Cu\(_A\) leads to an unpaired electron directly on the ligand which will have strong overlap thus AF coupling with the unpaired electron on Cu\(_B\). This produces a large excited state AF coupling, which greatly lowers the energy of the singlet relative to the triplet CT excited state. Thus while bridging a ligand between two metal centers will tend to stabilize the energy of the L donor orbital and increase the CT transition energy of both the singlet and triplet, the large CT excited state AF coupling will greatly lower the energy of the singlet CT relative to the triplet. The observed shift of the CT energy

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in a dimer relative to the corresponding monomer is then the net effect of these competing contributions.

This excited state AF coupling in the LMCT transition of a bridging ligand propagates into the ground state through the covalency of the bridging ligand-metal bond. In valence bond theory, covalency is introduced by the configuration interaction mixing of the CT excited state into the ground state, which is known as the valence bond configuration interaction (VBCI) model. Since the singlet and triplet CT states are greatly split in energy (due to the excited state AF coupling in Figure 23 bottom) their configuration interaction with the ground state splits the singlet and triplet ground state energies leading to the AF coupling observed in the ground state of Cu(II) dimers. Thus the ground state AF coupling is given by equation 14:

\[-J^{GS} = c^2 (-J^{CT})\]  \[14\]

where \(c^2\) is the amount of bridging ligand character that is covalently mixed into the ground state and \(J^{CT}\) is the singlet/triplet splitting of the bridging ligand CT transition. Further details of the VBCI model are given in reference 55, and it has been used in reference 54 to estimate the ground state AF coupling in oxy hemocyanin which is too large to measure experimentally using ground state methods (see Section 3.1.4).

2.3.2 Copper(II)Copper(I)—Mixed valent (MV) copper sites have been important in biology in CuZ in N2OR56 and in the half Met derivatives57 of the coupled binuclear copper proteins (also in the electron transfer site CuA 58,59). In a MV system, specifically a binuclear site labeled CuA and CuB, the extra electron can be on CuA \((\psi[\text{Cu}_A(I)\text{Cu}_B(II)])\) or on CuB \((\psi[\text{Cu}_A(II)\text{Cu}_B(I)])\) or partially to fully delocalized over the two coppers as given by equation 15.

\[
\psi_g = \sqrt{(1-\alpha^2)}\psi[\text{Cu}_A(I)\text{Cu}_B(II)] + \alpha \cdot \psi[\text{Cu}_A(II)\text{Cu}_B(I)] \]  \[15\]

In the Robin and Day classification scheme,60 when \(\alpha^2 = 0\), the system is a class I MV complex, with the extra electron (or hole) localized on one Cu. For \(0 < \alpha^2 < 0.5\) the system is class II MV and shows perturbed spectral features associated with interactions with the second copper. In EPR there will be more than four copper hyperfine lines (four hyperfine lines from CuB split by the hyperfine coupling constant of \(A_{\text{CuB}}\) that reflects the amount of electron spin density on this copper, each again split into four hyperfine lines with a hyperfine coupling, \(A_{\text{CuA}}\), reflecting the amount of electron spin density on CuA; this has had a powerful application in multifrequency EPR, particularly using S-band to resolve overlapping hyperfine contributions)58 and in the absorption spectrum there will be an intervalent transfer transition (IV) (vide infra). For \(\alpha^2 = 0.5\) the system is a class III, completely delocalized, MV site. This will exhibit \(2NI+1=7\) copper \((N=2\) equivalent Cu with I=3/2\) hyperfine lines split by a hyperfine coupling constant that is about half that of an equivalent Cu(II) monomer, and a \(\psi \rightarrow \psi^*\) transition in its absorption spectrum (vide infra).

The origin of the electron delocalization between the two copper centers is the electronic coupling matrix element, \(H_{\text{AB}}\), that, as shown in Figure 24 can reflect both direct d orbital
overlap between the Cu’s and the effects of a superexchange pathway associated with bridging ligands.

For two equivalent Cu’s this would lead to delocalization, however this electronic coupling is opposed by vibronic trapping. As shown in Figure 25A, when the hole is on CuA the ligands will contract to stabilize the hole on this Cu, while the opposite is the case for the hole localized in CuB. This antisymmetric combination of these ligand breathing modes on the two Cu’s is known as the Q− vibrational mode in MV theory.61

Adding H_{AB} to the vibronic trapping term leads to an interaction between the potential energy surfaces as shown in Figure 24B. When H_{AB} is small (H_{AB}(i)) the two surfaces split at the crossing point (Q_− = 0) but the minima are still localized (with some mixing into the ground state from the second copper). This is the class II MV situation with some hyperfine coupling from the second copper. As illustrated in Figure 24A, excitation from the ground into the excited state transfers an electron from one Cu to the second Cu and is called an intervalence (IV) transfer transition (at an energy \( \lambda \)). From the potential energy surfaces for H_{AB}(i) in Figure 25B the excited state distorts along the Q− mode and the rR spectrum will exhibit resonance enhancement in this vibration. For the class III case (H_{AB}(ii) in Figure 25B) the ground state minimum is at Q_− = 0, the complex is symmetric and both coppers contribute equally to the ground state wavefunction. Thus seven hyperfine lines will be observed in EPR, and excitation from the ground to the excited state involves a \( \psi \rightarrow \psi^* \) transition in that an electron transferred from a bonding to an antibonding molecular orbital between the coppers. From Figure 25B H_{AB}(ii), this produces no distortion in the Q− mode (the \( \psi \) and \( \psi^* \) minima are both at Q_− = 0) but does lead to elongation and thus rR enhancement in the Cu-Cu and bridging ligand vibrational modes, since it is a metal-metal bonding to antibonding transition.62

It should finally be noted that H_{AB} in MV systems can, through the VBCI model, be related to the AF coupling between two Cu(II) described in section 2.3.1.1.55,63 This is given by equation 16:

\[
-2J = \frac{H_{AB}^2}{U} \tag{16}
\]

where U is the Coulomb repulsion between two electrons on the same copper (i.e. 2Cu(II) \( \rightarrow \) Cu(III)Cu(I)). This is important because the electronic coupling matrix element also plays a key role in Marcus Theory of ET.64 Thus differences in J reflect differences in the rate of electron transfer that can control the nature of intermediates required for different biological functions65 (see Section 3.3.6)

2.4 Higher Nuclearity Sites: Spin Frustration and Antisymmetric exchange

The trinuclear copper cluster (TNC) active site in the multicopper oxidases (section 3.7.1) requires additional consideration of the interactions among three Cu(II) S=1/2 centers. As shown in Figure 26, the resting TNC site has a pair of Cu(II)’s (called Type 3, vide infra) that can antiferromagnetically couple leading to an S’=1 triplet state at 2J above the S’=0 singlet ground state. Allowing for the presence of the third (Type 2) non-bridged S = ½
Cu(II), the $S'=0$ couples to form an $S_{TOT}=\frac{1}{2}$ ground state and the $S'=1$ couples to form an $S_{TOT}=\frac{1}{2}$ and $3/2$ states, still at $2J$, since there is no bridging interaction of the Type 2 Cu(II) with the Type 3 Cu(II) pair. The wavefunction for the $S'=0$ $S_{TOT}=1/2$ ground state thus has the spin localized on the Type 2 Cu(II) and only exhibits paramagnetic properties of this Cu(II) center. If all three Cu(II)’s are now allowed to have equivalent bridging, therefore exchange interactions, the $S'=1$ $S_{TOT}=1/2,3/2$ split in energy leading to the diagram in Figure 26 top right, with the quartet at $3J$ above the now orbitally degenerate $2E$ ground state, where this degeneracy is associated with the $S'=0$ $S_{TOT}=1/2$ and $S'=1$ $S_{TOT}=1/2$ sublevels. The spin topology with three equivalent, exchange coupled $S=1/2$ Cu(II)’s is a spin frustrated system as all three pairs of $S=1/2$ Cu(II)’s cannot be simultaneously AF coupled in a triangular bridged system.66

The spin frustrated $2E$ ground state will undergo a large ZFS (by amount $\sqrt{3}$ G in Figure 26 bottom) due to a phenomenon known as antisymmetric exchange. This derives from in state SOC which can be large and mixes the $S'=1,0$ $S_{TOT}=1/2$ wavefunctions leading to an equivalent spin distribution over the three Cu(II)’s. It also has a major effect on the Zeeman splitting of the spin sublevels therefore the EPR spectrum of a spin frustrated system. In particular, as shown in Figure 26 bottom, when the magnetic field is applied perpendicular to the $z$ ($C_3$) axis of the Cu(II) trimer, there is a Zeeman interaction between the two ZFS levels of the $2E$. This leads to a non-linear dependence of the Zeeman splitting with increasing magnetic field. Therefore higher fields, corresponding to very low $g$ values, are required to satisfy the resonance condition of the EPR experiment.

As with anisotropic exchange, this antisymmetric exchange derived ZFS is due to SOC of a LF excited state into the ground state and thus can again be related to the ground state $g$ value deviation from 2.0050–52 (equation 17).

$$G=(g_\parallel - 2.00)(-2x^2-y^2) \quad [17]$$

$J_{x^2-y^2}$ involves both the ground state exchange interaction between $d_{x^2-y^2}$ orbitals on pairs of Cu(II)’s and the exchange coupling of the $d_{xy}$ LF excited state on one Cu(II) with the $d_{x^2-y^2}$ ground state on the second Cu(II). Therefore spin frustrated trinuclear Cu(II) states with large antisymmetric exchange hence low $g$ values ($< 2.00$, i.e. the native intermediate in the multicopper oxidases (MCOs), see Section 3.7.1) require bridging ligands between the three Cu(II)’s that provide good superexchange pathways for coupling both the ground and excited state of each Cu(II) to the ground state of the adjacent Cu(II).

From model studies this can be accomplished both by a $\mu_3$ oxo and by three $\mu_2$-OH bridges.66

### 2.5 Electronic Structure Calculations

Spectroscopy experimentally determines electronic structure and, therefore, it is important to correlate spectroscopic data to the results of electronic structure calculations. New developments in theoretical methods combined with the unprecedented computational resources available have provided chemists with powerful new tools for the study of electronic structure and reactivity of chemical systems. Thanks to the new advances in...
supercomputing, is now possible to study with Quantum Mechanics relatively large systems with multiple open shell metal centers and to further include, with the help of Molecular Mechanics, the whole protein and the solvent molecules. The popularity of these methods is reflected in the increasing number of electronic structure calculation software packages available and their impact on research. It is not the aim of this section to provide a detailed description of each theoretical method, but to present key essentials of the most popular methods in the study of transition metal complexes, in general, and copper sites in particular. We emphasize that different methods and basis sets give different results and it is critical to evaluate calculations with spectroscopic data. Calculations validated by data can then be used for insight into the transition states and frontier molecular orbitals, that are key to reactivity.

2.5.1 Ab Initio Wave Function Methods—Hartree-Fock (HF) theory describes a many-electron wave function \( \Psi \) as a linear combination of the products of independent one-electron wavefunctions, \( \chi \) molecular orbitals (MOs), which are linear combinations of atomic orbitals \( \varphi \) and these, in turn, are linear combinations of the basis functions \( \phi \).

The time independent Schrödinger equation can then be solved using the Hartree-Fock Self Consistent Field (HF-SCF) scheme

\[
\phi_i = \sum_{j=1}^{M} c_j \varphi_j \rightarrow \chi_i = \sum c_i \phi_i \rightarrow \psi_i (x_1, x_2, \ldots, x_n) = \chi_1 (\chi_2) \cdots \chi_n (\chi_n) \rightarrow \hat{H} \sum_i \psi_i = \sum_i E_i \psi_i \tag{18}
\]

where the hamiltonian that operates over the trial wavefunction usually has the following form,

\[
H = -\sum_{i} \frac{\hbar^2}{2m_e} \nabla_i^2 - \sum_{k} \frac{\hbar^2}{2m_k} \nabla_k^2 - \sum_{i} \sum_{k} \frac{e^2 Z_k}{r_{ik}} + \sum_{i<j} \frac{e^2}{r_{ij}} + \sum_{k<l} \frac{e^2 Z_k Z_l}{r_{kl}} \tag{19}
\]

The terms in the hamiltonian are the kinetic energy of the electrons \( i \), the kinetic energy of the nuclei \( k \), the potential energy attraction between electrons and nuclei, the interelectronic repulsion \( i,k \) and the internuclear repulsion \( k,l \). This expression is solved through the Hartree-Fock Self-Consistent Field approach, which is an iterative process to calculate the best single-determinant solution for the time-independent Schrödinger equation. Fock extended Hartree’s iterative process to Slater Determinant wave functions. The Fock operator is defined for each electron \( i \) as:

\[
f_i = -\frac{1}{2} \nabla_i^2 - \sum_{k} \frac{Z_k}{r_{ik}} + \sum_{i=1}^{n/2} \left[ 2J_i - K_i \right] \tag{20}
\]

where the first part of the operator is the one-electron core Hamiltonian, \( J_i \) is Coulomb operator and \( K_i \) the exchange operator. It is the last part of the operator that leads to “four center integrals”, which are the two electronic exchange and Coulomb interactions of the form

\[
\int dr_1 \int dr_2 \phi_\alpha^*(r_1) \phi_\beta^*(r_2) \frac{1}{|r_1 - r_2|} \phi_\alpha(r_1) \phi_\beta(r_2) \tag{21}
\]
where $\varphi_{p,q,r,s}$ are analytic functions on four different centers. Those integrals scale as $N^4$ where $N$ is the number of electrons of the system.

Basis functions are the set of mathematical functions from which the wavefunctions are constructed. There are several mathematical functions that can be chosen to build the wavefunction. Given the shape of the atomic orbitals, one must think in terms of Slater-type functions are a logical choice, but the fact that the four center integrals corresponding to the interelectronic repulsion in the Hamiltonian have to be solved by numerical methods limits their utility in medium to large size molecular systems. However, high-quality Slater-type orbital (STO) basis sets have been developed. Gaussian basis sets are the most widely used, given that these functions solve the four index integrals in an analytical way. Moreover, Gaussian basis sets can turn four center integrals into a finite sum of two center integrals, and in the next step, to finite sums of one-center integrals, speeding up the calculations by a factor of 4 to 5. The general functional form of a normalized Gaussian-Type orbital (GTO) in atom-centered Cartesian coordinates is:

$$
\varphi(x, y, z; \alpha, i, j, k) = \left(\frac{2\alpha}{\pi}\right)^{3/4} \left[\frac{(8\alpha)^{i+j+k}i!j!k!}{(2i)!(2j)!(2k)!}\right]^{1/2} x^i y^j z^k e^{-\alpha(x^2+y^2+z^2)} \tag{22}
$$

where $\alpha$ is an exponent controlling the width of GTO, and $i$, $j$, and $k$ are non-negative integers that dictate the nature of the orbital in the Cartesian notation.

There are a number of options in choosing a Gaussian basis set, depending on the balance between accuracy and computing time. Ideally, the basis set should be as large as possible, so the Hartree-Fock limit is achieved based on the Variational Principle. This can however involve excessive computational cost. For systems that have transition metal centers, the most common basis sets used in calculations are triple-$\zeta$, which means three Gaussian functions are used for each atomic orbital. Among the most popular basis sets of this type are the split-valence basis set of Pople and coworkers, the correlation-consistent polarized core basis set from Dunning, and the triple-$\zeta$ basis set from Ahlrichs and coworkers. In many cases it is necessary to include polarization functions on the metal center as well as the donor atoms to improve the description of the bonding interaction and add flexibility to the Gaussian basis set. Also diffuse functions often need to be included, which are shallow Gaussian functions that allow for a better description of the “tail” of the atomic orbital. The notation from the Pople group for a split valence basis set is usually of the type X-YZW for a triple-$\zeta$ basis set, where X denotes the number of primitive Gaussians (i.e., each of the Gaussian functions used to build up the orbital function) for each atomic core orbital, and YZW indicate that the valence orbitals are made of three Gaussian functions, the first a linear combination of Y primitive Gaussians, the second a linear combination of Z primitive Gaussians, and so on. Polarization functions are usually denoted by the symbol “*” and diffuse functions by the symbol “+”.

Aside from the Gaussian basis sets, the other two main approaches are the use of Effective Core Potentials (ECP, or pseudopotentials) and the use of plane waves. ECPs replace the core electrons and the nucleus with an effective potential, allowing for a large reduction in the size of the basis set and the number of electrons, speeding up the calculation. These also
allow inclusion of relativistic effects in the calculation when dealing with heavy elements. Plane waves are particularly useful in solid state calculations as these have translational symmetry.

The HF-SCF wavefunction for the electronic ground state is then a single Slater determinant build from occupied MOs.\textsuperscript{67} However, for a many electron system, the premise that the motion of each electron can be described by assuming that it moves in a static field provided by the nuclei and all the other electrons is an approximation. Electrons repel each other, therefore, they will adjust to minimize their mutual repulsion, thus the electron motion is correlated. As a result, HF theory overestimates the electron repulsion energy by an amount called the *correlation energy*, which becomes important in describing the covalency and calculating reaction energies and barriers. Thus the need for correlated *ab initio* methods. Electron correlation is usually divided into dynamic and static correlation. The dynamic correlation is the correlation of the movement of the electrons while the static correlation is important for molecules in which the ground state cannot be described by a single determinant. To improve the wave function use is made of the fact that after the SCF optimization of the MO coefficients for all the occupied orbitals, the coefficients for the virtual (i.e unoccupied) orbitals are also obtained. This allows one to construct additional Slater determinants that account for different electronic excited configurations (single, double, triple, etc, electron excitations into virtual orbitals).\textsuperscript{67} Mixing these additional determinants with the ground state gives a more flexible wavefunction that includes static electron correlation. When all the coefficients are optimized variationally for all possible configurations within a set of occupied and unoccupied MOs, the method is called Full Configuration Interaction (FCI). For biologically relevant systems, FCI is unaccessible, so a truncation of the wavefunction is necessary. However, the truncation of the CI space leads to the lost of size consistency (i.e., the energy of two noninteracting entities is different from the sum of their individual energies). Given that the FCI is not accessible for large systems other ways to achieve the inclusion of electron correlation in the wave function have been developed.

**2.5.1.1 Many Body Perturbation Theory:** In a single configurational approach, the electron correlation energy can be included using manybody perturbation theory (MBPT). Møller and Plesset proposed operator expressions for these energy corrections.\textsuperscript{73} The most popular MBPT level calculation is Møller-Plesset second order perturbation theory (MP2) with a HF determinant as the reference function. This allows an estimate of dynamic electron correlation for species with an electronic ground state well represented by a single Slater determinant. The increasing computational power available today allows for higher order interactions, typically MP3,\textsuperscript{74,75} MP4\textsuperscript{76} and MP5,\textsuperscript{77} within this formalism.

**2.5.1.2 Multiconfigurational Self-Consistent Field Method:** As indicated above, a way to improve the quality of the wave function is include additional possible determinants for excited electron configurations in the ground state. By selecting only a limited number of determinants from all possible, we can construct a multi-configurational wavefunction ($\phi_n$ are many electron single determinants),
for which the lowest energy is found by optimizing both the MO coefficients and the $c_n$ configuration interaction coefficients. This MC-SCF wave function is able to retrieve the static electron correlation energy for systems that can only be described with more than one (nearly)-degenerate determinant. The most popular way to implement this is the Complete Active Space (CAS) SCF method. For a given electronic state, the configurations with large $c_n$ use only a limited set of molecular orbitals. Therefore, for an $n$-electron system, the MO space can be split in three blocks. The inactive orbitals (those that are doubly occupied in all the configurations, containing $m$ electrons), the external orbitals (those that have zero occupancy in all the configurations) and the active orbitals, which contain the active electrons ($n-2m$). The wavefunction is defined as the Full Configuration Interaction of all the possible configuration state functions (CSF) having the spatial and spin symmetry imposed by the active electrons in the active orbitals. This method is convenient and overcomes many problems of Hartree-Fock calculations on open shell systems. However, it is difficult to recover a large portion of the dynamic correlation energy by expanding the active space. A better way to include the dynamic correlation energy is using MP2 with a CASSCF reference wavefunction (CASSCF/CASPT2).

The obvious limitation of this method in biological relevant systems is the size of the active space. To account for large systems, the Restricted Active Space (RAS) SCF method was developed. In this method, the active space is further divided into three subspaces. In parallel with CAS, RAS can account for dynamic correlation via MP2 in the RASSCF/RASPT2 scheme.

2.5.1.3 Coupled Cluster Methods: Coupled Cluster Methods (CC) are based on rewriting the Full Configuration Interaction wave function as:

$$\Psi_{CC} = e^{T_1} \Psi_{HF}$$  [24]

where the cluster operator $T_1$ operates over all the $n$ electrons and generates all the possible determinants having $i$ excitations from the reference wave function ($\Psi_{HF}$). In a sense, this method is a full CI, but the advantage relies on the truncation of the $T$ operator. Usually, the $T$ operator is approximated as $T=T_{1}+T_{2}$, defining the Coupled Cluster Singles and Doubles (CCSD), and a Taylor expansion of this operator shows that the method accounts for size consistency. This method is very computationally demanding, and inclusion of triple excitations is non accessible for large molecules. Triple excitations are therefore included via perturbation theory.

2.5.2. Density Functional Methods—While CAS and CC wavefunction methods are good when it comes to the inclusion of electron correlation, they are also very computationally demanding, and have a problem of size limitation. Bioinorganic systems usually involve large molecules that are often not accessible with these wavefunction methods. An alternative approach to this problem is the use of Density Functional Theory (DFT). Theoretically supported by the Hohenberg-Kohn theorems and within the Kohn-
Sham (KS) DFT framework, the intractable many body problem of interacting electrons in a static external potential is reduced to a tractable problem of non-interacting electrons moving in an effective potential. This effective potential includes the external potential and the effects of the Coulomb interactions between the electrons, therefore the exchange and correlation interactions.

In many body electronic structure calculations, the nuclei of the molecules are assumed to be fixed (the Born-Oppenheimer approximation), generating a static external potential \( V \) in which the electrons move. A stationary electronic state can be described by a wavefunction that satisfies the time independent Schrödinger equation for an \( n \)-electron system:

\[
\hat{H}\Psi = [\hat{T} + \hat{V} + \hat{U}] \Psi = \left[ \sum_{i=1}^{n} -\frac{\hbar^2}{2m} \nabla_i^2 + \sum_{i=1}^{n} V(\vec{r}_i) + \sum_{i<j}^{n} U(\vec{r}_i, \vec{r}_j) \right] \Psi = E\Psi \quad [25]
\]

where \( H \) is the hamiltonian, \( T \) the kinetic energy, \( V \) the potential energy from the external field due to the positive charges of the nuclei, and \( U \) is the electron-electron interaction energy. While \( T \) and \( U \) are universal, \( V \) depends on the system, and this equation is not separable into simpler single-particle equations because of \( U \). This equation can be solved as described above by expanding the wave function into Slater determinants, but DFT provides an alternative method to deal with the electron-electron interaction. In DFT the key variable is the particle density \( \rho(\vec{r}) \), which for a normalized wave function is given by

\[
\rho(\vec{r}) = \int d^3r_2 \int d^3r_3 \ldots \int d^3r_n \Psi^*(\vec{r}_1, \vec{r}_2, \ldots, \vec{r}_n) \Psi(\vec{r}_1, \vec{r}_2, \ldots, \vec{r}_n) \quad [26]
\]

where \( n \) is the number of electrons.

This relation can be reversed, this is, for a given \( \rho_0(\vec{r}) \) it is possible to calculate the corresponding ground state wavefunction \( \Psi_0(\vec{r}_1, \vec{r}_2, \ldots, \vec{r}_n) \Psi_0 \) is a unique functional of \( \rho_0 \), and any ground-state expectation value of an observable is also a functional of \( \rho_0 \). In particular, the ground state energy is a functional of \( \rho_0 \) as well as \( T, U \) and \( V \). The electronic density determines the potential and thus the Hamiltonian, which determines the wave function.

\[
E(\rho) = T(\rho) + U(\rho) + \int V(\vec{r}) \rho(\vec{r}) d^3r \quad [27]
\]

In principle, this approach is exact, and minimization of the \( E(\rho) \) will give the exact \( E(\rho_0) \) and therefore all the other ground state properties. In practice, the exact functional is not known and one still needs to approximate the small but important contribution to the single particle potential energy term, called the exchange-correlation (XC) energy. Depending on this approximation, the calculation will be more or less accurate. The present exchange-correlation functionals are itemized below.

2.5.2.1 Local Density Approximation (LDA): This is the simplest density functional and has been widely used by the solid state community. In the LDA, the XC energy depends only on the density at a point and is that of a uniform electron gas of that density. While the exchange energy can be computed exactly, no analytical derivation of the correlation energy
density has proven possible. Highly numerically accurate calculations of several different densities allowed Vosko, Wilk and Nusair to design local functionals by subtracting the analytical exchange density from the total calculated density in order to include the correlation part. The authors proposed several fitting schemes for the correlation density, the most popular being the VWN and VWN5 local exchange-correlation functionals. Unfortunately, LDA methods are not very accurate for molecular properties as the electron density is not uniform.

2.5.2.2 Generalized Gradient Approximations (GGA): A way to improve the correlation functional is to make it depend not only on the local value but on the gradient of the density. Inclusion of the gradient correction defines the Generalized Gradient Approximation (GGA) functionals. Originally labeled as “non local functionals”, GGAs correct the functional by adding the gradient correction to the LDA functional, this is,

\[ \varepsilon_{X/C}^{GGA}(\rho(\mathbf{r})) = \varepsilon_{X/C}^{LDA}(\rho(\mathbf{r})) + \Delta \varepsilon_{X/C}(\frac{\nabla \rho(\mathbf{r})}{\rho^{3/2}(\mathbf{r})}) \]  

One of the most popular GGA exchange functionals was developed by Becke, which includes a single empirical parameter, but other popular exchange functionals without empirical parameters are BP86 and PBE. For the correlation part, one of the most popular correlation functionals, LYP, does not correct the LDA expression using the above equation, but instead computes the total correlation energy. The GGA functionals are more accurate than the LDAs, and reduce the error in bond dissociation energies and transition-state barriers.

2.5.2.3 Meta-Generalized Gradient Approximations (MGGA): After the inclusion of the gradient, the next step is to include the dependence of the electron density on the second derivative, i.e. the Laplacian. This approach leads to the meta-GGA functionals, which in addition to the gradient at each point also include the dependence of the KS kinetic energy density. TPSS is an example of this kind of functional. Meta-GGA calculations are usually as computationally expensive as the GGA and more accurate for pure functionals.

2.5.2.4 Hybrid Functionals: A hybrid functional includes a certain amount of the exact Hartree-Fock exchange. By adding a fraction of exact exchange, one can often better reproduce the effects of static correlation. Mixing exact exchange however is not free of cost, because it is non-local, and depends both on the electron density and also the density matrix. The most popular hybrid functional is the B3LYP, which includes an empirically adjusted 20% of exact HF in the exchange part of the functional. Following this approach, any functional can be turned into a hybrid functional by mixing some amount of HF exchange.

2.5.2.5 Fully nonlocal functionals: These functionals require input from both the occupied and unoccupied KS orbitals. These are the equivalent of including post HF methods in DFT. Development of these functionals is still in progress and they are, in general, computationally expensive.
Due to the success of Density Functional methods, chemists have been trying to apply DFT to problems with increasing complexity. This has resulted in the development of new Density Functional methodologies aimed at overcoming the inherent limitations of “classic” DFT methods. The double hybrid functionals\cite{96,97} include a certain amount of HF exchange and a perturbational correlation correction. These functionals improve the calculation of kinetic barriers and dispersion forces due to the inclusion of the perturbational term, and have shown some success in the calculation of energy gaps between different spin states.\cite{98} However, the construction of the functional makes it more expensive than the usual DFT methods. Constrained DFT methods have successfully been applied to systems with long-range charge transfer states, which is of special interest when studying systems that involve proton-coupled electron transfer processes.\cite{99,100} Also, major advances have been recently made in the calculation of non-bonded interactions using dispersion corrected functionals, that provides an accurate energy calculation for van der Waals interactions, a key factor for the correct prediction of inter- and intramolecular noncovalent interactions.\cite{101,102}

2.5.3. Quantum Mechanics-Molecular Mechanics (QM-MM) Methods—In order to study chemical reactions at the active sites of enzymes, chemists usually simplify the system by focusing on the metal center and its surrounding residues. To mimic the effects of the enzyme and solvent, a dielectric continuum model (typically Polarized Continuum Model (PCM) or COSMO (Conductor like screening model)) is usually used. The alternative is to use a simplified model of the protein and solvent in the Quantum Mechanics-Molecular Mechanics (QM-MM) method.\cite{103} This approach treats the active site at a high level of theory (usually DFT, but wavefunction methods can also be used), and the protein surroundings at a lower level, usually Molecular Mechanics. This allows the inclusion of steric and electrostatic effects on the active site. In order to combine QM and MM one needs a proper treatment of the boundary region. There are two basic groups of coupling schemes, based on the treatment of electrostatic interactions.\cite{104}

2.5.3.1 Electrostatic Embedding (EE): In this method, the QM calculation is performed in the presence of the MM system by properly including terms that describe the electrostatic interaction between the QM and the MM regions. This method accounts for the polarization effect of the charge distribution of the MM region on the active site, which is key to the electronic structure of the active site.

2.5.3.2 Mechanical Embedding (ME): Here, the QM calculation is performed in the absence of the MM part, and all the interactions are treated at the MM level. The original ONIOM (our own n-layered integrated Molecular Orbital and Molecular Mechanics)\cite{105} coupling scheme belongs to this group, although the most updated version of this coupling scheme also allows for EE.

The connection of the QM and the MM region is a challenging problem. The two approaches to do this are the link atom and the local orbital schemes. The first introduces an extra hydrogen atom in order to saturate the QM-MM regions.\cite{106} The later uses localized bond orbitals to connect the two regions. The treatment of the boundary region is still a work in progress, where numerous and significant advances have been made in order to improve the quality of QM-MM calculations.\cite{107}
2.5.4 Application to Cu sites—As presented above, there are a wide range of methods to calculate electronic structures. Although the choice of method is highly influenced by the size of the system and the goal of the calculation (i.e., geometry optimization, physical properties, reaction profiles, etc), it is important to evaluate the predictive properties of these methods as well as their ability to provide insight into the electronic structure of the studied system. In order to do this, benchmark calculations and calibration protocols are required. In the following sections, we present well-studied copper systems where spectroscopic data, which provide the experimental electronic structure of the molecule, are used to evaluate Density Functional Theory methods. Spectroscopically calibrated methods that reproduce the experimental data provide insight into the frontier molecular orbitals that are key to reactivity and a solid methodology to evaluate reaction coordinates.

2.5.4.1 Calibration of DFT methods using [CuCl$_4$]$^{2-}$: One of the early applications of DFT in bioinorganic chemistry was the blue Cu site. However, this method had to first be evaluated experimentally. This was done on the [CuCl$_4$]$^{2-}$ complex, which has been studied with spectroscopic methods extensively over ten orders of magnitude in photon energy.$^{108–110}$ The bridge between experimental data and theoretical calculations was done within one of the earliest DFT methods, the X$\alpha$ scattered wave (X$\alpha$-SW) code.$^{86,111}$ The results from these calculations were found to be too covalent relative to experiment$^{36,112}$ thus the atomic sphere radii were adjusted to fit the [CuCl$_4$]$^{2-}$ experimental data. With adjusted parameters, the ground-state description was in good agreement both for [CuCl$_4$]$^{2-}$ and for the blue Cu active site. Following this approach modern GGA functionals have been evaluated using the [CuCl$_4$]$^{2-}$ complex as a benchmark.$^{113}$ A considerable amount of ground and excited state data are available for both tetragonal ($D_{4h}$) and flatted-tetrahedral ($D_{2d}$) [CuCl$_4$]$^{2-}$ complexes.$^{114,115}$ The spectroscopic data define the ground state spin density of the Cu(II) ion in the $D_{4h}$ structure as 0.62 ± 0.02, which can be directly compared with the results from electronic structure calculations, thus allowing experimental calibration of GGA functionals. Spin density is a parameter directly related to the electronic structure of the molecule, making it a better calibration guideline than bond distances or thermochemical data.

Before discussing functional effects, it is important to consider the effect of the size of the basis set on the calculated spin density. Both all electron and effective core potential basis sets were tested with BP86 (GGA pure functional), B3LYP (hybrid with 20% HF) and BHandHLYP (hybrid with 50% HF). The calculated spin density was found to be dependent on the size of the basis set. From these calculations is was concluded that at least a triple-$\zeta$ basis set with diffuse functions on the metal is required in order to achieve effective basis set saturation. Lower quality basis sets give an incorrect spin density on the copper atom; larger basis sets do not significantly improve the calculated spin density.

When the pure BP86 functional with a saturated basis set was used to calculate the electronic structure of the [CuCl$_4$]$^{2-}$ anion in a square planar geometry, the spin density on the Cu atom was calculated to be 0.43 (± 0.02).$^{113}$ This value indicated the presence of excessive LMCT mixing into the ground state. In other words, the bonding description for the ground state of [CuCl$_4$]$^{2-}$ in $D_{4h}$ geometry with this functional is too covalent (Figure 27 left), and includes too much ligand character in the singly occupied HOMO (SOMO, the $\beta$-
LUMO in spin unrestricted calculations). As a consequence of this, the ligand field transitions are shifted too high energy while the LMCT transitions are found to be too low in energy. Thus, a less covalent bonding description is required to increase the metal character in the β-LUMO. This increases the spin density on the Cu center and shifts the ligand field and charge transfer transitions to their correct range of energies based on experiment. This requires destabilizing the copper d-manifold with respect to the ligand orbitals (Figure 27 right), through a more ionic description of the Cu-Cl bond.

This can be accomplished within the Density Functional Theory by adding exact Hartree-Fock exchange into the exchange part of the functional by means of a hybrid functional. Two functionals were explored, B3LYP and BH and HLYP, which mix 20% and 50%, respectively, of HF exchange into the pure functional, at the basis set saturation limit. The B3LYP functional significantly improves the ground and excited state properties of the [CuCl₄]²⁻ in $D_{4h}$ geometry, but still gives a ground state that is too covalent, with a calculated copper spin density of 0.52. On the other hand, the BHandHLYP functional gives an overly ionic description, with a copper spin density of 0.67. Therefore the optimal amount of HF exchange was determined by systematically varying the local and non-local exchange and correlation functionals (Figure 28). Among the different combinations, 38% of the total Density Functional exchange (both local and non-local) was replaced with HF exchange to match the experimental spin density keeping the correlation part unchanged. This calibration was done for the BP86 functional, but the amount of HF is transferable to other pure functionals such as BLYP. This B(38HF)P86 hybrid functional, adjusted to the ground state, is also able to calculate the ligand field and charge transfer transition energies with reasonable agreement with respect to experiment. Although all the functionals overestimate the Cu-Cl bond length relative to crystallographic data, it is worth noting that the spectroscopically calibrated B(38HF)P86 functional is the one that gives the best agreement with the experimental value.¹¹³

Both solvent and crystal lattice effects on the calculated spin density have been evaluated and lead to small variations of less than 2%. However, the solvent has a larger effect on energy related parameters and bond dissociation energies, as expected for a negative charged molecule. Also the crystal lattice (H-bonding from the counterions) stabilizes the square planar geometry, preventing the distortion towards the $D_{2d}$ geometry observed in the gas phase calculations.

Comparison with higher levels of theory has also been performed. The electron density has been generated for the selected methods in Table 3. MP4 converges to a reasonable bonding description, and only Quadratic Configuration Interaction Singles and Doubles (QCISD) method with a saturated basis set is able to reproduce the experimental value. Coupled Cluster methods give a too ionic bonding description due to the lack of triple excitations. The good description of the ground state and the electronic excitation energies obtained with the B(38HF)P86 functional make it suitable to study biologically important Cu(II) systems. However, as discussed in the next section, environment effects on the HOMO and the presence of stronger covalent bonds require a detailed analysis of each class of copper complexes.
2.5.4.2 Blue Cu site: The first crystallographic determination of plastocyanin was accomplished in 1978 by Freeman and coworkers.\textsuperscript{116} The geometric structure of the copper(II) active site, distorted tetrahedral, raised the issue of the protein’s role in determining the geometric structure of the active site. The copper coordination sphere in plastocyanin also has two chemically interesting ligands: a thiolate sulfur of a Cys residue with a short bond length (~2.1Å) and a long thioether sulfur-Cu bond of a Met (~2.9Å). The coordination sphere is completed by two His ligands at normal bond distances (Figure 29A).\textsuperscript{116}

The unusual geometry and ligation are responsible of the unique spectral features of the blue Cu site.\textsuperscript{2,117} Blue Cu has an intense absorption band at 16000cm\(^{-1}\) (\(\varepsilon = 5000 \text{ M}^{-1} \text{ cm}^{-1}\))\textsuperscript{36}, and a ground state EPR signal with a hyperfine coupling (\(|A_|| = 53 \times 10^{-4} \text{ cm}^{-1}\))\textsuperscript{118} of the electron spin to the nuclear spin on the copper, reduced by more than a factor of two with respect to normal copper complexes. These spectral features reflect a novel ground state wave function that plays a key role in the biological ET function of this protein. From single crystal EPR studies\textsuperscript{43} the ground state has an unpaired electron in a \(d_{x^2-y^2}\) orbital (SOMO, \(\beta\)-LUMO), and the electronic z-axis of this site was found to be tilted only 5° with respect to the long thioether Cu-S bond. This axis orientates the \(d_{x^2-y^2}\) orbital to be within 15° of the plane defined by the three strong ligands (Cys and two His). Initially the low Cu hyperfine coupling value was attributed to the distorted tetrahedral coordination site. It was thought that this distortion would allow the Cu 4p\(_z\) orbital to mix into the \(d_{x^2-y^2}\) orbital, which would lower \(A_||\) due to the spin dipolar contribution to hyperfine coupling. However, Cu K-edge (Section 2.2.5) experiments clearly showed that only Cu 4p\(_{x,y}\) mixes into this orbital. These data led to the alternative explanation for the small hyperfine coupling of a highly covalent site. In fact, the experimental values for the ground state wave function through Cu L-edge (Section 2.2.6) and S K-edge XAS spectroscopies are 0.41 Cu-d and 0.38 S-p character respectively.\textsuperscript{43}

As with [CuCl\(_4\)]\(^{2-}\), Xo-SW calculations gave ground state description that was too covalent, and it was necessary to adjust the parameters of the calculation. This resulted in good agreement with the spectroscopic data on the blue Cu ground state showing a highly covalent site mostly delocalized between the \(\pi\) orbital of the Cys-S and the \(d_{x^2-y^2}\) orbital of the Cu (Figure 29B).\textsuperscript{112} When a small model for the active site including only the first coordination sphere was used with modern DFT calculations, the same qualitative description of the ground state was obtained. However, as with Xo-SW this was quantitatively too covalent and the adjusted functional, B(38HF)P86, was required to reduce the covalency through inclusion of significant Hartree-Fock exchange. However, for the blue Cu site when the model used for the calculations was expanded to include second sphere interactions, the H-bond of a nearby amide with the thiolate (which has been studied in detail for iron-sulfur proteins)\textsuperscript{119} and the protein dipoles, less HF exchange (the 20% of B3LYP) was required in the functional to reproduce experiment (Table 4). This has been experimentally evaluated for the blue Cu site in azurin.\textsuperscript{120}

It is important to observe that the protein effects on blue Cu site differs from than the inclusion of the crystal lattice effects in [CuCl\(_4\)]\(^{2-}\). In the [CuCl\(_4\)]\(^{2-}\), the counterions also interact through H-bonds to the chlorides, but while this stabilizes the square planar
geometry it has essentially no effect on the covalency and the Cu-Cl bonds. In the case of blue Cu, however, the site is more covalent and second sphere interactions do affect the covalency of the Cu-S bond. Therefore less HF exchange is required in the hybrid functional to reproduce the experimental nature of the bond.

In summary, correlation to experiments on the blue Cu site show that one must be careful in choosing a computational approach. Although the spectroscopically calibrated B(38HF)P86 functional is in excellent agreement for inorganic Cu(II) complexes, for the highly covalent blue Cu site second sphere interactions affect the covalency of the Cu-ligand bond and result in the need for less HF mixing into the ground state (but still the 20% HF in B3LYP for the blue Cu center).

2.5.4.3 CuO$_2$ species: There are two structural types in this class of oxy-Cu species: end-on and side-on bound O$_2$. For the latter, two limiting electronic structure descriptions are observed. From L-edge XAS and rR spectroscopies the side-on CuO$_2$ complex with the trispyrazolyl borate ligand is a superoxo-Cu(II) species, while for the β-diketiminate ligand the side-on CuO$_2$ complex has a peroxo-Cu(III) electronic structure. For all end-on CuO$_2$ complexes, the electronic structure description is that of a superoxo-Cu(II) species. Both side-on structures are ground state singlets while the end-on structures are triplets. It is important to evaluate how well the different types of DFT calculations (i.e., pure functionals and hybrids) do in correlating to experiment in order to use DFT calculations in evaluating possible reaction coordinates. Both the details of the spectroscopic elucidation of the electronic structures of these complexes and the evaluation of possible reaction coordinates are presented in section 3.3.

For the end-on CuO$_2$ complexes, both pure (BP86) and hybrid (B3LYP and B(38HF)P86) functional calculations give the experimentally observed triplet ground state. In correlating to the crystallographically defined [TMG$_3$trenCuO$_2$]$^+$ complex (Table 5), the hybrids give the best agreement with respect to the CuO$_2$ bonding. These appear to be appropriate for reaction coordinate calculations for the end-on CuO$_2$ structure observed in the non-coupled binuclear Cu site in peptidylglycine α-hydroxylating monooxygenase (see section 3.3). The origin of the triplet ground state can be seen from Figure 30A. For the end-on bound case, the π$_\sigma^*$ orbital of the O$_2'^-$ overlaps with a lobe of the d$_{z^2}$ orbital (highest energy d orbital) of the Cu(II) leading to a σ bonding/antibonding interaction. This raises the energy of the d$_{z^2}$ orbital while leaving the second O$_2'^-$ orbital, the π$_\gamma^*$, non-bonding and on the Cu. If their splitting, Δ, is not large, both the d$_{z^2}$ and the π$_\gamma^*$ will be half occupied predicting the triplet ground state observed experimentally.

In going to the side-on CuO$_2$ structure, the highest energy molecular orbital is now the d$_{x^2-y^2}$ orbital and it undergoes a stronger bonding/antibonding interaction with the O$_2'^-$ π$_\gamma^*$ due to the greater overlap in the side-on geometry (two Cu-O bonds). This leads to a larger value for Δ. When this is large enough, one gets a spin paired singlet ground state. This is the case for the trispyrazolyl borate CuO$_2$ complex, from SQUID magnetic susceptibility measurements. Correlations to DFT calculations (Table 6) show that only a pure functional (BP86) is capable of reproducing the singlet ground state. B3LYP gives a side-on triplet at 8.25 kcal/mol below the antiferromagnetic coupled singlet. It is interesting to
compare the contours of these two calculations (Figure 31). For the B3LYP calculation, both the triplet and antiferromagnetic coupled singlet have a $d_{x^2-y^2}$ and a $\pi^*$ orbital half occupied as their splitting $\Delta$ is not large enough to spin pair. For the BP86 singlet, the $\alpha$ and $\beta$ unoccupied orbitals have similar spatial distributions hence little spin polarization. Thus for the pure functional, $\Delta$ in Figure 30B is large enough to overcome electron-electron repulsion and spin pair, and the direct covalent interaction between the $\pi^*$ and $d_{x^2-y^2}$ orbitals is large enough to lead to little spin polarization.\textsuperscript{123}

From this correlation to experiment, it appears that when the metal-ligand bond gets very strong (i.e., covalent), there is less need for HF mixing in the functional as this tends to decrease the covalency. Finally from L-edge XAS, the appropriate energy diagram for the $\beta$-diketiminate complex is that shown in the Figure 30C where the strong $\beta$-diketiminate donor ligand raises the energy of the $d$ orbital and limits the amount of charge donation from the $O_2^{2-}$ to the Cu.\textsuperscript{122,126} This leads to the peroxy-Cu(III) electronic structure description with a singlet ground state. DFT calculations with a pure functional reproduce this, however when HF is included for this strong donor, $\beta$-diketiminate ligand the calculations predict a triplet ground state, while from experiments it is a singlet.\textsuperscript{126}

Therefore, it is clear that the nature of the copper oxygen bond (i.e., the covalency) determines the functional required to correlate electronic structure calculations to experimental data. End-on CuO$_2$ systems are less covalent, thus HF exchange mixing is needed, and the B3LYP functional correctly reproduces the covalency of the bond and the S=1 ground state. Alternatively, side-on CuO$_2$ systems are more covalent, thus requiring less HF exchange. As a consequence, the correct bonding description and S=0 ground state are well described by the pure functional BP86.

These correlations between different functionals and experiment for CuO$_2$ and those presented above for [CuCl$_4$]$^{2-}$ and Blue Cu suggest that the amount of HF mixing in the functional can vary, decreasing with increasing covalency of the bond, and the amount required is best established by correlation to experiment on the system being studied.

**2.5.4.4 Cu$_2$O$_2$ species:** Several enzymes involved in O$_2$ activation for the oxidation of organic substrates function with a coupled binuclear Cu active site. Much effort has been focused on their structure, spectroscopy and reactivity both in the metalloproteins and in model complexes aimed at understanding the nature of the copper-oxygen bond and the characterization of possible intermediates to provide mechanistic insights into reaction coordinates. These are described in Section 3.2. For some model systems, two types of isomers can be found in equilibrium,\textsuperscript{127} which are the $\mu$-$\eta^2$: $\eta^2$ side-on peroxy\textsuperscript{128} and the bis-$\mu$-oxo species\textsuperscript{129} (Figure 32). The interconversion and reactivities of these species will be discussed later. Here, we focus on their electronic structures.

The $\mu$-$\eta^2$: $\eta^2$ side-on peroxy\textsuperscript{128} species has been spectroscopically characterized by K-edge XAS, which shows a pre-edge peak at 8979 eV characteristic of Cu(II) (Section 2.2.5). Resonance Raman spectroscopy indicates that this species contains a peroxy moiety with a relatively weak O-O bond ($\nu_{O-O}$ ranging between 720 and 760 cm$^{-1}$). SQUID magnetic susceptibility measurements show it has a singlet ground state. The most direct experimental
probe into the \([\text{Cu}_2(\text{O}_2)]^{2+}\) bonding interaction is L-edge XAS (Section 2.2.6) that shows 52±4% Cu d character in the LUMO.\(^{130}\) To reproduce this experimental covalency, a hybrid functional containing 10–20% HF exchange is needed. B3LYP (20% HF exchange) gives a ground state wavefunction with 56.6% Cu d character. With BP86 the Cu covalency is underestimated to be 45.3%. Importantly, this difference in HF exchange produces fundamentally different electronic structure descriptions. The pure functional gives delocalized \(\alpha\) and \(\beta\) spin orbitals, thus no spin polarization (Figure 33 Left), whereas, B3LYP gives opposite spins localized on each Cu\(^{II}\) leading to an antiferromagnetically coupled ground state (Figure 33 Right). Thus, hybrid functionals with 10–20% HF exchange better capture the experimental covalency and lead to a different description of the ground state wavefunction compared to the pure functional BP86.

K-edge XAS performed on the bis-\(\mu\)-oxo\(^{11}\) isomer shows a pre-edge at \(\sim 8981\) eV that is characteristic of Cu(III). Magnetic susceptibility measurements have characterized this isomer as a singlet (S=0). L-edge XAS has again been used to quantitate Cu-O bonding giving 40±4% Cu d character per hole in the ground state wavefunction. This experimental covalency can only be reproduced with a hybrid functional containing 20–40% HF exchange (BP86 and B3LYP give 32.9% and 35.4% Cu covalency, respectively).

Thus, the experimental electronic structure of both the \(\mu-\eta^2: \eta^2\) side-on peroxo and the bis-\(\mu\)-oxo species are best reproduced using significant HF exchange. One might thus expect this spectroscopically calibrated hybrid functional to give the best thermodynamic description for the \(\mu-\eta^2: \eta^2\) side-on peroxo to bis-\(\mu\)-oxo equilibrium. However, the literature has indicated that pure functionals (e.g. BP86) seem to correctly estimate the experimentally observed Gibbs free energy of \(\sim 0–1\) kcal/mol that is associated with the isomer equilibrium for several ligand systems where both species are observed. B3LYP seems to overstabilize the \(\mu-\eta^2: \eta^2\) peroxo isomer by \(\sim 18\) kcal/mol.\(^{131}\) These calculations, however, do not include relativistic and dispersion corrections, both of which have been shown to significantly affect the total free energy.\(^{132}\) The inclusion of these effects are reported to modify the absolute energies of the \(\text{Cu}_2\text{O}_2\) systems by significant amounts that range from 10 kcal/mol for the bis-\(\mu\)-oxo to 30 kcal/mol for the for \(\mu-\eta^2: \eta^2\) peroxo isomer, the relativity correction being the dominant contribution to the total energy. Thus, significant changes are expected when calculating the thermodynamics of this equilibrium, which seems to indicate that hybrid functionals with HF exchange are in fact needed to best reproduce the experimental electronic structure and to provide good agreement to thermodynamic quantities when dispersion and relativistic corrections are included.

3. Copper Active Sites that Activate Dioxygen

3.1 Reversible \(\text{O}_2\) Binding: Overcoming the Spin Forbiddenness

3.1.1. Enzymology—Hemocyanin (Hc) is an extra-cellular oxygen transport protein that makes up the major component of the circulatory fluid (hemolymph) in arthropods and molluscs. Deoxy-Hc contains a pair of Cu(I) atoms which bind dioxygen yielding two Cu(II) centers that lack an EPR signal.\(^{133}\) The antiferromagnetic coupling that is responsible for this observation is a defining feature that classifies Hc as a coupled binuclear copper protein and distinguishes it from other binuclear copper proteins that lack magnetic interactions.
between the copper centers (Section 3.3). Oxy-Hc also has a unique absorption spectrum (Section 3.1.4) that imparts a blue color to the hemolymph, in contrast to the red blood of organisms that utilize hemoglobin for oxygen transport. However, Hc does not contain the heme prosthetic group of hemoglobin. While the spectral features and protein derived ligands of the binuclear copper site are highly conserved, the protein structure of Hc in molluscs and arthropods is unique.

Hc's from both organisms have quaternary structure that contains a significant number of binding sites as required for cooperative O\textsubscript{2} binding. Mollusc Hc subunits are ~400 kDa and contain eight functional units that each contain a single binuclear copper site (labeled a-h beginning from the amino terminus). The quaternary structure is a decamer of eight subunits or a dimer of decamers arranged as hollow cylinders that are roughly 18 nm high by 35 nm in diameter (Figure 34A).\textsuperscript{134–136} Within the cylinder, the individual peptides are arranged in an antiparallel fashion forming a major and minor groove. The structure of the wall is highly conserved and contains three tiers made up of functional units a-f (the ordering of the functional units in the decamer of Keyhole Limpet Hc\textsuperscript{134} is shown in Figure 34A). At one end of the cylinder, functional unit g and h make up the collar, which contains more structural variability across various organisms. Functional unit g (shown in light blue, Figure 34A right) forms dimers to make up the arc portion of the collar located on the inside of the cylinder while functional unit h (shown in gold, Figure 34A right) makes up the slab. In some cephalopods (octopus, squid, and cuttlefish), functional unit g is absent and the arc is symmetrically arranged. In other organisms, one of the g functional units in each dimer is shifted towards the slab reducing the overall symmetry of the decamer.\textsuperscript{135,136} These decamers can also dimerize at their open face, which occurs in organisms such as keyhole limpet.\textsuperscript{134} These structures have also been found to contain a diverse array of carbohydrate structures,\textsuperscript{137–141} however the physiological significance is not well understood. A variety of mollusc Hcs have been clinically used to fight carcinomas due to the antigenic potency from these unique glycosylation patterns.\textsuperscript{142}

Arthropod Hc subunits are ~75 kDa and contain one oxygen binding site. The quaternary structure is characterized by one, two, four, six, or eight hexamers depending on the species. Each individual hexamer is comprised of a heterogeneous mixture of bean shaped subunits arranged as two trimers in D\textsubscript{3} symmetry (a representative structure of P. interruptus\textsuperscript{143} is shown in Figure 34B). The two trimers are connected by two types of dimer interface; a tight dimer interface (between the blue and yellow subunits in Figure 34B) and a loose dimer interface (between the blue and green subunits). This hexameric structure also forms the building blocks for the oligohexamers in a variety of different conformations. While arthropod Hc is also a glycoprotein, the carbohydrate content is a smaller fraction of the protein weight and less chemical diversity has been observed across different arthropod species.\textsuperscript{144–146}

While the presence of Hc in chelicerata and crustacean (two subphyla of arthropods) are well known, it was believed that the tracheal system was sufficient for oxygen transport in centipedes, millipedes, and insects which belong to the subphyla myriapoda and hexapod. These organisms, though, contain hexamerins, which are evolutionary related to the arthropod Hc.\textsuperscript{147,148} The insect hexamerins lost their copper binding domains and hence...
oxygen binding capacity and instead function as amino acid storage proteins. Recently, Hc proteins that bind oxygen have been identified in myriapods and hexapods. While little is known about the O₂ binding properties of these Hc’s, Hc from myriapods have high oxygen affinity coupled with low cooperativity which is consistent with a primary function of oxygen storage rather than transport. However, higher cooperativity has been observed in a hexapod Hc suggesting a possible role in O₂ transport. Little is known about the evolutionary causes for the loss or retention of Hc oxygen transport in myriapoda and hexapod.

The evolutionary divergence of arthropod and mollusc Hc from a common copper dependent oxygen binding protein is believed to have happened approximately 550–600 million years ago (MYA) and 700–800 MYA, respectively. This divergence occurred early within the evolution of the phyla and is believed to have proceeded gene duplication that is responsible for the unique quaternary structure. While it is likely that Hc evolved from a primordial oxygen binding protein that preceded aerobic respiration, the identity of the common ancestral copper protein is currently disputed. Due to sequence and structural similarity at only one copper-binding site, some have proposed that the ancestral protein contained a mononuclear copper site. However, sequence similarity with tyrosinase (Ty) and catechol oxidase (CO) have lead others to speculate that the common ancestor was a binuclear copper protein and that differences in the second copper-binding site are a result of divergent evolution.

3.1.2. Thermodynamics and Kinetics—Hc is one of three oxygen transport proteins found in biology (a binuclear iron protein hemerythrin and the heme containing protein hemoglobin are the other two). While cooperativity is limited in hemerythrin, this feature is essential for the function of hemoglobin and Hc. In the absence of cooperativity, oxygen binds to isolated sites producing a hyperbolic saturation curve (shown in blue in Figure 35A). In the presence of molecular interactions between active sites, the binding of oxygen to one site can increase the affinity of the other active sites producing a sigmoidal binding curve (shown in red in Figure 35A) described by the following equation

\[ \theta = \frac{PO_2^n}{PO_2^n + P_{1/2}^n} \]  

where \( \theta \) is the fraction of bound sites, \( PO_2 \) is the pressure of O₂, \( P_{1/2} \) is the pressure where the concentration of oxy and deoxy sites are equal, and \( n \) (the Hill coefficient) is related to the strength of the interaction between the sites. For a hexamer, \( n \) can range from perfectly cooperative (\( n = 6 \)) to completely isolated (\( n = 1 \)). In Hc, the values of \( P_{1/2} \) have been shown to vary greatly with environmental factors such as ionic composition, pH, and the presence of organic molecules (vide infra). As a result, oxygen affinities (\( P_{1/2} \)) between species have been compared for various organisms measured in whole blood or in physiological saline and pH (Table 7). The average oxygen affinity of Hc in these organisms is 14 and 18 torr for arthropods and molluscs, respectively, which is only slightly higher than the oxygen affinity of adult hemoglobin (27 torr). While these organisms all have similar affinities despite living in different climates, organisms that live in extreme ocean environments such as hydrothermal vents (\( B. thermydron \)) or the oxygen minimum layer (\( G. ingens \)) have
very high O\textsubscript{2} affinity, similar to other organisms that live in these environments for which the physiological hemolymph pH has not been determined.\textsuperscript{162}

However, the high affinity of Hc is not the only feature of these proteins that allow them to reversibly bind O\textsubscript{2}. \textit{In vivo}, the presence of sigmoidal oxygen binding behavior is essential to efficiently transfer oxygen from the gills to the extremities. In the case of the crab \textit{Cancer magister}, hemocyanin in the hemolymph is characterized by a \( P_{1/2} \) of 20 torr and a Hill coefficient of 3 (Figure 35A).\textsuperscript{167} \textit{In vivo}, the O\textsubscript{2} pressure in the gills and extremities is 105 and 24 torr, respectively. For the physiological sigmoidal curve, the oxygen saturation changes from 99\% to 63\%, delivering 36\% of the O\textsubscript{2} capacity of the protein. However, a hypothetical non-cooperative O\textsubscript{2} carrier with the same affinity would only deliver 29\% of the O\textsubscript{2}. The difference between the cooperative and non-cooperative binding is greatly increased during exercise where the \textit{in vivo} pressures drop to 89 and 10 torr in the gills and extremities, respectively. During this time of metabolic demand, a cooperative oxygen transport protein is much more efficient (88\% delivery) than the non-cooperative one (48\% delivery), which binds less oxygen at the gills and releases less at the extremities.

This cooperative behavior has been shown to dependent on the multimeric quaternary structure of Hc,\textsuperscript{197–199} including molluscs that contain seven or eight functional units per protein.\textsuperscript{188,200,201} From equation 29, the magnitude of this cooperativity is quantified by the Hill coefficient. The largest measured Hill coefficients are 9.3\textsuperscript{202} and 8.6\textsuperscript{203} for arthropods and molluscs, respectively. While these large Hill coefficients assist with O\textsubscript{2} binding, they are much smaller than the number of active sites in each enzyme (24 and 160). These results indicate that the primary function of these large molecular assemblies is not to extend the cooperative unit throughout the quaternary structure. Rather the large molecular weight is believed to increase the number of O\textsubscript{2} binding sites in an extracellular protein without drastically increasing the osmotic pressure.\textsuperscript{204} Experimentally, the Hill coefficient is frequently determined from the linearization of the oxygen binding plots (Figure 35B), called a Hill plot. Here, the \( \log \left( \frac{\theta}{1-\theta} \right) \) is plotted as a function of the \( \log(PO_2) \) and the slope determines the Hill coefficient (\( n \)) while the intercept is the \( \log(P_{1/2}) \). In the case of non-cooperative oxygen binding, the slope is unity since \( n = 1 \) (shown in blue squares in Figure 35B). For cooperative binding, three distinct regions of the Hill plot can be identified (shown in red dots in Figure 35B). At high and low oxygen pressures, the slope is one while the slope between these extremes is greater than one.

Physical insight to these regions can be provided by the Monod, Wyman, Changeux (MWC) two-state model.\textsuperscript{205} In this model, oxygen binding is characterized by two quaternary confirmations of the enzyme, a low affinity tense state (the T-state) and a high affinity relaxed state (the R-state) related by the equilibrium constant L where \( L=[T]/[R] \). At low and high O\textsubscript{2} pressures, oxygen binds to only the T-state and R-state, respectively, resulting in a slope of one. At intermediate oxygenation, an increased number of deoxy sites are in the high affinity R-state, resulting in slopes greater than unity. From these plots, three important parameters can be determined. The affinity for the T and R states can be determined from non-linear fitting of the MWC two-state model or by extrapolation of the linear region of the Hill plot (Figure 35B), where the intercepts correspond to the \( P_{1/2} \) of the T and R states.
Additionally, the site-site interaction, \( \delta(\Delta G) \), can be determined from the difference in the Gibbs free energy of the T and R states and is related to their respective equilibrium constants where

\[
\delta(\Delta G) = \Delta G_R^{\text{O}_2} - \Delta G_T^{\text{O}_2} = RT \ln\left(\frac{P_{1/2}^{R}}{P_{1/2}^{T}}\right) \quad [30]
\]

This site-site interaction represents the thermodynamic change that resulted from the structural re-arrangement from the tense to the relaxed state. In hemocyanin, the average \( \delta(\Delta G) \) is significantly larger in arthropods (−2.5 kcal/mol) than mollusc (−1.4 kcal/mol) suggesting that the hexameric quaternary structure found in arthropods allows for a larger driving force (Table 8) for \( \text{O}_2 \) binding.

Yet only cooperative oxygen binding is generally not sufficient to efficiently function as an oxygen transport protein especially in organisms that live in dynamic aquatic environments. In many organisms, the modulation of oxygen affinity in response to environmental factors such as temperature changes and temporary hypoxia is essential to survival. The effect of temperature changes on Hc \( \text{O}_2 \) affinity can be curtailed by minimizing the magnitude of \( \Delta H \) since

\[
\ln(K) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad [31]
\]

However the value of \( \Delta H \) for Hc has been frequently calculated incorrectly in the literature. Numerous reports have utilized an integrated form of the van’t Hoff equation in the following form

\[
\Delta H = R \frac{\Delta \ln(P_{1/2})}{(T_1 - T_2)} \quad [32]
\]

The use of \( P_{1/2} \) rather than the equilibrium constant does not account for the temperature dependence of the solubility of \( \text{O}_2 \), resulting in \( \Delta H \) values that are significantly smaller due to the decreased solubility of \( \text{O}_2 \) at higher temperatures.\(^{220}\) As a result, thermodynamic parameters have been re-determined from the temperature dependence of \( P_{1/2} \) values by accounting for the solubility of oxygen at the experimental salinity\(^{221}\) from the temperature dependence of Henry’s law parameterized for \( \text{O}_2 \) solubility in sea water.\(^{222}\) Determining values for the thermodynamic parameters in this manner (Table 9) indicates that on average, \( \text{O}_2 \) binding by Hc is slightly exothermic (−4.5 and −8.2 kcal mol\(^{-1}\) for arthropods and mollusc, respectively) and \( \Delta S \) is slightly positive (6 and 5 cal mol\(^{-1}\) K\(^{-1}\)), however, large variability in \( \Delta H \) and \( \Delta S \) is observed. Some of the most exothermic binding energies exist in organisms (\( \text{G. antarcticus}^{177} \) and \( \text{A. monachus}^{223} \)) that live in cold environments that have little to no change in temperature. Similarly, organisms that live near hydrothermal vents have small or slightly positive values for \( \Delta H \), which is believed to be an advantageous adaptation as a direct result of an environment where temperatures change rapidly.\(^{160,161,224–246}\) However, physiological responses to temperature changes complicate the direct interpretation of thermodynamic data. For example, the oxygen affinity of blue crab (\( \text{C. sapidus} \)) Hc at low temperatures is very high (less than 4 torr), however the animals respond to this environmental condition by entering into a semi-hibernative state.\(^{165}\)

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Further insight into thermodynamic parameters can be determined from the T and R states of Hc. Since these parameters have only been determined for a smaller number of organisms (Table 10), general trends are difficult to define. For *P. interruptus*\(^\text{199}\) and *H. pomatia*\(^\text{227}\), both ΔH and ΔS are more positive in the high affinity R-state than the low affinity T-state. However, the opposite trend is observed for *L. hierosolima*.\(^\text{228}\) One important difference between *L. hierosolima* and *P. interruptus* is the affinity of the monomer. In *L. hierosolima*, monomeric Hc has identical thermodynamic parameters to the R-state,\(^\text{228}\) while the monomer in *P. interruptus* is that of the T-state\(^\text{199}\) (the affinity of the monomer in *H. pomatia* is unknown). This difference would necessitate differential subunit interactions to induce cooperativity and could explain the different trends in the thermodynamic parameters.

The effect of the protein on the thermodynamics can be determined from a comparison to the oxygenation of Cu(I) salts of binucleating and mononucleating synthetic ligands which form binuclear, side-on bridged copper peroxide complexes. For these model compounds, ΔH ranges from −28 to −7.7 kcal mol\(^{-1}\) and ΔS ranges from −65 to −21 cal mol\(^{-1}\) K\(^{-1}\) (Table 11). These ranges are a result of the coordinating ligand, the flexibility of the linker in the binucleating ligands, coordinating molecules in the Cu(I) complex, and the solvent. And while the rates of oxygenation for the binucleating ligands are faster due to their intermolecular nature, both the mononucleating and binucleating ligands have similar thermodynamic parameters. This is likely due to large geometric changes in the binucleating ligands since the Cu•••Cu separation in the Cu(I) salts is much longer (7.0 to 8.9 Å for the structurally characterized models\(^\text{250–252}\)) than after O\(_2\) binding (Cu•••Cu of ~3.6 Å). A comparison to monomeric Hc\(^\text{199,228}\) (ΔH ranges from −10.1 to −7.5 kcal mol\(^{-1}\) and ΔS ranges from −12.6 to −1.7 cal mol\(^{-1}\) K\(^{-1}\)) indicates that the protein environment causes the reaction to be more thermoneutral and decreases the entropic cost of binding dioxygen. A further comparison to thermodynamic parameters determined from cooperative O\(_2\) binding in Hc indicates that on average, ΔH is more thermoneutral and ΔS is slightly positive. Given an identical binuclear copper core, these results suggest that the protein environment functions to preorganize the active site for O\(_2\) binding and indicate that changes in the protein tertiary and quaternary structure overcome the intrinsic entropic cost of O\(_2\) binding. However, it is important to recognize that data determined from Hc also includes other factors such as the interactions of protein with ions or protons.

Of these various factors, Hc is generally most sensitive to changes in pH. For oxygen transport proteins (Hc and hemoglobin) a decrease in pH generally causes a decrease in O\(_2\) affinity, referred to as the normal Bohr effect.\(^\text{258}\) During metabolic demand, the concentration of CO\(_2\) (which acidifies the blood via conversion to carbonate) and acidic metabolic products such as L-lactate decrease the hemolymph pH.\(^\text{171,247,259–263}\) The normal Bohr effect causes a decrease in Hc O\(_2\) affinity delivering more oxygen under these conditions. The magnitude of this effect is quantified by the Bohr coefficient (Δlog(P\(_{1/2}\))/ΔpH). Bohr coefficients for arthropods and molluscs can be as large as −2.7\(^\text{225}\) and −2.2\(^\text{223}\), respectively. However, some Hcs exhibit a reverse Bohr effect where decreases in pH increase O\(_2\) affinity. While some of the measured reverse Bohr effects only occur at extreme pH values,\(^\text{228,237,248}\) snails,\(^\text{191–194,94,231,247,248,264–267}\) horseshoe crabs,\(^\text{268,269}\) and a species...
of chiton\textsuperscript{184} and a crayfish\textsuperscript{240} have reverse Bohr effects within a physiologically relevant pH window. For some snails\textsuperscript{191,192,270,271}, the pH of the blood is around 8.0 where the Bohr effect is very small. For horseshoe crabs\textsuperscript{272} and some snails\textsuperscript{193,194,247}, the physiological pH falls within the range of the reverse Bohr effect. However, more pronounced blood acidification results from large environmental changes such as decreased temperature or environmental hypoxia.\textsuperscript{191,192,248,265,266} This increased affinity allows for \(O_2\) transport under environmentally imposed \(O_2\) limiting conditions and is believed to be an adaptive feature of \(Hc\) in these organisms since both snails and horseshoe crabs enter into periods of extended hypoxia during their life cycle.

Within the framework of the MWC two-state model\textsuperscript{205}, external effectors, such as pH, that modulate \(Hc\) affinity are referred to as heterotrophic allosteric effectors in contrast to \(O_2\), which is a homotropic allosteric effector. Within the framework of this model, heterotropic allosteric effectors affect \(O_2\) affinity by preferentially stabilizing either the T or R state changing the value of the equilibrium constant \(L\). However, some experimental data in the presence of allosteric effectors, such as protons, cannot be fit to this model. As a result, different modifications of the MWC model have been employed. One modification is to allow the affinities of the T and R state to change as a function of pH.\textsuperscript{188,203,206,208,264} For \(Hc\)’s with a normal Bohr effect, the affinity of both the T and R state decreases with increasing pH. Another strategy that has been employed is the addition of other states to the MWC two-state model. In the three-state MWC model, a third allosteric state is invoked that has an \(O_2\) affinity intermediate between the T and R state.\textsuperscript{273} Another model employs nested interactions.\textsuperscript{274} In this model, a smaller allosteric unit (likely a hexamer in arthropod \(Hc\)) is affected by a larger allosteric unit (an example being a dimer of hexamers). Each of these structural units has a T and R state resulting in four states, tR, tT, rR, and rT, where the lowercase letter denotes the affinity of the smaller allosteric unit and the capital letter is that of the larger one. The fitting of these models to \(Hc\) \(O_2\) affinity for species with a normal Bohr effect indicates that protons stabilize the lower affinity states.\textsuperscript{212,214} In the case of organisms with a reverse Bohr effect such as snails, the application of a pH dependent MWC two-state model indicates that protons increase the affinity of either the T-state in \(H.\ pomatia\textsuperscript{264}\) or the R-state in \(L.\ stagnalis\textsuperscript{203}\). Data for the horseshoe crab \(L.\ polyphemus\) indicate that in the region of the reverse Bohr effect, protons stabilize the high affinity state decreasing the values of \(L\) in a nested model.\textsuperscript{275}

While both temperature and pH independently affect \(Hc\) \(O_2\) affinity, a decrease in temperature also results in an increase in hemolymph pH.\textsuperscript{272,276,277} For most \(Hc\)’s, the negative \(\Delta H\) and the normal Bohr effect both increase the \(O_2\) affinity of the protein. To minimize the change in \(Hc\) \(O_2\) affinity, some have hypothesized that the temperature sensitivity of \(Hc\) is inversely related to the Bohr effect.\textsuperscript{231} A large compilation of thermodynamic parameters and Bohr effects (Table 9) indicates that these two parameters are only weakly coupled (the Pearson product-moment correlation coefficient for the compiled arthropods and mollusc in Table 9 is \(-0.38\) and \(-0.19\), respectively, where 1 is a positive correlation, -1 is a negative correlation, and 0 is no correlation.).

For most hemocyanins, temperature and pH have the largest effect on \(O_2\) affinity. However, numerous other heterotrophic allosteric effectors are known including inorganic ions,
products of anaerobic respiration, and neuro-transmitters. In some mollusc Hc, cooperativity has been shown to depend on the presence of divalent cations such as calcium and magnesium. However, this behavior results from a lack of quaternary structure in the absence of these ions. In species where the Hc quaternary structure does not depend on divalent ions, cooperative oxygen binding is observed in their absence. The majority of Hc have an increased affinity and cooperativity in the presence of increasing concentrations of calcium and magnesium resulting from an increased affinity of the R-state. A second calcium binding site has also been observed that causes a decrease in O\textsubscript{2} affinity. However, the high concentrations of calcium required to bind in this second site are not observed in hemolymph under normal environmental conditions. Interestingly, at low concentrations calcium has been shown to decrease the affinity of horseshoe crab and snail Hc while increasing cooperativity resulting from changes to the affinity of both the T and R state, however exceptions are known.

Similar effects have also been observed with chloride. Most species, including horseshoe crabs, show an increased affinity and cooperativity in the presence of increasing chloride ions, however snail Hc experiences a decreased affinity and an increased cooperativity. While these properties of snail and horseshoe crab Hc are generally conserved within their species, the cause or adaptive function of these properties is not well understood. Unlike pH, there is currently no evidence that in vivo ionic environments are utilized to effect Hc O\textsubscript{2} affinity. Instead, changes in salinity in organisms that move between different environments must be combated. In general, organisms seem to utilize three strategies to modulate changes in oxygen affinity. Some organisms regulate the ionic concentration of their hemolymph. Other organisms possess Hc that is not greatly modulated by changes in ionic concentrations and some arthropods adapt to changing salinities by regulating Hc protein expression, which changes the ratio of the different subunits making up the hexameric quaternary structure.

In contrast, organic heterotrophic allosteric effectors are utilized to modulate Hc in response to changing environments. These organic effectors can be grouped into two broad categories; products of anaerobic metabolism (urate and L-lactate) and neurotransmitters (dopamine). Both the concentration of urate and L-lactate increase in the hemolymph when organisms are maintained in hypoxic environments, while significant increases in L-lactate and not urate occur during exercise. Generally, both urate and L-lactate increase the O\textsubscript{2} affinity of Hc, however exceptions are known. It is believed that the primary adaptive function of these effectors is to increase O\textsubscript{2} affinity during hypoxia. During exercise, it is believed that L-lactate functions to moderate the Bohr effect. Interestingly, land based arthropods are less sensitive to L-lactate which likely results from these organisms infrequently encountering hypoxic environments. The concentration of neurotransmitters in the hemolymph has also been shown to increase during exercise. However, its effect on Hc is less well understood since it has only been studied in a small number of species with mixed results. While the O\textsubscript{2} affinity of Hc from the crab C. magister increases in the presence of dopamine, other Hc are insensitive to changes in
However, the physiological concentration of dopamine in the hemolymph is very low suggesting that this effect is moderate in vivo.

Further molecular insight can be gained from exploring the kinetics of O\textsubscript{2} binding. In general oxygen-binding rates (k\textsubscript{on}) range from 1.3 to 44 \(\mu M^{-1} s^{-1}\) and dissociation rates (k\textsubscript{off}) range from 9 to 2750 \(s^{-1}\) (Table 11) These results indicate that changes in affinity result from changing only the dissociative rate. This change accounts for the difference in affinity between the R and T\textsuperscript{199,303} as well as in the cooperative state\textsuperscript{203,268} as pH\textsuperscript{203,215} or temperature\textsuperscript{199} is perturbed. However, Hirota and coworkers have proposed that changes in k\textsubscript{on} and not k\textsubscript{off} are responsible for the observed changes in O\textsubscript{2} affinity.\textsuperscript{304} However, in these experiments k\textsubscript{off} values were measured by laser flash photolysis while values of k\textsubscript{off} in Table 11 were determined from temperature jump or stopped flow measurements. The differences in technique are likely the cause of these different results with the temperature jump and stopped flow measurements being more reflective of the O\textsubscript{2} dissociation caused by O\textsubscript{2} depletion in vivo.

An extensive collection of various molecular properties (P\textsubscript{1/2}, thermodynamic parameters, responses to heterotropic effectors, etc.) on a large variety of species allows us to draw some general conclusions about the thermodynamic and kinetic properties of Hc. Most essential to its function, is the low O\textsubscript{2} affinity coupled to high cooperativity. This allows Hc to transport oxygen in organisms that live in diverse ecosystems, some that have very low O\textsubscript{2} concentrations. While the protein functions to minimize the entropic cost of O\textsubscript{2} binding, organisms in environments that are susceptible to rapid changes in temperature have adapted to minimize the magnitude of \(\Delta H\). In general, a decrease in the hemolymph pH is accompanied by a decrease in O\textsubscript{2} affinity (normal Bohr effect), which assists in delivering O\textsubscript{2} to the extremities during exercise-induced hypoxia. Yet some species of molluscs that experience extended periods of environmentally induced hypoxia have a reverse Bohr effects in response to lower O\textsubscript{2} concentrations. Similarly, divalent cations, chloride, and a number of small molecules that are the byproducts of metabolism have also been shown to be heterotropic allosteric effectors. Yet the concentration of ions is generally kept constant (either in the environment or via regulatory mechanisms) and the concentrations of metabolic products are generally low except in cases of extended environmental hypoxia. From kinetic measurements, changes in the equilibrium constant are attributed to changes in the off rate while the on rate generally remains constant. Yet, less is understood about how various conditions such as changes in temperature or pH affect the tense and relaxed states of Hcs. This is especially true when comparing organisms that have very different responses to the same effector, such as the reverse Bohr effect. This lack of knowledge hinders the development of more molecular models for these properties and a more thorough understanding of the difference properties of Hc among different species.

3.1.3. Structure—The tertiary structures of Hc from both arthropods and molluscs hemocyanins consist of three distinct domains. In arthropod Hc (a representative structure of \textit{L. polyphemus}\textsuperscript{306} is shown in Figure 36A) domains one and two (in yellow and red, respectively) are mainly \(\alpha\)-helical and the third domain is a \(\beta\)-barrel (in blue). While domain one shields the binuclear copper site contained in domain two, the third domain is believed to be the evolutionary remnant of a copper chaperone.\textsuperscript{307} This domain is structurally similar...
to cupredoxins and a bacterial CopC protein (an evolutionary link between copper-trafficking proteins and cupredoxins). However, the lack of a copper-binding site in domain three suggests that this function has been lost over time. Domain two contains the active site, which is located in a four-helix bundle. The tertiary structure of the mollusc Hc functional units a-g contain only two domains (the oxy structure of subunit g from *E. dofleini* is shown in Figure 36B); an α-helical domain one that contains the active site (in red) and a six-stranded β-sandwich (domain two in blue) that shields the active site. While the structure of these two domains area similar in all mollusc functional units, functional unit h contains an extra C-terminal domain that is structurally similar to domain three in arthropods and the copper chaperone CopC.

The central domain contains the copper active site, which is coordinated by three His residues per copper. Two copper centers are distinguished by their relative location where Cu_A is ligated by residues closer to the N-terminus than Cu_B. While the structure of the Cu_B site in both arthropod and mollusc Hc is conserved, the structure of Cu_A is rather unique (Figure 37). In arthropods, the four α-helices that contain the six His ligands are arranged in pseudo-two fold symmetry. However, in molluscs this symmetry is absent and one Cu_A ligand (His2562 in *E. dofleini*, Figure 37C) is located on a loop instead of an α-helix. However, this His is covalently tethered to a highly conserved Cys residue (2560 in *E. dofleini*, Figure 37C) at the ε1 carbon. From a structural standpoint, it is believed that this covalent linkage is critical to stabilizing His2562 in the active site yet any definitive role in O_2 binding is unknown. The binuclear active site in both arthropods and molluscs is surrounded by two highly conserved Phe residues (Phe200 and Phe360 in arthropods and Phe2569 and Phe2698 in molluscs Figure 37), which form a conserved Phe-X-X-His sequence pattern that makes up the coordination environment of both Cu_A and Cu_B in all known Hc. A steric interaction between these Phe residues and the copper coordinating His residues and hydrogen bonds from peptide carbonyl oxygens or waters to the histidine Nδ orients these residues for copper binding. Another highly conserved hydrophobic residue is also found in the vicinity of the active site that links two protein domains. In arthropods, domain one is linked to domain two by a single residue, Phe49 (shown in yellow in figure 37B). In molluscs, Leu2830 (shown in blue in figure 37C) connects domain two to the active site in domain one. Both residues are believed to be essential for allosteric regulation (*vide infra*).

A variety of atomic resolution structures from both arthropods and molluscs have been determined (Table 12). In deoxy-Hc, the Cu(I) is coordinated in a distorted trigonal environment (Figure 37A and Table 13). Dioxygen binds to the Cu(I) site and is reduced by two electrons to peroxide which binds in an μ-η^2:η^2 coordination mode (Figure 37B–C); a structural motif that was originally characterized in a tris(pyrazolyl)borate model complex. In *L. polyphemus*, peroxide binding results in a square planer coordination environment with two trans axial histidines (His204 and His328, Figure 37B) with bond lengths of 2.3 Å. In oxy-Hc from *E. dofleini* (Figure 37C), the His nitrogens adopt a staggered, trigonal, coordination environment and have similar Cu-N bond distances. In oxy-Hc, the Cu•••Cu distance is 3.5–3.6 Å, similar to the average Cu•••Cu distance in model complexes (3.51 Å). While the crystal structures do not have sufficient

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resolution to accurately determine the O-O bond length, binuclear Cu peroxy model complexes indicate that average O-O and Cu-O bond are 1.42 and 1.92 Å, respectively.\textsuperscript{312}

A comparison between the oxy and deoxy structure of \textit{L. polyphemus} reveals that the binding of O\textsubscript{2} shortens the distance between the two copper atoms from 4.6 to 3.6 Å.\textsuperscript{306} Despite this large structural change at the active site, the rest of the protein structure is mainly unperturbed. However, a larger geometric change between the two arthropod deoxy-Hc structures provides a clue to the molecular mechanism of cooperative oxygen binding.\textsuperscript{143,309} The Cu(I) coordination distorts from a trigonal planer environment in \textit{L. polyphemus} to a more pyramidal environment in \textit{P. interruptus} changing the Cu•••Cu distance from 4.6 to 2.9 Å (Table 13). This change in Cu•••Cu distance is accompanied by a rotation of domain one from a closed conformation in \textit{L. polyphemus} to an open one in \textit{P. interruptus}. This rotation of approximately 8° places a steric constraint on the active site via Phe49 in the closed position (found in \textit{P. interruptus}), which increases the Cu•••Cu distance. The movement of Phe49 (Figure 37A) has also been proposed to gate an O\textsubscript{2} diffusion tunnel assisting in cooperativity.\textsuperscript{306,310} Dioxygen affinity in the crystallization buffer indicates that the deoxy-Hc structure of \textit{L. polyphemus} is in the T-state while deoxy-Hc from \textit{P. interruptus} has no O\textsubscript{2} affinity.\textsuperscript{309} Despite the lack of a crystal structure of deoxy-Hc in the R-state, it is hypothesized that the domain rotation, which results in an increased Cu•••Cu separation, is responsible for the major mechanism of allosterism. A binding site for the allosteric regulator, chloride, is located between domain one and two,\textsuperscript{306} which stabilizes the closed conformation decreasing oxygen affinity for isolated subunits of \textit{L. polyphemus}.\textsuperscript{313} However, the native quaternary structure has an increased affinity for O\textsubscript{2} in the presence of chloride;\textsuperscript{287} an observation that currently has no molecular explanation.

In arthropod Hc, the rotation of domain one is proposed to be translated to the hexameric quaternary structure through the tight dimer interface (i.e. between the blue and yellow subunits in Figure 34B).\textsuperscript{309} The two trimers then rotate with respect to one another along the three-fold symmetry axis. The loose dimer interface (i.e. between the blue and green subunits) assist in the geometric rotation since this contact is located in a plane horizontal to the axis of rotation and is comprised of a small number of residue interactions. In molluscs, the effect of the domain rotation on the molecular structure is less well understood. Molecular modeling combined with electron microscopy suggest the major allosteric unit is located in the wall between the major and minor groove due to an increased number of inter-subunit contacts.\textsuperscript{134,135} This model suggests that the interfaces contain numerous histidine residues and salt bridges, which has been offered as an explanation for the observed effect protons have on cooperative oxygen binding.

These structures are consistent with a model where cooperative O\textsubscript{2} binding is modulated by the Cu•••Cu separation. In the low affinity state, the separation is larger (4.6 Å in the arthropod \textit{L. polyphemus}) than the high affinity state. The high affinity state is formed by a domain rotation that shortens the Cu•••Cu by placing a steric constraint on the active site via a conserved Phe in arthropods. This rotation can affect other active sites in the hexameric quaternary structure leading to cooperative oxygen binding. A similar model has been proposed for mollusc Hc due to the multidomain structure and a conserved Leu linking the
second domain to the active site. However, no direct structural evidence of this rotation has been observed in arthropod Hc.

**3.1.4. Electronic Structure**—Deoxy-Hc, owing to its closed shell, diamagnetic binuclear Cu(I) state, is spectroscopically silent to most methods. Nonetheless, the oxidation state of the Cu was firmly established as +1 based on its diagnostic 1s → 4p pre-edge transition at 8984 eV (Figure 38).\[^{304,314}\] From the Cu(I) K-edge XAS analysis presented in Section 2.1.1 the intense pre-edge reflects a site composed of a pair of three coordinate Cu(I) ions, consistent with that observed via crystallography.

Upon coordination of O\(_2\) to the deoxy-site to form oxy-Hc, a change in the Cu K-edge pre-edge spectrum is observed (Figure 38) indicating that both Cu(I) ions are oxidized to the +2 oxidation state.\[^{315,316}\] Oxy-Hc has an intense absorption band at ~350 nm (\(\varepsilon\): ~20 mM\(^{-1}\) cm\(^{-1}\)) with an associated resonance enhanced Raman vibration at 749 cm\(^{-1}\) that shifts to 708 cm\(^{-1}\) in the \(^{18}\)O\(_2\) isotopologue.\[^{317,318}\] The observation of this band indicates that when the two Cu(I) ions are oxidized (to +2) the O\(_2\) is reduced by two electrons to the peroxide level and the 350 nm absorption feature can be assigned as a peroxide → Cu(II) CT transition. For peroxide end-on bound to a single Cu(II) (Figure 39) there is a CT transition at ~10,000 cm\(^{-1}\) lower energy and with approximately one fourth the intensity of oxy-Hc.\[^{319}\] From the resonance enhanced Raman spectrum \(\nu\)_{O—O} is 803 cm\(^{-1}\); 50 cm\(^{-1}\) higher that that of oxy-Hc. Moreover, oxy-Hc has no EPR signal, and from SQUID magnetic susceptibility the two Cu(II)s are strongly antiferromagnetically coupled (-2 \(J\) > 600 cm\(^{-1}\)).\[^{320}\] From Section 2.3.1.1, this reflects the peroxide bridge between the two Cu(II) ions in oxy-Hc providing a superexchange pathway for the strong antiferromagnetic coupling (through the peroxide \(\pi^*\_\sigma\) orbital, vide infra). For an end-on peroxide bridged binuclear Cu(II) complex, the peroxide to Cu(II) CT transition intensity goes up by a factor of two and \(\nu_{O—O}\) increases by 30 cm\(^{-1}\) relative to the end-on peroxide bound mononuclear Cu(II) site (Figure 39B vs A). The CT intensity quantifies the donor strength of the peroxide to the Cu(II) and the two fold increase in intensity reflects the peroxo donor interactions with the two Cu(II) ions in the end-on bridged structure. This removes electron density from the HOMO of the peroxide that is a \(\pi^*\) orbital (antibonding with respect to the O—O bond) and \(\nu_{O—O}\) increases from 803 cm\(^{-1}\) to 832 cm\(^{-1}\). Thus the unique high energy and intensity of the peroxide to Cu(II) CT transition, and low \(\nu_{O—O}\) in Figure 39C, reflect a unique electronic structure associated with the geometric structure of oxy-Hc.

From an early model study of Kitajima\[^{321}\] and from the protein crystallography (Section 3.1.3), oxy-Hc has the peroxide bound side-on and bridged between the two Cu(II) (Figure 39C). This results in the unique electronic structure of oxy-Hc shown in Figure 40.\[^{128}\] The O\(_2^{2−}\) \(\pi^*\) HOMO set is doubly degenerate but split in energy upon binding to the Cu(II). The \(\pi^*\_\sigma\) orbital is oriented along the Cu–O (peroxide) bonds and is strongly \(\sigma\) bonding with the LUMO, the symmetric combination of \(d_{x^2−y^2}\) orbitals on the two Cu(II)s. The second peroxide \(\pi^*\) orbital (the \(\pi^*_v\), \(v = \text{vertical}\)) is perpendicular to the Cu–O bond and only weakly interacting with the Cu(II) ions. The \(\pi^*_\sigma\) donor dominates the bonding and is responsible for the intense 350 nm CT absorption band in oxy-Hc. As shown by the contour of the LUMO in Figure 40, the side-on peroxy bridge has four donor interactions (two with each Cu(II)) which stabilizes the \(\pi^*_\sigma\) donor orbital energy and results in the high energy and,
in particular, the high intensity of the peroxide $\pi^* \rightarrow \text{Cu(II)}$ LUMO CT of oxy-Hc (the 350 nm band). This strong donor interaction removes electron density from the peroxide $\pi^*$ orbital and $\nu_{O-O}$ would be expected to increase to well above the 830 cm$^{-1}$ of the end-on peroxide bridge structure in Figure 39B. However, the $\nu_{O-O}$ decreases to 750 cm$^{-1}$. This reflects the second important contribution to the bonding. The LUMO of peroxide is its $\sigma^*$ orbital. In the side-on bridged structure this undergoes a bonding interaction with the HOMO (antisymmetric contribution of the $d_{x^2-y^2}$ orbitals in the Cu(II)). This shifts a limited amount of electron density from the Cu(II) ions into the $\sigma^*$ orbital on the peroxide (a backbonding interaction) which is strongly O–O antibonding resulting in the low $\nu_{O-O}$. Finally, these $\pi^*$ donor bonding/$\sigma^*$ backbonding contributions produce a large HOMO/LUMO splitting and result in the strong antiferromagnetic coupling of oxy-Hc (see section 2.3.1.1).

A detailed description of the bond strengths and definitive assignments of the vibrational features of the side-on Cu$_2$O$_2$ peroxy core was obtained from a force field based on the resonance Raman data and the IR spectrum of the [{(Tp)Cu$_2$-(µ-η$^2$:η$^2$-O$_2$-2)}] model complex (Tp = trispyrazoyl borate). These data are shown in Figure 41 with the normal mode assignment in Figure 42 as obtained from a Urey-Bradley normal coordinate analysis (NCA) (Table 14). In order of increasing energy, the dominant feature in the resonance Raman spectrum associated with the 350 nm absorption is the 284 cm$^{-1}$ vibration. This has no $^{18}$O$_2$ isotope dependence and is assigned as the totally symmetric ($A_g$ in $D_{2h}$ with Cu–Cu along x and z perpendicular to the Cu$_2$O$_2$ plane) Cu•••Cu stretch ($v_{Cu•••Cu}$) which reflects the dominant excited state distortion in this CT transition. In the same energy region in Figure 41 are weaker Cu–N stretches of the ligand environment that varies among species and likely has new information yet to be uncovered. At 331 cm$^{-1}$ in the IR spectrum of the model is the $B_{2u}$ Cu–O stretch (Figure 42) that shifts to 321 cm$^{-1}$ in the $^{18}$O$_2$ isotopologue. In the proteins (but not in the more symmetric model complex) a weak resonance Raman feature is present at 572 cm$^{-1}$, which shifts to 549 cm$^{-1}$ with $^{18}$O$_2$ and is assigned as the $B_{3u}$ or $B_{1g}$ Cu–O stretch, both possibilities having been considered in the NCA. Neither mode is totally symmetric and therefore not allowed in resonance Raman but would gain some intensity in the distorted protein site. At 763 cm$^{-1}$ is the $v_{O-O}$ that is totally symmetric and allowed by resonance Raman and reflects a shortening of the O–O bond in the CT excited state. Finally the feature at 1144 cm$^{-1}$ shifts to 1098 cm$^{-1}$ with $^{18}$O$_2$ and is assigned as the first overtone of the 572 cm$^{-1}$ vibration. While the fundamental is not totally symmetric and therefore does not contribute to the resonance Raman spectrum in the model complex, the overtone of a non-totally symmetric vibration is symmetric and resonance Raman allowed. The NCA of oxy-Hc gives the $k_{O-O}$ of 2.4 mDyn/Å. For the end-on peroxy model (Figure 39A) and end-on peroxy bridged dimer (Figure 39B), the $k_{O-O}$ are 2.9 mDyn/Å and 3.1 mDyn/Å respectively. The NCA eliminates mechanical coupling and mode mixing and demonstrates that the O–O bond of oxy-Hc is indeed weak due to the $\sigma^*$ backbonding discussed above.

We now consider in more detail the electronic absorption spectrum of oxy-Hc. Figure 43A shows the low temp absorption and RT CD spectra of oxy-Hc. Also included is the low-temp absorption of met-Hc which has two Cu(II) ions that are antiferromagnetically coupled.
but with the peroxide replaced by a water derived ligand (likely a bridging OH− ligand).

This allows the absorption and CD features of oxy-Hc at ~700 nm (~14,000 cm−1) to be assigned as the LF transitions of the two Cu(II) ions. In addition to this and the intense 350 nm peroxo → Cu(II) CT transitions there is a weaker absorption band at ~600 nm (~16,700 cm−1) and a CD feature at ~480 nm (~21,000 cm−1). Neither are present in met-Hc. The excitation dependence of the resonance Raman (Figure 43B) shows that in the low energy (600 nm) absorption band, the νO=O is enhanced while in the ~350 band, the Cu•••Cu stretch at 280 cm−1, which reflects Cu–O distortion (vide supra), is dominantly enhanced. Thus, the low-energy absorption is assigned as the O22−π*ν→Cu(II) CT transition with the low intensity reflecting the limited overlap of the dx2−y2 acceptor orbital with the peroxide π*ν donor. The enhancement of νO=O by excitation into this band reflects the relative donation of the peroxide π*ν being less than that of the π*σ. Thus, CT excitation out of the π*ν leads to a greater distortion of the νO=O bond, while CT from the π*σ leads to dominant distortion in the Cu-O bond. The enhancement of the νO=O band also profiles the CD feature at 480 nm; therefore, it is a second component of the π*ν CT. In a series of binucleating model complexes where the Cu•••Cu distance was constrained by the length of the organic linker, a fourth CT band is present on the low energy side of the 350 nm absorption which increases in intensity and decreases in energy as the Cu•••Cu distance decreases (Figure 44).324 The Cu2O2 butterflies as the linker is shortened and the new absorption feature was assigned as a second component of the O22−π*σ to Cu(II) CT transition from the resonance Raman profile that shows enhancement of the 280 cm−1 Cu•••Cu vibration in this new absorption feature.

These spectral features of oxy-Hc and related model complexes can be explained based on a transition dipole vector coupling model (TDVC),323 which is a relative of exciton theory.316 Each peroxide π* orbital makes CT transitions to each of the two Cu(II) ions in the peroxide bridged site. In zeroth order, these transitions are degenerate. However, in a dimer their transition dipole moments, which are vectors along the change in electron density in the CT process, couple into symmetric and antisymmetric combinations. These coupled excited states thus split into two transitions for each of the π*σ and π*ν CT processes. Considering only the planar Cu2O2 D2h structure (with z perpendicular to the plane and x along the Cu-Cu vector) the O2−π*ν and π*σ transform as b3g and b1g respectively, and the symmetric and antisymmetric combinations of the dx2−y2 Cu orbitals transform as b1g and b2u respectively. Thus, the π*ν transitions split into a B1u (z) component that is electric dipole allowed and corresponds to the 600 nm absorption band and the B2g (Ry) that is electric dipole forbidden but magnetic dipole allowed. From section 2.2.3, the B2g component should contribute to the CD but not the absorption spectrum and thus is assigned to the 480 nm CD feature in Figure 43A. The π*σ CT also splits into two components (A3g and B3u), only one of which (the B3u (x)) is electric dipole allowed and can be assigned to the 350 nm absorption band of oxy-Hc. The butterfly distortion of the Cu2O2 core lowers the symmetry to C2v. In C2v symmetry, the two components of the π*σ become A1 (z) and B1 (x). Thus both are now electric dipole allowed wherein the A1 gains intensity in the out of plane direction due to the butterfly distortion. Thus, π*σ makes a CT transition to each Cu center and these transition dipole moment vectors couple. In the planar D2h structure, in one combination (B3u) the transition dipole vectors add giving intensity polarized along the
Cu•••Cu and in the other combination, these cancel and there is no intensity ($A_2$). In the butterfly distortion, the vectors shift out of the $xy$ plane. Now the antisymmetric ($A_1$) combination of transition dipoles gives a non-zero net dipole that is polarized along $z$ (Figure 44).

The TDVC model correlates the intensity of the $A_1$ and $B_1$ CT transitions in the Cu(II)-peroxo model complexes with the magnitude of the bending of the Cu$_2$O$_2$ core. This gives the intensity ratio as a function of the angle ($\Theta$) between the two transition dipole vectors ($\mu$):

$$\frac{I^{A_1}}{I^{B_1}} = \frac{(\mu_1 + \overline{\mu}_2)^2}{(\mu_1 - \overline{\mu}_2)^2} = \frac{\sin^2(\Theta/2)}{\cos^2(\Theta/2)} \quad [33]$$

From equation 33, it is evident that as the core distorts away from planar (i.e. the “butterfly” bending distortion), the intensity of the $B_1$ component decreases concomitant with an increase in the intensity of the $A_1$ component. This is just what has been observed in the spectra in Figure 44 and may be relevant in the CT absorption and CD spectra of the two phyla of hemocyanins. In Figure 45A comparing the mollusc $Busyccon$ with the arthropod $Cancer$ Hc, the latter shows increased absorption intensity in both $\pi^*$ peaks and a moderately intense low energy shoulder is at 400 nm. The EXAFS of oxy-Hc is rather unique showing an intense outer shell feature at ~3.7 Å corresponding to the Cu•••Cu interaction with a low Debye-Waller factor (arrows in Figure 45B and C). In going from mollusc to arthropod Hc, the Cu•••Cu distance decreases from ~3.7 to ~3.6 Å, an observation consistent with some butterfly distortion of the Cu$_2$O$_2$ core in arthropod hemocyanin. This structural difference in arthropod compared to mollusc may be relevant to reactivity, as catalase activity is only observed in mollusc hemocyanins.

As described in Section 2.3.1.2, CT transitions of bridging ligands in metal dimers provide the superexchange pathway for antiferromagnetic coupling in the ground state. Here we consider the correlation of the unique CT absorption spectrum of oxy-Hc in Figure 43A to the strong ground state antiferromagnetic coupling ($-2J^{GS} > 600 \text{ cm}^{-1}$). In the next section we explore how changes in this superexchange pathway overcome the spin-forbidden process of triplet dioxygen bonding to singlet deoxy-Hc to produce ox-Hc, an antiferromagnetically coupled singlet.

From Figure 46, the electron pair in the $\pi^*_{\sigma}$ orbital of the peroxide provides the superexchange pathway for the strong antiferromagnetic coupling between the two Cu(II) ions. As indicated at the top of Figure 46, a CT transition from the $\pi^*_{\sigma}$ of the bridging peroxide ligand to Cu$_A$ leaves net electron spin in the $\pi^*_{\sigma}$ orbital which directly overlaps the electron spin on Cu$_B$ and will have a very strong antiferromagnetic coupling as there is direct orbital overlap. This CT antiferromagnetism greatly lowers the energy of the singlet CT states of bridging ligands (by $-2J^{CT}$) and it is the mixing of this CT antiferromagnetic coupling into ground state that is responsible for $-2J^{GS}$. The high intensity of the O$_2^2- \pi^*_{\sigma} \rightarrow$ Cu(II) ($d_{x^2-y^2}$) CT reflects significant overlap of the $\pi^*_{\sigma}$ with the $d_{x^2-y^2}$ orbitals. Thus, both a large $-2J^{CT}$ and a significant CT mixing into the ground state contribute to a large $-2J^{GS}$.
A VBCI model was developed to describe this correlation of the bridging ligand CT excited states to the superexchange pathway of the ground state (i.e. the coupling of the CT absorption spectrum to the ground state magnetism). This is presented in Figure 47. The GS configuration has spins of $\frac{1}{2}$ on Cu$$_A$$ and Cu$$_B$$ and forms $\Gamma_+^1$ and $\Gamma_-^3$ states at zero energy in the absence of CI (+/− refer to symmetric and antisymmetric with respect to the two halves of the dimer). The bridging ligand to metal CT generates four states: $\Gamma_+^1$, $\Gamma_-^1$, $\Gamma_+^3$, $\Gamma_-^3$ given the electron can be excitated to Cu$$_A$$ or Cu$$_B$$, at an energy $\Delta$ above the ground configuration. States of the same symmetry and spin can undergo a configuration interaction due to the covalent ligand-metal overlap given by $h_{d\pi}$ that correlates to the intensity of the LMCT transition. As shown in Figure 47, singlet (left) and triplet (right) energy diagrams labeled CI GS-CT, the interaction between the singlet (triplet) ground state and CT state of the same symmetry splits the singlet (right) CT transition by $\Delta E_{HL}$, the energy splitting deriving from covalent overlap ($h_{d\pi}$) and can be related to the HOMO/LUMO splitting in a MO diagram. Importantly, at this level of CI the singlet and triplet GS are at the same energy (i.e. no antiferromagnetic coupling). However, these do split in energy upon inclusion of two more configurations: 1) the metal to metal charge transfer (MMCT) at an energy $U$ above the ground state where $U$ is the the Mott-Hubbard Columb potential of charge transfer insulators that is the repulsion of two electrons in the same Cu d orbital (~6.5eV for Cu), and 2) the double CT (DCT) where the two electrons on the bridging ligand orbital have been transferred to the two Cu(II) ions. The energy of DCT transitions are much higher than $U$ and can generally be neglected. Importantly, the MMCT and DCT transitions only produce singlet excited states and at energies above the LMCT states. These singlet excited states will mix and lower the energy of the singlet CT states relative to the triplet CT states through CI, an effect which propagates to the singlet ground state. As shown in Figure 47 it is the MMCT singlet mixing with the bridging singlet CT state that leads to the CT excited state antiferromagnetic coupling, $-2J^{CT}$, which in turn yields the ground state antiferromagnetism $-2J^{GS}$ observed experimentally. The energy matrices describing this CI are given by:

\[
\begin{array}{cccc}
    GS & CT(\pi_+^1) & MMCT & DCT \\
    0 & 0 & 0 & (h_{d\pi})_\nu \\
    CT(\pi_+^1) & h_{d\pi} & \Delta - E & h_{d\pi} & (h_{d\pi})_\nu \\
    MMCT & 0 & h_{d\pi} & U - E & (h_{d\pi})_\nu \\
    DCT & 0 & \sqrt{2}h_{d\pi} & 0 & E_{DCT} - E \\
    CT(\pi_+^1) & (h_{d\pi})_\nu & 0 & (h_{d\pi})_\nu & \Delta_\nu - E \\
\end{array}
\]

and the ground state antiferromagnetic coupling can be related to the excited state antiferromagnetic coupling by $-2J^{GS} = \lambda^2(-2J^{CT})$, where $\lambda^2$ is the amount of CT mixing into the ground state due to covalency. Given that $\lambda^2 \approx 10\%$, the excited state antiferromagnetic coupling will be an order of magnitude larger then the ground state antiferromagnetic coupling. Application of the VBCI model to the absorption spectrum of oxy-Hc gives
−25000 cm$^{-1}$ for $−2J_{CT}$ suggesting that the 350 nm band is significantly lowered in energy relative to its one electron orbital transition energy (high due to the high $Z_{eff}$ of the side-on peroxide as it has strong donation to the two Cu(II)) leading to a $−2J_{GS}$ of 2500 cm$^{-1}$. This value is consistent with the originally reported lower limit of > 600 cm$^{-1}$ obtained by SQUID magnetometry and reflects the only experimentally based estimate of the ground state exchange coupling; but more importantly demonstrates that the CT absorption spectrum is a distinct probe of the superexchange pathway for exchange coupling between the two Cu(II) ions.

A number of derivatives of Hc have been prepared as probes of the protein environment and the mechanism of O$_2$ binding. One particular derivative of oxy-Hc, metazide, results from the associative displacement of peroxide by azide in the active site while the oxidation state of both Cu ions is still +2. Met-azide Hc is EPR silent, which suggests that the azide (possibly along with a μ-OH) bridges the metals creating an overall antiferromagnetically coupled ground state. This is verified by the observation of three intense N$_3^−$ $→$ Cu(II) CT transitions in the absorption spectrum (Figure 48), whereas only two are possible with an azide bound to a single Cu(II) ion. These transitions are $π_{nb}$ and $π_{nb}$, analogs of the peroxide $π^*$ CT transitions described above but where the HOMOs of N$_3^−$ are non-bonding. The splitting and intensity of these transitions of the N$_3^−$ binding reflect the TDVC of CT transitions to two Cu(II) as described above for oxy-Hc and are quite dependent on the phylum, indicating structural distortions.

A second derivative of Hc is the “half-met” state generated by reacting deoxy with NO. Two initial equivalents of NO yield met-Hc and N$_2$O, which is then reduced by a third equivalent of NO to yield the half-met NO$_2^−$ derivative. The NO$_2^−$ binds tightly but can be displaced using excess N$_3^−$, which can in turn be displaced by other anions. Half-met Hc is an EPR active mixed-valence Cu(I)/Cu(II) state with an overall spin of $S = \frac{1}{2}$. The binding of a variety of monoanionic ligands to half-met Hc has been surveyed including N$_3^−$, F$^−$, Cl$^−$, Br$^−$, I$^−$, SCN$^−$, CN$^−$, NO$_2^−$, OCN$^−$, CH$_3$CO$_2^−$. The binding of these anions can be classified into two groups. The first group is composed of CH$_3$CO$_2^−$, OCN$^−$, SCN$^−$, NO$_2^−$, F$^−$, and CN$^−$ and result in a localized class I mixed-valence electronic structure. The second group is composed of the halides (not including F$^−$) and azide. Coordination of these anions results in a class II mixed-valence electronic structure as a result of N$_3^−$ and halides bridging the Cu centers. These second class of anions result in two particularly diagnostic spectral signatures as a result of delocalization of the unpaired spin over the two coppers. The first is the appearance of a broad intervalence-transfer (IT) transition ($λ_{max} = 1570$ nm, $ε = 1200$ M$^{-1}$ cm$^{-1}$ when $L = \frac{1}{2}$) in the UV/Vis spectrum 0 (Figure 49A), a feature that requires a bridging coordination mode. This was the first proof that the ligands (including O$_2$) bridge the two Cu(II) ions of Hc. The energy and intensity of this IT band are inversely related, with both the intensity and energy proportional to the efficiency of the bridge as an ET pathway. The second diagnostic spectral feature is the dramatic change in shape of the EPR spectrum (Figure 49B), which is a result of the delocalization of the unpaired spin. The unique EPR spectrum of azide bound half-met Hc was used for structural insight into cooperativity in dioxygen binding to Hc in the “spectral probe” studies described in the next section.

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3.1.5. Mechanism—The binding of dioxygen to deoxy-Hc requires the transfer of two electrons from the Cu(I) ions to dioxygen to form the bridged peroxide. Additionally, the triplet ground state of dioxygen must be converted to the antiferromagnetically coupled singlet of oxy-Hc; a formally spin forbidden process. DFT calculations have been performed to obtain insight to the manner in which the active site overcomes this spin forbiddenness. Throughout the reaction coordinate, the coordination mode of the dioxygen adjusts to maximize metal-ligand overlap. When the center of dioxygen is distant from that of the coppers (Figure 50 right), the bonding interaction of the O₂ is weak and the π* orbitals are not split in energy maintaining the triplet ground state, which minimizes electron-electron repulsion. As dioxygen more strongly interacts with the coppers, the coordination mode is converted to a bridging, butterflied µ-η²:η² one. This results in productive overlap of the Cu orbitals with dioxygen and the transfer of charge from the coppers to dioxygen, each copper interacting with a different π* orbital of dioxygen (becoming a peroxide bridge) producing orthogonal magnetic orbitals, which are ferromagnetic (Figure 22A middle) maintaining the triplet ground state. Importantly at this point, the two unpaired electrons are on the two coppers and the electron, electron repulsion is greatly reduced thus the singlet potential energy surface (PES; Figure 50 blue) is now close in energy to the triplet PES (red). Flattening of the butterfly distortion along the O₂ binding coordinate leads to the overlap of both metal centered magnetic orbitals with one superexchange pathway (the π*σ orbital on peroxide), resulting in strong antiferromagnetic coupling to produce the singlet state (Figure 50 left in blue). Analysis of the Mulliken charges along this O₂ binding coordinate indicates that the two electrons from the two coppers reduce dioxygen simultaneously, (in the bridged structure), and thus overcome the unfavorable one electron reduction of dioxygen. Thus the strong exchange coupling associated with the bridging peroxide that forms along the O₂ binding reaction coordinate overcomes the “spin forbiddenness” of this binding reaction and results in a concerted, 2-electron process.

Further insight to the mechanism of O₂ binding has come from a comparison to the coupled binuclear Type 3 (T3) site in the multi-copper oxidases (see Section 3.7.1). The O₂ binding site of the multi-copper oxidases (MCOs) contains a tri-nuclear copper cluster in its native form. Removal of the Type 2 (T2) copper (see Section 3.7.1.2) results in the isolation of a binuclear T3 copper site that is structurally similar to Hc (2 Cu ions each coordinated by 3 His ligands). The oxidized form of this site also lacks an EPR signal due to antiferromagnetic coupling and the reduced form binds small molecules such as CO but importantly does not react with oxygen in the absence of the T2 copper. A comparison between the XAS spectra of the deoxy forms of both enzymes indicates a substantial difference in their spectral features (Figure 51). The binuclear T3 copper in the MCO has a more intense pre-edge feature at 8983 cm⁻¹ corresponding to the Cu 1s → Cu 4pz transition due to a more planar coordination environment. In contrast, both arthropod and mollusc Hc have a lower intensity Cu 1s → Cu 4pz transition and lower energy and higher intensity Cu 1s → Cu 4px,y transitions due to a more trigonal pyramidal coordination environment.
The implications of the different coordination environments of Hc and the binuclear T3 copper site in the MCO on their respective O\textsubscript{2} reactivity have been computationally evaluated.\textsuperscript{333} Models for both sites were constructed that contained the six His ligands from representative crystal structures with frozen α carbon atoms on each of the ligands. Optimization of the deoxy-structures indicated that deoxy-Hc was 6.9 kcal/mol higher in energy than the deoxy form of the T3 site. Importantly, the geometry optimized deoxy sites also had different Cu•••Cu distances (4.2 and 6.5 Å for Hc and the binuclear T3 center in the MCOs, respectively) and a larger trigonal pyramidal distortion of the Cu(I) centers in Hc, consistent with the XAS spectra in Figure 51. While the binding of O\textsubscript{2} to the Hc model to form oxy-Hc is exothermic (ΔH = −2.9 kcal/mol), the binding of dioxygen, as peroxide in a side-on configuration to the binuclear Cu(I) site in the MCO is endothermic by 5.6 kcal/mol. This difference in binding energy mostly reflects the protein constraints on the reduced sites. The origin of the energy difference observed in the deoxy structures was determined by comparing an unconstrained potential energy surface (open symbols in Figure 52) along the Cu•••Cu vector to one where the α-carbons were frozen by the protein constrains (filled symbols in Figure 52). The slopes of the two unconstrained potential energy surfaces are very similar, indicating similar electrostatic repulsion in the two protein environments. The energy difference between a Cu•••Cu separation of 6.5 Å (the optimized Cu•••Cu distance in the constrained T3 model) and 4.2 Å (the optimized Cu•••Cu distance of the constrained Hc model) for the unconstrained T3 site is \approx 5.8 kcal/mol, which accounts for most of the energy difference between deoxy-Hc and deoxy-T3 MCO. This indicates that the larger electrostatic repulsion between the two Cu(I) atoms in deoxy-Hc is critical for its ability to bind dioxygen.

This electrostatic repulsion in Hc results from the protein constraint on the Cu•••Cu distance due to interactions between two α-helices that contain a His ligand for each Cu (His204 and 364 in Figure 53). In the absence of this electrostatic repulsion that destabilizes the deoxy site, as observed in the binuclear T3 copper site in the MCO, O\textsubscript{2} binding is lost. This protein derived constraint, due to the presence of salt bridges and π-π interactions between these two α-helices, results in an electrostatic repulsion between the Cu ions that functions as an “entatic” or “rack” state in Hc.\textsuperscript{333} Cooperativity between active sites can effect this electrostatic repulsion by modulating the Cu•••Cu distance in the deoxy site. In a structural model of the relaxed deoxy state (Figure 53 in orange and light blue), Phe49 is distant from Cu\textsubscript{B} and the Cu•••Cu separation is short (2.9 Å) increasing electrostatic repulsion.\textsuperscript{143} In the tense deoxy state (red and yellow in Figure 53), the rotation of domain 1 to the closed conformation (with Phe49 close to the copper site) results in a π-π stack between Phe49 and His328 causing the Cu•••Cu distance to increase to 4.6 Å, decreasing electrostatic repulsion.\textsuperscript{309} Dioxygen binding in a bridging mode shortens the Cu•••Cu distance, breaking this π-π interaction, which favors the open confirmation. This leads to a domain rotation that can be transmitted to other subunits via a variety of interdomain contacts.

Direct evidence for active site structural perturbations arising from O\textsubscript{2} binding to adjacent functional units has come from the preparation of a Hc derivative denoted the “spectral probe”, where a small fraction of the active sites in the quaternary structure have been converted to the EPR active half-met state.\textsuperscript{335} When the adjacent sites are oxygenated, the EPR spectrum of the half-met spectral probe in both arthropod and mollusc Hc is similar to...
the half-met monomer in the presence of excess azide (Figure 54). Upon deoxygenation, the spectral probe converts to a class II mixed-valent state, resembling the nonexcess azide spectra (Figure 54 deoxy, compare to Figure 49B red), which converts back to the excess azide spectra upon re-oxygenation. The changes in the spectral features of the half-met sites in the spectral probe indicate that when O₂ binds to a binuclear Cu⁺ site, the quaternary structure shifts (from T to R), which affects the O₂ binding through a change in the Cu•••Cu distance.

3.2 Oxygen Activation by Coupled Binuclear Active Sites: Polyphenol Oxidases and Phenol Oxygenases

3.2.1. Enzymology—Coupled binuclear polyphenol oxidases (CB-PPOs) contain a magnetically coupled binuclear copper active site that activates dioxygen to catalyze the production of quinones in a diverse range of organisms. While the term “polyphenol oxidases” has been used to describe the multi-copper oxidase, Laccase (Section 3.7.1), laccase performs one-electron oxidations of phenols, in contrast to the two-electron oxidation of o-diphenols by the coupled binuclear copper protein family. CB-PPOs can be further divided into two subsets of enzymes with differing reactivity: tyrosinase (Ty), also called monophenol monooxygenase, internal monooxygenase, or a mixed function oxidase, (EC 1.14.18.1) and catechol oxidase (CaOx) (EC 1.10.3.1). While both Ty and CaOx perform the two-electron oxidation of o-diphenols to quinones (referred to as two-electron oxidase or catecholase activity), only Ty can catalyze the conversion of phenols to o-diphenols (referred to as monooxygenase, phenolase, or cresolase activity). Ty catalyzes the conversion of the amino acid, L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to L-DOPAquinone (3-(3,4-dioxocyclohexa-1,5-dien-1-yl)-L-alanine) (Figure 55). Early oxygen labeling studies by Mason indicated that the oxygen atom incorporated into L-DOPAquinone originates from dioxygen. This, in combination with Hayaishi’s studies on a non-heme iron containing intradiol dioxygenase, demonstrated the presence of oxygenases in biology (monooxygenase for Ty and dioxygenase for pyrocatechase).

The enzymatic production of L-DOPAquinone by the CB-PPOs provides the monomers that nonenzymatically polymerize, forming eumelanin via the Mason-Raper pathway. First, L-DOPAquinone forms cycloDOPA via an intramolecular Michael addition (Figure 56). CycloDOPA is then oxidized by another molecule of LDOPAquinone forming DOPAchrome, regenerating a molecule of DOPA. DOPAchrome decomposes preferentially into 5,6-dihydroxyindole (DHI) via decarboxylation but can also form 5,6-dihydroxyindole-2-carboxylic acid (DHICA) via a tautomerization. These two dihydroxyindoles are then oxidized enzymatically by Ty or CaOx or nonenzymatically by L-DOPAquinone before auto-polymerizing, forming eumelanin. While eumelanin makes up the major component of melanin found in nature, pheomelanin can also be present (usually <25% of the total melanin polymer). Pheomelanin forms from the intermolecular addition of L-cysteine to L-DOPAquinone, forming 5-S-Cysteinyl dopa (Figure 56) or 2-S-Cysteinyl dopa (not shown). Similar to the Mason-Raper pathway, the cysteinyl dopa will be oxidized by L-DOPAquinone before cyclizing to a 1,4-benzothiazine intermediate that autopolymerizes to pheomelanin. While the chemical
formation of both the benzothiazine and dihydroxyindoles has been extensively studied, the molecular structures of both the eumelanin and phenomelanin polymer are less well understood.

Overall sequence similarity suggests that the CB-PPOs in arthropods are closely related to arthropod Hc (and hexamerins, see Section 3.1.1)\textsuperscript{351,352} while fungal, bacterial, plant, and vertebrate Ty is more closely related to molluscan Hc.\textsuperscript{351} One defining structural difference between arthropod and molluscan Hc is the presence, only in the latter, of a highly conserved His ligand on Cu\textsubscript{A} (the Cu atom that is coordinated by residues closer to the N-terminus) that has been cross-linked to a Cys residue (Section 3.1.3). However, sequence similarity,\textsuperscript{353,354} protein sequencing,\textsuperscript{355,356} and protein crystallography (\textit{vida infra}) indicate that plant and fungal CB-PPOs contain a Cys-His crosslink that is absent in bacterial Ty. While not conclusive, this structural feature suggests that the coupled binuclear copper proteins evolved from a common binuclear protein and that the observed differences are a result of divergent evolution. However, the function of this divergence is not well understood.

A variety of intra and extra cellular CB-PPOs have been characterized in fungi, gram-positive, and gram-negative bacteria. The production of melanin in these organisms has been correlated to the resistance to radiation, toxic metals, oxidants, free radicals, extreme temperatures, and fungal tissue damage.\textsuperscript{357–359} Melanin is also believed to increase virulence in pathogenic fungi and bacteria through a variety of mechanisms including protection from enzymatic lysis and hydrolysis, antimicrobial compounds, and phagocytosis (the process of cellular entrapment in a membrane vesicle).\textsuperscript{359,360} In soil bacteria, CB-PPOs polymerize otherwise toxic mono and \textit{o}-diphenols into a variety of polymers such as melanin, and humic acids.\textsuperscript{357} These physiological functions rely upon the presence of a copper chaperone that ensures the proper transportation and copper loading of Ty. The chaperone MelC1, in the genus \textit{Streptomyces}, is critical for copper loading\textsuperscript{361,362} and transports the enzyme through the twin-arginine translocation (Tat) pathway\textsuperscript{363,364} by forming a dimer with apo Ty.\textsuperscript{365} A copper chaperone (PpoB1) has also been identified in \textit{Marinomonas},\textsuperscript{366} however a CB-PPOs copper chaperone has not been reported in fungi.

In plants, CB-PPOs polymerize a variety of \textit{o}-diphenols (catechin, chlorogenic acid, dopamine, and caffeic acid are common in vivo substrates). While the majority of known COs are found in plants and fungi, some of these enzymes also possess monooxygenase activity.\textsuperscript{367–375} CB-PPOs are nuclear encoded proteins that are directed to the thylakoid lumen by a N-terminal signaling peptide via multiple pathways including the Tat pathway.\textsuperscript{376,377} While little is known about the function of the enzyme in the thylakoid lumen, it has been hypothesized that the CB-PPOs are involved in the reduction of dioxygen produced from photosynthesis.\textsuperscript{378,379} Yet most of the enzyme in the lumen is inactive due to the presence of the C-terminal domain (similar to molluscan Hc). In \textit{vivo}, this latent form can be activated by free fatty acids\textsuperscript{380} or proteolytic cleavage between the two domains.\textsuperscript{381} Upon tissue damage, phenols are released from the vacuole and the CBPPOs are activated resulting in the browning of plant tissue.\textsuperscript{382–385} CB-PPOs are also localized in the trichromes of plants and are critical for the entrapment of insects.\textsuperscript{282,386} Expression levels have also been correlated to defense mechanisms against insects, pathogens, and
herbivores.\textsuperscript{383,387–389} The plant signaling molecule methyl jasmonate has been shown to increase the expression levels of CB-PPO genes and corresponding protein product in many plants.\textsuperscript{384,390} A number of CB-PPOs also participate in synthesis of various natural products in plants. Ty related proteins possessing monoxygenase activity participate in the production of plant pigments such as aurone\textsuperscript{391} and betalin\textsuperscript{392} as well as antioxidants such as creosote bush 8–8' linked lignans.\textsuperscript{393}

In arthropods (most notably insects, crustaceans, and arachnids), the CB-PPO (sometimes called phenoloxidase or prophenoloxidase for the active and inactive forms, respectively) is mostly produced by hemocytes, an immune cell found in the hemolymph, and participates in clotting, encapsulation of parasites, and sclerotization of the cuticle.\textsuperscript{394,395} Similar to plants, arthropod CB-PPOs are transcribed in an inactive form that is activated by a cascade of serine proteases.\textsuperscript{394,395} However, some arthropods, such as the horseshoe crab, lack a CB-PPOs gene.\textsuperscript{395} Instead, it is believed that the immune system utilizes a clotting enzyme that induces CaOx activity in horseshoe crab Hc.\textsuperscript{396,397} A similar activity in Hc has been accomplished \textit{in vitro} with protein denaturants\textsuperscript{398,399} such as SDS\textsuperscript{400–403} or urea\textsuperscript{404} and by proteolytic cleavage;\textsuperscript{405,406} however, a more general physiological function of this activity in Hc has not been established.

In animals, the most common CB-PPOs is Ty, which is responsible for melanin formation. However, some specialized functions in animals have been characterized, such as the formation of ink in molluscs including octopus.\textsuperscript{407–409} Present in vertebrates are two Ty related proteins (Trps) that are believed to have formed from gene duplication.\textsuperscript{410} While other organisms, notably plants, contain multiple isoforms of CB-PPO, the Trps enzymatically control the polymerization of L-DOPAquinone creating a more diverse melanin polymer.\textsuperscript{347} One of the Trps, DOPAchrome tautomerase (Dct or Trp2), contains a zinc cofactor and catalyzes the tautomerization of DOPAchrome to DHICA (Figure 57).\textsuperscript{411} In the absence of enzymatic control, the ratio of DHICA to DHI is 1:70 at neutral pH.\textsuperscript{344} While the metal cofactor of Trp1 is still unknown,\textsuperscript{412} it is believed to be an isozyme of Ty given that mouse Trp1 has low monoxygenase and CaOx activity.\textsuperscript{413,414} However, the reactivity of Trp1 towards DHICA is less clear. In mice, only Trp1, and not Ty, has two-electron oxidase activity for DHICA\textsuperscript{415,416} while in humans, only Ty has this activity.\textsuperscript{417} Besides catalytic activity, Trp1 also has a structural function in stabilizing Ty.\textsuperscript{418,419}

The regulation of Ty expression levels in vertebrates is induced by environmental factors, such as UV light, which is translated to numerous signaling molecules including cyclic AMP.\textsuperscript{420} The majority of these signals lead to the production of the Microphthalmia associated transcription factor (MITF) causing the upregulation of Ty, Trps, and numerous other proteins that assist in the cellular processing of Ty.\textsuperscript{420,421} Ty, once synthesized in the cytosol, is processed in the endoplasmic reticulum (ER) and the Golgi apparatus, and is stabilized by both Trp1 and 2.\textsuperscript{418,419} In the ER, the N-terminal signaling sequence is cleaved and Ty is glycosylated, and folded with the assistance of multiple chaperones.\textsuperscript{422} Ty is then transported to the Golgi where the glycans are further modified before being exported to the melanosomal membrane that is spanned by a C-terminal domain (only in vertebrates). Copper is loaded by the metal chaperone ATP7A once Ty reaches the melanosome.\textsuperscript{423} There are numerous mutations in the signaling pathway, in Ty, or in related processing

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proteins that are responsible for various pigmentation disorders in humans.\textsuperscript{424} Hundreds of specific mutations of the Ty gene are known that range from point mutations to frame shift mutations cause Type I oculocutaneous albinism causing hypopigmentation and impaired vision with varying severity.\textsuperscript{424–426} Diminishing melanosome formation in hair follicle is believed to be one cause of hair graying with age.\textsuperscript{427} While numerous mechanisms have been proposed to account for these observations, recent evidence suggests that high hydrogen peroxide levels coupled to low expression of methionine sulfoxide reductase also cause grey hair by inactivating Ty via methionine oxidation.\textsuperscript{427,428} Some have also postulated that another function of Ty is the formation of neuromelanin in the brain,\textsuperscript{429–431} however others have proposed that phenols, such as dopamine, polymerize non-enzymatically.\textsuperscript{432} Even the presence and expression levels of Ty in neuronal cells are disputed.\textsuperscript{429,430,432,433} While little is known about the formation of neuromelanin, the cellular production of neuromelanin increases the likelihood of cell death in patients with Parkinson’s disease.\textsuperscript{434}

Recently, a new, fourth sub-class of the coupled binuclear copper enzyme family has been discovered (in addition to Hc, CaOx, and Ty) that converts \( o \)-aminophenols to nitrosophenols (hydroxyanilinase activity, Figure 57).\textsuperscript{435} In \textit{S. murayamaensis}, a hydroxyanilinase (NspF) performs the terminal step of the biosynthesis of 4-hydroxy-3-nitrosobenzamide. A similar enzyme from \textit{S. griseus} (GriF) has also been shown to possess hydroxyanilinase activity \textit{in vitro} on specific \( p \)-substituted phenols (\textit{vide infra}).\textsuperscript{435} \textit{In vivo}, GriF performs the two-electron oxidation (oxidoreductase activity) of \( 3 \)-amino-\( 4 \)-hydroxybenzaldehyde to the corresponding \( o \)-iminoquinone in the biosynthesis of grixazone A and B, a yellow pigment and parasiticide (Figure 57).\textsuperscript{436} Hydroxyanilinases have also been shown to possess CaOx and monophenolase activity indicating that these enzymes possess all of the Ty functions as well as the additional hydroxyanilinase function.\textsuperscript{435} With \( o \)-aminophenols as substrates, Ty only performs a two-electron oxidation to produce \( o \)-iminoquinones.\textsuperscript{435,437}

\textbf{3.2.2. Kinetics—}While a number of CB-PPOs have been characterized with various substrate specificities, Ty is the most prevalent and best characterized member. To convert phenols such as tyrosine to L-DOPAquinone, Ty binds dioxygen with a measured equilibrium binding constant of 16.5 – 46.6 \( \mu \)M,\textsuperscript{438,439} falling within the range of oxygen affinities found in mollusc Hc (Section 3.1.2). The rate of \( O_2 \) binding to deoxy-Ty (19–23 \( \mu \)M\(^{-1}\) s\(^{-1}\))\textsuperscript{438,440} is also similar to mollusc Hc (5–30 \( \mu \)M\(^{-1}\) s\(^{-1}\)).\textsuperscript{203,303,305} During catalysis, three states of Ty (and CaOx) have been characterized; deoxy ([Cu(I)]\(^{2+}\)), oxy ([Cu(II)]\(^{2+}\)), and met-Ty (two Cu(II)’s bridged and antiferromagnetically coupled by a hydroxide ligand) (Figure 58 and 59). Unlike in Hc where almost all of the active sites are oxygenated under air-saturated conditions, purified Ty contains approximately 85% met and 15% oxy-Ty.\textsuperscript{441,442} This can be converted to mostly oxy by a peroxide shunt reaction\textsuperscript{441} or reduction to deoxy followed by exposure to oxygen. While oxy-Ty is able to convert both phenols\textsuperscript{443} and \( o \)-diphenols to quinones,\textsuperscript{444} met-Ty is only able to oxidize \( o \)-diphenols to quinones (Figure 58 and 59). Despite similarities at the active site between oxy-Hc and oxy-Ty, the differential reactivity has been shown to result in part from differential access to the active site. Addition of small molecule ligands, such as azide, displace the peroxide from the
oxy site four orders of magnitude faster in Ty than in Hc.\textsuperscript{325,445} Additionally, a substrate analogue and competitive inhibitor, mimosine, readily displaces peroxide from oxy-Ty \((k = 162 \text{ h}^{-1})\) but not from oxy-Hc \((k \ll 10^{-4} \text{ h}^{-1})\).\textsuperscript{445} Yet, similar rates of \(O_2\) binding to deoxy-Ty and Hc suggest that the active sites have similar access for dioxygen, potentially due to the presence of an \(O_2\) tunnel, which in Hc is believed to close after oxygen binding (Section 3.1.3).

### 3.2.2.1 Catechol Oxidase Activity:

While the enzymatic control of the oxidation of \(o\)-diphenols is essential \textit{in vivo}, the oxidation of \(o\)-diphenol can occur \textit{in vitro} by a variety of oxidants including dioxygen \((k = 2.0 \times 10^{-5} \text{ s}^{-1}, \text{pH} = 7.6)\)\textsuperscript{446} and is catalyzed by copper chloride (the rate of oxidation more than doubles with 1.0 mM CuCl\(_2\)).\textsuperscript{447} While the oxidation of L-DOPA \((E_0' = 0.54 \text{ V vs SHE}, \text{pH} = 6.75, n = 2)\)\textsuperscript{448} is required to form melanin, L-DOPA also functions as the \textit{in vivo} reducing agent for met-Ty \((E_0' = 0.60 \text{ V vs SHE}, \text{pH} = 7.0, n = 2)\)\textsuperscript{336} regenerating deoxy-Ty (Figure 58). Under steady state turnover conditions, mushroom Ty oxidizes L-DOPA with a \(k_{\text{cat}}\) of \(~150 \text{ s}^{-1}\) (seven orders of magnitude faster than the uncatalyzed reaction) and a \(K_m\) value of \(~450 \mu\text{M}\).\textsuperscript{449–451} The \(K_m\) for dioxygen varies with the chemical structure of the substrate,\textsuperscript{449,452} suggesting that the binding of \(o\)-diphenols and oxygen is not ordered,\textsuperscript{14} however kinetic modeling can account for this observation assuming oxygen binds first.\textsuperscript{453} Thermodynamic parameters for CaOx activity have also been determined from \(k_{\text{cat}}\) giving \(\Delta G^\neq \sim 13–17 \text{ kcal/mol (25° C)}\) depending on the substrate (\(\Delta G^\neq = 14.5 \text{ kcal/mol for L-DOPA})\).\textsuperscript{449,454}

The CB-PPOs, especially the CaOx from different plant sources, have varied substrate specificity with respect to substituted \(o\)-diphenols such as 4-methylcatechol, catechin, pyrogallol, and caffeic acid.\textsuperscript{455–460} Ty also exhibits stereospecificity with respect to L-DOPA \((K_{m}^{D-\text{DOPA}}/K_{m}^{L-\text{DOPA}}=5.6\text{ for mushroom Ty})\).\textsuperscript{450} \textit{In vitro}, the specific activity of CaOx maximizes around neutral pH.\textsuperscript{449,461} A decrease in \(K_m\) from pH 4–7 corresponds to a \(\text{pK}_a\) of 4.9 – 6.0 depending on the substrate.\textsuperscript{449} However, the molecular identity of this \(\text{pK}_a\) or the cause of the substrate dependence is unknown. \textit{In vivo}, the pH of human melanosomes (~ 3.5)\textsuperscript{462} has been shown to affect pigmentation levels due to the pH dependence of Ty.\textsuperscript{463} Increased melanin production in individuals with darker skin results in part of a more neutral melanosome, increasing Ty activity.\textsuperscript{464} The substrate dependence of catalysis has also been used as a mechanistic probe of mushroom tyrosinase indicating that substrates with similar redox potentials have \(k_{\text{cat}}\) values that differ by an order of magnitude.\textsuperscript{438} However, substrates with no substituents such as pyrogallol \((k_{\text{cat}} = 1263 \pm 63 \text{ s}^{-1})\) are oxidized more rapidly than substrates with large, polar substitutents such as L-DOPA \((k_{\text{cat}} = 109 \pm 9.1 \text{ s}^{-1})\) consistent with the hypothesis that the rate-limiting step in the oxidase catalysis is affected by a steric interaction and not the redox couple of the diphenol.\textsuperscript{438}

To determine the rate-determining step, kinetic modeling coupled with transient phase kinetics have determined that the reaction of 4-\textit{tert}-butylcatechol with oxy-Ty is 20 times slower than its reaction with met-Ty.\textsuperscript{438} This model is also consistent with the observation of a steady state concentration of oxy-Ty.\textsuperscript{438} Additionally, a solvent deuterium isotope effect of \(1.67 \pm 0.1\) on \(k_{\text{cat}}\) during the oxidation of L-DOPA suggests the rate-limiting step includes a proton transfer.\textsuperscript{465,466} The observation of a linear free energy relationship
between the Hammett parameter and $K_m$ and $k_{cat}$ indicates that the resonance delocalization of a negative charge affects both the binding and the rate-limiting step ($\rho = -1.0$ and $-2.5$ for $K_m$ and $k_{cat}$, respectively). Assuming that the different enzyme sources and substrate substituents do not change the rate-determining step, these data indicate the slowest step in catalysis is the reaction of the oxy form with the diphenol. The solvent KIE and substrate dependence suggest that within this reaction, the rate-determining step is a structural rearrangement that is effected by the size of the substrate concurrent with the transfer of a proton. This proton transfer is then followed by a two-electron oxidation of the diphenol, which is not rate-determining.

3.2.2.2 Monoxygenase Activity: In mushroom Ty, the kinetics of the reaction with L-tyrosine is characterized by a ten-fold decrease in $k_{cat}$ ($\approx 10 \text{ s}^{-1}$) compared to the conversion of L-DOPA ($\approx 150 \text{ s}^{-1}$), while $K_m$ is similar ($\approx 300 \mu M$) for both reactions. In steady state turnover, the slowest elementary kinetic step of the monoxygenase and CaOx steps ($k_{MO} = 10^3 \text{ s}^{-1}$, $k_{CO} = 10^7 \text{ s}^{-1}$) have been estimated from comparing kinetic simulations to turnover frequency. During turnover with a non-native reductant, the $\alpha$-diphenol can be trapped by borate, indicating that the $\alpha$-diphenol is an intermediate in the catalytic cycle (Figure 59). Ty also exhibits preferential binding of L-tyrosine ($K_m = 0.270 \text{ mM}$) over D-tyrosine ($K_m = 1.870 \text{ mM}$). Similarly, Ty exhibits regiospecificity, reacting preferentially (lower $K_m$ and higher $k_{cat}$) with $p$-phenols over $m$-phenols and is unreactive towards $o$-phenols such as $o$-fluorophenol. The temperature dependence of $k_{cat}$ in an aqueous, organic solvent mixture gives a free energy barrier of 16.1 kcal/mol (25 °C), which is in excellent agreement with the turnover number measured in aqueous solution ($k_{cat} = 10 \text{ s}^{-1}$ at 25° C predicts $\Delta G^\ddagger = 16$ kcal/mol). The hydroxylation of monophenols proceeds most favorably at neutral pH. Over a pH range of 5–9, a decreased in $K_M$ corresponds to a $pK_a$ of 7.85, however the molecular origin of this protonation is currently unknown.

Unique to the reaction of Ty with monophenols is the presence of a lag period before steady state turnover is established. This lag phase depends on substrate concentration, enzyme concentration, and the presence of native ($\alpha$-diphenols) and non-native reductants. The lag phase has been attributed to the presence of met-Ty in the resting form of the enzyme, which is unreactive towards monophenols. As a result, met-Ty must be converted to deoxy-Ty before the system reaches steady state turnover. Absent any initial reductant to reduce met-Ty, it (~85% of resting Ty) does not participate in turnover until $\alpha$-diphenols such as L-DOPA are generated non-enzymatically from L-DOPAquinone (Figure 56). L-DOPA can then react with met-Ty forming deoxy-Ty to eventually achieve steady state turnover. Additionally, the lag phase has been shown to increase with increased substrate concentration. This has been attributed to phenols binding to met-Ty forming an off pathway complex, consistent with the observed substrate inhibition (Figure 59 bottom left).

To determine the mechanism and the rate-limiting step during turnover, substrate specificity and isotope effects have been measured. Steady state turnover of para-substituted monophenols has been shown to vary with respect to electron withdrawing substituents. A Hammett relationship between $\sigma^+$ and $k_{cat}$ has a negative slope ($\rho = 1.0$ for $K_m$ and $-2.5$ for $k_{cat}$, respectively).
−2.4 and −1.7 for *A. bisporus* and *A. oryzae*, respectively) indicating that a positive charge is created in the rate-determining step. The magnitude of ρ compared to binuclear copper model systems has been further used to support a mechanism of electrophilic aromatic substitution (EAS) (vide infra). Incorporation of deuterium at the ortho-carbon produces an inverse isotope effect (kHcat/kDcat = 0.9) supporting a mechanism of EAS where the hydroxylation is rate limiting. However a tritium isotope effect of 1.2 has also been measured on Ty from a different source that was originally proposed to support a mechanism of EAS where the proton transfer from the ortho-carbon was rate limiting. Yet others have reported being unable to kinetically distinguish between protium and deuterium or tritium. A large solvent deuterium isotope effect that is linear with respect to the concentration of D2O has been measured for various substrates (3.45 ± 0.3 for L-tyrosine). The strength of the isotope effect was correlated to chemical shift of the C1 carbon of the substituted phenol, consistent with the rate-limiting step being the transfer of the phenolic proton to a base upon coordination to the Cu. However, this interpretation is inconsistent with the Hammett relationship since this mechanism should produce a positive ρ value due to the creation of negative charge.

To probe the effect of second sphere residues on substrate binding, kinetic parameters of mutated variants have been determined. Most intriguing is a series of variants have a differential effect on the oxidase and oxygenase reactivity of Ty indicating that monophenols and o-diphenols have different interactions with the protein pocket. For example, the sterospecific recognition of L-DOPA in mouse Ty has been attributed to His389, which is adjacent to His390, a ligand for the Cu atom closest to the C-terminus (CuB) (See Section 3.2.3). Interestingly, the H389L variant affects the relative K_m for L and D-DOPA (K_mD-DOPA/K_L-DOPA = 9.9 for WT and 1.3 for H389L) without affecting the sterospecific recognition of L-tyrosine. A comparison between the kinetics of L-DOPA and dopamine suggest that His389 forms a hydrogen bond to the carboxylic group in L-DOPA. Another variant in mouse Ty (H378Q), which is also in the vicinity of CuB, affects the substrate K_m for the CaOx activity while the K_m of the monooxygenase activity is unaffected (Table 15). A similar improvement in the oxygenase/oxidase activity has also been observed in the CuB variant (R209H) of bacterial Ty from *B. megaterium*, which increases the k_cat for the Ty activity while decreasing the CaOx activity (Table 15). Interestingly, a variant of human Ty (A206T located near Cu_A) that is responsible for type 1 oculocutaneous albinism increases the oxygenase activity 204% while decreasing the oxidase activity to 15% of WT. These results have been interpreted to support a mechanism where phenols bind to Cu_A while o-diphenols bind at Cu_B.

Numerous inhibitors of the CB-PPOs have also been studied for various applications in medicine and agriculture. Correlations of inhibitor structure to function have generated insight into the nature of the active site pocket. Carboxylic acids have been shown to be competitive inhibitors of L-tyrosinase and L-dopa. Binding kinetics for various carboxylic acids indicate that aromatic acids (such as benzoic acid) have larger binding constants (K_eq = 800 M⁻¹) than aliphatic acids (such as acetic acid K_eq = 180 M⁻¹). This is due to an order of magnitude change in binding rate (k_on = 17 and 4 M⁻¹h⁻¹ for benzoic acid and acetic acid, respectively) while the dissociation rates are comparable (~1000 h⁻¹).
This kinetic difference, combined with spectroscopic studies (vide infra), suggests a favorable interaction between the aromatic inhibitor and the protein pocket. These results are also consistent with the weak competitive inhibition of the monoxygenase activity in Ty by toluene ($K_i = 23 \text{ mM}$ while the $K_i$ of benzoic acid is $0.018 \text{ mM}$). The large $\Delta S^o$ of binding of toluene ($54 \text{ cal mol}^{-1} \text{ K}^{-1}$) is also consistent with a hydrophobic interaction between toluene and the protein pocket.

To further probe the mechanism of binding to the active site, the pH dependence of various inhibitors has been determined. $P$-nitrophenol, a slow substrate and competitive inhibitor of L-tyrosine, is a better inhibitor at low pH with a $pK_a$ of 6.82 measured from the formation constant during L-tyrosine turnover. This $pK_a$ is similar to the $pK_a$ of the phenolic proton of $P$-nitrophenol under the same conditions, consistent with the protonated phenol inhibiting turnover. A similar experiment with the inhibitor benzoic acid indicated that the protonated form inhibits Ty. A proton inventory, which measured change in the formation constant with respect to the fraction of $D_2O$ in the solvent, is linear indicating that a single proton is transferred in the rate-determining step of inhibition. This result excludes protonated groups such as water or hydroxide as the proton acceptor since they would involve multiple protons and would give a non-linear relationship. From the slope of this plot, a product fractionation factor, which is the enrichment of deuterium in the product relative to the solvent concentration, was determined to be $0.64 \pm 0.02$. This fractionation factor is unique since amino acid residues generally have fractionation factors of one except cysteine which has a fractionation factor of 0.40 – 0.46. As a result, Strothkamp and coworkers propose that the identity of the base during inhibition could be the peroxide bridge and hypothesize that the peroxide could also function as a general base in catalysis. However, results from model complexes argue against this hypothesis since side-on copper peroxides are not basic. Additionally, low fractionation factors have been observed with amino acid residues other than cysteine due to the presence of low-barrier hydrogen bonds. These low-barrier hydrogen bonds occur when there are short heavy-atom, heavy-atom distances and similar $pK_a$'s of the two groups involved.

These results indicate that the reaction of oxy-Ty with a monophenol is slower than the subsequent reaction of the intermediate diphenol with met-Ty. Data suggest that the monophenol first binds CuA resulting in the formation of a $\pi$-protein interaction. Subsequently, attack at the ortho-carbon in an EAS mechanism generates the diphenolic intermediate. However, the rate-determining step of this reaction is currently disputed. Some have argued that the deprotonation of the substrate upon coordination to CuA is rate limiting while others have argued that the EAS (either the attack on the ring or the deprotonation of the $\sigma$ complex) is rate limiting. A similar controversy exists over the identity of the base that deprotonates the monophenol. While the pH dependence of inhibitors binding has resulted in a model where the bridging peroxide acts as the base, second sphere residues within the vicinity of the active site (vide infra) have also been suggested.

### 3.2.2.3 Activated Hc:

While Hc has a primary function as a dioxygen transport protein, native Hc has been shown to have weak CaOx activity. Significant enhancement of CaOx activity in both arthropod and mollusc Hc has been accomplished with protein denaturants such as SDS or urea, proteolytic cleavage, or via protein/
protein interactions. These results further support early kinetic studies that suggest the functional difference between Ty and Hc is due in part to substrate access to the binuclear active site. A more limited number of both arthropod and mollusc Hc has been shown to have inducible monoxygenase activity. For example, tarantula Hc has been shown to convert L-tyrosine to DOPA quinone, however kinetic parameters were not determined. The monoxygenase activity of mushroom Ty and subunit g of O. vulgaris Hc have been measured for a series of p-substituted phenols (Figure 60). The kinetic parameters indicate that activated Hc is an inefficient tyrosinase with a K_m that is two to three orders of magnitude larger in Hc (~10 mM) than in Ty (~ 0.010 mM) and a k_cat that is three orders of magnitude smaller (0.099 vs 120 for the reaction of Ty and Hc with 4-methyphenol, respectively). Given that Ty and Hc have identical oxy sites, these results indicate that second sphere residues in Ty both bind substrate more tightly and lower the activation barrier for hydroxylation more efficiently than in Hc. Yet, the Hammett plot (Figure 60) of k_cat for activated Hc is suggestive that both Ty and Hc operate via a similar mechanism (ρ = −1.6 and −2.4 for Hc and Ty, respectively). Incorporation of deuterium into the ortho positions of the substrate yields in an inverse deuterium isotope effect in subunit g of O. vulgaris Hc, which is suggestive of EAS where the hydroxylation of phenol is the rate-determining step.

3.2.2.4 Hydroxyanilinase Activity: The best-characterized hydroxyanilinase, NspF, has a k_cat and K_m of 260 s^{-1} and 0.72 mM, respectively, for its native substrate, 3-amino-4-hydroxybenzamide (Figure 57). Substitution of the functional group para to the o-aminophenol results in a decrease in k_cat for the hydroxyanilinase activity and for some substrates, such as the unsubstituted o-aminophenol, no hydroxyanilinase activity is observed. For this substrate, NspF instead performs the two-electron oxidation to form the corresponding o-iminiquinone (Figure 57). Interestingly, Ty only performs the two-electron oxidation of o-aminophenols. Turnover rates for the two-electron oxidase activity of S. murayamaensis NspF and N. crassa Ty with the same substrate are of the same order of magnitude indicating that the difference in selectivity between these two enzymes originates from a difference in k_cat for the hydroxyanilinase activity. From the magnitude of the rates, the ΔΔG‡ for this activity in NspF relative to Ty is expected to be greater than 4 kcal mol^{-1}.

3.2.3. Structure—High-resolution crystal structures of the CB-PPOs can be divided into four categories, plant CaOx (I. batatas, V. vinifera), bacterial Ty (S. castaneoglobisporus and B. megaterium), fungal Ty (A. bisporus) and insect Ty (M. Sexta) (Table 16). While the plant CaOx and bacterial and fungal Ty structures are evolutionarily and structurally similar to mollusc Hc, the structure of insect Ty is similar to arthropod Hc. All of the structures contain a central, globular domain that is mainly α-helical. This domain contains a 4 α-helix bundle that is highly conserved in all known CB-PPOs and Hc. In the plant CB-PPOs and bacteria, CB-PPOs, a highly conserved His-X(n)-His- (8) His sequence coordinates Cu_A while a His-X(3)-His-X(n) motif coordinates Cu_B. While in insect Ty (and arthropod Hc), the ligands have a His-X(3)-His-X(n) motif for both Cu atoms. Unique to plant CaOx and insect Ty is the presence of multiple domains that shield the active site.
Plant^377 and vertebrate^505 CB-PPOs also contain an N-terminal signaling peptide that directs transport of the protein to the thylakoid lumen and endoplasmic reticulum, respectively. While plant CB-PPOs are associated with the thylakoid membrane, only vertebrate CB-PPOs contain a transmembrane fragment found at the C-terminus. However, the structures of these domains are unknown since the plant CaOx structures are of the processed form that lacks this domain^498,499 and no vertebrate structures have been reported.

Crystal structures of four different forms of the binuclear copper active site in the CB-PPOs have been obtained; apo, deoxy, oxy, and met. The similarity between apo and deoxy Ty indicate that the His residues are pre-aligned for Cu binding. In the deoxy form, the copper coordination environment is characterized as a distorted tetrahedron with a fourth, bridging water derived ligand that is absent in the deoxy structures of Hc. Despite the presence of a bridging solvent ligand, the Cu(I) coordination environment is mostly planar resulting in a Cu•••Cu separation greater than 4.0 Å, similar to the T-state of deoxy Hc (Table 13 and Table 17). Oxygen binds the deoxy site as peroxide in an µ-η^2:η^2 coordination mode, decreasing the distance between the two copper atoms (3.8 Å and 3.4 Å in oxy-CaOx determined from EXAFS in I. batatas^498 and oxy-Ty determined from crystallography in S. castaneoglobisporus^500, respectively). (Figure 61A). The shorter Cu•••Cu distance in oxy-Ty from S. castaneoglobisporus is potentially influenced by the presence of a caddy protein required for crystallization.^500 A caddy protein residue (Tyr98) forms a hydrogen bond with the peroxide (O_Tyr98-O_peroxide = 3.0 Å) causing the core to butterfly, decreasing the Cu•••Cu distance. The coordination environment of Cu(II) with respect to the three His ligands in oxy-Ty from S. castaneoglobisporus is staggered trigonal (Cu-N_His bond lengths range from 2.1–2.2 Å), similar to oxy-Hc from E. dofleini157. Also relevant to the catalytic cycle is the active site structure of met CaOx and Ty (Figure 61B and Table 17). A variety of different crystal structures of met-CB-PPOs contain two Cu(II) atoms bridged by zero, one, or two solvent derived ligands. In these structures, the distance between the coppers is extremely flexible with Cu•••Cu distances ranging form 4.9 – 2.9 Å. However, two solvent derived bridges can only be accommodated by Cu•••Cu distances that are 3.1 Å or shorter due to steric repulsion between the two single atom bridges. A single bridge can accommodate Cu•••Cu distances up to 4.0 Å due to a Cu-O distance of ~2.0 Å. Yet, the inherent flexibility in the Cu•••Cu distance within these different structures assists with oxygen binding (Section 3.1.5) and the shorter Cu•••Cu distances suggest that other copper dioxygen isomers, such as a bis-µ-oxo (Cu•••Cu ≈ 2.8 Å in model complexes), would not be structurally restrained by the protein environment. However, this isomer has not been observed in any member of this enzyme family.

Due to their evolutionary relationship, the overall fold and Cu His ligands of bacterial and fungal Ty, plant CaOx, and mollusc Hc are very similar. While all of these structures have a Cu_A His ligand that is located on a flexible loop, a conserved Cys (92 in I. batatas^498 CaOx and 83 in A. bisporus^500) is crosslinked to a Cu_A His ligand (109 in CaOx and 85 in fungal Ty) (Figure 61B). The amino acid sequence of two other fungal Ty^355,356 and sequence similarity^353,354 indicates that the Cys-His crosslink is conserved in fungal Ty. In both fungal Ty^502 and mollusc Hc^157, the Cys-His crosslink occurs within a conserved Cys-X-X-
His motif while this crosslink is found in a His-X(4)-Cys motif in plant CB-PPOs. However, the Cys in plant CB-PPOs is not crosslinked to the adjacent His but rather a different Cu ligand (I. batatas, His88-X(4)-Cys92 forms the motif while Cys92 is crosslinked to His109). While this covalent linkage is absent in bacterial Ty (Figure 61), the presence of this structural motif in vertebrate Ty cannot be determined due to the presence of three Cys rich regions that have no sequence similarity to the structurally characterized CB-PPOs enzymes. Intriguingly, computational modeling of a human Ty structure predicts five Cys residues located on flexible loops that are unlikely to form disulfide bonds. While these regions are known to be essential for protein function, no direct evidence for this crosslink in vertebrate Ty has been reported. The formation of this crosslink has been shown to occur in vitro upon loading Cu(II). The formation of a disulfide linkage leads to the reduction of the coppers allowing oxygen to bind, forming oxy-Ty that is then reduced to met upon the formation of the crosslink. Mutation of this crosslinked Cys was also found to decrease kcat for L-tyrosine (59 s−1 vs 1.8 s−1 in wt vs enzyme variant) but does not completely abolish enzymatic function. Structural data indicate that this covalent linkage restricts active site flexibility since crystal structures of bacterial Ty, which lack the crosslink, have larger disorder (B factors) at CuA. Yet, how this flexibility impacts catalysis is not well understood.

The second sphere residues of all structurally determined bacterial Ty and CaOx contain a structurally conserved Glu (236 in I. batatas and 182 in S. castaneoglobisporus) that can act as an important acid/base residue during catalysis (Figure 61A). While Glu 236 is highly conserved in CaOx, the presence of a variable length, flexible loop in bacterial Ty has likely obscured the observation of this residue based on sequence comparisons. While three crystal structures of Ty (from S. castaneoglobisporus, B. megaterium, and A. bisporus) have a Glu in the vicinity in the active site, some have suggested that a His ligand could also act as a proton acceptor if it is displaced by substrate binding. However, NMR studies on a slow substrate, p-nitrophenol, indicate that all six His ligands remain coordinated to Cu(II) when this substrate binds to met-Ty bridged by a single chloride ion. Additionally, the Cys/His crosslink in fungal Ty would greatly restrict the conformational flexibility of this CuA His ligand making it unlikely to act as a base in these organisms.

Unique to CaOx is a large hydrophobic residue (Phe261 in I. batatas CaOx) that shields substrate access to the CuA site (Figure 61B). This residue is conserved in all plant CaOx except for two, which have a Leu at this position. In Ty, a smaller hydrophobic residue can be found at this position (Gly 204, Val 218, or Val 283 in S. castaneoglobisporus, B. megaterium, and A. bisporus respectively), which increases access to the CuA site (Figure 61A). This structural difference in CuA substrate access is consistent with the kinetic results from various Ty variants (Section 3.2.2). A structure of a variant with mutation to a residue in the vicinity of CuB (R209H variant in B. megaterium Ty) has also been determined. This residue is located one residue after a CuB His ligand and the wt structure with the inhibitor bound (Figure 62B vide infra) shows the position of Arg209. Kinetic characterization of this variant shows an increase in the oxygenase/oxidase activity ratio. The two different confirmations of Arg present in the wt structure suggest that this...
residue is more flexible than His209. Hence, His209 is believed to restrict access of diphenols to CuB supporting the hypothesis that monophenols bind CuA while o-diphenols bind CuB.

Three structures with inhibitors bound have also been determined. Phenylthiourea (PTU) (Figure 62) binds the two Cu atoms in CaOx from I. batatas via a bridging sulfur atom of the thiocarbamide while the nitrogen interacts with CuB (Figure 62A).498 The binding of PTU increases the Cu•••Cu separation to 4.2 Å, causes a slight rotation of Phe261, and a movement of His244 ligand to form a π-stack. The direct coordination of sulfur to the Cu in the crystal is also consistent with the observation of a sulfur to copper CT transition at 625 nm.508 Molecular modeling of o-diphenols in this orientation favors monodentate coordination of the substrate to CuB.498 Additionally, two inhibitors that both contain an α-hydroxyketone, kojic acid (KA), and tropolone (TP), have been soaked in crystals of B. megaterium501 (Figure 62B) and A. bisporus502 (Figure 62C) Ty, respectively. However, neither inhibitor interacts directly with the Cu active site, although a direct interaction with the metal centers is observed in solution with a similar inhibitor, mimosine (Figure 63), due to the observation of mimosine to Cu CT transition at 425 nm.509 Kojic acid binds to met-Ty crystals from B. megaterium with the hydroxymethyl ~7 Å from the active site, forming interactions with Phe197, Pro201, Asn205, and Arg209 (Figure 62B).501 Tropolone binds to met-Ty510 from A. bisporus in a similar hydrophobic pocket 3.0–3.5 Å from the Cu active site forming interactions with Val283, His263, and Phe264 (Figure 62C).502 However, the presence of a pre-binding site is consistent with kinetic measurements that indicate that both kojic acid and tropolone are slow-binding inhibitors.511,512 The most common mechanism that results in slow-binding inhibition involves the formation of a lower affinity enzyme-inhibitor complex that converts to a second, more stable complex.513 For these inhibitors, the structural characterized interaction is likely the non-convalent enzyme-inhibitor complex and the second binding site involves direct coordination to copper.

While the majority of CB-PPOs are structurally related to mollusc Hc, the crystal structure from an insect (M. sexta) has also been determined (Figure 64).503 The tertiary structure of insect Ty is different from either the bacteria Ty or the plant CaOx but is similar to the structure of arthropod Hc (RMSD 1.27 Å with 41.4 % amino acid identity to L. polyphemus).309 Unique to insect Ty (relative to other CB-PPOs) is the presence of three domains and a hetero dimer interface (between two isozymes, PPO1 and PPO2),503 similar to the three domains in arthropod Hc and the tight dimer interface that makes up the hexameric quaternary structure (Figure 34).309 Domain one shields the active site via Phe88 in PPO2 (Figure 64),503 which is in a similar location as the aromatic ring of PTU bound to CaOx in I. batatas498 (Figure 62A). To activate insect Ty, PPO activating protease cleaves within domain one of Ty at Arg51514,515 exposing a negatively charged surface, which is believed to dimerize with the clip domain of an auxiliary factor, the serine protease homolog.503 This interaction is hypothesized to cause a rearrangement of the tertiary structure of insect Ty, removing Phe88 from the active site,503 however the structure of this active form is unknown. Also within the vicinity of the active site in PPO2 is a basic residue (Glu395), similar to Glu236 in I. batatas CaOx (shown in Figure 61B).503 However, the structurally equivalent residues in L. polyphemus Hc309 and PPO1503 are Thr351 and
Ser393, respectively. Kinetic studies suggest that Glu395 in PPO2 may assist in the two-electron oxidase activity of insect Ty since $V_{\text{max}}$ for PPO2 is approximately five times larger that PPO1. Yet, similar tyrosinase activity is measured in the presence of small amounts of L-DOPA.

Interestingly, the proteolytic activation of plant and fungal CB-PPO occurs via a different mechanism than in insect Ty. Instead of cleaving within the shielding domain, the proteolytic cleavage of plant and fungal PPO occurs between the two domains.\(^{381}\) While the structures of the two domains in plant, fungal, and mollusc Hc are predicted to be similar from homology modeling,\(^{381,517}\) the two domains are believed to be connected by different secondary structure.\(^{381}\) In mollusc Hc, these two domains are connected by a 24 residue $\alpha$-helix,\(^{157}\) while the corresponding sequence in plant and fungal CB-PPOs is predicted to be disordered which is believed to favor proteolytic cleavage.\(^{381}\) Thus while both cleavage and a tertiary structure rearrangement are necessary to activate insect Ty, the shielding domain is directly removed by proteolytic cleavage in plant and fungal CB-PPO.\(^{381}\) A similar structural rearrangement has been proposed for the detergent induced oxidase activity in Hc.\(^{518}\) Molecular models from cryo-electron microscopy (8.0 Å resolution) suggest that domain one in arthropod Hc rotates in the presence of SDS, exposing the active site to allow substrate access.\(^{518}\)

### 3.2.4. Electronic Structure

Crystallography of Hc, CaOx, and Ty reveals a common binuclear copper active site. This common molecular structure leads to identical spectroscopic features for both $\text{oxy}$ and $\text{deoxy}$ states. The spectral features of $\text{oxy}$ and $\text{deoxy}$-Hc were described in Section 3.1.4. To summarize, the spectroscopic signature of $\text{deoxy}$ is an intense Cu(I) preedge feature at 8984 eV in the XAS. The spectroscopic signatures of $\text{oxy}$ are the $\text{O}_2^{2-} \rightarrow \text{Cu(II)}$ CT transitions at $\sim$345 nm ($\varepsilon \approx 20,000 \text{ M}^{-1} \text{cm}^{-1}$) and $\sim$580 nm ($\varepsilon \approx 1,000 \text{ M}^{-1} \text{cm}^{-1}$) which upon excitation with laser light leads to a low intraperoxide stretch at 755 cm$^{-1}$ that downshifts to 714 cm$^{-1}$ in the $^{18}$O$_2$ isotopologue, and a “$\nu_{\text{Cu} \cdots \text{Cu}}$” at 274 cm$^{-1}$ in the resonance Raman spectrum, consistent with a $\mu$-$\eta^2$: $\eta^2$ binuclear copper peroxy core (Figure 65).\(^{519}\) Both $\text{oxy}$ and $\text{deoxy}$ are EPR silent. As presented above, a third EPR silent state found during catalysis for Ty and CaOx that is not functionally relevant in Hc is called the $\text{met}$ or fully oxidized bicupric state.

$\text{Met}$-Ty and CaOx have been crystallographically characterized, and contain a water derived bridging ligand between the Cu centers (Figure 62B and 64). XAS of $\text{met}$-Ty indicates the Cu ions are in the 2+ oxidation state.\(^{520}\) However, $\text{met}$-Ty is EPR silent, indicating the Cu(II) ions are strongly antiferromagnetically coupled. Given the coupling, the water-derived bridge is likely a hydroxide anion, which would be a far more efficient superexchange pathway than a water bridge and the pKa of the proton to form a $\mu$-hydroxo bridge is estimated to be $<5$. A hydroxide anion bridge is supported by EXAFS on the $\text{met}$ form which indicates a 1.8 Å scatterer on Cu.\(^{508}\) $\text{Met}$-Ty does not absorb strongly in the visible region. A ligand field band is observed at 680 nm in the absorption spectrum, and a moderatly intense feature at $\sim$325 nm ($\varepsilon: 3500 \text{ M}^{-1} \text{cm}^{-1}$) is observed and assigned as the $\text{OH}^- \rightarrow \text{Cu(II)}$ CT transition. This feature is also present in the CD spectrum.\(^{445}\)
Despite the similarity between the active sites of Hc, CaOx, and Ty, substrate access to the sites is very different as described in Section 3.2.2. Access to the site is not the only issue, as the substrate interaction with the protein pocket at the active site of Ty and CaOx is also essential. From Section 3.2.3, crystallographic characterization of Ty with inhibitors bound display the inhibitor bound to the Cu directly or near the site in a secondary binding site. The structure of CaOx solved with the inhibitor phenylthiourea bound to the active-site in a bridging configuration between the Cu ions (Figure 62A). All other information regarding the interaction of the active-site with substrate and substrate analogs comes from spectroscopy. In a key experiment, the substrate analog mimosine was used as a probe of substrate binding in met-Ty. Upon addition of mimosine, an absorption feature was observed at 425 nm, which was assigned as a mimosine → Cu(II) charge transfer transition (Figure 66). The detection of a CT transition is significant, as it requires orbital overlap between the donor phenolate and the acceptor Cu, indicating that mimosine is bound directly to Cu. Further information comes from the EXAFS, wherein a slight increase in intensity of the dipole forbidden 1s → 3d transition was observed upon binding mimosine. This increase is consistent with a distortion of the tetragonal environment of the Cu.

As a detailed spectral probe of mimosine binding to the active site of Ty, a half-met derivative was created in an analogous manner to that for Hc (See Section 3.1.4). The half-met derivative consists of a Cu(I)/Cu(II) mixed-valent site with an overall S = ½ ground state. EPR spectroscopy of half-met Ty reveals a localized mixed-valent state, displaying a normal Cu(II) type spectrum. Upon addition of mimosine to half-met Ty, significant changes occur in the EPR spectrum (Figure 67). Specifically, the EPR spectrum becomes more rhombic with a splitting of $g_\perp$, the hyperfine coupling at $g_z$ decreases, and a large hyperfine coupling is observed on $g_x$ ($g_z = 2.290$, $g_y = 2.115$, $g_y = 2.023$, $A_z = 111.3 \times 10^{-4}$ cm$^{-1}$, $A_x = 15.0 \times 10^{-4}$ cm$^{-1}$, and $A_y = 72.0 \times 10^{-4}$ cm$^{-1}$). Taken together, these spectral changes require a substantial amount of $d_{z^2}$ mixing into the $d_{x^2-y^2}$ ground state. This mixing would occur if a trigonal bipyramidal structural perturbation accompanies the binding of mimosine to copper (Figure 7B).

In a similar manner, the binding of aliphatic and aromatic carboxylates was also investigated with the half-met Ty derivative. Whereas binding of aliphatic carboxylates produced no significant change in the EPR spectrum, binding of aryl carboxylates that are competitive inhibitors results in EPR spectral changes that are analogous to those observed in the mimosine adduct (Figure 67), which indicate a similar trigonal bipyramidal distortion. The carboxylate binding is also accompanied by the appearance of a positive CD transition at 10,000 cm$^{-1}$, the origin of which is a ligand field transition that gains intensity and lowers in energy further supporting a distortion of the Cu towards a trigonal bipyramidal geometry. Thus, substrate analogs bind directly to Cu, and when these contain aromatic substituents there is an additional interaction with the protein pocket that stabilizes a trigonal bipyramidal distortion of the site along the reaction coordinate. From the differences in binding constants (150 M$^{-1}$ for alkyl versus 1000 M$^{-1}$ for aryl substrates) the interaction with the pocket is worth ~1.3 kcal/mol. Two candidates for the origin of this stabilization are: 1) π-π interaction with a copper bound histidine or 2) interaction with the strictly
conserved Asn205 (Figure 62). However, significant efforts are required to develop an understanding of this protein pocket effect.

3.2.5. Mechanism

3.2.5.1 Diphenolase: The mechanism of Ty is described by two interpenetrating catalytic cycles that comprise monophenolase and diphenolase activity (Figure 68). The diphenolase cycle (the outer cycle in Figure 68) has a reductive and an oxidative phase and requires two substrates and one dioxygen for each turnover. In the reductive phase, the 1,2-catechol binds to met to generate met-D. The catechol substrate reduces the Cu(II) ions to Cu(I) concomitantly with release of the quinone to generate deoxy. In the oxidative phase, dioxygen binds to deoxy to generate oxy, which subsequently reacts with 1,2-catechol to generate oxy-D, finally releasing quinone and water to generate met. In essence, the enzyme is catalyzing the four-electron reduction of dioxygen to water in discrete 2 electron steps using the substrate catechol as a two electron donor without the release of hydrogen peroxide.

The structural requirements for diphenolase activity are less than those required for the monophenolase activity, as catechol oxidases are unable to perform monophenolase reactions. Nonetheless, information regarding the structural requirements of the diphenolase cycle is emerging. In the oxidative phase, oxy-D state has traditionally been drawn with catechol bridging the two Cu centers. However, in oxy, the Cu ions are efficiently antiferromagnetically coupled through the peroxide bridge, and thus the twoelectron oxidation of the catechol could proceed via coordination to a single copper. Support for this possibility comes from mechanistic studies on Ty variants (see Table 15), which distinguished effects associated with residues near the individual Cu ions. In these studies, mutations in the vicinity of CuA resulted in no effect on the catechol oxidase activity, whereas mutations to residues around CuB affected this activity. Taken together, these mutational studies suggest that the protein pocket is exerting some influence on the diphenolase reaction, and that binding to a single metal site should be considered.

3.2.5.2 Monophenolase: The monophenolase cycle (inner cycle of Figure 68) requires one phenol substrate and one dioxygen per turnover, and generates one quinone product and one water. The cycle begins with the addition of dioxygen to deoxy to generate oxy. Next, reaction of oxy with a phenol is believed to generate the enzyme-oxygen-phenol ternary complex oxy-T, which is the key intermediate in the catalytic cycle, although this has not yet been observed. Interpretations of mutagenesis studies favor coordination of the phenol to CuA since mutations of residues in the vicinity of CuA had an effect on the Ty activity (see Section 3.2.2). It is generally understood that the phenol substrates coordinate directly to a copper, rather then docking into the protein in the vicinity of the Cu2O2 core (as found for example in cytochrome P450) based on the spectral features observed upon addition of substrate analogs (Section 3.2.4) to met and half-met Ty. These spectral features require that the phenol-analogs coordinate directly to the Cu. Nonetheless, direct coordination of phenol to Cu in oxy-Tyr has not been demonstrated and model studies of Karlin and coworkers (vide infra) show that monoxygenation can occur without direct substrate coordination.
The intimate details of the attack of oxygen on the ring subsequent to oxy-T has been the subject of intense research efforts that have spanned decades. The consensus overall mechanism of the hydroxylation of the phenol ring is via EAS based on: 1) the negative slope of the Hammet parameter ($\rho$), which indicates positive charge buildup in the intermediate (Section 3.2.2), 2) correlation to the EAS mechanism of monooxygenation of phenols by cleaved mollusc Hc that proceeds with a similar Hammet parameter and an inverse $^2$H isotope effect, and 3) correlation to functional model studies (vide infra) which have been conclusively demonstrated to proceed via EAS. The remaining issue pertains to the nature of the Cu$_2$O$_2$ core in the ternary complex oxy-T, an issue raised based on observations in model studies.

Tolman and coworkers discovered that the reaction of dioxygen with certain Cu(I) model complexes resulted in a product wherein the O—O bond was broken to generate a bis-µ-oxo Cu$_2$(O)$_2$ core (Figure 69). X-ray diffraction studies demonstrated a long O⋯O (2.287 Å) and a short Cu⋯Cu distance (2.794 Å). This bis-µ-oxo core was in equilibrium with the “side-on” peroxo Cu$_2$(µ-η$^2$-O$_2$) isomer, an equilibrium that was sensitive to environmental factors including solvent and anions, indicating that these two species were necessarily close in relative free energy for the ligand systems studied. The isomerization from side-on peroxo to bis-µ-oxo is accompanied by significant spectral changes (Figure 70), which have facilitated an in depth study of the electronic structure change associated with this isomerization. An essential detail is that the pre-edge feature at 8979 eV in the side-on peroxo species that is characteristic of Cu(II) (see Section 2.2.5) shifts up in energy by 1.9 eV indicating that the oxidation state of the Cu has increased from Cu(II) to Cu(III). Thus, two electrons from Cu break the peroxo bond. The process can be understood from the correlation of the molecular orbitals upon isomerization (Figure 71). As described in Section 3.1.4, the side-on bridged peroxide species on the left has a peroxo group in which its $\sigma^*$ is accepting electron density from Cu $d_{x^2-y^2}$ HOMO (i.e. backbonding). As the O—O bond is lengthened from 1.4 Å to 2.8 Å, the $\sigma^*$ of the peroxo is decreased in energy and charge flows from the Cu(II) into this $\sigma^*$ orbital to cleave the O—O bond. In the bis-µ-oxo species on the right of Figure 69, complete transfer of a pair of electrons (one from each Cu(II)) has taken place leaving two µ-oxo groups and two Cu(III). This change in electronic structure leads to two additional diagnostic spectral changes. A new intense charge transfer transition appears at lower energy (400 nm in the bis-µ-oxo compared to 350 nm in the side-on peroxo) corresponding to an oxo → Cu(III) CT transition to the new LUMO on the Cu(III)s. Laser excitation into this band leads to a different resonance Raman spectrum, where the O—O stretch of the side-on peroxide is replaced by an intense peak at ~600 cm$^{-1}$, assigned as the symmetric Cu$_2$O$_2$ core expansion (Figure 70 bottom). These side-on peroxide and bis-µ-oxo structures provide distinct frontier molecular orbitals (FMOs), which are low lying unoccupied orbitals with significant oxygen character for good overlap with substrate, for electrophilic attack on the π density of the substrate ring. In a side-on peroxo case, the LUMO is Cu(II) based with significant O$_2^2-\pi^*_{\sigma}$ character and in the bis-µ-oxo case, the Cu(III) LUMO has significant (O$_2^2-\sigma^*$ character (Figure 71). The existence of this equilibrium in model studies raises the possibility of a bis-µ-oxo participating in Ty chemistry upon substrate coordination to the Cu in oxy-T in Figure 68.
Indeed, chemical precedence for this hypothesis has been generated by Stack and coworkers using the dbed (N,N-di-tert-butylethylenediamine) ligand framework (Figure 72).\textsuperscript{479} From resonance Raman spectra, the precursor complex is a $\mu$-$\eta^2$-$\eta^2$ peroxo $[\text{dbedCu}^{\text{III}}]_2(O_2)$ ($\nu_{\text{O-O}} = 730 \text{ cm}^{-1}$); reaction with phenolate at cryogenic temperatures (−120°C) results in phenolate binding which drives the formation of a discrete intermediate $[\text{dbedCuOPh}]^{-}(O)$-$[\text{dbedCu}]$, wherein the O—O bond is cleaved, resulting in a bis-$\mu$-oxo core, based on the loss of the 730 cm$^{-1}$ O—O feature of the side-on peroxide with concomitant growth of the 600 cm$^{-1}$ feature of a bis-$\mu$-oxo core (Figure 72 left). Upon warming, the phenol is hydroxylated; a process that has been attributed as an EAS mechanism based on its inverse isotope dependence ($k_{\text{H}}/k_{\text{D}} = 0.9$) and phenol substituent effects ($\sigma^+$ correlation, $\rho \approx 1.4$). Monitoring this process by resonance Raman spectroscopy indicates the loss of bis-$\mu$-oxo at 600 cm$^{-1}$ and the growth of the carbonyl stretch of the oxygenated product, demonstrating that the bis-$\mu$-oxo is directly responsible for oxygenation of the ring (Figure 72 right).

DFT calculations correlated to the spectral data have been performed to quantitatively assess the reaction coordinate.\textsuperscript{523} The phenolate binds axially to the side-on perooxo core, and undergoes a rotation into the equatorial plane, which drives the formation of the bis-$\mu$-oxo isomer. This orientation is suitable for proximal attack on the ortho position of the ring. Inspection of this process reveals that the frontier molecular orbital (the LUMO) that is available for EAS in bis-$\mu$-oxo complexes is the antisymmetric combination of the Cu 3d$_{x^2-y^2}$ orbital which contains the oxo $\sigma^*$ character (Figure 73 left). Two electrons from the ring are transferred to reduce the Cu(III) to Cu(II), forming the EAS sigma complex (Figure 74). Finally, the reaction trajectory is completed by deprotonation of the ring to yield a bidentate bridged catecholate species. Taken together, these studies demonstrate that it is feasible for a bis-$\mu$-oxo to be the reactive intermediate during tyrosinase catalysis, formed by coordination of phenolate to the Cu with substrate rotation into the equatorial plane.

Earlier, Karlin and coworkers demonstrated that Cu-peroxo adducts were also competent for EAS to yield phenols.\textsuperscript{524–526} They synthesized a scaffold based on a $m$-xylyl bridge to generate the $\mu$-$\eta^2$-$\eta^2$ peroxo complex $[\text{Cu}_2(\text{NO}_2-\text{XYL})O_2]^+$ (Figure 75). This complex, upon warming, hydroxylates the xylyl tether. The rate of oxidation is dependent on the substituents on the tether, which correlates to the Hammett $\sigma^+$ parameter with a $\rho$ value of ~2.1. When the reactive ring carbon position is methylated, an NIH shift was observed.\textsuperscript{525} These observations strongly indicate an EAS mechanism. The reaction has been monitored spectroscopically by resonance Raman (a 750 cm$^{-1}$ O—O stretch is observed in this $\mu$-$\eta^2$-$\eta^2$ peroxo; no 600 cm$^{-1}$ stretch of a bis-$\mu$-oxo is present, therefore there is no evidence for involvement of this species along the reaction), and as shown in Figure 76, the disappearance of the peroxo structure was coupled to the growth of C-O stretch of the phenolate product. These studies indicated that the $\mu$-$\eta^2$-$\eta^2$ peroxo core is also competent for EAS of exogenous substrates. Calculations were performed on this model system to evaluate the reaction coordinate. The side-on peroxo attacks the ring to generate an sp$^3$ hybridized carbon, followed by electron transfer from the ring directly in the peroxo. Importantly, the transfer of the electrons proceeds without intermediacy of a bis-$\mu$-oxo along the reaction coordinate.
Given these two model systems that demonstrate both bis-μ-oxo and side-on peroxo cores are competent for the EAS of phenols, the timely question is which does Nature utilize as the active oxidant in Ty and what are the factors that control this selection. The lack of a trapped intermediate state with phenol bound to the oxy core has motivated theoretical investigation into the likelihood of the peroxo/bis-μ-oxo equilibrium in Ty. DFT studies deemed the bis-μ-oxo to be prohibitively uphill from the μ-η²,η², and thus an unlikely intermediate. However, different functionals suggest this possibility is feasible. It is useful to consider the structural factors required to direct the core towards peroxo or bis-μ-oxo descriptions, and the impact of the protein pocket on structure. One factor that derived from the studies on the binuclear copper complexes in Figure 74 is the rotation of the phenolate donor ligand into the equatorial plane as being essential for bis-μ-oxo formation. It could be envisioned that the protein pocket controls the orientation of the phenol relative to the Cu₂O₂ core. In this context, two observations provide insight. First, in the binding of aliphatic versus aromatic carboxylates to met-Ty it was found that substrate binding is favorable in the aromatic case by an additional ~1.5 kcal/mol due to interactions of the phenol ring with the active site pocket. Second, the EPR spectrum of mimosine bound half-met Ty (Figure 67) showed a significant trigonal bipyramidal distortion about the copper, indicative of the active-site influence on the orientation of the phenol. These observations suggest that the pocket influences the orientation of the phenol prior to and during electrophilic attack and the key issue is whether a major rearrangement of the substrate occurs before the transition state of the EAS.

To complete the monophenolase catalytic cycle, the face of the phenol ring rotates towards the oxygen of the core to orient the ortho carbon into position for electrophilic attack. During the attack, the electrons are transferred to the dioxygen (or to the Cu’s directly in the case of a bis-μ-oxo oxidant) and the C—O bond is formed in a sigma complex. The proton is removed from the ring to the distal O to yield an orthodiphenolate that bridges the Cu(II) ions, perhaps asymmetrically as in Figure 74. Further protonation of the resulting μ-OH to H₂O leads to the reduction of the Cu ions and a release of quinone, thus reforming the deoxy state.

A final observation of the hydroxylation of phenols by synthetic complexes is that the hydroxylation occurs when phenolate, rather then phenol, is reacted with the Cu₂O₂ cores. Reaction of the synthetic copper dimers with phenol does not result in the hydroxylation product, but instead results in the radical coupling products, suggestive of H• abstraction competency of Cu₂O₂ cores in addition to EAS. The relevance of this synthetic observation for tyrosinase catalysis is unclear but suggests an intimate relationship between protons and mechanism in Ty catalysis. The identity of the associated active site base has been the subject of investigation, with three possible candidates emerging. First, it has been proposed that the bridging peroxo itself is basic, a hypothesis that is contradicted by model studies wherein side-on peroxo adducts have been shown to be electrophilic, rather then nucleophilic. Second, it has been proposed that a Cu bound His could dissociate and be the destination of the proton. However it is noteworthy that the His believed to be involved is in fact crosslinked to a nearby Cys residue in nearly a third of the Ty homologs, and a crosslink would be expected to rigidify this residue. In addition, the dissociation of His from the Cu is expected to be a thermodynamically uphill process. Finally, a third possible
base that is somewhat underexplored in the literature is a conserved glutamate in close proximity to the Cu$_2$O$_2$ core (see Section 3.2.3). Regardless of the identity of the base, the issue of proton movement is important, based on the observation of a solvent $^2$H isotope effect (Section 3.2.2).

### 3.2.5.3 Hydroxyanilinase:

Reaction of o-aminophenol with Ty and CaOx yields the two electron oxidation product, o-iminoquinone, in a reaction analogous to diphenol oxidation by CaOx. Recently it was found that a fourth member of the coupled binuclear copper family (NspF) catalyzes an alternative reaction, the oxygenation of o-aminophenol to nitrosophenol, reactivity coined hydroxyanilinase activity. Comparison of the hydroxyanilinase versus CaOx activities in NspF versus a close homolog GriF and Ty revealed that the difference in selectivity leading to the oxygenated nitrosophenol product was a result of differences in the oxygenase (hydroxyanilinase) activity rather than differences in the oxidase (CaOx) activity (see Section 3.2.2).

This observation was suggestive of protein secondary coordination impacts on the reaction landscape rather than intrinsic differences in the reactivity of the Cu$_2$O$_2$ cores, which appear indistinguishable spectroscopically. The reaction trajectories were evaluated by DFT and further supported this secondary coordination environment control hypothesis, in that the structural requirements for the hydroxyanilinase reaction differed from that of either oxygenation of monophenols or oxidation of diphenols (Figure 77). Specifically, the transition state for oxygenation of o-aminophenols by an electrophilic attack mechanism required a six member ring, whereas oxygenation of monophenols requires a five member ring. Thus, the substrate orientation is significantly different in the transition state, which would be expected to be a major factor in controlling the selectivity of the reaction as the protein pocket must accommodate this different in substrate orientation. Details regarding which specific residues direct the reaction landscape require a crystal structure of NspF, but the coupled binuclear Cu family of enzymes provide an important series where the protein pocket different types of reactivity utilizing equivalent Cu$_2$O$_2$ active sites.

### 3.3 O$_2$ Activation for H-atom abstraction: Non-coupled Binuclear Copper Enzymes (role of exchange coupling in reactivity)

#### 3.3.1 Enzymology—

In addition to the coupled binuclear copper enzymes Tyrosinase, Catechol Oxidase and NspF, there exists another class of binuclear copper proteins where the coppers are at a distance of ~11 Å with no bridging ligands such that they do not electronically couple. These enzymes, the non-coupled binuclear copper enzymes, accomplish activated C-H bond hydroxylation for the synthesis of physiologically important neurotransmitters and hormones. Three enzymes comprise this class: the well studied eukaryotic proteins dopamine $\beta$-monoxygenase (D$\beta$M; EC 1.14.17.1) and peptidylglycine $\alpha$-hydroxylating monoxygenase (PHM; EC 1.14.17.3) and the recently identified insect tyramine $\beta$-monoxygenase (T$\beta$M).

The non-coupled binuclear copper enzymes catalyze the regio- and stereospecific hydroxylation of secondary C-H bonds (Figure 78). This hydroxylation is a two-electron oxidation, which is coupled to the reduction of dioxygen to yield one H$_2$O and the incorporation of the second oxygen into the substrate therefore these enzymes are classified...
as monooxygenases. Two electrons come from the substrate and the second two electrons come from the oxidation of two equivalents of ascorbate to semidehydroascorbate. These enzymes have two coppers per subunit. On the basis of structural and spectroscopic studies (vide infra), the coppers have different coordination environments, either Cu_H (or Cu_A) and Cu_M (or Cu_B). Cu_M is the site where dioxygen is activated and substrate binding occurs while Cu_H serves as an electron transfer site.

DβM, a glycoprotein found in mammalian neurosecretory vesicles of the adrenal gland, carries out the catalytic conversation of dopamine to norepinephrine (Figure 78) in the catecholamine biosynthetic pathway. DβM activity is physiologically critical for the regulation of dopamine and epinephrine. DβM genetic deficiency in humans results in patients being unable to synthesize norepinephrine and epinephrine resulting in severe hypotension. Additionally, in mouse studies it has been found that knocking out the DβM gene results in fetal death in utero, verifying that DβM is also essential for fetal development. Inhibitors have been developed for DβM as antihypertensive agents in effort to limit the production of norepinephrine. The first compound to reach clinical testing was fusaric acid, but most modern inhibitors are based on an imidazole-2-thione scaffold and are significantly more potent. DβM is also capable of carrying out hydroxylation chemistry on a series of phenylethylamine compounds similar to dopamine. Interestingly, the presence or absence of the phenol –OH group does not impact turnover suggesting that aryl alcohol group does not constructively interact with the protein (Figure 78). DβM is also capable of catalyzing various non-native oxidation reactions including sulfoxidation, selenoxidation and epoxidation of olefins. The protein has been isolated from a number of mammalian sources, most studied of which are bovine and rat. The protein exists in both soluble and membrane-bound forms. Soluble DβM can be either a dimer or tetramer consisting of two and four identical 73-kDa monomeric subunits, respectively. The membranebound enzyme is exclusively a tetramer and is composed of two soluble-like subunits of 73-kDa and two other distinct 77-kDa subunits. It is thought that this protein exists in equilibrium between the membrane-bound and soluble forms where the soluble form is released into the blood and cerebrospinal fluid.

TβM catalyses the hydroxylation of tyramine to octopamine (Figure 78), a crucial neurotransmitter that is responsible for a wide variety of physiological functions including neuromuscular transmission, behavioral development and ovulation. TβM is a homologue of DβM exhibiting 39% and 55% sequence identity and similarity, respectively. It has been isolated from Lobster, Moth and Drosophila, but little is understood about the enzyme in vivo. Recombinant TβM (rTβM), from Drosophila, has been expressed and used in site-directed mutagenesis studies (vide infra). rTβM exists in solution primarily as a ~69 kDa monomer with small amounts of dimer.

PHM exhibits 32% sequence identity with DβM and is one component in the bifunctional membrane-bound enzyme peptidylglycine α-amidating monoxygenase (PAM), which facilitates hydrolytic amidation of peptide hormones. PAM is capable of carrying out alternative monooxygenase reactions including sulfoxidation, amine N-dealkylation and O-dealkylation. Peptide amidation occurs in two steps. The first is accomplished by PHM that hydroxylates the α-carbon of a C-terminal glycine-extended peptide (Figure 78). This is
followed by hydrolytic amidation of the geminal aminoalcohol by peptidyl-α-hydroxyglycine-α-amidating lyase (PAL) yielding the activated N-terminal peptide hormone. This process is crucial in the activation of hormones since over half of all peptide hormones must be α-amidated to be bioactive. PAM has been isolated from a number of mammalian sources including bovine, mouse, and rat and is found in Drosophila. Deletion of the PAM gene in Drosophila results in the absence of amidated peptides and leads to late embryo or early larva death. Additionally, PAM knockout mice died midgestation consistent with the amidated peptides being crucial for fetal development.

Rat PAM (~120 kDa) undergoes endoproteolytic cleavage to give a soluble PHM domain of 45 kDa and a membrane-associated (70 kDa) or soluble (50 kDa) PAL domain where each domain was catalytically active in their respective reactions. This recombinant form has been used in site-directed mutagenesis experiments (vide infra). An important development in the study of this enzyme came from the development of a recombinant form of PAM from rat. A 35 kDa protein was isolated, exhibiting only PHM activity, denoted as the PHM catalytic core (PHMcc). This is the predominant form used in kinetic, crystallographic and spectroscopic studies (vide infra).

As stated above, these proteins contain two copper ions per subunit in different coordination environments, which are suggested to have different roles in catalysis. Blackburn has succeeded in producing a ‘half-apo’ form of PHM. This was accomplished by using CN− to remove Cu from the protein with CO bound selectively to CuM (vide infra). For DβM the tetrameric form can exist as either an 8 Cu or 4 Cu form, where the 4 Cu form is thought to contain only CuM. Whether this form is catalytically active has been the subject of debate. Another human protein was found to exhibit high sequence similarity to these enzymes and classified as a member of this family. Named monoxygenase-X (MOX), this enzyme is the least well-understood member as its chemistry and function are still unknown. MOX is not secreted, as is DβM, but membrane bound in the endoplasmic reticulum of both endocrine and non-endocrine cells and highly expressed in the salivary gland and ovaries.

Together, these enzymes exhibit distinct physiological functions but are complimentary in their stereospecific hydroxylation chemistry. The following sections deal with the similarities and differences in reactivity of these proteins and how they relate to the mechanism of substrate hydroxylation and dioxygen activation by the non-coupled binuclear copper sites. We then correlate their reactivity to that of the coupled binuclear Cu enzymes and consider the role of exchange coupling in determining reactivity.

3.3.2 Kinetics—The enzymes in this group exhibit similar kinetics and therefore equivalent mechanisms. Generally, two molecules of ascorbate reduce the coppers of the fully oxidized resting state in a ping-pong mechanism (vide infra)–giving the reduced enzyme and two molecules of semidehydroascorbate. Substrate and dioxygen then bind to the reduced enzyme yielding a ternary complex from which dioxygen activation and substrate hydroxylation occur to give product and oxidized enzyme.

3.3.2.1 Steady State Kinetics: Extensive steady-state kinetic studies, including isotope effects, have been performed on all three of the enzymes in this group. Under conditions of
excess ascorbate where the reduction of the enzyme is facile, the two steady-state parameters are the catalytic efficiency ($k_{cat}/K_m$), which accounts for all reversible steps leading to the first irreversible step (i.e., the C-H abstraction step) and turnover number ($k_{cat}$), which includes all steps after the first irreversible step leading to product and resting enzyme. The perturbation of these kinetic parameters is important for the discussion of isotope effects later in this section.

These proteins turnover in the presence of substrate, ascorbate and dioxygen. Dioxygen dependent steady-state kinetics for these enzymes are comparable where all enzymes exhibit $K_m(O_2) \approx 50–500 \, \mu$M. The dependencies on substrate will be discussed in terms of the most studied substrates of the respective enzymes. The substrate dependent steady-state parameters are enumerated in Table 18. It is found that these enzymes have comparable $k_{cat}$ and $K_m$ values. It should be noted that hippuric acid is the smallest substrate and most versatile for isotope substitution for PHM (vide infra), however, many glycine terminal peptides are found to be catalytic for PHM (e.g., Tyr-Val-Gly). These extended peptides bind tighter to PHM ($K_m \sim 10 \, \mu$M), which may be due to constructive protein-substrate interactions absent in hippuric acid. Additionally, the substrate dependence on enzyme activity is found to exhibit Michaelis-Menten steady-state kinetics for DβM and PHM, while TβM is inhibited ($K_i = 3.5 \, \text{mM}$) by substrate at tyramine concentrations greater than 250 µM. It is suggested for this enzyme that tyramine binds to the oxidized state since enzyme inhibition diminishes with increasing ascorbate, generating more reduced enzyme. This is also supported by an increase in inhibition with additional dioxygen, generating more oxidized enzyme. These enzymes exhibit similar $K_m$ values for ascorbate and a ping-pong mechanism for enzyme reduction. PHM exhibits an apparent ascorbate $K_m(asc) = 0.25 \, \text{mM}$, where increasing the ascorbate concentration leads to an increase in both $k_{cat}$ and $K_m$ for substrate (Tyr-Val-Gly). Therefore, the rate of enzyme reduction increases the rate of substrate turnover. Interestingly, no ascorbate is found to be bound in the reported crystal structures for PHM, where reductant is in significant excess (vide infra). In contrast to PHM, DβM turnover velocities decrease with increasing ascorbate fitting to negative cooperatively with a Hill coefficient in the range of 0.15 – 0.30. This is reflected in the increase of apparent $K_m(asc)$ of 0.05 – 0.10 mM at high ascorbate concentration to > 10 mM under limiting reductant concentration. Due to the substrate inhibition of TβM, only an estimated $K_m(asc) \geq 16 \, \text{mM}$ was obtained. Additionally, it was found that $k_{cat}/K_m(asc)$ decreases with added tyramine, which is not observed in the other two enzymes. These results, along with the substrate inhibition, indicate that tyramine competes with ascorbate for binding to the oxidized enzyme. Therefore, an ascorbate binding site can be invoked for TβM where no such a binding site has been found for PHM and DβM.

Significant mechanistic insight has come from the substrate primary isotope effect on $k_{cat}$ and $V_{max}/K_m$. (Table 19). The isotopic perturbations do not significantly alter $k_{cat}$ indicating that C-H activation is not rate limiting. Furthermore, dioxygen $^{18}$O kinetic isotope effects on $k_{cat}/K_m$ values (table 20) values are consistent with dioxygen reduction also not being rate limiting since they are below the maximum equilibrium isotope effect for the formation of a superoxo (1.0339). Interestingly, with deuteron-substrate, the
measured $^{18}$O isotope effect for both PHM and DβM increases implying that changes in the dioxygen bond order are coupled reversibly to the C-H activation step. If dioxygen activation were irreversible, the magnitude of the $^{18}$O isotope effect would be independent of substrate deuteration. In other words, O-O bond cleavage can only occur after H-atom abstraction (vide infra).

Since the isotope perturbations do not significantly impact turnover, in contrast to the expected behavior for an enzymatic process where C-H and/or O-O activation would be rate limiting, dissociation of product has been proposed as the likely rate determining process in catalysis. This is consistent with the fact that enzyme reduction and reoxidation can be ruled out as rate determining based on single turnover studies, where the latter process includes the intramolecular electron transfer step (vide infra). 578

### 3.3.2.2 Dioxygen and Substrate Order:

To determine the order of the reaction of substrate and dioxygen in catalysis, two general methods were employed. The first comes from the interpretation of the intercepts of reciprocal (1/ vs 1/[O$_2$]) plots where varying amounts of reactants change the intercepts depending on the formation of the ternary complex. The other, developed by Klinman measured the substrate primary isotope effect on $k_{cat}/K_m$(substrate) as a function of dioxygen concentration. Since this parameter contains all rates up to the first irreversible step ($k_5$ in Figure 79), which is the step that exhibits the isotope effect, the effect of varying dioxygen on the isotope effect will reflect the binding of dioxygen to the ternary complex relative to the labeled substrate.

For PHM, the substrate and dioxygen binding order was found to indicate an equilibrium ordered mechanism (Figure 79 top). The intercepts of the initial velocity measurements in the plot of 1/ vs 1/[O$_2$] showed no variance with substrate consistent with a mechanism where dioxygen binds to the enzyme after substrate. The isotope effect on $k_{cat}/K_m$(hippuric acid) was invariant with respect to dioxygen (and $k_{cat}/K_m$(O$_2$) showed no variation with hippuric acid). This was also consistent with an equilibrium ordered mechanism where the dissociation of substrate ($k_2$) is faster than that of dioxygen binding ($k_3$O$_2$).

Alternatively, the mechanism was found to be random for DβM and TβM (Figure 79 bottom). For both proteins, the plots of 1/ vs 1/[O$_2$] intercept change with varying substrate concentrations all to the left of the 1/ axis, consistent with either substrate or dioxygen having the ability to bind first to the reduced enzyme Additionally, the isotope effect on $k_{cat}/K_m$(substrate) ($^2$H for tyramine with TβM and $^3$H for dopamine with DβM) varies with dioxygen concentration and remains above 1.0 at maximal O$_2$ concentration. Since the isotope effect remains above 1.0, it is implied that both dioxygen and substrate are capable of dissociating from the ternary complex, consistent with a random mechanism.

### 3.3.2.3 C-H hydroxylation:

As mentioned above, the primary isotope effect on $k_{cat}$ is minimal and implies that C-H hydroxylation is not rate limiting in catalysis. However, it is still possible to obtain the ‘intrinsic’ kinetic isotope ($^3$D) effect for this step in a method developed by Northrop using the tritium and deuterium isotope effects on V/K under identical conditions (equation 36).
The intrinsic isotope effects for PHM and DβM (table 21) are remarkably similar and imply that the complex for C-H activation is identical in both enzymes. Using this intrinsic primary isotope effect, the microscopic rate for C-H activation can be modeled and the values for both enzymes are comparable. The intrinsic secondary deutero α-isotope effects are also found to be essentially identical. Additionally, using the temperature dependence on the intrinsic isotope effect ($D_k$) in PHM afforded an estimation of the $\Delta H^{\ddagger}$ for C-H cleavage to be $\sim 13$ kcal/mol and a $\Delta G^{\ddagger}$ can be calculated from transition state theory for C-H cleavage to be $\sim 14$ kcal/mol. Using $k_{\text{cat}}$ for these overall reaction conditions, an activation free energy of $\sim 17$ kcal/mol for the overall rate determining process (likely product release).

### 3.3.2.4 Dioxygen and Substrate Coupling:

The kinetic relationship between substrate oxidation and dioxygen reduction came from a study on DβM where substrates with a $\sim 10^3$ fold range of $k_{\text{cat}}/K_m$ all showed $\sim 1.0$ dioxygen consumption per product formed. This establishes that dioxygen and substrate are ‘tightly coupled’ in catalysis and suggests that no partially reduced dioxygen species (e.g., hydrogen peroxide) are ‘leaked’ during turnover, which would result in values $< 1.0$. It was argued on this basis that a Cu-hydroperoxo species is unlikely the primary oxidant in catalysis. This is strongly supported by electronic characterization of a Cu-hydroperoxo model complex where the results clearly indicate that this species is not activated towards C-H abstraction (vide infra).

### 3.3.2.5 Single Turnover:

Further insight concerning the mechanism of these enzymes came from single turnover experiments on DβM. Kinetics on the reduction of the copper sites in DβM came from EPR freeze quench studies. Reduction of DβM fit to a first order rate of 250 s$^{-1}$. Steady state measurements at these conditions (16 s$^{-1}$), and those noted previously are an order of magnitude slower and therefore enzyme reduction is not rate limiting in catalysis.

In order to examine the kinetics of enzyme reoxidation, DβM was stoichiometrically reduced with ascorbate and then combined with tyramine substrate. It is noted that pre-reduced enzyme does reoxidize in air, but on the minute time scale. It was measured by EPR that in the presence of substrate the enzyme reoxidizes at a maximal rate of 82 s$^{-1}$. This confirms two aspects of the oxidation of the enzyme and substrate hydroxylation: (1) in the absence of substrate the enzyme reacts with dioxygen nearly 1000-fold slower confirming that substrate accelerates dioxygen reduction, and (2) that enzyme reoxidation is also not rate limiting in catalysis (Table 18).

### 3.3.2.6 Possible Cu-O$_2$ species:

The species responsible for C-H abstraction has been a debated topic for many years. To this end, the aforementioned kinetics studies are able to eliminate a few possibilities. The first intermediate proposed to perform this reaction was a Cu(II)-hydroperoxo species. From the kinetics on dioxygen-substrate coupling, the argument that no decoupling of dioxygen reduction and product formation has provided evidence that a Cu(II)-hydroperoxo is likely not the primary oxidant. Furthermore, a
Cu(II)-hydroperoxo species has been studied experimentally in a model complex and is not activated towards C-H abstraction. Another relevant intermediate discussed recently is a [Cu-O]⁺ oxyl species. From O-O cleavage bond energetics, it is expected that the formation of this is very energetically unfavorable and likely catalytically inaccessible. Furthermore, the ¹⁸O isotope effects increase with deutro-substrate indicating that dioxygen reduction is coupled to C-H activation. Therefore, the O-O bond cannot be cleaved prior to C-H activation. The intermediate that best fits the kinetic and experimental models (vide infra) is a Cu-superoxo species.

In summary, kinetic studies mostly by Klinman have been paramount in the development of the mechanism of the non-coupled binuclear copper enzymes. Overall, the kinetics derived mechanism (Figure 80) of these enzymes involves initial reduction (k_red) of the resting enzyme by ascorbate to give the reduced state. This then binds substrate and dioxygen, random in DβM and TβM and equilibrium ordered in PHM, to give the ternary complex. From here the dioxygen bond is activated to form a putative Cu(II)-O₂⁻ (vide infra) and is coupled to the abstraction of a hydrogen atom from substrate (k_C-H). The following step is either (pathway 1) hydroxyl transfer to form product (i.e. O-O bond homolysis) followed by electron transfer from Cu_H to Cu_M (k_et) (vide infra) or (pathway 2) electron transfer that occurs with protonation and heterolytic O-O cleavage followed by substrate radical coupling to a Cu-O. radical species. The pathway for the electron transfer has yet to be determined and has been subject of debate (vide infra). Both mechanistic pathways yield the oxidized enzyme with substrate still bound, to Cu_M in pathway 2 versus protein bound in pathway 1. The dissociation from the enzyme (k_off) is suggested to be the rate determining process in catalysis. This mechanism will be further elaborated with information from spectroscopic and electronic structure studies (section 3.3.4 and 3.3.5). From a physiological standpoint, a rate determining product release implies that these proteins function not only to produce their respective biological products but also in product regulation.

3.3.3 Structure—Important advances in the understanding of these enzymes came with the determination of various crystal structures of PHMcc by Amzel and coworkers and are summarized in Table 22. Due to the high sequence identity among these enzymes, the structure of PHMcc has been interpreted generally for all of the proteins in this class.

The overall structure of PHMcc has two domains consisting of about 150 residues each. The protein secondary structure is mostly composed of β-strands (Figure 81 top) with one copper cofactor per domain. The two coppers are separated at a distance ~ 11 Å, which is consistent with the spectral features indicating no magnetic coupling (Figure 81 bottom). Interestingly, both coppers are solvent exposed where a water filled cleft separates the metal sites and provides easy access for substrate to the active site. The coppers are differentiated on the basis of their protein derived coordinating ligands. Cu_H is bound by three histidines (107, 108 and 172) while the other copper, Cu_M, is bound by two histidines (242 and 244) and one methionine (314) (Figure 81 bottom). Both oxidized and reduced forms of PHMcc have been crystallized and show changes at the copper centers. In the oxidized form, Cu_M is also coordinated by a solvent derived aquo ligand, presumably hydroxo, which is absent in the reduced form. Cu_H also has an aquo derived ligand in the oxidized state based...
on spectroscopic studies\textsuperscript{539} (\textit{vide infra}); this ligand is not observed in the reduced structure, which has a trigonal planar geometry. The overall protein structure does not change considerably between the reduced and oxidized forms. Interestingly, the reduced structure, obtained with high ascorbate concentration, shows no evidence for ascorbate bound to the protein. The structure of these sites will be further refined by spectroscopic studies in the following section.

Another series of crystal structures have described the nature of substrate interaction with the protein.\textsuperscript{584} Oxidized PHMcc was crystallized in the presence of a substrate, N-\textit{\textalpha{}}-acetyl-3,5-diiodothyrosylglycine (Ac-DiI-YG, IYG). The resulting crystal structure (PDB code 1OPM) shows the substrate bound near the Cu\textsubscript{M}, which supports the idea that this copper is the site of substrate hydroxylation. More recently, the crystal structure of a pre-catalytic complex (Figure 82A) was solved at a resolution of 1.85 Å (PDB code 1SDW) with the slow substrate N-acetyl-diiodothyrosyl-D-threonine (IYT) bound near Cu\textsubscript{M}.\textsuperscript{125} Interestingly, a dioxygen derived moiety is observed bound to Cu\textsubscript{H} in an end-on mode (Figure 82 B, O-O of 1.23 Å). The IYT substrate is anchored to the protein via a salt bridge to Arg240, a hydrogen bond to nearby Tyr318 and a hydrogen bond from the side chain amide of Asn316 (Figure 82C). Although the Cu(I) oxidation state was thought to be appropriate as the crystal structure was under reducing conditions, since the crystal was exposed to molecular oxygen, the oxidation state of the copper and dioxygen derived moiety are ambiguous. Additionally, the dioxygen moiety is poised, upon rotation by 110\degree of its distal oxygen, at a distance of ~ 2.2 Å to abstract the \textit{Pro-S} hydrogen of the glycine residue (Figure 82D). The findings in this structure support the idea that an end-on Cu(II)-superoxo activated species is formed at Cu\textsubscript{M} and is oriented such that it can abstract the relevant H-atom in catalysis.

Recent investigations have lead to crystal structures of PHMcc with small molecules bound to Cu\textsubscript{M}.\textsuperscript{601} Crystals of PHMcc were soaked with excess NO\textsubscript{2}\textsuperscript{\textendash}, N\textsubscript{3}\textsuperscript{\textendash} and CO (3 atm), and show the exogenous ligands only bound to Cu\textsubscript{M}. These results are consistent with FTIR studies of CO binding to the reduced enzyme, where CO only binds to Cu\textsubscript{M}\textsuperscript{574}. It is suggested that, since Cu\textsubscript{H} functions to transfer electrons to Cu\textsubscript{M}, Cu\textsubscript{H} must maintain its redox potential in a narrow range and therefore does not bind exogenous ligands. However, these results are not consistent with the spectroscopic findings from solution samples that show both N\textsubscript{3}\textsuperscript{\textendash} and NO\textsubscript{2}\textsuperscript{\textendash} bind to both the Cu\textsubscript{M} and Cu\textsubscript{H} sites.\textsuperscript{539} This likely reflects the higher concentration of exogenous ligands used in the spectroscopic studies and/or the difference in sample preparation between the spectroscopic studies and the crystallization.

These crystal structures have provided (1) the identity of the endogenous ligands that bind the copper centers, (2) insight into the distance between the two coppers, (3) the substrate binding mode, and (4) the possibility of having an activated Cu/O\textsubscript{2} intermediate poised to perform H-atom abstraction in catalysis. The next section reviews the electronic structure of these sites and possible reactive Cu/O\textsubscript{2} intermediates as candidates in the enzyme mechanism.
3.3.4 Electronic Structure and Spectroscopy

3.3.4.1 Enzyme Spectroscopy: First, the spectroscopy of the reduced Cu(I) state will be considered. As mentioned in section 2, this is limited to only a few methods (XAS, XES and FTIR of carbon monoxide binding). EXAFS on reduced DjβM indicates that the protein has two coppers with an average of ~ 2–3 histidines at ~ 1.93 Å with one Cu bound to a methionine at 2.25 Å and ~ 1–2 aquo derived ligands.602 These results are analogous to those observed for PHM603 and TβM.604 Additionally, copper pre-edge and EXAFS data on the reduced M314I variant in PHM indicate the loss of a copper, likely CuM.605 This implies that the methionine ligand is essential for stabilization of the reduced CuM site. This is consistent with the observation of no activity of the M314I variant. Carbon monoxide (CO) binds the Cu(I) ions in both PHM and DjβM with a Cu:CO stoichiometry of 1:0.5, indicating that CO only binds to one copper.574 EXAFS of this form shows that CO binds to CuM. The enzyme-CO complexes exhibit analogous C-O stretches at ν(CO) = 2089 cm\(^{-1}\) and 2093 cm\(^{-1}\) for DjβM and PHM, respectively, which show only limited backbonding from Cu into the CO.574,603 A reduced half-apo derivative of DjβM has also been characterized (prepared by dialysis of the CO bound form in the presence of CN\(^-\)). EXAFS on this derivative shows one copper with 2 histidines at 1.99 Å, one methionine at 2.25 Å and a solvent derived ligand weakly bound at 2.53 Å. The presence of the methionine ligand indicates that the copper remaining is CuM.574 EXAFS on this form also shows that CO binding displaces the weakly bound ligand, and the FTIR spectrum shows a C-O stretch at ν(CO) = 2089 cm\(^{-1}\), which is identical to the fully metalated enzyme.574 These results confirm that CO binds to CuM, the dioxygen binding site. Using the XAS data and DFT calculations based on the crystal structures, reduced Cu(I) resting state geometries were obtained.539 The resulting CuM structure is tetrahedral with two histidines, an axial methionine and a water ligand (Figure 83A). CuH is in a trigonal planar geometry ligated by three protein derived histidines and no solvent ligand (Figure 83B).

The oxidized state of these enzymes contains two Cu(II) ions and is accessible to a larger range of spectroscopic studies (Section 2.2). EXAFS on the oxidized state of DjβM exhibits an average of ~ 2–3 histidines at 1.99 Å and ~ 1–2 solvent derived O ligands per copper at 1.94 Å.602 Interestingly, there is no observed methionine bound to copper, implying that the Cu-SMet bond has elongated or been broken upon oxidation. Ligand field (LF) transitions (i.e., d→d transitions) are essential in describing the geometry of Cu(II) sites. The LF region of the CD and MCD spectra of resting PHMcc gives two transitions at 12,200 cm\(^{-1}\) and 16,700 cm\(^{-1}\) that form a pseudo-A term (derivative shape) in the MCD spectrum (Figure 84A).539 These transitions do not shift upon binding of substrate (acetyl-Tyr-Val-Gly, AcYVG) implying that substrate binding does not perturb the copper ions, consistent with the EXAFS of substrate bound to DjβM and the IYG bound crystal structure. The EPR spectrum of oxidized PHMcc exhibits only one resolvable signal (g\(_z\) = 2.288; A\(_z\) = 157 × 10\(^{-4}\) cm\(^{-1}\) (Figure 84B)) that integrates to 2 Cu(II) ions and exhibits g\(_z\) > g\(_x,y\) > 2.0, confirming that both coppers have d\(_{x^2-y^2}\) ground states. In attempt to differentiate the Cu centers, nitrite (NO\(_2^-\)) and azide (N\(_3^-\)) were bound to the Cu(II) sites.539 Azide binding slightly shifts the two LF transitions (Figure 84C) and new transitions are observed in the charge transfer (CT) region at 21,000 cm\(^{-1}\) to 30,000 cm\(^{-1}\) (Figure 85). Azide bound to a single copper can only exhibit two CT features from the highest occupied π\(^{nb}\)_Γ and π\(^{nb}\)\(_\sigma\)
MOs of azide. Since the CT spectrum resolves into three bands (29,600 cm\(^{-1}\), 25,550 cm\(^{-1}\) and 21,950 cm\(^{-1}\)), azides must bind to both coppers. The 29,600 cm\(^{-1}\) and 25,550 cm\(^{-1}\) features are assigned to \(\pi_{nb}\) (the non-bonding MO of the azide that \(\sigma\)-bonds to the copper) to Cu(II) CT transitions for Cu\(_M\) and Cu\(_H\), respectively (since Cu\(_M\) has a higher coordination number and therefore higher energy Cu d orbitals then Cu\(_H\)). The third transition at 21,950 cm\(^{-1}\) is of lower intensity and can be assigned to a \(\pi_{nbv}\) (where the \(v\) indicates the azide orbital perpendicular to the Cu-azide plane) CT to one of the Cu(II) sites due to the limited orbital overlap. Thus the absorption/CD/MCD spectra show that azides are bound to both Cu centers. The X-band EPR spectrum of azide bound PHMcc (Figure 84D) shows only one tetragonal Cu(II) EPR signal with a smaller \(g_z = 2.246\). NO\(_2^-\) binding results in significant perturbation of the MCD spectrum (Figure 84E). Six transitions were observed in the LF region and since a single Cu(II) can only have 4 d\(\rightarrow\)d transitions, this confirms that NO\(_2^-\) binding leads to different geometric and electronic structures for both Cu\(_M\) and Cu\(_H\). This observation is consistent with changes in the EPR spectrum. Addition of NO\(_2^-\) to the PHM enzyme leads to the resolution of two EPR signals with \(g_z = 2.265\) and 2.298 (Figure 84F). Neither corresponds to that of the resting oxidized state and indicate that both two copper sites have bound nitrite. This change in the EPR indicates different binding modes for nitrite to Cu\(_M\) and Cu\(_H\), presumably due to differences in the exchangeable coordination positions for each copper. Over all, these spectroscopic findings show that azide and nitrite bind to both the Cu\(_H\) and Cu\(_M\) sites in solution.

With insight from spectroscopy\(^{539}\) and crystallography\(^{538,601}\), structures of the oxidized Cu(II) sites were obtained from DFT calculations (Figure 83C and D). The Cu\(_M\) site is in a distorted square pyramidal geometry ligated by a long axial methionine, two histidines and two aquo derived ligands, one hydroxo and one water (Figure 83C). Oxidized Cu\(_H\) is in a D\(_{2d}\) distorted square planar geometry ligated by three histidines and one aquo ligand (Figure 83D)\(^{539}\).

It is interesting to consider how these very structurally different resting Cu(II) sites exhibit practically identical EPR and LF spectroscopic features. This was explained with geometric/electronic structural correlations between Cu\(_H\) and Cu\(_M\) (Figure 86). The square pyramidal Cu\(_M\) structure has a LF with the d\(_{x^2-y^2}\) orbital highest in energy and d\(_{xy}\), d\(_{z^2}\), and d\(_{xz}/d_{yz}\) in decreasing order (Figure 86, right). The LF of the Cu\(_H\) D\(_{2d}\) distorted square planar structure has d\(_{x^2-y^2}\) highest in energy with d\(_{xy}\), d\(_{xz}/d_{yz}\), and d\(_{z^2}\) in decreasing order (Figure 86, left). Removing the axial methionine of Cu\(_M\) shifts the d\(_{x^2-y^2}\) and d\(_{xy}\) orbitals up in energy while the d\(_{z^2}\) orbital shifts down. Replacing a hydroxo ligand with a histidine significantly stabilizes the d\(_{x^2-y^2}\) and d\(_{z^2}\) due to the stronger donation of the hydroxo relative to histidine. Distortion (D\(_{2d}\)) of the ligands from square planar destabilizes the d\(_{x^2-y^2}\) orbital while the d\(_{xy}\) is stabilized resulting in the Cu\(_H\) site. Its LF is similar to that of Cu\(_M\) where the only significant difference is the energy of the d\(_{z^2}\) orbital, which is higher in the latter case due to bonding with the methionine. Therefore, the square pyramidal distortion and the axial methionine oppose the effects of the strong hydroxo ligand yielding an electronic structure that produces analogous spectral features for Cu\(_M\) and Cu\(_H\).\(^{539}\)

3.3.4.2 Cu/O\(_2\) Model Complexes: Thus far, no dioxygen intermediates have been spectroscopically characterized for these enzymes in solution leading to much speculation.
about their nature. Alternatively, synthetic model chemistry has produced a series of structurally defined and, in some cases, reactive mononuclear Cu-oxygen complexes, including Cu(II)-superoxo, Cu(III)-peroxo and Cu(II)-hydroperoxo species that have been used to evaluate the plausibility of each intermediate in this reaction.

Early kinetics investigations of DJM and PHM proposed that a Cu(II)-hydroperoxo was the likely candidate for the intermediate responsible for H-atom abstraction. This prompted the spectroscopic study of Fujisawa’s tris(pyrazolyl)borate Cu(II) hydroperoxo model complex (Figure 87), in addition to analogous alkylperoxo complexes, in effort to evaluate its electronic structure and potency for H-atom abstraction. The EPR spectrum of this complex has $g_∥ = 2.380, g_⊥ = 2.090$ consistent with a $d_{x^2-y^2}$ ground state where the small hyperfine splitting $|A_∥| \approx 40 \times 10^{-4}$ cm$^{-1}$ is consistent with a ground state with ~62% Cu character (note from equation 2 the high $g_// value brings down the $A_// value). Absorption and MCD spectra (Figure 88) exhibit LF transitions at < 13,000 cm$^{-1}$ and two CT transitions at ~16,500 cm$^{-1}$ and ~18,000 cm$^{-1}$ associated with the hydroperoxo $\pi^*_v$ and $\pi^*_\sigma$ peroxo orbitals respectively, where $\sigma$ and $\pi$ indicate that the $\pi^*$ orbitals are perpendicular to and within the Cu-O-O plane, respectively. The former transition is more intense than the latter, indicating that the dominant bonding interaction is between the $d_{x^2-y^2}$ and the $\pi^*_v$ hydroperoxo orbitals. This complex exhibits a resonance Raman (rR) enhanced O-O stretch at 843 cm$^{-1}$, which shifts to 799 cm$^{-1}$ upon $^{18}O$ substitution, and a Cu-O stretch at 624 cm$^{-1}$, which shifts to 607 with $^{18}O$ substitution (Figure 89). The high O-O stretch frequency for the peroxo is indicative of a strong O-O bond due to polarization of the hydroperoxo by the proton, which mixes the $\pi^*_v$ and $\pi^*_\sigma$ orbitals. Extending this hydroperoxide intermediate to the CuM active site of PHM produces a half occupied FMO mostly on the Cu site with a $d_{x^2-y^2} - \pi^*_\sigma$ ground state of 61% Cu and only 2% distal O character (Figure 90 right). This electronic structure will be considered with respect to its relevance in the reaction mechanism in the next section.

As presented in the kinetics and structure sections, further research on these enzymes led to the postulation that a Cu(II)-superoxo intermediate is responsible for C-H abstraction. In efforts to understand this intermediate, a number of synthetic complexes have been reported as reported (Table 23) along with their Cu-O and O-O vibrational frequencies. The first such structurally and spectroscopically defined side-on bound ($\eta^2$) Cu(II)-$O_2^{2-}$ model complex was synthesized by Fujisawa and coworkers (Figure 91). Resonance Raman spectra of the complex exhibited a diagnostic O-O vibration of a superoxo at 1043 cm$^{-1}$ accompanied by the symmetric Cu-O vibration at 554 cm$^{-1}$ (Figure 92). The presence of a pre-edge feature at ~8979 eV in the K-edge XAS confirmed that the copper oxidation state was Cu(II). This complex exhibits no effective magnetic moment ($\mu_{\text{eff}}$) from SQUID magnetic susceptibility below 150 K indicating an $S = 0$ ground state (Figure 93) (consistent with its lack of an EPR signal). As the temperature is increased, $\mu_{\text{eff}}$ increases consistent with Boltzmann populating a low-lying $S = 1$ excited state, fit to ~1500 cm$^{-1}$ above the ground state. The absorption spectrum reveals four weak (< 400 M$^{-1}$cm$^{-1}$) d $\rightarrow$ d transitions at 10,200 cm$^{-1}, 14,300$ cm$^{-1}, 22,100$ cm$^{-1}$ and 26,100 cm$^{-1}$, consistent with a square pyramidal Cu(II) LF. The onset of an intense band occurs at an energy of > 30,000 cm$^{-1}$ was assigned to a dimeric Cu$_2$O$_2$ component in the CuO$_2$ complex while no LMCT
transition from the superoxo Cu(II) is observed (Figure 94A). The O-O vibration in the resonance Raman spectrum profiles the 22,100 cm\(^{-1}\) feature implicating d \(\rightarrow\) d transition mixed with the superoxo \(\pi^* \rightarrow\) Cu CT and therefore is assigned as \(d_{yz} \rightarrow d_{xy}\) since the \(d_{yz}\) is the only orbital with the correct symmetry to mix with the superoxo \(\pi^*\) orbital. The Cu \(d_{xy}\) is the highest energy metal orbital in square pyramidal complexes while the two highest occupied superoxo MOs (in the singlet side-on copper complex) are the \(\pi^*_{\sigma}\) and \(\pi^*_{\pi}\) orbitals. Thus, two LMCT transitions would be expected from the \(\pi^*\) orbitals to the Cu \(d_{xy}\) orbital. The \(\pi^*_{\sigma}\) orbital is located in the CuO\(_2\) plane and has considerable overlap with the \(d_{xy}\), which would lead to an intense CT feature at high energy. Since no CT feature is observed experimentally (Figure 94A), this transition must be higher than the spectral cutoff (i.e. >32,500 cm\(^{-1}\)). DFT calculations predicted a low-lying singlet excited state would produce a low energy LMCT transition and, indeed, this was observed at low temperature at 4,200 cm\(^{-1}\) (~200 M\(^{-1}\)cm\(^{-1}\) at 10 K, Figure 94B). This CT arises from the superoxo \(\pi^*_{\pi}\) orbital, which is oriented out of the CuO\(_2\) plane and minimally overlaps the \(d_{xy}\) orbital leading to its weak intensity. The \((xy)^1(\pi^*_{\pi})^1\) CT excited configuration associated with this singlet would lead to a triplet excited state at lower energy. This can be assigned to the paramagnetic excited state that is experimentally observed by SQUID magnetic susceptibility (Figure 93) at ~1500 cm\(^{-1}\) above the singlet ground state. Consequently, the singlet ground state must derive from a different electronic configuration compared to the lowest energy triplet state.

Correlation of these data to DFT calculations of the singlet ground state and the triplet excited state provides further insight into the electronic structure of this complex. The singlet ground state MO diagram consists of a pair of opposite spin electrons delocalized over the Cu \(d_{xy}\) and the \(\pi^*_{\sigma}\) antibonding orbitals (Figure 95). The calculated spin distributions have very little polarization (small net spin densities) on the Cu and superoxo moieties due to the highly covalent spin delocalized nature of the ground state. The computed triplet excited state is comprised of the two orthogonal \(\beta\)-LUMO orbitals: the superoxo \(\pi^*_{\pi}\) and the Cu \(d_{xy}\)-\(\pi^*_{\sigma}\). These two states reflect different configurations where the singlet ground state is highly covalent with no spin polarization and the triplet excited state involves excitation of one e\(^-\) from the \(\pi^*_{\pi}\) into the \(d_{xy}/\pi^*_{\sigma}\) orbital.\(^{121}\)

The high covalency between the copper and superoxo of the ground state of this Cu(II)-superoxo species is significant. When a side-on superoxo is modeled into the active site for PHM, an \(\alpha, \beta\) unoccupied FMO is obtained that is delocalized over the superoxo and the Cu (Figure 96). Due to this high oxygen character in the FMO, this intermediate is more activated for H-atom abstraction than the Cu(II)-hydroperoxo therefore strongly implying the relevancy of the Cu\(^{II}\)-superoxide for reactivity.\(^{600}\)

Elucidation of the PHMcc crystal structure showing dioxygen bound to Cu\(_{M125}\)\(^{125}\) in an end-on fashion focused interest on end-on bound Cu(II)-superoxo complexes. Although end-on bound species are observed as transient intermediates in the formation of binuclear copper trans-peroxo complexes, eight stabilized end-on bound mononuclear Cu(II)-superoxo model complexes have been reported (Table 23). The first and only structurally characterized complex is end-on Cu(II)-superoxo, [TMG\(_3\)trenCuO\(_2\)]\(^+\) (where TMG\(_3\)tren is 1,1,1-tris[2-[\(\pi^2-(1,1,3,3\text{-tetramethylguanidino})\text{ethyl}]\)-amine], of Sundermeyer and Schindler (Figure
This complex has been subjected to rigorous spectroscopic study. According to the NMR, the model complex is paramagnetic. Variable temperature, variable field (VTVH) MCD shows that this complex has a triplet ground state. The complex exhibits one intense absorption band at 22,500 cm$^{-1}$ (3,500 M$^{-1}$ cm$^{-1}$) attributed to a LMCT ($\pi^*\sigma$ of the superoxo to the $d_{z^2}$ of the copper; note the trigonal bipyramidal geometry of TMG$_3$tren leads to a $d_{z^2}$ ground state) based on its low MCD to absorption intensity (Figure 98A and B). Three bands at 10,100 cm$^{-1}$, 11,670 cm$^{-1}$ and 14,820 cm$^{-1}$ exhibit intense MCD signals but limited absorption intensity and thus were assigned as $d\to d$ transitions. There is also a weak transition at ~13,000 cm$^{-1}$ with no MCD intensity but is apparent from the rR excitation profile (Figure 98C). The rR spectrum exhibits resonance enhancement of both the Cu-O stretching mode at 435 cm$^{-1}$ and O-O stretching mode at 1120 cm$^{-1}$ consistent with a superoxo-level species. The rR profiles of these two vibrations are significantly enhanced over the aforementioned CT transition while only the O-O stretch is enhanced within the envelope of the low energy transitions (Figure 98C). The former is consistent with the assignment of a superoxo to Cu(II) CT, but the second is unique. The profile reaches a maximum corresponding to the weak absorption feature at 13,000 cm$^{-1}$ implying considerable distortion of the O-O, with little distortion of the Cu-O bond, in the excited state associated with this transition. From analysis of possible excited state distortions using TD-DFT calculations, the 13,000 cm$^{-1}$ was assigned as a superoxo intraligand (IL) transition from the $\pi^*_\sigma$ to the $\pi^*_v$ orbital (Figure 99), the first observed for any superoxo bound metal complex.

From the above discussion, side-on superoxo Cu(II) complexes are singlets while end-on superoxo-Cu(II) complexes are triplets. The separation of the $\pi^*_v$ and the $d_{z^2}$ orbitals ($\Delta$ in Figure 99) is the significant contributor in determining the ground state of these complexes. For the triplet to be the ground state, $\Delta$ must be smaller than the electron spin pairing energy. In going from end-on to side-on the binding of the superoxo with the Cu(II), the $d\cdot\pi^*_\sigma/d^*\pi^*_v$ splitting ($\Delta$) increases due to the strong $\sigma$ bonding in the side-on case causing the spins to pair (Figure 100). From the experimentally observed $\pi^*_\sigma$ to $\pi^*_v$ (IL) and superoxo $\pi^*_\sigma$ to copper $d_{z^2}$ (LMCT) transitions in the end-on [TMG$_3$trenCuO$_2$]$^+$ complex, an upper limit of $\Delta$ in the end-on structure can be established (Figure 99) to be 9,500 cm$^{-1}$, which is ~1/2 of the energy required for spin pairing. A calculated end-on structure from Cu$_M$ in PHM also has a triplet ground state with two orthogonal half occupied orbitals: the Cu $d_{x^2-y^2}$ and the superoxo based $\pi^*_v$. Importantly, the half occupied $\pi^*_v$ orbital is at low energy (Figure 100) with near 100% character on the superoxo (Figure 101; it would therefore be an optimal FMO for H-atom abstraction by a metal bound superoxo species).

### 3.3.5 Molecular Mechanism

#### 3.3.5.1 Dioxygen Intermediates:
Initial mechanistic hypothesis for the non-coupled binuclear Cu enzymes suggested that H-atom abstraction is accomplished by a Cu$_M$(II) - hydroperoxo intermediate. The electronic structure of this species was characterized in the aforementioned spectroscopic study of the tris-pyrazolyl borate Cu(II)-hydroperoxo complex. Computationally, an end-on bound hydroperoxo was modeled into the active site of Cu$_M$ (Figure 90) where the electronic structure consists of a singly occupied FMO that has essentially no character on the distal oxygen of the hydroperoxo (~2%).
FMO is not activated for H-atom abstraction since it lacks oxygen orbital character to overlap with the substrate H-atom donor orbital. Consistent with this, there is a high computed activation barrier for H-abstraction by a Cu(II)-OOH intermediate (~ 40 kcal/mol) (Figure 102, left in blue). Therefore, a Cu(II)_M-hydroperoxo is not suitable for H-atom abstraction in catalysis.

Spectroscopic, kinetic and crystallographic studies have alternatively suggested that the key reaction intermediate for H-atom abstraction is a Cu(II)-superoxo species. Modeling a side-on Cu(II)-superoxo at the active site of Cu_M results in an electronic structure with an α, β unoccupied FMO that is non-polarized and covalently delocalized over the Cu and πσ* with considerable oxygen character (Figure 96). Therefore, this FMO is more activated towards H-atom abstraction than the Cu(II)-hydroperoxo model. However, the PHMcc pre-catalytic crystal structure exhibits an end-on bound dioxygen. Building on the results from the spectroscopically and structurally defined end-on superoxo model complex [TMG₃trenCuO₂]⁺, which has a triplet ground state, an end-on superoxo species was modeled at the Cu_M active site. This computed intermediate also has a triplet ground state with two half-occupied orthogonal magnetic orbitals: one being the Cu dₓ²₋ᵧ² that is antibonding with the πσ* and the other is the πv* orbital with little Cu d character (Figure 101). The latter orbital is highly activated for H-atom abstraction due to its low energy and considerable oxygen character. Extending this one-electron reduced end-on triplet Cu(II)_M-superoxo species into the PHM active site, a Gibbs free energy activation barrier for H-atom abstraction is calculated to be 19.7 kcal/mol, which is much lower than that for the Cu(II)-hydroperoxo model and is in reasonable agreement with the experimental value of ~ 14 kcal/mol. Thus, the one-electron reduced Cu(II)-superoxo species is a viable intermediate in the non-coupled binuclear copper enzymes.

Other reactive dioxygen intermediates have been proposed based on calculations as the primary oxidant in these enzymes. Yoshizawa and coworkers modeled the homology of the PHM crystal structure to construct a three-dimensional structure of DβM. QM-MM calculations were then used to generate reaction coordinates. The H-atom abstraction by a Cu(II)-superoxo species (modeled as an antiferromagnetic coupled singlet state) was calculated to have a barrier of 16.9 kcal/mol; while a Cu(III)-oxo species was shown to accomplish H-atom abstraction with a barrier of +3.8 kcal/mol (modeled as a triplet state). The Cu(III)-oxo intermediate is actually a Cu(II)-oxyl with a triplet ground state from DFT calculations. However, this intermediate is not feasible due to the thermodynamic cost of forming this strong oxidant (~ 37 kcal/mol from Cu(II)-OOH homolytic cleavage). Another QM-MM calculation by Crespo and coworkers invokes the formation of the Cu(II)-hydroperoxo species but predicted that it will spontaneously form a [Cu(IV)O]²⁺ intermediate upon protonation where this high valent species would be responsible for H-atom abstraction. This reaction parallels that of P450 mechanism where compound I (formally a Fe(V)=O species) forms via protonation and heterolytic cleavage of a Fe(III)-hydroperoxo intermediate. This is also unreasonable as the energy required to form this intermediate is thermodynamically inaccessible. The spontaneous cleavage of the O-O bond upon protonation of the Cu(II)-OOH was performed by adding a proton to the system without compensating the for the energy of removing the proton from solution (~260 kcal/mol). Additionally, both of these proposals are inconsistent with the kinetics result of
Klinman and coworkers where the dioxygen $^{18}O_2$ kinetic isotope effect was found to increase with substrate deuteration establishing that the O-O bond must be intact prior to H-atom abstraction \textit{(vide supra)}.$^{533}$

Synthetic model complexe experiments have also supported a Cu(II)-superoxo species in H-atom abstraction. Two end-on Cu(II)-superoxo model complexes have been shown to activate C-H bonds. Karlin \textit{et al.} have synthesized an end-on Cu(II)-superoxo species, from the reaction of the Cu(I) complex with dioxygen at $-125 \degree C$, that is competent towards C-H activation via H-atom abstraction from N-Benzyl-1,4-dihydronicotinamide (BNAD).$^{606}$ This reaction is first order in both Cu(II)-superoxo complex and the substrate, and exhibits a primary KIE of 12.1, consistent with enzyme kinetic data. Additionally, Itoh and coworkers have synthesized a mononuclear compound that is spectrosocopically characterized to be an end-on Cu(II)-superoxo, in the ligand system $L^X$ ($L^X=1-(2-p-X$-phenethyl)-5-(2-pyridin-2-ylethyl)-1,5-diazacyclooctane; $X=\text{o-CH}_3, \text{-H}, \text{-NO}_2$).$^{609}$ Upon first-order decomposition, it is found that the benzyl C-H bond of the ligand has been hydroxylated in $\sim$ 30% yield where an $^{18}O$ substitution experiment confirms that the origin of the hydroxyl is from molecular oxygen. The Sundermeyer end-on Cu(II)-superoxo [TMG$_3$trenCuO$_2$]$^+$ has also been found to perform H-atom abstraction from TEMPO-H and phenols producing a Cu(II)-hydroperoxo species that is proposed to go on to hydroxylate a ligand methyl group.$^{616}$ Calculations investigating this hydroxylation found the barrier to abstract the H-atom by the Cu(II)-hydroperoxo species to be 23.5 kcal/mol. This barrier is much lower than that calculated for a Cu(II)-hydroperoxo species in the enzyme system ($\sim$ 40 kcal/mol) and appears to reflect an incorrect transition state.$^{600}$

Finally, the significant issue remaining in the research on this mechanism is the lack of definition of the oxygen intermediates in the enzymes. The PHMcc pre-catalytic crystal structure has defined an end-on oxygen bound species whose electronic structure is unclear and requires experimental definition.

\textbf{3.3.5.2 Electron Transfer (ET):} One puzzling aspect of the mechanism of these enzymes is the process by which the electron is transferred from Cu$_H$ to Cu$_M$. As observed in the crystal structure of PHMcc, the coppers are separated at a distance of 11 Å through a water filled cleft.$^{538}$ It has been suggested that inter-domain motion may help to diminish the distance between the coppers, but this is considered unlikely due to the fact that PHM in the crystal phase, where conformational rearrangement is limited, is catalytically active.$^{584}$ An ET pathway provided by the substrate and protein residues has also been proposed for the enzyme-substrate complex. Interestingly, in the IYT bound form of the pre-catalytic complex of the PHMcc, the enzyme shows a $\sim$ 20 Å pathway, which has been considered viable for ET.$^{584}$ This pathway consists of H108 bound to Cu$_H$ that is hydrogen bonded to Gln170 where the electron would be delivered to substrate via a water mediated hydrogen bond (Figure 82A). It is important to note that this electron transfer would be to the glycyl $\alpha$-carbon, which is conserved for all substrates. This is supported by molecular dynamics calculations by Moliner.$^{617}$ However, a mutational study by Eipper and coworkers showed that mutation of Gln170 to Ala or Asn resulted in no change in enzymatic activity suggesting that this residue does not play an important role in ET.$^{618}$ These findings have been evaluated by the previously mentioned calculations, which do not agree as they predict...
significant loss of activity for the Asn variant. Klinman has also studied the effects of peptide substrates of different lengths on turnover. The $k_{cat}$ was found to be higher (~2 times) for the shorter substrate. This implies the electron transfer step is independent of substrate length where the difference in rate was attributed to a difference in product dissociation from the enzyme (vide supra). This is presented as evidence for a water-mediated pathway and against a through protein-substrate ET pathway.

### 3.3.5.3 Completion of the Reaction Coordinate:

After the hydrogen atom from the substrate has been abstracted, the resulting intermediate is proposed to be a Cu(II)-hydroperoxo species and a substrate radical. Two mechanisms, a rebound mechanism and a heterolytic cleavage mechanism, have been suggested as shown in Figure 80. Calculations suggested a mechanism of hydroxyl rebound to the substrate radical to yield a Cu(II)-oxyl species. Proton coupled electron transfer from CuH to this then results in the fully oxidized form with a hydroxo bound to CuM, which can be reduced to react with dioxygen (Figure 102 green). Klinman and coworkers proposed an alternative hydroxylation mechanism where electron transfer from CuH is coupled to the hydroperoxo cleavage to form a Cu(II)-oxyl species, which then recombines with the substrate radical to give a copper bound hydroxylated product complex. The relative energetics of these and other possibilities are presently being evaluated.

### 3.3.6 Exchange Coupling Contribution to Reactivity

The key electronic difference between the "coupled" and "non-coupled" binuclear copper enzymes is the different magnetic interactions between the Cu(II)’s that leads to consideration of the impact of exchange coupling on determining the individual reaction mechanisms. The exchange coupling term $J$ of binuclear cupric sites can be related to the electronic coupling matrix element for electron transfer ($H_{AB}$) between a Cu(I) and Cu(II) in Marcus Theory via equation 16 in Section 2.3.2, where $U$ is the repulsion between two electrons on the same copper. For the coupled binuclear copper sites (sections 3.1 and 3.2) this exchange coupling, therefore $H_{AB}$, is large and results in the simultaneous two electron reduction of dioxygen to peroxide, where the resulting $[\text{Cu}_2\text{O}_2]^{2+}$ complex has an electronic structure (i.e., an FMO) capable of 2 e$^-$ electrophilic aromatic substitution (section 3.2). This contrasts the non-coupled binuclear enzymes where the exchange coupling is immeasurably weak and thus $H_{AB}$ is small. This would allow for initial single electron reduction of dioxygen (as opposed to two electron reduction) at CuM to form a superoxo species with the appropriate electronic structure ($\pi^*$ FMO in Figure 101) for 1 e$^-$ H-atom abstraction. Electron transfer from CuH would then occur at a later stage in the reaction where an intermediate is formed with a large driving force to allow for ET even at low $H_{AB}$ (e.g., the Cu-oxyl species that would form upon substrate oxygenation (Figure 102, right green).

### 3.4 O₂ activation by a mononuclear Cu site: cofactor biogenesis in Galactose Oxidase

The enzymes Galactose Oxidase and Amine Oxidase catalyze the two-electron oxidation of substrates with a single copper center. This is accomplished with an additional covalently bound redox active cofactor present at the active site. The formation of this cofactor (known as a biogenesis reaction) involves the post-translational modification of a Tyrosine residue at the active site. In this section we consider the cofactor biogenesis reaction of Galactose Oxidase.
Oxidase, with Cu(I) and Cu(II), with the former being more efficient and potentially relevant to the reduction of O$_2$ by Cu(I) in the non-coupled binuclear copper enzymes discussed in the last section.

3.4.1. Enzymology—The active form of Galactose Oxidase (D-Galactose Oxidase EC 1.1.3.9, GO) has a radical containing copper site that catalyzes the stereospecific oxidation of primary alcohol substrates into aldehydes, coupled to the two electron reduction of one dioxygen molecule to hydrogen peroxide (Figure 103 top).$^{620,621}$ Although the natural substrate for the enzyme is D-Galactose, GO accepts a broad range of substrates, from sugars to aromatic alcohols. Alcohol oxidation is strictly regioselective and no secondary alcohols are oxidized. The enzyme can also oxidize aldehydes to carboxylates, but at a much slower rate (Figure 103 bottom).$^{622,623}$

GO is a secretory fungal enzyme that has been isolated from *Dactylium dendroides*, *Gibberella fujikuroi* and *Fusarium graminearum*. Mature GO is a monomer of 68 kDa molecular mass, containing one copper center per protein. The protein has three distinct domains, and the active site is located on the surface of the second domain, which is the largest. It has been reported that the mature protein contains a small amount of carbohydrate (less than 10% of the total mass), although this has not been observed in the crystal structures.$^{624}$

The GO oxidation reaction (Figure 103 top) makes this enzyme ideal for bioanalytical applications, due to the stoichiometric relation between substrate oxidation and oxygen reduction. The highly clinically important determination of galactose uses this type of chemistry.$^{625}$ GO is also used in synthetic chemistry to generate complex aldehydes and carboxylic acids from compounds with a single primary alcohol as a functional group. In addition, GO has recently been used because of its selectivity towards galactose, among other hexoses, to modify D-galactose-β-[1,3]-N-acetylgalactosamine, an important tumor marker. The presence of this disaccharide is strongly correlated with colon cancer, and GO provides a rapid method for its early detection.$^{626}$

The two-electron reduction of dioxygen to hydrogen peroxide involves the two-electron oxidation of the substrate by a site that contains only one copper center. Therefore, nature has evolved an additional cofactor at the active site, a Cys-Tyr covalently linked radical center bound through the phenoxyl oxygen and antiferromagnetically coupled ($J < -100$ cm$^{-1}$ for $H = -2JS_1\cdot S_2$) to the copper(II) center. The redox potential for this cysteinated-tyrosine ligand has been measured to be 0.45 V, and is thus stabilized relative to a free tyrosine radical (0.95 V) through the covalent linkage with the cysteine residue in the ortho position and through π-π stacking with a nearby tryptophan residue (W290). Among the variants that have been developed for this enzyme, those that involve W290 (W290G, W290H and W290F), show profound effects on the galactose binding constant and the catalytic rate of the enzyme, suggesting an additional role of W290 residue in substrate binding and activation.$^{627}$

The catalytic mechanism of GO has been studied with a range of approaches, including enzyme model studies and calculations.$^{628-633}$ The kinetics of GO are complex due to the
coexistence of three oxidation states of the enzyme and the possibility of reversible activation/inactivation by the interconversion of these states (section 3.4.5). The overall reaction consists of separate reduction and oxidation steps, i.e. a ping-pong mechanism (Figure 104). While \( k_{\text{red}} \) has a broad range of values depending on the substrate (0.8–2.7 \( \times \) 10\(^4\) M\(^{-1}\)s\(^{-1}\)), the \( k_{\text{ox}} \) is more or less constant (0.98–1.02 \( \times \) 10\(^7\) M\(^{-1}\)s\(^{-1}\)). Steady state kinetics gives a \( K_{\text{m}} \) of 82 mM for D-Galactose substrate at saturated \( O_2 \) concentrations,\(^6^{35}\) that varies a little depending on the organism from which the enzyme is obtained. This large \( k_{\text{ox}} \) value might reflect an enzyme strategy for broad substrate specificity.

There is no crystal structure of the active site with the D-galactose bound, but the fact that the open coordination site of Cu(II) in the crystal structures is usually occupied by water or an anion of the crystallization buffer indicates that the galactose coordinates to replace the labile equatorial ligand (see section 3.4.3).\(^6^{36}\) The proposed mechanism involves the deprotonation of the OH group of the sugar by an axial tyrosine (Y495) upon coordination to the Cu(II), a stereospecific pro-S hydrogen atom abstraction by the Cys-Tyr radical cofactor, an inner sphere electron transfer by the substrate radical reducing Cu(II) to Cu(I) and finally the dissociation of the aldehyde product (Figure 105).\(^6^{22}\)

Glyoxal oxidase (GLOX) is a related enzyme that performs the oxidation of aldehydes to carboxylic acids coupled to a two electron reduction of \( O_2 \) to \( H_2O_2 \). This enzyme is found in the fungus \textit{Phanerochaete chrysosporium}, an organism extensively studied for its lignin-degrading ability. GLOX shares many properties with GO despite lacking obvious sequence identity (less than 20%). No crystal structure has been solved yet for GLOX, but the spectroscopic data on the active site (mostly EPR and rR) indicate that the active site is very similar to that of GO.\(^6^{37}\) Although these two enzymes are related in many ways (including similar molecular weights), the redox potentials for the radical cofactors are different (0.66 V in GLOX vs. the 0.45 V in GO, both relative to NHE). The biological function of GLOX is clearly the generation of \( H_2O_2 \). The enzyme only works under ligninolytic conditions, when lignin peroxidase and manganese peroxidase are also produced, and both of these enzymes require hydrogen peroxide to function. The fact that the main biochemical role of GLOX is the generation of \( H_2O_2 \) has raised the issue of as to whether this might also be the \textit{in vivo} role of Galactose Oxidase.

In order to be fully active, Galactose Oxidase has to go through a series of post-translational modifications that results in three protein forms. The pre-sequence form has 680 amino acids and a molecular weight of 70.2 kDa. The first cleavage removes the 24 amino acid pre-sequence leader peptide in the secretory pathway, and seems to be copper independent, because fungi can produce both cleaved and pre-sequence forms of GO under metal deprivation stress.\(^6^{27}\) A second cleavage that is dependent on the Cu(II) cuts the C-terminus at an arginine and removes 17 amino acids to generate the preprocessed form of the enzyme (without the Cys-Tyr crosslink), with 639 amino acids and 68 kD molecular weight. Finally, the biogenesis reaction takes place, generating the processed Cu(II)-Tyr-Cys radical antiferromagnetically coupled active site. In copper-limited conditions, heterologous expression of galactose oxidase results in the three forms of the protein, identifiable as distinct bands on SDS/PAGE.\(^6^{38}\) The other enzymes that use a metal catalyzed biogenesis reaction to modify a tyrosine residue (cytochrome \( c \) oxidase, with a histidine ligand
covalently linked to a tyrosine residue, and Amine Oxidase) use the modified Tyr along with the copper to react with O$_2$. Here we focus on the biogenesis reaction to form the radical cofactor in GO, which involves dioxygen activation by a single copper center without the involvement of an additional redox cofactor.

3.4.2 Kinetics—Preprocessed GO from *Pichia pastoris* can be expressed without the crosslink between Cys228 and Tyr272, allowing for kinetic studies of the biogenesis reaction. This can be accomplished by following the formation of the radical cofactor, which has been studied using both redox states of copper (Cu(I) and Cu(II)) and under both aerobic and anaerobic conditions. Studies of copper(I) biogenesis are more challenging as this d$^{10}$ metal ion is spectroscopically silent (section 2.1).

Cu(I) performs the biogenesis reaction about $10^4$ times faster than Cu(II).$^{639}$ with a $t_{1/2}$ of 3.9 s compared with a $t_{1/2}$ of 5.1 h for Cu(II). The Cu(I) reaction (studied at low copper loading) uses 1.8eq of O$_2$ consistent with the generation of two molecules of H$_2$O$_2$ per active site formed. For Cu(I), three reactions are observed depending on pH. Reaction 1 (Figure 106) dominates at the biologically relevant pH (pH ~7.0). Stopped-flow kinetic experiments monitoring the reaction of Cu(I) GO with O$_2$ leads to the formation of the characteristic bands of the fully oxidized form, with the crosslink between Tyr272 and Cys228, at 445 nm and 850 nm (see section 3.4.4) with a $k_1 = 1.78\times10^{-1}$ s$^{-1}$, which is well described by a single exponential. This rate constant increases with increasing pH to $k_2 = 4.15\times10^{-1}$ s$^{-1}$ at pH = 8.35, at which point reaction 2 (Figure 106) dominates. The increase in rate constant with pH is interpreted as the deprotonation of the mechanistically important Cys228 proton (section 3.4.5). The increased rate at pH 8.35 can also be described using a single exponential. The spectral features observed at pH < 7.0 are different from the ones observed at pH ≥ 7.0 (Reactions 1 and 2 in Figure 106), lacking the 850 nm band and showing a blue shift of the 445 nm band (a phenolate to Cu(II) charge transfer band, see section 3.4.5). Reaction 3 proceeds in two steps, in which the GO-Cu(I) first reacts with O$_2$ in the presence of a proton with $k_3 = 4.47\times10^{-2}$ s$^{-1}$ to oxidize the metal and release HO$_2$. The GO-Cu(II) then turns into a species ($k_4 = 1.8\times10^{-5}$ s$^{-1}$) that has spectral features similar to those of GO anaerobically loaded with Cu(II) and lacking the Tyr272 and Cys228 crosslink (406 nm band, see section 3.4.4), suggesting a possible Cu(II)-Cys bond. Further characterization of this species is yet to be reported. The solvent kinetic isotope effect (SKIE) has been studied for this reaction over a broad range of pH, showing a maximum $k_{H_2O}/k_{D_2O}$ of 1.51 at pH 7.00 that drops to 1.00 at pH 8.35. The pH dependence of the kinetic constants and the SKIE between 6.5 ≤ pH ≤ 8.5 show the same behavior, implying that the trend observed in both is due to the ionization of an unique proton that plays a mechanismically important role in the biogenesis reaction (see section 3.4.5).

While the Cu(I) biogenesis reaction is much faster, there has been discussion as to whether a Cu(II) might be involved in forming the crosslink *in vivo*, possibly in the absence of O$_2$ to avoid premature cell deterioration due to radical reactions and is thought to be viable in the formation of the thioether crosslink in the trans-Golgi network.$^{636}$ To study the possible role of Cu(II) in biogenesis, both aerobic and anaerobic loading experiments have been done with preprocessed GO and Cu(II).
When 0.8 eq of Cu(II) and O₂ are added simultaneously to preprocessed GO, the biogenesis reaction takes place with a kinetic constant of \( k = 3.8 \times 10^{-5} \text{ s}^{-1} \) (Figure 107a), which increases by two orders of magnitude to \( k = 3.5 \times 10^{-3} \pm 5 \times 10^{-4} \text{ s}^{-1} \) when 3.5 fold excess Cu(II) is added, indicating a copper concentration dependence. Under excess copper conditions, densitometry measurements on stained gels from samples taken during the reaction gave a rate constant for the formation of the Cys228-Tyr272 bond of \( k = 5 \times 10^{-3} \pm 8.3 \times 10^{-4} \text{ s}^{-1} \), which is comparable to the biogenesis rate under the same copper concentration conditions. These results suggest that the rate limiting step for the formation of the fully oxidized mature GO is the formation of the thioether crosslink. This reaction consumes 1.5 equivalents of O₂, determined by the concomitant formation of H₂O₂.

When Cu(II) is added anaerobically to preprocessed GO, a three phase process is observed (Figure 107b). The first occurs upon addition of 0.8 eq of Cu(II) to preprocessed GO. Based on EPR data, the copper(II) loads completely in seconds into the active site, showing one very weak absorption band at 600 nm. Anaerobic incubation of this species leads to the formation of a second species with an intense absorption feature at 406 nm. The kinetics for the formation of this absorption band can be fit with a single exponential to give a \( k_{406} = 3.2 \times 10^{-4} \text{ s}^{-1} \). Longer incubation times (up to 24 h) leads to a third bleached species indicating reduction to Cu(I). Crystallographic data and SDS-PAGE analysis show that this species contains the covalent bond between the Cys228 and the Tyr272, indicating that Cu(II) is able to crosslink these two residues in the absence of O₂. The decay of the 406 nm band gives \( k_{406, \text{decay}} = 5 \times 10^{-5} \text{ s}^{-1} \), which is comparable to the aerobic rate of formation of the mature form of the enzyme when stoichiometric Cu(II) is added (vide supra). This may indicate that the rate-determining step is the anaerobic formation of the thioether crosslink by the single Cu(II) center.

There is also an anaerobic Cu(II) concentration dependence on the kinetic constants as addition of a 3.5 eq excess Cu(II) leads to the immediate formation of the 406 nm species, followed by its rapid decay to the bleached species \( k_{406, \text{decay}} = 4.08 \times 10^{-5} \text{ s}^{-1} \). SDS-PAGE analysis shows that this species contains the thioether crosslink. Interestingly it has also been detected that the excess copper does not act as the additional electron acceptor required in the formation of the crosslinked, bleached species. The nature of the extra electron acceptor remains unclear (section 3.4.5).

The effect of anaerobic Cu(I) addition was also studied and the amount of thioether bond formed quantified by SDS-PAGE analysis. The rate of thioether bond formation with Cu(I) was estimated to be \( k_{Cu(I)} = 7.8 \times 10^{-5} \text{ s}^{-1} \), which is on the same order as the kinetic constant of the anaerobic crosslink formation with stoichiometric copper(II). From the experimental data it remains unclear if the similar rate is because both Cu(I) and Cu(II) can perform the anaerobic crosslink or their similar rates simply reflect a faster conversion of Cu(I) to Cu(II).

When the second species (406 nm) is mixed with oxygenated buffer, the crosslinked radical site is generated with \( k_{406,O_2} = 3.9 \times 10^{-5} \text{ s}^{-1} \) for stoichiometric Cu(II) loading. This rate is very similar to the rate reported for biogenesis by the addition of Cu(II) in oxygenated buffer, and is in the same order of magnitude as the kinetic constant \( k_{406,O_2} \) for the anaerobic decay of the 406 nm species to the bleached one. These rates suggest that the 406
nm species is the reactive species that forms the crosslink anaerobically *in vivo*, and that the rate-determining step is the formation of the thioether bond.

From the above rates is can be concluded that the Cu(I) performs the aerobic biogenesis reaction most rapidly with a rate dependent on the protonation state of the nearby cysteine residue, while Cu(II) can also generate the crosslink in a slow, but O$_2$ independent reaction.

### 3.4.3 Structure

X-ray crystal structures for GO have been reported from a variety of organisms, including *Dactylium dendroideum*, *Aspergillus nidulans* and *Fusarium Graminearum*. Most of the crystal structures are on processed forms of the enzyme (i.e., with the thioether crosslink or with the radical cofactor present); little crystallographic data are available for the preprocessed enzyme (Table 24).

Processed Galactose Oxidase (with the radical cofactor) is a monomer with one copper center per protein, three distinct domains and a total molecular weight of 68 kD with 639 aminoacids (Figure 108). The three domains are composed mostly of beta sheets, with almost no turns. The N-terminal domain (domain 1, red in Figure 108) contains the first 155 amino acids, and forms a globular unit with eight strands of antiparallel $\beta$-sheets folded into a sandwich. This domain contains a metal binding site that is not the active site. It is formed by Asp32, Asn34, Thr37, Glu142 and the peptide carbonyls of the Lys29 and Ala141. These amino acids define a pseudo octahedral coordination site that contains a Na$^+$ cation from the crystallization buffer (PIVES). However, based on its −2 negative charge, the natural metal might be a divalent cation (e.g. Ca$^{2+}$). In addition, this domain contains a carbohydrate binding site that may function in targeting extracellular carbohydrates. A hydrophobic patch connects domain 1 with domain 2. The second domain is the largest (residues 156 to 532, yellow in Figure 108) and contains the copper active site. This domain is formed by groups of four-stranded antiparallel $\beta$-sheets in pseudo sevenfold symmetry (Figure 108B). This second domain contains three of the four metal ligands of the active site Tyr272 from the Cys-Tyr radical cofactor, Tyr495 and His496. Domain 2 contains a large hole along the sevenfold axis, which is filled by two $\beta$-strands of the third domain and a number of water molecules, one of this $\beta$-strands, belonging to the third domain, contains the fourth copper ligand (His581). The third domain (residues 533 to 639, blue in Figure 108) lies behind the second domain, and is formed by a pack of seven, mostly antiparallel, $\beta$-strands surrounding a hydrophobic core.

The active site of the enzyme is located in the second domain, and the copper center is located on the solvent accessible surface of this domain, close to the pseudo-sevenfold axis. The copper ion has a square pyramidal geometry, with Tyr495 in the axial position and the rest of the ligands in the equatorial plane, including the crosslinking cofactor formed by the Tyr272 and Cys228. The open equatorial coordination position is usually filled by a water molecule or a negative counterion from the crystallization buffer. Once the cofactor is formed, a Tryptophan residue from domain 2 interacts with the crosslinked Cys-Tyr residue through a $\pi-\pi$ stacking interaction (Figure 109). This interaction has been shown to provide structural and electronic stability to the active site of GO through the effects on the catalytic reaction of a series of Trp290 variants.
Dooley and coworkers have reported the only crystal structure of apo-preprocessed Galactose Oxidase. The structure largely resembles the mature GO, with the exception of the active site region. The thioether bond is not present, and the Tyr272 is disordered and modeled in two conformations. Trp290 is also not found in its stacking position (Figure 110A). The authors were also able to obtain crystallographic data on a 3 minute Cu(II) soaked apo crystal (Figure 110B). The electronic density suggests that the copper binds to His496 and His592, and that Cys228 rotates around its carbon-carbon bond to coordinate to the copper. The later observation is based on a negative density peak from the Cys228 sulfur and a positive peak adjacent to the copper. The Cu-S bond is estimated to be 2.2 Å and the copper(II) is in a very unusual three coordinate T-shaped geometry. After 24 h, the anaerobic Cu(II) soaked crystals show that the Cu-S bond is broken, the thioether crosslink is formed, and Tyr272 is coordinated to the Cu(II) in the equatorial plane (Figure 110C).

Given the important role that Cu(I) seems to play in the biogenesis reaction, it would be highly desirable to have structural information on its coordination environment in the preprocessed enzyme. However, no crystallographic or XAS information on this species is currently available.

### 3.4.4 Electronic structure—

Preprocessed copper bound GO, is an important target for spectroscopic studies that would provide insight into the metal ion activation for the biogenesis reaction. As indicated in section 3.4.2, this reaction is performed by both Cu(I) and Cu(II), and it is clear that the cofactor biogenesis is $10^4$ times faster with Cu(I). As shown in section 2.1, Cu(I) is unaccessible to most spectroscopic techniques, and requires the use of X-ray absorption and emission spectroscopy at the Cu K-edge and crystallographic studies. However such studies have not yet been reported.

From section 3.4.2, when Cu(II) is loaded anaerobically into preprocessed GO, a series of three species is observed. The first species appears almost immediately after anaerobic Cu(II) loading. This species has a characteristic EPR signal ($g_\parallel = 2.21, g_\perp = 2.05, A^{Cu_\perp}_{Cu_\parallel} = 170$ Gauss, Figure 111A black), indicative of coordination of the Cu(II) to the protein, and shows a weak absorption feature at 600 nm ($\epsilon_{600} = 200$ M$^{-1}$cm$^{-1}$, Figure 111B, first spectrum) that likely reflects ligand-field transitions. After a few hours of incubation, the spectral signature of the second species is fully formed, although it is important to note that the 600 nm absorption band does not decay. This second species has been studied with several spectroscopic methods. Its EPR spectrum shows basically the same features as the short time species ($g_\parallel = 2.225, g_\perp = 2.005, A^{Cu_\parallel}_{Cu_\perp} = 175$ Gauss, Figure 111A blue and red), which are characteristic of an axial Cu(II) site) and lacks a detectable free radical feature. Its most distinctive feature is its yellow color, which reflects the intense ligand to metal charge transfer (LMCT) at 406 nm ($\epsilon_{406} = 3790$ M$^{-1}$cm$^{-1}$, Figure 111B). The CD spectrum of the 406 nm species shows features at 434 nm and 748 nm (both bands positive, Figure 111C). The 406 nm absorption band was used to resonance enhance the Cu-ligand Raman vibrations associated with this feature. The rR spectrum shows vibrational features at 257 and 343 cm$^{-1}$, assigned to Cu-S stretches (Figure 111D). Based on these vibrations and the lack of Tyr ring modes, the 406 nm band has been assigned as a Cys to Cu(II) charge transfer transition. At longer incubation times (24 h), the bleached species is formed.
This third species has the thioether crosslink, and from the lack of an absorption and EPR features is likely reduced. Thus, from spectroscopy, a Cu(II)-thiolate complex forms and this then decays to form the crosslink between Tyrosine and Cysteine. The crosslink involves a two-electron oxidation. The first electron is accepted by the Cu, while the acceptor of the second electron is not clear. Experiments with 3.5 excess Cu(II) shows the rapid formation of the 406 nm feature and a broad and weak band at 625 nm. The band at 625 nm has tentatively been assigned to aqueous Cu(II), and does not decay with time. With 3.5 fold excess Cu(II), the 406 band decays to a species with absorbance at 435 nm and lower intensity.

The reaction of anaerobic processed reduced GO with O$_2$ generates mature GO. Mature GO has some distinctive spectral features not observed in any other copper metalloenzyme, reflecting the Cys-Tyr radical and its interaction with the Cu(II). The fully oxidized form shows no EPR signal, due to antiferromagnetic coupling between the Cu(II) and the Cys-Tyr crosslink radical cofactor ($J < -100$cm$^{-1}$ for $H = -2J\cdot S_1\cdot S_2$). A small sharp signal around the free electron value corresponds to a small (~0.1 spins/protein) fraction of crosslinked apoenzyme in the sample that is able to form a stable free radical (Figure 112, upper right). In addition to the lack of EPR signal, the holo enzyme has distinctive features in its absorption spectrum. This is dominated by two intense bands: one centered at 445 nm ($\varepsilon_{445} = 5500$ M$^{-1}$cm$^{-1}$) the second is very broad and centered at 850 nm ($\varepsilon_{850} = 3400$ M$^{-1}$cm$^{-1}$) (Figure 112, upper left). The CD spectrum resolves these bands into at least 8 different transitions (Figure 112, left). rR experiments with excitations at 659 nm and 875 nm on the 850nm band show vibrations that correspond to ring mode frequencies (Figure 112 lower right). The peaks at 1382, 1487, 1479, 1595 and 1185 cm$^{-1}$ are assigned to the tyrosyl radical, while the peaks at 1170, 1246, 1499 and 1603 cm$^{-1}$ are assigned to the tyrosinate ligand. From these the 445 nm band has been assigned as a LMCT from the Cys-Tyr radical cofactor to the Cu(II). Based on absorption and MCD data, The 850 nm band is assigned as a ligand to ligand charge transfer (LLCT) between the axial tyrosinate and the equatorial Tyr-Cys ligand radical mediated by the d$_{xy}$ orbital of the Cu(II). TD-DFT calculations disagree with this assignment and suggest an intraligand transition of the cysteinated Tyrosine with no contribution from Tyr495. 

**3.4.5 Mechanism**—In contrast to the biogenesis mechanism for the formation of TPQ in Amine Oxidase (see section 4.2), very little is known about the cofactor biogenesis mechanism in GO. The ortho covalent linking of the cysteine sulfur to the tyrosine is indicative of oxidative radical coupling reactions and would be consistent with the nucleophilic addition of a thiol to a phenoxyl radical or the addition of a phenol to a thyl radical. The proposed mechanisms for the radical cofactor biogenesis are often based on the product and involve a high degree of speculation. For the more efficient Cu(I) aerobic mechanism the radical would have to be generated by reaction with O$_2$, while for the less efficient but anaerobically viable mechanism the radical would be generated by ligand coordination to the Cu(II). This ligand could either be the Tyr272 or the Cys228, and the radical character in the Cu-ligand is thus thought to reflect a resonance contribution to the ground state.
Dooley and coworkers have proposed two possible pathways for the cofactor biogenesis with copper(II), one for aerobic and another for anaerobic conditions. The aerobic proposal (Figure 113) invokes the copper(II) coordination to the Tyr272 in the active site, and some radical character in the ground state, very similar to a proposal for the first step in the biogenesis reaction of Amine Oxidase (section 4.2). This tyrosyl radical character would react with O\(_2\), generating a peroxo-bridged quinone species. The thiolate would then attack at the ortho position with the loss of H\(_2\)O\(_2\). The loss of the ortho hydrogen atom as H\(^+\) recovers the aromaticity of the ring and a second oxidation with another O\(_2\) molecule generates the final Cu(II)-Tyr(•) cofactor with the concomitant formation of another H\(_2\)O\(_2\) molecule. Firbanks, Dooley and coworkers have outlined an alternate aerobic mechanism, which involves the monoxygenation of the Cys228 to a sulfenate group.\(^{638}\)

Both Firbank and Dooley have also proposed a possible route for the anaerobic copper(II) dependent biogenesis.\(^{636,638}\) This mechanism (Figure 114) uses the rR data that show a copper-thiolate bond (i.e. the absorption band at 406 nm, Figure 111B). The authors propose that under anaerobic conditions, the copper(II) binds to the cysteine (and not the tyrosine), generating a copper(II)-thiolate species which has a resonance form in which one electron is transferred from the sulfur atom to the copper(II) to generate a Cu(I)-thiyl radical species. The thiyl radical would attack the phenol ring, and with the loss of an electron (acceptor unknown) generate a carbonium ion intermediate. Proton loss would then lead to the final species in which the thioether bond has been formed (i.e. the Cu(I) containing bleached species in Figure 114).\(^{636}\)

These mechanisms, although consistent with the formation of a crosslinked product, have issues. In the aerobic route, the proposed resonance contribution of the radical form to the ground state complex will be very small, because it corresponds to a charge transfer excited state of the phenolate-copper(II) complex (see section 4), and the EPR data show no contribution from a radical. In the anaerobic route also no radical is observed and the final electron acceptor is unknown.

Finally, as indicated above, the biogenesis reaction with copper(I) is orders of magnitude faster than with copper(II). Whittaker and coworkers have proposed a mechanism for the copper(I) aerobic biogenesis reaction (Figure 115). The copper(I) coordinates to His496, His581 and Tyr272, and reacts with O\(_2\) to generate a superoxide radical via one electron transfer from copper to dioxygen. This would parallel the discussion in Section 3.3 concerning the non-coupled binuclear Cu enzymes and would likely involve end-on triplet CuO\(_2\) species.

This bound radical is thought to perform hydrogen atom abstraction from the Cys228 to form a thiyl radical, reducing the superoxide to hydrogen peroxide. The next step would be an electrophilic addition of the thiyl radical to the Tyr272 ring and the formation of the thioether bond. Loss of the ortho proton with concomitant lost of an H\(_2\)O\(_2\) molecule would then lead to a reduction of the copper(II) to copper(I). Addition of another equivalent of O\(_2\) generates the final Cu(II)-Tyr(•) species (Figure 115). Within this model the measured rate constant at high pH (8.5, see section 3.5.2) is associated with the deprotonation of the Cys228. The thiolate group has a lower reduction potential (E\(^0\)(RS\(^+\)/RS\(^-\)) = 0.80 V vs. 0.00 V vs. NHE).
$E^0(\text{RSH}/\text{RSH}) = 1.37 \text{ V both vs. NHE}$ and would react more rapidly (via electron transfer) to generate a thyl radical and a coordinated peroxide. Exchange of the cysteine sulfhydryl group in D$_2$O would also account for the observed SKIE. However, this mechanism does not deal with the spin along the biogenesis reaction, particularly as end-on Cu(II)-O$_2$(−) complexes are triplets while formation of the crosslinked product results in a singlet. Also the one electron reduction of O$_2$ is energetically unfavorable and for this to occur the potential of the Cu(I) preprocessed site needs to be tuned down and the resultant superoxide-Cu(II) bond needs to be strong.

In summary, a lot is known about the catalytic reaction of GO, but less is known about the biogenesis reaction to form the unique Cys-Tyr radical cofactor. Although the in vitro biogenesis reaction with Cu(I) is four orders of magnitude faster than with Cu(II), there is still a debate as to which copper redox state is involved in the biogenesis reaction in vivo. Both processes clearly involve different mechanisms as O$_2$ is the oxidant with Cu(I) in the aerobic route while the Cu(II) would be the oxidant in the anaerobic mechanism. Also, the aerobic reaction appears to involve coordination of a Tyr to the Cu(I) while the anaerobic reaction appears to involve activation of the Cys by coordination to the Cu(II). Clearly, interesting chemistry needs to be understood in the formation of this unusual covalent crosslinked-Cu site.

3.5 Particulate methane monooxygenase and related systems: H-atom abstraction from methane

3.5.1 Enzymology—Methane monooxygenases (MMOs) present in methanotrophic bacteria accomplish a formidable task in nature: oxidation of the inert C-H bond of methane (bond dissociation energy of ~104 kcal/mol). It is this capacity which constitutes the enzyme’s functional significance and application. Methane gas is an underutilized source of global energy; understanding the mechanism of methane oxidation by MMOs can greatly advance the molecular design and synthesis of functional catalysts with the ability to carry out the methane to methanol conversion at ambient temperature and pressure. This would allow for the efficient storage and use of methane as an independent source of carbon-derived energy. This is attractive as current methods which perform the methane to methanol conversion involve high temperatures and pressures and are not energy efficient. In industry, the first step in methanol synthesis from methane gas is steam-methane reformation (SMR) (CH$_4$ + H$_2$O $\rightarrow$ CO + 3H$_2$O) over a nickel catalyst, which is highly endothermic. The products of this reaction are referred to as synthesis gas (syngas). Syngas is further reacted over a different catalyst (Cu/ZnO/AL$_2$O$_3$), also at high temperature and pressure, to form methanol (CO + 2H$_2$ $\rightarrow$ CH$_3$OH). Methane is also a major contributing greenhouse gas. In this respect, overall methane reduction in the atmosphere is important and could be facilitated by MMOs. Also important from the standpoint of bioremediation is the ability of MMOs to oxidize other alkanes and alkenes, including chlorinated compounds.

There are two types of MMOs, a soluble (sMMO) and particulate (pMMO), membrane bound form. Methane oxidation in sMMO occurs at a binuclear non-heme iron site, while for pMMO this has been controversial but appears to occur at a binuclear copper
It should be noted that the only other enzyme currently known to oxidize methane is ammonia monoxygenase (AMO) and is found in ammonia oxidizing bacteria. These bacteria are obligate chemo-lithoautotrophs which oxidize ammonia in two steps. NH\textsubscript{3} is oxidized to NH\textsubscript{2}OH by AMO and NH\textsubscript{2}OH is oxidized to NO\textsubscript{2}\textsuperscript{-} by hydroxylamine oxidoreductase (HAO). Like pMMO, AMO is membrane bound and has three subunits (Section 3.5.3). Unfortunately, the structure and metal content of AMO remain unknown due to the lack of pure active enzyme, which retains activity. Evidence exists that supports either a copper or iron or copper/iron metal containing cofactor in AMO.

Methanotrophic bacteria currently constitute 13 genera, all of which are within α and γ proteobacteria. These bacteria thrive in diverse, and sometimes extreme, aerobic environments which consist of sediments, soils, peat bogs, freshwater, seawater, and hot springs. Methane, utilized as a sole source of carbon and energy in these bacteria, is oxidized in the first step of their metabolic pathway (Figure 1). Proceeding methane oxidation is the oxidation of methanol to formaldehyde by methanol dehydrogenase, formaldehyde oxidation to formate by formaldehyde dehydrogenase (FalDH), and, finally, the pathway completes with formate oxidation to CO\textsubscript{2} by formate dehydrogenase. Carbon utilized for biosynthesis of cellular material is assimilated at the level of formaldehyde. Further oxidation of formaldehyde to CO\textsubscript{2} is dissimilatory, generating metabolic reducing equivalents. The division of these bacteria into types is based upon their mode or pathway of formaldehyde assimilation. Type I (genera Methylomicrobium, Methylomonas, Methylospheara, Methylocaldum, and Methylobacter) methanotrophs use the ribulose monophosphate (RuMP) pathway and type II (genera Methylosinus, Methylocella, and Methylocystis) use the serine pathway. Type X (e.g. Methylococcus) utilizes the RuMP pathway, while also containing enzymes involved in the serine pathway. Type X also grow at higher temperatures than Types I and II. Researchers within the bioinorganic community largely study MMOs derived from the organisms Methylococcus capsulatus (Bath) (Type X) and Methylosinus trichosporium OB3b (Type II).

Apart from the genus Methylocella, all types of methanotrophs produce particulate forms of MMO (pMMO), which are housed in intracytoplasmic membranes. The genus Methylocella utilizes only sMMO. However, both types II and X are capable of expressing either pMMO or a cytoplasmic, sMMO. Which MMO is expressed is dependent upon the growth media, with sMMO being expressed under copper starvation conditions. When copper is present in sufficient concentrations the ‘copper switch’ is thrown, a currently unknown mechanism, and pMMO is expressed.

Given the importance of copper concentration in MMO expression, an efficient mechanism for copper uptake and acquisition is essential. Recent progress has been made concerning this subject. The main player has been identified as methanobactin (mb), originally coined, more generally, as copper binding compounds (CBCs) or copper binding ligands (CBLs), although the latter compounds were previously poorly identified and of variable sizes. Recently, mb has been isolated and more thoroughly characterized using XAS, XPS, and EPR, and is compared to the iron siderophores, the iron-binding compounds responsible for the cellular uptake of iron. Interestingly, copper bound mb is
reduced (Cu(I)) upon either Cu(I) or Cu(II) loading. The source of the reducing equivalent has yet to be determined. Cu(I) and Cu(II) binding to mb have been proposed to take place via different pathways. Cu(II) binding is proposed to proceed through multiple steps, which may involve dimeric and tetrameric forms, while Cu(I) binding appears to take place through a monomer species. Affinity for Cu(II) has been reported for various Cu:mb ratios, and can be as high as $3.25 \times 10^{34} \pm 3.0 \times 10^{11} \text{M}^{-1}$ for Cu:mb ratios between 0.07 and 0.2. A value of $>8 \times 10^{18} \text{M}^{-1}$ was reported upon fitting the isothermal titration calorimetry data. The affinity of mb for Cu(I) has been reported to be $(6–7) \times 10^{20} \text{M}^{-1}$.

pMMO and sMMO, although performing the same function, show large differences in their structural features. sMMO contains three components: a hydroxylase (MMOH), a regulatory protein termed B (MMOB), and a reductase (MMOR). All of these components must be present for methane oxidation. pMMO is trimERIC: each monomer is composed of three polypeptide subunits, $\alpha$ (pmoB), $\beta$ (pmoA), and $\gamma$ (pmoC), ~47, ~24, and ~22 kDa, respectively, in an $\alpha_3\beta_3\gamma_3$ polypeptide arrangement. This differs from MMOH of sMMO. MMOH is a heart-shaped dimer; the protomers are each made up of three subunits, and form an overall $\alpha_2\beta_2\gamma_2$ structure. X-ray crystallographic studies (Section 3.5.3) indicate that the pmoB subunit is made up of two cupredoxin-like $\beta$-barrels, which constitute the soluble domain, and two transmembrane helices. The pmoA and pmoC subunits constitute the majority of membrane-spanning region. Although the metal content and the location and nature of the active site for methane oxidation have been highly controversial, X-ray crystallography, in combination with a reactivity study involving site-directed mutagenesis has recently allowed for significant headway. It is now considered that the pmoB subunit contains the active site for methane oxidation and consists of a binuclear copper center ligated by highly conserved residues. Furthermore, in the Bath enzyme, the pmoB subunit also contains a mononuclear copper site, but this is not conserved across all pMMOs and is not observed in the crystal structure of the OB3b enzyme. The pmoC subunit houses another metal binding site. In M. capsulatus Bath it is occupied by zinc from the crystallization buffer, but houses copper in the OB3b enzyme. The function of the mononuclear metal sites is not currently understood. A more detailed discussion of the structure of pMMO is presented in Section 3.5.3.

3.5.2 Kinetics—Unlike the well-studied sMMO, only limited kinetic data exist for pMMO. Data that are currently available show a high degree of variability depending on preparation and purification procedure. The variations across different preparations have been previously discussed and tabulated. While sMMO is capable of oxidizing a wide range of substrates including $n$-alkanes, $n$-alkenes, aromatic and alicyclic compounds, pMMO is far less promiscuous and only shows the capability of oxidizing short alkane and alkene chains up to five carbons in length. Additionally, pMMO has been shown to hydroxylate $n$-butane and $n$-pentane enantioselectively to (R)-2-butanol and (R)-2-pentanol, respectively. Both pMMO and sMMO can oxidize chlorinated substrates.

The activity of pMMO samples is typically determined using the propylene epoxidation assay either with NADH (used for membrane bound pMMO) or duroquinol (used for solubilized pMMO) as a reductant. As mentioned, values obtained from these assays show a
high degree of variation. Nevertheless, specific activities of ~10–200 nmol propylene oxide (mg protein · min)\(^{-1}\) have been reported for the membrane bound form of \textit{M. capsulatus} (Bath), while values of ~2–126 nmol propylene oxide (mg protein · min)\(^{-1}\) have been reported for purified preparations.\(^{656,695}\) Purified \textit{M. trichosporium} OB3b pMMO had a reported activity of ~3–4 nmol propylene oxide (mg protein · min)\(^{-1}\). A more confusing aside is that, in one report, when pMMO was isolated under anaerobic conditions, a specific activity of 290 nmol propylene oxide (mg protein · min)\(^{-1}\) was measured, the highest yet observed.\(^{670}\) This is inconsistent with another report that anaerobic purification yields an enzyme with little to no activity.\(^{694}\)

Although it is typical to use NADH or duroquinol (only quinols can reduce solubilized pMMO)\(^{675,694–69}\) in determining the enzymatic activity, the \textit{in vivo} electron donor is unknown. Some data exist that suggest various possible physiological electron donors for pMMO. pMMO activity is inhibited by diphenyliodonium (DPI). DPI is known to inhibit type 2 NADH:quinone oxidoreductase (NDH-2), which is proposed to catalyze the reduction of pMMO through the reduction of a pool of endogenous quinones. These reducing equivalents may arise from oxidation of formaldehyde and formate during metabolism (Figure 116). Alternatively, methanol dehydrogenase and the cytochrome \(bc_1\) complex have also been implicated as having possible involvement in the electron transfer process. The former proposal is based on the observation of increased pMMO activity when copurified with 63 and 8 kDa polypeptides, where the 63 kDa polypeptide may be methanol dehydrogenase. The cytochrome \(bc_1\) complex may reduce pMMO through ubiquinone 8, a hypothesis based on the copper induced coexpression of pMMO and the cytochrome \(b\)-linked FalDH. A putative docking site for electron transfer agents, which consists of several negatively charged residues, has been observed by X-ray crystallography (Section 3.5.3).\(^{685}\)

pMMO from \textit{M. trichosporium} OB3b is inhibited by the aerobic addition of \(\text{H}_2\text{O}_2\).\(^{691}\) This was determined by the observation that the addition of catalase to pMMO reduced with duroquinol increased the activity of pMMO. \(\text{H}_2\text{O}_2\) inhibition could also be reversed by the subsequent addition of catalase.

Studies of sMMO have benefited from a large body of kinetics data using radical clock type probes; however, little data of this type have been accumulated for pMMO. Furthermore, single-turnover reactivity has yet to be addressed for pMMO, and therefore little is known about the catalytic cycle. Using chiral ethanes and butanes,\(^{697}\) Chan and coworkers have observed complete retention of stereochemistry upon hydroxylation and a \(k_{\text{H}}/k_{\text{D}}\) kinetic isotope effect of 5.2–5.5 at 30 °C.\(^{698}\) Further study indicated that, for multicarbon alkane chains, pMMO preferentially oxidizes at the C-2 position.\(^{699}\) Also, no intermolecular \(^{12}\text{C}/^{13}\text{C}\) kinetic isotope effect was observed for pMMO initiated propane hydroxylation at the secondary carbon (no \(^{12}\text{C}/^{13}\text{C}\) isotope effect was found for sMMO either), which was interpreted as indicating that there is very little transition state structural change at the carbon center involved in the oxidation.\(^{700}\) Alkene epoxidation of \textit{cis}- and \textit{trans}-but-2-ene by pMMO yielded only the \textit{meso} and \textit{d,l}-2,3-dimethylloxirane products, which led the authors to favor an electrophilic syn addition across the double bond.\(^{699}\) With these data, Chan and coworkers argue that methane oxidation by pMMO occurs via a concerted, direct O-atom (or oxene) insertion mechanism (\textit{vide infra}, Section 3.5.5).\(^{700,701}\)
In order to unravel the mechanism of pMMO, further single turnover kinetics data, activation energies, and trapping intermediates will be important.

3.5.3 Structure—X-ray crystal structures of pMMO have been reported for both the *M. capsulatus* (Bath)\(^68^5\) and *M. trichosporium OB3b\(^65^8\) enzymes at resolutions of 2.8 and 3.9 Å, respectively. We first address the Bath structure and then highlight important differences between the *M. capsulatus* Bath and *M. trichosporium OB3b* structures. pMMO is found to crystallize as a trimer, as mentioned above, consisting of three copies each of the pmoB, pmoA, and pmoC subunits, which together form an \(\alpha_3\beta_3\beta_3\) polypeptide arrangement (Figure 117). The trimer is \(-105\) Å in length and \(-90\) Å in diameter with a large hole at its center. The three pmoB subunits form the soluble region, which consists of six \(\beta\)-barrels, two from each protomer, and extends \(-45\) Å from the membrane region (Figure 117). The pmoA and pmoC subunits comprise the majority of the membrane region, which is spanned by 42 transmembrane helices, 14 from each protomer. The hole is \(-11\) Å in diameter in the soluble region and expands to \(-22\) Å on its way through the intramembrane space. Hydrophilic residues (e.g., glutamic and aspartic acid and arginine) line the inside of the hole in the soluble region while hydrophobic residues line the hole in the membrane region. The hydrophilic residues are not conserved, however.

A single protomer is shown in Figure 118a. It contains one copy each of pmoA, pmoB, and pmoC. A single subunit of pmoB, shown in Figure 118b, consists of two anti-parallel \(\beta\)-barrels (one seven stranded, the other eight) located at both the N-terminus and C-terminus. These \(\beta\)-barrels are roughly perpendicular to one another and are separated by a \(\beta\)-hairpin and two transmembrane helices. pmoA and pmoC are shown in Figure 118c and Figure 118d, respectively. pmoA is made up of seven transmembrane helices, which pack against the two from pmoB. Some of these helices are parallel to the membrane while others are tilted. The five transmembrane helices of pmoC are parallel to the membrane.

pmoB was found to contain two distinct metal sites: one mononuclear copper center and one binuclear copper site. The mononuclear copper center is located \(-25\) Å above the membrane near the N-terminal \(\beta\)-barrel surface (Figure 118a and b) and is coordinated via \(\delta\)-N atoms of His48 and His72 in a close to linear fashion (Figure 119A). Another residue, Gln404, is within \(-3\) Å of copper. No solvent ligands could be discerned at 2.8 Å resolution. Neither His48 nor Gln404 are conserved across all pmoB subunits, and this results in different metal contents across pMMOs (vide infra). Also contained within the pmoB subunit is a binuclear copper site (Figure 119B), found to be \(-10\) Å from the lipid bilayer interface (arrow, Figure 118a). The Cu-Cu distance was refined to \(-2.6\) Å, in good agreement with EXAFS experiments (vide infra, Section 3.5.4). One copper is coordinated by both the \(\delta\)-N and N-terminal amino nitrogen of the N-terminus His33 residue (the first 32 residues have been proposed to be part of a leader sequence) while the other copper is coordinated by both the \(\delta\)-N and \(\varepsilon\)-N of His137 and His139, respectively—two highly conserved residues in various pMMOs. pmoC contains the third metal binding site in the crystal structure. This site, \(-19\) Å from the binuclear copper site, contains zinc (Figure 118a, just beneath arrow). The Zn\(^{2+}\) coordination environment is represented by a distorted tetrahedron made up of the conserved residues Asp156, His160, and His173 from pmoC and Glu195 from pmoA (Figure 119c). The authors contribute the presence of zinc in this site to zinc acetate derived
from the crystallization buffer because of the virtual absence of zinc measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) before crystallization. The possibility of other metal binding sites was not fully ruled out. Chan et al have used the crystal structure to model a trinuclear copper cluster in a site ~13 Å from the ‘zinc site’, termed ‘Site D’ (Figure 120). The presence of this cluster was considered on the basis of metal stoichiometry and spectral data (vide infra, Section 3.5.4). This putative metal binding site is composed of a number of hydrophilic residues from the pmoA subunit (His38, Met42, Met45, Asp47, Trp48, Asp49, and Glu100) and one from the pmoC subunit (Glu154). Chan et al proposed that the lack of metals in this site in the crystal structure was due to loss of copper during purification. In summary, the X-ray crystallographic results for M. capsulatus (Bath) pMMO show that the enzyme contains both a mononuclear and a binuclear copper site located within the soluble pmoB (spmoB) subunit, a possible third metal binding site in pmoC, and a hydrophilic pocket that may house other metals.

The X-ray crystal structure of M. trichosporium OB3b shows interesting correlations to that of M. capsulatus (Bath). There was also a Cu-Cu interaction in this structure (~2.52 Å); however, the mononuclear copper site which was present in M. capsulatus (Bath) (in the pmoB subunit) is not found in pMMO from M. trichosporium OB3b as the ligands of this metal site are not conserved. The third metal binding site (zinc site) present in M. capsulatus (Bath) was found to be occupied by copper in M. trichosporium OB3b. Here, copper is coordinated by the same ligands found for the ‘zinc site’ in M. capsulatus (Bath) (ligands are conserved). Lastly, similar to the Bath enzyme, no copper was found in site D.

Recently, a 2.68 Å X-ray crystal structure of Methylocystis species strain M (type II) has appeared and is the highest quality structure yet determined. It shows structural similarities to both M. capsulatus (Bath) and M. trichosporium OB3b in that it contains a binuclear copper site (although not all pmoB subunits bind two copper ions, but instead contain only one (vide infra), a ‘zinc site’ (pmoC), and no mononuclear site (pmoB subunit) (Figure 121A). Furthermore, the overall trimeric structure is maintained; however this structure has led to revised models of the pmoA and pmoC subunits and the coordination geometry of the ‘zinc site’ in the M. capsulatus (Bath) structure. The pmoB subunit structure in Methylocystis species strain M is very similar to other pMMOs; however, two of the three binuclear active sites of the trimer were modeled as containing one copper ion. The lack of the second copper ion in these sites was proposed to be due to the increased lability of copper. The structure also revealed that part of pmoA in the Bath enzyme structure was actually from pmoC. An overlay of pmoA from the Methylocystis species strain M and the original M. capsulatus (Bath) structures are shown in Figure 121B. A difference was observed at Thr196 (Thr191 in M. capsulatus (Bath) pmoB), which is labeled in Figure 121B, where it extends along the membrane (on the periplasmic side) and is connected to a transmembrane helix (residues 217–244) and terminates at residue 252 (on the cytoplasmic side of the membrane). This is different from the structure from Bath, in which Thr191 is modeled as continuing through the membrane to the cytoplasmic side and winds up toward the periplasmic side (overlaid with residues 217–244 of Methylocystis species strain M). This difference in traces indicated that residues 195–215 in the M. capsulatus (Bath) structure belonged to pmoC, which now makes them residues 178–197 of Methylocystis
species strain M pmoC. Furthermore, the residues 225–252 of pmoC from Methylocystis species strain M (Figure 121C) were proposed to derive from a yet to be identified polypeptide. Because the extra helix is not observed in the Bath enzyme (Type X) and is also observed in the OB3b enzyme, it was suggested that it may be related to type II methanotrophs. The \textit{M. capsulatus} (Bath) structure was resolved using this model as a starting point and resulted in a higher quality fit. Also, instead of Asp156, His160, and His173 from pmoC and Glu195 (\textit{M. capsulatus} (Bath) numbering) from pmoA being bound to zinc, this structure showed that zinc was bound by only Asp129, His133, and H146 from pmoC. Glu200 (\textit{Methylocystis} species strain M numbering) from pmoA is H-bonded to pmoB residues. A fourth ligand was modeled as water, but possible partial occupation of this ligand binding site by a disordered side chain was not ruled out because of the poor electron density in this region and the fact that residues 197–225 could not be modeled. This structure shows that the ‘zinc site’ is now exposed and resides along the wall of the hole in the center of the trimer.

The hydrophobicity of a protein pocket adjacent to the binuclear site has led to the suggestion that it may support methane binding. This pocket is lined with hydrophobic residues (Pro94 from pmoB and Leu78, Ile163, and Val164 from pmoC). Other aspects of the protein structure have been used to make observations concerning putative electron donors. As mentioned in Section 3.5.2, the \textit{in vivo} electron donor is not known for pMMO. It was noted previously that there was a section of non-protein derived, strong electron density found close to the interface of the pmoB \(\beta\)-barrels (Figure 122, Left). This density was overlaid with the structure of a duroquinol molecule, and it was suggested that quinols derived from exogenous quinones could bind in this region. Also, Figure 122, Right shows a surface representation of the electrostatic potential of a pMMO protomer. A negative (red) patch made up of acidic residues from both the pmoB and pmoC subunits is located in the soluble region. This patch of density is near the binuclear copper site in the pmoB subunit and was compared to the proposed cytochrome \(c\) docking site in cytochrome \(c\) oxidase.

It is also interesting to compare the binuclear copper site found in pMMO to those observed for the other coupled binuclear copper sites in hemocyanin and tyrosinase (Section 3.1 and 3.2), as well as the T3 site in the MCOs (Section 3.7.1). In pMMO, the protein only provides two nitrogen-based ligands to each copper, whereas three histidine-derived ligands to each copper are present in the other binuclear copper sites. Furthermore, the Cu-Cu distance in pMMO is very short (~2.6 Å) relative to hemocyanin (~4 Å), tyrosinase (~3.5 Å), and the T3 site in the MCOs (~6 Å) in their deoxy states. The function of protein constraints on the ligand field geometry of the coppers and the initial Cu-Cu separation has already been shown to play a functional role in the differences in \(O_2\) activation (or reversible binding) across these related active sites (e.g. the close Cu(I)-Cu(I) distance in hemocyanin relative to the T3 site provides the driving force for \(O_2\) binding in the former). Some experimental data concerning \(O_2\) (and/or \(H_2O_2\)) activation at the binuclear site in pMMO exist and are summarized in Section 3.5.4.

As mentioned in Section 3.5.1, copper uptake and acquisition is accomplished by mb. Despite being difficult to isolate and purify, several different types of mbs have been identified, although only a couple have been fully structurally and chemically characterized.
The 0.92 Å resolution X-ray crystal structure of mb from *M. trichosporium* OB3b is shown in Figure 123.684 The exact mass has been determined from mass spectrometry to be 1215.1781, with the molecular structure of 1-(N-[mercapto-{5-oxo-2-(3-methylbutanoyl)oxazol-(2)-4-ylidene}methyl]-Gly\(^1\)-L-Ser\(^2\)-L-Cys\(^3\)-L-Tyr\(^4\))-pyrrolidin-2-yl-(mercapto-[5-oxo-oxazol-(2)-4-ylidene]methyl)-L-Ser\(^5\)-L-Cys\(^6\)-L-Met\(^7\) (C\(_{45}\)H\(_{56}\)N\(_{10}\)O\(_{16}\)S\(_{5}\)Cu\(^−\)).678,681 Other forms of fully structurally characterized mbs vary slightly.683 Cu(I) is bound in a distorted tetrahedron by two nitrogen (~2.0 Å) and two sulfur ligands (~2.4 Å) provided by the two non-amino acid moieties (oxazolone rings), which each contribute one nitrogen and one sulfur ligand (Figure 123). The sulfur ligands are modeled as thionyl groups (C=S-Cu) as opposed to thiolates (C-S-Cu).

### 3.5.4 Electronic Structure

In this section, we first briefly review the spectral features of mb, then focus on the spectral data of pMMO. Because of the controversial nature of the active site and its rationalizations based on the reported spectroscopy, we briefly summarize these spectral data and the proposed structures, but focus more on the data that directly relate to the *M. capsulatus* (Bath) and *M. trichosporium* OB3b crystal structures (Section 3.5.3).

To date, absorption data for apo-mb have been reported for *M. trichosporium* OB3b from various preparations with only subtle differences.678,679,683,684,705,706 They show several features in the 200–400 nm region. From low to high energy, these features have been assigned to the oxazolone rings and the thionyl functionalities. Small contributions in the higher energy region (<300 nm) may also occur from the disulfide linkage as well as the phenolic group of the tyrosine residue.679 The absorption spectra do show variability with addition of Cu(II), pH, and UV light exposure.678 Fluorescence data at various wavelengths have been reported as well, and have been shown to be quenched upon addition of Cu(II).678,706 As mentioned in Section 3.5.1, Cu(I) is bound in mb, despite Cu(II) loading. The source of the reducing electron is not currently known, but structural, analytical, and spectroscopic methods have been able to rule out the S(Cys) residues or disulfide linkages of the copper binding molecule or a ‘sacrificial’ pool of mb molecules which form disulfide linkages.679,680,684 In addition, the origin of high binding affinity for Cu has not been determined and no electronic structure calculations have appeared. A bonding analysis for copper mb would be important in determining the origin of the high affinity for copper and, possibly, whether or not the ligand donates the reducing equivalent upon Cu(II) binding.678 mb can also bind other metals, but with a reduced affinity relative to Cu.707

As opposed to sMMO, the study of pMMO has not benefited from a large body of spectroscopic data on different forms of the enzyme, and no catalytic intermediates have yet been observed. This is due mostly to the difficulties associated with handling and purifying pMMO, which has led to a number of different preparation protocols to be implemented and reported in the literature. Not surprisingly, the metal contents and stoichiometries of these preparations vary considerably. Tabulations provide a summary of these various preparations and the resultant metal analyses.656,686 Some spectroscopic data do exist on these different samples, and they have been used to generate hypotheses of the active site structure of pMMO. Chan and coworkers, with samples containing 13.6 Cu per 100 kDa, observed a broad, isotropic EPR signal at g ~2.1. This signal was superimposed with a type
2 EPR signal ($g_\parallel = 2.24$ and $g_\perp = 2.059$), which exhibited hyperfine coupling ($190 \times 10^{-4}$ cm$^{-1}$). The $g = 2.1$ signal was assigned to a ferromagnetically coupled trinuclear Cu cluster, while the type 2 signal was proposed to arise from a separate trinuclear Cu(II) cluster containing two antiferromagnetically coupled Cu(II)'s. When combined with the Cu stoichiometry, Chan and coworkers proposed pMMO housed five trinuclear Cu clusters: 3 E-clusters, which function as ET conduits, and two catalytic C-clusters. Note that the appearance of the X-ray crystallographic data led to the revision of this original assignment to a single catalytic trinuclear Cu center, which was modeled into the M. Capsulatus (Bath) crystal structure (vide supra, Section 3.5.3). Chan and coworkers also pointed out that the other C-cluster could also be mononuclear or binuclear copper. Other groups have not been successful in reproducing the broad, isotropic $g = 2.1$ signal, but do observe the type 2 EPR signal. Alternative interpretations of the $g = 2.1$ EPR signal have been suggested. Other propositions for the active site structure in pMMO include a Cu/Fe site, a binuclear iron site, or a binuclear copper site. The Cu/Fe proposition by DiSpirito and coworkers was due to the observation that pMMO activity increased upon addition of Fe(III) to M. trichosporium OB3b membrane preparations isolated from cells grown at low iron concentrations. Mössbauer data indicated the presence of a binuclear Fe(III) center, which was observed to be diamagnetic and thus antiferromagnetically coupled. The binuclear iron site was proposed to reside in the crystallographic ‘zinc site’ in M. Capsulatus (Bath).

Before the crystal structure in 2005, Rosenzweig et al suggested pMMO from M. Capsulatus (Bath) contained both a mononuclear copper site and a copper containing cluster. In that study, the XANES exhibited features indicative of both Cu(I) and Cu(II) in as-isolated pMMO (pMMO$_{iso}$). The Q-band EPR data for purified and membrane bound pMMO are given in Figure 124, Left. The spin Hamiltonian parameters were reported to be $g_\parallel = 2.24$, $g_\perp = 2.05$, $A_\parallel = 177 \times 10^{-4}$ cm$^{-1}$. Integration of the X-band EPR signal indicated that this species accounted for 40–50% of the total copper present (Note a variety of methanotrophs display this type 2 EPR signal). The intensity of this EPR feature did not increase upon ferricyanide addition, but did go away entirely upon addition of dithionite. The EXAFS data indicated a Cu-metal interaction, which was best fit to a 2.57 Å Cu-Cu interaction. Another study has provided more information on the nature and oxidation state of the metal sites in purified pMMO. Again, the XANES indicated the presence of both Cu(I) and Cu(II) in the as-isolated sample (Figure 124, Middle; note that the 8984 eV feature is characteristic of Cu(I) (see Section 2.1). The intensity of the Cu(I) feature increased upon reduction and decreased slightly with the addition of H$_2$O$_2$, although it did not disappear entirely. EPR spin quantitation indicated that ~40% of copper in pMMO$_{iso}$ and oxidized (pMMO$_{ox}$) was EPR active. As observed before, this type 2 signal went away upon reduction. It also did not increase in intensity upon H$_2$O$_2$ addition. EXAFS data for pMMO$_{ox}$, pMMO$_{iso}$, and pMMO$_{red}$ are given in Figure 124, Right. Single first shell fits to these data gave Cu-O/N bond lengths of 1.95, 1.97, and 2.11 Å for pMMO$_{ox}$, pMMO$_{iso}$, and pMMO$_{red}$, respectively. Addition of a split first shell Cu-O/N ligand environment improved the fits. The bond distances for pMMO$_{red}$ and pMMO$_{ox}$ were within the range of 1.93–2.11 Å. However, the Cu-O/N ligand environment in pMMO$_{iso}$ was best fit with inclusion of a bond at 2.22 Å in addition to 1.97 Å. Fits were further improved when scattering.
interactions at >2.5 Å were included. This resulted in a Cu-Cu bond length of 2.51 Å (a refinement of the previously reported 2.57 Å) for pMMO\textsubscript{iso} and pMMO\textsubscript{ox}. This increased to 2.65 Å in pMMO\textsubscript{red}. Furthermore, the Fe EXAFS data reported in this study did not yield an iron-metal scattering in the 2.5–2.65 Å range, which ruled out a Cu/Fe site. The very low intensity (~3 % of the Cu edge) observed in the Zn fluorescence region was used to rule out the presence of zinc in the ‘zinc site’ observed in crystallography, which indicated that this site was loaded with either copper or another metal. The EPR data (Q-band, 2K) also indicated that the iron present in these samples corresponded to high-spin ferric heme impurities ($g_\perp = 6.00$ and a $g_\parallel$ feature that could not be observed due to overlap with the copper-based signals).

Spectral data relative to the OB3b enzyme crystal structure have been reported (i.e. purified \textit{M. trichosporium} OB3b, three Cu/protomer and no iron or zinc).\textsuperscript{658} The X-Band EPR spectra are given in Figure 125, Left. These contained contributions from two species: a major component ($g_\parallel = 2.247, g_\perp = 2.052, A_\parallel^{Cu} = 196 \times 10^{-4} \text{ cm}^{-1}$) and a minor component ($g_\parallel = 2.225$ and $g_\perp = 2.060$ with an $A_\parallel^{Cu}$ very similar to the major component). The relative amount of the two components was not determined, but it was estimated that the major component made up at least 80% of the total copper present. Cu XANES data are given in Figure 125, Middle, and showed that purified \textit{M. trichosporium} OB3b contained a mixture of Cu(I) and Cu(II). EXAFS data (Figure 125, right) were best-fit with a Cu-O/N ligand environment with 1.97 Å bond lengths and a Cu-Cu distance of 2.52 Å.

A limited set of spectroscopic data do exist on variants (obtained by site-directed mutagenesis) available in pMMO from a heterologous expression system in \textit{E. coli} (\textit{vide infra}, Section 3.5.5).\textsuperscript{659} These are given in Figure 126, Left. Fits to the EXAFS data for spmoB (Figure 126, Left, A and B) yielded a Cu-O/N environment with bond lengths of 1.95 Å and a Cu-Cu interaction at 2.53 Å. The XAS edge data on spmoB (Figure 126, Right) indicate that all copper is present in the reduced state, despite loading Cu(II). The source of the electron was not determined, nor was the possibility of photoreduction ruled out. The Cu-Cu interaction at 2.53 Å in spmoB is shorter than that found in pMMO\textsubscript{red} (2.65 Å).

Some spectral data exist for the enzyme variants spmoB\_H48N and spmoB\_H137,139A (Figure 126, Left, (C,D) and (E,F) and Figure 126, Right, (B,C)). The former was designed to eliminate the mononuclear copper site (this is the copper site that is not present in the OB3b enzyme, but is present in the Bath enzyme) and the latter to eliminate the binuclear copper center (\textit{vide infra}, Section 3.5.5). The XAS data on the these variants indicate that copper is present as a mixture of Cu(I) and Cu(II) (Figure 126, Right). The EXAFS data for spmoB\_H48N were best fit with a Cu-O/N ligand environment of 1.96 Å and a Cu-Cu distance of 2.52 Å. The presence of a binuclear copper site in spmoB\_H137,139A was ruled out by the fact that, upon including a second shell, the fits were best when carbon was used as a scatterer rather than copper.

Thus, the current, most favored situation for the oxidation states of the metal ions in pMMO\textsubscript{iso} from the combination of the X-ray crystallography and spectroscopy is that the binuclear copper site is in a localized mixed-valence Cu(I)Cu(II) state.\textsuperscript{657} Since reduction eliminates the type 2 EPR signal and the Cu-Cu distance increases from 2.51 to 2.65 Å, this signal is thought to be associated with the mixed-valent binuclear copper site. Thus, the
binuclear copper site would be in a Cu(I)Cu(I) state in the fully reduced enzyme, which is important for further development of the mechanism of methane oxidation (vide infra, Section 3.5.5). The copper bound in the mononuclear and ‘zinc site’ would then be either Cu(I) or Cu(II). Note that the assignment of the Cu(II)Cu(I) site as localized mixed-valent (vide supra) is difficult to rationalize due to the short Cu-Cu distance (2.51 Å). This short distance leads to a direct overlap of the Cu 3d orbitals and will strongly favor a delocalized Cu(1.5)Cu(1.5) site. It is also interesting to note that the assignment as localized mixed-valent comes from the lack of the observation of a seven-line hyperfine pattern. An analogous situation occurs in CuA, which is a fully delocalized mixed-valent Cu(1.5)Cu(1.5) site (vide infra, Sections 3.7.2 and 5.2), where upon lowering the pH from 7 to 5 the seven-line hyperfine pattern collapses into a four-line pattern. Originally, this was thought to reflect a transition from a delocalized to a localized mixed-valent state; however, in CuA, lowering the pH from 7 to 5 resulted in the loss of a His ligand due to protonation and allowed a small amount of 4s mixing on one of the coppers. This mixing is responsible for the change in the EPR spectrum, but the site remains fully delocalized (from ENDOR and rR spectroscopy) due to the strong Cu-Cu interaction. If the binuclear site in pMMO is mixed-valent, a possibility such as this has yet to be ruled out. Further spectroscopic study of pMMO in various oxidation states should further elucidate the nature of the metal sites in pMMO.

Some spectroscopic data suggest H₂O₂ and/or O₂ may bind at the binuclear copper site. Anaerobic reaction of reduced pMMO (solubilized) with H₂O₂ (incubated overnight at room temperature) resulted in the formation of an absorption band at 345 nm (ε not reported) (Figure 127 (left)). This absorption band does not form using O₂ instead of H₂O₂. The same experiments were carried out in spmoB and the spmoB_H48N, spmoB_H137,139A, and spmoB_H48N_H137,138A variants. For reduced spmoB, addition of O₂ and H₂O₂ both result in the formation of 345 nm absorption feature (ε ~10 000 M⁻¹cm⁻¹) (Figure 127 (middle)). Titration data indicated binding of two molecules of H₂O₂ per spmoB. Only the spmoB_H48N variant was capable of forming the 345 nm band with O₂ and H₂O₂ (Figure 127 (right)). Furthermore, incubation of CH₄ and the spmoB samples in which the 345 nm band was formed (1h and 45 °C) resulted in a loss of absorption intensity, which was proposed to indicate that the 345 nm species is involved in the CH₄ oxidation pathway. However, it should be noted that protein degradation occurred during this process and inhibited the monitoring of the absorption feature with time. The results of a similar experiment without CH₄ were not reported. The absorption band energy and ε of these Cu₂-H₂O₂/O₂ derived species are all consistent with the formation of the following: 1) μ-η²-η²-peroxo-Cu(II)₂; 2) met-Cu(II)₂; and 3) hydroxo-bridged Cu(II)₂ type 3 site. These results support the fact that Cu₄/O₂ species can be formed at the binuclear copper site in both solubilized pMMO and spmoB. Further studies are needed to determine whether they are involved in catalysis. Lastly, also interesting are possible comparisons between the results presented here and those for the O₂ activation of Cu-ZSM-5, the only binuclear copper model capable of methane oxidation, where a μ-η²-η²-peroxo-Cu(II)₂ is the precursor to a mono-(μ-oxo) dicopper(II) reactive intermediate (vide infra, Section 3.5.5).
3.5.5 Mechanism

3.5.5.1 Methane Oxidation by pMMO: Recently, a study by Rosenzweig and coworkers has addressed the location and nature of the active site for CH₄ oxidation in pMMO, and builds on this group’s report of the 2.8 Å X-ray crystal structure of pMMO from *M. capsulatus* (Bath) (*vide supra*, 3.5.3). Using a series of variants from site-directed mutants, the authors showed that methane oxidation occurs at the binuclear copper site in the soluble domain of the pmoB subunit.

Specifically, the as-isolated pMMO sample in membranes had a specific activity of 50–200 nmol propylene oxide mg⁻¹ min⁻¹. Cyanide treatment to remove metal ions eliminated all activity in the propylene epoxidation assay. Reconstitution of apo pMMO with 2–3 equivalents of copper/100 kDa pMMO protomer restored ~70 % of the propylene epoxidation activity. The ability for these samples to oxidize methane was also investigated. The as-isolated pMMO samples had a specific activity of 22.9 ± 6.1 nmol methane mg⁻¹ min⁻¹. Reconstitution with copper after cyanide treatment restores ~90 % of the activity (21.7 ± 3.5 nmol methane mg⁻¹ min⁻¹). Addition of iron under either aerobic or anaerobic conditions to the apo pMMO, however, does not form an active enzyme. This was taken as evidence that copper, not iron, is contained in the active site of pMMO.

With the knowledge that copper is the metal in the active site, the authors showed that the location of the active site is in the spmoB subunit, specifically, spmoBd1 (the amino (N)-terminal cupredoxin domain; residues 33 – 172, contains both the binuclear and mononuclear copper sites). The soluble domains of the pmoB subunit were expressed in *E. coli*. spmoB, spmoBd1, and spmoBd2 (the carboxy (C)-terminal cupredoxin domain; residues 265 – 414) load copper in quantities of 2.84 ± 0.66, 1.59 ± 0.84, 0.24 ± 0.09, respectively. spmoB loads 0.17 ± 0.1 iron ions per protein. (The low copper loading value of 1.59 ± 0.84 for spmoBd1 is attributed to protein instability due to loss of stabilizing contacts (i.e., H-bonds and hydrophobic contacts) between spmoBd1 and spmoBd2). Both propylene epoxidation and methane oxidation activity were measured for these samples. The results are summarized in Figure 128, in which activity is given as the percentage of activity as compared to the activity of as-isolated, membrane-bound *M. capsulatus* (Bath) pMMO. The specific activity for propylene epoxidation by spmoB was reported to be 30.2 ± 10.5 nmol propylene oxide µmol⁻¹ min⁻¹ (note change in units), while the same assay under similar conditions for as-isolated pMMO yielded 51.1 ± 11.3 nmol propylene oxide µmol⁻¹ min⁻¹. For methane oxidation, spmoB yielded 203.1 ± 20.2 nmol methanol µmol⁻¹ min⁻¹, and the as-isolated pMMO yielded 325.1 nmol methanol µmol⁻¹ min⁻¹, spmoBd1, which houses both the mononuclear and binuclear copper sites, showed activity in both assays: 8.1 ± 3.7 nmol propylene oxide µmol⁻¹ min⁻¹ and 19.3 ± 4.7 nmol methanol µmol⁻¹ min⁻¹. Neither the spmoBd2 nor the iron loaded spmoB showed activity for either assay. These data eliminated the possibility that a binuclear iron site in the zinc crystallographic site or a trinuclear cluster in the intramembrane space could represent active sites for methane oxidation in pMMO.

To distinguish between the mononuclear and binuclear site being responsible for methane oxidation, the authors also studied several site directed mutants of spmoB. The
The spmoB_H48N variant, which eliminates the mononuclear copper site, binds 1.86 ± 0.52 copper ions per protein and had specific activities for propylene epoxidation and methane oxidation of 2.3 ± 0.4 and 14.8 ± 9.2, respectively. The spmoB_H137,139A and spmoB_H48N_H137,139A double and triple variants, which eliminate only the binuclear and both the binuclear and mononuclear copper sites, respectively, both bound copper ions (0.73 ± 0.15 and 0.82 ± 0.36, respectively), yet had no measurable activity. Interestingly, the authors note removal of the mononuclear copper site does not completely eliminate activity, even for the methane oxidation assay. These results show that the binuclear copper site can oxidize methane to methanol in pMMO. Now, it remains to determine the mechanism for this important reaction in pMMO.

Since no intermediates have been identified in pMMO to date, the current insight into the mechanism of pMMO has largely been derived from models—both experimental and computational. Below, we first summarize the computational studies that have appeared and follow with a description of the one well defined copper active site model for the methane to methanol conversion—specifically the zeolite Cu-ZSM-5.

### 3.5.5.2 DFT Calculations of C-H Activation of Methane by Cu$_x$O$_y$ Complexes

Quantum chemical calculations related to pMMO have mainly focused on evaluating the reactivity of a few Cu$_x$O$_y$ species. These structures are encompassed by trinuclear (5, considered to be a copper cluster where O$_2$ is reduced to a µ3 and a µ2 oxo), binuclear (2, 3, and 4), and mononuclear (1) copper complexes and are presented in Table 25. Methane oxidation has been modeled with two reaction mechanisms: concerted oxene insertion and hydrogen atom abstraction (Figure 129). The experimental data presented in Section 3.5.2 that indicate the presence of a KIE (5.2 ± 0.4 for [1-3H$_1$,2H$_1$]ethane and 5.5 ± 0.7 for [1-3H$_1$,2H$_1$,2-2H$_3$]ethane at 30 °C) and complete retention of stereochemistry for alkane hydroxylation have been used to argue for a concerted oxene insertion. However, these data cannot rule out a hydrogen atom abstraction mechanism. Of the structures in Table 25, 1, 3, and 4 were used to evaluate a hydrogen atom abstraction mechanism. Of these, 4 had the lowest calculated activation energy at 9.5 kcal/mol. 4 is disfavored relative to 3, however, because of the higher energy of formation, and 1 can be eliminated as it is formed at the mononuclear copper site, which is not conserved across pMMOs. Therefore, a hydrogen atom abstraction mechanism is most viable for intermediate 3. However, higher energy transition states are found in the complete reaction profile for 3. These represent C-O bond formation and rebound, which predicts a rate-limiting step other than C-H bond cleavage, in disagreement with the KIE for ethane hydroxylation.

A concerted oxene insertion has been evaluated for 2, 3, and 5. The activation energy for 2 is 44.0 kcal/mol$^{[19]}$ (a barrier of 21.0 kcal/mol has also been reported;$^{[20]}$ however, these calculations were performed using a spin restricted methodology, so the number is not likely reliable as the transition state should be spin polarized), which is much too high to represent the reactive species in the mechanism of pMMO. Activation energies of 21.7 and 15.0 kcal/mol have been reported for 3 and 5, respectively (a barrier of 40.5 kcal/mol has also been reported for 3. Here, upon formation of the transition state, a Cu-NH$_3$ bond is lost.
while a Cu-CH$_3$ bond is formed, which likely contributes to the high energy of this
transition state). Thus, the oxene insertion mechanism favors 5.

It is difficult to draw conclusions based on the computational results reported thus far. In
only one case are the activation energies for both mechanisms reported for one structure (3).
More specifically, the hydrogen atom abstraction mechanism has yet to be considered for 2,
which is known to abstract hydrogen from alkyl C-H bonds. Furthermore, low reaction
barriers can be obtained when high energy reactants are generated, which is the case for 4.

In summary, computational studies have yet to establish insight into the activation of a
binuclear copper site for reaction with inert C-H bonds of substrate. That is, what geometric
and electronic properties of the reactant, transition state, and product contribute to the
thermodynamics (i.e., O-H bond strengths) and the activation energy of the reaction (i.e.,
FMOs).

3.5.5.3 pMMO Active Site Models: Only very recently have copper containing molecular
models been made that can oxidize methane to methanol. A series of tricopper complexes
have been shown to oxidize hydrocarbons,\textsuperscript{722,723} including methane.\textsuperscript{724} Heterogeneous
systems, such as zeolites, offer alternatives for the study of the methane to methanol
conversion by copper active sites.\textsuperscript{39,725,726} Zeolites are microporous materials that can be
impregnated with metal ions. Dioxygen (as well as N$_2$O and NO) can be used as a reagent
for the generation of reactive, yet well isolated, M$_x$/O$_y$ species in the zeolite channels. In
many ways, this resembles the strategies adopted by Nature in the function of
metalloenzymes. The protein structure directs substrate access and guidance to a reactive
species, the formation and isolation of which is well controlled in order to prevent
deleterious side reactions with the protein. This active site isolation allows for the direct
spectroscopic study of molecular structures that would be very difficult to generate, trap, and
study in solution. This approach makes heterogeneous structures such as the zeolites or
metalorganic frameworks (MOFs) a powerful approach to studying reactive M$_x$/O$_y$ species.
The interaction of O$_2$ with various transition metal ion-containing zeolites has recently been
reviewed.\textsuperscript{727} Despite these attractive properties, however, one problem in studying zeolites,
as well as heterogeneous catalytic systems in general, is that the active site usually
represents a minority species, constituting only ~5% of the total metal content. This presents
a unique problem relative to the spectroscopic study of metalloproteins, in which the metal
cofactor is present in only ~0.2 weight % but in a spectroscopically silent (between 280 nm
and 2 µm and diamagnetic) protein background. In order to gain insight into the active site in
these systems, a direct spectroscopic handle on the active site and its reactivity is required
(vide infra).

The zeolite frameworks which are currently known to selectively oxidize methane include
ZSM-5, mordenite (MOR), ferrierite (FER), and beta (BEA).\textsuperscript{726} Both O$_2$ as well as N$_2$O
activated Cu-ZSM-5 and N$_2$O activated Fe-ZSM-5 (O$_2$ does not activate Fe-ZSM-5) can
oxidize methane into methanol at low temperature (100–150°C). Cu-MOR can oxidize
methane at 150°C as well, but there is an increase in methanol yield when reaction
temperatures reach 200 °C. This result has been ascribed to the presence of a second, less
reactive core in Cu-MOR. Both the Cu-FER and Cu-BEA require reaction temperatures
above 200°C. The nature of the reactive species in zeolite-based systems has remained elusive; however, recently the formation and reactivity of the reactive species in Cu-ZSM-5 have been elucidated in some detail.\textsuperscript{39,718} We review these results here.

Reduced Cu-ZSM-5 reacts with O\textsubscript{2} at 450°C and produces an intense absorption feature at 22,700 cm\textsuperscript{-1} (~440 nm) (Figure 130 Inset A) due to the formation of a Cu\textsubscript{y}/O\textsubscript{y} species. This O\textsubscript{2} derived active oxygen species was shown to stoichiometrically oxidize methanol into methanol at temperatures as low as 100°C. Upon exposure to CH\textsubscript{4}, the 22,700 cm\textsuperscript{-1} band is eliminated and CH\textsubscript{3}OH is produced. The low energy of the absorption band combined with EXAFS data (Cu-Cu distance of 2.87 Å) were initially used to assign this species as a bis(µ-oxo)dicopper(III,III) core (see Section 3.2).\textsuperscript{728} However, from the analysis of the amount of methanol formed, it was estimated that only ~5% of copper existed in the activated form,\textsuperscript{725,726} thus making the EXAFS results irrelevant as this is a bulk spectroscopic method. Also, an absorption band does not elucidate the nature of a species. In order to definitively define the methane oxidizing active core in Cu-ZSM-5 an appropriate spectroscopic method was needed. Laser excitation into the 22,700 cm\textsuperscript{-1} absorption band of O\textsubscript{2} activated Cu-ZSM-5 resulted in a very rich and informative resonance Raman spectrum. The data obtained after \textsuperscript{16}O\textsubscript{2} and \textsuperscript{18}O\textsubscript{2} (which generates CH\textsubscript{3}\textsuperscript{18}OH) activation are given in Figure 130. Isotope sensitivity is observed for features at 237 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 3 cm\textsuperscript{-1}), 456 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 8 cm\textsuperscript{-1}), 870 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 40 cm\textsuperscript{-1}), 974 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 10 cm\textsuperscript{-1}), 1725 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 83 cm\textsuperscript{-1}), and 1852 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 52 cm\textsuperscript{-1}). These vibrational data immediately ruled out the previous assignment of a bis(µ-oxo) dicopper(III,III) species due to the lack of an intense resonance enhanced 600 cm\textsuperscript{-1} vibration (See Table 26 for a compilation of the vibrational data of known Cu\textsubscript{y}/O\textsubscript{y} species) and are also inconsistent with either a µ-η\textsuperscript{1}−η\textsuperscript{1} dicopper(II) (ν(Cu-Cu) at ~270 cm\textsuperscript{-1}, ν(O-O) at ~750 cm\textsuperscript{-1}) or Cu(II)-superoxo (ν(O-O) at ~1,000–1,150 cm\textsuperscript{-1}) species. However, due to the observation of the 870 cm\textsuperscript{-1} isotope sensitive vibration, the \textsuperscript{16,18}O\textsubscript{2} data alone allowed the possibility that the Cu\textsubscript{y}/O\textsubscript{y} core has an O-O bond as η\textsuperscript{1}-hydroperoxide Cu(II), trans-µ-1,2-peroxo Cu(II), and hydroperoxo 2Cu(II) species can contribute in this region (Table 26). However, these alternatives were eliminated by using mixed-isotope O\textsubscript{2} to activate Cu-ZSM-5 (a statistical (1:2:1) mixture of \textsuperscript{16}O\textsubscript{2}–\textsuperscript{16/18}O\textsubscript{2}–\textsuperscript{18}O\textsubscript{2}). An O-O bond would show three vibrational features with a 1:2:1 intensity distribution pattern with the frequencies of the \textsuperscript{18}O\textsubscript{2}, \textsuperscript{16,18}O\textsubscript{2}, and \textsuperscript{18,16}O\textsubscript{2}, and \textsuperscript{16}O\textsubscript{2} at 830, ~850, and 870 cm\textsuperscript{-1}, respectively, for Cu-ZSM-5. Activation of Cu-ZSM-5 with mixed-isotope O\textsubscript{2}, however, resulted in the rR data in Figure 130 Inset B green. These are identical to the 1:1 normalized sum of \textsuperscript{16}O\textsubscript{2} and \textsuperscript{18}O\textsubscript{2} (black), which indicates that there is no O-O bond in the active species. Thus, the rR data ruled out all known Cu/O\textsubscript{2} structures in inorganic chemistry.

The most intense isotope sensitive feature was observed at 456 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 8 cm\textsuperscript{-1}). The 870 cm\textsuperscript{-1} feature (Δ(\textsuperscript{18}O\textsubscript{2}) = 40 cm\textsuperscript{-1}) had low intensity, while its second quantum, the 1725 cm\textsuperscript{-1} (Δ(\textsuperscript{18}O\textsubscript{2}) = 83 cm\textsuperscript{-1}) vibration, had an intensity that is six times higher than the fundamental. This supported the assignment of the 456 and 870 cm\textsuperscript{-1} vibrations as symmetric (ν\textsubscript{s}) and antisymmetric (ν\textsubscript{as}) stretches, respectively, of a mono-oxo group bridging two copper centers. Note that even quanta of antisymmetric modes are symmetric and rR allowed. Also, the energy of 2ν\textsubscript{as} in \textsuperscript{16}O\textsubscript{2} activated Cu-ZSM-5 was not exactly two times ν\textsubscript{as} (1740 cm\textsuperscript{-1} instead of 1725 cm\textsuperscript{-1}). This difference is due to a Fermi resonance
between the $^{18}\text{O}$-isotope sensitive fourth quantum of $\nu_s$, which is predicted to be at 1,824 cm$^{-1}$, and the $^{18}\text{O}$-isotope sensitive $2\nu_{as}$. Thus, the combination of an intense symmetric and a weak antisymmetric stretch was used to unambiguously assign the active species in Cu-ZMS-5 as a bent [Cu-O-Cu] core. The energy splitting of these symmetric and antisymmetric vibrations and their isotope shifts via normal coordinate analysis gave a Cu-O-Cu angle of $\sim 140^\circ$. This represented the first definitive characterization of a mono-oxygen bridged binuclear copper site in any system, synthetic, bioinorganic, or heterogeneous. It should be noted that, from UV-Vis, rR, and reactivity data, the identical site also forms with N$_2$O.

Monitoring the disappearance of the 22,700 cm$^{-1}$ absorption feature as a function of temperature allowed for the determination of an activation energy of 15.7 ± 0.5 kcal/mol (from Arrhenius plots of the reaction with CH$_4$ between 110 and 200 °C). Reaction with C$_2$H$_4$ resulted in an increased $E_a$ of the reaction by 3.1 ± 0.5 kcal/mol. These data show that O$_2$ activated Cu-ZSM-5 performs H-atom abstraction of the strong C-H bond of methane with a low barrier. The electronic structure contributions to the high reactivity of this core were defined using spectroscopically calibrated DFT calculations. A Cu$_2$O core with an angle of $\sim 140^\circ$ was modeled in the 10-membered ring of the ZSM-5 lattice and fit with two Al T-sites separated by two Si spacers (Figure 131). Both Cu(III)$_2$O and Cu(II)$_2$O models were considered; however, the former was eliminated due to the fact that the singlet wavefunction contained an internal instability. This relaxed the formally copper based holes into the zeolite lattice to form a Cu(II)$_2$O core and an electron deficient lattice. Thus, calculations were conducted using a [Cu$_{\text{AI}}$O-Cu$_{\text{BII}}$]$^{2+}$ species in the ferromagnetically coupled triplet state (predicted to be $\sim 2$ kcal/mol lower in energy than the antiferromagnetically coupled singlet state). DFT calculations on this Cu(II)$_2$O core accurately reproduced the vibrations observed from rR spectroscopy as well as the energy of the intense absorption feature (i.e. an oxo to Cu$^{\text{II}}$ CT). A transition state for hydrogen atom abstraction was found using this model. It had a calculated $E_a$ of 18.5 kcal/mol, which increased by 1.3 kcal/mol in the reaction with C$_2$H$_4$. These were in reasonable agreement with experiment. It was therefore shown that the computational Cu(II)$_2$O model in Figure 132 reproduced the low $E_a$ determined experimentally for H-atom abstraction from CH$_4$. Complete hydrogen atom abstraction (addition of both a proton and an electron) generated a delocalized [Cu$_{\text{A}}$-OH-Cu$_{\text{B}}$]$^{2+}$ intermediate and a carbon centered radical. This step was calculated to be endothermic by only 13.8 kcal/mol. This indicates that a strong O-H bond is formed in this intermediate ($\sim 90$ kcal/mol, calculated), which contributes to the low barrier. Further insight into the contributions to the reactivity of this core came from an analysis of the electronic structures of the transition state. It was found that there are two low lying singly-occupied orbitals (SOMOs) of the Cu(II)$_2$O core which contribute to the reactivity and the low activation barrier for C-H bond cleavage (Figure 132A). Upon formation of the transition state the frontier molecular orbitals (FMOs) of the [Cu$_{\text{AI}}$O-Cu$_{\text{BII}}$]$^{2+}$ core becomes strongly polarized to a [Cu$_{\text{A}}$O$^-$-Cu$_{\text{BII}}$]$^-$ “oxyl” species. This type of species, although yet to be observed experimentally in model chemistry, would be highly reactive as it has a low-energy unoccupied orbital with large O-character for overlap with the C-H bond. Indeed, the top SOMO in Figure 132B reflects this, showing 24% O and 60% CH$_4$ character, which is delocalized along the O-H-C vector. This strong orbital polarization at
the transition state is responsible for the low activation energy for methane oxidation. Thus, both Cu-ZSM-5 and pMMO oxidize CH\textsubscript{4} at a binuclear copper active site, which makes Cu-ZSM-5 a very promising molecular model of the reactivity of the enzyme.

A precursor to the mono(µ-oxo) dicopper(II) species in Cu-ZSM-5 has also been observed and spectroscopically characterized, which allows for a direct correlation of the zeolite model to binuclear copper enzyme chemistry\textsuperscript{718}. Reaction of reduced Cu-ZSM-5 with O\textsubscript{2} at room temperature yields a different absorption spectrum than when this reaction is carried out at 450°C. Under low temperature conditions, a band at ~29,000 cm\textsuperscript{-1} was rapidly formed (Figure 133A). Subsequent heating resulted in the direct transformation of the ~29,000 cm\textsuperscript{-1} band into the ~22,700 cm\textsuperscript{-1} band observed for the [Cu\textsubscript{2}O]\textsuperscript{2+} mono(µ-oxo) (Figure 133B). The ~29,000 cm\textsuperscript{-1} feature does not form with N\textsubscript{2}O; only the ~22,700 cm\textsuperscript{-1} band is formed, even at RT. rR spectroscopy with laser excitation at 363.8 nm on a RT, \textsuperscript{16}O\textsubscript{2} activated sample yields a spectrum with vibrational features at 269 and 736 cm\textsuperscript{-1} (Figure 134A, blue). Only the 736 cm\textsuperscript{-1} feature showed isotope sensitivity (Δ(\textsuperscript{18}O\textsubscript{2}) = 41 cm\textsuperscript{-1}). The absorption and rR data are characteristic of a µ(η\textsuperscript{2}-η\textsuperscript{2})peroxo dicopper(II,II) species with the ~29,000 cm\textsuperscript{-1} band assigned as the π* σ CT and the 269 and 736 cm\textsuperscript{-1} vibrations assigned to the Cu-Cu and O-O stretch, respectively. This is very similar to what is observed upon \textsubscript{2}O binding in Hc and Ty (Sections 3.1 and 3.2). Furthermore, upon heating the RT, \textsuperscript{16}O\textsubscript{2} sample, the 368.3 nm resonance enhanced features associated with the µ(η\textsuperscript{2}-η\textsuperscript{2})peroxo dicopper(II,II) species disappear while the 457.9 nm resonance enhanced features of the [Cu\textsubscript{2}O]\textsuperscript{2+} species grow in (Figure 134B). Thus, this coupled binuclear µ(η\textsuperscript{2}-η\textsuperscript{2})-[Cu\textsubscript{2}(O\textsubscript{2})]\textsuperscript{2+} species is a precursor complex and converts directly into the [Cu\textsubscript{2}O]\textsuperscript{2+} species, which oxidizes methane into methanol. Temperature programmed desorption (TPD) experiments showed that the second oxo atom transfers to the ZSM-5 lattice. The two electrons required to cleave the peroxo O-O bond appears to derive from spectator Cu\textsuperscript{+} located in ion-exchange sites. The resultant cycle for active site formation and reaction is summarized in Figure 135.

The reactivity cycle for Cu-ZMS-5 may have implications for the catalytic cycle of pMMO. Like ZSM-5 and sMMO, the cycle for pMMO may begin with a precursor type of complex in which O\textsubscript{2} is activated to a peroxo level species (\textit{vide supra}, Section 3.5.4). The Cu-Cu separation of ~3.3 Å in the zeolite likely drives O\textsubscript{2} binding and the reduction of dioxygen to the peroxo level in a µ(η\textsuperscript{2}-η\textsuperscript{2}) structure as in oxy Hc/Ty. Here, branching between the zeolite relative to the coupled binuclear copper sites may occur. In the zeolite, the Cu-Cu separation is not likely to change to a large extent during formation of the precursor and its subsequent transformation into the mono(µ-oxo) dicopper(II) species. This geometric constraint (i.e. an entatic type state associated with the zeolite lattice) on the Cu-Cu distance may play a role in driving the formation of a mono-(µ-oxo) as opposed to a bis(µ-oxo) species.

### 3.6 Structure/Function Correlations of O\textsubscript{2} Activation by Copper Sites

From Sections 3.1 – 3.5 we have observed that O\textsubscript{2} is activated by mononuclear copper sites in the non-coupled binuclear copper enzymes (the Cu\textsubscript{M} center) and in cofactor biogenesis by GO and by binuclear copper sites in the coupled binuclear copper enzymes and pMMO.
For the mononuclear copper sites, research efforts to this point have strongly indicated that a Cu(II)O$_2^-$ (i.e., cupric-superoxide) end-on triplet catalyzes a one-electron H-atom abstraction reaction. For both the non-coupled binuclear copper enzymes and the biogenesis reaction in GO, the X-H bonds are activated at ~87 kcal/mol, which would be accessible based on the [Cu(II)O$_2$]$^{2+}$ → [Cu(II)O$_2$H]$^+$ homolytic bond strength. Depending on the ligation and protein environment, this bond strength would be in the range of ~80 kcal/mol. The interesting issue in these mononuclear copper sites is the activation of O$_2$ by one electron reduction. The reduction potential for this process is −330 mV vs SHE depending on the conditions, and thus the ligation at the copper would have to tune down the potential and stabilize the Cu(II)-O$_2$$^{•−}$ bonding interaction (i.e., allow formation of an inner sphere complex). There can also be an entropic contribution depending on whether the O$_2$ replaces a coordinated H$_2$O.

In the coupled binuclear copper enzymes a [Cu(II)$_2$O$_2$$^{2−}$]$^{2+}$ peroxo intermediate species is clearly generated. The substrates are aromatics with strong C-H bonds (~110 kcal•mol$^{-1}$), and thus this reaction proceeds through an electrophilic aromatic attack, a two-electron process. This involves the transfer of the two electrons into the peroxide σ* orbital to cleave the O-O bond, either directly from the substrate or from the coppers if the bis-µ-oxo species is in fact formed along the reaction coordinate. Again this reaction is energetically accessible as the two-electron reduction potential of peroxide is ~300 mV vs NHE.

pMMO is rather unique in that the C-H bond is strong yet the enzyme uses a binuclear copper site to perform a one-electron H-atom abstraction. This would seem to require the formation of a strong O-H bond in the initial product as found to be the case in the Cu-ZSM-5 [Cu(II)$_2$O$_2$$^{2−}$]$^{2+}$ active site where the strength of the O-H bond formed is ~92 kcal•mol$^{-1}$. If an oxo (bridged or terminal) intermediate were indeed involved in pMMO, this would require that the 2Cu(I) reduced site react with O$_2$ to proceed to the 2Cu(III) state to reductively cleave the O-O bond. In this regard, it is interesting to note the significant structural differences in pMMO relative to the coupled binuclear copper enzymes where the 3 His/Cu of the latter are replaced by two endogenous ligands/Cu in pMMO, and one of these is a terminal amine. This strong equatorial donor ligation could promote further oxidation of the two coppers and the short Cu-Cu distance (~2.6 versus ~3.6 Å in the coupled binuclear copper enzymes) would also favor O-O cleavage. Clearly, it is important to define the factors in the pMMO binuclear copper site that activate it for H-atom abstraction from a strong C-H bond and in fact whether this is the correct description of the mechanism. As a final point, in the only intermediate in the CH$_4$ → CH$_3$OH reaction thus far characterized, the [Cu(II)$_2$O]$^{2+}$ FMO has the important property of polarizing to a Cu$^{1+}$$O$$^{•−}$ (i.e., oxyl) species at the transition state. This is also the case for Fe(IV)=O intermediates at their transition states and is likely a key requirement for H-atom abstraction from strong C-H bonds.$^{729}$

### 3.7.1 Multicopper Oxidases

Multicopper oxidases (MCOs) constitute a large and diverse family of enzymes, where oxidation of various substrates is coupled to the four-electron reduction of dioxygen generating two water molecules.$^{730}$ Common to all MCOs, this activity is carried out by a
minimum of four copper ions, arranged in a mono-nuclear Type 1 (T1) Cu site, which is the entry point of electrons from substrate, and a trinuclear Cu cluster (TNC), which accepts the substrate electrons and utilizes these in the 4 e⁻ reduction of dioxygen.⁷³⁰ The diversity of MCOs is inferred primarily at the T1 Cu site, where differences in coordination environments and solvent accessibility result in different substrate specificities.⁷³¹,⁷³²,⁷³³,⁷³⁴,⁷³⁵ Based on this, MCOs can be divided into two classes where members of the first target a variety of phenolic substrates, with broad specificity, while members of the second has high substrate specificity towards one- or two metal substrates (see Table 27). Some broad substrate MCOs have traits of both classes, with high specificity towards a single substrate, e.g. ascorbate or bilirubin, while still showing significant activity towards a broad range of phenolic substrates.⁷³⁶,⁷³⁷ The following sections will provide an overview of the enzymological (see also Table 28), kinetic, spectroscopic, and mechanistic properties of the MCOs.

3.7.1.1 Enzymology

3.7.1.1.1 Fungal laccases: The most abundant group of MCOs is the so-called laccases (EC 1.10.3.2), which are found throughout nature, and classified as broad substrate MCOs. Fungal laccases are the most well described MCOs, with over 100 enzymes purified and characterized.⁷⁵⁷ Apart from being easily accessible, this is evidently related to their interest from a commercial standpoint, where many functions have been proposed and several already implemented.⁷⁵⁸,⁷⁵⁹ Fungal laccases have primarily been identified in basidiomycetes, with white-rot fungal species being particularly abundant, and brown-rot fungi only described at a limited level.⁷⁶⁰ Fungal laccases are also present in ascomycetes and deuteromycetes, whereas no enzymes have been identified in zygomycetes or chytridiomycetes.⁷⁶⁰ Fungal laccases are usually present in several isoforms within the same organism, mainly identified by a genetic approach or by iso-electric focusing. The typical fungal laccase is a monomeric protein, with a molecular weight of approx. 60–70kDa, including a carbohydrate content of 10–25%. The pIs of fungal laccases fall in the acidic range, with most enzymes between 3–5.⁷⁶⁰,⁷⁶¹ An interesting property of fungal laccases is their heat tolerance, with optimum activity often reported at more than 55°C.⁷⁶²,⁷⁶³,⁷⁶⁴,⁷⁵⁷ This is one of the features that make fungal laccases interesting from an industrial perspective. In addition to activity, fungal laccases are also relatively stable at elevated temperatures, with half lives for decay reported to be several hours.⁷⁶²,⁷⁶⁵,⁷⁵⁷ Fungal laccases are most often extracellular, but several intracellular enzymes have been identified.⁷⁶⁰,⁷⁶⁶,⁷⁶⁷ Due to their ability to oxidize various poly-phenolic compounds, fungal laccases have long been suggested to participate in lignin degradation.⁷⁶⁸ Conclusive evidence for this functionality was presented in the late 1990’s by Bermek and Eriksson, who showed that laccase mutants from Sporotrichum pulverulentum were incapable of degrading lignin, in contrast to the wild-type enzyme.⁷⁴² Despite the relatively early elucidation of lignin-degrading functionality, the precise mechanism by which this occurs in fungal laccases is still not known.⁷⁶⁹ Other proposed biological functions of fungal laccases include pathogenesis and detoxification as well as development and morphogenesis of fungi.⁷⁴³
3.7.1.2 Plant Laccases: Although not as abundant as fungal laccases, plant laccases have played a pivotal role in the study of MCOs (vide infra). In particular laccase from the Japanese lacquer tree, *Rhus vernicifera* laccase (RvL), has been subject to numerous studies, both with respect to biological function and molecular mechanism.

Plant laccases are glycoproteins, with a carbohydrate content ranging from ~20–45%. Molecular weights have been reported in the range from ~60–130kDa, whereas pI values vary from 7.0–9.6. Most plant laccases are secreted proteins, with a few exceptions predicted to be targeted to the mitochondria. 

Previously, plant laccases were believed to be scarcely distributed, but recent studies have shown that they are in fact widely distributed in higher plants, particularly in angiosperms, but also in gymnosperms. An investigation of laccase gene sequences in *Arabidopsis thaliana* among others, showed that plant laccases can be divided into six divergent phylogenetic groups. Interestingly, groups 1–3 contain both gymnosperms and angiosperms, indicating that these genes had evolved prior to the division of gymnosperms and angiosperms some 300 MYA.

A characteristic feature of plant laccases, is the high degree of isoform occurrence in different plants, with 17 laccase genes being identified in *Arabidopsis thaliana*. Along with the complexity of plant extracts in general, this provides a significant challenge in determining the biological function of plant laccases.

One of the most well established functions of plant laccases, is wound healing in *R. vernicifera*. In response to stem cuts, an oxidative polymerization reaction of the alkylcatechols in the latex sap, is initiated by laccase. This produces a hard seal over the wound that serves to protect the stem. A more controversial role of plant laccases, is the involvement in lignin formation. This issue has been heavily debated over the years, but so far no conclusive evidence has emerged, although several strong indications have been reported. The first indication of a possible role in lignin formation came in 1992 when Sterijijades reported on the oxidative capability of a maple laccase on the three lignin precursors, sinapyl, coniferyl, and *p*-coumaryl alcohols. This finding was in contrast to previously conducted studies on *R. vernicifera* laccase, which indicated that this enzyme was not involved in lignin formation. Recent studies indicate that some laccase isoforms in *Arabidopsis thaliana* are involved in lignin formation, but that the majority are not. In contrast, strong evidence has been presented that links the formation of lignin to the class III peroxidases. An interesting hypothesis, in that respect, is the involvement of laccases in formation of the early primitive forms of lignin, whereas the later emerging, more complex, lignin structures are proposed to have been synthesized by peroxidases. Other putative functions of plant laccases include flavonoid oxidation, and cell wall formation, which also involves oxidation of phenols. Finally, it should be mentioned that Hoopes and Dean reported ferroxidase activity of a plant laccase from *Liriodendron tulipifera* in a 2004 study. However, no further information has been presented on this subject.

3.7.1.3 Prokaryotic Laccases: Bacterial laccases were first described in *Azospirillum lipoferum* in 1993. Several other bacterial laccases have been purified, expressed and
characterized since then.779 In addition, a significant number of bacterial laccase genes have been identified in a variety of bacterial strains.757 Recently, prokaryotic occurrence of laccase was extended to archae, with the reported purification and characterization of a glycosylated enzyme showing activity towards laccase substrates like syringaldazine, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 2,2’-dimethoxypropane (DMP).780 Furthermore, the archael laccase showed activity towards bilirubin. Based on this, the enzyme would more correctly be grouped with other Bilirubin Oxidases (BODs), as described below. This is also the case for the best studied bacterial laccase, CotA.751 From an evolutionary standpoint, fungal BOD structures actually group with the bacterial laccases,781 and it is likely that several of the putative prokaryotic laccases may indeed turn out to be BODs. Whereas bacterial laccases seem to be widely distributed in bacteria; only a few archael enzymes have been proposed based on genetic analyses.782 This may be related to the mostly anaerobic nature of archae.

Compared to fungal laccases, only limited information is available on the properties of prokaryotic laccases. Molecular masses have been reported from 50kDa to 250kDa.779 In addition to “traditional” laccase structures, prokaryotic laccases also include the so-called two-domain or small laccases (SLACs), which are significantly different with respect to size and structure.783 The molecular weight per active site is as low as 30kDa and the enzymes have two domains rather than three.784

With respect to other properties, prokaryotic laccases differ significantly from fungal laccases. Generally, they have been reported to have higher pl’s. Salt tolerance is another featured property of prokaryotic laccases. Lastly, it should be mentioned that prokaryotic laccases are generally expressed intracellular, again in contrast to most fungal laccases.779

The functionality of prokaryotic laccases have so far been elucidated only for a few species, and include melanin formation, spore resistance against UV-light and hydrogen peroxide,744,745 and morphogenesis.785 Studies on the laccase functionality of CotA revealed that the mutated enzyme were incapable of forming spore pigment.786 Similar conclusions were made in a study of Azospirillum lipoferum mutants.787 In a study on EpoA from S.griseus, morphogenesis was shown to be inhibited in mutated protein.785 However, upon incorporation of an epoA-induced plasmid, activity was restored.

**3.7.1.1.4 Insect Laccases:** Laccase-like activity has been known in insects for more than 50 years.788 Despite this, only limited information on insect laccases has been gathered and only a few enzymes have been purified and characterized.789 Studies have, however, identified laccase-like genes in a number of insects, and shown these genes to be present in a variety of tissues.789 Further, laccase-like activity has been identified in more or less purified samples from a number of insects, where the active enzymes showed properties such as acidic pH-optimum and thermal stability of up to 60–70°C.789

As for other laccases, the biological function of insect laccases is difficult to elucidate, primarily due to the complexity of samples. One function has, however, been unambiguously assigned for insect laccases, namely cuticle formation in Tribolium castaneum.746 Knock-out of the so-called laccase 2 gene resulted in a lethal phenotype with
soft, unpigmented cuticle, observed in larval, pupal, and adult stages. The same gene has been identified in the cuticle of several other insect species.\textsuperscript{790–793}

3.7.1.1.5 Ascorbate Oxidase: The existence of a unique ascorbic acid oxidizing enzyme was proposed more than 80-years ago,\textsuperscript{794} and finally established in 1941.\textsuperscript{795} Over the years, ascorbate oxidase (AOx) has attracted much interest both with respect to elucidation of the biological function, but also in various structural and mechanistic studies. In 1989, Messerschmidt et al. published the first crystal structure of a multicopper oxidase; that of AOx from green zucchini.\textsuperscript{796}

Ascorbate oxidase is most commonly found in higher plants,\textsuperscript{750} but has also been identified in fungi.\textsuperscript{781} AOx is characterized by having a high activity towards ascorbate, whereas “normal” laccase substrates are less efficient. Based on this activity, AOx is defined as a unique subclass of enzymes (E.C. 1.10.3.3), separate from laccase and other multicopper oxidases. This is consistent with a phylogenetic analyses of MCOs that group plant and fungal AOxs together, clearly separate from laccases and ferroxidases.\textsuperscript{781}

In plants, AOx forms homodimers with molecular weights of approx. 130kDa,\textsuperscript{797} whereas the sole fungal AOx characterized is a monomer with a molecular weight of 80kDa, including a carbohydrate content of approx. 11%.\textsuperscript{798} Another interesting property is the conserved functionality of fungal AOx at pH’s as low as 3.\textsuperscript{798} Furthermore, the fungal enzyme is thermostable up to ~60°C,\textsuperscript{798} whereas plant AOxs has a t\textsubscript{1/2} of only 20min at 55°C.\textsuperscript{799}

Despite intense investigation, the biological function of plant and fungal AOxs has still not been fully elucidated.\textsuperscript{800} Numerous studies have led to different proposed functions, among those, involvement in plant growth, which was initially proposed by Mertz based on cellular localization of maize AO.\textsuperscript{747} Later studies on this hypothesis have been somewhat contradictory. Lin and Varner,\textsuperscript{801} and Kato and Esaka\textsuperscript{802} supported the hypothesis by showing increased levels of AOx in expanding cells in zucchini, and elevated AOx activity in elongating tobacco cells, respectively. On the other hand, Sanmartin et al. did not detect any effect of AOx overexpression on plant growth.\textsuperscript{803} Other proposed functions of AOx includes a role as a terminal oxidase in an electron transport chain,\textsuperscript{748} and as a plant defense system against insects, decreasing the level of available ascorbic acid and thereby the level of antioxidants in the insect.\textsuperscript{749} A common feature of the above listed functionalities, is the importance of the reducing substrate, ascorbate. Recently, Tullio et al. suggested a novel functionality, which focuses on the oxygen reducing ability of AOx. The authors propose that AOx can establish an O\textsubscript{2} gradient across the cytoplasmic membrane, preventing hyperoxia by releasing excess O\textsubscript{2} to the apoplast.\textsuperscript{750}

3.7.1.1.6 Bilirubin Oxidase (BOD): Bilirubin oxidases (EC 1.3.3.5) are multicopper oxidases, categorized based on their ability to oxidize bilirubin to biliverdin, using dioxygen as an electron acceptor.\textsuperscript{804} This reactivity has been known for a long time, and has traditionally been observed in fungi.\textsuperscript{804} In addition to bilirubin activity, BODs also show activity towards the traditional phenolic laccase substrates.\textsuperscript{804} Recently, the bacterial laccase CotA from Bacillus subtilis was shown to have significant BOD activity,\textsuperscript{751} extending the
occurrence of BODs to the prokaryotic kingdom. BODs are interesting in several respects, first due to their diagnostic properties in medical examination of the liver. Also, they are candidates for the cathodic reaction of biological fuel cells, where they show important advantages compared to the fungal laccases, such as pH optima close to 7, and high tolerance to NaCl. From a mechanistic point of view, BODs have provided significant insight into the MCO family, primarily from mutational studies on *Myrothecium verrucaria*. Recently, the first crystal structure of a BOD was published, and it revealed an overall structure very similar to that of fungal laccases. Differences were, however, observed in proximity to the T1 Cu binding site, which may provide insight into the redox properties of this site.

Very little is known about the physiological function of BODs, but due to their similarity to fungal laccases, it’s hypothesized that the enzymes are involved in lignin degradation.

3.7.1.1.7 Phenoxazinone Synthase (PHS): Phenoxazinone synthase (EC 1.10.3.4, PHS) is a bacterial MCO identified only in *Streptomyces antibioticus*. Other enzymes, classified in the same group, have been found in other organisms, but none of these incorporate the four copper motif characteristic for MCOs. The particular interest in PHS stems from its ability to oxidize o-aminophenol to phenoxazinone, which is a precursor to the antibiotic Actinomycin. A pivotal study from 2000 showed that depletion of PHS from *S. antibioticus* had no affect on the formation of Actinomycin, indicating that this is not the biologically relevant function of the enzyme. Instead it has been proposed that PHS is involved in spore pigment production or spore morphogenesis. A crystal structure of PHS revealed that the active form of the enzyme is a hexamer, which in addition to the four catalytically relevant Cu ions per monomer, incorporates a structurally important fifth Cu which is proposed to link together the individual monomers. This motif has not been observed in any other MCOs (see section 3.7.1.3).

3.7.1.1.8 Ferroxidases: Fet3p, Ceruloplasmin, and Hephaestin: Ceruloplasmin (Cp, EC 1.16.3.1), particularly the human enzyme, has been intensely studied since its discovery more than 60 years ago. The enzyme displays a variety of fascinating properties, among them binding ~95% of serum copper, a unique six domain structure, and ferroxidase activity. Ceruloplasmin has been described in several organisms, including rat and mouse, which showed amino acid identity of up to 90%. The crystal structure of ceruloplasmin has been resolved, and it revealed a unique structure compared to other multicopper oxidases. First, the enzyme consists of six domains per active site, significantly different from the traditional three-domain MCOs and the newly discovered two-domain SLACs. Furthermore, ceruloplasmin has six coppers distributed into three T1 sites (one of which is permanently reduced and possibly an evolutionary remnant) and one trinuclear copper cluster. The six domain structure was recently proposed in another enzyme showing ferroxidase activity, namely Hephaestin (Hp). This enzyme has 50% sequence homology with ceruloplasmin, including conservation of copper ligating amino acid residues. Hp has not been studied as intensively as ceruloplasmin, and so far no crystal structure has emerged. Significant insight into the properties of this enzyme has, however, been obtained from heterologous expression of the human protein in hamster.
study, the recombinant enzyme showed significant ferroxidase activity, but only 3.13 Cu/molecule. The electronic absorption spectrum revealed a band at 607 nm, consistent with oxidized T1 Cu, as generally observed for multicopper oxidases. The molecular mass of the recombinant Hp is 130 kDa. Hp is a membrane-bound protein that has been found in a variety of tissues including the small intestine, colon, and brain. A 582 base pair deletion of Hp results in sex-linked anemia, a condition characterized by severe iron deficiency. The exact function of Hp has not yet been determined, but has been proposed to be linked to iron metabolism, with a possible role of oxidizing Fe(II), to the transferrin receptive Fe(III) form. However, no direct interaction between Hp and transferrin has yet been discovered. Cp is a glycoprotein with a molecular weight of ~130 kDa, with small variations due to differences in glycosylation. Two forms of Cp are expressed. The best characterized isoform being serum Cp, which has been identified in spleen, lung, and testis. The second isoform is a glycoposphatidylinositol (GPI)-anchored protein, occurring in astrocytes of the Central Nervous System (CNS) and the epithelial cells of the choroid plexus. The precise function of the anchored isoform is not known, but it is believed to be involved in iron-mobilization on the blood-brain, blood-testis barriers.

One of the main reasons for the interest in serum Cp is due to the mutational disease, aceruloplasmenia. This fatal inherited genetic disorder leads to progressive neurodegeneration (often diagnosed as dementia) in addition to diabetes caused by iron accumulation in the liver and pancreas. Mutations in the ceruloplasmin gene prevents loading of the trinuclear copper domain and thereby prevents ferroxidase activity significantly hindering iron metabolism, which leads to rapid degradation of the protein in blood plasma. Interestingly, the aceruloplasmin specific mutations in Cp does not lead to significant abnormalities in copper metabolism, which is somewhat surprising considering the high level of serum copper incorporated in Cp.

In addition to the iron homeostatic functionality, Cp has also been suggested to be involved in anti- or prooxidant actions, with somewhat contradicting studies supporting each case.

Fet3p is the functional homolog of Cp in yeast, in that it is critical for iron homeostasis. The enzyme has been studied primarily in Saccharomyces cerevisiae, but genetic analyses have revealed its presence in a range of fungal species. In comparison to Cp, the structure of Fet3p resembles the traditional three-domain MCOs, with a total of four Cu ions involved in the active site. Fet3p is a membrane-bound glycoprotein with a molecular weight of approx. 100–120 kDa. In the regulation of iron homeostasis, Fet3p has been shown to work in conjunction with a transmembrane iron transporter, Ftr1p, together facilitating the transport of iron across cellular membranes. The role of Fet3p is to oxidize Fe(II) to Fe(III), which is the substrate for Ftr1p. Several lines of evidence have been presented to support this functionality, among them the presence of Fet3p being required for the correct targeting of
Ftr1, the intracellular accumulation of Ftr1p upon Fet3p depletion, and the co-expression of Fet3p and Ftr1p.733,752,841,842

In addition to ferroxidase activity, Fet3p also exhibits significant cuprous oxidase activity.843 Mutational studies on Fet3p revealed that the enzyme plays a significant role in copper homeostasis, by regulating the amount of free Cu, and thereby preventing the toxic effects of this metal.753

3.7.1.1.9 Cuprous oxidase: CueO: CueO is a multicopper oxidase involved in copper homeostasis in Escherichia coli.754 The periplasmic enzyme is co-expressed with a membrane-bound ATPase, CopA, which together functions as a copper efflux system leading to copper tolerance in the organism.754 Roberts et al. reported the crystal structure of E.coli expressed CueO, highlighting, in addition to the general three-domain motifs, a 42 residue insert in domain three with a high occurrence of methionine residues.844 This region was later found to ligate a labile fifth copper ion, a novel feature of CueO compared to other MCOs.845 CueO has a high affinity for Cu(I), which is oxidized to Cu(II). This property has been hypothesized to diminish the availability of toxic Cu(I), a metal ion involved in Fenton chemistry, forming highly reactive radicals.755 CueO also shows considerable phenol oxidase activity, especially in the presence of excess Cu.846 The role of the phenol versus cuprous oxidase activity has long been debated, and was actually shown to be heavily influenced by the aforementioned met-rich insert. In a mutational study of CueO, Kataoka et al. investigated the influence on activity upon removal of the met rich region. It was shown that cuprous oxidase activity was significantly diminished upon removal, whereas the phenol oxidase activity was enhanced.847 In a more recent study, elegant experiments led to the conclusion that CueO is solely a cuprous oxidase in vivo, and that phenol oxidase activity is prevented due to the selective affinity of the met rich region for Cu(I) over Cu(II).848

3.7.1.10 Manganese oxidases: Mn is an important trace metal in the environment as well as in living cells. It occurs in a soluble reduced form, Mn(II), and in the insoluble forms, Mn(III,IV). Mn(II) is highly stable, and is only slowly converted to the oxidized forms.849,850 Certain, primarily bacterial strains, have been shown to enzymatically enhance the oxidation of Mn, an interesting property for bioremediation. Little is known about the oxidative process by which these bacteria operate, but it has been suggested that MCOs play a pivotal role.756,849 Several bacterial MCOs have been identified as putative manganese oxidases. None of these enzymes have, however, been purified or expressed.851 The most compelling evidence of involvement of MCOs in Mn oxidation came in a study on marine Bacillus species, where a gel-band with Mn oxidase activity, was determined to have a putative MCO, namely MnxG, based on mass spectrometry analysis.756 Interestingly, the enzyme was proposed to convert Mn(II) to Mn(III), but also Mn(III) to Mn(IV).

3.7.1.2 Kinetics: The MCOs can be divided into two categories depending on their native substrate either being a redox active organic molecule (organic oxidases) or a metal ion (metalloxidases; Table 27). Comparing the parameters from steady-state kinetics in Table 29, the organic oxidases have variable $K_M$ values with the highest being the tree Lc from Rhus vernicifera852 while the metallooxidases Fet3p843 and Cp have very low $K_M$ values.
(CueO<sup>853</sup> is comparable to CotA<sup>854,855</sup> and AO<sup>856</sup>) Therefore, the metalloxidases bind their substrate significantly tighter than to the organic oxidases with a specific metal binding site (<i>vide infra</i>). Additionally, the generally higher K<sub>M</sub> values of the organic oxidases are consistent with their low substrate specificity and reflect the fact that the substrate binding sites are more accommodating to various substrates. The turnover numbers for the metalloxidases are generally slower compared to those of the organic oxidases. The organic oxidases exhibit a wide range of optimal pH values (5.0 – 9.4) under catalytic conditions while the metalloxidases have optima within pH = 6.0 – 6.8. This large pH variance in the organic oxidizers has been shown to correlate with the isoelectric point of the protein.<sup>857</sup>

The mechanism for substrate oxidation at the T1 site has been investigated from kinetics. k<sub>cat</sub> for a selection of laccases have been shown by Xu to correlate linearly with the difference between the single electron reduction potential of the substrate and the T1 copper site<sup>857</sup>. This is consistent with substrate reduction of the T1 in organic oxidizers being an outer-sphere electron transfer mechanism and the rate-determining-step in turnover. Furthermore, the K<sub>M</sub> values for the laccases were shown to be invariant with respect to reduction potential differences between the T1 and substrate. In steady-state kinetics, Fet3p exhibited a significant increase in K<sub>M</sub> while leaving k<sub>cat</sub> unaffected, upon sight-directed mutation of key Fe(II) binding residues near the T1 site, implying that tight binding of the metal ion is essential for its oxidation.<sup>753,860</sup> Single turnover kinetics on these variants exhibit marked rate depression of T1 reduction from >1200 s<sup>−1</sup> in WT to ~141–2 s<sup>−1</sup> in the variants, establishing that these residues also provide a path for the electron to be transferred to the T1<sup>861</sup>. It is interesting to note the extremely fast reduction rate of the T1 Cu in Fet3p, which is much faster than the turnover rate of the enzyme<sup>843</sup>, and therefore not involved in the rate determining step, in contrast to the organic oxidases. A similar fast ET between Fe<sup>2+</sup> and T1 Cu<sup>2+</sup> is seen for Cp, the mammalian analog of Fet3p.<sup>862</sup> However, Cp differs markedly from Fet3p, in that it has a total of three T1 Cu sites, two of which are redox active, while the third is permanently reduced.<sup>863</sup> Kinetic and spectroscopic studies have shown that the redox potentials of both of the active T1 Cu’s in Cp are almost identical, and that ET between the two occurs at a rate of > 150 s<sup>−1</sup>.

Steady-state kinetics on dioxygen reduction by the MCOs, show similar values for K<sub>M</sub>(O<sub>2</sub>) ~ 20 µM and k ~ 6.0 × 10<sup>6</sup> M<sup>−1</sup>s<sup>−1</sup> at 25 °C suggesting that the dioxygen reaction in the reoxidation of the enzyme is comparable among all MCOs.<sup>852</sup>

Further understanding of the mechanism of the MCOs and their intermediates has come from extensive single turnover kinetic studies. The best-characterized and most extensively studied MCO, RvL, is considered here. Reduction of fully oxidized resting laccase proceeds via a multiphasic process.<sup>864,865</sup> Using hydroquinone as the reductant at physiological pH, the T1 exhibits an initial reduction rate of 1.58 × 10<sup>3</sup> M<sup>−1</sup>s<sup>−1</sup> and a final rate of 800 M<sup>−1</sup>s<sup>−1</sup> while the T3 reduces initially at 1.45 × 10<sup>3</sup> M<sup>−1</sup>s<sup>−1</sup> and finally at a substrate independent rate of 0.40 s<sup>−1</sup>. A key observation here is that the T1 is reduced faster than the T3 implying that there exists a slow intramolecular electron transfer between the T1 and T3 sites. Post-steady-state kinetics of RvLc, where oxygen is limiting with respect to substrate, exhibits second order decay at physiological pH of the T1 band to be 800 M<sup>−1</sup>s<sup>−1</sup>, while the decay at 340 nm (corresponding to the TNC Cu’s) exhibits biphasic decay with a substrate dependent
rate of 1400 M\(^{-1}\) s\(^{-1}\) and a substrate independent rate of 0.3 s\(^{-1}\). It is important to note that this contrasts the reduction kinetics of the resting laccase, where the T1 reduces faster than the 340 nm species and therefore must be different from the process in catalysis. Pulse radiolysis experiments have measured the intramolecular T1 to T3 electron transfer process to be 1.1 s\(^{-1}\) in resting RvLc and were independent of the amount of reductant and extent of reduced T1. This further suggests that the reduction of resting laccase is not relevant under catalytic conditions since it is orders of magnitude slower than the maximal turnover rate (560 s\(^{-1}\)).

Reaction of reduced native RvL with dioxygen rapidly forms the “native intermediate” (NI) at a bimolecular rate of 1.7 \times 10^6 M\(^{-1}\) s\(^{-1}\) at 3 °C and 6.0 \times 10^6 M\(^{-1}\) s\(^{-1}\) at 25 °C, in agreement with steady-state measurements. A derivative of the native enzyme was generated where the T1 Cu has been eliminated either via the mutation of the T1 Cys ligand to a Ser to give the T1 depleted form (T1D) or replaced chemically with a redox and spectroscopic inactive Hg(II) ion to give the T1 mercury derivative (T1Hg). Both of these derivatives contain a valid, reactive trinuclear Cu cluster that upon reduction reacts with dioxygen at a comparable bimolecular rate with that of the native enzyme (2.2 \times 10^6 M\(^{-1}\)s\(^{-1}\) for reduced T1Hg Lc), however, yielding a spectroscopically distinguishable intermediate from NI (vide infra) known historically as the “T1Hg intermediate” or more recently as the “peroxide intermediate” (PI). Without the T1 Cu present, PI slowly decays to NI, while in the native enzyme PI is not observed. Therefore, in the native enzyme, PI converts to NI at an estimated unimolecular lower-limit rate of > 350 s\(^{-1}\). In the decay of PI to NI, there is an observed \(^{18}\)O\(_2\) KIE of 1.11, which indicates that O-O bond cleavage occurs in this step and is rate limiting. Another relevant derivative of Lc, is the T2 depleted (T2D) form, where the T2 Cu of the trinuclear cluster has been reversibly removed leaving the T3 and T1 Cu’s. Exposure of dioxygen to reduced T2D Lc shows no reactivity therefore establishing that the T3 site in the MCOs is different from the coupled binuclear Cu center found in Hc, Tyr & CaOx (vide supra).

The NI intermediate decays slowly in the absence of reductant to the resting fully oxidized form with a first order rate constant of \(k = 0.05\) s\(^{-1}\) at 25 °C. This rate is significantly slower than that of the maximal turnover rate (560 s\(^{-1}\)) and therefore NI must reductively decay in the presence of substrate in catalysis. Therefore, NI is the catalytically relevant fully oxidized intermediate, not the resting state of the enzyme (vide infra). Additionally, since the intramolecular electron transfer from the T1 to the T3 in resting is slow, this electron transfer must be greatly accelerated in the reduction of NI, to be consistent with turnover.

3.7.1.3 Structure: Since the first report of a MCO crystal structure, that of ascorbate oxidase published in 1989 by Messerschmidt, several structures have emerged, covering almost all classes of MCOs. Invaluable insight has been obtained from these structures, both with respect to overall structure of the enzymes and with respect to the active copper sites. In this section we present the general three-domain MCO structure, as well as the deviations from this motif, observed in ceruloplasmin, and more recently the two-domain MCOs. We also provide a description of the various active site features, highlighting similarities and differences for the T1 and trinuclear sites, respectively.
3.7.1.3 Overall structure: The most widely distributed structure of MCOs is a globular, three cupredoxinlike domain structure. Domain 1 incorporates the T1 copper site, whereas the trinuclear site is situated between domains 1 and 3, with ligated histidines from both domains (Figure 136A). This structure is observed in laccases (except for the two-domain laccases), CueO, BOD, Fet3p, and ascorbate oxidase. Generally, two substrate channels are identified, one presumably facilitating dioxygen delivery to the trinuclear site, terminating close to the binuclear T3 site, and a second channel, presumably serving as an exit channel for the active site generated water molecules, originating at the T2 site (Figure 137).

In contrast to the three-domain MCOs, Cp has a unique six-domain structure, which incorporates a total of six copper ions in three T1 sites and one trinuclear site. The mononuclear T1 sites reside in domain 2, 4, and 6, respectively, whereas the trinuclear site is situated between domain 1 and 6 (Figure 136B), with ligands from both domains, similar to the three-domain MCOs.

Recently, a new type of MCO has emerged, namely the two-domain laccases. So far, three two-domain structures, all from bacterial sources, have been published. The structures consist of six domains, arranged in three two-domain units, each pair incorporating a T1 and a trinuclear cluster. As for other MCOs, the trinuclear cluster is situated in between two domains, with the T1 site incorporated in either domain 1 or 2 (Figure 136C).

3.7.1.3.2 Active site structures: From the first MCO crystal structure published in 1989, the unique active site including the T1 site and the spectroscopically predicted (in 1985), trinuclear Cu cluster was finally confirmed. Furthermore, the structure strongly supported a mechanistic model of substrate oxidation in the vicinity of the T1 Cu, electron transfer via a Cys-His pathway from the T1 Cu to the T3 Cu’s, and finally oxygen reduction to water at the trinuclear center, also first demonstrated by spectroscopy.

3.7.1.3.3 Substrate binding and the T1 Cu site: The unique T1 Cu site in MCOs, single domain blue Cu proteins, and nitrite reductase (see section 5.1), has been intensely studied. Much knowledge has been gathered from a combination of structural, spectroscopic, and biochemical experiments, and many of the properties determining electron transfer rates and redox potentials of these sites have been elucidated. A common trait of the T1 Cu’s in MCOs is the ligation by two His and one Cys, with the latter being responsible for the intense blue color of these enzymes. Furthermore, a fourth ligand, in the form of a weakly coordinating axial Met, is often observed (Figure 138A). Alternatively, the axial position can be occupied by non-coordinating residues e.g. Leu or Phe (Figure 138B), which tend to result in a high redox potential for these T1 coppers.

Solved structures of fungal laccases from M. albomyces, T. versicolor and T. trogii with phenolic substrates and analogues have described the binding sites for substrate oxidation. In general, the T1 sites in the organic oxidases are significantly solvent exposed and the substrate binding sites near the T1 site are ~5–8 Å deep comprised predominantly of hydrophobic residues. Therefore, the T1 site is particularly accessible to hydrophobic...
substrates. In the 2,6-DMP bound structures of *M. albomyces*, the oxygen of the phenol is within hydrogen bond distance of H508 (Figure 139A), which is a ligand to the T1 copper and therefore the principle electron acceptor in the oxidation reaction. This is further supported by the structures of xylidine bound to *T. versicolor* and *p*-toluate bound to *T. trogii* where the nitrogen and oxygen of the respective molecules are in similar orientation to the T1 histidines. Additionally, these atoms bound to the T1 histidines are also within close proximity to a nearby carboxylate residue, the only hydrophilic residue in each pocket. This carboxylate is suggested to be the proton acceptor in phenol oxidation. The nature of these substrate binding sites is consistent with the fact that these proteins are non-specific in terms of their oxidase activity since the sites only contain two key interactions for substrate oxidation.

Structural data of the metalloxidases Fet3p, Cp and CueO show that the T1 sites are far less solvent exposed compared to the sites for the organic oxidases. Structures of divalent cations bound to Cp show a substrate binding site composed of solvent exposed carboxylates (E272, E935 & D1025) and H940 in near proximity (~8 Å) to the buried T1 copper (Figure 139B).\(^{734}\) In Cp, this T1 connects to the TNC via the Cys-His pathway. Ion bound structures of Fet3p have not been reported, but a similar site comprised of solvent exposed carboxylates is evident from the structure and kinetic experiments coupled to site-directed mutagenesis suggesting that carboxylates D283, E185 and D409 bind ferrous substrate in Fet3p.\(^{733,860–862}\) Spectroscopic studies of Fe(II) bound to Cp and Fet3p confirm that the iron is bound in a six coordinate octahedral geometry in both enzymes.\(^{861}\) Therefore, both Fet3p and Cp display specific ferrous ion binding to a carboxylate rich substrate binding site that can facilitate e.t. to the T1.

A similar ion binding site has been structurally elucidated for CueO.\(^{845}\) Here, a fifth labile copper is present at a site located ~8 Å away from the T1 and bound in a trigonal bipyramidal fashion by two methionines (M355 and M441), two aspartate carboxylates (D360 and D439) and an aquo ligand. D439 hydrogen bonds to the nearby T1 histidine and therefore provides a pathway for electron transfer.

### 3.7.1.3.4 Trinuclear copper site:

Common to all crystal structures of the MCOs, is the coordination to a total of 8 His ligands of the three Cu ions in the trinuclear cluster, arranged in four HXH motifs.\(^{877–879}\) Each of the T3 Cu’s are ligated to three His and the T2 Cu is ligated to two (Figure 140). A water derived ligand is a third T2 ligand in all structures except for *M. albomyces*, which has a Cl⁻ coordinated in the similar position.\(^{887}\) Furthermore, all crystal structures contain a highly conserved 2\(^{nd}\) sphere carboxylate residue, hydrogen bonded to His residues from the T2 and T3β Cu (D77 in Figure 140). The significance of this residue has been demonstrated in independent mutational studies, where it was shown to be required for enzymatic activity.\(^{806,888}\) Another well-conserved carboxylate residue is identified at the bottom of the trinuclear cluster, hydrogen bonded to solvent channel water molecules (D456 in Figure 140). This residue has been demonstrated to play a significant role in the protonation of the dioxygen-derived molecules generated in the trinuclear cluster.\(^{889–892}\)
The redox states in the crystal of the individual Cu’s in the trinuclear cluster are difficult to assign. In addition, solvated electrons generated during X-ray irradiation may result in further reduction of the trinuclear Cu’s.\textsuperscript{893,894} A general feature of reported MCO crystal structures is the high variability observed for Cu-Cu distances in the TNC, in particular between the T3 Cu’s. This variability was shown, early on, to be related to the redox state of the TNC Cu’s. Messerschmidt et al. obtained a crystal structure of AOx grown under anaerobic conditions, with excess reductant, and found a T3 Cu-Cu distance of 5.1Å, significantly longer than the spectroscopically calibrated fully oxidized resting form of AO, that was found to be 3.7Å (Figure 141).\textsuperscript{796} In most crystal structures reported for MCOs, the T3 Cu-Cu distance falls within this range, with a few examples of shorter distances, e.g. in the structure of NI recently published.\textsuperscript{894} Another feature of the fully reduced structure of the TNC was the lack of any di- or monooxygen derived electron density between the two T3 Cu’s, consistent with the two T3 Cu’s having close to trigonal planar geometry (formed by the three His ligands), a preferred geometry of Cu(I). In the fully oxidized structure, for comparison, the geometry of the T3 Cu’s have trigonal pyramidal geometries (three His and one oxygen derived bridging ligand), with open coordination positions oriented towards the center of the TNC (Figure 140), consistent with the spectroscopically calibrated DFT structure (vide infra).\textsuperscript{895} Interestingly, very little difference is observed in the reduced versus oxidized geometry of the T2 Cu, with the two His ligands and a water derived ligand (OH\textsuperscript{-} from spectroscopy)\textsuperscript{895} resulting in a square planar geometry, with an open coordination position oriented towards the center of the TNC (Figure 140).

In most MCO crystal structures, mono- or dioxygen assigned electron density is observed in the TNC, showing a range of different confirmations. Only in very few cases, however, have the investigators made any attempt to spectroscopically calibrate the grown crystals either before or after exposure to radiation.\textsuperscript{893,894,896} A number of studies have reported changes in the oxygen derived species upon increasing radiation doses, most systematically in a study by Halkulinen and coworkers, where at low dose, a dioxygen was observed, which, upon increased radiation resulted in observation of single oxygen species.\textsuperscript{896} Several groups have employed the observations of different oxygen derived species to propose solution-relevant mechanisms for MCOs, often with obvious contradictions to spectroscopically well defined reaction intermediates.\textsuperscript{893,897,898} This illustrates that although much information can be obtained from X-ray crystallography, there is a need for more rigorous evaluation of the crystal growing conditions, as well as the effect of radiation on a given crystal structure.

### 3.7.1.4 Electronic Structure

#### 3.7.1.4.1 Resting Enzyme:

The resting oxidized state of the MCOs exhibits spectral features associated with all four of its coppers. The absorption spectrum (Figure. 142, top) exhibits two main features, a band at 330 nm (30,300 cm\textsuperscript{-1}), assigned as µ\textsubscript{2}-OH to T3Cu(II) CT transitions and another at ~ 600 nm (16,700 cm\textsuperscript{-1}) assigned as the S\textsubscript{Cys},π to T1Cu(II) CT. The EPR spectrum shows contributions from two $S = \frac{1}{2}$ Cu(II) ions, the T1Cu(II) ($g\parallel \sim 2.30, A\parallel = 40 – 95 \times 10^4\text{cm}^{-1}$) and the T2Cu(II) ($g\parallel \sim 2.24, A\parallel = 140 – 200 \times 10^4\text{cm}^{-1}$) (Figure 142, bottom). The T3 coppers are EPR silent due to antiferromagnetic coupling via a µ\textsubscript{2}OH superexchange pathway leading to an $S = 0$ ground state with an observed lower limit of $-2J > 550\text{cm}^{-1}$ from SQUID magnetic susceptibility experiments.\textsuperscript{730}
The spectroscopic features of the T2/T3 trinuclear cluster of the resting state have been further refined in the T1Hg derivative where the T1 Cu does not contribute and the TNC is unperturbed.\textsuperscript{871,895} This allows the LF transitions to be observed; the T2 in LT MCD as it is paramagnetic, and the T3 in CD. From a LF analysis of the $d \rightarrow d$ transitions observed in LT MCD (Figure 143A), and its EPR signal (Figure 142, bottom), the T2 copper has a $d(x^2-y^2)$ ground state in an approximately square planar geometry with a vacant equatorial position pointed towards the center of the TNC. Additionally, the T2 Cu(II) is coordinated by a solvent derived hydroxo ligand external to the cluster, which (from ESEEM) remains in this protonation state over its functional pH range.\textsuperscript{895} The spectral features of the T3 Cu(II)’s are resolved in the RT CD spectrum (Figure 143B). More than four LF transitions are observed indicating the two T3 Cu(II)’s are inequivalent, likely related to the $\varepsilon$ coordination of one His residue on T3$\alpha$ (vide supra). The LF’s of the T3 Cu’s are best described as trigonal bipyramidal with $d(z^2)$ ground states with the $z$-axis of each half occupied orbital oriented toward the $\mu_2$OH giving the AF coupling, and open coordination positions in the equatorial plane on each T3 Cu oriented toward the center of the cluster.\textsuperscript{895}

Density functional theory derived surface plots of the trinuclear cluster also show LUMOs on the T3 Cu-$\alpha$/$\beta$ and the T2 Cu directed towards the center of the cluster, where small molecules can bind in a bridging mode (Figure 144).\textsuperscript{895}

Key observations of the properties of the resting oxidized state of the MCOs, came from spectroscopic characterization of exogenous ligand binding to the trinuclear cluster. In a study from 1985, Allendorf et al. provided the first evidence, based on rigorous spectroscopic evaluation of azide and fluoride binding to native RvL, that the T2 and T3 Cu sites form a trinuclear copper cluster (TNC).\textsuperscript{884} These data were further elaborated upon in a subsequent study by Spira-Solomon et al.\textsuperscript{899} From room temperature absorption and LT MCD experiments, azide was found to bind in a high affinity form ($K \geq 10^4\text{M}^{-1}$) and a low affinity form ($K \sim 200\text{M}^{-1}$). Two features from the high affinity bound azide were resolved in LT MCD at 445 and 510 nm. The intensity of these features maximized at less than one enzyme equivalent of azide, and this high affinity form was eliminated upon treatment with peroxide. XAS studies showed that purified resting tree laccase contains $\sim 25\%$ reduced T3 Cu, which is oxidized by addition of peroxide. Based on this, the high affinity feature was assigned as T2 Cu(II) bound azide, formed in the presence of a reduced T3 Cu site.

Upon additional titration of native laccase with azide, several new features were observed in Abs, LT MCD, and LT EPR. Figure 145A shows the Abs. and LT MCD spectra in the azide to Cu(II) charge transfer region, baseline corrected by subtracting the intensity of the high affinity azide binding features. Several important features were observed. First, two prominent transitions are present in the Abs spectrum; an intense feature at 400nm and a shoulder at 500nm. Both transitions increase in intensity with the addition of azide. Comparing these transitions with transitions in the LT MCD spectrum revealed that only the 500nm shoulder has a corresponding paramagnetic contribution. The negative feature in LT MCD at 485nm is seen to increase with addition of azide, whereas the 385nm positive MCD feature first increases and then decreases with increasing azide concentration, inconsistent with the intensity pattern of the 400nm band in Abs. Based on this, the 400nm absorption
band was assigned as an azide to T3 Cu(II) CT transition (AF coupled so no corresponding feature in LT MCD), whereas the 500nm absorption (485nm LT MCD) band was assigned as a to T2 Cu(II) CT transition. Additional features in LT MCD at 330nm (−) and 440nm (−) showed intensity patterns identical to those of the 385nm LT MCD band. Finally, azide titrated laccase was also probed by LT EPR. Here, a characteristic new feature emerged at g = 1.86 (Figure 145B). The spin integrated intensity of this signal, correlated with that of the 340, 385, and 440nm LT MCD transitions. Importantly, these LT MCD transitions as well as the LT EPR signal showed a pH-dependent behavior, with increasing intensity at low pH, consistent with protonative uncoupling of the μ₂-OH T3 bridge. This uncoupled species has been investigated in significant detail in subsequent studies (vide infra).

Returning to the 400nm Abs with no corresponding LT MCD intensity, and the 485/500nm LT MCD/Abs features, complimentary fluoride binding experiments were conducted in order to obtain more information on exogenous ligand binding to the different copper sites. When fluoride was added to native laccase, pre-treated with 9 equivalents of azide, doublets were observed in the hyperfine EPR signal, indicating that fluoride bound to the T2 Cu(II) (vide infra). This F⁻ binding, significantly decreased the intensity of all the azide CT transitions, both to the T2 and to the T3 Cu(II)’s. Importantly, the stoichiometry of the reaction revealed that only one fluoride bound to the enzyme, i.e., one fluoride bound to the T2 eliminated azide binding at both the T2 and the T3 Cu(II)’s. This led to the conclusion that the T2 and T3 Cu sites of MCOs are arranged in a trinuclear cluster. This was later confirmed in all reported crystal structures of MCOs.

A further resolution of the above mentioned spectroscopic features of azide binding, was obtained in a study from 1990 by Cole et al.⁸⁷¹ Here, azide titrations were performed on the T1Hg derivative of RvL, eliminating the spectral complications of the T1 Cu. This study showed that the low affinity binding, associated with the 400 and 500nm bands, could be further resolved into two different binding events, with binding constants of 780M⁻¹ (500nm feature) and 270M⁻¹ (400nm feature), respectively. Also, the protonative uncoupled species was shown to correlate with binding of the first azide, and upon binding of the second azide, these features, including the 385nm MCD band and the LT EPR signal at g = 1.86, decreased in intensity (Figure 145A and B), indicating that the second azide binding perturbs the first azide bound trinuclear structure and thus its corresponding spectroscopic features.

Additional information of the nature of the uncoupled species has been obtained from more recently conducted experimental and computational studies.⁹⁰⁰ Here, a species with identical spectroscopic features to those of the uncoupled species but much more intense, was generated by azide addition to the native intermediate (NI). NI is the fully oxidized, catalytically relevant species in turnover of MCOs (vide infra, section 3.7.1.4.3). This azide bound NI (NIₐz) was found to decay to the one azide bound resting species (Rₐz, the first azide species) elucidated by Cole et al.⁸⁷¹ Also, interconversion of NIₐz and Rₐz is reversibly obtained by changing the pH, consistent with previous findings (vide supra). Figure 145C presents a structural model for this interconversion, obtained through DFT optimized structures calibrated by the spectroscopic data.
Further insight into the properties of the TNC came from spectroscopic and computational studies of T1Hg Lc in its fully oxidized resting form as well as a fully oxidized fluoride bound form. Fluoride is known to bind to the TNC with >10^5 higher affinity than for mononuclear Cu(II) sites. Since F^- has I_n = \frac{1}{2}, this results in characteristic doublet EPR features (Figure 146). A correlation with RT Abs, CD, and LT MCD showed that one fluoride binds to the open coordination positions at the center of the cluster, with only a weak perturbation of the LF features (T2 in LT MCD and T3 in CD) and without influencing the T3-OH bridge or the T2 coordinated hydroxide.

These experimental results were correlated to DFT calculations, and binding energies were calculated for F^- and the possibility of water binding as OH^- to the open coordination positions of the center of the TNC. The results showed that F^- binding was highly exothermic (−69 kcal/mol), explained by a favorable electrostatic interaction between the anion and the highly positive TNC, large enough to overcome the energy loss from desolvation of the fluoride. A similar electrostatic interaction was observed in binding of OH^- to the TNC, again resulting in an exothermic process. However, due to the energetically unfavorable deprotonation of water to produce the hydroxide, this process was calculated to be less favorable by 48kcal/mol compared to fluoride binding. However, water binding as OH^- at the center of the TNC is still calculated to be an exothermic process which is inconsistent with the coordination unsaturation of the resting enzyme (vide supra). However, there are four negatively charged residues found within 10Å of the TNC in most MCOs, counteracting the high positive charge of the resting TNC (+4 from 3 Cu(II)'s and 2 OH's). Including this charge contribution to the active site lowers the net positive charge and thus the energy gain of anion binding by ~27kcal/mol, enough to make the binding of OH^- unfavorable as observed experimentally. Thus, the protein environment facilitates open coordination sites in the center of the TNC, thereby tuning the redox properties of these Cu's. This is highly important in that it drives electron transfer from the T1 to the TNC, a requirement for activation of the enzyme towards dioxygen reduction (vide infra).

3.7.1.4.2 Peroxy Intermediate: The intermediate formed upon dioxygen exposure to a fully reduced TNC with the T1 redox inactivated (T1D and T1Hg) is the peroxy intermediate (PI). This species has now been identified in a number of different MCOs. PI forms rapidly (vide supra) to give an EPR silent species exhibiting strong CT transitions at 340 nm (29,400 cm^{-1}) and 470 nm (21,300 cm^{-1}) (Figure 147). In the T1Hg derivative of RvL, PI decays slowly (k = 0.013 min^{-1}) to an intermediate with an MCD signal of an NI-like species (vide infra) and finally to a species with an EPR signal identical to that of the T2 Cu in the resting enzyme. The decay of PI generated with ^{18}O_2 shows the appearance of two H_2^{18}O's, monitored via isotope ratio mass spectrometry (IRMS) indicating that both atoms of ^{18}O_2 must be present in PI since this labeled oxygen appears concomitantly with PI decay (Figure 148). The CD spectrum of PI (Figure 149 left) exhibits LF features of Cu(II)'s corresponding to the band observed in the Abs. spectrum at 600–800nm (Figure 147B), while no LT MCD intensity is observed (Figure 149, right). The absence of an MCD signal is consistent with the EPR silence of PI and indicates that the two Cu(II)'s are AF coupled (S = 0) with the third Cu in the TNC reduced. This confirms that PI is a two-electron reduced peroxo level species. Importantly, the CT Abs spectrum of PI greatly
differs from that of oxy-Hc, requiring that the peroxide bridging motif of PI is different from the \( \mu-\eta^2: \eta^2 \)-peroxo structure of Oxy-Hc (Figure 150). SQUID magnetic susceptibility studies further demonstrated AF coupling between the two Cu(II)’s in PI with a lower limit of \( -2J > 200 \text{ cm}^{-1} \). Finally, the Fourier transform of the EXAFS spectrum of PI (Figure 151) shows a new strong outer shell peak consistent with a tightly bridged (ie. low Debye-Waller factor) Cu-Cu vector at 3.4 Å, whereas this feature is absent in the EXAFS spectrum of the resting TNC.

A geometric and electronic structure model of PI has been developed from extensive DFT correlations to the spectroscopic data (Figure 152). When \( \text{O}_2 \) is bound to the reduced trinuclear cluster, the geometry optimized structure observed is that of the \( \mu-\eta^2: \eta^2 \)-peroxo between the two oxidized T3 Cu’s, a structure very similar to that of oxy-Hc in section 3.1 (Figure 152A) This, however, is inconsistent with the absorption features of PI compared to oxy-Hc (Figure 150). Mutagenesis studies, in Fet3p, have shown that reaction with dioxygen does not occur upon substitution of an MCO-conserved carboxylate residue, near the T2/T3\( ^{\beta} \) edge (D94 in Fet3p, \( ^{\beta} \) indicates the T3 closest to D94) with charge neutral Ala/Asn. Since a carboxylate residue at this position is required for dioxygen reactivity, it was included in the computational model. Optimization of a D94-included active site yielded a \( \mu_3^{-1,1,2} \)-peroxo species bridged between the oxidized T3\( ^{\beta} \) (\( \eta^2 \)), oxidized T2 (\( \eta^1 \)), and reduced T3\( ^{\alpha} \) (\( \eta^1 \)). These predicted oxidation states of the TNC Cu’s in PI have recently been experimentally confirmed (vide infra). The T2 and T3\( ^{\beta} \) Cu’s are close to D94, suggesting that the negative charge of the carboxylate residue lowers the redox potentials of the T2 and T3\( ^{\beta} \) Cu, resulting in favorable two-electron transfer to dioxygen from these Cu’s. This is seen in the electronic structure of PI (Figure 152B) where the T2 and T3\( ^{\beta} \) Cu(II)’s have their half occupied \( d_{x^2-y^2} \) orbitals strongly overlapping the \( \pi^*\sigma \) orbital of the peroxo, providing the superexchange pathway leading to their antiferromagnetic coupling in the \( \mu_2^{-1,1,2} \) bridged structure (Figure 152B). The LF of the T2 Cu(II) is lower than the LF of T3\( ^{\beta} \) Cu(II), therefore the CT transitions to the T2 Cu(II) will be lower in energy. Also, as described in section 3.1, the \( \text{O}_2^{2-} \) has a low energy \( \pi^*\nu \) and high energy \( \pi^*\sigma \) CT transition, which lead to the assignment of the \( \text{O}_2^{2-} \) to T2 and T3\( ^{\beta} \) Cu(II) CT transitions of PI, given in Figure 150.

3.7.1.4.3 Native Intermediate: As described above, PI is generated in the absence of a redox active T1 Cu. When the fully reduced native enzyme (T1 and TNC are all present and reduced) reacts with dioxygen, a different intermediate, NI is formed. This is characterized in Abs. by intense charge transfer bands at 318 and 365nm associated with the TNC, and also a 614nm band (Figure 153A top), the latter indicating that the T1 site has been oxidized. Therefore, at least one additional electron has been transferred to dioxygen, relative to PI. The 77K EPR spectrum only shows features resembling those of the T1 Cu(II). This led Reinhammar, who first reported NI, and others, to assign NI as a three electron reduced hydroxyl radical species, with electron transfers from the T1 Cu and two type 3 Cu’s. However, Cu K-edge XAS and MCD spectroscopies were not consistent with a hydroxyl radical species (vide infra).
Although NI studies have mainly been conducted in RvL, due to favorable kinetic properties, its occurrence in Cp, BOD, and CueO indicate that this intermediate is part of the reaction cycle in MCOs in general.

NI displays a range of interesting spectroscopic features, summarized in Figure 153, all contributing to the final determination of its geometric and electronic structure. From Cu K-edge XAS the lack of a 8984eV feature characteristic of Cu(I) showed that all Cu ions in NI are oxidized (Figure 153B), clearly inconsistent with a three electron hydroxyl species. Instead, dioxygen is reduced to the water level by four electrons. As mentioned, the 77K EPR spectrum shows the typical T1 Cu signal with narrow A, but no contribution from the TNC. However, upon lowering the temperature, new rhombic features emerge with g-values of 2.15, 1.86, and 1.65, drastically different from “normal” Cu(II) signals (e.g. the T2 Cu in the resting MCOs) that generally do not have g values below 2.00 (Figure 153D). The new features also show markedly different power saturation behavior with temperature relative to the T1 Cu, indicating a low lying excited state at the TNC in NI. The LT MCD spectrum of NI shows an intense pseudo-A term signal (i.e. derivative shaped) with features at 318nm (31,780cm<sup>-1</sup>) (+) and 365nm (27,560cm<sup>-1</sup>) (−), corresponding to the two CT Abs features (Figure 153A bottom). Furthermore, saturation behavior of the LT MCD signal with varying magnetic field at low temperature shows the unusual g values of NI are associated with an S<sub>TOT</sub> = ½ ground state (Figure 153C). Finally, the temperature dependence of the MCD intensity at high field shows an unusual behavior. Normally, the MCD intensity of paramagnetic species follows a 1/T Curie type behavior. From Figure 153E, the intensity at 25,000cm<sup>-1</sup> does initially decrease with increasing temperature up to 30K. However, when the temperature is raised further the intensity increases, a non-Curie behavior indicative of population of the low-lying excited state with a more intense positive MCD feature at this energy, compared to the ground state. By fitting the intensity at 25,000cm<sup>-1</sup> to a Boltzmann population model, an excited state energy of ~150cm<sup>-1</sup> is obtained (Figure 153F). Furthermore, by simulating the temperature dependence at each wavelength, the MCD spectrum of the excited state can be modeled (vide infra). The excited state energy of 150cm<sup>-1</sup> is supported by the relaxation studies (power at variable temperature) of the EPR signal at g = 1.65 signal indicating an Orbach process. In summary, there are three unique spectroscopic features of NI: a low lying excited state at 150cm<sup>-1</sup>, a ground S<sub>TOT</sub> =1/2 with g-values below 2.00, and a CT pseudo-A term in MCD.

An additional important spectroscopic input was obtained from EXAFS, which revealed that at least two Cu’s of the TNC in NI are tightly bridged together at a Cu-Cu distance of 3.3Å (Figure 154A). From magnetostuctural correlations, this distance is consistent with a pair of Cu’s with a singlet/triplet splitting of ~520cm<sup>-1</sup> for a singly bridged species (Figure 154B). Accounting for the presence of a third Cu(II) in the TNC 155, left), a singly bridged structure should have a ground doublet with excited doublet and quartet states at 520cm<sup>-1</sup>. When a bridge is introduced to the third Cu(II), this excited degeneracy is lifted (Figure 155, middle), but the lowest energy excited state remains at ~500cm<sup>-1</sup> above the ground state, inconsistent with the 150cm<sup>-1</sup> observed experimentally. Introducing a third bridge to close the triangle leads to spin frustration that significantly lowers the energy excited state transitions. As presented in section 2.4, spin frustration arises when all three bridging
interactions are antiferromagnetic and of similar magnitude. In that limit, each of the Cu(II) pairs want to be antiferromagnetically coupled, but this is obviously not possible in an all bridged triangle. The significant lowering of the doublet excited state energy is consistent with the low lying excited state observed in NI. From the constraints of the bridging interaction of 520 cm\(^{-1}\) and the 150 cm\(^{-1}\) low lying excited state, limits of the two remaining exchange couplings can be estimated to be 440–520 cm\(^{-1}\) and 435–445 cm\(^{-1}\), respectively. These limits can be further refined by evaluating the contribution of each Cu(II) in the TNC to the ground- and excited state MCD spectra. The MCD C-term has contributions from each of the three Cu’s of the TNC, representing the coupling coefficients of the individual Cu(II)’s to the overall ground- and excited state wavefunctions of NI.

These are given by:

\[ C_0(1/2, 0) = c_1C'_0(1) + c_2C'_0(2) + c_3C'_0(3) \]

\[ C'_0(1/2, 1) = e_1' C'_0(1) + e_2' C'_0(2) + e_3' C'_0(3) \]

where \( C_0 \) and \( C'_0 \) are the total C-term intensity of the ground and first excited state MCD spectra, respectively, and \( C_{0(i)} \) is the C-term contribution from Cu\( i \). The coupling coefficients, \( c_{(i)} \), are dependent solely on the J couplings, and from these, ground and excited state coupling coefficients can be evaluated within the above mentioned limits (Figure 156B). Inspection of the ground and excited state MCD spectra of NI (Figure 156A) reveals three different behaviors with respect to intensity and sign of the transitions. Bands labeled 8, 11, and 12 are negative in the ground state but have large positive intensities in the excited state. Band 9 is large and positive in the ground state with very limited intensity in the excited state. Finally, band 10 is positive in both the ground and excited state. Evaluating these behaviors in connection with the possible coupling coefficients of Fig 156B, gives AF exchange interactions of 520 (fixed from EXAFS distance), 470, and 430 cm\(^{-1}\) (Figure 156C). Importantly, this analysis shows that the different Cu centers contribute to the different CT transitions in the MCD spectrum of NI (labeled in Figure 156A) and the three large J-values (Figure 156C) require that all 3 Cu’s of the TNC are bridged.\(^{909}\)

As discussed in section 2.4, a spin frustrated trinuclear Cu(II) center can undergo a zero field splitting due to antisymmetric exchange which would lead to g-values well below 2.00 as observed for NI in Figure 153D. From section 2.4, antisymmetric exchange requires good ground state exchange coupling between two Cu(II)’s, SOC to an excited state on one Cu(II), and good exchange coupling between this excited state and the ground state on the adjacent Cu(II). There are two possible allbridged structures of NI, and model complexes exist for both.\(^{914,915}\) On possibility, TrisOH as shown in Figure 157A, would result from reduction of O\(_2\) to H\(_2\)O to produce two bridging OH-’s, and a third bridge would derive from the H\(_2\)O solvent. In the alternative µ\(_3\)-oxo stucture (Figure 157B), the O would derive from full reduction of O\(_2\). Both structures have the appropriate orbital arrangements for ground to ground and ground to excited state exchange coupling to have significant antisymmetric exchange (Figure 157C) and indeed we have observed this experimentally.\(^{66,916,917}\) Single crystal EPR experiments on TrisOH, Figure 158, show that very low g-values are in fact observed for this structure, when the field is perpendicular to its z-axis.\(^{918}\) From Figure 157C, for TrisOH two d\(_{x^2-y^2}\) ground state orbitals on two Cu(II)’s strongly antiferromagnetically couple, and SOC on one Cu gives a d\(_{xy}\) excited state on that
Cu, that can strongly couple to the $d_{x^2-y^2}$ ground state of the adjacent Cu, thus fulfilling the requirements for efficient antisymmetric exchange. A similar mechanism is realized for $\mu_3$-oxo when the $\mu_3$-bridging oxo group resides in or close to the plane of the three Cu(II)‘s. In this case, each $d_{x^2}$ ground state can SOC to a $d_{xz}$ excited state on an adjacent Cu(II) via the $L_z$ orbital angular momentum operator. Thus both TrisOH and $\mu_3$-oxo are possible structures for NI.

In order to distinguish between these models and determine the geometric and electronic structure of NI, we performed an analysis of the LT excited state CT MCD features of these trinuclear model complexes and applied the results to NI. TrisOH (Figure 159A), $\mu_3$-oxo (Figure 159B), and NI (Figure 153A) all share the common feature of an intense derivative shaped pseudo-A term in the charge transfer region of their MCD spectra. In order to have pseudo-A term MCD intensity, two perpendicular CT transitions are required, that can spin orbit couple in a third mutually perpendicular direction. Due to the $1/r^3$ dependence of spin-orbit coupling, this can be approximated as a single center, one electron operator. Thus for two CT transitions to SO couple, they must have the same donor and different acceptor orbitals (or vice versa) that share a common metal- or ligand based center. These requirements give rise to two different scenarios: CT from two ligands to a given Cu (TrisOH, Figure 159C) with metal based SOC, or CT from an oxo bridge to two different Cu’s ($\mu_3$-oxo, Figure 159D) with oxo centered SOC.

From Figure 159A, an intense symmetric pseudo-A term feature is observed for TrisOH in the energy region between 27,000 cm$^{-1}$ and 35,000 cm$^{-1}$. VTVH MCD of this feature shows four individual transitions, all x,y polarized, which then requires the spin-orbit coupling to be in the z-direction. Analyzing the orbital origin of these transitions indicates that only metal-based SOC can lead to significant pseudo-A intensity, coupling transitions from two different OH-ligands to the same metal site. This is possible due to the highly covalent Cu-OH $\sigma$-bonds that allow for significant metal character in the charge transfer states. As observed in Figure 159A, the pseudo-A term of TrisOH consists of a negative lower energy and a positive higher energy band. The signs of these bands can be theoretically predicted by evaluating the signs of the transition moment dipoles and the SOC operator as they relate to the MCD C-term. The sign of the transition moment dipole is obtained from the transition densities, which are the products of the donor and acceptor wavefunctions, whereas the sign of the SOC operator is given by ligand-field theory. From these assignments, the predicted signs of the pseudo-A term in TrisOH are a negative low energy and a positive high energy band, consistent with the experimental data in Figure 159A.

For the $\mu_3$-oxo complex, only ligand-based SOC is possible (Fig 158D). The MCD spectrum of $\mu_3$-oxo shows an intense pseudo-A term in the CT region (Figure 159B), with the low energy band positive and the high energy band negative. (Note: these transitions are eliminated by protonation of the bridge to $\mu_3$-OH). While this complex has an $S = 3/2$ ground state, DFT calculations of a more planar $S = \frac{1}{2}$ $\mu_3$-oxo structure, predict that the corresponding pseudo-A term will also have a positive low energy and negative high energy band, as observed experimentally in Figure 159B, and opposite to that of the TrisOH complex.
Finally this analysis was applied to the MCD features of NI. From Figs. 156A ground state MCD spectrum, the characteristic pseudo-A term has a positive low energy (27,560 cm\(^{-1}\)) and a negative high energy band (31,780 cm\(^{-1}\)). The VT MCD data further show that the pseudo-A term consists of CT transitions to the different Cu(II) centers (Figure 156A bottom). The fact that more than one Cu center is involved in the pseudo-A term, unambiguously assigns NI as having a \(\mu_3\)-oxo-like structure, since only the ligand-based SOC mechanism allows coupling of CT’s to two different Cu centers (Fig 159D). This assignment is further supported by the sign of the pseudo-A term of NI (Figure 153A and 156A ground state), with the positive band lower in energy than the negative band, consistent with the \(\mu_3\)-oxo but not the TrisOH spectrum in Fig 158B and A, respectively.

One issue remains regarding the structure of NI, namely whether the second oxygen atom of O\(_2\) reduction stays bound to the TNC as in Figure 160 (the origin of the proton on the \(\mu_2\)-OH will be resolved in the next section). EXAFS and DFT calculations were conducted in order to address this issue. In EXAFS, NI was compared to an azide-bound NI derivative (NI\(_{az}\)), where a single azide molecule is internally bridged to all three TNC Cu’s, with no additional bridging interactions. The first shell Cu-N/O interaction shows a higher coordination number in NI vs. NI\(_{az}\), indicative of a second bridging interaction between the two T3 Cu’s. Also, DFT calculations predict that protonative loss of the \(\mu_2\)-OH T3 bridge in Figure 160, will rotate one of the T3 Cu orbitals away from the x,y plane, resulting in ferromagnetic coupling with the other Cu ions, clearly inconsistent with the above described spin frustration features of NI. Finally, it is in fact calculated to be ~17 kcal/mol more favorable to protonate the \(\mu_3\)-oxo than the \(\mu_2\)-OH. These results allow the assignment of NI as a \(\mu_3\)-oxo species, with an additional \(\mu_2\)-OH bridge between the two T3 Cu(II)’s (Figure 160). This structure for NI is also consistent with QM/MM calculations.

In summary, extensive spectroscopic and theoretical studies have identified two fully oxidized TNC structures in MCOs. The resting enzyme with AF-coupled T3 Cu’s via a \(\mu_2\)-OH and a magnetically isolated T2 Cu (Figure 144), and NI with an all-bridged \(\mu_3\)-oxo structure (Figure 160). The all-bridged nature of NI tunes its redox properties and allows for facile e.t. from the T1 Cu to each of the TNC Cu’s through \(\mu_3\)-oxo superexchange pathways; a significant feature in terms of the reactivity of the MCOs (vide infra).

3.7.1.5 Molecular Mechanism

3.7.1.5.1 Molecular mechanism of O\(_2\) reduction in MCOs: Reduction of dioxygen to water by the MCOs occurs at the TNC, with four electrons being transferred in two two-electron transfer steps. A generally accepted mechanism, as depicted in Figure 161, has been formulated. The starting point is the fully reduced enzyme where O\(_2\) binds and gets reduced by two electrons from the T2 and T3\(\beta\) Cu, forming the spectroscopically characterized peroxo intermediate, PI. Two additional electrons are then rapidly transferred to PI (one from the T3\(\alpha\) of the TNC and one from the T1 Cu), resulting in O-O bond cleavage and formation of NI, where the oxygen atoms of the original O\(_2\) are reduced to the water level. In the presence of reducing substrate, NI will undergo fast reduction back to the fully reduced enzyme. Alternatively, with no excess reductant, NI will slowly decay to the thermodynamically more stable resting oxidized enzyme. The resting TNC is only slowly
reduced by the T1 at a rate inconsistent with turnover. This mechanism is a result of many years of spectroscopic, kinetics, and computational studies by our group and others that are summarized in the following sections.

3.7.1.5.2 Reactivity of the T3 binuclear Cu site: A significant achievement in the elucidation of the mechanism by which MCOs reduce $O_2$ to water, has been in determining the individual role of each of the Cu’s in the TNC. From Figure 161, the T2/T3β Cu(I)’s deliver the electrons in the first two-electron transfer step. This was shown experimentally as well as computationally (vide supra). Prior to this, the T3 binuclear Cu’s were assumed to be the active Cu’s in this step, mainly due to the resemblance of the site with that of the coupled binuclear Cu enzymes like hemocyanin, tyrosinase, and catechol oxidase (section 3.1 and 3.2), which in their reduced state bind and reduce dioxygen in a two-electron reversible step. Experimental insight into the reactivity of reduced T3 Cu sites in Hc (deoxy-Hc) and T2D RvL (deoxy-Lc) with dioxygen, was obtained by probing the redox state of the T3 Cu’s, by Cu K-edge XAS. Spectra of reduced enzymes were compared to those of these enzymes shortly after exposure to dioxygen. As seen in Figure 162A, a reduced T3 Cu site in deoxy Hc has significant intensity in the 8984eV region, consistent with the spectra of Cu(I) model complexes. After exposure to dioxygen, however, this intensity is eliminated showing oxidation of the two Cu ions in Hc. In contrast, no change is observed in the XAS spectrum of T2D deoxy-Lc after exposure to $O_2$ (Figure 162B), showing a lack of reactivity of the reduced T3 Cu site in the MCOs in the absence of the T2 Cu. This is interesting considering the apparent similarity in ligand environments, where both the Hc T3 and RvL T3 Cu sites have 6 highly conserved 1st sphere His ligands. A recent study by Yoon et. al, provided insight into this reactivity difference (cf. section 3.1.5). This was based on computational evaluation of energies of the deoxy forms of the enzymes, as well as binding energies of $O_2$ to the fully reduced sites. Models for each binuclear deoxy site were generated, imposing frozen αC positions of the His ligands as dictated by crystallography of Hc vs. MCOs. By varying the Cu-Cu distance, Potential Energy Surfaces (PES) were generated (Figure 52, filled symbols), with the most energetically favored structures obtained at 4.2 and 6.5Å for deoxy-Hc and T2D deoxy-Lc, respectively. A significant destabilization of ~6.9kcal/mol was observed for deoxy-Hc compared to deoxy-Lc. Subsequently, PES with the αC constraints removed, were generated (Figure 52, open symbols), revealing that the main contribution to the difference in energy is the electrostatic repulsion of the closely spaced Cu ions in deoxy-Hc. Additionally, $O_2$ binding energies of the two deoxy sites were evaluated on S=0 and S=1 reaction coordinates, generated by introducing an $O_2$ molecule into the center of the binuclear sites. For deoxy-Hc, $O_2$ binding was observed to be exothermic by 2.9kcal/mol, with a spin cross-over from S=1 to S=0 occurring at ~4.0Å with no additional barrier, resulting in a final μ-η2,η2 peroxo-level (S=0) structure with a Cu-Cu distance of ~3.6Å, consistent with experimental data (vide supra). In contrast, $O_2$ binding to T2D deoxy Lc was energetically unfavorable by 5.6 kcal/mol (Figure 163), where a peroxo structure was obtained, similar to that of oxy-Hc (Cu-Cu distance of 3.7Å), with spin cross-over occurring at ~4.0 Å and an energy barrier of ~9.0 kcal/mol. Thus, while $O_2$ binding to deoxy-Hc is exothermic with a barrier-free spin crossover, $O_2$ binding and reduction by deoxy T3 in T2D Lc is thermodynamically as well as kinetically, unfavorable. It is interesting to note, that the
calculated total energy of the O$_2$ bound structures of Hc and RvL, respectively, only differs by 1.9 kcal/mol, but due to the energy difference of the deoxy forms, only deoxy-Hc is capable of facile reaction with O$_2$. This difference is a property of the backbone constraints imposed on the T3 Cu coordinating His residues, as illustrated in Figure 164. Here, it is observed that most of the His residues, in Hc, reside at the end of rigid α-helical motifs, whereas, in MCOs, the corresponding residues are mainly situated on long flexible loops. The flexibility in the MCOs is reflected in reduced and oxidized crystal structures, respectively, where T3 Cu-Cu distances are seen to vary from ~3.4 to >5 Å (see section 3.7.1.3).

3.7.1.5.3 T2 and T3 Cu’s deliver electrons in the first two-electron step: While the T2D MCOs do not react with O$_2$, the T1 depleted forms, with full TNCs, do rapidly react with O$_2$ to form PI. From spectroscopic experiments, two electrons are transferred from the TNC to O$_2$ to form a peroxide bridged structure. The stabilization energies of possible PI structures of the TNC were evaluated. With a relatively small model, including only the three TNC Cu’s and their first coordination sphere (8 His and one T2 Cu OH), a side-on peroxy structure, bridging the two T3 Cu’s was found (Figure 152A, left). This is clearly inconsistent with reactivity- and spectroscopic results (vide supra). Therefore, an extended version, including a highly conserved aspartate residue (D94 in Fet3p, see Figure 165), was included in the model. A μ$_3$-1,1,2 peroxy structure was now found, with the peroxide bridging all three coppers, bidentate to T3β and monodentate to T2 and T3α (Figure 152A, right). Furthermore, calculated spin densities revealed that the T2 and T3β Cu were oxidized, and thereby responsible for the first two-electron reduction of dioxygen.

Experimental studies, supporting these calculations, have been carried out, focusing primarily on variants of Fet3p, including first and second sphere residues of the TNC Cu’s. In one study, the conserved aspartate residue (D94) was mutated to Glu, Gln, and Asn, in the holo forms of the enzyme, as well as the T1D forms. All mutated varients were expressed and purified with intact TNCs, but only D94E/T1DD94E showed facile reactivity towards dioxygen. While no reactivity was observed upon reaction of reduced T1DD94A/N with O$_2$, for T1DD94E, a peroxy intermediate with almost identical spectroscopic features to those of PI, was rapidly formed. This indicates that having a negatively charged residue in the vicinity of the T2 and T3β Cu’s is critical in the O$_2$ reduction process of MCOs. Similar results were later presented for bilirubin oxidase variants.

Experimental verification of the role of each of the Cu’s in the first two-electron transfer step, was obtained from a mutational study focusing on H126Q and H483Q variants of Fet3p (ligands to the T3α- and T3β Cu, respectively) (Figure 165). These two residues mirror each other in that they both connect to T2 Cu residues via a backbone N-H H-bond to C=O. The effect of the mutations was probed by XAS, CD, MCD, and EPR. Compared to the WT enzyme, small variations in energies and intensities were observed in the CD spectra of T1DH126Q and T1DH483Q, reflecting the change in electronic structure of the T3α- and T3β Cu. Furthermore, the observed changes in the EPR and MCD spectra, which selectively probe the paramagnetic mononuclear T2 Cu (vide supra), highlighted the above mentioned connectivity due to the H-bonding network in the vicinity of the TNC. Whereas only small perturbations were observed in the resting forms of the mutated enzymes, large
structural changes of the reduced forms were observed in Cu K-edge XAS spectra. The 8984 eV feature, probing the geometry of the reduced Cu’s, were observed to be more intense in the mutated enzymes compared to the WT. This is consistent with the mutated residues not being able to bind to their respective Cu’s in the reduced state, resulting in a close to linear geometry of the remaining ligated His residues. In comparing the reactivity of the reduced TNCs of T1DH126Q and T1DH483Q with that of WT T1D, only T1DH126Q was capable of facile reaction with dioxygen, as observed in the resulting absorption spectra in Figure 166. Importantly, in the CD spectra, which can resolve different components, the T1DH126Q O₂-generated species is identical to PI (Figure 167). This verifies that the T3α Cu is reduced in PI, since the mutated H126Q clearly perturbs the T3α Cu in the resting form. Despite the similarities of the T3α- and T3β Cu’s of the TNC, it is evident that these Cu’s possess an inherent asymmetry with respect to reactivity; mutating His to Gln on T3α- and T3β Cu, respectively, results in similar spectroscopic perturbations. However, only mutation of a T3α residue (stabilized in a redox inactive form) still allows facile two-electron reduction of dioxygen.

A closer look at the environment of the two Cu ions provides insight into the origin of this asymmetry. The conserved aspartate (D94), is close to the reactive T2-T3β Cu edge of the TNC (Figure 165), thereby lowering the potential of these Cu’s. Secondly, from crystal structures of fully reduced MCOs and corresponding XAS Cu K edge features of T2D Lc, Figure 162 (more intense 8984 eV feature in deoxy-Lc compared to deoxy-Hc), it is observed that the T3α Cu is situated in the plane of the coordinating His-N’s, whereas the T3β Cu is held out of the plane by as much as 0.3 Å. This further stabilizes the T3α Cu(I) relative to the T3β Cu(I) (verified by potentiometric redox titrations of T1DH126Q and T1DH483Q), thus deactivating T3α towards oxidation. This asymmetry leads to irreversible O₂ binding and its activation for reductive cleavage of the O-O bond by the TNC.

3.7.1.5.4 Second two-electron step- cleavage of the O-O bond: The second two-electron transfer step in the reduction of O₂ by the MCOs, involves cleavage of the O-O bond and formation of NI. As for the first two-electron step, insight into this step has been obtained from experimental as well as computational studies. On the experimental side, T1 inactive MCOs (primarily T1D in Fet3p and T1Hg in RvL) has been critical in evaluating the PI to NI conversion, due to the altered kinetic properties of the reaction. In a study from 1996, Shin et. al showed that T1Hg-generated PI slowly decayed, eventually regenerating the resting form of the enzyme. Monitoring the decay process by MCD established that PI decays via NI, before regenerating the resting form. This was significant because it allowed the study of the PI to NI conversion, which is not accessible in the holo enzyme, where this conversion is extremely fast and not rate limiting (vide supra). This study found a pH dependence of PI decay, with the decay rate at low pH being ~10 fold higher than at high pH. This was ascribed to protonation of a carboxylate residue in close vicinity to the TNC (vide infra). In a subsequent study, Palmer et. al investigated the Kinetic Isotope Effect (KIE) of 16O₂/18O₂ generated PI decay, and found a k₁₆/k₁₈ of 1.11, verifying that O-O bond cleavage is an integral part of this process. Furthermore, an inverse SKIE was observed at low pH, while no SKIE was observed at high pH, indicating that a proton assists in the O-O
bond cleavage at low pH, but is not required at high pH. Finally, Palmer et al. evaluated the more than $10^6$ decrease in rate of PI to NI conversion, observed in the redox inactive T1 enzyme compared to holo enzyme. By employing a model for dissociative electron transfer, developed by Saveant and coworkers, activation energies for one electron (T1 depleted enzyme) and two electron (holo enzyme) cleavage of the O-O bond were predicted at 8.5–12.5 kcal/mol and 2–3 kcal/mol, respectively, consistent with the difference in experimentally determined activation energies. This difference correlates to the redox potentials of the one and two electron reductions of peroxide, ~0.38 and ~1.37 V, resulting in the Morse potential energy surfaces in Figure 168. The high driving force of the two-electron reduction results in a low activation barrier, in contrast to the one electron process.

To obtain further insight into the origin of the pH effect on PI decay, mutational studies on Fet3p were conducted, focusing on the two highly conserved carboxylate residues (D94 and E487, Figure 165) in the second coordination sphere of the TNC. D94E, E487D, and E487A type 1 depleted variants were generated and analyzed with respect to their PI decay rates at high and low pH, and their SKIEs. PI decay rates are shown in Figure 169 and summarized in Table 30. All variants, except for E487A, show accelerated PI decay rates at low pH. This unambiguously ascribes the low pH accelerating effect to E487. The position of this residue, close to the T3 binuclear Cu’s, indicates that this pH effect is connected to the formation of the $\mu_2$-OH bridge, which, as previously described, is an important feature of the NI structure. Secondly, the SKIE of PI decay shifts from inverse (~0.89) in the WT enzyme to normal (2.0) in E487D. By employing the Westheimer model, the extent of $\mu_2$-O-H bond formation in the transition state of O-O bond cleavage can be evaluated. An inverse SKIE is indicative of a strong O-H bond being mostly formed in the transition state, whereas a normal SKIE indicates that the proton is further away from the product position. This is consistent with the shorter carboxylate side chain of Asp in E487D, compared to Glu in WT, being less efficient in proton transfer to the T3 bridging oxygen. This is also reflected in the decreased rate enhancement at low pH in E487D vs. WT (3-fold vs. 10-fold). Finally, from Figure 169 and Table 30, D94E has a normal SKIE at low pH, and the rate of decay is slower at all pH’s. In the WT T1D, changes in pH led to changes in the MCD spectrum. This was ascribed to perturbation of the T2 Cu OH ligand, induced by protonation of the D94 residue; a property of the aforementioned hydrogen bonding network in the vicinity of the T2 Cu. This connectivity is lost in the D94E variant, where no change in the MCD spectrum of the resting enzyme is observed upon changes in pH. The H-bonding network has a mechanistic role. In the WT enzyme, D94 is capable of removing a proton from the H$_2$O ligand at the T2 Cu in PI. This results in increased electron density at the T2 Cu, thereby enhancing the electron transfer from this Cu to the bound peroxide. This enhancement is lost in the D94E variant, resulting in the significantly lowered decay rate, compared to the WT enzyme.

We combine the above results into an overall model for the O-O bond cleavage step in native MCOs, as presented in Figure 170. The starting point (hypothetical), is a one electron reduced PI, PI’, where an electron has been transferred from the T1 Cu to the T2 Cu (vide infra), setting up a two electron reduced TNC capable of facile reduction of PI to NI. At low pH we assume that E487 is protonated, while D94 is deprotonated as required for reactivity (vide supra). The reaction coordinate towards the transition state involves proton...
transfer from E487, via a water molecule to the proximal oxygen atom of PI`, and a concerted deprotonation of the T2 Cu-bound water molecule via a H-bonded water molecule to D94. The combined effect of these proton rearrangements is to weaken the O-O bond of PI and decrease the redox potential of the T2 Cu, both of which will promote the O-O bond cleavage. At high pH, both of these proton transfer mechanisms are disrupted, resulting in the decreased reaction rate and less involvement of the μ2-O-H bond in the transition state, as reflected in the lack of a SKIE.889

The above spectroscopically defined structures of PI and NI, as well as the experimental kinetic and mechanistic insight, led to a computational evaluation of the energetics of O-O bond cleavage.906 The starting point of the reaction is ‘PI + e-‘ (Figure 171), where electron transfer from the T1 to the T2 Cu generates a structure with the T2-PI-O bond broken, and two TNC electrons available for reduction of PI to NI, at the T2 and T3α Cu’s. As shown in Figure 171, PI + e− to NI conversion involves O-O bond cleavage and protonation by E487 of the μ2-O2− bridge. Based on this, a pH potential energy surface plot was generated (Figure 172) where individual points on the grid correspond to varying O1-O2 and O2-H bond distances ranging from 1.45 and 1.85Å to 2.4 and 1.0Å, respectively. The 2D plot reveals a highly exothermic reaction. Two separate pathways can be identified, both with low activation barriers. Following the dashed line from PI + e− in the rear corner along the O-O bond cleavage coordinate leads to transition state 1 (TS1) (at ~1.70Å), with a low activation energy of ~5.9 kcal/mol, consistent with the experimentally estimated value. Subsequent proton transfer, results in NI formation with no additional activation barrier. Alternatively, following the H+ transfer coordinate leads to PI + e− + H+ formation, where the proton from E487 has been transferred to the T3 bridging oxygen, with no significant change in O1-O2 bond distance. Subsequent elongation of the O-O bond results in NI formation, via TS2, obtained at ~1.70Å, with a barrier of ~5.4kcal/mol relative to PI + e− + H+. It is interesting to note that no intermediate reaction pathway, with concurrent O-O elongation and O-H contraction, is observed in the 2D energy plot. This correlates well with experimental data, where the inverse SKIE observed at low pH is indicative of proton transfer before the transition state, resembling the PI + e− + H+ pathway, TS2, whereas a SKIE of 1, at high pH, is consistent with the PI + e− pathway, TS1, where proton transfer occurs after the transition state.889

Geometry optimized structures of both pathways, highlight a molecular orbital picture of O-O bond cleavage, in MCOs, that leads to the low barriers at both high and low pH.906 First, evaluation of the spin densities of the TNC Cu’s (Figure 173) shows that the T3β Cu remains oxidized throughout the reaction, whereas the T2 and T3α Cu’s simultaneously gain spin density as the O-O bond is elongated, verifying that the reduction of PI occurs via a concerted two electron transfer mechanism. A closer look at the frontier molecular orbitals of the TNC involved in the O-O bond cleavage, shows how the site is optimized for facile electron transfer, due to significant overlap of the HOMO of the donors (T2/T3α), with the LUMO of the acceptor (peroxide σ*), Figure 174. The overlap is enhanced as the energy of the LUMO is lowered with O-O bond elongation, resulting in strong orbital mixing at the transition state. The energy lowering is also enhanced by interaction of the oxidized T3β Cu with the peroxide σ* orbital, thereby further lowering the barrier and defining a critical role for this Cu in the reductive cleavage of the O-O bond. These features emphasize the
significance of the triangular topology of the TNC (Figure 174) in the reductive cleavage of the O-O bond resulting in an effective four electron transfer in the native enzyme.

3.7.1.5.5 Decay of NI to resting enzyme: The analysis of O-O bond cleavage, obtained from experimental as well as computational studies, concludes the description of the O₂ reductive part of the catalytic cycle of MCOs. Now we address the decay of NI to the resting enzyme. This occurs in the dioxygen reaction of the fully reduced enzyme in the absence of an electron from a reducing substrate, see Figure 161. As for the PI to NI conversion, spectroscopically defined structures of NI and resting oxidized enzyme, provide the scaffold for computational evaluation of this process. This process includes two proton transfers and release of one water molecule from the TNC, with the second oxygen from O₂ bound to the T2 in the resting form (Figure 175A). Two different starting structures of NI were evaluated, only differing in the protonation state of the T2 Cu bound water derived ligand (NI\textsubscript{H₂O} and NI\textsubscript{OH}). These structures include both the μ₃-oxo and the T3 μ₂-OH bridge, as dictated by spectroscopy and computations (vide supra). The first step of NI decay involves protonation (from solvent) of either the μ₃-O or the μ₂-OH. Protonation of the μ₃-O was found to be energetically favored by 17 and 25 kcal/mol for NI\textsubscript{H₂O} and NI\textsubscript{OH}, respectively. Also, protonation of the μ₂-OH results in loss of the bridging interaction, thereby generating a ferromagnetic structure, inconsistent with spectroscopic observations (vide supra). A second proton can then be transferred, either to the μ₃-OH or to the μ₂-OH. Both lead to the same result; protonative decoupling of the μ₂-OH bridge, generating an isolated T3β Cu with a water molecule bound, with the T2 and T3α Cu’s still bridged via the μ-OH (Figure 175A). The T3β Cu-bound water molecule is subject to facile exchange with solvent water molecules, while the second oxygen atom of the original dioxygen remains bound to the T2 and T3α Cu’s of the TNC. The subsequent steps in the decay of NI, involve rearrangement of this oxygen from its position inside the cluster to outside the cluster, terminally at the T2 Cu, as suggested from \textsuperscript{17}O experiments (vide supra). As shown in Figure 175B, this rearrangement occurs via proton transfer from the T3β Cu-bound water molecule to the rotating OH⁻, allowing for regeneration of the μ₂-OH bridge between the two T3 Cu’s. Transfer of this H₂O from inside to outside the cluster has a significant activation barrier, with a lower limit of 8.5 kcal/mol, consistent with the experimental value of 8.8–13.9 kcal/mol making this step rate determining in the conversion of NI to resting oxidized enzyme. As mentioned earlier, this process is too slow to be in the catalytic cycle and therefore NI reduction must be catalytically relevant.

3.7.1.5.6 Reduction of NI: Recently, the first report of kinetics on the reduction of NI showed that the rate of intramolecular electron transfer from the T1 to the TNC is > 700 s\(^{-1}\), consistent with turnover. Additionally, and under identical conditions, the rate of intramolecular electron transfer from the T1 to the TNC in the reduction of the resting enzyme was found to be 0.11 s\(^{-1}\), consistent with the aforementioned slow kinetics in section 3.7.1.2. The reduction of the resting TNC also exhibits a deuterium solvent kinetic isotope effect, which implies that both the reduction of resting and NI are a proton coupled electron transfer (PCET) processes. Importantly, these results indicate that the reduction of the resting TNC that is observed crystallographically, is not in the catalytic
cycle and establishes the reduction of NI as the relevant process in the rereduction of the MCOs.

This difference of more than three orders-of-magnitude in ET rate produces a unique opportunity to determine the molecular factors that control electron transfer rates in the MCOs. In Marcus Theory, these rates are dependent on the thermodynamic driving force, reorganization energy, and the electronic coupling matrix element between donor and acceptor. Calculations were performed on the reduction and protonation of these TNC active sites (resting and NI in Figure 176) to determine which of these contributions account for the large rate difference. It was computed that the proton coupled electron transfer driving force was more favorable for NI than resting by 7 kcal/mol, while estimates of reorganization energy were comparable; since the T3a copper was reduced in both T1 to T3a ET processes, the electronic coupling matrix element (through the T1 Cys-His pathway) can be considered equivalent in both processes. Therefore, the kinetic difference originates from a more favorable driving force for proton-coupled electron transfer to the TNC of NI relevant to resting. On the basis of the structures (Figure 176), this is attributed to the µ3-oxo ligand of NI since this group is significantly more basic than the µ2-OH of the resting site.

This new insight presents a unifying mechanism for both O2 reduction and enzyme rereduction since this µ3-oxo ligand originates from the reduction of O2 and the protonation of this group supplies the driving force for the rapid rereduction. Current studies focus on understanding the molecular mechanism of all the three ET steps in the reduction of NI to the fully reduced enzyme in the catalytic cycle.

### 3.7.2. Heme-copper respiratory oxidases

#### 3.7.2.1. Enzymology:

Heme-copper oxidases (HCOs), also referred to as heme-copper oxygen reductases, are a superfamily of membrane-bound enzymes that are located in the inner mitochondrial membrane of eukaryotes and in the cytoplasmic membrane of many prokaryotes. These catalyze the terminal step of respiration, the complete reduction of dioxygen to water. ~90–95% of all dioxygen is processed through HCO. During this process low energy electrons are removed from the respiratory chain. The overall dioxygen reduction is exergonic in nature. This reaction (O2 + 4H+ + 4e− → 2H2O) at 815 mV versus NHE per e− coupled to the oxidation of the reducing agent (Fe2+-cytochrome c → Fe3+-cytochrome c + 1e− at 235 mV) provides an overall driving force of 580 mV per electron (13 kcal/mol). This driving force is used to pump protons across the cytoplasmic (or mitochondrial) membrane (~5 kcal/mol required per proton) from the negative to the positive side (Equation 37).

\[
4e^- + 8H^+_{in} + O_2 \rightarrow 2H_2O + 4H^+_{out} \quad [37]
\]

Thus, for every electron reduction of O2, two protons can be translocated. The resulting proton gradient is used by ATP synthase to convert ADP and inorganic phosphate to ATP. These dioxygen reduction, electron transfer and proton pumping functions performed by HCO are illustrated in Figure 177A.
In all HCO, the fast reduction of dioxygen to water takes place at a bimetallic heme-copper site (Figure 177B) without generating toxic partially reduced oxygen species. This site is uniquely designed to overcome the first, one electron reduction of dioxygen, which is uphill (∼−350 mV at pH 7.0 vs NHE). The copper center, known as CuB, is positioned ~4.3–4 Å from the high-spin heme center depending on the exogenous ligation and oxidation states of the metals. The heme iron has a conserved axial histidine ligand. CuB is ligated to three histidines. Most HCO undergo a post-translational modification resulting in a covalent bond between one of the ligating histidines and a tyrosine residue. This cross-link is thought to be functionally relevant.

In addition to the bimetallic site, HCO contain between one to three other redox cofactors, which include a bimetallic CuA site and/or heme(s). These redox sites function in electron storage and transport from the external reductants to the heme-copper catalytic site. All HCO contain a second low-spin heme site (Figure 177A). In all HCO, this low-spin heme has two conserved axial histidines that are connected to the protein backbone. HCO may also contain a CuA center that can exist in an oxidized delocalized mixedvalent state. Each copper ion of CuA is bound to two bridging cysteines and one histidine. In addition, one copper has a weakly bound axial methionine whereas the other copper has an axial backbone carbonyl oxygen from a glutamate forming a long Cu-O ligand distance.

The HCO superfamily is quite diverse in terms of electron donors, heme types, and subunit composition leading to different electron and proton transfer channels. The HCO superfamily can be subdivided into three major functional and evolutionary families: A (containing subfamilies A1 and A2), B, and C (Figure 178). Mitochondria contain only type A1 oxidases, whereas prokaryotes contain all types of HCO. Among the prokaryotes, bacteria are known to contain all families of HCO, whereas archaea contain types A1 and B. Type C is observed only in two bacterial groups: 1) purple bacteria and 2) Flexibacter, Bacteroides and Cytophaga.

The A and B HCO require in addition to the central subunit (I), at least one other subunit (II). The type C HCO contain a central subunit (N) and either one (O) or two (O, P) additional subunits that are completely different from type A and B subunits. HCO belonging to type A use at least two proton pathways (D- and K-channels), whereas type B and C HCO require only one (a modified K-channel). Type A is further divided into A1 and A2 subfamilies depending on the residues along the D-channel. The details of the proton pathways (channels) will be discussed later. The proton pumping stoichiometry is not 1 H+/e− for all families. Family A generally has a stoichiometry closer to 1 whereas families B and C have ∼0.5 H+/e−. The lowered proton pumping stoichiometry in B and C families is energetically disadvantageous compared to the A family, requiring more O2 to be consumed to generate an equivalent membrane potential. Organisms encoding the B or C HCO have high apparent affinities for O2 and are usually expressed in organisms under low O2 or microaerobic conditions. It is not clear why nature would select for a less efficient enzyme in environments in which oxygen is limiting.

Nitric oxide reductases (NOR) have a close evolutionary link to HCO based on structural similarities. Subunit I in NOR is reminiscent of subunit I in HCO with the six histidines that
bind the metal centers. The other subunit of NOR resembles subunit O (that contains a mono-heme) of the C type HCO (Figure 178). NOR’s, however, catalyze a different reaction—the reduction of NO to N$_2$O. In addition, this enzyme has an Fe instead of Cu$_B$ at the catalytic site. Also, there is no tyrosine crosslinked to a histidine of this non-heme iron center. Interestingly, type C $cbb_3$ oxidase can also reduce NO to N$_2$O. It is not surprising, therefore, that NOR’s have the closest homology with C type HCO.

In addition to the three families, the HCO can be further divided into two subgroups, cytochrome $c$ oxidase (CcO or COX) and quinol oxidase (QO), depending on the source of the electrons required to reduce dioxygen. CcO, located in the mitochondrial membrane of all eukaryotes and in the cytoplasmic membrane of certain bacterial prokaryotes, use water soluble ferrocytochrome $c$ as the source of electrons whereas QOs, present only in some bacterial and archaeal prokaryotes, use membrane soluble ubiquinol or menaquinol as their electron source. Ferrocytochrome $c$ and ubiquinol are the most oxidizing electron carriers of the respiratory chain. CcO contain the mixed-valent Cu$_A$ site that is not present in QO. CcO span across all three HCO families; QO is present in only type A1 and B families.

There are three types of heme sites present in HCO’s: heme $a$, heme $b$, and heme $o$ (Figure 179). A subscript 3 denotes the O$_2$-binding heme site whereas lower case letters denote protein-bound hemes. The high-spin heme can be $a_3$, $b_3$ or $o_3$, whereas the low-spin heme can be either $a$ or $b$. Heme $a$ contains a formyl group in position 8 and a farnesyl tail of unsaturated isoprenoid groups in position 2, both of which are not present in heme $b$. Heme $o$ has the same structure as heme $a$ except for a methyl group in place of the formyl group. Only heme $a$ is present in most mitochondrial CcO. Heme $b$ has also been identified in submitochondrial particles. In bacteria, all three heme groups $a$, $b$ and $o$ can be present.

In addition to the structural subunits, a large number of accessory factors are involved in the assembly and maintenance of active HCO’s. Loss of function of some of these auxiliary proteins or mutations in the mitochondrially-encoded core subunits, although relatively rare, lead to CcO deficiency and are associated with a wide spectrum of clinical phenotypes ranging from isolated myopathy to multisystem disease. Most isolated CcO deficiencies are inherited as autosomal recessive disorders, which generally have a very early age of onset and a fatal outcome. Patients afflicted with these diseases present heterogeneous clinical phenotypes, including Leigh syndrome, hepatic failure and encephalomyopathy.

HCO’s are susceptible to inhibition by a variety of substrates including CO, sulfide (H$_2$S), cyanide (HCN), azide and formate (HCOOH). Of these, H$_2$S have been implicated in normal cellular signaling events because it can be readily metabolised by oxidative processes within CcO resulting in the enzyme acting as a physiological detoxifier of sulfide. Inhibition of CcO can also lead to toxic effects. Inhibition by cyanide and sulfide leads to acute respiratory failure, whereas with azide poisoning, the proximal cause of death is suggested to be circulatory collapse. CO is known to competitively bind to the reduced cytochrome $a_3$ and prevent its oxidation by oxygen; azide and formate un-competitively bind to the oxidized forms and prevent the reduction of this group; cyanide and sulfide
produce non-competitive inhibition towards oxygen. The five inhibitors all compete for the same or different oxidized forms of the enzyme, which are reversibly connected.

The endogenous production of NO in cells is also known to inhibit CcO activity. Low concentrations of NO for a short period of time has been proposed to be involved in the physiological and/or pathological regulation of respiration rate by extending the availability of O\textsubscript{2} either to cells at different distances from capillaries or within the mitochondrion. Higher NO levels and prolonged exposure, or conversion of NO to one of its more reactive oxides (peroxynitrite, nitrogen dioxide or nitrosothiols) results in the irreversible inhibition of respiration and other damage to mitochondria. It is known that NO inhibition of mitochondrial respiration has a role in cell death, by either necrotic or apoptotic mechanisms.

Two alternative pathways have been identified for the reaction of NO with CcO that depend on the rate of electron flux through the respiratory chain. At high electron reflux (i.e. high ferrocytochrome c concentrations), NO undergoes heme binding at the binuclear center in competition with O\textsubscript{2}, whereas at low electron reflux, NO binds to oxidized Cu\textsubscript{B}. Both pathways lead to effective, fully reversible inhibition of respiration and can be discriminated on the basis of the differential photosensitivity of the inhibited state. The oxygen sensitive NO binding to the reduced heme Fe to form a six-coordinated structure causes immediate inhibition of O\textsubscript{2} consumption and the formation of a light-sensitive nitrosyl derivative (Fe\textsuperscript{2+}-NO). The inhibition of O\textsubscript{2} consumption is greater at lower O\textsubscript{2} levels. The process is reversed when the NO is dissociated as a free, reactive radical. This pathway has been proposed to function both in regulating the formation of hydrogen peroxide from the respiratory chain and in signal transduction and controlling O\textsubscript{2} gradients in complex organs such as the liver or heart. Maladaptation in this pathway, in the presence of normal or enhanced levels of O\textsubscript{2}, leads to a mitochondrial dysfunction that has some of the characteristics of hypoxia, including a deficit in ATP. NO binding to the oxidized Cu\textsubscript{B} site of resting CcO, and to the Cu\textsubscript{B} site of intermediates P, F and O formed during turnover (see section 3.7.2.2.3) yields a light-insensitive Cu(I) nitrosonium (NO\textsuperscript{+}) complex. This Cu(I) nitrosonium can be oxidized to give nitrite, which can in turn inhibit the oxidase. The oxidase then recovers activity upon reduction, releasing nitrite into the bulk. Therefore, of relevance to cell pathophysiology, this pathway involving the oxidative degradation mechanism, disposes toxic NO in the form of less toxic nitrite.

3.7.2.2. Kinetics and Thermodynamics

3.7.2.2.1 Resting versus pulsed: The as-isolated, fully oxidized CcO has been shown to be different from the fully oxidized species formed after the four electron oxidation of the fully reduced enzyme. These species are commonly referred to as the resting and the pulsed forms, respectively. The interconversion between pulsed and resting forms at low pH (pH<8) is fairly slow. Armstrong and colleagues have shown that the pulsed form is converted to the resting enzyme within ~3 hrs at pH 7.2 (half-life of 40 min at 2 °C). Complete reduction of the resting form followed by complete oxidation produces the pulsed form. O\textsubscript{2} is physiologically involved but not necessary for the production of the pulsed state. The reduction potential of cytochrome a\textsubscript{3} in the resting enzyme is lower than that of the

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more physiologically relevant pulsed enzyme with the rate of intramolecular reduction of $a_3$ being significantly faster in the latter. The initial velocity for the steady-state reaction at pH 7.4, starting from the pulsed form is 4 to 5 fold faster than with the resting form with roughly stoichiometric additions of CcO and reduced Cyt c. Thus, the pulsed enzyme reaches steady state turnover sooner than the resting enzyme (pH 7.4). When Cyt c is in excess, the two forms proceed with roughly the same overall velocity. These observations are consistent with the model that the resting enzyme is not an intermediate in catalysis but is transformed into an active species after being fully reduced in the presence of O$_2$. Thus, the pulsed enzyme is the catalytically competent form that slowly decays to the resting form in the absence of additional reducing equivalents.

3.7.2.2 Slow versus fast: The resting form was originally taken to be the enzyme as-isolated. The as-isolated form can be inhomogeneous and contain a mixture of a fast and a slow form. Some authors have equated the resting form only with the slow form. The fast and slow forms are defined according to their rate of reaction with exogenous anionic ligands such as cyanide (CN$^-$), fluoride (F$^-$), and formate (HCOO$^-$). Alternately, the resting form has also been equated to the chloride-ligated fast form, which binds cyanide at a rate slower than the fast form but faster than the slow form. The term resting has thus become ambiguous. A fast form predominates when the enzyme is isolated under mildly alkaline conditions, whereas a slow form dominates in preparations at low pH. The fast form can be converted to the slow form upon incubation at low pH (pH <8) or with prolonged dilution of the enzyme. The reversal of the process is controversial. The slow form of bovine CcO exhibits a weak derivative shaped EPR signal with a $g' = 12$ in X-band that is not observed for the fast form, the difference arising from different magnetic interactions between the Cu$_B$ and heme $a_3$ (the precise nature of the spin coupling in each case is not known). The slow form with the $g' = 12$ EPR signal was not observed in the mitochondrial membrane and is suggested to have been produced as a consequence of one or more of the manipulations employed during purification. The slow form is, thus, considered an artifact of purification and not physiologically relevant. Spectroscopic properties of the fast and pulsed forms are very similar, which result in some authors incorrectly equating the two to be the same species. However, the pulsed form is metastable whereas the fast form is stable. The two forms also have different rates of reduction (discussed below). The metal ion ligation and coordination geometries of the fast and slow forms are not known. It appears that enzyme X-ray structural studies have not yet revealed differences between the two forms because all the crystal structures seem to be of the fast form (crystallographic studies, that do not explicitly mention whether they have a fast form, used enzyme purified at slightly alkaline conditions, which would suggest a fast form). Therefore, it is important to identify which form of the as-isolated enzyme, fast or slow, is present in a reaction, especially when dealing with single turnover kinetics. However, neither of these forms is the catalytically competent fully oxidized form that is the pulsed enzyme.

3.7.2.2.3 Single turnover kinetics: The catalytic cycle of HCO consists of two phases (Figure 180)—an oxidative phase where the reduced enzyme undergoes 4e$^-$ oxidation by O$_2$, and a reductive phase where the oxidized enzyme is reduced before it can bind the next
O₂ molecule. Two protons are translocated during the oxidative phase while two more protons are translocated during the reductive phase.

The four electron reduction of dioxygen has been extensively studied in two forms of CcO—the fully reduced (R or R₄) enzyme and the two electron reduced mixedvalence (MV or R₂) enzyme. The MV enzyme is formed by incubating the fully oxidized CcO with CO. The CO reduces the CuB and heme a₃ sites of the enzyme by two electrons and binds to the binuclear site. Dioxygen reduction has also been studied in the three electron reduced (R₃) enzyme, which is obtained by titrating the MV form with one electron equivalent of a reductant (such as TMPD, ascorbate, cytochrome c or NADH). In the MV+e form, heme a and Cuₐ are partially reduced and in an intramolecular redox equilibrium. Kinetic rates of the intermediate states formed in single turnover of the enzyme with O₂ have been studied by flow-flash photolysis of the bound CO in the presence of O₂ by a variety of biochemical and spectroscopic methods including Abs, rR and EPR.

Several kinetic intermediates have been resolved for the reaction of fully reduced, R-CcO with O₂. Transient absorption spectroscopy in the visible region (830 nm) suggests that O₂ first binds to the CuB site in the binuclear center with a rate constant of 10⁸ M⁻¹s⁻¹. The O₂ then weakly binds to heme a₃ with Kᵱ of ~0.3 mM, forming intermediate A (Figure 181) with a pseudo-first-order microscopic rate constant, k₁ = 1.2×10⁵ s⁻¹ at [O₂] = 625 µM. When the Cuₐ and heme a sites are reduced, as in R-CcO, intermediate A converts to Pₑ with an electron transfer from heme a to the binuclear center with a rate constant k₂ of 1–3×10⁴ s⁻¹ (Figure 181). The formation of Pₑ is independent of pH in the range 6.5–9.0, with no proton uptake. Nevertheless, the rate is slowed in D₂O (kᵱ/kD of 1.4–1.9), which suggests that transfer of an internal proton or hydrogen atom is involved in the rate-limiting step of Pₑ formation.

The decay of Pₑ to form F is the first step that shows a pH dependence with lower rates observed at higher pH values with a pKa of 7.9 and kₑ/kD of 1.4 at pH 7.4. This suggests that a proton is taken up during this kinetic phase with a rate constant k₃ of ~1×10⁴ s⁻¹ (time constant τ = 80 µs) resulting in the formation of F. A tryptophan residue (Trp272 for P. denitrificans) has also been suggested by de Vries and colleagues as an electron donor to the binuclear center after the formation of F to form Fₑ with a rate constant of ~8×10⁵ s⁻¹. The final intermediate is formed with the transfer of the second electron from the Cuₐ/heme a site leading to the formation of O₂H (the pulsed form of the HCO discussed above) with k₄ of ~6–9×10⁵ s⁻¹ (τ = 1.2 ms). The formation of O₂H is pH dependent, slower at higher
pH, with a $k_H/k_D$ of 2.5 measured at pH 7.4 and is rate-limited by proton uptake. The transmembrane voltage generated is biphasic with rate constants of ~830 s$^{-1}$ (coincident with electron transfer coupled to proton transfer) and 220 s$^{-1}$ (associated with proton transfer) with relative amplitudes of 1:3. The second electron transfer to the binuclear center, thus, completes the oxidative phase of the enzyme in Figure 181. The $O_H$ intermediate can then be re-reduced to form R with the injection of four electrons from cytochrome $c$ that is coupled to the uptake of four additional protons (two chemical and two pumped protons) as discussed in below.

The overall proton pumping for the oxidative phase of the cycle starting from the fully reduced R state to $O_H$ has been measured by Hallén et al. at pH 7–8. They report that 2.6 protons were released on oxidation of the fully reduced $CcO$. Mitchell found a similar number of 2.4 protons in the pH range of 7.5–8.5, which is in reasonable agreement.

Starting from the MV form of $CcO$ (as well as the three electron reduced form), the fast $O_2$ binding to CuB is again observed with a similar rate constant ($10^8$ M$^{-1}$ s$^{-1}$) as with R (or R$_4$). The $O_2$ then proceeds to bind to heme $a_3$ forming intermediate A. The amplitude of this phase has been shown to decrease with a decrease in the initial degree of reduction of $CcO$ (R$_4$ to R$_3$ to R$_2$/MV).

Intermediate A decays to P$_M$ (in MV-CcO) with $k_2$ ~5×10$^3$ s$^{-1}$ where the O-O bond has been cleaved (Figure 182). The formation of P$_M$ is ~5–6 times slower than the formation of P$_R$ (formed from R$_4$). Intermediate P$_M$ has been the subject of extensive controversy. Only three of the four electrons required to cleave the O-O bond are readily available in the binuclear site. In MV-CcO the source of the fourth electron has been suggested to be the tyrosine crosslinked to the CuB imidazole ligand or a highly conserved tryptophan residue that π-stacks with one of the CuB histidine ligands. Palmer and coworkers have provided arguments against the porphyrin ring of heme $a_3$ being a potential electron donor. With MV-CcO, the reaction stops at P$_M$ that is found to decay with a rate constant of 1.5×10$^2$ s$^{-1}$. No pH dependence is detected in any of the phases with MV-CcO. A kinetic isotope effect ($k_H/k_D$ of 1.4–1.8) is observed during the A→P$_M$ conversion, which is similar to that observed in the formation of P$_R$.

For the fully reduced (R$_4$) and three electron reduced (R$_3$) forms of the enzyme, it is possible that the P$_M$ state also forms but is not observed because it is rapidly converted to P$_R$. The rate constant for the conversion of P$_M$ to P$_R$ is then required to be significantly faster (half-life of 1 ns) than the rate constant for the formation of P$_M$ (half-life of 32 µs).

Single electron reduction of the P$_M$ state (formed from MV-CcO) has been studied by laser-flash excitation of a ruthenium complex (e.g. tris(2,2'-bipyridyl)ruthenium(II)), which is a photo-activatable electron donor. The P$_M$→F transition occurs upon rapid electron transfer from heme $a$ to the binuclear center (presumably to the Y244 radical) with a time constant of 0.3 ms (rate constant of 4000 s$^{-1}$). This electron delivery is coincident with the
generation of a transmembrane voltage, which is triphasic with time constants of 0.3 ms, 1.3 ms, and 7 ms (rate constants 3300 s\(^{-1}\), 770 s\(^{-1}\), and 140 s\(^{-1}\), respectively). The relative amplitudes are 1:1.3.\(^{961}\) Hence, a portion of the voltage generation is coincident with electron transfer, while the rest of the charge transfer across the membrane occurs after the ET step. A charge movement from heme \(a\) to the binuclear site is parallel to the plane of the membrane and will, therefore, not directly result in the generation of a transmembrane voltage. It is proposed that electron transfer is accompanied by the transfer of a proton from residue E242 at the gate site (to be discussed in section 3.7.2.3.) to the active site (a chemical proton, vide infra) along with reprotonation of E242 during the 0.3 ms phase. In the slower 1.3 ms phase, it is proposed that a second proton is transferred from E242 to the proton loading site or translocated to the P-side of the membrane followed by a rapid second protonation of E242.

The \(P_M\rightarrow F\) conversion is significantly slower than the \(P_R\rightarrow F\) conversion in the fully reduced enzyme (0.3 ms for \(P_M\), ~80 \(\mu\)s for \(P_R\)). The \(P_R\rightarrow F\) conversion has been assigned to a monophasic proton uptake.\(^{961}\) The final F state formed is identical in both cases. In the three electron reduced form of the enzyme, the \(P_R\rightarrow F\) conversion is also observed but to a lower extent.\(^{946}\)

A second single electron transfer (by flash photolysis of tris(2,2'-bipyridyl)ruthenium(II)) from heme \(a\) to the binuclear site results in an \(F\rightarrow O_H\) conversion with a similar rate constant as seen for the fully reduced CcO.\(^{961}\) This electron transfer step (1.5 ms \([\sim 7\times10^2 \text{ s}^{-1}\]) is 4–5 fold slower than the electron transfer in the \(P_M\rightarrow F\) transition (0.3 ms). Associated with the \(F\rightarrow O_H\) transition there is an electrogenic proton transfer that occurs in two phases with time constants of 1.2 ms and 4.5 ms. The first protonic phase corresponds roughly to the electron transfer to the binuclear site (1.5 ms). The ratio of the amplitudes of the two phases is 1:1.2, which is close to the value for the \(P_M\rightarrow F\) transition.\(^{961}\)

The second half of the catalytic cycle after the four electron oxidation of CcO by \(O_2\) is the four electron reduction of the fully oxidized enzyme \(O_H\). The \(O_H\), which is the \textit{pulsed} form described above, formed at the end of the oxidative part of the catalytic cycle is metastable and decays to the \textit{resting} form of the oxidized enzyme. Bloch et al estimated the lifetime of the \(O_H\) state to be at least ~30 s at room temperature and at pH 8.0 for \textit{P. denitrificans} to decay to the \textit{fast} form.\(^{962}\) The reduction of the oxidized enzyme in the \(O_H/pulsed\) form is different compared to the non-pulsed, \textit{fast} form. Reduction of \(O_H\), but not of the \textit{resting} form of the enzyme, leads to two successive proton-pumping events (Figure 183).

Proton pumping has been best documented for \(P_M\rightarrow F\) and \(F\rightarrow O_H\) transitions as described above, where a large part of coupled proton transfer occurs after electron transfer is complete. The proton pumping process observed during the \(O_H\rightarrow E_H\) and \(E_H\rightarrow MV\) steps (Figure 183) are less clear because the reduction kinetics depend on the way the oxidized enzyme was prepared. Only the \(O_H/pulsed\) form pumps a proton coupled to electron transfer to the binuclear site.

Brand et al has demonstrated that photoinjection of an electron into the \(O_H\) form of bovine CcO, results in rapid reduction of heme \(a\) (ET from Cu\(_A\) to heme \(a\) in bovine \(O_H\) has a rate
constant of $2 \times 10^4 \text{ s}^{-1}$, followed by biphasic electron transfer from heme $a$ to the binuclear center with rate constants of $750 \text{ s}^{-1}$ and $110 \text{ s}^{-1}$ (time constants of 1.3 ms and 9 ms, respectively), with relative amplitudes of 25% and 75%. The heme $a$ and Cu$_A$ are 63% reoxidized. It is proposed that the first phase of electron transfer is coupled to proton translocation while the second phase of electron transfer is coupled to the uptake of a chemical proton. The midpoint potential of Cu$_B$ is significantly more positive in the O$_H$ state compared to the resting, fast form of the enzyme. The one electron injection results in the electron residing mostly on Cu$_B$. In contrast, when O$_H$ is allowed to decay to the fast, resting form, O (Figure 183), one electron injection leads to form E (Figure 183) by monophasic reoxidation of heme $a$ with a slower rate constant of $90 \text{ s}^{-1}$, with only 30% of heme $a$/Cu$_A$ being reoxidized. In addition, no proton pumping is observed. Thus, there is a significant difference between the kinetics of the pulsed and fast resting forms of the bovine enzyme. It should be noted that in a separate study, Jancura et al observed no difference in the kinetic properties of the pulsed versus the fast oxidized bovine CcO. In addition, they saw a monophasic electron transfer from heme $a$ to the binuclear center with a rate constant of $80 \text{ s}^{-1}$ without the small fast phase at $750 \text{ s}^{-1}$. The reason for the difference is not clear.

When the bacterial CcO’s from *P. denitrificans* and *R. sphaeroides* are used instead of bovine CcO, only a monophasic electron transfer from heme $a$ to the binuclear center is observed during the O$_H$→E$_H$ transition with rate constants of $3000 \text{ s}^{-1}$ and $760 \text{ s}^{-1}$, respectively. The heme $a$ reoxidation was only ~25% in both cases. The non-pulsed, fast resting form of these bacterial enzymes gave similar rate constants as their pulsed counterparts for the O$_H$→E$_H$ transition. Belevich et al found a biphasic reduction of heme $a$ during the O$_H$→E$_H$ transition (rate constants of 6700 s$^{-1}$ and 1250 s$^{-1}$, with equal amplitudes) with bacterial *P. denitrificans*. The reason for the difference is not known. Ruitenberg et al found time constants of ~20 µs (50,000 s$^{-1}$) associated with the reduction of heme $a$ and a single slower phase of 175 µs (6000 s$^{-1}$). The slower phase is deuterium sensitive with $k_H/k_D$ of ~2 that is assigned to the uptake of a proton. They also provide evidence that this proton uptake occurs via the K pathway and not the D pathway (vide infra). A remaining question is whether electron transfer from Cu$_A$ to heme $a$ or the subsequent reduction of the binuclear center leads to proton uptake. The study by Ruitenberg et al on *P. denitrificans* suggests the former. Thus, there is some discrepancy about the exact electron transfer rates during the O$_H$→E$_H$ transition and its coupling to proton uptake. In addition, the difference in rates between the pulsed (O$_H$) and the non-pulsed fast (O) form of CcO is controversial. What is known is that only the pulsed form pumps a proton coupled to electron transfer. Further studies are needed to elucidate the structural basis for the functional difference observed between the two oxidized forms.

The one-electron reduction of state E$_H$ was studied by a stopped-flow flash technique. Single electron injection from a reduced ruthenium dimer results in biphasic electron transfer from heme $a$ to heme $a_3$ to form the MV state (Figure 183) with rate constants of $1100 \text{ s}^{-1}$ and $90 \text{ s}^{-1}$, and relative amplitudes of 11% and 89%. The second electron reduction of the binuclear site is also coupled to the uptake of one chemical proton and one pumped proton. This proton translocation is also not observed with the non-pulsed O...
enzyme (Figure 183). The two electron reduced MV form can then react with O₂ to form PM (Figure 182).

Ruitenberg et al generated EH by chemical treatment. First, F was generated by adding excess H₂O₂ to O. After removing the excess H₂O₂, CO was added to reduce F by two electrons to generate state EH. Injection of a single electron to EH by laser-flash of a bound ruthenium complex, generated a membrane potential that had three phases: τ ~27 µs (40,000 s⁻¹), ~0.2 ms (~5000 s⁻¹), and ~1.5 ms (~700 s⁻¹), with relative amplitudes of 0.26:0.25:0.49. The fast phase corresponds to electron transfer to heme a, the intermediate phase corresponds to the uptake of a charge-compensating proton, while the slow phase has been identified as pumping one proton across the membrane. There is also evidence that the charge-compensating proton is taken up via the K-pathway while the pumped proton is taken up via the D-pathway (discussed in section 3.7.2.3).

3.7.2.4 Thermodynamics: Study of the thermodynamic properties of the different redox sites provides important information about the level of reduction of each site during steady-state turnover (considered below). This is challenging because the redox properties of the four metal sites in CcO interact with each other anticooperatively and do not exhibit the same redox potentials as the isolated sites. The Cyt a and a₃, in particular, are influenced by more than one inter-site interaction. Spectroelectrochemical studies monitoring changes in absorbance, MCD and EPR features with changing potential have been commonly used to estimate the extents of reduction of Cyt c, a and a₃, and Cuₐ. Cuₐ potentials have been more challenging to study because they cannot be monitored optically and thus, these potentials have been obtained indirectly by their effect on the absorption of hemes with changing amounts of charge injected. More recently, ATRFTIR has also been used to study the redox properties of all metal sites.

Dutton and others have shown that the midpoint reduction potential (Eₘ) of Cyt c is 235 mV at pH 7.2 when bound to phospholipid vesicles, inside mitochondria or in submitochondrial particles and increases to 283 mV when isolated and solubilized (Table 31). Thus, Cyt c binding inside beef heart submitochondrial particles was found to lower the Eₘ by as much as 60 mV. Lindsay et al estimated the mean midpoint potential of Cuₐ to be 340 ± 10 mV independent of pH (Table 31). Contrary to this alternatively, Mitchell et al reported that the potential of Cuₐ has a pH dependence approaching ~60 mV/pH. A pH dependence of ~52 mV/pH was observed in the alkaline region with a midpoint redox potential of 366 mV at pH 8.0. Potentiometric titrations of the isolated CcO with Cyt c present by Tiesjema et al showed that the Cuₐ, heme a and a₃ sites titrate as single-electron acceptors with midpoint potentials of 280 mV, 230 mV and 370 mV, respectively, at pH 7.1 (Table 31). Dutton and others have reported that the hemes of CcO in intact mitochondria and in isolated CcO titrate with similar Eₘ values (195–220 mV for heme a and 340–390 mV for a₃ at pH 7.2) (Table 31). Note that these potentiometrically separable Eₘ values of Cyt a and a₃ may be influenced by heme-heme interactions.

Higher levels of heme a reduction were obtained in studies using detergent-solubilized CcOs. Detergents, such as Triton give relatively slow enzyme turnover rates that lead to higher heme a redox potentials and result in a higher steady-state reduction of heme a.
compared to \(a_3\). In such cases, heme \(a\) appears to be approximately in equilibrium with Cyt \(c\) with an \(E_m\) of 305 mV for pulsed CcO.\(^{775}\)

Alternatively, stoichiometric reductive titrations performed in the absence of exogenous ligands under anaerobic conditions revealed that Cyt \(a\) and \(a_3\) are reduced to approximately the same extent at all points in the titration starting with the fully oxidized enzyme.\(^{980}\) Thus both hemes have similar redox potentials when both hemes are oxidized. EPR data of the half-reduced CcO (isolated CcO and submitochondrial particles) show two forms of high-spin ferric hemes with a ~1:1 ratio. These signals appear with a half-reduction potential of about 380 mV in anaerobic redox. This behavior agrees well with the appearance of the high potential component in optical titrations. The data have been interpreted in terms of an equilibrating 1:1 mixture of \(a^3+a_3^2+:a_3^2+:a_3^3+: (K_{eq} = a^3+a_3^2+:a_3^2+:a_3^3+: = 1)\). The reduction of Cu\(_A\) initially lags the reduction of the two hemes but in the final stages of the titration is completely reduced prior to either heme \(a\) or \(a_3\). This indicates that reduction of one of the hemes lowers the redox potential of the second heme making the second heme harder to reduce, hence the buildup of reduced Cu\(_A\).\(^{978,980}\) Further reduction of hemes take place with \(E_m\) of ~220 mV. From these apparent \(E_m\) values of 380 mV and 220 mV and the \(K_{eq}\) of 1, the true \(E_m\) values of heme \(a\) and \(a_3\) are calculated to initially be quite positive and similar (362 mV). Reduction of one of the hemes, decreases the redox of the other by ~124 mV to be 238 mV (Table 31).\(^{978}\)

In addition to heme \(a-a_3\) interaction, heme \(a\) participates in anticooperative thermodynamic interactions which involve the two Cu sites.\(^{968}\) Spectroelectrochemical studies show that the interaction manifested at heme \(a\) is about 110 mV. The interaction between heme \(a\) and Cu\(_A\) is roughly 20–40 mV in CO adducts of the oxidase where heme \(a_3\) and Cu\(_B\) are stabilized in the reduced form.\(^{981}\) Thus, interaction between Cu\(_A\) and heme \(a\), causes the reduction potential for one of these sites to be decreased by approximately 40 mV upon reduction of the other.\(^{981}\) Studies with cyanide inhibited CcO, with heme \(a_3\) stabilized in the oxidized form, show 10–45 mV interaction between heme \(a\) and Cu\(_B\). The remaining interaction manifested at heme \(a\) is from heme \(a_3\). The interaction evident at heme \(a_3\) is about 70 mV and involves the Cu\(_B\) and heme \(a\) sites.

The redox potential of heme \(a\) is only moderately dependent upon pH (less than - 30 mV/pH unit) when all other sites are oxidized.\(^{968}\) A 30 mV decrease with an increase of one pH unit implies that the reduction of heme \(a\) is linked to the uptake, on the average, of only about 0.5 protons at pH 7.0, and significantly less at the higher pH values relevant to the mitochondrial matrix. These values are similar when the enzyme is inhibited with cyanide where heme \(a_3\) is oxidized. When the other redox sites are reduced, the pH dependence is weak (less than ~10 mV/pH unit, on average). This is similar to the value obtained with the CO-adduct where heme \(a_3\) and Cu\(_B\) are reduced. The thermodynamic properties of heme \(a_3\) decreased steeply with increasing pH (about - 56 mV/pH unit), indicating stoichiometric (1 H+/e−) coupling of protonation to reduction.\(^{978}\) The pH dependence of the heme \(a\) and \(a_3\) sites have important implications on the mechanism of proton pumping that will be discussed later.
3.7.2.2.5 Steady-state turnover: In turnover kinetic measurements, a reducing agent such as ascorbate or N,N,N′,N′-tetramethyl-1,4-phenylenediamine (TMPD) is commonly added to the reaction mixture containing reduced Cyt c and oxidized OcO in the presence of O₂ to recycle oxidized Cyt c formed during reaction. The sequence of reduction is described by the reaction sequence:

Reducing agent → Cytochrome c → OcO → O₂

The general mechanism that is used to describe the kinetic data observed is given in Equation 38:

\[
E+S \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES \overset{k_2}{\underset{k_1}{\rightleftharpoons}} O_2 \overset{k_2}{\underset{k_1}{\rightleftharpoons}} EP \overset{k_{-1}}{\underset{k_1}{\rightleftharpoons}} E+P \quad [38]
\]

where, E is OcO, S is Cyt c²⁺, and P is Cyt c³⁺. The dissociation constants \(K_D = k_{-1}/k_1\) for oxidized and reduced Cyt c from the enzyme are found to be roughly equal.

The rate of enzyme turnover \(V_{\text{max}}\) increases with the concentration of both the enzyme and Cyt c. At high enzyme concentrations, the overall activity is limited by the rate of re-reduction of Cyt c. The reaction between Cyt c²⁺ and OcO between pH 6.0–8.0 has an apparent first-order rate constant with respect to Cyt c. The \(K_D\) (enzyme-substrate dissociation constant, Equation 38) calculated for the kinetics of the overall reaction was found to be 30 µM for isolated OcO and 30–40 µM for particulate OcO. Gibson and others found the reduction of OcO by Cyt c to be second-order giving a value for \(k_1\) of \(10^6–7\) M⁻¹s⁻¹ between pH 6.0–8.0. Using this value of \(k_1\), the values of \(k_{-1}\) and \(k_2\) for Equation 38 were found to be 1.2×10³ s⁻¹ and 3×10² s⁻¹, respectively.

Sinjorgo et al studied the steady-state reaction of detergent-solubilized OcO over a broad range of [Cyt c] (1–6 orders of magnitude in excess of [OcO]) at different pH values (pH 5.4–8.6) and ionic strengths \(I = 25–200\) mM. The concave Eadie-Hofstee plots obtained at low ionic strengths \(I < 100\) mM, pH 7.8 at 25 °C, were described by two reactions between Cyt c and OcO with apparent \(K_m\) values of 40 µM (low-affinity reaction) and 40 nM (high-affinity reaction) with maximum velocities \(V_{\text{max}}\) of 230 s⁻¹ and 33 s⁻¹, respectively. At high \(I (> 100\) mM), which is close to the physiological value, the Eadie-Hofstee plots were linear and reflect only one reaction (with \(K_m = 20\) µM and \(V_{\text{max}} = 210\) s⁻¹ at \(I = 200\) mM, pH 7.8). Both the values of \(K_m\) and \(V_{\text{max}}\) with excess Cyt c are sensitive to the enzyme concentration. Maximal turnover numbers along with the apparent \(K_m\) were also affected by pH, with a decrease in both with increasing pH. With increasing ionic strength the apparent \(K_m\) and \(V_{\text{max}}\) values increased implying that the dissociation rate of Cyt c from OcO is enhanced. No optimum \(K_m\) or \(V_{\text{max}}\) is observed. These kinetic data are consistent with the presence of only one catalytic binding site for Cyt c with OcO.

There are four ET steps relevant to the reduction of the O₂ binding site summarized in Equation 39

\[
\text{Cytc → Cu₄ → hemeα → hemeα₃ → Cu₃} \quad [39]
\]
Steady-state turnover experiments on intact mitochondria best capture physiological properties exhibited by the five redox sites in Cyt c and CcO. Andreasson and others have shown that the initial rate of electron transfer from Cyt c to CcO is $\sim 1 \times 10^6$–$7 \times 10^7$ M$^{-1}$s$^{-1}$. The nature of the binding between Cyt c and CcO is unclear and may be ratelimiting. Heme c and CuA are found to be approximately in redox equilibrium in turnover (where ascorbate is employed as the reductant and TMPD as the redox mediator) while heme a remains relatively oxidized. The level of reduction of Cyt c and CuA do not increase linearly with enzyme turnover, whereas the reduction of heme a tracks the rate of enzyme turnover ($k_2 = 3 \times 10^2$ s$^{-1}$, Equation 38). This suggests that the reduction of heme a is the rate-limiting step with a rate constant of about $2 \times 10^4$ s$^{-1}$. The inherent ET from CuA to heme a in single turnover experiments is $2 \times 10^4$ s$^{-1}$. Therefore, the reduction of heme a is possibly rate-limited not by ET but by a structural change or a coupling to proton transfer during turnover.

The heme a ↔ heme $a_3$ redox equilibrium constant is calculated to be roughly 4, with forward and reverse rate constants for electron transfer of $k_F = 2.4 \times 10^5$ s$^{-1}$ and $k_R = 6 \times 10^4$ s$^{-1}$. The equilibrium constant of electron transfer between the two hemes is pH-independent between pH 6.5–9.0. The forward rate constant (thought to be a lower limit of the true ET rate) is much faster than any observed process in the (flow-flash) reoxidation of the fully reduced enzyme by O$_2$. Thus, electron transfer from heme a to the binuclear site cannot be significantly rate-determining during catalysis and heme a is not fully reduced during steady state turnover. This suggests that physiologically the enzyme reacts with O$_2$ without reaching the fully reduced R$_4$ state, has important implications regarding the O$_2$ reduction mechanism and proton pumping.

**3.7.2.3. Structure:** A detailed description of the crystallography of the HCOs is given in this thematic issue by Yoshisawa et al. Here is presented a structural overview that enables correlation to electronic structure and mechanism in the next sections. To date X-ray crystallographic structures of HCO’s from six species have been determined (Table 32). The crystal structures along with amino acid sequence alignment studies show that there is close structural similarity among A and B family CcO’s from different organisms (mammalian versus bacterial) and between CcO and QO from these families. The eukaryotic enzyme is a dimer in its active form and is composed of seven (in Dictyostelium discoideum) to 13 (in mammals) subunits. The prokaryotic enzyme contains fewer subunits (generally 4 subunits). Subunits I-III (referred to as the core subunits) are present in most A and B type HCO’s. A CcO has been shown to be fully active with respect to O$_2$ reduction to H$_2$O and proton pumping with only subunits I and II, suggesting that these form the essential catalytic core. The type C HCO’s, comprising $\sim 20\%$ of all HCO’s, have very different amino acid sequence from types A and B. These contain two core subunits, N and O. In this section, we will present an overview of the structure of bovine heart CcO that has been most widely studied and where mutagenic studies have aided in elucidating electron and proton transfer pathways and compare and contrast this to the other classes of HCO’s.

The overall structure of bovine heart CcO consists of 13 subunits (Figure 184A). There are four metal binding sites in bovine CcO. The binuclear center, heme $a_3$ and CuB, and the third metal center, heme a, are in subunit I (yellow), whereas the fourth metal domain...
containing CuA is positioned in subunit II (green). The four metal sites and their ligation are shown in Figure 184B. CuB is ligated to three histidines (His240, His291 and His290). One of the histidines (His240) is covalently crosslinked at the epsilon nitrogen to a tyrosine residue (Tyr244). Heme a3 has an axial histidine ligand (His376). The second heme a site, where the Fe is low-spin, has two axial histidines (His61 and His378). Both the hemes are non-covalently bound to the protein. A similar binuclear center and a second low-spin Fe heme site are present in all HCO’s consisting of all the same coordinating ligands to the redox centers. The cytochrome c (substrate) docking site is located on the surface of subunit II relatively close to the CuA site. Each of the Cu sites in CuA is bound to one His and two Cys residues. In addition, one Cu is coordinated to one Met whereas the second Cu is ligated to the backbone carbonyl oxygen of a Glu residue. The overall CuA site is approximately symmetric with respect to its two His and the two Cys ligands. The two thiolate groups act as bridging ligands between the copper atoms. In place of CuA, cbb3 oxidase from P. stutzeri contains three heme c molecules each with two axial ligands—one His and one Met.

The two hemes in bovine CcO are buried within the enzyme approximately 15 Å from the periplasmic surface. The hydroxyethylfarnesyl group of the high-spin heme is positioned into the lipid bilayer such that protons can enter the binuclear site from the cytoplasmic side of the membrane. The hydroxyethylfarnesyl side chain of the low-spin heme points into a hydrophobic region that blocks access to the heme from the cytoplasm. In QO that contains a low-spin heme b, the space occupied by the hydroxyethylfarnesyl tail of heme a in CcO is filled with bulky hydrophobic residues.

3.7.2.3.1 Enzyme-substrate interactions: The HCO superfamily use two types of electron donating substrates—CcO uses a water soluble protein, cytochrome c, whereas QO use a membrane soluble ubiquinol as its electron donor. The binding of the substrate to HCO is thought to be due to the electrostatic interactions between the substrate and enzyme. There is still a lack of microscopic detail concerning these interactions and little is known about the orientation adopted by the substrate when it docks at the enzyme binding site and the number and identity of the intermolecular interactions formed.

The substrate docking site in bovine CcO is next to subunit II on top of subunit I (Figure 185, right). The acidic amino acid residues, Glu109, Asp119, Glu127, Asp139, Glu157, and Asp158 of subunit II (shown in green), in this region could interact with the basic cytochrome c, to stabilize the enzyme-substrate complex. Among these amino acids Glu109 and Asp139 are well conserved even in bacterial enzymes, suggesting a strong role in binding of cytochrome c. Site-directed mutagenesis of CcO isolated from Paracoccus denitrificans confirmed that a patch of negatively charged residues on subunit II are indeed critical for the catalytic activity of the enzyme. These studies detected two carboxylic residues (Glu246 and Asp206) corresponding to Glu198 and Asp158 respectively of subunit II of bovine CcO that are of particular significance. With regard to the substrate, cytochrome c, little is known about the identity of their electrostatic “partners” with the exception of Lys13.
In contrast to the cytochrome c oxidases, subunit II of QO does not contain a substrate binding site. Instead, the membrane-spanning region of subunit I (Figure 185 left, yellow) contains a cluster of polar residues. Although a high degree of homology is observed between subunit I of CcO and QO, the sequence in this polar cluster is completely different. Mutation studies show Arg71 and Asp75 in the C-terminal half of helix I, and His98 and Gln101 in the N-terminal half of helix II (green), exposed to the interior of the lipid bilayer that is not present in the cytochrome c oxidase, form the quinone binding site.\textsuperscript{991} The presence of a second, lower affinity site in QO remains uncertain.

### 3.7.2.3.2 Active site:

The crystal structure of CcO from bovine heart\textsuperscript{997} was determined in 1995 to a resolution of 2.8 Å and was later improved to 1.8 Å\textsuperscript{998}. Yoshikawa et al also determined the structures of the bovine enzyme at different reduction states and with different ligands bound to the binuclear center.\textsuperscript{999} The binuclear center of bovine CcO, shown in Figure 177B, is located in subunit I and is 13 Å and 30 Å from the P- and N-sides, respectively. The epsilon nitrogen of His240 is covalently bonded to a tyrosine residue (Tyr244) at the ortho carbon of the ring as shown in Figure 177B. The Cu\textsubscript{A} site is naturally present in CcO (except for in cbb\textsubscript{3} from P. stutzeri) but not in QO (bo\textsubscript{3} from E. coli). The Cu\textsubscript{A}-heme \textit{a} and Cu\textsubscript{A}-heme \textit{a}\textsubscript{3} distances are 20.6 and 23.2 Å, respectively, when measured from the central point of the two copper atoms of the Cu\textsubscript{A} to the iron atoms.\textsuperscript{997}

### 3.7.2.3.3 Resting oxidized and reduced CcO:

Crystal structures of resting oxidized CcO show a continuous electron density between the heme iron and Cu\textsubscript{B} (Figure 186). There has been some controversy about the identity of this bridging species. Recent X-ray data have enabled interpretation of this density as a peroxide dianion (O\textsubscript{2}\textsuperscript{2−}) bridge between the two metals with a long O–O distance (1.7 Å).\textsuperscript{1000,1001} The Cu\textsubscript{B}-O\textsubscript{1} distance is 2.17 Å whereas the Heme \textit{a}\textsubscript{3} Fe-O\textsubscript{2} distance is 2.24 Å.\textsuperscript{1000} Table 33 lists crystallographic parameters of all the oxidized and reduced bovine CcO characterized to date. The Cu···Fe distance in the oxidized enzyme is between 4.34–5.02 Å whereas in the reduced enzyme the distance increases to between 5.00–5.19 Å. No electron density is observed between the binuclear center in the reduced enzyme. Another notable change that occurs on reduction is a 4.5 Å movement of the carboxylic group of Asp 51 along with the segment from Gly49-Asn55 towards the cytosolic surface in bovine CcO.\textsuperscript{1002} Apart from these, no other significant changes are observed. In the case of CcO from R. sphaeroides, there is an additional redox change that is not apparent in bovine CcO. The O-O distance between the Y288-OH and the OH group of the heme \textit{a} hydroxyl farnesyl tail decreases from 4.05 Å in the reduced state to 2.63 Å in the oxidized state (Figure 187. dotted [oxidized], dashed [reduced]).\textsuperscript{1003} The implications of this redox change to proton pumping will be discussed below.

X-ray crystallographic structures of several ligands bound to the binuclear active site have been solved.\textsuperscript{999,1000,1002} The main structural parameters are summarized in Table 34. These structures shed light into the possible motifs of O\textsubscript{2} binding. Analyses of the fully reduced CO- and NO-bound CcO (with CO and NO bound to Fe\textsubscript{a,3}) reveal a close to linear coordination structure for CO (Fe-C-O angle is 164 degree) and a bent, end-on coordination structure for NO (Fe-N-O angle is 131 degree; Figure 188 A and C).\textsuperscript{999} After being photo-dissociated from Fe\textsubscript{a,3}, the X-ray structure of the CO derivative determined at 100 K
indicates that CO is now bound to CuB in a side-on fashion (Figure 188 B). The Cu-C and Cu-O distances are 2.4 and 2.7 Å indicative of a weak CuB-CO bond. The 3.0 Å distance between CO and Fea3 suggests that no significant bonding interaction exists. The most dramatic change occurs upon binding cyanide to the binuclear center. The crystal structure of the fully reduced CN-bound form indicates that CN− is located roughly equidistant (2.3–2.4 Å) from Fea3 and CuB (Figure 188 D). Upon binding CN−, His290 is displaced and moves to a distance of 2.8 Å away from CuB.

3.7.2.3.4 Electron transfer pathways: The crystal structures enable identification of possible electron transfer pathways between the metal active sites inCcO. The location of CuA is consistent with its role as the initial electron acceptor from cytochrome c. In aa3-type CcO’s from Paracoccus denitrificans, bovine heart, yeast, and Rhodobacter sphaeroides it is now generally accepted that electrons are transferred from cytochrome c to CuA and then from CuA to the low-spin heme a. The electron transfer pathway between cytochrome c and CuO is less well understood due to the absence of any enzyme-substrate crystal structure. The crystal structure of bovine heart CcO revealed two efficient, conserved possible electron transfer pathway between CuA and heme a.989 Pathway 1 through His204 via Arg438 to heme a, shown in Figure 189 blue, consists of 14 covalent and 2 hydrogen bonds (one short H bond at 1.87 Å and one longer H bond at 2.35 Å).1009 Pathway 2 (Cys200 to heme a), shown in Figure 189 green, involves a total of 17 covalent bonds and travels from Cys200 to Ile199, through a short 1.83 Å H-bond to Arg439, then through a 1.92 Å space jump to the propionate chain and into the heme. The covalent Cu-S bond activates this path making it ~20 fold more efficient and competitive with pathway 1.1009 A third pathway (pathway 3, Figure 189 red) through Cys196 via Tyr440 and Arg439 has also been explored.1009 This path involves 24 covalent bonds and 2 hydrogen bonds and has a calculated rate 270 times less efficient than that of pathway 1, indicating that this pathway is not a major contribution in the CuA to heme a ET path.

The electron is subsequently transferred from heme a to heme a3. The proximity of heme a to heme a3, shown in the crystal structures of bovine heart and bacterial enzymes, suggests that heme a serves as an effective electron donor to heme a3. The two hemes are nearly perpendicular to one another, and while the Fe-Fe distance is ~13.4 Å, the edges of the hemes approach each other to ~4.5 Å (dashed line in Figure 190) with 14 covalent bonds between the two Fe’s. A fully covalent path also exists composed of 16 bonds between the two Fe’s through His378, Phe377 and His376. In addition, the phenyl plane of Phe377 observed half way between the two planes of the heme a3 and one of the imidazole ligands of heme a (His378) has been proposed as an electron transfer pathway (Figure 190). The distances between the phenyl plane and the two heme planes on both sides are as short as 3.5 Å. Any change in the orientation and position of the phenyl group, induced by changes in the oxidation state of hemes, could affect the electron transfer between Fe a and Fe a3.

In the case of QO, the CuA site is not present and electrons are directly transferred from ubiquinol to low-spin heme b and subsequently to the heme-CuB binuclear center. The fast electron transfer from ubiquinol to heme b can be achieved through the conserved hydrophobic residues Phe103 and Met79 that are situated between the two moieties and within van der Waals contact distance of heme b (Figure 191).991
3.7.2.3.5 Proton transfer pathways: Electron transfer and O$_2$ reduction are coupled to proton pumping. The coupling mechanism will be considered in section 3.7.2.5.2. Here the proton transfer pathways are summarized. HCO’s pump protons from the cytoplasm or the inner matrix of mitochondrial membrane (negative N-side) to the periplasm or mitochondrial intermembrane space (positive P-side). The unidirectionality of proton transfer is thought to be achieved by a gating mechanism that prevents backflow of protons to the cytoplasm (Section 3.7.2.5.2).$^{1010,1011}$

The protons to be translocated (pump protons) as well as those used for the chemical reaction (chemical protons) are transported, to some extent, through common channels. Protons are transferred most effectively through H-bonded chains of water and amino acid residue systems via Grotthuss shuttling.$^{1012}$ The water molecules are present inside cavities within the protein matrix. These cavities provide space for conformational changes of side chains that can induce a new H-bond system. Any conformation change induced by changes in redox or ligand binding states could control proton transfer. Thus a conformationally controlled network composed of hydrogen bonds could function as a proton pump coupled with dioxygen reduction.

X-ray structures, along with site-directed mutagenesis and sequence alignments have revealed two candidates for proton uptake, the D and K channels, for the mitochondria and mitochondria-like enzymes (Figure 192). Table 35 lists the important residues along the D and K proton pumping pathways for bovine CcO and will be summarized below. A third pathway has been identified in several HCO’s (H channel in bovine and $P$. denitrificans, E channel in $P$. denitrificans, and Q channel in $T$. thermophilus) but these are less well understood. Additionally, the proton exit pathway for pump protons en route to the outer periplasmic surface (P-side) remains enigmatic. This channel is thought to start from the site that accepts protons from the gate site. The likely candidates for the acceptor site are a cluster of heme propionates, two arginines, and associated waters.

The D-pathway is thought to be used for the transfer of all four pumped protons as well as the uptake of two chemical protons during reaction of the fully reduced CcO with O$_2$.$^{1013,1014}$ This pathway starts at residue Asp91 and ends at Glu242 in bovine CcO. Mutation of Asp91 to Asn has been shown to abolish enzyme activity suggesting that the D channel is involved in the uptake of chemical protons (Figure 192 left).$^{1007,1015}$ Mutation of the E286 residue to Q in $R$. sphaeroides CcO, which is the equivalent of E242 in bovine CcO (at the end of the D pathway), results in almost total loss of activity and inhibition of proton transfer during oxygen reduction (reaction starting with O$_2$ and the fully reduced enzyme stops at the $P_R$ intermediate).$^{992}$ Chemical protons can flow from E242 to Cu$_B$, at a distance of ~10–12 Å, through a short series of waters that can be modeled into a hydrophobic cavity between E242 and the active site. E242 has been invoked to be a key residue and also function as the gate site that minimizes leakage of protons to the cytoplasm.$^{1016}$

The K-channel in bovine CcO spanning from Lys265 at the entrance to the cytoplasmic N-side, via Tyr244 ends at His240, a ligand to Cu$_B$, and therefore is thought to be a channel for chemical protons (Figure 192 right). The K-pathway is not used during oxidation of the
reduced enzyme with O₂. During the reduction of CcO (O→R), one to two chemical protons are taken up through the K-pathway. The strong hydrogen bonding between Y244-OH (corresponds to Y288 in R. sphaeroides) and heme a₃ farnesyl-OH (Figure 187) is proposed to function as a closed K path gate, since its presence prohibits proton transport via this path to the active site. The K-channel is considered to involve residues K319 and T316 of subunit I in bovine CcO (corresponds to K362 and T359, respectively, in R. sphaeroides, Figure 187) (Table 35). This is because the mutation of K362 and T359 in R. sphaeroides results in a major loss of activity (up to 99.95% for K362M). Despite important details that are now known, the mechanism by which ET is coupled to proton transfer at the molecular level remains unclear.

The H-pathway proposed in bovine CcO, defined first by crystallographically, conducts only pumped protons and extends from the N-side of the membrane surface to the P-side via a water channel and a H-bond network (Figure 193). The pathway starts with a water channel that allows access from the N-side. The pathway then extends to the formyl group of the low spin heme a, which is hydrogen bonded to R38. The hydrogen bond network from R38 extends to D51, which is located near the intermembrane surface of CcO, via the peptide bond between Y440 and S441. Blockage of either the water channel by a double mutation (V386L and M390W) or proton transfer through the peptide by a S441P and D51N mutation was found to abolish the proton pumping activity but not the O₂ reduction activity. Mutation of D51 was also found to result in loss of pumping ability. Note that the amino acid residues of the hydrogen bond network and the structures of the low spin heme peripheral groups are not completely conserved amongst members of the HCO superfamily.

**3.7.2.4. Electronic Structure:** Cytochrome oxidase (cytochrome aa₃) contains three metal sites: 1) a binuclear copper electron transfer site (Cu_A, gₓ = 2.18, gᵧ = 2.02, and gᵦ = 1.99 in the oxidized state), 2) a bis-His ligated low-spin heme (heme a, gₓ = 3.0, gᵧ = 2.2 and gᵦ = 1.5 in the oxidized state), and 3) the hetero-binuclear heme-copper active site (heme a₃ – CuB) in which the heme, with its open coordination site, is high-spin. As previously described, QO (cytochrome bo₃) lacks the Cu_A site, transferring electrons from ubiquinol to the ET heme directly. The reaction of dioxygen with heme-copper oxidases is extremely fast, and thus definition of the early sequence of transient intermediates during the catalytic cycle comes from spectroscopic measurements on microsecond freeze-hyperquench prepared samples or from in situ time-resolved spectroscopic measurements based on the flash-flow method developed by Greenwood and Gibson. The kinetic scheme developed in Section 3.7.2.2 is R → A → P → F → O_H → E_H → R, of which only a single state, fully (four electron) reduced state R⁴, has been crystallographically defined (Resting CcO has also been crystallographically characterized but is not on the catalytic pathway).

**3.7.2.4.1 Compound R:** The UV/Vis spectrum of fully reduced CcO is dominated by the heme spectral features at 443 and 605nm. Reduced CcO is EPR silent. XAS has been performed on Compound R in both the cytochrome oxidases and the QOs (cytochrome bo₃, and aa₃-600) as a probe of the copper coordination environment and oxidation state. As the QOs contain a single copper ion, spectral interpretation is simpler. In the Cu K-edge XAS spectrum, a pre-edge feature was observed at 8983 eV, and was assigned as the
diagnostic $1s \rightarrow 4p$ transition, consistent with a Cu(I) oxidation state (Figure 194). The intensity of the Cu first shell scatterer in the EXAFS of Compound R is markedly decreased from that in the resting (oxidized) form of the enzyme, an observation that has been interpreted as significant weakening (or loss) of one of the Cu histidine ligands upon reduction. However, this observation has not been made in the crystallography of reduced forms of the enzyme (see Section 3.7.2.3) and thus possibly reflects some sample heterogeneity, with substoichiometric amounts of bound Cl$^-$ or CO having been observed in the A. ambivalens aa$_3$ QO.$^{1030}$

3.7.2.4.2 Compound A: The primary intermediate observed upon exposure of reduced enzyme to dioxygen is compound A. Given its short lifetime, information on this state has come from time-resolved UV/Vis and resonance Raman measurements. In the UV/Vis difference spectrum, the Soret of compound A is shifted to ~422 nm from that observed in the fully reduced enzyme (443 nm).$^{948,1031-1033}$ Time-resolved resonance Raman interrogation of this optical change results in enhancement of a feature at 568 cm$^{-1}$, which downshifts to 547 cm$^{-1}$ in the $^{18}$O$_2$ isotopologue (Figure 195).$^{1034-1038}$ Based on comparison of the energy and isotopic shift of this feature to those observed in oxymyoglobin and oxyhemoglobin, the feature was assigned as a \(\nu_{Fe-O}\) stretching mode of an analogous Fe-O$_2$ intermediate.

3.7.2.4.3 Compound P: Rapid decay of compound A yields compound P. While initially compound P was thought to have an intact O—O bond (P stood for peroxy), it is now known that the O—O bond has already broken in this state. This observation was first reported by Weng and Baker based on the comparison of UV/Vis spectral features in P to cytochrome c peroxidase compounds I and II,$^{1039}$ and subsequently by room temperature MCD studies of Thomson and coworkers.$^{1040,1041}$ The absorption spectrum of P (in cytochrome aa$_3$) is dominated by a Soret band at 428 nm, and a 607 nm feature in the difference spectrum with resting. Resonance Raman spectral interrogation of P (in cytochrome aa$_3$) produced either during catalysis (using time-resolved methodology), or artificially (by reaction with partially reduced MV-CO with dioxygen or fast oxidized with H$_2$O$_2$) results in observation of an isotope sensitive feature at 803 cm$^{-1}$ (769 cm$^{-1}$ in the $^{18}$O isotopologue) consistent with assignment as a ferryl-heme \(\nu_{Fe-O}\) (Figure 196).$^{957,1042,1043}$ Unequivocal demonstration that the O—O bond was indeed broken in Compound P comes from reaction of fast oxidized with H$_2^{16/18}$O$_2$, in which no other oxygen isotope sensitive bands were observed.$^{1044}$

Despite the similarities in the UV/Vis absorption and resonance Raman spectra of Compound P generated by different methods (differentiated as P$_R$ from the fully reduced enzyme plus dioxygen, P$_M$ from the mixed-valent enzyme plus dioxygen, and P$_H$ from the oxidized enzyme plus hydrogen peroxide), it is noteworthy that these states are in fact different, as they are generated from reactants with different redox states, and thus have different numbers of electrons available. Specifically, in the generation of P$_R$, four electrons are available within the metal sites (1 in heme $a$, 2 in heme $a_3$, 1 in Cu$_B$) to cleave the O—O bond. In contrast, the generation of P$_M$ (and P$_H$) only has three electrons available and thus is deficient by one electron (P$_M$: 0 in heme $a$, 2 in heme $a_3$, 1 in Cu$_B$; P$_H$: 0 in heme $a$, 1 in heme $a_3$, 0 in Cu$_B$, 2 in H$_2$O$_2$). Thus, P$_M$ (and P$_H$) must obtain a fourth electron from a non-
metal site of the protein in order to cleave the O—O bond. Careful analysis of the UV/Vis spectral features of P_R and P_M by principle component analysis (specifically by singular value decomposition) has revealed slight spectral differences. One method that could potentially resolve the issue of the fourth electron is EPR. Indeed, a variety of tryptophan and tyrosine radicals have been observed in PH. However, the current consensus is that these radicals are related to generation of P with hydrogen peroxide, and that the on-pathway P_M heme $a_3$-$Cu_B$ site is EPR silent due to magnetic coupling of the tyrosine radical with the copper ion, implying the His-Tyr crosslink functioning as a superexchange pathway for the antiferromagnetic interaction. A magnetically coupled Y•-Cu(II) state in P_M is supported by the study of a model complex bearing a phenol functionalized imidazole ligand, [Cu^{II}(BIAIP)], that was demonstrated to be EPR silent; however, this proposal has been challenged by an alternate model study with a similar ligand architecture wherein the Cu(II) and phenoxy radical were uncoupled. We note that in our own computational studies, the coupling of a Cu(II) and the radical phenolate through an imidazole is dependent on the phenolate-imidazole dihedral angle, varying from anti- to ferromagnetic as the ring (and thus the magnetic orbital of the ligand) is oriented in and out of plane, an observation that perhaps could explain some of the empirical differences. While the P_M state is EPR silent, the P_R state has a characteristic Cu EPR signal, which definitively indicates the Cu is in the +2 oxidation state.

Chemical evidence for the Tyr as the origin of the fourth electron, and thus being a neutral radical in state P_M, comes from a radioactive iodide experiment followed by degradative mass spectroscopic analysis of Babcock and coworkers. In this experiment, reaction of P_M with iodide led to the observation of labeled Tyr-His in state P_M (albeit in 3 % yield) – the reaction of iodide with other states (F and O) did not lead to labeled tyrosine. The assertion of a Tyr244• radical in P_M is further supported by FT-IR data of Wikström and coworkers, in which the difference spectrum of P_M with O shows a band at 1519 cm$^{-1}$, consistent with a neutral tyrosine radical (Figure 197). P_M further lacks a band at 1311 cm$^{-1}$, observed in P_R, and attributed to a tyrosinate anion. The lack of the 1311 cm$^{-1}$ band in P_M also indirectly supports a neutral tyrosine in P_M and directly suggests a deprotonated tyrosinate in P_R.

3.7.2.4.4 Compound F: The rate of decay of Compound P is associated with isotope effects (Section 3.7.2.2) and thus a proton translocation is involved in the P → F conversion. The UV/Vis absorption spectrum of Compound F is quite different from that observed for Compound P, yet remains dominated by the heme spectral features with a Soret band at 428 nm and Q band features at 580 nm and 535 nm in the difference spectrum (cytochrome aa$_3$). The resonance Raman spectrum of Compound F displays two oxygen isotope sensitive features at 786 cm$^{-1}$ and 355 cm$^{-1}$, which downshift to 749 cm$^{-1}$ and 339 cm$^{-1}$ in the $^{18}$O isotopologue. These features have been assigned as the vFe—O and $\delta$N—Fe—O of an Fe(IV)=O (Figure 198). FT-IR of Compound F shows a feature at 1308 cm$^{-1}$, which has been ascribed to a tyrosinate anion (analogous to the tyrosinate in P_R) which suggests that the proton that is taken up (see Section 3.7.2.2) generates a copper-bound aquo ligand.
3.7.2.4.5 Compound $O_H$: The absorption spectrum of $O_H$ is dominated by heme features, and has a Soret at 424 nm, consistent with an oxidized ferric-heme cofactor. The resonance Raman spectrum of Compound $O_H$ is associated with an isotope sensitive feature at 450 cm$^{-1}$ (under single turnover conditions in cytochrome aa$\textsubscript{3}$), which downshifts to 425 cm$^{-1}$ in the $^{18}$O isotopologue. This feature is sensitive to deuterium substitution and thus $O_H$ has been assigned as having a hydroxide bound to the heme a$\textsubscript{3}$.

3.7.2.5. Molecular Mechanism: Heme-copper oxidases catalyze the oxidation of cyt c using dioxygen as the terminal electron acceptor. This process is thermodynamically favorable, and some of the excess free energy gained from this redox process is used to pump protons. It has been shown in specific mutated CcO variants that the proton pumping function can be eliminated without loss of the oxygen reduction activity. Thus, the elementary kinetic steps of the mechanism of oxygen reduction can be studied decoupled from those of pumping.

3.7.2.5.1 Reductive cleavage of $O_2$: The general landscape for $O_2$ reduction that has been outlined in Section 3.7.2.2 comes from time resolved single turnover studies, which yield the following simplified sequence of intermediates R → A → P → F → $O_H$. The identities of these intermediates have been explored spectroscopically in Section 3.7.2.4 and have been assigned as Y-Cu(I)-Fe(II) → Y-Cu(I)-Fe(III)-$O_2$•− → Y•-Cu(II)OH-Fe(IV)=O → Y•-Cu(II)OH$\textsubscript{2}$-Fe(IV)=O → Y•-Cu(II)OH$\textsubscript{2}$-Fe(III)-OH (Figure 199). Thus, the O—O bond is broken in the A → P step concomitant with the proposed net $H•$ abstraction from the crosslinked tyrosine. In CcO, this A → P transformation is generally considered from the O—O bond cleavage perspective. It is also interesting to consider the A → P step from the net $H•$ perspective to draw insight into the factors required for O—O bond cleavage and parallels to other heme oxidases (i.e. cytochrome P450). Both CcO and cytochrome P450 react with dioxygen to form an early superoxo complex, which is followed in a subsequent step by a high-valent Fe(IV)=O with a ligand centered radical (P$\textsubscript{M}$ in CcO and compound I in cytochrome P450). A major difference between these parallel reactions is that in cytochrome P450, an additional intermediate has been observed — a ferric-hydroperoxo called compound 0 — while no additional intermediate has been observed in CcO. This observation stimulates the question as to whether the ferric-superoxo in compound A of CcO is the oxidant or whether an unobserved intermediate peroxo functions as oxidant. The question can only be answered experimentally at present by model studies since no intermediate peroxo has been observed in CcO. While an emerging body of evidence suggests ferric-superoxo complexes may be competent for net $H•$ reactions, it is noteworthy that only one ferric-heme-superoxo complex has been reported to be competent for reaction with phenols (Figure 200). In this work of Collman and coworkers employing a picket fence porphyrin derivative with a copper binding site, the authors demonstrated both inter- and intra-molecular reactivity with phenols to yield high-valent Fe(IV)=O adducts and phenoxy radicals. The reaction requires copper to proceed, highlighting its parallels to the CcO active site.

Alternatively, a system has recently been reported by Karlin and coworkers wherein a ferric-heme-peroxo-copper complex is competent for reaction with phenols to yield high-valent...
Fe(IV)=O and phenoxy radicals (Figure 201). A common structural motif between the functional Collman and Karlin systems is the presence of an axial imidazole ligand, analogous to the axial His present in heme a₃ of CcO. The axial ligand is essential because in the Karlin system, the spin of the heme is modulated by the presence or absence of the axial ligand, and only the low-spin peroxo bridged Cu(II)-O₂⁻⁻Fe(III) complex with the axial ligand bound is reactive, perhaps suggesting spin as a factor in catalysis. The effect of spin on O—O bond rupture has been assessed in studies on non-heme ferric-hydroperoxo complexes, and the higher reactivity of the LS versus the HS complex was attributed to kinetic control; during O—O bond rupture the reaction on the low-spin surface proceeds with a lower barrier as a result of good overlap of the redox active orbitals. It should be noted that while the heme of CcO is ligated by an axial His residue suggestive of a low-spin oxidant, it has been proposed that in the evolutionarily related NO reductases (see Section 3.7.2.1), the axial His dissociates from the heme during catalysis. This observation has not been addressed in CcO. Nonetheless, the issue of active oxidant has been explored theoretically. Calculations that reproduce both the barrier and driving force of O—O bond cleavage invoke intermediacy of a bridging peroxy level adduct (Iₚ). Further calculations extended from a magnetostructural correlation observed in the low-spin functional heme-peroxo-copper model of Karlin and coworkers suggest that a bridging peroxide allows for O—O bond cleavage without spin surface crossing (Figure 202). To break the O—O bond of a putative peroxide intermediate in CcO, a pair of electrons must be transferred into the σ* orbital of the peroxide. If the consensus mechanism in Figure 199 is correct, these electrons originate from the Fe and the tyrosine. The electron from the Fe must be spin down, to generate the S=1 Fe(IV)=O, and therefore the electron that comes from the tyrosine must be spin-up, thus the residual unpaired electron on the Tyr• is spin down. If the Tyr• and Cu(II) in Pₘ are antiferromagnetically coupled (Section 3.7.2.4), the unpaired spin on the Cu(II) must then be spin up. Since the electrons on the copper would not be involved in a Iₚ → Pₘ transformation, the electron on the Cu(II) would start spin up, implying that the putative Iₚ in CcO consists of a ferromagnetically coupled Fe—O—O—Cu core if the O—O bond is to be broken without a spin surface crossing. The origin of this expected ferromagnetic coupling comes from a magnetostructural correlation developed in the low-spin heme-peroxo-copper model complex. Specifically, if the protein enforces a Cu•••Fe distance of greater than 4.6 Å (Section 3.7.2.3), this would require that a putative intermediate Fe-O-O-Cu peroxo intermediate adopt a dihedral angle > ~150°. This large dihedral would result in a ferromagnetic (S = 1) ground state as a result of orthogonal magnetic orbitals leading to the lack of a superexchange pathway between the magnetic orbitals of the low-spin ferric heme and the Cu(II), in contrast to an unconstrained model complex, in which a more acute Fe-O-O-Cu is accessible and results in an antiferromagnetic (S = 0) ground state. A key question that remains is how large an effect this spin topology plays during catalysis. This is likely dependent on the magnitude of the coupling between the Cu(II) and Y•, a value which has not been measured at present.

While the balance of experimental and theoretical observations suggest an unobserved bridged peroxo as the key intermediate, it is paradoxical that the resting “as-isolated” form of CcO has been crystallographically defined as containing a peroxide group spanning the heme and copper, as sufficient electrons are available to allow the O—O bond...
in this state to spontaneously cleave. This issue has not been resolved, but we note that in resting CcO, the heme \( a_3 \) is high-spin \((S = 5/2)\) based on 1) the \(\lambda_{\text{max}}\) of the heme Soret band in the absorption spectrum of resting CcO that appears at 424 nm, indicative of a high-spin electronic structure,\(^{1077,1078}\) 2) SQUID magnetization studies that have demonstrated that the heme \(a_3-Cu_B\) pair is antiferromagnetically coupled to yield an overall \(S = 2\) spin system,\(^{1079,1080}\) and 3) EPR and MCD on resting ubiquinol oxidase that further indicates a small, anisotropic superexchange parameter \((J \approx 1\text{ cm}^{-1})\) and a large positive zero-field splitting \((D = 5\text{ cm}^{-1})\) for heme \(a_3\).\(^{1081}\) These spectroscopic observations are contradictory to those for the heme-peroxo-copper model complex, wherein the analogous end-on bridging peroxide causes the heme to be LS.\(^{1069}\) A HS peroxide in resting CcO would suggest a weak interaction of the peroxide with the heme which would necessitate poor overlap of the redox active orbitals needed to cleave the O—O bond. Alternatively, given the observation of a spin correlation to reactivity in the heme-peroxo-copper model complex, perhaps the spin state is the reason that the peroxo in resting CcO does not cleave. These issues of spin and active oxidant thus remain unresolved in understanding the O—O bond rupture process in CcO.

Given the lack of certainty regarding the active oxidant, it is difficult to ascertain the order in which protons and electrons enter the A \(\rightarrow\) P transformation. Nonetheless, based on the evidence for a neutral Tyr• in \(P_M\),\(^{1057,1082}\) it is likely that the electron comes from the fully conserved Tyr, which makes the active site Tyr-His crosslink of central interest. Mutated CcO variants in which the Tyr244 was eliminated failed to bind copper in an appreciable amount, and thus the crosslink was believed to perform a structural role.\(^{1083}\) However an alternative role of the crosslink could be as an electron transfer pathway to get the fourth electron to the oxygen derived ligand to facilitate cleavage.\(^{1075}\)

The crosslink functioning as an electron transfer pathway hypothesis only finds utility in the case of a bridging intermediate peroxo. In the case where the Fe(III)-superoxo is the active oxidant, it is more likely that to break the O—O bond the three electrons are taken from the heme, followed by electron backfilling from the crosslinked Tyr through the heme farnesyl substituent-crosslinked tyrosine hydrogen bond.\(^{1074}\) In either case, the factor that would determine the origin of the electron is the redox couple of the Tyr versus the redox couple of the heme, for which the protonation state of the tyrosine would be of central importance (i.e. a deprotonated tyrosine would have a more favorable redox couple for oxidation). Based on FT-IR, Compound P contains a deprotonated Tyr, which supports direct electron transfer from the Tyr. As the Tyr hydroxyl substituent would be greater than 5Å from a dioxygen bound to heme \(a_3\), an active site water relay may be needed as a means to transmit the proton. The issue is particularly relevant because it signifies the entry of protons into the O—O cleavage, and thus of the coupling of O—O cleavage to proton pumping.

3.7.2.5.2 Proton Pumping: A detailed description of the proton pumping mechanism is presented by Wickstrom et al. in this thematic issue. Here, we present a brief overview for correlation to the mechanism of dioxygen reduction. In general, proton pumping by CcO is driven by the alternating redox states of the cofactors coupled to protein motion.\(^{1084}\) The movement of electrons from site to site provides an electrostatic driving force for translocation of protons in order to compensate the charge of the electrons.\(^{1085}\) Thus, proton
movement is strongly coupled to electron transfer. In order to move the large distances through the protein, the protons shuttle rapidly through hydrogen bonded chains of water and amino acid residues, called Grotthuss shuttling. Detailed mechanistic understanding of the factors in proton pumping is an area of significant active research and a diversity of scientific opinion. There are, however, a few concepts regarding the minimal requirements for pumping that have emerged:\footnote{1086} 

1) the free energy to pump the protons comes from the large redox potential drop of the reduction of dioxygen to water relative to cyt c, which primarily provides an electrostatic driving force to maintain electroneutrality among high proton affinity sites, 2) the existence of a proton loading site in which the affinity for protons dramatically changes depending on redox states and protein conformations, 3) proton channels constructed of protein residues and water as a means to conduct the protons, and 4) a gating mechanism to prevent backflow of protons.

While the free energy used to pump protons in heme-copper oxidase comes from the large redox potential drop in the reduction of oxygen to water, it is nonetheless possible to decouple the pumping of protons from the oxidation of cyt c and concomitant reduction of oxygen to water, which allows for the study of electron transfer and proton translocation independently. One specific variant that highlights this feature of heme-copper oxidases is the N139D variant (\textit{R. sphaeroides}), a mutation along the D- pathway that eliminates proton pumping and increases the rate of O$_2$ reduction. The role of the dioxygen reduction is to provide a driving force for the stepwise transfer of electrons from heme a. The changes in the electrostatic environment by movement of the electrons between heme a and heme a$_3$ drives the movement of protons. It is interesting to consider that the electron transfer from heme a to heme a$_3$ is perpendicular to the translocation of pumped protons which indicates that sidechain and water reorganization during the modulation of the electrostatic environment must play a role. We note in passing that the area of decoupling of proton pumping from the oxygen reduction there is an emerging body of evidence that suggests that regulation of pumping by the organism is a viable aspect of catalysis, including variability in the number of protons pumped per electron. Two mechanisms under consideration for the attenuation of pumping are either via leakage of protons back across the membrane, or dissipation of the excess free energy from the oxygen reduction as heat. The regulation of proton pumping, while an area in early development, has significant physiological implications for organisms that have a need for heat as well as ATP.

The identity of the pump site within heme copper oxidases has not been resolved. Because pumping is driven by redox catalysis, it is likely that the pumping site is located in close proximity to redox sites. Current proposals implicate functional groups on the P side of the heme a$_3$/Cu$_B$ site, including the heme a propionates,\footnote{1087} the heme a$_3$ propionates, and residues in their immediate vicinity as the leading candidates. The key attribute of the proton loading/pump site is that the affinity for the proton must vary in order to either take up or release a proton at the appointed time during the sequence of electron transfer events. The local electrostatic environments over the series of redox states have been investigated computationally,\footnote{1088–1090} and suggest that the dominant contributors to the change in proton affinity of a potential proton loading site is electrostatics and coupled protein motions.
Based on crystallography (see Section 3.6.2.3) and a variety of mutational studies, the proton entrance channels are moderately well defined (the K- and D-channels) while the other channels, in particular the H-channel, is somewhat less well understood. Early on, it was appealing to define the K- and D-channels as responsible for translocating pumped and chemical protons respectively. However, mutational studies of uncoupled variants indicate that the K-channel provides two chemical protons and the D-channel provides two chemical and all four pumped protons. It is not known what the advantage of two uptake channels would be. Current mechanistic thought requires barriers to separate chemical from pumped protons. The D-channel would appear to violate this idea, but does contain a residue believed to function as a branch point (E286 \textit{R. sphaeroides}, Section 3.6.2.3) to separate the chemical and pumped proteins. There has further been discussion regarding the role that water plays in the channels to facilitate temporal separation of pumped versus chemical protons.\textsuperscript{1091,1092} While the description of the H-channel was based on crystallography (Section 3.6.2.3), recent mutagenesis work on the H-channel of bovine CcO has revealed that D51X variants turn off pumping while retaining the ability to reduce dioxygen.\textsuperscript{1023} This suggests that in bovine CcO the H-channel plays a major role in proton pumping.

Much progress on the mechanism of gating has been made recently. The starting point of these studies comes from X-ray crystallography. Specifically, the E286 residue at the interior end of the D-channel that has been found in a “down” and “up” configuration (Figure 203),\textsuperscript{1093} with the “down” state preferred by 2 kcal/mol with a 6 kcal/mol barrier to interconversion.\textsuperscript{1094,1095} MD simulations of this residue are found to vary significantly depending on the hydration in the cavity and the choice of membrane environment around the enzyme in the model. Recent simulations by Voth and coworkers that explicitly allow for proton transport (multi-state empirical valence bond methodologies) reveal that deprotonation of the “up” conformer leads to rapid transition to the “down” state only when heme a and heme a\textsubscript{3} are reduced and oxidized respectively, providing a means to reorient E286 into the proton channel to rapidly accept another proton and prevent back-leaking.\textsuperscript{1089} It should be noted an equivalent residue to E286 has not been identified in \textit{T. Thermophilus} CcO (cytochrome ba\textsubscript{3}). Instead, it is thought that the ba\textsubscript{3} and caa\textsubscript{3} oxidases use a tyrosine and serine residue (called YS pair) to perform an equivalent gating function.\textsuperscript{1096} Alternative gating schemes have been proposed, one particularly intriguing recent suggestion is the dual gating proposed in bovine CcO along the H-channel,\textsuperscript{1087} in which the heme a farnesyl sidechain and formyl block proton exchange of the heme a propionate on the negative side, and Y440-S441 peptide bond blocks the positive side. Oxidizing the heme a tautomerizes the Y440-S441 peptide bond to allow the proton to escape. Reduction opens the heme a farnesyl and formyl interactions to allow for protonation of the heme a propionate. More general structural changes have also been observed experimentally, in particular in the crystal structures of oxidized and reduced states of CcO. In resting CcO, a tight hydrogen bond is present between the CuB bound His-Tyr ligand and the heme a\textsubscript{3} farnesyl hydroxide (Figure 204A). This hydrogen bond is lacking in a reduced structure, and the lack of this hydrogen bond is correlated to increased hydration in the K-channel (Figure 204B).\textsuperscript{1004} The tight hydrogen bond in the oxidized form thus blocks entrance of protons into the heme a\textsubscript{3}/CuB from the K-channel, allowing for alternating access of protons from the K-and D-channel to the binuclear site. It should be
noted however, that the fully reduced enzyme may not be an intermediate during catalysis (see Section 3.6.2.2).

The sequence of events of proton pumping are coupled to the electron transfer steps in the transitions of states $P \rightarrow F$, $F \rightarrow O$, $O \rightarrow E$, and $E \rightarrow R$. Fadda and coworkers have described a mechanism for each of the individual pumping events by three states defined by the charge on the heme cofactors written as $[0|1]$, $[1|0]$, and $[1|1]$, where the digit on the left and right are the overall charge on heme $a$ and heme $a_3$ sites respectively (Figure 205). Starting from the $[0|1]$ state wherein the heme $a$ is reduced thereby raising the p$K_a$ of the pump/proton loading site (PLS), a proton is delivered to the PLS which raises the redox couple of the binuclear site such that an electron is transferred from heme $a$ to generate the $[1|0]$ state. Next, a chemical proton is recruited to the binuclear site to generate a $[1|1]$ state, in which the p$K_a$ of the PLS changed resulting in proton release from the PLS. Finally heme $a$ is rereduced by Cu$_A$ to generate a $[0|1]$ state. This sequence of events is capable of describing the kinetics of ET and proton release. In the steady state $P \rightarrow F$ transition, examination by single-turnover studies reveals the transition occurs in two steps, $P_M \rightarrow P_R \rightarrow F$, in which an electron is transferred in $P_M \rightarrow P_R$ and the proton in $P_R \rightarrow F$. Immediately prior to $O_2$ cleavage, the heme-copper site is electron rich, and drives the loading of a proton in the pump site. Dioxygen reacts to generate the superoxidized $P_M$, a $[0|1]$ state with a presumably loaded PLS site. Next, an electron is transferred from heme $a$ to the heme-copper site to create $P_R$, a $[1|0]$ state. This electron rich $P_R$ state then recruits a chemical proton generating a $[1|1]$ state, equivalent to state $F$, which then modulates the p$K_a$ of the PLS to eject the proton to yield intermediate $F^*$. In a similar manner, these three states are repeated four times per pumped proton. This scenario also implies a rich set of protonation microstates during the $F \rightarrow O$, $O \rightarrow E$, and $E \rightarrow R$ transformations. However, while this overall mechanistic sequence provides a coarse picture of proton pumping, many of the molecular level details are still unresolved. In particular, aspects of the architecture of the binuclear site including the Tyr-His crosslink, heme $a_3$ axial imidazole coordination, role of the copper, and identity of the proton loading site and gate sites remain among the open questions. Details are emerging regarding these structural components and their role for $O_2$ reduction; presumably these structures are integral components for the coupling of oxygen reduction to proton pumping as well.

### 3.8 Reduction of $O_2$ to $H_2O$

While many enzymes formally reduce $O_2$ to $H_2O$ in their catalytic cycles (for example as presented in section 3.2-catechol oxidase couples $O_2$ reduction to $H_2O$ to two, two-electron oxidations of catechol) only the MCOs and HCOs are capable of an effectively concerted four electron reduction of $O_2$. In both classes, the second two-electron reduction (of peroxide) is fast indicating a low activation barrier for O-O bond cleavage; thus far only in the case of variants of the MCOs where electron transfer from the T1 is eliminated can a two electron peroxide intermediate be observed. The low barrier for this second two-electron reduction of $O_2$, derives from the trinuclear nature of the catalytic sites in both classes (the TNC in the MCOs and the heme$_{a_3}$/Cu$_B$/Tyr in the HCOs, Figure 206). In the MCOs, the second two-electrons derive from two Cu$^+$’s that, in the triangular topology of the TNC, have good overlap with the peroxide $\sigma^*$ orbital, and the third Cu$^{II}$ of the TNC acts like a
proton in lowering the energy of the σ* LUMO. In the HCOs the second two-electrons derive from heme$_{a3}$Fe$^{III}$ and the covalent Tyr (through a superexchange pathway involving the Cu$_B$) with oxidation of Tyr coupled to proton transfer to the peroxide to promote O-O bond cleavage. In both classes of oxidases there is thus a low intrinsic overpotential for O$_2$ reduction, and ~800 mV/electron (at pH = 7) is available for either oxidizing high potential one-electron substrates (in MCOs), or low potential Cyt c with the excess energy used in proton pumping (in the HCOs).

A second parallel between these two classes is that the trinuclear centers are buried in the protein and electrons enter from the substrate via a T1 or Cu$_A$ center near the surface of the oxidase and are transferred rapidly over 13–18Å to the catalytic site. A key difference is that Cu is only capable of one-electron redox while the heme$_{a3}$Fe$^{II}$ is capable of two-electron redox and the covalent Tyr is also redox active. Thus in the MCOs all four Cu’s are oxidized in the four-electron reduction of O$_2$ to H$_2$O which provides an efficient mechanism for substrate oxidation in that the T1 at the surface of the enzyme is immediately available for redox. Alternatively in the HCOs all four electrons derive from the catalytic site, therefore four holes are stored here for proton pumping. The HCOs therefore have the additional feature of the buried heme$_{a3}$Fe$^{II}$ for transfer of each of the four electrons that are coupled to the proton pumping in the reduction of the heme$_{a3}$/Cu$_B$/Tyr active site.

A final interesting correlation is that in both classes of four-electron oxidases the oxidized form of the enzyme obtained in turnover (NI in the MCOs, the pulsed enzyme in the HCOs), but not the resting form, is catalytically active. In the MCOs it is clear that NI loses its μ$_3$-oxo in going to the resting enzyme and this drastically slows electron transfer from the T1 Cu. Thus, the crystallographically defined resting TNC is not involved in catalysis. In the HCOs the crystallographically defined resting enzyme form has a peroxide bridging the heme$_{a3}$/Cu$_B$ center. It is important to understand the origin of this bridging peroxide and why it is inactive rather than spontaneously undergoing O-O bond cleavage to generate the four-hole heme$_{a3}$Fe$^{IV}$=O/Cu$^{II}$/Tyr$^*$ catalytic state.

4. Substrate Activation by Cu$^{II}$ Sites

Reactions between triplet O$_2$ and singlet organic substrates are spin-forbidden, leading to very slow rates despite the reactions being thermodynamically favorable. Two possible catalytic strategies for overcoming this barrier using a mononuclear metal center are observed in bioinorganic chemistry: O$_2$ activation by a reduced metal and substrate activation by an oxidized metal. These complementary strategies are observed in mononuclear iron enzymes, where Fe$^{II}$-dependent enzymes such as the extradiol dioxygenases activate O$_2$ while Fe$^{III}$-dependent enzymes perform substrate activation in two possible ways: the intradiol dioxygenases by binding the substrate as a ligand and promoting the reaction with O$_2$ via a concerted LMCT mechanism where Fe$^{III}$ acts as a spin buffer; the lipoxygenases by using an Fe$^{III}$-OH site to perform H-atom abstraction from the substrate (to form an Fe$^{II}$-OH$_2$), resulting in a substrate radical activated for reaction with O$_2$. A similar pattern is observed for the two different redox states of copper in mononuclear copper enzymes. In Sections 3.3 and 3.4, O$_2$ activation by mononuclear copper sites in the reduced Cu$^+$ state was discussed. The alternative strategy utilizing mononuclear Cu$^{II}$ for
substrate activation is employed by the enzyme quercetinase, in the cofactor biogenesis reaction of the copper-containing amine oxidases (including lysyl oxidase) and also in one possible biogenesis mechanism in the enzyme galactose oxidase as described in Section 3.3.4. In this section, what is known about the reactivity of Cu\textsuperscript{II} in quercetinase and in amine oxidase cofactor biogenesis will be discussed, concluding with thoughts on the nature of substrate activation by Cu\textsuperscript{II} active site.

4.1 Quercetinase

4.1.1 Enzymology—Quercetinase (quercetin 2,4-dioxygenase, 2,4-QD, EC 1.13.11.24 [named “quercetin 2,3-dioxygenase”, but this does not reflect the reaction done by the enzyme]) is an extracellular enzyme expressed by some microorganisms when grown on rutin, a quercetin precursor (Figure 207), as the sole carbon source.\textsuperscript{1099,1100} It catalyzes the degradation of quercetin and related flavonols to the depside (diphenolic ester) 2-protocatechuoylphloroglucinol and CO (Figure 207).\textsuperscript{1101} Quercetin and other flavonols are nutrients present in foods such as onions, red leaf lettuce, and tea and are of interest for their antioxidant and anti-inflammatory properties.\textsuperscript{1102} They are used as herbal medicines and as part of traditional Chinese medicine.\textsuperscript{1103} Quercetin is being investigated as a treatment for a variety of diseases,\textsuperscript{1104} including cardiovascular disease,\textsuperscript{1105} and a variety of cancers.\textsuperscript{1106,1107} The mechanism of action of fungal and bacterial quercetinases may be related to the metabolism of quercetin by microflora in the intestinal tract.\textsuperscript{1108} In addition, quercetinase has been investigated as a food preservative.\textsuperscript{1109}

Quercetinases have been isolated from the fungi Aspergillus flavus,\textsuperscript{1099} Aspergillus niger,\textsuperscript{1110} Aspergillus japonicus,\textsuperscript{1111} and Penicillium olsonii\textsuperscript{1112} and the prokaryotes Bacillus subtilis\textsuperscript{1113,1114} and Streptomyces sp. FLA.\textsuperscript{1115} All quercetinase enzymes are glycoproteins with 16–47\% sugar content (w/w). Sequence data for the quercetinases from A. japonicus, P. olsonii, B. subtilis, and Streptomyces have been reported and there is a high degree of sequence similarity between enzymes from different source organisms (compared to P. olsonii, 61\% similarity with A. niger, 58\% with A. japonicus, but only 15\% with B. subtilis and 30\% with Streptomyces).\textsuperscript{1112} Despite this sequence similarity, there are large differences in quaternary structure between 2,4-QDs from different sources. All 2,4-QDs belong to the cupin superfamily, but 2,4-QDs from A. japonicus, P. olsonii, and B. subtilis are bicupins while Streptomyces 2,4-QD is a monocupin.\textsuperscript{1115} The 2,4-QDs from A. japonicus and B. subtilis are homodimers\textsuperscript{1111,1116} while the P. olsonii 2,4-QD is a monomeric protein\textsuperscript{1112} and the A. niger 2,4-QD is a heterotrimer.\textsuperscript{1110}

The metal ion content of quercetinases from different organisms is a topic of ongoing research. All quercetinases isolated from fungal sources (the Aspergillus and Penicillium enzymes) contain \~1 Cu atom per monomer,\textsuperscript{1111,1112,1117} excepting A. niger 2,4-QD, which binds \~2 Cu per trimer.\textsuperscript{1110} The copper is in the Cu\textsuperscript{II} redox state, since a cuproine assay by Oka and Simpson detected very little Cu\textsuperscript{I} in the as isolated enzyme.\textsuperscript{1117} A. niger 2,4-QD is also isolated with variable Ni content in addition to Cu\textsuperscript{II}, but the amount of nickel present does not affect the enzyme activity.\textsuperscript{1110} In contrast, the prokaryote quercetinases from B. subtilis and Streptomyces are expressed in E. coli and bind many different divalent metals, depending on the metal content of the culture media.\textsuperscript{1118,1119} B. subtilis 2,4-QD was
originally isolated containing a mixture of Fe$^{II}$ and Fe$^{III}$, but bound Fe can be removed by DDTC and EDTA chelation and full activity is only restored by incubation with Cu$^{II}$ or Co$^{II}$ (Mn$^{II}$ and Fe$^{III}$ also show some activity). Experiments to determine the divalent metal that gave the highest activity in crude cell extracts of B. subtilis and Streptomyces 2,4-QD indicate that Mn$^{II}$ and Co$^{II}$ are most active in B. subtilis 2,4-QD while Ni$^{II}$ and Co$^{II}$ are most active in Streptomyces 2,4-QD. When 2,4-QD is isolated with these metals, the metal stoichiometry is not consistent with tight binding to the proposed active site (1 site per subunit) as Co$^{II}$ and Ni$^{II}$ bind in less than stoichiometric amounts (0.65 Co$^{II}$ per subunit in B. subtilis, ~0.5 Co$^{II}$ or 0.5 Ni$^{II}$ per subunit in Streptomyces) and Mn$^{II}$ binds in excess (1.8 Mn$^{II}$ per subunit). Despite the work that has been done on the metal content and activity of the bacterial quercetinases, it is not clear which metal is bound in the native enzyme in these systems. Alternatively, it is clear that Cu$^{II}$ is the native metal in the fungal quercetinases. Further arguments can be made from comparing the kinetics of quercetinases isolated with different metals (vide infra) that quercetinase should be considered to be a Cu$^{II}$ enzyme and the incorporation of different metals is due to the E. coli expression system.

4.1.2 Kinetics—Early experiments on 2,4-QD by Simpson et al. used atom labelling to identify the precise reaction performed by 2,4-QD. Labelling the C3 carbon in rutin, which is transformed to quercetin in vivo by the enzyme rutinase, with $^{14}$C shows that the C3 carbon of quercetin is released as CO by 2,4-QD from A. niger (Figure 207). When the reaction is carried out with $^{18}$O$_2$ by A. flavus 2,4-QD, the CO produced does not contain $^{18}$O while the depside product contains both atoms from $^{18}$O$_2$, incorporated at C2 and C4 (detected by MS and IR spectroscopy). Performing the reaction in H$_2$-$^{18}$O resulted in no change in the amount of $^{18}$O in the water, so $^{18}$O from H$_2$-$^{18}$O is not incorporated into either product and the O of the CO released comes from the original C3-OH group of the substrate. On the basis of these results, 2,4-QD can be identified as a 2,4-dioxygenase.

Experiments by Oka et al. show that 2,4-QD substrates bind to anaerobic resting 2,4-QD from A. flavus and react upon addition of O$_2$. Kooter et al. and Steiner et al. have reproduced this result for A. japonicus 2,4-QD. In contrast, Hund et al., using 2,4-QD from A. niger, find that no difference is observed in the EPR spectrum of the enzyme upon anaerobic incubation with a threefold excess of quercetin. They propose that the presence of O$_2$ may be required for substrate binding. This discrepancy may be related to the insolubility of quercetin in aqueous solutions, which was overcome in the experiments of Oka et al. and Kooter et al. by the addition of DMSO (10% (v/v) Oka, 5% (v/v) Kooter), an additive not present in the EPR experiment of Hund et al. Oka et al. have investigated the structural requirements for substrates of 2,4-QD. 2,4-QD will dioxygenate a variety of 3-hydroxyflavones where a C3-OH group, a double bond between C2 and C3, and a substituent on C2 are all found to be required for substrate reactivity.

Maximum specific activities and K$_M$ values have been reported for several copper containing 2,4-QDs (182 µmol min$^{-1}$ mg$^{-1}$, 5.2 µM quercetin, and 0.12 mM O$_2$ for A. flavus, 73 µmol min$^{-1}$ mg$^{-1}$ and 6.6 µM quercetin for A. niger, 175 µmol min$^{-1}$ mg$^{-1}$ and 1.1 µM quercetin for P. olsonii) but full Michaelis-Menten kinetics with several different substrates are reported only for 2,4-QD from P. olsonii (Table 36). Various substitution patterns on the B and C rings of the flavonoid result in variations in the K$_M$ and relative
rates of reaction, due to the structural constraints of binding the substrate in the active site pocket.

The higher $K_M$ values reported for 2,4-QD from *P.olsonii* in Michaelis-Menten kinetics studies are due to a higher % DMSO used in the assay. Tranchimand et al. investigated the effect of % DMSO and found that it increases the apparent $K_M$ (19 µM with 20% DMSO v.s. 1.1 µM with 1% DMSO for quercetin).\(^{1112}\) There is also a pH effect on the enzyme activity that varies with the enzyme source: while *A. flavus* shows maximum activity pH 5.5–6.0,\(^{1123}\) *P.olsonii* shows an optimum pH of 6.5\(^{1112}\) and *A. niger* shows no pH effect on activity pH 2.0–6.7.\(^{1110}\) The origin and significance of the pH profile of the enzyme activity of 2,4-QD remain to be studied. Single turnover experiments on the 2,4-QD ES complex with O\(_2\) have not been reported and are an interesting future direction for kinetics studies.

2,4-QD is inhibited by reducing agents such as sodium dithionite, 2-mercaptoethanol and dithiothreitol but recovers from this inhibition after exposure to air or mixing with airsaturated buffer. This reactivation is not retarded by *p*-chloromercuribenzoate, so it results from reduction of the copper site rather than from reduction of disulfide bonds in the protein.\(^{1120}\) This establishes that Cu\(^{II}\) is the active redox state in 2,4-QD. Pre-incubation of the enzyme with substrate prevents inactivation by a reducing agent, suggesting that substrate binding makes the copper site more difficult to reduce (either by decreasing E\(^{5+}\) or by blocking access to the metal site).\(^{1120}\) Various copper-specific chelating agents inhibit Cu-loaded 2,4-QD, most commonly ethylxanthate, diphenylthiocarbazone, toluene-3,4-dithiol, and diethylthiocarbamate (DDC).\(^{1110,1120}\) Attempts to use copper chelators like DDC to remove the copper from quercetinase were unsuccessful. Reactivation of quercetinase by divalent metals after inactivation and attempted copper removal with DDC is ascribed to removal of the DDC inhibitor from the copper site by competitive binding to excess free divalent metal, rather than to reconstitution of the active site with another metal.\(^{1110}\)

Several recent studies report activity for the bacterial quercetinases with alternative divalent metals, including Fe\(^{II}\), Ni\(^{II}\), Co\(^{II}\), and Mn\(^{II}\). The initial studies of a bacterial 2,4-QD by Bowater et al. and Barney et al. identify 2,4-QD from *B. subtilis* as an iron-containing enzyme.\(^{1113,1114}\) However, the as-isolated Fe-loaded 2,4-QD has an activity that is 2–3 orders of magnitude lower than that observed for Cu-loaded 2,4-QD while having similar $K_M$(substrate) values (3.8 µM and 0.8 µM respectively for quercetin).\(^{1118}\) This result could reflect the activity of Fe-loaded 2,4-QD being due to 1% or less copper present while the majority of the active sites contain iron and are inert. However, Barney et al. rule this out by showing that Fe-loaded 2,4-QD is not inhibited by copper-specific chelators (ethylxanthate, diethylthiocarbamate, kojic acid) but instead by 1,10-phenanthroline and 2,2’-dipyridyl, which are better iron chelators.\(^{1118}\) 2,4-QDs from *B. subtilis* and *Streptomyces* purified in *E. coli* could also be isolated containing Mn\(^{II}\), Co\(^{II}\), or Ni\(^{II}\).\(^{1118,1119}\) These enzymes show $K_M$ values in the µM range, $k_{cat}$ values at least an order of magnitude lower than those reported for Cu-loaded 2,4-QD, but $k_{cat}/K_M$ values similar to those reported for *P.olsonii* 2,4-QD (8.8x10\(^6\) M\(^{-1}\) s\(^{-1}\) for Cu-loaded 2,4-QD from *P.olsonii*;\(^{1112}\) 7.9x10\(^6\) M\(^{-1}\) s\(^{-1}\) and 7.0x10\(^6\) M\(^{-1}\) s\(^{-1}\) for Mn-loaded and Co-loaded 2,4-QD from *Streptomyces*\(^{1119}\) with quercetin as the substrate). However, since the $K_M$ for *P.olsonii* was artificially lowered by
having a higher % DMSO than was used in the other studies, the correct comparison gives at least a 20 fold higher $k_{\text{cat}}/K_M$ for Cu-loaded 2,4-QD.

Considering that copper is known to be the native metal in fungal quercetinases, that Cu-loaded 2,4-QD has higher activity, $k_{\text{cat}}$ and catalytic efficiency than 2,4-QDs containing other metals, and that in systems with other metals residual copper may be responsible for some of the activity, quercetinase should be regarded as a copper enzyme unless further work establishes the presence of another metal in a native enzyme system.

4.1.3 Structure—Crystal structures of 2,4-QD from *A. japonicus* have been solved for the resting enzyme at pH 5.2 (PDB ID: 1JUH), the enzyme with the substrates quercetin and kaempferol (Table 36) bound anaerobically (PDB IDs: 1H1I, 1H1M) and the enzyme with the inhibitors diethydithiocarbamate and kojic acid bound (PDB IDs: 1GQG, 1GQH). The crystal structure of *B. subtilis* 2,4-QD with iron as the active site metal has also been reported, but will not be discussed here (PDB ID: 2H0V).

The crystal structure of resting *A. japonicus* 2,4-QD at pH 5.2 was determined at 1.6 Å resolution by Fusetti et al. after deglycosylation by endoglycosidase-H. 2,4-QD is a ~100 kDa homodimeric protein with 350 amino acid residues. An ordered heptasaccharide chain that was not removed by deglycosylation treatments spans the dimer interface and forms significant surface contacts between the monomers. Each monomer is composed of two similarly structured domains, comprising 2 antiparallel $\beta$ sheets, 8 strands forming a $\beta$ sandwich and two $\alpha$ helices (Figure 208). The domains are joined by a linker of 60 amino acid residues, one part of which is disordered in the crystal structure. In each domain there is a large hydrophobic cavity, which in the N-terminal domain contains the copper site. The active site is 10 Å from the protein surface and is solvent-exposed, due to disorder in a 9 amino-acid stretch of the interdomain linker, which would otherwise act as a lid for the hydrophobic active site cavity. Consistent with elemental analysis, only the N-terminal domain of 2,4-QD binds Cu; in the C-terminal domain, the positions occupied by the three His residues which ligate copper in the N-terminal domain are replaced by other non-coordinating residues.

The protein-derived copper ligands are three histidine residues (His66, His68, and His112) and a glutamate residue (Glu73), the first known case of an endogenous carboxylate residue serving as a ligand in a copper protein. Two geometries are observed for the copper site in resting 2,4-QD (Figure 209A and B). The major form (70%, Figure 209A) is a tetrahedral site where the copper is ligated by His66, His68, His112 and a solvent-derived ligand at a bond distance of 2.2 Å from the copper, with Glu73 in an off-copper conformation and H-bonding to the solvent-derived ligand. The minor form (30%, Figure 209B) is a distorted 5 coordinate site in which Glu73 is an additional monodentate ligand to the copper center and the solvent derived ligand shifts to an equitorial position 2.4 Å from Cu and is H-bonded to the non-coordinating O of Glu73. The solvent derived ligand and His68 are the axial ligands of the 5 coordinate site and His66, His112 and Glu73 are the equatorial ligands. The presence of two different forms of the copper site is consistent with EPR studies of the resting enzyme, which show a mixture of two copper species in a pH-dependent equilibrium (*vide infra*). Modeling quercetin into the active site cavity of quercetinase shows that the
shape of the hydrophobic cavity imposes an orientation on quercetin that requires monodentate coordination to copper through its C3-OH group, rather than the bidentate coordination via the C3-OH and C4=O groups that is observed in model complexes.  

The crystal structures obtained for the ES complex of 2,4-QD with the substrates quercetin (5,7,3',4'-tetrahydroxy flavonol) and kaempferol (5,7,4'-tri hydroxy flavonol) anaerobically bound confirm the binding mode proposed by modelling. The substrates bind through the C3-OH group, displacing the solvent-derived copper ligand (Figure 209C). No disorder is observed in the coordination environment of the copper site in the ES complex. The copper site is a 5-coordinate distorted square pyramid, with His66, His68, His112, and Glu73 as protein-derived ligands (His68 is the axial ligand). The non-coordinating O of the Glu73 ligand is within H-bonding distance of the C3-OH group of the substrate (2.66 Å for quercetin and 2.43 Å for kaempferol), suggesting that the proton of the C3-OH group is retained in a hydrogen bond and that in addition to being a ligand Glu73 may have a mechanistic role as an active site base. Steiner et al. propose that the C3-OH proton is transferred to Glu73 upon substrate binding to quercetinase and this assignment is supported by a DFT calculation of the ES complex performed by Siegbahn. While the pKa’s of the two groups – 4 for Glu and 7 for quercetin – are more consistent with the proton remaining on the quercetin hydroxyl group, that of the C3-OH would be lowered by coordination to the CuII. When bound to quercetinase, the B ring of the substrate is not coplanar with the rest of the molecule, but is instead bent out of the plane by 10–12°, consistent with some sp3 character on the C2 position. While this could indicate radical character on the C2 carbon, EPR spectra of the ES complex do not show the presence of an organic radical or reduction of the copper site (vide infra). Instead, the shape of the active site cavity could account for this distortion, because positioning the B ring to be coplanar with the A and C rings would result in significant steric clashes with two protein sidechains, Phe75 and Phe114. DFT calculations on quercetin show that the bending observed in the crystal structure only costs 1 kcal/mol. The enzyme-substrate binding is further stabilized by van der Waals interactions with many residues in the active site pocket. Particularly, a van der Waals interaction between the A ring of the substrate and Pro164, part of the flexible interdomain linker, leads to ordering of the linker to form a lid on the active site cavity and further stabilize substrate binding. There are no protein-derived hydrogen bond donors in the hydrophobic active site pocket, but two ordered water molecules supply hydrogen bonds to the O7 and O4' hydroxyl groups of quercetin.

Crystal structures of quercetinase with two different inhibitors have also been reported: diethyldithiocarbamate (DDC, Ki ~ 10⁻⁷ M) and kojic acid (KOJ, Ki ~ 10⁻³ M), the latter a weak inhibitor whose structure resembles the B ring of quercetin. The overall structure of the protein, including the disorder of the linker region that covers the active site, is not changed by the binding of these inhibitors. As in the substrate-bound crystal structures, only one coordination environment for the copper site is observed in the inhibitor-bound structures, but the coordination observed depends on the inhibitor. Both inhibitors bind to the copper site as bidentate ligands, displacing the solvent-derived ligand. DDC coordination yields a 5-coordinate site, with copper ligated by His66, His68, His112 and the two sulfur atoms of DDC. DDC is bound asymmetrically (Cu-S1 2.2 Å, Cu-S2 2.9 Å) in a distorted
square pyramidal geometry. Glu73 is rotated away from the copper site to H-bond to a conserved water molecule. In contrast, KOJ yields a 6-coordinate structure with His66, His68, His112, Glu73 (monodentate) and the two oxygens of KOJ as ligands. KOJ coordination is also asymmetric (Cu-O3 1.89 Å, Cu-O4 2.58 Å) due to differences in the nature of the two oxygen ligands (O3 being the alkoxide and O4 the carbonyl). Both inhibitors bind with their molecular planes perpendicular to the observed orientation for quercetin in the active site cavity, blocking access to the portion of the active site pocket that binds the B ring of quercetin. Blocking the active site pocket so that quercetin cannot bind to the enzyme is proposed as a common mode of inhibition for chelating inhibitors.\textsuperscript{1125}

4.1.4 Spectroscopy and Electronic Structure—Absorption, EPR, and XAS spectroscopies have been used to investigate the copper-containing quercetinases from the Aspergillus sources in their resting forms, upon anaerobic substrate binding, and in the product-bound state observed after reaction of the substrate-bound enzyme with O\textsubscript{2}. As with the X-ray crystallography, the most complete studies have been performed on 2,4-QD from A. japonicus. The spectroscopy of these species in A. japonicus and A. flavus will be discussed first and then compared to the spectroscopy of A. niger, which has some spectroscopic differences.

The absorption spectrum of the resting form of 2,4-QD has been reported by Oka et al. for the A. flavus enzyme. The absorption spectrum contains no absorption peaks in the 350–800 nm range,\textsuperscript{1120} although very concentrated solutions of the enzyme (2.7 mM in protein) are faintly green colored.\textsuperscript{1117} This indicates that the resting copper site has no significant ligand to metal charge transfer transitions (Figure 210A), which is consistent with a normal copper site (Type 2) with the ligand set of histidine, water, and possibly a 30% contribution from a carboxylate as reported by X-ray crystallography. The EPR spectrum of the resting enzyme from A. japonicus, reported by Kooter et al., shows a mixture of two species in an approximate 70:30 ratio at pH 6.0 (Figure 211A).\textsuperscript{1121} The 70\% component has $g_\parallel = 2.330$ and $A_\parallel = 149\times10^{-4}$ cm\textsuperscript{-1} while the 30\% component has $g_\parallel = 2.290$ and $A_\parallel = 134\times10^{-4}$ cm\textsuperscript{-1}. The $g$ and $A$ values of the minor species can be resolved by a pH perturbation of the resting enzyme, since at pH 10.0 the enzyme is reversibly converted to the minor form (Figure 211B, $g_\parallel = 2.289(4)$, $A_\parallel = 128\times10^{-4}$ cm\textsuperscript{-1}; $g_\parallel = 2.178(5)$, $A_\parallel = 61\times10^{-4}$ cm\textsuperscript{-1}; $g_x = 2.011(3)$, $A_x = 56\times10^{-4}$ cm\textsuperscript{-1}).\textsuperscript{1121} Kooter et al. compare these parameters to copper model complexes and suggest that the high pH form of the active site has a trigonal bipyramidal coordination geometry. However, this would have a $dz^2$ ground state with $a_{g_{\perp}} > g_{\parallel} = 2.0$, whereas the reported EPR parameters of the high pH site ($g_x > g_y > g_z \approx 2$) are characteristic of a distorted tetragonal site with significant $dz^2$ mixing into a $dx^2-y^2$ ground state. This is consistent with the two active site geometries observed in the crystallography, performed at pH 6.0, where a tetrahedral form contributes 70\% and a distorted 5 coordinate site contributes 30\% (\textit{vida supra}).\textsuperscript{1111} Cu K edge XAS spectroscopy performed by Steiner et al. is also consistent with this assignment.\textsuperscript{1122} The XAS spectrum of the resting enzyme at pH 6.0 shows no features in the pre-edge region. Steiner et al. fit the EXAFS spectrum to each of the two possible coordination models observed in the crystal structure (4 coordinate 3His+O at 2.00 Å or 5 coordinate 3His+2O at 2.01 Å) and determined that both models fit the EXAFS spectrum equally well. They use a valence bond sum analysis to suggest that a
coordination number intermediate between four and five is consistent with the data, corresponding to a heterogeneous coordination environment with one four-coordinate and one five-coordinate component as observed in both the EPR spectroscopy and X-ray crystallography of resting A. japonicus 2,4-QD.\textsuperscript{1122}

Upon anaerobic incubation of the resting enzyme with the flavanol substrates: quercetin, morin, and 3-hydroxyflavone, the absorption spectrum is dominated by the absorption band of the substrate, shifted to lower energy into the 300–500 nm region due to binding to the enzyme (free quercetin absorption Figure 210 B, ES complex Figure 210C).\textsuperscript{1120} The shift in the wavelength of substrate absorption upon addition to the enzyme does not occur if the enzyme had been preincubated with a copper chelating agent (e.g. ethylxanthate). This is consistent with substrate binding to the copper site of A. flavus 2,4-QD under anaerobic conditions. Additionally, EPR spectra have been reported after anaerobic incubation of A. japonicus 2,4-QD with a variety of flavonol substrates: quercetin, kaempferol, galangin, myricetin, morin, datiscetin, fisetin, 7-hydroxy flavonol, and flavonol.\textsuperscript{1121} A single Cu\textsuperscript{II} species is observed in these EPR spectra with $g_{//} = 2.310–2.337$ and $A_{//} = 110–142$ G, depending on the substrate (quercetin yields $g_{//} = 2.336$ and $A_{//} = 124 \times 10^{-4}$ cm$^{-1}$, Figure 211C). These parameters are similar to those observed for the dominant species in the resting enzyme, characterized by X-ray crystallography as a tetrahedral site without Glu73 bound to Cu. However, the substrate-bound site characterized by X-ray crystallography is 5-coordinate with Glu73 bound, nominally more similar to the minor component of the resting enzyme.\textsuperscript{1124} No reduction of Cu\textsuperscript{II} to Cu\textsuperscript{I} or evidence of an organic radical is observed upon substrate binding in EPR spectra recorded at 77 K, which contrasts a substrate radical suggestion based on the pyramidalization of the substrate C2 carbon in substrate-bound crystal structures.\textsuperscript{1121} Steiner et al. also report the XAS spectra of 2,4-QD with the substrates quercetin and myricetin bound.\textsuperscript{1122} The absence of a pre-edge feature at 8984 eV (characteristic of Cu\textsuperscript{I} complexes) and the consistent position and shape of the K-edge in the resting enzyme and the substrate-bound enzyme additionally demonstrate that there is no reduction of the copper site upon substrate binding, consistent with the EPR data. The EXAFS data for the substrate bound 2,4-QD are best fit as a single species with a single shell of 3 His and 2 O ligands with an average distance of 2.00 Å. Attempts to refine the fit to include two shells of ligands yielded a possible fit to shells at 1.96 Å and 2.05 Å, but Steiner et al. set aside these results because of insufficient resolution in their data.\textsuperscript{1122} These EXAFS results agree with the conclusions from X-ray crystallography and EPR spectroscopy of the ES complex that substrate coordination imposes order on the coordination environment of the copper site (vide supra).

When samples of the substrate-bound enzyme are exposed to oxygen, turnover occurs and the absorption band of the bound substrate decays, resulting in a featureless absorption spectrum similar to that of the resting enzyme (Figure 210D).\textsuperscript{1120} However, the EPR spectrum of the product when EPR samples of the ES complex are exposed to O$_2$ is different from that of the resting enzyme (Figure 211D, a single species with $g_x = 2.295(4)$, $A_x = 131 \times 10^{-4}$ cm$^{-1}$; $g_y = 2.169(5)$, $A_y = 43 \times 10^{-4}$ cm$^{-1}$; $g_z = 2.014(3)$, $A_z = 63 \times 10^{-4}$ cm$^{-1}$).\textsuperscript{1121} The spectrum after turnover is very similar to a spectrum of the product-bound enzyme obtained by adding the depside product to resting 2,4-QD and spectral features of the resting...
enzyme could be recovered by repeated buffer exchange. On this basis, Kooter et al. identify the species after turnover as the enzyme with the product bound at the copper site.\textsuperscript{1121} However, the spectrum after turnover is also very similar to the minor component of the resting site (observed cleanly at high pH) and this similarity should be investigated. A detailed spectroscopic study is required to elucidate the electronic structure of the resting, substrate-bound, and product-bound 2,4-QD.

There are large differences between the spectroscopic data reported for 2,4-QD from \textit{A. japonicus} (discussed above) and those reported by Hund et al. for 2,4-QD from \textit{A. niger}.\textsuperscript{1110} The EPR spectrum of resting 2,4-QD from \textit{A. niger} shows contributions from only one species, which has $g_{||} = 2.293$ and $A_{||} = 166 \times 10^{-4}$ cm$^{-1}$ and a superhyperfine contribution to the $g_{\perp}$ region. Simulation of the $g_{\perp}$ region without the superhyperfine contribution lead to values of $g_{y} = 2.055$, $g_{x} = 2.01$ and $A_{y} = A_{x} = 9 \times 10^{-4}$ cm$^{-1}$. The superhyperfine contribution is a pattern of nine lines split by 13 G in the $g_{\perp}$ region, indicating coupling with 4 equivalent nitrogen ligands. However, \textit{A. niger} and \textit{A. japonicus} 2,4-QD have 66\% sequence similarity and there are no additional His residues in the vicinity of the \textit{A. niger} 2,4-QD active site. Indeed, all the key active site residues (copper ligands and key second sphere residues involved in substrate binding) are conserved.\textsuperscript{1112} This raises the possibility that there may be an exogenous ligand bound in \textit{A. niger} 2,4-QD. The EPR spectrum does not change upon anaerobic addition of substrate, which would be expected if the active site were blocked by the binding of an exogenous ligand.\textsuperscript{1110} Alternatively, the lack of change in the EPR spectrum upon anaerobic addition of quercetin may be due to substrate insolubility in the absence of added DMSO (\textit{vide supra}). These differences in EPR spectroscopy between 2,4-QD from \textit{A. niger} and \textit{A. japonicus} have been previously interpreted by Kooter et al. as an indication that there is a significantly different coordination environment in the \textit{A. niger} resting enzyme and an alternative mechanism for 2,4-QD activity may be required.\textsuperscript{1121} However, in view of the high degree of sequence similarity between the enzymes, the origin of the spectroscopic differences needs to be clarified further by structural and spectroscopic studies of \textit{A. niger} 2,4-QD before any conclusions can be drawn about whether there are in fact significant structural or mechanistic differences from \textit{A. japonicus} 2,4-QD.

\subsection*{4.1.5 Molecular Mechanism—}A general mechanism has been proposed for quercetinase, on the basis of the structural and spectroscopic studies of the enzyme ES complex (Figure 212).\textsuperscript{1124} The resting copper site exists in equilibrium between a Glu73-off form (1A) and a Glu73-on form (1B). Substrate binds to the copper site as a monodentate ligand via the C3-OH group (2A), which is deprotonated and hydrogen bonds with Cu-on Glu73. The Cu$^{II}$-substrate complex is thought to be in an equilibrium with a Cu$^{I}$-substrate radical complex (2B) formed by an intramolecular electron transfer, but this equilibrium is shifted far to the left, so the Cu$^{I}$-substrate radical form is not experimentally observed (alternatively, this step has been described by Siegbahn as the formation of an excited state of the Cu$^{II}$-substrate complex, \textit{vide infra}). After formation of the Cu$^{I}$-substrate radical, O$_{2}$ could react at the substrate radical (3A) or at Cu$^{I}$, forming a Cu$^{II}$-superoxo species (3B). Formation of the C2-O bond is followed by formation of the second C-O bond at C4, forming an endoperoxide.
intermediate (4). This endoperoxide intermediate decays in a concerted step, which includes O-O bond cleavage and the release of CO, to form the depside product (5).

Siegbahn has used DFT calculations to evaluate this reaction pathway and particularly distinguish between the two possible sites where O2 could react in a CuI-substrate radical complex.1126 His results show that the formation of the CuII-superoxo is 14 kcal/mol lower in energy than O2 attack on the substrate radical. Reaction between the ES complex and O2 is the rate limiting step of the reaction, with ΔH = 9.6 kcal/mol (a transition state and barrier for this step were not calculated). The entropy loss in this step is estimated to be ~5 kcal/mol, yielding AG = 14.6 kcal/mol (entropy contributions were not included in the calculations of the other steps of the reaction). Upon formation of the CuII-superoxo, the quercetin radical dissociates from the copper site (Figure 212 3B) and rotates relative to copper so that attack of the superoxide on C2 from the top becomes possible with ΔH‡ = 1.1 kcal/mol and ΔH = −18.3 kcal/mol. Formation of the second C-O bond to yield the endoperoxide intermediate then proceeds with ΔH‡ = 7.1 kcal/mol and ΔH = 1.7 kcal/mol. In this step, the hydrogen bond from Glu73 shifts from O3 to O4, stabilizing the extra charge on C4. The subsequent concerted O-O bond cleavage and CO release has ΔH‡ = 0.2 kcal/mol and is highly exothermic, with ΔH = −101.8 kcal/mol. The whole reaction is exothermic by 96.7 kcal/mol.1126 The point at which these calculations substantially disagree with experimental results is in the attack of the CuII-superoxo on the quercetin radical. The dissociation of the quercetin radical from CuII and its repositioning prior to superxo attack may also not be possible given the constraints of the substrate binding pocket. Indeed, Steiner et al. suggest that the CuII-superoxo attack on C2 may not be geometrically possible if quercetin is constrained in the position observed in the crystal structure of the ES complex.1124

Some mechanistic insight into the reaction performed by 2,4-QD has been obtained from model complex studies. The dioxygenation of the simplest flavonol, 3-hydroxyflavone, occurs in the presence of O2 under many conditions, including base-catalyzed dioxygenation in protic and aprotic solvents,1127,1128 radical-initiated dioxygenation,1129,1130 stoichiometric dioxygenation of ZnII,1131 CoII,1132 CuII,1133 and CuI1134 flavonolate compounds, and dioxygenation catalyzed by CuII model complexes with nitrogen donor ligands.1133,1134 Several recent reviews cover this model chemistry.1132,1135 When the flavonol is deprotonated, the free flavonolate anion reacts with O2, yielding the same products as the enzymatic reaction. A study of the reaction of potassium flavonol with O2 has shown that in the absence of a redox-active metal this reaction proceeds by a single electron transfer pathway in which the deprotonated flavonolate directly transfers an electron to O2 in a rate-limiting outer sphere electron transfer step.1128 The reaction has a first order dependence on O2 and free superoxide has been detected by nitro blue tetrazolium in these reactions while the flavonolate radical has been observed by EPR. Radical coupling of the flavonolate radical and superoxide produces a peroxide which goes to the product via an endoperoxide intermediate, resulting in incorporation of both atoms of O2 into the depside product (verified by 18O2 labelling). However, the rate of the reaction of flavonolate with O2 is rather slow, as indicated by a second order rate constant for non-enzyme potassium flavonolate dioxygenation of 0.33±0.01 M−1 s−1 at 80°C in DMF.1128 This
contrasts with a high catalytic efficiency for the enzyme reflected by a $k_{\text{cat}}/K_M$ on the order of $1 \times 10^{8} \text{ M}^{-1} \text{s}^{-1}$ at room temperature and pH 6, where the flavonol rather than the flavonolate is present (in 20% DMSO, vide supra).\textsuperscript{1112} Thus the reaction catalyzed by 2,4-QD involves an activation of the flavonol substrate towards reaction with O$_2$ rather than simple deprotonation of the flavonol by 2,4-QD followed by direct reaction of the flavonolate with O$_2$.

Attempts have been made to obtain further insight into the Cu$^{\text{II}}$ substrate activation process that is involved in enzymatic catalysis by studying Cu$^{\text{II}}$ flavonolate complexes of the form Cu$^{\text{II}}$L(fla), where L is a chelating nitrogen donor ligand. Interestingly, a comparison of the rates of dioxygenation for representative Cu$^{\text{II}}$, Cu$^{\text{I}}$ and Zn$^{\text{II}}$ model complexes (Table 37) shows that the transition metal-containing complexes react with O$_2$ three orders of magnitude slower than potassium flavonolate under similar conditions and that there is little difference between the rates observed for similar Cu$^{\text{II}}$, Cu$^{\text{I}}$, and Zn$^{\text{II}}$ complexes. For each of these reactions, a second order rate constant for the dioxygenation reaction was obtained from the rate law $k_2[\text{fla}][O_2]$, showing that a bimolecular reaction with O$_2$ is the rate-limiting step.

Thus, coordination of the flavonolate to a transition metal decreases its reactivity towards O$_2$. This can be rationalized by considering that coordination to a metal will lower the energy of the flavonolate HOMO through bonding interactions with the metal (a Lewis acid interaction, in the case of Zn$^{\text{II}}$ or Cu$^{\text{I}}$), making the flavonolate harder to oxidize. Therefore, the coordinated flavonolate will be less reactive towards the single electron transfer mechanism that leads to dioxygenation of the free flavonolate. It is possible that the reported reactivity of these complexes is due to ligand dissociation, yielding a small amount of free flavonolate in solution which then reacts with O$_2$, a mechanism that has not been directly tested.

This leads to an interesting issue as to the nature of the substrate activation performed by the 2,4-QD enzyme and how the enzyme differs from the Cu$^{\text{II}}$ model complexes, such that coordination to Cu$^{\text{II}}$ in the enzyme activates the flavonol substrate rather than deactivating it by making the flavonolate harder to oxidize. A key structural difference between the 2,4-QD ES complex and known Cu$^{\text{II}}$ flavonolate model complexes may contribute to this difference. The model complexes all bind flavonolate as a bidentate ligand via the C3 hydroxyl and the C4 carbonyl groups, in contrast to the enzyme, which binds the substrate as a monodentate ligand. These different coordination modes will lead to different orbital interactions and monodentate coordination may lead to an electronic structure that is more favorable for O$_2$ attack. Pap et al. argue that a monodentate or asymmetric bidentate coordination mode will increase the radical character on the flavonolate ligand, allowing direct reaction with O$_2$ without requiring an initial slow outer-sphere electron transfer as in the free flavonolate reaction.\textsuperscript{1135}

Additionally, some Cu$^{\text{II}}$ flavonolate model complexes, typically those with phenanthroline or bipyridyl ligands or of the form Cu$^{\text{II}}$L(fla)$_2$, perform a different dioxygenation reaction from the enzyme.\textsuperscript{1137} These yield an O-benzoylsalicylic acid product, produced by a 2,3-rather than a 2,4-dioxygenation of 3-hydroxyflavone. This alternative dioxygenation
proceeds through a 1,2-dioxetane intermediate which decays via chemiluminescence, rather than the endoperoxide intermediate proposed for the enzyme. Thus, there are two possible dioxygenation pathways from a proposed intermediate alkylperoxide: attack on the C4 carbonyl, leading to an endoperoxide intermediate, or peroxidation of the double bond by attack on C3 to form a 1,2-dioxetane intermediate (Figure 213). The factors in the active site of 2,4-QD that direct the enzymatic reaction toward the endoperoxide rather than the 1,2-dioxetane route (and therefore perform 2,4-dioxygenase rather than 2,3-dioxygenase chemistry) are an interesting topic for future study.

**4.2 Cofactor Biogenesis in the Copper Amine Oxidases**

**4.2.1 Enzymology**—The copper-containing amine oxidases are a family of enzymes found in all forms of life excepting *Archaea* (EC 1.4.3.21 and EC 1.4.3.22; note that not all amine oxidases are copper-containing). Amine oxidase (AO) performs the oxidation of primary amines and diamines to aldehydes and ammonia, producing H$_2$O$_2$ as a byproduct (Figure 214). AO is a quinoprotein, containing a protein derived organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (topaquinone, TPQ), which is the key reactive functionality of the active site. The active site also contains a Type 2 or normal copper site. A wide variety of roles have been discovered for AO in biochemical processes. In prokaryotes and yeast, AO allows the organism to use amines as the sole source of carbon and nitrogen for cell growth. In plants, AO is associated with cell wall formation and wound healing. In mammals, the role of AO is less clear and multiple roles are likely, including amine metabolism, cell signaling (via H$_2$O$_2$), and amine-promoted apoptosis. High levels of AO in serum are associated with a variety of diseases, including type 1 and type 2 diabetes, liver disease, hypertension, atherosclerosis, and congestive heart failure. AO is being investigated as a treatment for anaphylaxis (for its anti-histamine properties) and has been considered as an antitumor agent (due to its role in apoptosis). AO has been isolated from eubacteria (*Escherichia coli* ECAO, *Arthrobacter globiformis* AGAO), yeasts (*Hansenula polymorpha*, HPAO; *Pichia pastoris*, PPLO), plants (pea seedling, PSAO; lentil seedling, LSAO), and mammals (bovine serum, BSAO; pig kidney, PKAO; human vascular adhesion protein-1, VAP-1; human diamine oxidase, hDAO). AOs from different sources have relatively low sequence similarity, despite having similar tertiary and active site structures (vide infra). The sequence similarity typically falls in the range of 20–30% of residues identical, excepting BSAO and VAP-1, which have 84% of residues identical. However, several key active site residues are strictly conserved in all AOs, including a consensus sequence containing the covalently bound cofactor (Thr-X-X-Asn-Tyr-(TPQ)-Asp/Glu-Tyr), an Asp proposed to be the active site base involved in catalysis, and a Tyr residue that hydrogen bonds to the cofactor.

It was recognized early in the study of AOs that the enzymes contain a novel chromophore, a cofactor that contains a carbonyl group that reacts with phenylhydrazine. This chromophore was originally proposed to be the known cofactor pyridoxyl phosphate, but this was ruled out by comparison of the resonance Raman spectra of the phenylhydrazine...
Following the suggestion that the cofactor was a quinone, a covalently bound form of pyrroloquinoline quinone (PQQ) was proposed, since protein digestion of phenylhydrazine-derivatized protein gave a low molecular weight molecule identical by HPLC to the phenylhydrazine derivative of pyrroloquinoline quinone. However, attempts to confirm this identity for the cofactor were unsuccessful, and finally Janes et al. were able to isolate the cofactor-containing peptide fragment and show by MS and NMR that the cofactor was not PQQ but rather the wholly novel protein-derived cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ). Subsequent work by Mu et al. showed that the cofactor is formed through a post-translational modification reaction (cofactor biogenesis) where a precursor Tyr residue is modified to TPQ. It was further demonstrated that cofactor biogenesis of TPQ from the precursor Tyr occurs by a self-catalyzed reaction involving the mononuclear Cu$^{II}$ site and O$_2$ rather than by a chaperone protein.

The active site of AO therefore performs two quite different reactions: the first turnover (the cofactor biogenesis reaction), which processes Tyr to TPQ, and, after the cofactor has been formed, the catalytic oxidation of amines. The cofactor biogenesis reaction involves substrate activation by Cu$^{II}$, while in catalytic turnover the copper site plays a role in activating O$_2$ for reduction during regeneration of the cofactor. A comprehensive review of cofactor biogenesis in a series of enzymes is presented in this thematic issue by Klinman et al. Here we focus on the biogenesis reaction as it relates to the broad theme of substrate activation by an oxidized Cu$^{II}$ active site.

The catalytic turnover of AO proceeds in two half reactions, the reductive half reaction, in which amines are converted to aldehydes and the TPQ cofactor is reduced to an aminooquinol (TPQ$_{N\text{-red}}$, Figure 215A) and the oxidative half reaction, in which O$_2$ reacts with the active site to regenerate the oxidized TPQ cofactor and release hydrogen peroxide and ammonia (Figure 215B). The mechanism of the reductive half reaction is well established. The primary amine substrate reacts with the C5 carbonyl of TPQ, forming a Schiff base. Deprotonation of the α carbon of the amine substrate converts this to a product Schiff base, reducing the cofactor. Hydrolysis of this Schiff base releases the product aldehyde, leaving TPQ$_{N\text{-red}}$. There is continuing controversy, however, over the mechanism of the oxidative half reaction and the role that the copper site plays in this process. The resting TPQ$_{N\text{-red}}$ site obtained after reduction of the protein by the substrate amine exists in equilibrium between a Cu$^{II}$-aminooquinol form and a Cu$^{I}$-semiquinone form (Figure 215B), which can be observed directly in the absorption and EPR spectra of the resting substrate reduced AO from certain sources (PSAO, AGAO) but is not observed in AOs from other sources (HPAO, BSAO). In AOs where the Cu$^{I}$-semiquinone form has been observed, a mechanism for the reductive half reaction has been proposed where O$_2$ is activated by the Cu$^{I}$ site in the Cu$^{I}$-semiquinone form of the reduced site and subsequently oxidizes the cofactor back to TPQ$_{ox}$, releasing NH$_3$. Single turnover kinetics experiments on the reaction of O$_2$ with amine-reduced AGAO, performed by Shepard et al., confirm that the Cu$^{I}$-semiquinone reacts rapidly with O$_2$ and oxidation of the aminooquinol cofactor proceeds through the Cu$^{I}$-semiquinone. In contrast, the reaction of O$_2$ with amine-reduced HPAO, where the Cu$^{I}$-semiquinone form is not observed, proceeds at the same rate in Cu$^{II}$-reconstituted and Cu$^{II}$-reconstituted enzyme. This suggests that the oxidation of the cofactor proceeds by an outer sphere electron transfer to O$_2$, with the metal site later
involved in stabilizing the superoxide generated in this step. Thus, there is ongoing controversy as to whether the oxidative half-reaction in the catalytic turnover of AO proceeds by an inner sphere or an outer sphere mechanism and whether copper is involved in redox chemistry or just provides a charge stabilization effect.

Lysyl oxidase (LOX) is a specific AO involved in the cross-linking of collagen and elastin during the maturation of connective tissue. LOX has been purified from *Drosophila*. LOX from *Drosophila* has been shown to be a 38 kDa monomeric protein that contains a similar mononuclear copper active site to other AOs but contains a different covalently-bound cofactor, lysine tyrosylquinone (LTQ). As with other AOs, the biogenesis of the cofactor is a self-processing reaction requiring CuII and O2 to transform a Tyr and a Lys residue to LTQ. The mechanism of LTQ biogenesis is closely related to that of TPQ biogenesis and very little independent study of it has been performed. Instead, the majority of studies have been done on TPQ biogenesis and can be related to LTQ biogenesis (vide infra).

Protein-derived cofactors have also been observed in other enzyme systems. Most are crosslinked amino acid residues closely associated to a metal site, like the Cys-His crosslink found in some binuclear copper proteins (mollusc hemocyanin, plant catechol oxidase and fungal tyrosinase, mentioned above), the His-Tyr crosslink in the active site of CcO and the Tyr-Cys radical cofactor in GO (vide supra). These crosslinked cofactors can perform a structural role in the protein or perform redox chemistry in catalysis by donating or accepting an electron (CcO, GO). Quinone cofactors like TPQ and LTQ add an electrophilic site in the protein and are involved in the catalysis of reactions with amines. These are the only quinone cofactors that do not require an external mechanism but instead have self-catalyzed biogenesis reactions involving CuII. This copper dependent biogenesis reaction is the focus of this section.

4.2.2 Kinetics—Apo-AO (protein lacking TPQ and copper cofactors) can be prepared in metal free conditions and is isolated with the precursor Tyr instead of with TPQ. The apo enzyme can be reconstituted with divalent metals to study the kinetics of TPQ biogenesis (apoAO reconstituted anaerobically with CuII is referred to as “preprocessed AO” and contains the copper cofactor but lacks TPQ). Defining the preprocessed CuII site and the initial reaction of this site with O2 are key to understanding the nature of the substrate activation involved in AO cofactor biogenesis.

The stoichiometry of the TPQ biogenesis reaction has been measured by Ruggiero et al. using a spectrophotometric assay to monitor H2O2 production (measuring formation of a quinoneimine dye) and monitoring O2 consumption using a Clark electrode. These experiments confirmed that 2 moles O2 were consumed per TPQ produced and 1 mole H2O2 was generated in the process. Nakamura et al. have determined that the C2 carbonyl of TPQ is derived from solvent, because the stretching frequency of this carbonyl observed in the resonance Raman spectrum shifts when the biogenesis reaction is performed in H218O. The origin of the C5 carbonyl could not be determined, because this oxygen undergoes exchange with the solvent, but it is presumed to come from O2. Ruggerio et al. have also demonstrated that the rate of TPQ biogenesis is not affected by the concentration of CuII.
above stoichiometric amounts, indicating that only copper bound in the active site participates in the biogenesis reaction.\textsuperscript{1,181} In some biogenesis experiments reported in HPAO, only a third of the available sites are oxidized to TPQ. This may reflect incomplete copper loading in these experiments (\textit{vide infra}).\textsuperscript{1,182}

Initially, it was assumed that Cu\textsuperscript{II} was the redox state active in TPQ biogenesis because, upon reconstitution of the apo-enzyme with Cu\textsuperscript{II} in the presence of O\textsubscript{2}, the TPQ biogenesis reaction proceeds spontaneously.\textsuperscript{1,166} Cu\textsuperscript{II} has been confirmed to be the active redox state by Samuels and Klinman by comparing the rate of TPQ biogenesis in apo-HPAO reconstituted with Cu\textsuperscript{II} with the rate observed in apo-HPAO reconstituted with Cu\textsuperscript{I}.\textsuperscript{1,183} When apo-HPAO is reconstituted with Cu\textsuperscript{II} and exposed to air, TPQ forms with $k_{TPQ} = 0.08\pm0.03 \text{ min}^{-1}$, while when the apo enzyme is reconstituted with Cu\textsuperscript{I}, the rate of biogenesis is slower by a factor of 17, with $k_{TPQ} = (4.7\pm0.2)\times10^{-3} \text{ min}^{-1}$. Samuels and Klinman also used EPR spectroscopy to monitor the redox state of Cu during the biogenesis reaction of Cu\textsuperscript{I}-reconstituted HPAO. No Cu\textsuperscript{II} intermediate species is observed and the rate of oxidation to Cu\textsuperscript{II} obtained from the EPR experiment is identical to the rate of TPQ formation, indicating that oxidation of Cu\textsuperscript{I} to Cu\textsuperscript{II} is rate-limiting. At the end of the reaction, only 26% of the active site has been transformed to TPQ, which corresponds 1:1 with the amount of loaded Cu\textsuperscript{I} that has been oxidized to Cu\textsuperscript{II}. This result further confirms that oxidation from Cu\textsuperscript{I} to Cu\textsuperscript{II} is required for the biogenesis reaction to proceed. This definitively establishes that the biogenesis reaction involves a Cu\textsuperscript{II} substrate activation process.

During the biogenesis of preprocessed HPAO, an intermediate with an absorption band at 350 nm ($\epsilon \approx 3200 \text{ M}^{-1} \text{ cm}^{-1}$) is observed.\textsuperscript{1,184} The intermediate forms with a rate constant of $0.51\pm0.01 \text{ min}^{-1}$ and decays with a rate constant of $0.076\pm0.003 \text{ min}^{-1}$, which is identical to the rate of TPQ formation in air ($k_{TPQ} = 0.08\pm0.03 \text{ min}^{-1}$). An isosbestic point is observed between the 350 nm intermediate and the 480 nm band of the product TPQ, indicating that the decay of the 350 nm intermediate is rate-limiting in TPQ formation. The amount of the 350 nm intermediate that accumulates is greater by a factor of 3 when the biogenesis reaction is performed at pH 8.5 relative to when it is performed at pH 7. This is associated with a 5-fold increase in the rate of biogenesis going from pH 7 to pH 9, with a pK$_a$ of 8.45. The 350 nm intermediate has been tentatively assigned as a Cu\textsuperscript{II}-tyrosinate species (\textit{vide infra}) and the pK$_a$ of 8.45 attributed to the precursor Tyr, deprotonation of which would lead to greater accumulation of a Cu\textsuperscript{II}-tyrosinate species. Despite this central role proposed for the 350 nm intermediate in biogenesis in HPAO (its decay is rate-limiting and it is assigned as the key intermediate in Cu\textsuperscript{II} substrate activation, see section 4.2.5), the 350 nm intermediate is not observed in biogenesis in AGAO.\textsuperscript{1,181} The rate of biogenesis is also an order of magnitude slower in HPAO than in AGAO ($k_{TPQ}$ (HPAO) = 0.16\pm0.008 \text{ min}^{-1}, $k_{TPQ}$ (AGAO) = 1.5\pm0.2 \text{ min}^{-1}) at saturating O\textsubscript{2}. The dependence of $k_{TPQ}$ on the concentration of O\textsubscript{2} (\textit{vide infra}) accounts for the difference between the value of $k_{TPQ}$ (HPAO) = 0.08\pm0.03 \text{ min}^{-1} reported above for experiments in air and the value for $k_{TPQ}$ (HPAO) reported for saturating O\textsubscript{2} ($k_{TPQ} = 0.16\pm0.008 \text{ min}^{-1}$). Since the rate of TPQ formation is faster in AGAO than in HPAO, DuBois and Klinman suggest that the 350 nm intermediate may occur in AGAO, but on a faster timescale that could potentially be observed with stop-flow techniques.\textsuperscript{1,185} Another option is that the relative rates of different
steps in the biogenesis reaction may differ between HPAO and AGAO, so that the 350 nm species does not accumulate in experiments with AGAO. The observation of this key intermediate in biogenesis in HPAO but not in other AOs is an issue that remains to be resolved in refining the mechanism for TPQ biogenesis.

The effect of a range of variables on the kinetics of TPQ formation has been evaluated in order to identify the nature of the rate-limiting step in TPQ biogenesis or at least rule out certain possibilities. The consumption of O₂ during the reaction can be measured directly and exhibits single phase kinetics. Since two molecules of O₂ are consumed in this reaction, single phase kinetics for O₂ consumption indicates that reaction of the first molecule of O₂ is slow relative to reaction of the second O₂. Deuteration of the precursor Tyr ring at the C3 and C5 positions has no effect on the rate of biogenesis, which rules out decay of a proposed aryl-peroxide intermediate as a possible rate-limiting step (since this would involve deprotonation of the ring at the C5 position). These results are consistent with the proposal of Klinman et al. that the rate limiting step is attack of the first molecule of O₂ on the preprocessed site, after prebinding of O₂ to a binding pocket in the protein (required by a faster rate for O₂ consumption than TPQ formation, vide infra). A rate-limiting step of O₂ attack on a Cu⁺-tyrosinate species is also consistent with DFT calculations performed by Prabhakar and Siegbahn on the biogenesis reaction (see Section 4.2.5). The temperature dependence of the rate of biogenesis has been measured by Schwartz et al., who obtain an Arrhenius activation energy of $E_a = 8.4\pm0.5$ kcal/mol for the rate-limiting step in TPQ biogenesis. Definitive identification of the rate-limiting step is required in order to interpret the chemical significance of this activation energy.

In a variety of kinetic experiments performed on TPQ biogenesis, particularly those that measure the dependence of rates on the concentration of O₂, the biogenesis reaction is initiated by adding Cu²⁺ to a solution of the apo-enzyme that has been preequilibrated with a known concentration of O₂. This contrasts with the method previously described for studying biogenesis (vide supra), in which apoAO is anaerobically pre-loaded with Cu²⁺ and TPQ biogenesis is initiated by exposing the preprocessed enzyme to O₂. Several kinetic studies directly compare rate constants and kinetic parameters obtained by these two methods, which requires the assumption that Cu²⁺ binding to the active site of AO (an additional kinetic step in the aerobic Cu²⁺ addition method) is fast. To establish that this assumption is valid, Schwartz et al. report identical rates for TPQ biogenesis measured using the two methods, which implies that Cu²⁺ binding is fast relative to biogenesis. However, in biogenesis experiments initiated with Cu²⁺ addition, the 350 nm intermediate previously observed when preprocessed HPAO reacts with O₂ does not accumulate, suggesting that the rate limiting step changes when biogenesis is initiated by adding Cu²⁺ aerobically.

Instead, in experiments where TPQ biogenesis is initiated by adding Cu²⁺, a species with a 380 nm absorption band is observed to accumulate rapidly and decay with a rate constant of $k_{380} = 0.06\pm0.01 \text{ min}^{-1}$, which is identical to the rate of TPQ formation ($k_{\text{TPQ}} = 0.08\pm0.03 \text{ min}^{-1}$ under these conditions). However, this species is observed when copper is added to HPAO even when the active site is already occupied by Cu²⁺ or Zn²⁺. In addition, in mutated active site variants of HPAO in which the rate of biogenesis is significantly
decreased, the kinetics of growth and decay of the 380 nm species are not altered. Therefore, Dove et al. conclude that the 380 nm species is not involved in the biogenesis reaction since it is not an active site copper species. DuBois and Klinman have subsequently proposed that the 380 nm absorption feature may be due to a transient copper binding site involved in Cu loading into the active site. The rate of copper binding in AOs other than HPAO has also not been investigated and whether CuII binding contributes to the kinetic and spectroscopic data for TPQ biogenesis in AGAO is not known.

The rate of TPQ biogenesis is dependent on the concentration of O2. This dependence has been measured by DuBois and Klinman, enabling a comparison between the rates of TPQ formation and O2 consumption (CuII addition to apoHPAO was used to initiate biogenesis in these experiments). The dependence of kTPQ on the concentration of O2 is hyperbolic with kmax = kTPQ(sat’d O2) = 0.16±0.008 min⁻¹ and KM= 233±35 µM (these do not correspond to Michaelis-Menten kcat and KM values because the biogenesis reaction is a single-turnover process). The kinetics of O2 consumption during the biogenesis reaction have also been measured and saturation kinetics are observed with a kmax(O2) = kO2(sat’d O2) = 0.27±0.02 min⁻¹ and KM= 216±45 µM. An interesting feature of these results is that O2 is consumed faster than TPQ is produced. DuBois and Klinman propose that O2 binds in an O2 binding pocket before reaction with the preprocessed active site during TPQ formation. Another interesting observation that can be made from comparison to previous experiments is that the 350 nm intermediate is formed faster than O2 is consumed. If the 350 nm species is formed without the participation of O2 but is triggered by O2 being present (as proposed by Dove et al.), this suggests that the 350 nm intermediate is a CuII-tyrosinate complex formed due to an O2 triggered conformational change of the active site. However, the faster rate of 350 nm intermediate formation relative to O2 consumption could also be due to the different experimental conditions, since the O2 consumption was initiated by adding CuII to aerobic apoHPAO while the spectroscopic experiments were initiated by adding O2 to preprocessed HPAO with CuII preloaded.

Attempts to chemically trap a CuI-tyrosyl radical intermediate in biogenesis have been unsuccessful. Addition of DEANO (a reagent used to generate NO in situ) to the biogenesis reaction had no effect on TPQ formation, indicating that no tyrosyl radical accumulates during the reaction.

Several variants of first and second sphere active site residues have been used to gain insight into the cofactor biogenesis reaction. Mutation of His ligands at the CuII site (vide infra) to non-coordinating residues in AGAO (H431A, H433A, H592A) leads to no formation of TPQ, underlining the importance of the copper site in TPQ biogenesis. A variant of one of these His ligands to Cys in HPAO (H624C) does perform TPQ biogenesis, but at a greatly reduced rate (kTPQ = 0.07±0.04 hr⁻¹). The intermediate previously observed in WT HPAO also accumulates in H624C, but the absorption band is shifted to 390 nm with an order of magnitude slower formation and decay (kform = 0.042±0.003 min⁻¹, kdecay = kTPQ = 1.2×10⁻³ min⁻¹). Interestingly, the mutation of a second sphere Met residue which forms part of the putative O2 binding pocket in the holo enzyme (M634) shifts the intermediate absorption maximum to energy as well as retarding the rate of biogenesis. The magnitude of this effect on the rate is linearly related to volume of the hydrophobic

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sidechain that replaces M634, which DuBois and Klinman interpret to mean that M634 forms part of binding pocket for O₂ in the active site. Finally, mutation of a conserved active site Tyr residue, which H-bonds to the TPQ cofactor in the holo enzyme (Y305A, Y305F), results in an alternative product for the biogenesis reaction, identified by an absorption feature at 400 nm. The formation of TPQ relative to the alternative product is pH dependent, with TPQ primarily formed at low pH, while the 400 nm product forms at high pH. The 400 nm product exhibits no reactivity with phenylhydrazine and is, therefore, not a quinone. The 400 nm species was originally proposed to be an alkyl-peroxide and it has recently been confirmed by X-ray crystallography that the product is a doubly-peroxidated ring (see Section 4.2.3).

Contrary to early reports about TPQ biogenesis, metals other than copper are also competent to perform the reaction, albeit at greatly reduced rates. Co²⁺, Ni²⁺, and Zn²⁺ bind tightly to apo-AO and cannot be displaced by subsequently added Cu²⁺. Zn²⁺ is inert with respect to TPQ biogenesis, but TPQ is slowly formed in Co²⁺-AGAO and Ni²⁺-AGAO in the present of saturating O₂ with \( k_{TPQ}(Co^{II}) = 0.079\pm0.002 \) hr⁻¹ and \( k_{TPQ}(Ni^{II}) = 0.075\pm0.001 \) hr⁻¹. Biogenesis with an alternate metal is also observed in Ni²⁺-HPAO with \( k_{TPQ}(Ni^{II}) = 0.044\pm0.001 \) hr⁻¹ and Ni remains EPR-silent throughout the reaction. The rate for TPQ biogenesis with an alternate metal is over three orders of magnitude slower than the rate of biogenesis with Cu²⁺ and thus could be due to trace copper in the metal salt. Okajima et al. address this by using high purity Co²⁺ and Ni²⁺ salts and additionally showing that intentional doping of 5% Cu²⁺ in the Co²⁺ or Ni²⁺ does not perturb the rate of biogenesis, indicating that trace Cu²⁺ is not responsible for the observed reaction. Instead, the large difference in rates may indicate that biogenesis proceeds by a different mechanism with other metals. It is interesting to note that redox activity may not be necessary for cofactor biogenesis, since Ni²⁺ and Co²⁺ will perform the reaction, but the metal must also be more than just a Lewis acid, since Zn²⁺ is inert. The nature of substrate activation by metals, and particularly by Cu²⁺, which is most active in this reaction, becomes an even more interesting question in light of these results.

To summarize, the kinetics studies that have been performed on TPQ biogenesis have shown that; a) Cu²⁺ performs this reaction, not Cu¹⁺, and Cu²⁺ is significantly better than other divalent metals; b) an intermediate can be observed in biogenesis in HPAO that is proposed to be a tyrosinate Cu²⁺ LMCT species; c) decay of this intermediate is the rate limiting step in biogenesis and d) O₂ prebinding in an O₂ binding pocket occurs during biogenesis. The rate-limiting step of biogenesis must involve the first equivalent of O₂ but is not the decay of an aryl-peroxide. These kinetics suggest that the ratelimiting step of cofactor biogenesis is the step involving the Cu²⁺ substrate activation process. Spectroscopic and structural studies that identify the observed intermediate and the rate-limiting step would be valuable in elucidating the mechanism. In addition, differences observed in the kinetics of biogenesis between HPAO and AGAO and between the two different methods for studying biogenesis in HPAO (Cu²⁺ initiated and O₂ initiated) need to be resolved. Additionally, Cu²⁺ binding to the protein could have a significant effect on some of these kinetics results but has yet to be thoroughly investigated.
### 4.2.3 Structure

X-ray crystal structures of copper amine oxidases have been reported from a variety of organisms, including from eubacteria (e.g. AGAO), yeasts (e.g. HPAO), plants (e.g. PSAO) and mammals (BSAO and VAP-1). The majority of these crystal structures are of the processed enzyme, including wild-type and mutared variant structures, inhibitor complexes, and structures with Xe to characterize potential O$_2$ channels. Only the few crystal structures relevant to understanding the Cu$^{II}$ substrate activation involved in cofactor biogenesis will be discussed here. A summary of the available crystal structures of processed copper amine oxidases is presented in Table 38.

The overall protein structure of AO is similar in all crystal structures, regardless of the state of the active site (apo, preprocessed, or processed). The significant sequence differences between AOs from different sources do not affect the overall fold of the enzyme or the structure of the active site. AO is a homodimer, with each monomer containing three subunits (Figure 216). The largest subunit, containing 394 residues in AGAO, is an 18-stranded $\beta$ sandwich (colored in light blue and yellow). The smaller subunits (100 and 82 residues in AGAO) pack on the surface of the large subunit, in slightly different positions depending on the source organism. Two $\beta$ ribbon arms extend from each monomer to the surface of the large subunit of other monomer, stabilizing the dimer. The active site is buried in the large subunit, near to a large, solvent filled cavity on the dimer interface and close to where the $\beta$ ribbon arm from the other monomer is positioned.

In processed AO, two possible conformations of the TPQ cofactor are observed. In the on-Cu conformation, TPQ is a ligand to the copper site through its C4-OH group and three His residues additionally coordinate to Cu, two Ne-coordinated and one N$\delta$-coordinated (Figure 217B). The geometry of the copper site in this conformation is tetrahedral distorted towards trigonal pyramidal (where the Cu-O$_{TPQ}$ bond length is long, 2.4 Å). AO obtained by dissolving these crystals is inactive. The alternative conformation of TPQ, observed in active enzyme, involves rotations around the C$\alpha$-C$\beta$ and C$\beta$-C$\gamma$ bonds so that TPQ is positioned away from the copper into a pocket in the active site lined by Tyr284, Asp298, and Tyr296 (Figure 217A, residue numbers from AGAO). In this conformation, TPQ hydrogen bonds with Tyr284, a conserved residue which has been shown to be chemically important (vide infra), and the reactive C5=O carbonyl of TPQ is positioned towards the substrate entrance channel gated by Tyr296 and towards Asp298, the proposed active site base. In this active conformation, the copper site is bound by the same three His residues and additionally two solvent-derived ligands in a square pyramid. These general features of the active site of processed AO have been observed in AOs from all the source organisms studied, although the number of localized solvent molecules bound to the copper site varies between different structural determinations. In some AGAO structures, two conformations are observed for the residue His592, which is N$\delta$-coordinated to the copper. This conformational flexibility, which is not observed in AOs from other sources, is proposed to be due to incomplete occupancy of the site by copper and likely is not significant.

Several crystal structures relevant to AO cofactor biogenesis have been obtained and are summarized in Table 39.
These include structures of apo AO (lacking both Cu$^{II}$ and TPQ, from AGAO and HPAO$^{1194,1230}$ and of apo AO reconstituted with Zn$^{II}$, Co$^{II}$, and Cu$^{II}$ (metals that do not catalyze the biogenesis reaction or, in the case of Co$^{II}$, perform it slowly) showing the position of the precursor Tyr residue in the presence of a metal cation. Two different stages of the biogenesis reaction have been directly observed in crystals of preprocessed AGAO (containing Cu$^{II}$ but prior to TPQ biogenesis); one of the preprocessed enzyme with Cu$^{II}$ anaerobically bound and one of a dopaquinone (DPQ) intermediate in the biogenesis reaction.$^{1195}$ In addition, crystal structures have been obtained of the HPAO variant Y305F, which reacts with O$_2$ but does not form the mature TPQ cofactor and instead shows a unique partially processed form of the substrate Tyr residue where the ring is multiply-peroxidated,$^{1190}$ and the AGAO variant D298K, which forms the LTQ cofactor that is present in lysyl oxidase.$^{1233}$ The overall structure of the AO enzyme in these forms is the same and the only significant differences are observed at the active site.

The crystal structure of apoAO, lacking Cu$^{II}$ and TPQ, was first reported by Wilce et al. and later by Klema et al. for HPAO (see Table 39).$^{1194,1234}$ In these structures, the precursor Tyr residue (Tyr382 in AGAO) is pointing towards the site that will bind Cu$^{II}$ (defined by the 3 His residues that are copper ligands in the processed enzyme, Figure 218A). From the apo structure, it appears that the ligands are positioned so that copper binding will result in a tetrahedral site in which the precursor Tyr residue is coordinated to Cu$^{II}$. This geometry is indeed observed in the active site of preprocessed AOs reconstituted with Zn$^{II}$ and Co$^{II}$, where the metals are coordinated by the 3 His residues and the precursor Tyr in a tetrahedral geometry. In the Zn$^{II}$ and Co$^{II}$ structures, the metal-Tyr distances are clearly consistent with a M$^{II}$-phenolate bond (2.02 Å and 1.87 Å for the two Zn$^{II}$ structures$^{1231}$ and 2.19 Å for the Co$^{II}$ structure,$^{1234}$ Table 39). However, in the sole available crystal structure of apo AGAO reconstituted anaerobically with Cu$^{II}$ (1.9 Å resolution), the copper is bound to the three His residues and the precursor Tyr points towards Cu$^{II}$, but the Cu-O distance of 2.58 Å is too long to be described as a Cu-phenolate bond and the geometry of the site is not tetrahedral (Figure 218B).$^{1195}$ Several explanations have been proposed for the long Cu-O distance observed in the anaerobic Cu$^{II}$ bound structure. This may reflect reduction to Cu$^{I}$ in the experiment, although at low temperature in the crystal the tyrosine should be limited in its ability to change position. This hypothesis is supported by a crystal structure obtained for a Cu$^{I}$ loaded form of preprocessed HPAO, which shows a very similar site geometry to that reported by Kim et al. for Cu$^{II}$ in preprocessed AGAO.$^{1230}$ Alternatively, the long Cu$^{II}$-tyrosine distance may reflect the tyrosine remaining protonated at the pH used for crystallography (pH 6.8, pK$_a$(Tyr) $\approx$ 10), resulting in a weak Cu$^{II}$-tyrosine interaction rather than a Cu$^{II}$-tyrosinate bond, although the presence of a bond with Cu$^{II}$ would be expected to drive the deprotonation of Tyr. Finally, it could be that tyrosine is not a copper ligand in anaerobic preprocessed AO and only binds to copper after O$_2$ is added to initiate cofactor biogenesis, a hypothesis supported by the possible assignment of the 350 nm intermediate observed by Klinman et al. as a Cu$^{II}$-tyrosinate species ($\textit{vide supra}$). Further resolution of the structure of anaerobic Cu$^{II}$-bound preprocessed AO by complimentary spectroscopic studies is necessary to unambiguously determine the starting point of the biogenesis reaction, in which the tyrosine is activated for reaction with O$_2$. 

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Further insight into TPQ biogenesis can be obtained from crystal structures that contain partially processed forms of the cofactor. When crystals of the preprocessed Cu-bound enzyme from AGAO are briefly exposed to O₂ and then frozen, it is observed that 50% monooxidation of the tyrosine ring has occurred (from 50% occupancy of an oxygen atom at C5 of the ring), generating a dihydroxyphenylalanine or dopaquinone intermediate (Figure 218C), the oxidation state of which cannot be determined due to the low resolution of the crystal structure (2.1 Å). The C4-O group of the intermediate is directed towards Cu, possibly indicating that it is bound to the copper site, but again the Cu-O distance is long for a bond (2.6 Å). After longer exposure to O₂, the crystals contain a ring with oxygen atoms at the C2, C4, and C5 positions and coordinates to the Cu²⁺ site in the geometry observed for the inactive conformation of TPQ with a Cu-O bond length of 2.3 Å (Figure 218D). A short distance is observed between the C2-O group of the ring and Thr403 (O-O 2.3 Å) that has been described as a hydrogen bond. This together with the absence of an absorption feature at 480 nm in this crystal suggest that the ring, while oxygenated, has not yet been oxidized from the reduced (TPQ₁₀₀) to oxidized (TPQ₉₀) form of the cofactor in this crystal. The crystals could be further incubated with O₂ to generate the processed active site with the active off-Cu conformation of TPQ and the 480 nm absorption band corresponding to TPQ₉₀ (Figure 218E). Therefore, Kim et al. propose that oxidation of the cofactor is required to promote the conformational change from the inactive on-Cu (Figure 218D) to the active off-Cu TPQ conformation (Figure 218E). However, since independent evidence shows that the fully oxidized cofactor can adopt an on-Cu conformation and that enzyme exhibiting this structure is inactive, this conformation may not be relevant to cofactor biogenesis.

Partially processed forms of TPQ have also been observed in some AO variants. A crystal structure of the Y305F variant of HPAO (Tyr305 in HPAO corresponds to Tyr284 in AGAO) has been reported showing that an alternative product is obtained in the biogenesis reaction. Instead of the quinone ring formed in the native biogenesis reaction, a doubly peroxidated ring is present in this variant. The crystal structure data required the presence of two different peroxidated ring structures, each at 50% occupancy: 5-hydroxo-2,4-peroxophenylalanine and 5-hydroxo-3,4-peroxophenylalanine. In addition, a second sphere residue near the copper site, Met634, had been oxygenated. These results indicate that the absence of Y305 blocks cleavage of the O-O bond in biogenesis, leading to uncontrolled oxidation of the precursor Tyr. Another mutation performed in AGAO, D298K, allows trapping of covalent trapping of the dopaquinone intermediate in biogenesis. Nucleophilic attack of the variant Lys residue on the dopaquinone intermediate leads to formation of a crosslink similar to the one present in lysyl oxidase. This is proposed to be the mechanism for formation of LTQ, the lysyl oxidase cofactor, since lysyl oxidase does contain a Lys residue at the appropriate position in its sequence.

### 4.2.4 Spectroscopy and Electronic Structure

Three key species in AO cofactor biogenesis are targets for spectroscopic study: anaerobic preprocessed AO, the 350 nm intermediate, and processed AO. Spectroscopy of the anaerobic preprocessed enzyme can give an electronic structure description of the species that reacts with O₂ to initiate cofactor biogenesis. A key intermediate in the biogenesis reaction, defined by an absorption feature...
at 350 nm, can be characterized by spectroscopy to reveal its geometric structure and thus its role in the biogenesis reaction. Despite the importance of a spectroscopic and electronic structure definition of these early stages of biogenesis to define that nature of the activation of the precursor Tyr by Cu$^{II}$, relatively little spectroscopic characterization of these first two species has been performed. Alternatively, a large amount of spectroscopic characterization has been performed on the processed enzyme, which provides a starting point for understanding the limited spectroscopic data available for the preprocessed enzyme.

The copper site in processed AO had been elucidated by spectroscopy well before the first crystal structure of the enzyme was published. The EPR spectrum of the copper site in a resting processed AO was first reported by Yamada et al. in 1963. The spectrum was further analyzed by Suzuki et al., who reported an axial EPR spectrum with $g_{∥} = 2.29$, $g_{⊥} = 2.06$, $A_{∥} = 175 \times 10^{-4}$ cm$^{-1}$. These results are consistent with more recently reported EPR parameters for processed AGAO (Figure 219C, red line, $g_{∥} = 2.29$, $g_{⊥} = 2.07$, $A_{∥} = 187 \times 10^{-4}$ cm$^{-1}$), BSAO ($g_{∥} = 2.28$, $g_{⊥} = 2.06$, $A_{∥} = 172 \times 10^{-4}$ cm$^{-1}$), and HPAO ($g_{∥} = 2.30$, $g_{⊥} = 2.06$, $A_{∥} = 163 \times 10^{-4}$ cm$^{-1}$). The EPR spectrum of BSAO by Suzuki et al. also showed superhyperfine coupling in the $g_{⊥}$ region with 2–3 $^{14}$N donor ligands (5–7 lines, $A_{N} = 14$ G). EXAFS studies on BSAO identified 3–4 histidine donor ligands based on a comparison to Cu(imid)$_4^{2+}$, and a square pyramidal structure with three equatorial His ligands and two water ligands was proposed for the processed copper site. Studies using pulsed EPR techniques (ENDOR and ESEEM) agreed with this structure and further determined that the equatorial water ligand is more tightly bound than the axial water ligand. The optical absorption spectra of processed AO are dominated by contributions from the oxidized TPQ cofactor. The absorption feature at 460–480 nm (depending on the enzyme source) with $\varepsilon \approx 3300$ M$^{-1}$ cm$^{-1}$ can be assigned to the first $\pi \to \pi^*$ transition of the quinone cofactor and gives AO its characteristic pink color (Figure 219A, red). The CD spectrum of processed AGAO has a positive band at 370 nm and a negative band at 430 nm which are associated with TPQ$^{ox}$, while a broad negative band at 700 nm has been assigned as a d-d transition (Figure 219B, red). This spectroscopic analysis accurately predicted the structure of the copper site in processed AO, which was later confirmed by X-ray crystallography.

Preprocessed AGAO has been studied by absorption, CD, and EPR spectroscopy. The preprocessed site has no significant absorption feature (Figure 219A, black spectrum). The CD spectrum shows a negative band in the 700–800 nm region which has been assigned as a Cu$^{II}$ d-d transition, but the other d-d transitions of the site have not been resolved (Figure 219B, black). A feature at 380 nm in the reported CD spectrum of preprocessed AGAO is not reproduced in our unpublished work. The EPR spectrum of the preprocessed site is axial with $g_{∥} = 2.32$, $A_{∥} = 153 \times 10^{-4}$ cm$^{-1}$, $g_{⊥} = 2.07$ and superhyperfine coupling with 3 N donor ligands with $A_{N} = 14$ G (Figure 219C, black). Spectroscopic work recently performed in our lab suggests that the features that have been reported for the preprocessed site in fact have a significant contribution from copper bound to a secondary copper binding site in the protein. Thus, the contributions of several copper species must be resolved to obtain the spectroscopic features of the active site copper in preprocessed AO. This work is the focus of ongoing study.
Very little spectroscopy has been performed on the 350 nm intermediate observed in HPAO.\textsuperscript{1184} The intermediate has been identified as a Cu\textsuperscript{II} LMCT species because the absorption maximum shifts from 350 nm to 390 nm when the copper ligand His624 is mutated to Cys, suggesting that the copper site contributes to the electronic structure of the intermediate. Dove et al. note that the absorption feature of the intermediate ($\lambda_{\text{max}} = 350\text{ nm}$, $\varepsilon \approx 3200\text{ M}^{-1}\text{ cm}^{-1}$) is consistent with a variety of chemically plausible Cu\textsuperscript{II} species, including a Cu\textsuperscript{II}-tyrosinate complex, a Cu\textsuperscript{II}-superoxide, and a Cu\textsuperscript{II}-aryl peroxide.\textsuperscript{1184} Attempts to perform resonance Raman into the 350 nm band to identify the species were unsuccessful and trapping of the intermediate for study by other spectroscopic techniques has not been reported. While a tentative assignment of this intermediate as a Cu\textsuperscript{II}-tyrosinate has been made by ruling out the other plausible options using kinetics data, further spectroscopic investigation to confirm its identity would be valuable.\textsuperscript{1185}

In the absence of detailed spectroscopic and electronic structure information on either the anaerobic preprocessed site or the 350 nm intermediate, tetrahedral model complexes of the form $[\text{CuL(OPh-4-F)}]^+$, where L represents a tridentate N-donor ligand ($L_1 = \text{HB(3,5-iPr}_2\text{pz)}_3$ or $L_3 = \text{HB(3-tBu-5-iPr}_2\text{pz)}_3$), have been studied (Figure 220A and B, respectively).\textsuperscript{1239} These models closely represent the tetrahedral $[\text{Cu(His)}_3\text{Tyr}]^+$ structure that has been proposed for the preprocessed site based on X-ray crystallography and for the 350 nm intermediate, but do not display reactivity with O\textsubscript{2} (perhaps due to sterics). However, the Cu-phenolate bond lengths in these models are significantly shorter than what has been observed in the preprocessed enzyme\textsuperscript{1195} ($L_1\text{ Cu-O 1.731 Å, } L_3\text{ Cu-O 1.837 Å}$), confirming that the Cu-Tyr interaction in the preprocessed enzyme is not a tyrosinate-copper bond. The key difference between these two complexes is the orientation of the phenolate ring relative to the N-donor ligation (Figure 220). In the $L_1$ complex the phenolate ring is oriented perpendicular to the Cu-O-C plane, which is close to the orientation of the Tyr ring in the crystal structure of preprocessed AGAO obtained by Kim et al.,\textsuperscript{1195} while for $L_3$ the phenolate ring is approximately in the Cu-O-C plane. A summary of the spectroscopic features of these complexes is presented in Table 40 and the spectra are given in Figure 221 and Figure 222.

The rhombic splittings of the g values observed in the EPR spectra (Figure 221) reflect significant $dz^2$ mixing into the $dx^2-y^2$ ground states of these complexes (6.9% for $L_1$, 11.5% for $L_3$ from DFT calculations). The spin density found on the phenolate ring in the ground state wavefunctions, 14% for $L_1$ and 9% for $L_3$, reflect only a small amount of radical character on the phenolate ring resulting from binding to Cu\textsuperscript{II}. The different orientations of the phenolate ring lead to different orbital contributions to bonding in the $L_1$ and $L_3$ complexes. The LUMO of the $L_1$ complex has a $\pi$-bonding interaction between the $dx^2-y^2$ orbital on Cu\textsuperscript{II} and the phenolate out-of-plane (oop) $\pi$ valence HOMO (Figure 223A), while the $L_3$ complex has a $\sigma$-bonding interaction between the $dx^2-y^2$ orbital and the phenolate in-plane (ip) $\pi$ valence HOMO-2 (Figure 223B). Therefore, the nature of the phenolate-Cu\textsuperscript{II} bond depends on the orientation of the phenolate ring relative to the $dx^2-y^2$ orbital. This difference in bonding is reflected in the significant differences in the energies and intensities of the phenolate to Cu\textsuperscript{II} LMCT transitions in the two complexes. In the $L_1$ complex, the two phenolate to Cu\textsuperscript{II} LMCT transitions are split by 11,420 cm\textsuperscript{-1} (bands 6 and 10 in Figure 222).
left top) relative to a splitting of 23,166 cm\(^{-1}\) (band 8 and a band too low in energy to observe in Figure 222 right top) in the L3 complex. This large energy difference reflects the fact that in the L1 complex the phenolate oop orbital is lowered in energy by bonding, decreasing the energy splitting of the oop and ip phenolate orbitals, while in the L3 complex the energy of the phenolate ip orbital is lowered by bonding, increasing this energy splitting. The observed CT transitions (Figure 222) result from both of these phenolate donor interactions with the dx\(^2\)-y\(^2\) acceptor orbital. In addition, in the L1 complex the lower energy oop LMCT is the more intense transition (\(\varepsilon = 2363 \text{ M}^{-1} \text{ cm}^{-1}\) compared to \(\varepsilon = 842 \text{ M}^{-1} \text{ cm}^{-1}\) for the ip LMCT) because of better overlap between the oop phenolate HOMO with the singly occupied dx\(^2\)-y\(^2\) orbital, while in the L3 complex, the higher energy ip LMCT is more intense (from TD-DFT calculations, since the oop LMCT is predicted to be too low in energy to be experimentally observed in the L3 complex) due to better overlap of the ip phenolate HOMO-2 with the dx\(^2\)-y\(^2\) acceptor orbital in the CT process (Figure 223B). Thus in the absorption spectrum of the L1 complex (Figure 222 left top) the most intense transition is band 6, the oop phenolate to Cu CT, while in the absorption spectrum of the L3 complex (Figure 222 right top) the most intense transition is the ip phenolate to Cu CT, band 8.

The electronic structure description obtained from these model complexes leads to the following conclusions about preprocessed AO and the nature of Cu\(\text{II}\) substrate activation. First, the spectroscopy currently reported for preprocessed AO (\textit{vide supra}) is not consistent with the Cu(His)\(_3\)Tyr ligand set suggested by X-ray crystallography, since there is no intense LMCT feature in the preprocessed AO absorption spectrum and the EPR spectrum is axial rather than rhombic. This can also be deduced from the large difference in Cu-O bond length between the preprocessed site in the enzyme (2.58 Å) and the model complexes (1.731 Å and 1.837 Å). Insufficient spectroscopic information is available on the enzyme to fully evaluate whether this could be a reasonable structure for the 350 nm intermediate. However the similarity between the ring orientation of the L1 complex and the positioning of the precursor Tyr suggests that there should be an intense low energy phenolate to Cu CT in the enzyme, at an energy lower than the observed 350 nm band if this intermediate were a Cu(His)\(_3\)Tyr complex. Second, while some radical character is present in the ground state of Cu\(\text{II}\)-phenolate complexes, this radical character in itself is insufficient to allow the complex to react with O\(_2\). A lack of reactivity was predicted using DFT calculations, which give a large, positive \(\Delta G^0\) value for the formation of the Cu\(\text{I}\)-superoxoquinone complex, produced by O\(_2\) attack on the phenolate ring of the Cu\(\text{II}\) complex (51 kcal/mol for the L1 complex and 80 kcal/mol for the L3 complex).\(^{1239}\) These large \(\Delta G\) values reflect the energies of the phenolate to Cu\(\text{II}\) CT transitions, which reduce Cu\(\text{II}\) to Cu\(\text{I}\) (L1 14,730 cm\(^{-1}\) = 42 kcal/mol, L3 27,850 cm\(^{-1}\) = 80 kcal/mol), and the reaction of the resultant phenoxyl radical with O\(_2\). Therefore, additional studies are necessary to understand the ability of the Cu\(\text{II}\) site in preprocessed AO to catalyze a reaction between tyrosine and O\(_2\).

4.2.5 Molecular Mechanism—A mechanism for TPQ biogenesis in AO has been proposed based on the kinetic work of Klinman et al.\(^{1185}\) and the X-ray crystallography of Kim et al.\(^{1195}\) (Figure 224). Starting with the preprocessed site (1), O\(_2\) binds in a pocket in the protein (2) triggering a conformational change that causes the precursor Tyr to bind to.
Cu$^{II}$ (3A). This Cu$^{II}$-tyrosinate complex is thought to have some tyrosyl radical character (3B), so O$_2$ can react with the ring to form a Cu$^{II}$-coordinated peroxoquinone (4). O-O bond cleavage of this intermediate is promoted by Tyr305, and yields the crystallographically observed DPQ intermediate (5). Hydrolysis of DPQ is promoted by the Cu$^{II}$ site (6), yielding the reduced form of the TPQ cofactor (7) which can react directly with O$_2$ to generate fully oxidized TPQ (8).

Key steps of this mechanism have been evaluated by DFT calculations and studies of model complexes. Prabhakar and Siegbahn have calculated the reaction coordinate for this proposed mechanism, starting from the Cu$^{II}$-tyrosinate complex formed after O$_2$ binding to the protein.\[1187\] The complex has a spin population of 0.7 on the tyrosinate ring and only 0.3 on Cu, which suggests dominant tyrosyl radical character. O$_2$ reaction at Cu$^{I}$ in this species, forming a Cu$^{II}$-superoxide-tyrosyl radical complex, has a $\Delta G$ of 6.0 kcal/mol (including a loss of entropy upon binding O$_2$ worth 8.4 kcal/mol), which is 11.7 kcal/mol more favorable than direct reaction with the tyrosyl radical to form the Cu$^{I}$-superoxoquinone (transition states and barriers for this step were not reported). The barriers calculated for the succeeding steps are 8.4 kcal/mol for formation of the Cu$^{II}$-coordinated peroxoquinone, 11.4 kcal/mol for O-O bond cleavage, 16.0 kcal/mol for the hydration of DPQ, 12.6 kcal/mol for aromatization of hydrated DPQ to form TPQ$_{red}$, and 2.1 kcal/mol for oxidation of TPQ$_{red}$ to TPQ$_{ox}$ by O$_2$. According to these results, hydration of DPQ should be the rate-limiting step, but Prabhakar and Siegbahn argue that the barrier for this step is overestimated because the step as calculated did not involve direct attack by the Cu$^{II}$-coordinated hydroxide on the DPQ ring, but instead a long range proton transfer through a chain of water molecules to form free hydroxide that attacks the C5 carbon of DPQ and B3LYP does not accurately calculate the barriers of long-range proton transfer reactions. Instead, it is argued that the first two steps calculated should be considered as a unit, making binding of O$_2$ to Cu and attack by the superoxide product at the C5 carbon of the tyrosyl radical the rate limiting step with a barrier of 6+8.4 = 14.4 kcal/mol. Overall, the TPQ biogenesis reaction is calculated to be exothermic by 69 kcal/mol.\[1187\] These calculations indicate that the key intermediate in the TPQ biogenesis reaction having an absorption band at 350 nm should have significant Tyr radical character and that reaction of O$_2$ with this species should occur at the Cu$^{I}$ rather than directly with the tyrosyl ring.

The calculations by Prabhakar and Siegbahn contrast recent calculations by Ghosh et al. inspired by [Cu$^{II}$L((OPhen))]+ complexes which model the Cu$^{II}$((His)$_3$(Tyr)) structure proposed for the 350 nm intermediate.\[1239\] These calculations show only a small amount of radical character on the phenolate ring (14%-9% spin density) relative to 70% in the calculations of Prabhakar and Siegbahn. This is not due to large differences in the structures of the models relative to the protein active site, but instead reflects the difference in the choice of functional (BP86 with 38% HF exchange for Ghosh et al. and B3LYP for Prabhakar and Siegbahn). The smaller amount of HF exchange in B3LYP (20%) is known to result in more spin density on the ligands of metal complexes, and this effect on the spin densities has in fact been observed in calculations on the L1 and L3 model complexes using B3LYP.\[1239\] In Ghosh et al., a comparison of different functionals in the model systems was made and BP86 with 38% HF was selected as the functional that best reproduces the electronic structural parameters determined from spectroscopy, therefore giving the best description of these...
systems. In addition to the spin density differences, the $\Delta G$ for $O_2$ attack on the phenolate ring to form a non-coordinating $Cu^{I\text{-I}}$-superoxoquinone species calculated by Ghosh et al. is at least 50 kcal/mol (depending on the phenolate ring orientation) compared to 17.7 kcal/mol for Prabhakar and Siegbahn. This large energy difference may be due to the spin density differences, to the differences in functional, or to other methodology differences. These differences between the two sets of calculations result in significantly different mechanistic conclusions in the two studies. While Prabhakar and Siegbahn favor formation of a $Cu^{II\text{-I}}$-superoxo as the initial reaction of the preprocessed site with $O_2$ ($\Delta G = 6$ kcal/mol), Ghosh et al. favour a concerted attack of $O_2$ on both the $Cu$ and C5 of the phenolate ring to form a coordinated $Cu^{II\text{-I}}$-peroxoquinone ($\Delta G = 11$ kcal/mol, see Section 4.3). Ultimately, a comparison of the electronic structures from calculations with the spectroscopy of the preprocessed active site is necessary to calibrate the calculation methodology before accurate mechanistic conclusions can be drawn about the nature of substrate activation by the $Cu^{II}$ site.

The step in which DPQ is hydrated to form $TPQ_{red}$ ($6 \rightarrow 7$ in Figure 224) is proposed to be copper-catalyzed because hydration of $o$-quinones does not occur via general base catalysis in aqueous solution. To demonstrate that $Cu^{II}$ can catalyze this step, model complexes have been studied by Ling et al. which have a $M^{II}$ site with a tethered catechol group that can be oxidized in situ to a DPQ-like functionality. When $M^{II}$ is $Cu^{II}$, $Co^{II}$, $Ni^{II}$ or $Zn^{II}$ these complexes undergo a subsequent hydration to form a TPQ-like hydroxyquinone product (after further oxidation by periodate), albeit with an order of magnitude slower rates for $Ni^{II}$ and $Zn^{II}$ compared to $Cu^{II}$. The rate-limiting step is proposed to be attack of a metal-coordinated hydroxide on the substrate quinone.

Attempts to chemically trap the DPQ intermediate in TPQ biogenesis by the mutation of the adjacent Asp residue to Lys (D298K) result in the formation of a cofactor identified by absorption, resonance Raman, and X-ray crystallography to be a tautomer of the LTQ cofactor observed in lysyl oxidase (LOX). Stabilization of the iminoquinol tautomer of LTQ (Figure 224, 10) over the aminoquinone form (Figure 224, 9) is influenced by hydrogen bonding from another second sphere residue, Y284, and so some of the second sphere residues in LOX must also play a role in the stabilization of the active form of the cofactor. These results establish a mechanistic proposal for LTQ biogenesis, where the early steps of the cofactor biogenesis reaction ($Cu^{II}$ substrate activation, $O_2$ attack to form a $Cu^{II\text{-I}}$-peroxoquinone and O-O bond cleavage to form DPQ) are the same in both TPQ and LTQ biogenesis and the only difference is what nucleophile adds to the DPQ intermediate – $Cu^{II\text{-I}}$-coordinated hydroxide (TPQ) or a Lys residue (LTQ biogenesis pathway, Figure 224).

### 4.3 The Nature of $Cu^{II}$ Substrate Activation

The proposed mechanisms for 2,4-dioxygenation in 2,4-QD and TPQ biogenesis in AO involve a common description of the nature of $Cu^{II}$ substrate activation. Both mechanisms invoke a step in which $Cu^{II}$-coordinated substrate transfers an electron to $Cu^{II}$, forming a $Cu^{I\text{-I}}$-substrate radical species. Three descriptions of this step have been put forward which attempt to invoke the presence of a substrate radical even though reduction of the $Cu^{II}$ site upon substrate binding is not observed: 1) The $Cu^{I}$-substrate radical is in equilibrium with
the CuII-substrate site, via an intramolecular electron transfer, but the equilibrium is shifted far to the left (as per Steiner et al.\textsuperscript{1124}); 2) The reaction is performed by a substrate→Cu CT excited state of the CuII-substrate complex (as per Seigbahn\textsuperscript{1126}); 3) The ground state of the CuII-substrate complex contains radical character which makes the substrate reactive towards O\textsubscript{2} (often written as a resonance hybrid of CuI-substrate radical with CuII-substrate complex\textsuperscript{1185}). It is an open issue as to whether any of these descriptions for the activation of substrate as a radical is correct and there is evidence against each, primarily from the AO cofactor biogenesis reaction. In the case of the equilibrium description (1), addition of reagents that react with radicals or CuI (such as NO) might be expected to trap the substrate radical by driving the equilibrium, but no substrate radical has been observed in these systems by chemical trapping.\textsuperscript{1184} In the case of the excited state reactivity description (2), the LMCT energy is included in the activation barrier for the O\textsubscript{2} attack step. The lowest energy determined for phenolate-CuII LMCT in a model complex, with a similar geometry to the active site of preprocessed AO, is 14,727 cm\textsuperscript{−1} (41 kcal/mol), which is significantly higher than the measured ΔH\textsuperscript{‡} for TPQ biogenesis (8.4 kcal/mol).\textsuperscript{1239} In the case of the ground state radical character description (3), only a small amount of radical character is observed on the rings of CuII-phenolate model complexes.\textsuperscript{1239} In addition, significant radical character would be expected to yield observable spectral effects. While there may be some evidence for this third description, given the pyramidalization of the C2 carbon of quercetin in the ES complex of quercetinase observed by X-ray crystallography,\textsuperscript{1124} even in this ES complex of 2,4-QD no reduction of CuII to CuI or CuI-radical species is observed by EPR and XAS.\textsuperscript{1121,1122}

Given the lack of evidence for the CuI-substrate radical required by current proposals, alternative proposals for the nature of substrate activation by CuII coordination should be explored. Calculations inspired by the CuII-phenolate model complexes have shown that while direct attack by O\textsubscript{2} on the phenolate ring at C2 has a high barrier (ΔG = 51 kcal/mol), concerted attack on both the phenolate C2 and CuII to form a coordinated peroxoquinone has a much lower barrier (ΔG = 11 kcal/mol).\textsuperscript{1239} In this concerted process, triplet O\textsubscript{2} approaches the site oriented to bind bridging the CuII and the phenolate with an antiferromagnetic alignment of the spins as in Figure 225 right and α spins from both the phenolate HOMO and the Cu are transferred to the απ orbitals of O\textsubscript{2}. This is facilitated by the fact that one O\textsubscript{2} π* orbital has σ overlap with the HOMO of the phenolate (Figure 225A) and the other O\textsubscript{2} π* orbital has π overlap with the half occupied CuII dx\textsuperscript{2}-y\textsuperscript{2} orbital (Figure 225B). The transfer of two α electrons to O\textsubscript{2} occurs concertedly with the LMCT of an electron with β spin from the phenolate HOMO to CuII to maintain the electron density on CuII, effectively flipping the unpaired spin on CuII (Figure 225C). Thus the CuII acts as a spin buffer that overcomes the spin-forbiddenness of the reaction without reduction of CuII or the need to invoke a phenoxy radical-CuI intermediate. This may be an attractive alternative to the current mechanistic proposals. Another possibility is that the resting CuII-OH could perform hydrogen atom abstraction from the Tyr, generating a tyrosyl radical that can react with O\textsubscript{2}. However, this mechanism would also involve a tyrosyl radical intermediate, which has not been observed. Further attempts to obtain evidence for a substrate radical in the course of a CuII substrate activation reaction and extension of the concerted CT spin-buffering proposal in Figure 225 to cofactor biogenesis in AO and the
mechanism of quercetinase are required to understand the nature of Cu\textsuperscript{II}-substrate activation for the spin forbidden reaction of singlet organic substrates with triplet O\textsubscript{2}.

### 5.0 Copper Sites in Bacterial Denitrification

Thus far this review has focused on copper containing enzymes that exclusively use molecular O\textsubscript{2} as the oxidant for performing redox chemistry. There are other important biochemical pathways involving copper enzymes that do not use O\textsubscript{2} or its other redox states (such as peroxide) as oxidants. One such pathway is the global nitrogen cycle, which involves a number of important and unique metalloenzymes that perform redox chemistry on dinitrogen, ammonia, and nitrogen oxides (Figure 226).\textsuperscript{1241} Nitrogen fixation (N\textsubscript{2} reduction to NH\textsubscript{3}) is catalyzed by nitrogenase, a binary protein system that involves an iron-sulfur cluster containing reductase and an enzyme containing two large clusters, the [Fe\textsubscript{8}S\textsubscript{7}] P-cluster implicated in electron transfer and the catalytic [MoFe\textsubscript{7}S\textsubscript{9}-Chomocitrate] FeMoco cluster. The Mo in this cluster can be substituted by V and Fe in other homologues of the nitrogenase enzyme. The six electron reductive process of ammonification (NO\textsubscript{2}\textsuperscript{−} reduction to NH\textsubscript{3}) involves a multiheme enzyme known as cytochrome c nitrite reductase.\textsuperscript{1242}

Alternatively, denitrification, the multi-step reductive pathway that completes the nitrogen cycle by reducing nitrogen oxides back to gaseous dinitrogen (Figure 226), involves four interesting metalloenzymes, two of which contain copper.\textsuperscript{1243} The two electron reduction of nitrate to nitrite is performed by a molybdopterin enzyme which can also contain an iron-sulfur cluster (for membrane-bound nitrate reductases) or heme site(s). The one electron reduction of nitrite to nitric oxide, the first dedicated step of denitrification, can be performed by two alternative nitrite reductases: a copper containing metalloenzyme with a type 1 (blue) copper electron transfer site and a type 2 (or normal) copper active site (CuNiR) and a heme cd\textsubscript{1} metalloenzyme (heme NiR). Denitrifying organisms express exclusively either CuNiR or heme NiR depending on whether nitrite reduction is required under aerobic conditions (heme NiR is less sensitive to O\textsubscript{2} than CuNiR). Nitric oxide reduction, which involves the two electron reductive coupling of two equivalents of nitric oxide to form nitrous oxide, is performed by an enzyme that contains a unique heme/non-heme iron binuclear active site closely structurally related to the active site in the heme-copper oxidases (but without the Tyr crosslink, see Section 3.7.2). Finally, the terminal step in denitrification, a two electron reduction which converts nitrous oxide to dinitrogen and water, is performed by nitrous oxide reductase, which contains two copper sites, a binuclear Cu\textsubscript{A} electron transfer site and a tetranuclear copper sulfide cluster called Cu\textsubscript{Z}.\textsuperscript{1243}

Organisms are considered to be denitrifiers if they convert nitrogen oxides to a gaseous product.\textsuperscript{1244} Not all denitrifying organisms possess the complete denitrification pathway and some terminate denitrification by releasing gaseous nitrous oxide instead of dinitrogen. These organisms lack the gene cluster responsible for nitrous oxide reductase expression.\textsuperscript{1244} Denitrification can serve a number of cellular functions, including detoxification of nitrogen oxides, particularly nitrite, and redox balancing (taking up excess reducing equivalents when O\textsubscript{2} and CO\textsubscript{2} are not available).\textsuperscript{1245} However, the main purpose of denitrification in most bacterial denitrifiers is respiration, the use of nitrogen oxides as the terminal electron acceptors for the cell to conserve energy for growth in environments with little or no oxygen, like the soil or deep ocean. The reductive steps of denitrification occur in...
the periplasm of denitrifying bacteria and electron transport across the cytoplasmic membrane by the obligatory upstream source of electrons for all the denitrification enzymes, the cytochrome bc$_1$ complex, translocates protons across the membrane and establishes the electrochemical gradient necessary to drive ATP synthesis. This method of establishing a proton gradient is less efficient than O$_2$ respiration using cytochrome c oxidase so denitrifying organisms have regulatory mechanisms to switch over to O$_2$ respiration when O$_2$ is available. Several enzymes in denitrification, including copper nitrite reductase and nitrous oxide reductase, are thought to be deactivated by O$_2$.

In this review we will provide a brief summary of the chemistry of copper nitrite reductase (Section 5.1) and focus in more detail on nitrous oxide reductase because of its unique tetranuclear copper active site, unprecedented in either copper biochemistry or in synthetic copper-sulfur chemistry, and the number of remaining questions about how this site reduces N$_2$O (Section 5.2).

### 5.1 Copper Nitrite Reductase

Copper nitrite reductase (CuNiR) performs the one electron reduction of nitrite to nitric oxide and water (with uptake of two protons). The enzyme contains two mononuclear copper sites, a type 1 (T1) or blue Cu site involved in electron transfer and a type 2 (T2) or normal copper site where substrate binding and reduction occurs. Three states of CuNiR - the resting state (both oxidized and reduced), the oxidized nitrite-bound state, and an NO bound state - are structurally and spectroscopically well characterized and a large number of X-ray crystal structures, including atomic resolution structures, are available for all three states. In each of these states, the coordination of the T1 site is not perturbed and differences are due to ligand binding at the T2 site. While the physiological function of CuNiR is the reduction of nitrite to NO, CuNiR also catalyzes the reverse reaction, converting NO to nitrite. The equilibrium constant for the conversion of nitrite to NO by CuNiR is pH dependent and nitrite reduction is thermodynamically favoured (i.e. $K_{eq} > 1$) below pH 6.2 (pH 6.2 is also the optimum pH for nitrite reduction in steady-state kinetics studies). In addition, in the presence of excess NO, reduced CuNiR can catalyze the reductive coupling of two NO molecules to form N$_2$O. While this coupling reaction is unlikely to be relevant in vivo, it has been observed to complicate in vitro attempts to characterize a product bound T2 Cu-NO complex obtained by treating reduced CuNiR with excess NO (vide infra).

CuNiR is a homotrimer where each monomer contains a T1 and T2 copper site separated by 12.5 Å distance (one hexameric variant is known). The T1 site is ligated by His95, His145, Cys136, and Met150 (Figure 227). The T2 copper site is located at the interface between the monomers and is ligated by three His ligands (Figure 227). Two His residues of the T2 site come from one monomer (His100 and His135) and the third from another monomer (His307), indicating that the trimer structure is necessary for function. Cys136, a ligand of the T1 site, is adjacent to His135, a ligand of the T2 site, forming a Cys-His pathway for rapid through-bond electron transfer as also present in the MCOs (Section 3.7.1). In the oxidized resting state of CuNiR, the T2 site is additionally coordinated by a solvent derived ligand and has a tetrahedral geometry (Figure 228A)
state of CuNiR is reduced, this solvent derived ligand is lost while the remainder of the coordination environment of the T2 site is not perturbed.\textsuperscript{1250} In the oxidized resting state, the solvent-derived ligand is hydrogen bonded to a second sphere residue Asp98 which additionally hydrogen bonds through a localized water molecule to another second sphere residue, His255.\textsuperscript{1250,1251} These two residues have been identified by mutagenesis studies as essential for enzyme activity.\textsuperscript{1252} They have also been implicated in the pH profile of the activity of CuNiR, which has a maximum at pH 6.2 and decreases at low pH with a pKa of 5 (assigned as the pKa of Asp98) and at high pH with a pKa of 7 (assigned as the pKa of His255).\textsuperscript{1253} Study of the pH dependence of the nitrite bound form of the T2 site has shown that the pKa of Asp98 is 6.4 when nitrite is coordinated (\textit{vide infra}).\textsuperscript{1254} Mutation of a nearby hydrophobic residue, Ile289, decreases the rate of catalysis but does not totally eliminate nitrite reduction.\textsuperscript{1253} The redox potentials of the T1 and T2 sites have been measured in resting \textit{Alcaligenes xylosoxidans} NiR as 255±3 mV and 244±18 mV, respectively, at pH 7.\textsuperscript{1255} The redox potential of the T2 site decreases to 137 mV at pH 8.4 (from 218 mV in NiR from \textit{Rhodobacter sphaeroides}), turning off electron transfer between the T1 and the T2 sites at high pH, when Asp98 is deprotonated.\textsuperscript{1256} Additionally, site-directed mutagenesis has been used to create a variant of CuNiR in which electron transfer from the T1 to the T2 site is disrupted by mutation of one of the His ligands of the T1 site to alanine (H145A), resulting in a high potential T1 site that remains permanently reduced.\textsuperscript{1257} Thus, three strategies are available for studying nitrite binding to the T2 site without T1 to T2 electron transfer, which would lead to reduction of nitrite: 1) nitrite binding to fully oxidized CuNiR (where the T1 site is oxidized), 2) nitrite binding to CuNiR at high pH (pH > 8.4, where the T1 site can be selectively reduced, and 3) nitrite binding to the T2 site in the H145A variant, which contains a permanently reduced T1 site.

Almost all of the structural studies of the nitrite bound state of CuNiR have employed the first of these strategies: nitrite addition to fully oxidized resting CuNiR. A large number of available crystal structures show that nitrite binds to the oxidized T2 site, displacing the solvent derived ligand observed in resting and coordinating as a bidentate ligand with both oxygens bonding to Cu (Figure 228B).\textsuperscript{1246} Nitrite is asymmetrically bound to the T2 site, with the Cu-O bond closer to Asp98 being shorter by 0.2–0.6 Å (Table 41).

There is a hydrogen bond between Asp98 and the closer oxygen of nitrite (O-O distance of 2.8–3.4 Å). There is also some degree of variability in the orientation of the nitrite ligand relative to the T2 copper (variable Cu-N distance, Table 41). In the one known crystal structure where nitrite is added to a crystal of reduced NiR, the coordination geometry of nitrite bound at the reduced T2 site does not differ substantially from the coordination observed to the oxidized T2 site, except that the Cu-O bond lengths are slightly longer (by 0.05 Å and 0.27 Å, Table 41).\textsuperscript{1250} Mutated variants of CuNiR where the second sphere residues His255 or Ile257 are perturbed show a monodentate coordination geometry for nitrite, which coordinates to the T2 site through one oxygen atom.\textsuperscript{1264} Spectroscopic characterization of the nitrite-bound state of wild-type CuNiR has been performed at both low pH with the T1 site oxidized and at high pH with the T1 site either oxidized or reduced.\textsuperscript{1254} EPR spectroscopy of nitrite bound T2 site at pH 8.4 with the T1 site either oxidized (Figure 229B) or reduced (Figure 229C) shows that nitrite binding is not perturbed...
by the redox state of the T1 site. However, a significant change in the nitrite-bound T2 site is observed upon comparing EPR spectra at pH 8.4 and pH 5.5 (Figure 229B and Figure 229A, respectively). Thus, the low pH form of the nitrite bound T2 site, which is the species present at the pH of maximum enzyme activity, has one more proton than the high pH form. DFT calculations have been used to evaluate models for both the high pH and low pH nitrite-bound T2 sites to identify where these protons reside in the active site. These calculations favor a model where at high pH His255 is protonated and Asp98 deprotonated while at low pH, both His255 and Asp98 are protonated, with a hydrogen bond between Asp98 and the coordinated nitrite. Thus, protonation of Asp98 triggers electron transfer from the T1 to the nitrite-bound T2 site. Further, when the T1 reduced, nitrite-bound oxidized T2 state at pH 8.4 is rapidly lowered to pH 5.6 (a pH drop experiment), NO is evolved and the absorption spectrum shows that the T1 site is rapidly oxidized. This indicates that protonation of Asp98 upon going from high pH to low pH triggers electron transfer from the T1 to the nitrite-bound T2 site, resulting in turnover.

The structure of CuNiR complexed with the product of nitrite reduction, NO, was first reported by Tocheva et al. in 2004 from crystals of reduced CuNiR that had been exposed to excess exogenous NO. There are several unusual features of this structure. First, the NO molecule is bound side-on to the T2 copper site (Figure 228C). Second, the EPR spectrum reported after identical NO addition to reduced CuNiR in solution shows an oxidized T2 copper signal with a nine line pattern in the $g_{\perp}$ region due to superhyperfine coupling with four nitrogen donor ligands (Figure 230A). A further study by Tocheva et al. shows that this side-on NO species with a putative Cu$^{II}$ EPR spectrum is observed when starting from both wild-type CuNiR and the reduced and oxidized forms of the H136A variants (in which T1 to T2 ET does not occur). This leads to an initial assignment of the CuNiR NO complex as a side-on Cu$^{II}$-NO$^{−}$ species, different in both geometric and electronic structure from all known mononuclear Cu-NO complexes, which are end-on coordinated and have Cu$^{I}$-NO$^{•}$ radical EPR spectra. Subsequent spectroscopic investigations of this putative Cu$^{II}$-NO$^{−}$ species in wild type CuNiR by EPR, MCD, and $^{14}$N and $^{1}$H ENDOR show that in fact the Cu$^{II}$ species observed in solution is identical to the nitrite-bound oxidized T2 site (with the T1 site reduced, Figure 230B). At short times after NO addition to reduced CuNiR in solution, a Cu$^{I}$-NO$^{•}$ species is observed by EPR (Figure 230C), which subsequently converts to the nitrite-bound resting form of CuNiR. In wild-type CuNiR, this occurs via reductive coupling of two equivalents of NO forming N$_2$O, which was detected directly by an assay using nitrous oxide reductase. This process oxidizes the T1 and T2 sites and subsequently the reverse reaction of CuNiR proceeds, oxidizing NO to NO$_2^{−}$. This generates the observed nitrite-bound oxidized T2 site and a reduced T1 site. How this proceeds in CuNiR variants where T1 to T2 ET is affected has not been described, however identical Cu$^{II}$ EPR spectra are observed in these variants as in wild type, so formation of the nitrite-bound T2 site is likely the process that also occurs in solution. Thus, the NO adduct of CuNiR that forms in solution has a Cu$^{I}$- NO$^{•}$ electronic structure, not the Cu$^{II}$-NO$^{−}$ electronic structure that was initially proposed. This Cu$^{I}$-NO$^{•}$ species can also be observed upon nitrite reduction by CuNiR and has been characterized by EPR and ENDOR. Analysis of the orientations of the $g$- and $A$-tensors obtained from the EPR indicate that the NO ligand is bound end-on with a ~160° Cu-N-O angle. $^{1}$H coupling features are observed...
in the ENDOR spectrum that arise from a magnetic interaction with the C81 protons of the sidechain of Ile289, indicating that the end-on NO molecule is oriented towards Ile289 in the active site. These studies reveal an intriguing difference between how NO interacts with CuNiR in solution versus in crystals. In solution an end-on bound Cu\(^{\text{I}}\)-NO\(^{\cdot}\) species is formed that further reacts, eventually forming the nitrite bound oxidized T2 site, while in crystals a side-on NO bound T2 site is stable. Several computational studies have been undertaken to determine the interactions responsible for the stability of the side-on NO binding geometry observed in CuNiR crystals and all show that the end-on structure is energetically favoured by 3–10 kcal/mol. Recent calculations by Merkle and Lehnert which include the second sphere residues Asp98 and Ile289 show that the side-on bound geometry is a local minimum while the end-on geometry is the global minimum, with a small barrier of 1.0 kcal/mol required for conversion from the side-on to the end-on structure.\(^{1267}\) Interactions that contribute to stabilizing the side-on coordination geometry include hydrogen bonding from Asp98, which is 1–2 kcal/mol stronger in the side-on case, and steric constraints from Ile289, which restricts the geometry of the end-on NO complex. Thus, the side-on coordination of the CuNiR NO complex observed in crystals may represent a metastable form that relaxes to end-on coordination in solution when the positions of the sidechains of Ile289 and Asp98 are more flexible.

A consensus mechanism for the reduction of nitrite by CuNiR is presented in Figure 231. The first two steps of the mechanism are binding of NO\(_2^\cdot\) to the T2 site and electron transfer from the T1 to the T2 site (producIng the T2 Cu\(^{\text{I}}\) Site). Whether these two steps are ordered or if a random-sequential mechanism operates has been a matter of long debate in the literature. However, both a recent single-molecule study\(^{1268}\) and a steady-state kinetics study considering the substrate dependence of turnover support a random-sequential mechanism,\(^{1269}\) where either ET or substrate binding could occur as the first step (Figure 231, pathways A and B) and this view has become generally accepted. When both nitrite binding and T1 to T2 ET have occurred, the mechanism converges at a T2 reduced nitrite bound intermediate. This is the active intermediate for N-O bond cleavage, which occurs via transfer of a proton from Asp98 and oxidation of the T2 site, resulting in NO formation. Computational studies of this step by Ghosh et al. suggest that the lowest energy pathway involves first protonation of the bound nitrite followed by electron transfer to break the N-O bond.\(^{1254}\) Computational investigations of three possible geometries for the Cu\(^{\text{I}}\) intermediate with a protonated nitrite ligand (bidentate \(\eta^2\), monodentate \(\eta^1\kappa N\), and monodentate \(\eta^1\kappa O(H)\)) show that N-O bond cleavage only has a low barrier when the protonated nitrite is a bidentate ligand, similar to the crystallographically observed geometry for nitrite binding to the oxidized T2 site (Figure 230A). In this geometry, the reaction proceeds by direct release of NO with a calculated \(\Delta E^\ddagger\) of 16 kcal/mol, which agrees well with the experimental activation energy of 15 kcal/mol for nitrite reduction.\(^{1253}\) Thus the role of the experimentally observed NO bound species in the catalytic reactivity of NiR remains to be resolved. Possible roles of this species include as a product-inhibited form of the enzyme or as an intermediate not in nitrite reduction but in the reductive coupling reaction catalyzed by NiR in the presence of excess NO.
5.2 Nitrous oxide reductase

5.2.1 Enzymology—Nitrous oxide reductase (N\textsubscript{2}OR) is the terminal enzyme of bacterial denitrification and reduces N\textsubscript{2}O by two electrons, breaking the N-O bond to release N\textsubscript{2} and H\textsubscript{2}O. This reaction is exergonic by 81 kcal/mol\textsuperscript{1244} but has an activation barrier of 59 kcal/mol in the gas phase.\textsuperscript{1270} N\textsubscript{2}O release into the atmosphere is an environmental concern, since N\textsubscript{2}O is the primary source of NO\textsubscript{x} in the stratosphere, leading to ozone depletion,\textsuperscript{1271} and is a greenhouse gas with 300 times the global warming potential of CO\textsubscript{2}.\textsuperscript{1272} Agricultural use of nitrogen based fertilizers is the primary source of anthropogenic N\textsubscript{2}O emissions, as both nitrifying and denitrifying soil bacteria that lack a N\textsubscript{2}OR can generate N\textsubscript{2}O from these chemicals.\textsuperscript{1273} N\textsubscript{2}O is also released as a byproduct of industrial processes (particularly the production of adipic and nitric acids), by the combustion of biomass and fossil fuels, and from wastewater treatment.\textsuperscript{1271} Combined, anthropogenic sources of N\textsubscript{2}O account for 40% of all N\textsubscript{2}O emissions.\textsuperscript{1274} Capture and destruction of N\textsubscript{2}O through chemical catalysis is a possible strategy for mitigating the effects of N\textsubscript{2}O on the ozone layer and the global climate.\textsuperscript{1271,1273}

The requirement of copper for nitrous oxide reduction in denitrifying bacteria was identified in 1980 when it was shown that copper is essential for growth of the denitrifier Alcaligenes faecalis under an N\textsubscript{2}O atmosphere, where N\textsubscript{2}O respiration is the sole source of energy for growth.\textsuperscript{1275} This was later confirmed when it was shown that in the absence of copper, N\textsubscript{2}O is the terminal product of nitrate-induced denitrification in Pseudomonas stutzeri.\textsuperscript{1276} The role of copper as a cofactor in nitrous oxide reductase was identified when a previously isolated copper protein from Pseudomonas stutzeri was shown to reduce N\textsubscript{2}O in vitro with reduced methyl viologen as the electron source.\textsuperscript{1277} Since this discovery, N\textsubscript{2}OR had been purified and biochemically characterized from 11 denitrifying bacteria: Pseudomonas stutzeri (PsN\textsubscript{2}OR),\textsuperscript{1278} Rhodopseudomonas sphaeroides f.sp. denitrificans,\textsuperscript{1279} Paracoccus denitrificans (PdN\textsubscript{2}OR),\textsuperscript{1280} Wolinella succinogenes (WsN\textsubscript{2}OR),\textsuperscript{1281} Achromobacter cycloclastes (AcN\textsubscript{2}OR),\textsuperscript{1282} Pseudomonas aeruginosa,\textsuperscript{1283} Paracoccus pantotrophus (PpN\textsubscript{2}OR),\textsuperscript{1284} Thiobacillus denitrificans (TdN\textsubscript{2}OR),\textsuperscript{1285} Alcaligenes xylosoxidans,\textsuperscript{1286} Hyphomicrobium denitrificans,\textsuperscript{1287} and Pseudomonas nautica (PnN\textsubscript{2}OR, later reclassified as Marinobacter hydrocarbonoclasticus).\textsuperscript{1288} A further 66 denitrifying prokaryotes have thus far been identified through genomic analysis to contain the NosZ gene associated with N\textsubscript{2}OR.\textsuperscript{1244}

N\textsubscript{2}ORs are homodimers with molecular weights of 120–160 kDa, a copper content of ~12 Cu per dimer, and a sulfide content of ~2 S\textsuperscript{2−} per dimer (Table 42).\textsuperscript{1289}

N\textsubscript{2}OR contains two copper sites: Cu\textsubscript{A}, a binuclear copper site with two Cys, two His, one Met and the backbone carbonyl of a Trp residue as ligands, which acts as an electron transfer site (as in the heme-copper oxidases, see Section 3.7.2), and Cu\textsubscript{Z}, a tetranuclear \(\mu^4\)-sulfide bridged cluster liganded by seven His residues, which is thought to be the site of N\textsubscript{2}O binding and reduction (see Section 5.2.3).\textsuperscript{1292} The ligands of the Cu\textsubscript{A} site were identified from mutagenesis studies and its structure was determined by analogy to the structurally characterized Cu\textsubscript{A} site in the heme-copper oxidases, which has close to identical properties to Cu\textsubscript{A} in N\textsubscript{2}OR.\textsuperscript{58,1293,1294} In contrast, the structure of Cu\textsubscript{Z} was determined by
X-ray crystallography and is still a matter of active study (*vide infra* and discussion in Section 5.2.3). The residues binding the CuA and CuZ sites are strictly conserved in all the N2ORs, showing that the copper sites are present even in N2ORs which have not been structurally or spectroscopically characterized.1244 There is a high degree of similarity between N2ORs isolated from different sources (Table 42). The sequence similarity of all purified N2ORs with PsN2OR is greater than 70%, with the noted exceptions of TdN2OR and WsN2OR. TdN2OR is a membrane bound protein,1285 while other N2ORs are periplasmic, and WsN2OR contains an additional domain with a heme cofactor,1281 which accounts for the sequence differences. Despite the high sequence similarity between N2ORs, AcN2OR and RsN2OR were initially reported to be monomeric based on gel filtration results that did not show a band for the native dimer.1279,1282 However, in view of the conclusion drawn from X-ray crystallography that the dimerization is necessary for function (see Section 5.2.3), and a subsequent crystal structure showing that AcN2OR is in fact a dimer,1295 it is likely that both AcN2OR and RsN2OR are dimers. In fact, the largest biochemical difference between N2ORs from different sources is the large variability in their analytical copper content, which can vary significantly between sources and for the same source organism, depending on the protein purification conditions (*vide infra*). The initial purifications of N2OR reported a low copper content of 8 Cu per dimer1278,1280 compared to more recent results of ~10 Cu per dimer, but even these recent analytical results do not show full copper loading (12 Cu per dimer, based on X-ray crystallography).1288,1289 This is attributed to incomplete loading of CuZ in vivo or to loss of some CuZ during the protein purification. Analytical sulfide quantitation in N2OR does not show the same variability, although a sulfide concentration assay that is not susceptible to sulfide oxidation and using the copper content to account for CuZ occupancy is required to accurately determine the sulfide count per CuZ site.1289,1296

A gene cluster has been identified that is required for N2O reduction, which encodes the N2OR protein and several ancillary proteins required for its expression, maturation, and maintenance.1297,1298 The core of this cluster, which is the minimum required for N2O reduction, contains six genes (NosRZDFYL) and is sometimes associated with a further gene, NosX.1299 It is also associated with the Tat transporter system, which is responsible for transporting the N2OR apo-protein into the periplasm, where the maturation of N2OR is completed.1300 Preliminary investigations have described the characteristics and putative functions of the protein products of the Nos gene cluster. The NosZ gene encodes the N2OR apo protein.1301 In mutant strains that lack the NosDFY or NosL proteins or contain a modified form of the NosR protein, the biogenesis of the CuA site is unaffected.1244 CuA can be reconstituted in N2OR by exogenous copper in vitro while CuZ cannot be reconstituted,1278 underlining the importance of a chaperone assisted biogenesis mechanism for CuZ but not for CuA. CuA is thought to be loaded in vivo by the same route used for the loading of CuA in the hemecopper oxidases, by transfer of CuI from a ubiquitous copper chaperone.1244 The ancillary genes of the Nos gene cluster are therefore associated with the biogenesis of the CuZ site and its maintenance in vivo or with regulation of NosZ expression. NosDFY encodes an ABC type transporter where NosY is a membrane-spanning protein, NosF is a cytoplasmic ATPase, and NosD is a periplasmic protein from the carbohydrate binding and sugar hydrolase protein family.1298 Mutant strains lacking

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NosDFY express CuZ deficient N₂OR, indicating that NosDFY is essential for CuZ biogenesis. The exact role of this transporter system is not known, but it is proposed to be the sulfur transporter that supplies the sulfide required for CuZ biogenesis. NosL encodes a lipoprotein which preferentially binds a single Cu and is thought to be the copper transporter associated with CuZ assembly. However, active N₂OR containing both copper sites can be obtained in the absence of NosL, so an alternative Cu chaperone must exist and NosL is not essential. NosR encodes a transmembrane protein that contains a flavin binding site in the N-terminal (periplasmic) domain and two [4Fe-4S] ferrodoxintype iron-sulfur clusters in the C-terminal (cytoplasmic) domain. In the absence of NosR, the NosZ protein is not expressed, indicating that NosR has a regulatory role. However, in the presence of modified forms of NosR where the flavin binding domain is deleted or the ferrodoxin sites are modified, N₂OR is obtained that contains both CuA and CuZ, but the spectroscopic and redox properties of CuZ are modified (vide infra). A similar phenotype is obtained in the absence of the NosX gene product for organisms that contain NosX, which codes for another periplasmic flavoprotein. This suggests that NosR and NosX are not involved in CuZ biogenesis but play a role altering the state of the CuZ site during turnover and sustaining the catalytic activity of N₂OR. If NosR and NosX are in fact required to sustain N₂O reduction in vivo, further study of the process of CuZ biogenesis and maintenance will be necessary to define the full catalytic mechanism of N₂OR.

Both whole cell and in vitro studies have been used to discover the identity of the physiological electron donor to N₂OR, not least so that its reactivity can be studied with the native electron donor rather than with a non-physiological reductant. Whole cell studies in a variety of bacteria confirm that a periplasmic cytochrome c is oxidized with the initialization of N₂O reduction. In some cases the specific cytochrome c that is the physiological electron donor has been identified. Strains that knock out particular cytochrome c proteins in vivo have been used to identify cytochrome c2 as the electron donor to N₂OR in R. capsulatus. In vitro reactivity studies have identified the endogenous electron donor of PnN₂OR as cytochrome c₅₅₁ and that of PpN₂OR as cytochrome c₅₅₂. However, PpN₂OR, AcN₂OR, and WsN₂OR will also turn over in vitro with mammalian cytochrome c as the electron donor and PpN₂OR will also accept electrons from pseudoazurin. In contrast, PnN₂OR is only reactive with the native cytochrome c from P. nautica. The interaction between cytochrome c and N₂OR has been studied using molecular dynamics docking simulations. These show cytochrome c binding to a hydrophobic portion of the surface of N₂OR near the CuA site, with a 10–14 Å distance between the heme and CuA.

An interesting issue is that N₂OR is observed to have different spectroscopic features, redox properties, and reactivity depending on whether it is purified aerobically or anaerobically. In the absence of the NosR and NosX proteins accessory proteins, N₂OR purified under anaerobic conditions has identical characteristics to aerobically purified protein. These differences take on new significance with the recent publication of a crystal structure of anaerobically prepared PsN₂OR which shows a different structure for the CuZ site than that observed for aerobically prepared protein (see Section 5.2.3). Early biochemical studies and the recent X-ray structure agree that...
aerobically and anaerobically purified N2OR have identical molecular weights and protein structure. Instead the observed differences result from changes in the CuZ site. While most N2ORs have only been purified under one set of conditions, three different studies have performed parallel anaerobic and aerobic purifications. These show that there is a lower copper content in aerobically isolated protein (first three preparations in Table 42). This is attributed to sensitivity of the CuZ site towards O2, leading to a loss of CuZ upon aerobic handling, which accounts for some differences in the relative reactivity and spectral features of aerobically and anaerobically prepared N2OR (see Sections 5.2.2 and 5.2.4). However, the majority of the differences cannot be explained only by changing relative occupancies of the CuA and CuZ sites. The CuZ site is also isolated in a different redox state depending on the purification conditions. The CuH3CuI (1-hole) redox state is the resting state of the CuZ site obtained from aerobic purifications and the 2CuII2CuI (2-hole) redox state is obtained from anaerobic purifications (the assignment of these redox states will be discussed in Section 5.2.4). Further, the CuZ site has different redox properties in anaerobically and aerobically prepared N2OR. For anaerobically prepared N2OR, a reversible redox couple between the 2-hole and 1-hole states of the CuZ site can be observed with E' = +60 mV versus NHE in addition to the CuAox (Cu1.5-Cu1.5) to CuAred (CuI-CuI) redox couple at E' = +260 mV versus NHE. However, for aerobically prepared N2OR the CuZ site is redox inert under all conditions except reduction with methyl viologen, which has been shown to reduce CuZ to the fully reduced 4CuI redox state (see Section 5.2.2). These differences in redox properties lead several studies to propose that there are structurally different CuZ sites in anaerobically and aerobically prepared N2OR, which have been named CuZ and CuZ* respectively. CuZ* is then considered to be an inactive form of the catalytic center generated by reaction with O2. Prior to the recent crystal structure, this structural difference between CuZ and CuZ* was thought to be a minor perturbation, perhaps due to a second sphere residue interaction, since the spectroscopic properties of CuZ and CuZ* are very similar when they are compared in the 1-hole redox state (see Section 5.2.4). However, the recent X-ray crystal structure of anaerobic N2OR in fact indicates a significant structural difference, with CuZ containing two bridging sulfide ligands while the previously described structure of CuZ* contains only one. These results will be discussed in Section 5.2.3. In light of this new structural information, interpretation of the spectral features and electronic structure of CuZ is required and the relationship between CuZ and CuZ* and their involvement in the catalytic cycle must be addressed. Additionally, the roles of CuZ and CuZ* in vivo require definition, since the relative abundance of these two forms of the cluster depends not only on O2 but also on the ancillary proteins NosR and NosX. With the recent definition of the structure of CuZ relative to CuZ*, the stage is set to address the questions that remain about the differences between anaerobically and aerobically purified N2OR and the relevance of these differences to N2OR reactivity.

5.2.2 Kinetics—N2OR reactivity is measured with a standard assay system that employs reduced methyl viologen or benzyl viologen as the electron donor, which is present in large excess. The viologen can be reduced using clostridial hydrogenase, a stoichiometric amount of dithionite or photochemically by a flavin and UV light. The reaction is initiated either by adding N2OR to the reductant solution presaturated with N2O or by

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injecting N₂O into a mixture of the reductant and N₂OR. The reactivity can then be monitored spectrophotometrically by following the decay of the absorption of the reduced viologen or by GC analysis of the amount of N₂O present over time. The rate of N₂O consumption is reported as a specific activity in µmol N₂O min⁻¹ mg⁻¹ N₂OR. A summary of the activities of N₂ORs isolated from various source organisms is presented in Table 43.

The specific activities of N₂ORs isolated from different sources can be compared to the activity of N₂OR in vivo. This activity has been estimated in P. stutzeri by measuring the consumption of N₂O by whole cells and estimating the amount of N₂OR in the cell immunochemically. This yields an activity range of 48–72 µmol min⁻¹ mg⁻¹ as the minimum specific activity of PsN₂OR in vivo. In comparison, the activity of N₂ORs assayed as isolated is in the range of 1–10 µmol min⁻¹ mg⁻¹, well below the minimum activity in vivo, showing that N₂OR from both aerobic and anaerobic preparations is obtained in an inactive state and a chemical activation step is necessary to restore N₂O to full activity. Thus, comparisons of the activities of as isolated N₂ORs have limited usefulness for understanding the kinetics and mechanism of N₂O reduction by N₂OR. These values likely reflect the reactivity of a small amount of active N₂OR present in the assay. Instead, considering the conditions required to activate the enzyme and its reactivity in an activated form are required to understand the kinetics and molecular mechanism of N₂O reduction by N₂OR. An exception to this is WsN₂OR, which has a specific activity of 160 µmol min⁻¹ mg⁻¹ without prior activation. The presence of the additional heme site in WsN₂OR gives it unique reactivity, including the ability to perform turnover with dithionite as the sole electron donor, while other N₂ORs are inactive unless methyl viologen or cytochrome c are used as the electron source.

Two procedures have been discovered that activate as isolated N₂OR, increasing its specific activity by 1–2 orders of magnitude to values equal or greater than the minimum activity expected based on whole cells. The first is a base activation procedure in which as-isolated N₂OR is dialyzed overnight against a high pH buffer (pH 9–10). First reported for PsN₂OR, base activation results in a 14-fold increase in the activity of anaerobically isolated PsN₂OR but only a 6-fold increase in the activity of aerobically isolated PsN₂OR. It has also been observed for anaerobically isolated PaN₂OR (54-fold increase in activity) and aerobically isolated PpN₂OR (4-fold increase in activity). The molecular basis for the activation process is not known and no structural or spectroscopic studies have been reported on N₂OR after prolonged dialysis at high pH. Spectroscopic studies of anaerobically purified AcN₂OR and aerobically purified PnN₂OR at pH 10 show only small differences in the CuZ⁺ site due to pH (see 5.2.4). Whether there is a high pH effect on the CuZ site or on CuA has not been investigated. Further study of the base activation process has the potential to yield new insight into the reactivity of N₂OR.

The second activation procedure is a reductive activation in which N₂OR undergoes a prolonged preincubation with reduced methyl viologen (k_activation=0.07 min⁻¹), which is already present in the activity assay. This increases the activity of both anaerobically and aerobically isolated protein by 2 orders of magnitude over the asisolated values, to maximum values of 122 µmol min⁻¹ mg⁻¹ (anaerobic AcN₂OR) and 275 µmol min⁻¹ mg⁻¹ (aerobic PnN₂OR). The origin of this increase in activity was elucidated by Ghosh
et al., who showed that the activation is correlated to a decrease in the EPR spin intensity observed for the 1-hole CuZ$^*$ site (Figure 233). Thus, the mechanism of activation is reduction of the CuZ$^*$ site, initially in the resting 1-hole redox state (S=½, see Section 5.2.4), to the fully reduced 4Cu$^I$ state (S=0), which is the active redox state for reaction with N$_2$O. The 4Cu$^I$ state can also react directly with N$_2$O in the absence of additional reductant to generate a two electron oxidized Cu$_A^{ox}$ 1-hole CuZ$^*$ state of N$_2$OR, with the reduction of N$_2$O to N$_2$ confirmed by gas chromatography mass spectrometry detection of isotopically labeled $^{15}$N$_2$ produced from $^{15}$N$_2$O during the single turnover. High steady-state activity with methyl viologen is not observed for any redox states of the CuZ site (with the 4Cu2S site rather than the CuZ$^*$-4Cu1S site). When the [Cu$_A^{ox}$ CuZ 2-hole], [Cu$_A^{red}$ CuZ 2-hole], and [Cu$_A^{red}$ CuZ 1-hole] states are prepared from PpN$_2$OR, their observed activities are low for all three redox states in both aerobically and anaerobically prepared enzyme (8 and 2 μmol min$^{-1}$ mg$^{-1}$, respectively). Thus, the 4Cu$^I$ redox state is the active state of CuZ$^*$ in the methyl viologen assay. It is likely that the small activities reported for other redox states of N$_2$OR are a result of reduction of a small amount (1–5%) of the N$_2$OR by the methyl viologen present in the activity assay, which then accounts for all of the observed activity.

Despite this evidence that the 4Cu$^I$ state is the active redox state of CuZ$^*$, the reduction of the resting 1-hole state of CuZ$^*$ to the 4Cu$^I$ state is too slow to be part of the catalytic cycle ($k_{\text{red}} = 0.07$ min$^{-1}$ in the presence of a 500-fold excess of reduced methyl viologen, compared to $k_{\text{cat}} = 275$ s$^{-1}$ in the steady state turnover of PnN$_2$OR). Several explanations for this discrepancy have been proposed, including that the slow rate and requirement for a non-physiological reductant rule out the involvement of the 4Cu$^I$ redox state in vivo or that an alternative reduction step available in vivo rapidly converts resting 1-hole CuZ$^*$ to the fully reduced state. A more attractive explanation is that the resting 1-hole state of CuZ is itself not part of the catalytic cycle. Indeed, an alternative 1-hole intermediate state of CuZ$^*$ has been observed in the single turnover of PnN$_2$OR when fully reduced [Cu$_A^{red}$ 4Cu$^I$ CuZ$^*$] N$_2$OR is reacted with a stoichiometric amount of N$_2$O. Cu$_A$ and CuZ$^*$ are rapidly oxidized, but while CuZ$^*$ is observed to be in a 1-hole redox state, its spectral features differ from those of the resting 1-hole state. In the absence of further reaction, this intermediate species, designated CuZ$^o$, slowly converts to resting 1-hole CuZ$^*$ ($k_{\text{obs}} = 0.3$ min$^{-1}$). CuZ$^o$ has high specific activity, equal to that of the 4Cu$^I$ state, indicating that it is kinetically competent and can be rapidly reduced in turnover. The decay of this activity occurs with the same rate constant as the decay rate of CuZ$^o$ obtained spectroscopically ($k_{\text{decay}} = 0.3$ min$^{-1}$), indicating that N$_2$OR activity is dependent on the presence of CuZ$^o$, not the resting 1-hole form of CuZ$^*$. This resolves the issue of the slow rate of reduction of the resting 1-hole state, since turnover can proceed from the 4Cu$^I$ redox state through 1-hole CuZ$^o$ without requiring a slow reduction step. It is important now to determine how the 1-hole form of CuZ$^o$ relates to that of CuZ$^*$ to understand the effectiveness of this intermediate in turnover.

Steady state kinetic studies on reductively activated protein have been used to investigate the dependence of N$_2$OR reactivity on substrate concentration, reductant concentration, pH and solvent deuteration. Usually these have been done without controlling for the ratio of CuZ$^*$ to CuZ present, but the values reported for aerobically purified N$_2$OR should...
correspond solely to CuZ* reactivity. The N₂O concentration dependence of steady state turnover, performed for anaerobically isolated AcN₂OR and aerobically isolated PnN₂OR, yields similar k_cat and K_M value, with k_cat on the order of 200 s⁻¹ and K_M(N₂O) values on the order of 20 µM, indicating weak binding of N₂O (Table 43).

The reductant concentration dependence gives a K_M(methyl viologen) of 12 µM. For PnN₂OR containing only CuZ*, the maximum specific activity is reported in the pH range of 8 to 9, with decreasing activity at low pHs with a pK_a of 6.6. Anaerobically isolated AcN₂OR shows a more complex dependence on pH. The pH profile of activity shows two maxima, indicating four separate pK_a’s significant to turnover. The position and intensities of these maxima also shift depending on the pH used for the preceding reductive activation step. This complex dependence of N₂OR reactivity on pH has not been fully explained and how it relates to the possible presence of CuZ due to anaerobic isolation is unclear. The dependence of the rate of the reductive activation step on pH has been separately investigated in aerobically isolated PnN₂OR and is 4-fold slower at high pH (pK_a = 9.0±0.2). This pK_a has been assigned to a Lys residue that protonates and hydrogen bonds to a hydroxide ligand on the Cu_I-Cu_IV edge, raising the reduction potential of the CuZ* site and making it easier to reduce (vide infra). A solvent deuterium isotope effect has also been observed for AcN₂OR with k_cat = 163 s⁻¹ and K_M= 25 µM in H₂O and k_cat = 97 s⁻¹ and K_M= 35 µM in D₂O. The isotope effect on k_cat indicates the involvement of a solvent-exchangeable proton in the rate-determining step of N₂O reduction, while the difference observed in K_M is consistent with a role for hydrogen bonding in the N₂O binding step.

Kinetic studies that use cytochrome c as the electron donor have been performed for AcN₂OR, PnN₂OR, PpN₂OR, and WsN₂OR. These show that cytochrome c is indeed capable of reducing the Cu₄ site and under appropriate conditions N₂OR can turnover in vitro with a cytochrome c (either the native cytochrome or mammalian cytochrome) as the electron donor. However, significant kinetic differences are observed when cytochrome c is used as the electron donor instead of methyl viologen. Anaerobically isolated AcN₂OR has a relatively high specific activity without prior reductive activation when two equivalents of horse heart cytochrome c are used as the electron donor (45 µmol min⁻¹ mg⁻¹ as isolated compared to 124 µmol min⁻¹ mg⁻¹ after reductive activation with methyl viologen). However, for aerobically isolated PnN₂OR turnover with cytochrome c₅₅₁ requires prior reductive activation and occurs with a very low specific activity, comparable to the activities observed for the inactive state of N₂OR (2 µmol min⁻¹ mg⁻¹ with cyt c₅₅₁ compared to 157 µmol min⁻¹ mg⁻¹ with methyl viologen). The rate of this turnover of PnN₂OR with cyt c₅₅₁ is independent of N₂O concentration, indicating that there is a different rate limiting step than for turnover with methyl viologen, and the pH dependence of the activity also differs, showing a maximum at pH 7 (pKa’s 5.5 and 8.3). The rate of electron transfer from cytochrome c₅₅₁ to PnN₂OR has been separately measured by cyclic voltammetry (k = 5.5±0.9 × 10⁵ M⁻¹ s⁻¹) and is consistent with the reported k_cat, so under the conditions of the PnN₂OR study electron transfer from cytochrome c₅₅₁ to N₂OR is rate-limiting. Rate limiting intermolecular electron transfer from cytochrome c is also consistent with a study of anaerobically isolated

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PpN₂OR which measured the rate of electron transfer from horse heart cytochrome c to PpN₂OR in the [Cu₄⁺² 2-hole Cu₇] state to be 150 s⁻¹ when saturating concentrations of cytochrome c are used. Comparing this value to the kcat values for N₂ORs with methyl viologen shows that kₚₑₜ (c → N₂OR) < kcat(methyl viologen) and thus electron transfer from cyt c should be rate-limiting in turnover when cyt c is the electron donor. Additionally, the [Cu₄red 2-hole Cu₂⁻] state of PpN₂OR will react with N₂O in the presence of stoichiometric reduced cytochrome c. A lag phase in this reaction may involve reduction of the [Cu₄red 2-hole Cu₂⁻] state and may indicate that an allosteric effect on N₂OR, induced by cytochrome c binding, is necessary for reduction of Cu₇. 

Since there are significant differences between N₂ORs purified anaerobically and aerobically, comparisons of the specific activities of N₂ORs obtained under different purification conditions have been used to attempt to argue which form is physiologically relevant. Studies comparing N₂ORs that have been purified from the same organism under two sets of conditions and then assayed identically have been performed, but the results are ambiguous. When aerobic and anaerobic PsN₂OR are assayed as isolated, the anaerobic enzyme is slightly more active than the aerobic enzyme, but both have only low activity. After base activation, anaerobic PsN₂OR has 10-fold higher activity than the aerobic form. In contrast, PpN₂OR assayed as isolated and in different redox states shows lower activity for the anaerobic enzyme, but again these activities were low compared to reductively activated PpN₂OR. Aerobically isolated AcN₂OR is reported to have a tenth of the activity of anaerobically isolated AcN₂OR after reductive activation, but this is attributed to a significantly lower copper content and active site occupancy in the aerobically isolated protein. The maximum specific activities reported for anaerobic and aerobically purified N₂ORs from any source organism following reductive activation are comparable (122 µmol min⁻¹ mg⁻¹ for anaerobic AcN₂OR and 157 µmol min⁻¹ mg⁻¹ for aerobic PnN₂OR). In addition, all of these comparisons assume the same occupancy of the tetranuclear copper site in N₂ORs after difference isolation procedures. A further assumption is the enzyme being studied is a pure species rather than a mixture of two active site forms, Cu₇ and Cu₇⁺, present in different relative amounts depending on the purification conditions, as has been determined by spectroscopic study of anaerobically and aerobically purified PpN₂OR. Due to the absence of controls for active site occupancy and the ratio of Cu₇ to Cu₇⁺ present, it is not possible to distinguish the differential reactivity of the anaerobic and aerobic forms of N₂OR from the currently available data and further study of the relative reactivities of anaerobically and aerobically purified N₂OR is required.

A variety of small molecules that inhibit N₂OR have been identified, both from in vitro and in vivo studies. Acetylene is a widely recognized inhibitor of N₂OR activity in vivo but is only weakly inhibiting in vitro. Other in vivo inhibitors, such as hydrogen sulfide, have not been studied in purified N₂OR so it is difficult to distinguish whether they directly inhibit N₂O reduction by interaction with N₂OR or through a regulatory process. Some inhibitors which have been tested in vitro include nitric oxide, nitrite, fluoride, isocyanate and carbon monoxide. While these molecules have been shown to perturb the steady-state activity of N₂OR, the type of inhibition, the mode of interaction of these molecules...
with the copper sites and which redox state they target has not been determined. Cyanide, 2-
mercaptoethanol, azide and some chelators irreversibly inhibit N$_2$OR due to decomposition
of the active site.\textsuperscript{1278,1280} One crystallographic study indicates that iodide is an inhibitor of
N$_2$OR, but the kinetic data are not reported.\textsuperscript{1295}

5.2.3 Structure—A key turning point in the study of N$_2$OR came with the publication of
crystal structures of PnN$_2$OR and PdN$_2$OR in 2000 by Brown et al., who discovered that the
Cu$_Z$ site is a tetranuclear copper cluster, rather than a binuclear copper site, as had been
previously proposed (\textit{vide infra}).\textsuperscript{1292,1326} Crystal structures for N$_2$OR have since been
reported from four sources, PnN$_2$OR (PDB ID 1QNI),\textsuperscript{1292,1326} PdN$_2$OR (PDB ID
1FWX),\textsuperscript{1327} AcN$_2$OR (PDB ID 2IWF)\textsuperscript{1295} and PsN$_2$OR (PDB ID 3SBP, 3SBQ).\textsuperscript{1296} The
first three structures were obtained from aerobically purified and crystallized protein while
the recent PsN$_2$OR structure is the sole example of a structure for anaerobically purified and
crystallized N$_2$OR. In addition, two crystal structures have been reported with small
molecules bound to the active site, a structure of AcN$_2$OR with the inhibitor iodide bound to
the Cu$_Z^*$ cluster (PDB ID 2IWK)\textsuperscript{1295} and a structure of PsN$_2$OR showing the substrate N$_2$O
bound near Cu$_Z$ (PDB ID 3SBR).\textsuperscript{1296}

The initial crystal structure of resting PnN$_2$OR, obtained at 2.4 Å resolution, shows that
N$_2$OR is a homodimer, with each monomer containing an N-terminal domain with a 7-
bladed $\beta$-propeller fold, containing the Cu$_Z^*$ cluster, and a C-terminal domain with a
cupredoxin fold, containing the Cu$_A$ site (Figure 234).\textsuperscript{1326} The monomers are associated in a
head-to-tail fashion, with the C-terminal domain of one monomer forming contacts with the
N-terminal domain of the second monomer. The distance between the Cu$_A$ and Cu$_Z^*$ sites in
the same monomer is 40 Å, while the distance between the Cu$_A$ site in one monomer and the
Cu$_Z^*$ site in the second monomer is only 10 Å. This indicates a functional role for
dimerization in N$_2$OR, since the 40 Å distance is too great for electron transfer between Cu$_A$
and Cu$_Z^*$ in the same subunit, but electron transfer across the dimer interface is possible.\textsuperscript{1326}

The Cu$_A$ site contains two copper atoms bridged by two Cys residues (Cys565, Cys561),
with one copper further ligated by His526 and Met572 while the other is ligated by His569
and the backbone carbonyl of Trp563 (Figure 235). Both His526 and His569 are equitorial
and N$\delta$-coordinated to Cu. The distance between the two copper atoms in Cu$_A$ is 2.48 Å,
consistent with a Cu-Cu bond. The Cu$_A$ site in N$_2$OR is highly similar the Cu$_A$
electron transfer site in cytochrome c oxidase, as had previously been predicted by comparison of the
spectroscopy of N$_2$OR and CcO (see Section 5.2.4).\textsuperscript{1326,1328,1329}

Cu$_Z^*$ has been shown to be a tetranuclear copper cluster, unprecedented in biology or in
synthetic copper complexes.\textsuperscript{1326} The four copper atoms in the cluster are arranged in a
butterfly structure with a single atom bridging all four copper, leading to a cluster with
approximately C$_2$ symmetry where three of the coppers (Cu$_I$, Cu$_II$ and Cu$_IV$) and the $\mu_4$
bridging ligand are coplanar (Figure 236A). In the initial report of the crystal structure of
PnN$_2$OR, the $\mu_4$ bridging ligand in the cluster was assigned as an oxo (O$^{2-}$).\textsuperscript{1326} However,
after spectroscopic and biochemical results that established that there are sulfur isotope
sensitive stretches in the resonance Raman spectrum of the Cu$_Z^*$ site and inorganic sulfide
quantification in N$_2$OR giving a 1:6 ratio with Cu,\textsuperscript{1289} the structure of Cu$_Z^*$ was revised to

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be a $\mu_4$-sulfide bridged tetraneuclear copper cluster. Subsequent higher resolution refinement of crystal structures from PdN$_2$OR and AcN$_2$OR (1.6 Å and 1.86 Å resolution, respectively) corroborated this assignment. The copper-copper distances in the Cu$_Z^*$ cluster are not equal, with shorter Cu$_{II}$-Cu$_{IV}$ and Cu$_{II}$-Cu$_{III}$ distances than Cu$_{I}$-Cu$_{IV}$ and Cu$_{I}$-Cu$_{III}$ distances (Table 44, copper numbering from Figure 236).

The cluster is ligated by seven histidine residues, with two His ligating each copper atom with the exception of Cu$_{IV}$, which has only one His ligand. Five of the seven ligating His residues are Ne-coordinated, while His437 and His79, bound to Cu$_{IV}$ and Cu$_{II}$ respectively, are Nδ-coordinated. An important difference between the known structures of the Cu$_Z$ cluster is the number and identity of the ligands on the Cu$_{I}$-Cu$_{IV}$ edge. In PnN$_2$OR and PdN$_2$OR, one solvent derived ligand is observed on the Cu$_{I}$-Cu$_{IV}$ edge, occupying a bridging position in PdN$_2$OR$^{1327}$ (Figure 236A) but closer to Cu$_{IV}$ in PnN$_2$OR.$^{1292}$ In AcN$_2$OR, two solvent derived ligands are observed, one bound to Cu$_{I}$ and the other to Cu$_{IV}$ (Figure 236B).$^{1295}$ The Cu$_{I}$-Cu$_{IV}$ edge is the most open edge of the Cu$_Z^*$ cluster and therefore is proposed to be the site of N$_2$O binding.$^{1326,1330}$ The variability in solvent coordination at this edge is consistent with labile solvent derived ligands that can be easily replaced by N$_2$O. A crystal structure of AcN$_2$OR with the inhibitor iodide bound to Cu$_Z^*$ has also been reported (Figure 236C).$^{1295}$ Iodide binds as a bridging ligand on the Cu$_{I}$-Cu$_{IV}$ edge, displacing the solvent-derived ligands and blocking the proposed site of N$_2$O binding.

The proposed mechanism of inhibition for I$^-$ is thus competing with N$_2$O for binding to the Cu$_{I}$-Cu$_{IV}$ edge of the cluster.

The recent publication of a crystal structure of anaerobically purified and crystallized PsN$_2$OR challenges the role of the Cu$_{I}$-Cu$_{IV}$ edge as the N$_2$O binding site and also sheds new light on the differences between anaerobically and aerobically purified N$_2$OR (containing Cu$_Z$ and Cu$_Z^*$, respectively).$^{1296}$ The overall protein fold and dimer structure of anaerobic PsN$_2$OR does not differ significantly from previous crystal structures. However, significant changes are observed in the copper sites. The Cu$_Z$ site has an additional sulfur ligand bridging the Cu$_{I}$-Cu$_{IV}$ edge, blocking the proposed N$_2$O binding site (Figure 236D). This edge ligand has been described as a sulfide but definitive assignment of its protonation state is beyond the scope of X-ray crystallography. The remainder of the structure of the site, including the bond lengths and angles between the copper atoms and the $\mu_4$-sulfide, is conserved relative to the previously described 4CuS site in aerobically purified N$_2$OR (Table 45). This establishes the key structural difference between the copper sites in anaerobically and aerobically purified N$_2$OR: Cu$_Z$ is a 4Cu2S cluster while Cu$_Z^*$ is a 4Cu1S cluster with a solvent derived edge ligand. A change also occurs in the Cu$_A$ site. His583, the His ligand of the Cu atom further from Cu$_Z$, is not bound to the Cu$_A$ site in this structure but instead is swung away from the copper, leaving it three-coordinate. This difference is not observed in cytochrome c oxidase but has been proposed in N$_2$OR to be a switching mechanism for triggering electron transfer from Cu$_A$ to Cu$_Z$.$^{1296}$

A structure has also been determined for anaerobic PsN$_2$OR with N$_2$O bound near the Cu$_Z$ site, obtained by pressurizing the anaerobic crystal with 15 bar of N$_2$O followed by flash freezing.$^{1296}$ One localized N$_2$O molecule is observed adjacent to the Cu$_Z$ site, positioned above the Cu$_{IV}$- $\mu_4$S-Cu$_{II}$ face of the cluster and hydrogen bonding to two second sphere...
residues, Met627 and through a localized water molecule to His626, which is also a Cu_A ligand (Figure 237). The shortest distance between N_2O and the Cu_Z site is 3.1 Å (N_2O-Cu_{II}), which is too long to represent a coordination interaction. The N_2O molecule is linear, indicating that there is no significant back-bonding from the Cu_Z cluster. It is possible that N_2O is present in the protein channel through which it accesses the Cu_Z site but the redox state of Cu_Z present in the crystal is not the correct one to coordinate N_2O and activate it for reduction. In an interesting detail, when N_2O is bound between the Cu_A and Cu_Z sites the His583 residue again ligates the more distant Cu of Cu_A. The significance of this conformational change of the Cu_A site to reactivity and how N_2O binding can trigger this change on the side of Cu_A that is more distant from its binding site needs future investigation.

5.2.4 Spectroscopy and Electronic Structure—N_2OR is a protein for which a rich array of spectroscopic information is available. Early in the study of N_2OR, different states of the protein were qualitatively differentiated based on their color (e.g. anaerobically isolated N_2OR was identified as “the purple form” while aerobically isolated N_2OR was identified as “the pink form”). These colors reflect the rich absorption spectra of the Cu_A, Cu_Z* and Cu_Z sites, which contain intense absorption bands in the visible region due to S→Cu ligand to metal charge transfer transitions from the sulfide ligand in the Cu_Z and Cu_Z* clusters and the bridging Cys ligands in Cu_A. These intense LMCT transitions provide an opportunity to study the vibrations of these sites by resonance Raman spectroscopy and further result in increased intensity for the copper d-d transitions, which borrow intensity from the LMCT transitions. Significant insights have also been derived from electron paramagnetic resonance and X-ray absorption spectroscopy. Key insights that have been obtained by spectroscopy include the identification of the μ_4 sulfide ligand in Cu_Z*, the elucidation of the resting redox states of Cu_Z and Cu_Z*, and the role of a second sphere Lys residue that hydrogen bonds to the solvent derived ligand on the Cu_I-Cu_III edge of Cu_Z*. Some ongoing areas of study are the spectroscopic differences between Cu_005A, Cu_Z*, and Cu_Z, the insight these give into the electronic structures of these sites given their different structures, and how these differences relate to reactivity.

The spectroscopic features and electronic structure of Cu_A have been extensively described through the study of the Cu_A containing domain of CcO, spectroscopic studies of an N_2OR mutant deficient in Cu_Z, and synthetic constructs of Cu_A in azurin. The key spectral features of Cu_A^{ox} contribute to the spectroscopy of resting N_2OR prior to reduction and therefore are summarized here, while Cu_A^{red} is spectroscopically silent. Cu_A^{ox} is a class III MV Cu^{1.5}-Cu^{1.5} site with a σ_u ground state and a low-lying π_u excited state (Figure 238). Cu_A^{ox} exhibits an axial EPR signal with g_∥ of 2.18 and g_⊥ of 2.02 and a sharp 7 line hyperfine pattern in the g_∥ region with A_∥ = 44×10^{-4} cm^{-1} (Figure 239A, spectra shown from a Cu_Z deficient mutated variant of PsN_2OR known as N_2OR V). The 7 line hyperfine pattern shows that the unpaired spin is delocalized over two equivalent Cu nuclei (I_{Cu} = 3/2) and the small A_∥ value indicates high covalent delocalization of the unpaired spin onto the Cys thiolate ligands (26±3% S character by S K-edge XAS). The g_∥ value of 2.18 is achieved through mixing with the low-lying π_u excited state at an energy ~3,500 cm^{-1} above the G.S. Cu_A^{ox} has an absorption spectrum...
with three transitions at 20,800 cm\(^{-1}\) (480 nm, \(\varepsilon \approx 4000\) M\(^{-1}\) cm\(^{-1}\)), 18,500 cm\(^{-1}\) (540 nm, \(\varepsilon \approx 4000\) M\(^{-1}\) cm\(^{-1}\)), and 12,800 cm\(^{-1}\) (780 nm, \(\varepsilon \approx 3000\) M\(^{-1}\) cm\(^{-1}\)) (Figure 239B).\(^{1334}\) The absorption transitions at 20,800 cm\(^{-1}\) and 18,500 cm\(^{-1}\) correspond to an intense pseudo A feature in the MCD spectrum of Cu\(_A\)^{ox} (Figure 239C) and are assigned to the perpendicularly polarized S(p\(_x\))→SOMO and S(p\(_y\))→SOMO transitions. In a pure \(\sigma_u\)^{+} G.S., only the S(p\(_x\)) orbital would have overlap with the SOMO, so the observation of two S(p) →SOMO transitions is another result of the \(\pi_u\) mixing into the G.S. Upon laser excitation into the CysS(p\(_x\))→SOMO CT transitions, dominant Cu-S(Cys) stretching modes at 259 cm\(^{-1}\) (mixed Cu-S(Cys)/Cu-N(His) stretching mode, \(\Delta ^{34}S = -3.4\) cm\(^{-1}\)), 276 cm\(^{-1}\) (out of plane “twisting” Cu-S streching mode, \(\Delta ^{34}S = -1.9\) cm\(^{-1}\)) and 347 cm\(^{-1}\) (Cu\(_2\)S\(_2\) core breathing mode, \(\Delta ^{34}S = -1.6\) cm\(^{-1}\)) are enhanced.\(^{1318}\) In N\(_2\)OR, the 347 cm\(^{-1}\) Cu\(_2\)S\(_2\) stretch is split into seven total \(34^S\) isotope sensitive modes ranging from 323–408 cm\(^{-1}\) in energy, which arises from coupling with the Cys and His sidechain vibrations. The transition at 12,800 cm\(^{-1}\) in both the absorption and MCD spectra has been assigned as the intervalence \(\psi \rightarrow \psi^\ast\) CT transition associated with the mixed valent nature of the 2Cu\(_A\)^{δ} site.\(^{62}\) Laser excitation into this transition resonance enhances the three vibrations discussed above and additionally enhances an ~130 cm\(^{-1}\) core “accordion” mode involving motions of the two Cu nuclei along the Cu-Cu bond vector, characteristic of a \(\psi \rightarrow \psi^\ast\) CT for a class III mixed valent site. The relatively high energy of this \(\psi \rightarrow \psi^\ast\) transition indicates direct Cu-Cu orbital overlap, due to the short 2.48 Å distance between the coppers in the Cu\(_A\)^{ox} site. The mixed valent \(\sigma_u\)^{+} G.S. of Cu\(_A\)^{ox} with its direct Cu-Cu bonding (Figure 238) results in significant electronic delocalization that is essential for a low site reorganization energy, leading to rapid and efficient electron transfer.\(^{62}\)

All of the spectral features of Cu\(_A\)^{ox} can be observed in the absorption, MCD, and EPR spectra of resting N\(_2\)OR (Figure 240), whether it is isolated anaerobically (A) or aerobically (B).\(^{1290}\) The 7 line hyperfine pattern of Cu\(_A\) appears significantly sharpened in N\(_2\)OR that is prepared anaerobically. This sharper hyperfine pattern is also seen for the Cu\(_Z\)^{+} variant N\(_2\)OR V (Figure 240 A).\(^{1334}\) In both cases, the sharpness of the Cu\(_A\) signal is due to the absence of spectral overlap with an additional S=½ signal, assigned to resting Cu\(_Z^\ast\), that is present in aerobically isolated N\(_2\)OR but absent in anaerobically isolated N\(_2\)OR.\(^{1290}\) Thus, no significant differences in the spectroscopy of Cu\(_A\)^{ox} in resting N\(_2\)OR are observable between anaerobically and aerobically isolated N\(_2\)OR despite crystallographic evidence that in anaerobically isolated N\(_2\)OR Cu\(_A\) has one less His ligand.\(^{1296}\) Absence of a His ligand, which is a strongly bound equatorial Cu ligand, would be expected to significantly perturb the spin distribution of the site, which should be reflected in the EPR spectrum (as has been observed for a His→Ala variant in a construct of Cu\(_A\) in azurin).\(^{716}\) The absence of such a perturbation of the Cu\(_A\)^{ox} EPR spectrum in resting anaerobically isolated N\(_2\)OR has yet to be explained and developing an understanding of this is necessary to evaluate the effect of the His(off)→His(on) conformational change on electron transfer by Cu\(_A\) and thus its contribution to N\(_2\)OR reactivity.

Spectroscopic studies of the Cu\(_Z^\ast\) site, which assigned the spectral features and used these to elucidate the electronic structure of the active site, were performed on the dithionite reduced state of resting aerobically isolated PtN\(_2\)OR.\(^{1330,1331}\) Under these conditions, Cu\(_A\) is selectively reduced so it does not contribute and the remaining spectral features are due to
CuZ*. Both the EPR spectrum and the magnetic saturation behavior of the MCD spectrum of CuZ* indicate that the resting state of this site has a total spin of $\frac{1}{2}$.\textsuperscript{1331} Two redox states with $S=\frac{1}{2}$ are possible for a tetranuclear copper site: the 3-hole 3Cu\textsuperscript{II}Cu\textsuperscript{I} state (where two of the oxidized coppers are antiferromagnetically coupled) and the 1-hole Cu\textsuperscript{III}3Cu\textsuperscript{I} state. Fitting the intensity of the feature at 8984 eV in the pre-edge of the Cu K-edge X-ray absorption spectrum, a feature characteristic of Cu\textsuperscript{I} complexes, was used to distinguish between the two possibilities. The intensity at 8984 eV in the Cu K-edge XAS spectrum of dithionite reduced PnN\textsubscript{2}OR (Figure 241) establishes the 1-hole state (i.e. Cu\textsuperscript{III}3Cu\textsuperscript{I}) as the resting redox state of CuZ*.\textsuperscript{1331}

The dominant absorption feature of 1-hole CuZ* is a broad absorption band at 15,600 cm\textsuperscript{−1} (640 nm, $\varepsilon \approx 4000$ M\textsuperscript{−1} cm\textsuperscript{−1}) that gives dithionite-reduced N\textsubscript{2}OR its blue color (Figure 242A).\textsuperscript{1330} This corresponds to an intense pseudo-A feature in the LT MCD spectrum (bands 5 and 6 in Figure 242B).\textsuperscript{1330} The presence of a pseudo A feature requires two perpendicularly polarized transitions that can spin-orbit couple in a mutually perpendicular direction on a single center. In CuZ*, the only options that satisfy these conditions are the $S \rightarrow$ Cu CT transitions from the p orbitals of the $\mu_4 S^{2−}$ to different copper centers (Figure 243). Upon laser excitation into the 640 nm absorption band, three intense S isotope sensitive vibrational modes are resonance enhanced: 366 cm\textsuperscript{−1} ($\Delta^{34}$S = −2.1 cm\textsuperscript{−1}), 386 cm\textsuperscript{−1} ($\Delta^{34}$S = −5.8 cm\textsuperscript{−1}), and 415 cm\textsuperscript{−1} ($\Delta^{34}$S = −7 cm\textsuperscript{−1}) (Figure 244A).\textsuperscript{1318} These vibrations profile differently (Figure 244C) and the profile of the 386 cm\textsuperscript{−1} vibration requires the presence of a third S$\rightarrow$Cu CT transition (band 7 in Figure 242), in addition to the two S$\rightarrow$Cu CT transitions in the MCD pseudo A (bands 5 and 6).\textsuperscript{1330} On the basis of the relative intensities of these three S$\rightarrow$Cu CT transitions, band assignments rationalized from an orbital overlap model indicate that band 6 (the most intense) is the Sp\textsubscript{x}$\rightarrow$Cu CT transition, which has the greatest overlap between donor and acceptor MOs, band 5 is the Sp\textsubscript{z}$\rightarrow$Cu CT transition, and band 7 is the Sp\textsubscript{y}$\rightarrow$Cu CT transition (where the orientations of the different $\mu_4 S^{2−}$ p orbitals are given in Figure 243). The resonance enhanced vibrations can be assigned as mixtures of the four Cu-S stretching modes based on normal coordinate analysis and considerations of their enhancement profiles (Figure 244D). The 415 cm\textsuperscript{−1} is thus assigned as the Cu\textsuperscript{I} S stretch, the 386 cm\textsuperscript{−1} vibration as the symmetric breathing mode of the cluster, and the 366 cm\textsuperscript{−1} as dominantly the Cu\textsuperscript{II} S stretch. A total of thirteen electronic transitions are required for a simultaneous fit of the low temperature absorption, CD, and MCD spectra (Table 45).

Six of these transitions, including the three S(p)$\rightarrow$Cu charge transfer transitions (bands 5–7), have significant metal character based on their C/D ratios (bands 3–8 in Figure 242). The remaining transitions with high C/D ratios (bands 3–4, 8) are assigned as d-d transitions of the dominantly oxidized copper atom (vide infra) while the transitions with low C/D ratios are assigned as His$\rightarrow$Cu CT transitions (bands 9–13) and the intravalence Cu\textsuperscript{II}$\rightarrow$Cu\textsuperscript{I} CT transition (band 2).\textsuperscript{1330} The EPR spectrum of resting 1-hole CuZ*, determined at both X-band and Q-band, is axial with a $g_{//}$ of 2.16 and $g_{\perp}$ of 2.04 and hyperfine contributions in the $A_{//}$ region from two copper nuclei in a ~5:2 ratio ($A_{//1} = 61 \times 10^{-4}$ cm\textsuperscript{−1}, $A_{//2} = 24 \times 10^{-4}$ cm\textsuperscript{−1}) (Figure 245).\textsuperscript{1331} The low $g_{//}$ value of 2.16 is due to the high energy of the dxy$\rightarrow$dx\textsuperscript{2}−y\textsuperscript{2} transition (18,000 cm\textsuperscript{−1}, band 8).\textsuperscript{1330} The axial nature of the EPR spectrum shows that
the unpaired electron spin is in an orbital with dominant Cu dx^2-y^2 character and the presence of two unequal A// values indicates that the unpaired spin is delocalized over two copper atoms in a 5:2 ratio."Thus 1-hole CuZ* is a class II mixed-valent site. This correlates well with a ground state wavefunction determined from DFT using a model of the active site with hydroxide as the CuI-CuIV edge ligand (Figure 246) in which the unpaired spin is dominantly localized on CuI (33%) but there is a significant contribution from CuIV (10%). The spin is more localized on CuI because it has a higher coordination number than CuIV (4 ligands rather than 3). The dominant bonding interaction in CuZ* involves the μ4 sulfide pσ orbital overlap with the dx^2-y^2 orbitals of CuI and CuII. This σ bonding interaction provides a superexchange pathway between CuI and CuII, which may be important for the ability of the CuZ* site to perform 2 electron chemistry. CuII may also be on the electron transfer pathway between CuA and the CuZ* site, via a solvent bridge between His79, a CuII ligand, and His569, the proximal His ligand of CuA, which are 5.68 Å apart across a solvent-filled cavity.

The effect of pH on 1-hole CuZ* was further studied to confirm the protonation states of the μ4 S ligand and the solvent derived ligand on the CuI-CuIV edge. The pH dependence of the electronic structure of 1-hole CuZ* was studied by comparing the spectroscopy of dithionite reduced PnN2OR and AcN2OR at pH 6.0 and pH 10.5. The optical transitions of 1-hole CuZ*, determined from the LT absorption and MCD spectra, are not perturbed by pH. The EPR spectra at these two pHs are also virtually identical, indicating that the ground state wavefunction is not perturbed. Thus there is no change in the protonation states of the solvent derived CuI-CuIV edge ligand or the μ4 S ligand over this pH range, since protonating or deprotonating these ligands would significantly perturb the spin density distribution of the cluster. Additionally, the S K-edge XAS spectrum is unaffected by pH, confirming that the protonation state of the μ4 S does not change over this pH range. This strongly favors the assignment of the sulfur ligand as a μ4-sulfide and the CuI-CuIV edge ligand as hydroxide over the whole pH range.

While the electronic structure of the 1-hole CuZ* site is unaffected by pH, there is some perturbation of the resonance Raman spectrum of 1-hole CuZ* at high pH. At pH 10.5 the CuI-S stretch shifts up in energy to 424 cm^-1 (from 415 cm^-1 at pH 6.0) and shows an H2 18O isotope shift of 9 cm^-1, compared to pH 6.0 where all three Cu-S stretching vibrations show no solvent 18O isotope sensitivity (Figure 244B). This can be attributed to a shift in the position of the hydroxide ligand on the CuII-CuIV edge, leading to kinematic coupling between the CuI-S stretch and the CuI-OH stretch at high pH. This pH effect has a pKa of 9.2 and is attributed to the deprotonation of a second sphere Lys residue, Lys397, which is 3.6 Å from the CuI-CuIV edge. Either indirect hydrogen bonding, through a localized water molecule bridging between the hydroxide ligand and Lys397, or rotation of Lys397 to form a direct hydrogen bond to the hydroxide are possible. In the DFT optimized structure used to describe the ground state wavefunction of 1-hole CuZ* (vide supra), a direct hydrogen bond with Lys397 is included. With this hydrogen bonding interaction, the hydroxide ligand does not bridge the CuI-CuIV edge, but instead is only bound to CuI (CuI-O 1.95 Å, CuIV-O 3.30 Å).
While the electronic structure of the 4Cu2S CuZ site has yet to be defined, a number of previous studies have qualitatively described its spectral features, including the information necessary to deduce its resting redox state. Anaerobically purified N2OR contains an oxidized CuA site as well as the CuZ site. When CuA is selectively reduced with ascorbate, leaving only the spectral features of resting CuZ, the dominant spectral feature of the site is an intense absorption band at 17,900 cm\(^{-1}\) (~560 nm, \(\varepsilon \approx 4000\) M\(^{-1}\) cm\(^{-1}\)) with a weaker shoulder at ~15,900 cm\(^{-1}\) (Figure 240A, red).\(^{1290}\) The MCD spectrum of anaerobically prepared N2OR after ascorbate reduction shows only the features of some amount of 1-hole CuZ\(^*\) (30% CuZ\(^*\) according to Rasmussen et al.)\(^{1290}\) and no new features due to CuZ, indicating that resting CuZ is diamagnetic. This is supported by EPR, which shows that the resting state of CuZ is EPR silent (Figure 240A, red; a weak, featureless residual signal that represents <2% of the spin of one Cu\(^{II}\) is again attributed to the presence of some CuZ\(^*\)).\(^{1290}\)

In principle, three redox states with S = 0 are available for a 4Cu2S site: fully oxidized 4Cu\(^{II}\) (where two pairs of oxidized coppers are antiferromagnetically coupled), 2-hole 2Cu\(^{II}\)2Cu\(^{I}\) (with two antiferromagnetically coupled Cu(II)'s), and fully reduced 4Cu\(^{I}\). The fully reduced state can be ruled out because of the presence of intense absorption transitions for the site. While no spectroscopic method has yet been used to experimentally distinguish between the fully oxidized and 2-hole redox states (e.g. XAS), the 2-hole redox state is generally considered to be the resting redox state of CuZ, as it is likely to be more energetically accessible than the highly charged [4Cu2S]\(^{4+}\) fully oxidized state.\(^{1338}\)

The reducing agent dithionite can be used to reduce anaerobically isolated N2OR to a state containing CuA\(^{red}\) and 1-hole 4Cu2S CuZ. Its absorption spectrum is dominated by an intense, broad feature at 14,900 cm\(^{-1}\) (~670 nm, \(\varepsilon \approx 4000\) M\(^{-1}\) cm\(^{-1}\); Figure 240A, blue) that has a corresponding intense pseudo A feature at low temperature that is qualitatively similar to that of 1-hole CuZ\(^*\) (Figure 240A, blue).\(^{1290}\) The EPR spectrum of 1-hole CuZ is axial with a g\(_{\perp}\) of 2.055 and a g\(_{\parallel}\) region that is poorly resolved in X band EPR (Figure 240A, blue). The resonance Raman spectrum obtained from laser excitation into the dominant band of 1-hole CuZ shows two intense S isotope sensitive vibrations at 363 cm\(^{-1}\) and 382 cm\(^{-1}\).\(^{1318}\) Thus the spectroscopic features reported for 1-hole CuZ are qualitatively quite similar to those of 1-hole CuZ\(^*\). Prior to the recent crystallographic definition of a 4Cu2S core structure for CuZ (see Section 5.2.3), this high degree of spectroscopic similarity between the 1-hole forms of CuZ and CuZ\(^*\) led to the conclusion that the difference between the two sites was a matter of the protein environment or second sphere interactions.\(^{1290,1318}\) In light of the significant structural differences between CuZ and CuZ\(^*\) that have recently been elucidated, further evaluation of the electronic structure of 1-hole CuZ is required to understand both the similarities and the differences between the clusters. Assignment of the protonation states of the two sulfur ligands in CuZ will be the necessary first step towards assigning its spectroscopic features and elucidating its geometric and electronic structure in both of its accessible redox states.

### 5.2.5 Molecular mechanism

On the basis of kinetic and spectroscopic studies of the reaction of N2O with CuZ\(^*\) that have identified the active redox state of this site and rate information for a number of steps, a mechanistic proposal for the reaction of the 4Cu1S CuZ\(^*\) site with N2O in turnover has been developed.\(^{1322}\) Since much of this information is...
not known for the 4Cu2S CuZ site, particularly its active redox state, a proposed mechanism for N2O reduction by the anaerobic CuZ enzyme is lacking. The mechanism for N2O reduction by CuZ* will be discussed here. Further work to develop a mechanistic understanding of the relative reactivity of the 4Cu2S CuZ site with N2O is an important future direction for N2OR research.

The proposed mechanism for N2O reduction by CuZ*-containing N2OR is presented in Figure 247.1322,1339 The active redox state of CuZ* has been identified as the fully reduced 4CuI state (see Section 5.2.2).1317 Thus, the reaction begins with the fully reduced state of N2OR, in which both CuA and CuZ* are reduced (A). N2O then binds to the 4CuI CuZ* site in a µ-1,3 bridging mode on the CuI-CuIV edge (B).1339 In the next step of N2O reduction, a 2 electron transfer from fully reduced CuZ* to N2O induces N-O bond cleavage, releasing N2 and leaving the oxygen as an O2− ligand bound bidentate on the CuI-CuIV edge of a putative 2-hole intermediate (C). This N-O bond cleavage is then followed by two electron transfer and two proton transfer steps to regenerate fully reduced CuZ* and complete the catalytic cycle. Based on DFT calculations (vide infra), alternating protonation and reduction steps are favored, yielding a possible hydroxobridged 2-hole intermediate (D) which is reduced by electron transfer from CuAred to generate the CuAox/CuZo 1-hole intermediate that is observed experimentally in single turnover (E).1339 The first protonation and reduction steps are rapid, since there is no observed accumulation of a 2-hole CuZ* intermediate (C or D).1322 After reduction of CuAox by the physiological reductant, cytochrome c, CuZo is rapidly reduced by CuAred to complete the catalytic cycle.1309 In the absence of an external reductant, a slower non-reductive decay converts CuZo to the inactive resting 1-hole state of CuZ* (F), which then must undergo a slow reductive activation process to re-enter the catalytic cycle (vide supra).1322 Given the kinetics of electron transfer to N2OR by cytochrome c (see Section 5.2.2),1309,1310 cytochrome c reduction of N2OR is thought to be the rate limiting step in vivo, while with a faster, non-physiological reductant such as methyl viologen the reduction of CuZo by CuAred is likely to be rate-limiting.

A number of the details of the mechanism of N2O reduction have been evaluated using DFT calculations, including the binding mode of N2O to the fully reduced state of CuZ*, the role of backbonding in N-O bond cleavage, the role of hydrogen bonding in lowering the barrier for N-O bond cleavage, and the thermodynamics of protonation and reduction of potential CuZ* intermediate species.1339 DFT calculations using the BP86 functional and 6–311G* basis set show that µ-1,3 binding of N2O is the lowest energy structure for N2O coordination to 4CuI CuZ* (Figure 247, B).1339 In the optimized geometry (Figure 248A), the N2O ligand is bent, with a 139° N-N-O angle. This bent binding allows significant backbonding from the 4CuI site into the unoccupied π* orbital of N2O; the LUMO has 61% N2O π* character (mostly on N) and 22% Cu character (Figure 248B). Backbonding results in the elongation of the N-O bond from 1.184 Å in free N2O to 1.321 Å in the complex, indicating that N2O is activated for the key N-O bond cleavage step. These DFT calculations yield an upper limit for the activation barrier for N-O bond cleavage of 18 kcal/mol. This barrier could be further lowered to 9–13 kcal/mol by hydrogen bonding to the O atom of N2O, which carries significantly more charge in the transition state of N-O bond cleavage than in the initial N2O.
bound complex. However, a model complex study by Bar-Nahum et al. suggests that a \( \mu-1,1-O \) binding mode for \( N_2O \) coordination to \( Cu_Z^* \) might be relevant and this possibility should be compared to the \( \mu-1,3 \) binding geometry.

DFT calculations have also been used to investigate the thermodynamics of protonation and reduction of potential \( Cu_Z^* \) intermediates (Figure 249). Protonation of an oxo-bridged 2-hole intermediate is thermoneutral (\( \Delta G = 0 \text{ kcal/mol} \)), which suggests that proton transfer could be concerted with N-O bond cleavage. Subsequent reduction of a hydroxide-bridged 2-hole intermediate is downhill, suggesting that sequential protonation and reduction steps are possible. In contrast, discrete protonation or reduction of a hydroxide-bridged 1-hole intermediate is uphill (\( \Delta G = +17 \text{ kcal/mol} \) or \( \Delta G = +15 \text{ kcal/mol} \), respectively), indicating that PCET is required to generate the thermodynamic driving force for formation of the fully reduced state. In the absence of the exogenous electron donor required to perform the second reduction step (via reduction of \( Cu_A^{ox} \), \textit{vide supra} ), a hydroxide-coordinated 1-hole intermediate would be thermodynamically favoured over an oxo-bridged 1-hole species (\( \Delta G = +47 \text{ kcal/mol} \)) or a water-coordinated 1-hole species (\( \Delta G = +17 \text{ kcal/mol} \)). This suggests that the experimentally observed \( Cu_Z^0 \) intermediate should be a hydroxide-coordinated 1-hole species. However, further spectroscopic characterization is required to definitively assign the structure of \( Cu_Z^0 \) and determine the factors responsible for its rapid reduction in turnover.

Four interesting issues must be clarified to fully understand the mechanism of \( N_2O \) reduction: 1) determining which of \( Cu_Z \) (4Cu2S) and \( Cu_Z^* \) (4Cu1S) is, in fact, the reactive form of the cluster in vitro and in vivo, thus evaluating the importance of the \( Cu_{1-IV} \) edge as an \( N_2O \) binding site, 2) describing the mechanism by which the 4Cu2S \( Cu_Z \) site reacts with \( N_2O \), 3) clarifying the nature of the 1-hole \( Cu_Z^0 \) intermediate in the turnover of \( Cu_Z^* \) with \( N_2O \) to elucidate its differences from resting \( Cu_Z^* \) that account for its rapid reduction and role in turnover, and 4) determining the nature of the two electron reduction of \( N_2O \) by trapping a 2-hole intermediate form of \( Cu_Z^* \) in turnover before electron transfer from \( Cu_A \), perhaps in an \( N_2O \) variant lacking the \( Cu_A \) site. Thus a number of important directions for research remain to fully understand nitrous oxide reductase and its unique tetranuclear \( Cu_Z \) cluster active site.

### 6.0 Concluding Comments

In the different sections presented above, we have attempted to define the present state of Cu active site catalysis and biogenesis. A number of topics in Cu biochemistry have not been presented here. The blue Cu and \( Cu_A \) sites are described in passing in the sections on the multicopper oxidases, nitrite reductases, \( N_2O \) reductases, and cytochrome C oxidases but not presented in detail. Recent reviews are available on their geometric and electronic structures and their contributions to electron transfer. Also, Cu/Zn superoxide dismutase, which plays a key role in detoxifying superoxide, and its mutations that can lead to amyotrophic lateral sclerosis (ALS) (also known as Lou Gehrig’s disease) has been the focus of a number of reviews and is presented in the Valentine et al chapter of this issue. We have also not covered the large field of copper homeostasis, transport, and storage which usually involves Cu(I) binding to Cys ligands, which have been thoroughly reviewed.
Two particularly interesting areas of Cu bioinorganic chemistry are not explored here as present knowledge is too limited. The GH61 enzymes or polysaccharide monooxygenases (PMOs) have a single Cu center that activates O$_2$ for H-atom abstraction from crystalline cellulose embedded in lignin to break it into smaller soluble saccharides that can be used as precursors for biofuel production (Figure 250A). Crystal structures are now available which show a Cu active site with two His (one methylated at the N-$\varepsilon$ position), a terminal amine, and a water-derived ligand. In one structure, the x-ray radiation has reduced the site which has reacted with O$_2$ to form what is proposed to be an end-on bound superoxide (Figure 250B). A mechanism parallel to that of the non-coupled binuclear Cu enzymes in section 3.3, where the Cu(II) superoxide H atom abstracts from the C1 or C4 carbons of cellulose has been invoked, and it will be interesting to see how the ligation differences in GH61s relative to those of the Cu$_M$ center in the non-coupled binuclear Cu enzymes relate to new reactivities. Also, Cu sites have been strongly correlated to neurodegenerative diseases. Cu(II)’s tightly bound to His, and perhaps deprotonated amide ligands, are present in the prion proteins related to transmissible spongiform encephalopathies, amyloid $\beta$ proteins related to Alzheimer’s disease, and $\alpha$-synuclein in Parkinson’s disease. The functional and misfunctional roles of the Cu(II) are unclear but appear to relate to protein folding, aggregation, and perhaps redox to generate reactive oxygen species.

The field of Cu bioinorganic chemistry has developed far in the enzymology, modeling, spectroscopy, and geometric and electronic structure correlation with reactivity. However, as we have tried to emphasize throughout this review, there is still much to be understood in the different classes of Cu enzymes described above and in new areas that are ripe for exploration.

**Acknowledgments**

EIS would like to thank his past students and collaborators as indicated in the references cited for their outstanding contributions to this field and Dr. Ryan Cowley for thoughtful comments. The funding for this research by NIH grant DK31450 is gratefully acknowledged.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>DOPAquinone</td>
<td>3-(3,4-dioxocyclohexa-1,5-dien-1-yl)-L-alanine</td>
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<tr>
<td>DHI</td>
<td>5,6-dihydroxyindole</td>
</tr>
<tr>
<td>DHICA</td>
<td>5,6-dihydroxyindole-2-carboxylic acid</td>
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<td>Abs</td>
<td>Absorption</td>
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<td>AO</td>
<td>Amine oxidase</td>
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<tr>
<td>AF</td>
<td>Antiferromagnetic</td>
</tr>
<tr>
<td>AGAO</td>
<td><em>Arthrobacter globiformis</em> Amine Oxidase</td>
</tr>
<tr>
<td>AOX</td>
<td>ascorbate oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>BOD</td>
<td>Bilirubin Oxidase</td>
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<tr>
<td>CaOx</td>
<td>catechol oxidase</td>
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<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>CT</td>
<td>Charge Transfer</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CI</td>
<td>Configuration Interaction</td>
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<td>CuNiR</td>
<td>Copper Nitrite Reductase</td>
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<tr>
<td>CB-PPOs</td>
<td>Coupled Binuclear Polyphenol Oxidases</td>
</tr>
<tr>
<td>CeO</td>
<td>Cytochrome c oxidase</td>
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<tr>
<td>DFT</td>
<td>Density Functional Theory</td>
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<td>DβM</td>
<td>Dopamine β-monooxygenase</td>
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<td>DPQ</td>
<td>Dopaquinone</td>
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<tr>
<td>ECP</td>
<td>Effective Core Potentials</td>
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<td>Z_{eff}</td>
<td>Effective nuclear charge</td>
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<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
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<td>ESEEM</td>
<td>Electron Spin Echo Envelope Modulation</td>
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<td>ET</td>
<td>Electron Transfer</td>
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<td>ENDOR</td>
<td>Electron-Nuclear Double Resonance</td>
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<tr>
<td>H_{AB}</td>
<td>Electronic Coupling Matrix Element</td>
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<td>EAS</td>
<td>Electrophilic Aromatic Substitution</td>
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<td>EXAFS</td>
<td>Extended X-ray absorption fine structure</td>
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<td>FMOs</td>
<td>Frontier molecular orbitals</td>
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<td>FCI</td>
<td>Full Configuration Interaction</td>
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<td>GO</td>
<td>Galactose Oxidase</td>
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<td>GTO</td>
<td>Gaussian-Type orbital</td>
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<td>Glyoxal oxidase</td>
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<td>Zero Field Splitting</td>
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<td>Hartree-Fock</td>
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<td>Hp</td>
<td>Hephaestin</td>
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<tr>
<td>Abbreviation</td>
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<td>HOMO</td>
<td>Highest Occupied Molecular Orbital</td>
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<td>Kinetic Isotope Effect</td>
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<td>Kohn-Sham</td>
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<td>Ligand to Ligand Charge Transfer</td>
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<td>LMCT</td>
<td>Ligand to Metal Charge Transfer</td>
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<td>LDA</td>
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<td>LUMO</td>
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<td>Lysine Tyrosylquinone</td>
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<td>MGGA</td>
<td>Meta-Generalized Gradient Approximation</td>
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<td>MMO</td>
<td>Methane monooxygenase</td>
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<tr>
<td>MYA</td>
<td>million years ago</td>
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<tr>
<td>MV</td>
<td>Mixed Valint</td>
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<td>MO</td>
<td>Molecular Orbitals</td>
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<td>MCO</td>
<td>Multicopper Oxidase</td>
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<tr>
<td>IVT</td>
<td>N-acetyl-diiodotyrosyl-D-threonine</td>
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<td>dbed</td>
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<td>NI</td>
<td>Native Intermediate</td>
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<tr>
<td>NOR</td>
<td>Nitric oxide reductase</td>
</tr>
<tr>
<td>N$_2$OR</td>
<td>Nitrous oxide reductase</td>
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<tr>
<td>PAL</td>
<td>peptidyl-$\alpha$-hydroxylglycine-$\alpha$-amidating lyase</td>
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<td>PAM</td>
<td>peptidylglycine $\alpha$-amidating monooxygenase</td>
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<td>PHMcc</td>
<td>peptidylglycine $\alpha$-hydroxylating monooxygenase catalytic core</td>
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<td>Peroxy Intermediate</td>
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<td>Phenoxazinone synthase</td>
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<td>PMOs</td>
<td>polysaccharide monooxygenases</td>
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<td>PES</td>
<td>Potential Energy Surface</td>
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<td>rR</td>
<td>Resonance Raman</td>
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<td>RvL</td>
<td><em>Rhus vernicfera</em> laccase</td>
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<td>RT</td>
<td>Room Temperature</td>
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<td>SKIE</td>
<td>Solvent kinetic isotope effect</td>
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<td>Spin-orbit Coupling</td>
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<td>SQUID</td>
<td>Superconducting Quantum Interference Device</td>
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<td>Time-dependent Density Functional Theory</td>
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<td>Trinuclear Copper Cluster</td>
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<td>Type 1</td>
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<td>Ty</td>
<td>Tyrosinase</td>
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<td>VBCI</td>
<td>Valence bond configuration interaction</td>
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<td>VTVH</td>
<td>Variable temperature, variable field</td>
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<td>X-Ray Absorption Spectroscopy</td>
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<td>X-ray magnetic circular dichroism</td>
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<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<tr>
<td>XANES</td>
<td>X-ray Absorption Near Edge Structure</td>
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<tr>
<td>Xα-SW</td>
<td>Xα Scattered Wave</td>
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Biographies

EIS

Edward I. Solomon grew up in North Miami Beach, Florida, received his Ph.D. at Princeton (with D.S. McClure) and was a postdoctoral fellow at The Ørsted Institute (with C.J. Ballhausen) and then at Caltech (with H.B. Gray). He was a Professor at the Massachusetts Institute of Technology until 1982 when joined the faculty at Stanford University, where he is now the Monroe E. Spaght Professor of Humanities and Sciences and Professor of Photon Science at SLAC National Accelerator Lab. He has been an Invited Professor in Argentina, Australia, China, France, India and Japan. Professor Solomon’s research is in the fields of Physical-Inorganic and Bioinorganic Chemistry with emphasis on the application of a wide range of spectroscopic methods combined with QM calculations to elucidate the electronic structure of transition metal sites and its contribution to physical properties and reactivity. He has received a wide range of medals and awards and is a member of the National Academy of Sciences, the American Academy of Arts and Sciences and a Fellow in American Association for the Advancement of Science and the American Chemical Society.

DEH

David E. Heppner was born and raised in Chicago, Illinois. He earned a B.S. in Chemistry summa cum laude from the University of Minnesota pursuing computational and synthetic studies of copper-containing bioinorganic systems in the labs of Christopher J. Cramer and William B. Tolman. He is currently a doctoral candidate at Stanford University where he is advised by Edward I. Solomon, working on mechanistic studies of the multicopper oxidases.

EP

Esther M. Johnston received her B.Sc. from the University of Toronto. She did summer research at the Université de Montréal with Professor Christian Reber. She is currently
working towards her Ph.D. in inorganic chemistry at Stanford University under the direction of Edward Solomon. Her research interests focus on using spectroscopy to understand substrate activation by oxidized copper sites in biology and characterizing the electronic structure and reactivity of the tetranuclear copper sulfide cluster in nitrous oxide reductase.

JWG

Jake W. Ginsbach grew up outside of Casper, WY. He attended Whitman College where he performed undergraduate research with Frank M. Dunnivant on the desorption kinetics of chlorinated pesticides from sediments. After graduating from Whitman in 2009, he began working on his Ph.D. at Stanford University with Edward I. Solomon. His research focuses on the reactivity and structure/function correlations of the coupled binuclear copper proteins family and related copper, dioxygen model compounds.

JC

Jordi Cirera (Barcelona, Spain, 1979) graduated in chemistry from the University of Barcelona (2002) and received his doctorate (with honors) from the same university in 2006 under the supervision of prof. Santiago Alvarez and prof. Eliseo Ruiz. After obtaining his degree, he made a postdoctoral stage at Stanford University in prof. Edward I. Solomon’s group working in copper mediated biogenesis in metalloenzymes. He has been visiting researcher at the Max-Planck Institute for solid state research, working with Prof. Jens Kortus in the field of Single Molecule Magnets. He is currently a postdoctoral fellow at University of California, San Diego, working in Prof. Francesco Paesani’s group in the computational modeling of electronic structure properties in Metal-Organic Frameworks. His research interests focus on the electronic structure of transition metal complexes and the implications that this has on the stereochemistry, spectroscopic parameters and reactivity of these molecules, using electronic structure methods combined with spectroscopic data.

MQ
Munzarin F. Qayyum grew up in Dhaka, Bangladesh, received her B.A. in Chemistry and Economics from Wellesley College in Massachusetts in 2006. Her undergraduate research was done under the supervision of Nolan T. Flynn and focused on investigating the drug delivery properties of Poly(N-Isopropylacrylamide) hydrogels loaded with gold nanoparticles. She is currently working towards her Ph.D. in Chemistry at Stanford University under the direction of Edward I. Solomon and Keith O. Hodgson. Her research focuses on the use of different X-ray absorption techniques to investigate the geometric and electronic structure of copper-containing metalloproteins and correlating them to reactivity.

MTKE

Matthew T. Kieber-Emmons was born in 1980 in Buffalo, NY. He received a B.S. in 2002 from Saint Joseph’s University in Philadelphia, PA. He pursued graduate studies with C.G. Riordan at the University of Delaware receiving a Ph.D. in 2007 studying oxygen and sulfur activation by monovalent nickel, and was an NIH postdoctoral fellow at Stanford University with E.I. Solomon studying heme-copper oxidases and type III copper sites. He has accepted a faculty position in the Department of Chemistry at the University of Utah and will continue studies on problems in bioinorganic and bioinspired catalysis.

CK

Christian H. Kjaergaard was born in Copenhagen, Denmark. He earned a B.Sc. in Agricultural Science and a M.Sc. in Biology/ Biotechnology from the University of Copenhagen. He is currently doing his Ph.D. in Chemistry in Prof. Edward I. Solomon’s research group at Stanford University. His research focuses on the Multicopper Oxidases and how their unique active sites allow for the efficient reduction of dioxygen.

RGH

Ryan G. Hadt was born in Eau Claire, Wisconsin. He received his B.S. and M.S. in inorganic chemistry from the University of Minnesota Duluth (with Prof. Victor N.
Nemykin). During this time he also worked on a summer research project at Tohoku University in Sendai, Japan (with Prof. Yoshiyuki Kawazoe). He is currently working toward his Ph.D. in inorganic chemistry at Stanford University (with Prof. Edward I. Solomon) as an ARCS scholar and Gerhard Casper Stanford Graduate Fellow. His current research uses a broad range of spectroscopic methods coupled to electronic structure calculations to elucidate structure-function relationships in transition metal ion mediated catalysis and biological electron transfer.

LT

Li Tian obtained her PhD degree with Prof. Richard Friesner on QM/MM study of P450 catalysis mechanism and developing empirical correction parameters in DFT calculation. She did her first postdoc with Prof. Edward Solomon on spectroscopy study and quantum chemistry simulation of Cu proteins and model complexes that mimic the protein active site. Her research was focused on the catalysis mechanism of the non-coupled binuclear copper enzymes. She is currently working for Dr. Eric Martin at Novartis on protein family based virtual screening methods.

References

10. Penner-Hahn, JE.; Ward, JD. Personal communication.

Chem Rev. Author manuscript; available in PMC 2015 April 09.
147. Telfer WH, Kunkel JG. Annual review of entomology. 1991

Chem Rev. Author manuscript; available in PMC 2015 April 09.

Chem Rev. Author manuscript; available in PMC 2015 April 09.
220. For a hypothetical case where P1/2 = 6 and 11 torr at 10° and 20° C, respectfully, in a solution at 32‰ salinity, equation(3.1.1) determines that ΔH = −10.0 kcal/mol. When only the temperature dependence of O2 solubility is accounted for, ΔH = −6.4 kcal/mol. Accounting for both temperature and salinity gives the most accurate value of −6.7 kcal/mol.
221. To convert salt concentrations to salinity, 1‰ salinity was assumed to be equivalent to 1 gram of salt per liter.


238. Powell ML, Watts SA. Comparative Biochemistry And Physiology AMolecular & Integrative Physiology. 2006; 144:211.


245. Terwilliger RC, Terwilliger NB. Comparative Biochemistry And Physiology AMolecular & Integrative Physiology. 1987; 87:683.


Chem Rev. Author manuscript; available in PMC 2015 April 09.


510. While the original report assigned the structure of A. bisporus as deoxy-Ty due to the long Cu•••Cu distance, the lack of an intense 8984 eV feature in the XAS spectra (Ismaya WT, Rozeboom HJ, Schurink M, Boeriu CG, Wichers H, Dijkstra BW. Acta crystallographica. Section F, Structural biology and crystallography communications. 2011; 67:575.) of the crystals indicates that the Cu’s are oxidized.
521. Casella and coworkers have claimed to observe the formation of oxy-T Ty based on a small charge transfer band at ~410 nm. However, the result is controversial.

Chem Rev. Author manuscript; available in PMC 2015 April 09.

Chem Rev. Author manuscript; available in PMC 2015 April 09.


Chem Rev. Author manuscript; available in PMC 2015 April 09.
897. Bento I, Silva CS, Chen ZJ, Martins LO, Lindley PF, Soares CM. Bmc Structural Biology. 2010; 10

Chem Rev. Author manuscript; available in PMC 2015 April 09.

Chem Rev. Author manuscript; available in PMC 2015 April 09.


1091. Wikstrom M, Verkhovsky MI. Biochimica et biophysica acta. 2003

Chem Rev. Author manuscript; available in PMC 2015 April 09.


Chem Rev. Author manuscript; available in PMC 2015 April 09.


Figure 1.
Copper active sites in biology.
Figure 2.
Latimer Diagram for Oxygen Reduction at pH = 7.0 Adapted from References 5 and 6.
Figure 3.
(Left) Ligand field splitting of Cu(I) 4p orbitals as a function of site geometry. (Right) K-edge XAS of two-coordinate Cu(I) [Cu₂(EDTB)](ClO₄)₂ (red), three-coordinate Cu(I) [Cu(L₁-pr)](BF₄) (green), and four-coordinate Cu(I) [Cu(py)₄]ClO₄ (blue). (Reprinted with permission from Ref. 8. Copyright 1987 American Chemical Society.)
Figure 4.
(Left) Schematic of Cu K-β emission. The ligand ns and np (where n=2,3 depending on ligand type) to metal 1s transitions occur via LMCT. L(np) and L(ns) = ligand p and s hole, respectively. (Right) XES of ZnO (a d^{10} system).
Figure 5.
XES spectra showing the satellite region of d⁹ Cu(II) complexes.
Figure 6.
XES spectra showing the valence region of different d^{10} Zn^{2+} complexes. (A) Ligation to Zn is varied from C, O, to F. (B) Ligation to Zn is changed from H_{2}O to OH\(^{-}\). (Used with permission from Ref. 10.)

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Figure 7.
Ligand Field Splitting of the d manifold. A. Octahedral to square planar to tetrahedral. B. Square pyramidal to trigonal byparimidal. Rearrangements through Berry pseudorotation (C2v) and associated ligand displacement (Cs) coordinates.
Zeeman effect on an $S=1/2$, $M_S=\pm 1/2$ system. The degenerate $M_S$ values split in a magnetic field by $g\beta H M_S$. This splitting equals the microwave energy $hv$, at a magnetic field where microwave absorption occurs.
Figure 9.
(top) A frozen solution EPR spectrum gives a powder pattern with all orientations ($g\parallel(H_1)$ →$g_{\perp}(H_2)$) weighted by the number of molecules with specific orientations. Thus the intensity increases towards the field associated with the perpendicular direction. (middle) The EPR spectrum is taken as the first derivative to enhance sensitivity. (bottom) Anisotropic hyperfine coupling of the electron spin to the Cu nucleus spin $I=3/2$. 

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Figure 10.

Delocalization of the electron spin onto the ligand $L_i$ by an amount $(\beta_i^{L_i})^2$. The ligand valence orbital is a hybrid with $s$ and $p_z$ character.

*Chem Rev*. Author manuscript; available in PMC 2015 April 09.
Figure 11. ENDOR experiment for an $S=1/2$, $M_S = \pm 1/2$ metal site. Left, Levels for protons ($I=1/2$, nuclear Zeeman large related to metal-ligand hyperfine) and Right, levels for other nuclei (in particular $N$, $I = 1$, nuclear Zeeman smaller than metal-ligand hyperfine coupling).
Figure 12. The ESEEM spectrum: (top) Modulated decay of the spin echo and (bottom) The Fourier Transform of the modulation.\textsuperscript{25,26}
Figure 13.
The differences in $\sigma$ and $\pi$ overlap between the ligand and the copper valence orbitals leads to low energy weak $\pi$ and higher energy intense $\sigma$ charge transfer transitions.
Figure 14.
Resonance Raman spectroscopy: Excitation energy dependence of resonance intensity gives the excitation profile. (Reprinted with permission from Ref. 39)
Figure 15.
Cu K-edge spectra of Cu$^{II}$ (black line) and Cu$^{III}$ (blue line) model complexes. The inset amplifies the pre-edge region.
Figure 16.
(Left) Orientation averaged Cu K-edge spectra of $D_{4h}\text{CuCl}_4^{2-}$ and plastocyanin. Insets show the expanded pre-edge region. (Right) Polarized Cu K-edge spectra of plastocyanin. Inset shows the expanded pre-edge region. (Reprinted with permission from Ref. 43. Copyright 1993 American Chemical Society.)
A) Ground-state wave functions

B) Final-state wave functions

Figure 17.
Configuration interaction formalism for the Cu K-edge near-edge analysis. A) Ground-state wave functions determined by $T$ and $\Delta$ parameters. B) Final-state wave functions with the $1s\rightarrow4p + LMCT$ shakedown final-state ($\Psi_s$) separated from the main $1s\rightarrow4p$ final-state ($\Psi_m$) by the splitting $W$. 
Figure 18.
Z-polarized Cu K-edge spectra of $D_{2d}$ $\text{CuCl}_4^{2-}$. Data (---) and fit (-----). The intensity of the main transition is not well established due to the rising-edge background. (Reprinted with permission from Ref. 43. Copyright 1993 American Chemical Society.)
Figure 19.
Cu L₃-edge XAS spectra for D₄h-CuCl₄²⁻ and plastocyanin. Values listed are the amount of Cu d character in the half occupied HOMO. (Reprinted with permission from Ref. 45. Copyright 1993 American Chemical Society.)
Figure 20.
Cu L-edge XMCD spectra for A) mononuclear blue Cu site in plastocyanin measured at ~0.3 K, and B) Cu center in [(F₈TPP)Fe³⁺-O-Cu²⁺(TMPA)](ClO₄) heme-Cu dimer measured at 2.2 K. C) Structure of [(F₈TPP)Fe³⁺-O-Cu²⁺(TMPA)](ClO₄) heme-Cu dimer. (Reprinted with permission from Ref. 47. Copyright 2004, American Institute of Physics.)
Figure 21.
EPR spectrum of an S=1 system including the characteristic $ΔM_S = 2$ transition and the Zero Field Splitting D of the $ΔM_S = 1$ transition which are anisotropic.
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phenylthiourea (PTU)  kojic acid  mimosine  tropolone
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Figure 236.
A comparison of the structures of CuZ from different N2OR crystal structures. A) PdN2OR,1292 B) AcN2OR,1295 C) AcN2OR with iodide bound,1295 and D) anaerobically purified PsN2OR,1296 Copper is colored in green; sulfide in yellow; iodine in purple; and oxygen in red.
Figure 237.
The structure of N$_2$O bound at the Cu$_Z$ site in a crystal of anaerobic PsN$_2$OR. Distances to Cu$_Z$ and key residues labelled in Å.
Figure 238.
DFT optimized ground state wavefunction ($\sigma_u^*$) and low-lying excited state wavefunction ($\pi_u$) of Cu$_A$ modeled as [Cu$_3$(SCH$_3$)(imz)$_2$]$^+$ (B3LYP/TZVP(Cu,S)/6-31G*). (Reprinted with permission from Ref. 1335. Copyright 2003 American Chemical Society.)
Figure 239.
Spectral features of CuA from the N2OR V variant of PsN2OR (lacking the CuZ cluster). A) 77 K EPR spectrum. B) Room temperature absorption spectrum. C) Low temperature MCD spectrum.
Figure 240.
Spectroscopic comparison of anaerobically and aerobically purified PsN₂OR. A) Room temperature absorption, low temperature MCD (7T, 5K), and low temperature EPR (77 K) of anaerobically isolated N₂OR in the fully oxidized state (black), ascorbate reduced state (red) and dithionite-reduced state (blue). B) Room temperature absorption, low temperature MCD (7T, 5K), and low temperature EPR (77 K) of aerobically isolated N₂OR in the fully oxidized state (black), ascorbate reduced state (red) and dithionite-reduced state (blue).
Figure 241.
Copper K-edge X-ray absorption spectrum of N₂OR, with fits predicted for a 3Cu²⁺Cu⁺ site (dashed) and a Cu²⁺3Cu⁺ site (dotted).
Figure 242.
A) 10 K absorption spectrum of 1-hole Cu$_Z^*$ from PnN$_2$OR. B) MCD spectrum of 1-hole Cu$_Z^*$ from PnN$_2$OR (+7T, 5K).
Figure 243.
Orientation of the S p orbitals in CuZ⁺. Selected MOs from a DFT calculation on an early model of the CuZ⁺ site (BP86 38%HF, 6-311G*/6-31G*). (Reprinted with permission from Ref. 1330. Copyright 2002 American Chemical Society.)
Figure 244. Resonance Raman spectrum of 1-hole CuZ* with pH and H2O18 dependence. A) Resonance raman spectrum of 1-hole CuZ* from PnN2OR at pH 7 (77 K, 629 nm excitation). B) Resonance raman spectrum at pH 10.5 (77 K, 629 nm excitation). C) Excitation profiles of the three resonance enhanced vibrations of 1-hole CuZ* profiled at pH 7, revealing three S to Cu charge transfer transitions (5, 6, 7). D) Normal modes of the CuZ* core assigned by normal coordinate analysis. (Reprinted with permission from Ref. 1330. Copyright 2002 American Chemical Society.)
Figure 245.
EPR spectra of 1-hole Cu$^{+*}$ from PnN$_2$OR. A) X-band EPR at pH 7 and 77 K (simulated spectrum top, experimental bottom). B) Q-band EPR at pH 7 and 77 K (simulation top, experimental bottom). (Reprinted with permission from Ref. 1320. Copyright 2007 American Chemical Society.)
Figure 246.
Lowest unoccupied molecular orbital of 1-hole Cu$_Z^+$, labeled with Mulliken Atomic Spin Density for core atoms (B3LYP/6-311++G** on Cu$_{4}$SO/6-31G* on remaining atoms).
Figure 247.
Proposed molecular mechanism for N₂O reduction by Cu₂⁺ containing N₂OR. MV abbreviates methyl viologen.
Computationally predicted geometry and electronic structure of N$_2$O binding to fully reduced Cu$_Z$$^*$. A) Optimized geometry of the lowest energy structure of N$_2$O bound to 4Cu$^1$ Cu$_Z$$^*$ (BP86, 6-311G*). B) LUMO of the N$_2$O complex. (Reprinted with permission from Ref. 1339. Copyright 2006 American Chemical Society.)
Figure 249.
Computational evaluation of sequential protonation and reduction steps to reduce 2-hole oxo-bridged Cu_{Z}^{*} to fully reduced aquo-Cu_{Z}^{*} (B3LYP/6-311++G** on Cu_{SL}/6-31G* otherwise).
Figure 250.
(A) Reaction of cellulose chain opening by the polysaccharide monooxygenases, via H-atom abstraction from the 1 position, as proposed by Phillips et al. (B) End-on bound superoxide structure of PMO-2 from *Neurospora crassa* (pdb: 4EIR). Gold sphere: Cu, Red spheres: oxygen. Second sphere residues Y168 and Q166 (<4Å from the Cu) are included.
Table 1

Effect of the \( L_i (i = x,y,z) \) operator on the real d functions

<table>
<thead>
<tr>
<th>( L_i d_j )</th>
<th>( \hat{L}_i d_j )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L_x d_y = -i d_y )</td>
<td>( \hat{L}<em>x d_y = i\sqrt{3}d</em>{x^2 - y^2} )</td>
</tr>
<tr>
<td>( L_y d_x = i d_x )</td>
<td>( \hat{L}<em>y d_x = i d</em>{x^2} )</td>
</tr>
<tr>
<td>( L_z d_x = -i d_x )</td>
<td>( \hat{L}<em>z d_x = i\sqrt{3}d</em>{x^2} )</td>
</tr>
<tr>
<td>( L_x d_y = i\sqrt{3}d_{x^2 + y^2} )</td>
<td>( \hat{L}<em>x d_y = i\sqrt{3}d</em>{x^2 + y^2} )</td>
</tr>
<tr>
<td>( L_y d_z = i d_z )</td>
<td>( \hat{L}<em>y d_z = i d</em>{x^2} )</td>
</tr>
<tr>
<td>( L_z d_y = -i d_y )</td>
<td>( \hat{L}<em>z d_y = -2i d</em>{x^2 - y^2} )</td>
</tr>
<tr>
<td>( L_x d_z = -i\sqrt{3}d_{y^2} )</td>
<td>( \hat{L}<em>x d_z = -2i d</em>{x^2} )</td>
</tr>
<tr>
<td>( L_y d_z = -i\sqrt{3}d_{x^2 - y^2} )</td>
<td>( \hat{L}<em>y d_z = -2i d</em>{x^2} )</td>
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<tr>
<td>( L_z d_z = 0 )</td>
<td>( \hat{L}_z d_z = 0 )</td>
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Table 2

Biologically relevant nuclear spins, g values and hyperfine interactions with electron spin\(^{20}\)

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<thead>
<tr>
<th>Ligand</th>
<th>( I^a )</th>
<th>Natural Abundance(%)</th>
<th>( g_N^b )</th>
<th>( A_{iso}^L/\text{gauss} )</th>
<th>( A_{aniso}^L/\text{gauss} )</th>
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</thead>
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<tr>
<td>( ^1H )</td>
<td>( \frac{1}{2} )</td>
<td>99.9844</td>
<td>5.58536</td>
<td>508</td>
<td>-</td>
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<tr>
<td>( ^2H )</td>
<td>1</td>
<td>0.0156</td>
<td>0.857386</td>
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<tr>
<td>( ^13C )</td>
<td>( \frac{1}{2} )</td>
<td>1.108</td>
<td>1.40440</td>
<td>1119</td>
<td>63.8</td>
</tr>
<tr>
<td>( ^14N )</td>
<td>1</td>
<td>99.635</td>
<td>0.40358</td>
<td>557</td>
<td>33.5</td>
</tr>
<tr>
<td>( ^15N )</td>
<td>( \frac{1}{2} )</td>
<td>0.365</td>
<td>-0.56608</td>
<td>-781</td>
<td>-46.6</td>
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<tr>
<td>( ^17O )</td>
<td>5/2</td>
<td>0.037</td>
<td>-0.75720</td>
<td>-1659</td>
<td>-102</td>
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<tr>
<td>( ^33S )</td>
<td>3/2</td>
<td>0.74</td>
<td>0.42849</td>
<td>975</td>
<td>56.6</td>
</tr>
<tr>
<td>( ^35Cl )</td>
<td>3/2</td>
<td>0.74</td>
<td>0.54727</td>
<td>1672</td>
<td>102</td>
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<tr>
<td>( ^37Cl )</td>
<td>3/2</td>
<td>24.6</td>
<td>0.45553</td>
<td>1391</td>
<td>84.8</td>
</tr>
</tbody>
</table>

\(^a\) Nuclear spin is in multiples of \( \hbar/2\pi \)

\(^b\) \( g_N \) is in multiples of \( (e/2Mc) \)
### Table 3

Calculated Mulliken Spin Densities for Cu(II) in \([\text{CuCl}_4]^{2-}\ D_{4h}\) complex \(^{113}\)

<table>
<thead>
<tr>
<th>Method</th>
<th>TZV%6-31G*</th>
<th>ECP(SDD)</th>
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<tr>
<td>HF</td>
<td>0.83</td>
<td>0.85</td>
</tr>
<tr>
<td>MP2</td>
<td>0.70</td>
<td>0.70</td>
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<tr>
<td>MP4</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>CCD</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>CISD</td>
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<td>0.74</td>
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<tr>
<td>QCISD</td>
<td>0.60</td>
<td>0.58</td>
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</tbody>
</table>
Table 4

Effect of second sphere interactions on the covalency of the Cu-S bond in blue Cu sites. Inclusion of protein dipoles and H-bond interactions decreases the HF exchange needed in the hybrid functional.\textsuperscript{117}

<table>
<thead>
<tr>
<th>Model</th>
<th>Functional</th>
<th>Cu-spin</th>
<th>S\textsubscript{Cu}-spin</th>
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</thead>
<tbody>
<tr>
<td>Experiment</td>
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<td>0.41 d</td>
<td>0.38 p</td>
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<tr>
<td>33 atom</td>
<td>BP86</td>
<td>0.27</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>B3LYP</td>
<td>0.33</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>B(38HF)P86</td>
<td>0.46</td>
<td>0.44</td>
</tr>
<tr>
<td>217 atom</td>
<td>B3LYP</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>QM/MM (all protein)</td>
<td>B3LYP</td>
<td>0.44</td>
<td>0.42</td>
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</tbody>
</table>
Table 5

Crystal structure bond lengths and absolute difference with respect calculated bond length in DFT optimized structures. All distances in Å.

<table>
<thead>
<tr>
<th>Bond Lengths</th>
<th>Crystal</th>
<th>BP86</th>
<th>B3LYP</th>
<th>B(38HF)P86</th>
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</thead>
<tbody>
<tr>
<td>Cu-O(proximal)</td>
<td>1.927</td>
<td>0.113</td>
<td>0.079</td>
<td>0.027</td>
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<tr>
<td>Cu-O(terminal)</td>
<td>2.842</td>
<td>0.153</td>
<td>0.083</td>
<td>0.020</td>
</tr>
<tr>
<td>O-O</td>
<td>1.280</td>
<td>0.077</td>
<td>0.068</td>
<td>0.066</td>
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</tbody>
</table>
Table 6
DFT calculation (6-311G* basis set) for hydrotris(3-tertbutyl-5-isopropyl-1-pyrazolyl)-borate CuO₂. Ground state geometries are all side-on bound superoxo-Cu(II). Energies relative to the lowest singlet state (kcal/mol) after correction for spin contamination. Adapted from ref 121

<table>
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<tr>
<th>Functional</th>
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<th>ΔE</th>
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<td>BP86</td>
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<tr>
<td>BP86</td>
<td>3</td>
<td>1.23</td>
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<tr>
<td>B3LYP</td>
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<tr>
<td>B3LYP</td>
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<td>−8.25</td>
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Table 7
Oxygen affinities (P1/2) of hemocyanin at under physiological conditions.

<table>
<thead>
<tr>
<th>Arthropod</th>
<th>Organism</th>
<th>P1/2 (torr)</th>
<th>pH</th>
<th>T (° C)</th>
<th>Sample</th>
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</thead>
<tbody>
<tr>
<td>Crab</td>
<td><em>Atelecyclus rotundatus</em></td>
<td>5.6</td>
<td>7.8</td>
<td>10</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Birgus latroz</em></td>
<td>21</td>
<td>7.6</td>
<td>29</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Bythograea thermydron</em></td>
<td>3.0 to 5.3</td>
<td>7.7</td>
<td>2 to 20</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>Callinectes sapidus</em></td>
<td>1.4 to 15</td>
<td>7.5</td>
<td>5 to 25</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Cancer borealis</em></td>
<td>1.2 to 11</td>
<td>7.7 to 8.1</td>
<td>5 to 15</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Cancer magister</em></td>
<td>20</td>
<td>7.7</td>
<td>10</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Cardisoma carnifex</em></td>
<td>11</td>
<td>7.6</td>
<td>29</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Coenobita brevimanus</em></td>
<td>15</td>
<td>7.7</td>
<td>29</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Corystes cassivelaunus</em></td>
<td>3.1 to 4.5</td>
<td>7.8 to 7.9</td>
<td>10</td>
<td>WB</td>
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<tr>
<td></td>
<td><em>Gecarcinus lateralis</em></td>
<td>18</td>
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<td>7.5</td>
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<tr>
<td></td>
<td><em>Goneplax rhomboides</em></td>
<td>4.1</td>
<td>7.8</td>
<td>10</td>
<td>WB</td>
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<td><em>Holthuisana transversa</em></td>
<td>2.6 to 12</td>
<td>7.4</td>
<td>15 to 35</td>
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<td><em>Leptograpsus variegatus</em></td>
<td>22</td>
<td>7.8</td>
<td>15 to 20</td>
<td>WB</td>
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<td></td>
<td><em>Liobunia emarginata</em></td>
<td>9 to 17</td>
<td>7.4 to 7.8</td>
<td>25</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>Liocarcinus depurator</em></td>
<td>11</td>
<td>8.1</td>
<td>10</td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td><em>Menippe mercenaria</em></td>
<td>8.2 to 15</td>
<td>7.5 to 7.7</td>
<td>15 to 25</td>
<td>WB</td>
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<td></td>
<td><em>Pagurus bernhardus</em></td>
<td>24 to 70</td>
<td>7.8 to 8.0</td>
<td>5 to 25</td>
<td>WB</td>
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<td></td>
<td><em>Ocypode quadrata</em></td>
<td>7 to 11</td>
<td>7.9 to 8.1</td>
<td>25</td>
<td>S</td>
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<tr>
<td>Crayfish</td>
<td><em>Astacus leptodactylus</em></td>
<td>9.6 to 17</td>
<td>7.4 to 8.2</td>
<td>10 to 20</td>
<td>WB</td>
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<td></td>
<td><em>Oropectes rusticus</em></td>
<td>7.0 to 8.4</td>
<td>7.7 to 7.9</td>
<td>15</td>
<td>WB</td>
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<td></td>
<td><em>Pacifastacus leniusculus</em></td>
<td>6.2 to 12</td>
<td>7.6 to 7.8</td>
<td>10 to 25</td>
<td>S</td>
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<td>Isopod</td>
<td><em>Glyptonotus antarcticus</em></td>
<td>10</td>
<td>8.0</td>
<td>0</td>
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<tr>
<td>Krill</td>
<td><em>Meganystiphanes norvegica</em></td>
<td>18 to 50</td>
<td>7.9</td>
<td>5 to 10</td>
<td>WB</td>
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<td>Lobster</td>
<td><em>Galathea strigosa</em></td>
<td>12</td>
<td>8.0</td>
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<td><em>Jasus edwardsii</em></td>
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<td>20</td>
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<td><em>Nephrops norvegicus</em></td>
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<td></td>
<td><em>Callianassa subterranea</em></td>
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<td>7.8</td>
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Chem Rev. Author manuscript; available in PMC 2015 April 09.
### Arthropod

<table>
<thead>
<tr>
<th>Organism</th>
<th>$P_{1/2}$ (torr)</th>
<th>pH</th>
<th>T ($°$ C)</th>
<th>Sample$^a$</th>
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<tbody>
<tr>
<td>Calocaris macandreae$^{182}$</td>
<td>0.9</td>
<td>7.8</td>
<td>10</td>
<td>WB</td>
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<tr>
<td>Gnathophausia ingens$^{181}$</td>
<td>1.0 to 1.2</td>
<td>8.0</td>
<td>2 to 10</td>
<td>S</td>
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<tr>
<td>Jawa nocturna$^{182}$</td>
<td>1.5</td>
<td>7.7</td>
<td>10</td>
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<tr>
<td>Macrobrachium rosenbergii$^{183}$</td>
<td>6.7 to 35</td>
<td>7.5 to 7.7</td>
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<td>Upogebia deltaura$^{182}$</td>
<td>6.5</td>
<td>7.8</td>
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<td>Upogebia stellate$^{182}$</td>
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<td>10</td>
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### Mollusc

<table>
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<tr>
<th>Organism</th>
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<th>Sample$^a$</th>
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<td>Nautilus pompilis$^{187}$</td>
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<tr>
<td>Buccinum undatum$^{191}$</td>
<td>8.9 to 59</td>
<td>7.5 to 8.1</td>
<td>10</td>
<td>WB</td>
</tr>
<tr>
<td>Busycon canaliculatum$^{192}$</td>
<td>11 to 14</td>
<td>7.9 to 8.1</td>
<td>22</td>
<td>WB</td>
</tr>
<tr>
<td>Fasciolaria tulipa$^{193}$</td>
<td>7</td>
<td>7.7 to 7.9</td>
<td>25</td>
<td>WB</td>
</tr>
<tr>
<td>Fusitrition oregonensis$^{193}$</td>
<td>7</td>
<td>7.7 to 7.9</td>
<td>25</td>
<td>WB</td>
</tr>
<tr>
<td>Haliotis iris$^{194}$</td>
<td>3.9</td>
<td>7.0</td>
<td>15</td>
<td>WB</td>
</tr>
<tr>
<td>Squid</td>
<td></td>
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</tr>
<tr>
<td>Illex illecebrosus$^{195,196}$</td>
<td>7.9</td>
<td>7.4 to 7.5</td>
<td>15</td>
<td>WB</td>
</tr>
<tr>
<td>Loligo pealei$^{195,196}$</td>
<td>7.9 to 13</td>
<td>7.2 to 7.5</td>
<td>15</td>
<td>WB</td>
</tr>
</tbody>
</table>

$^a$WB (whole blood) or S (physiological saline solution)
Table 8

Site-site interaction energy $\delta(\Delta G)$ from model fits to oxygen binding data.

<table>
<thead>
<tr>
<th>Arthropod</th>
<th>$P_{O_{1/2}}^T$ (torr)$^a$</th>
<th>$P_{O_{1/2}}^R$ (torr)$^a$</th>
<th>$\delta(\Delta G)$ (kcal/mol)</th>
<th>T (°C)</th>
<th>$P_{O_{2}}$ (torr)</th>
<th>$n_H$</th>
<th>Heterotrophic Affectors</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crab</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>Callinectes sapidus</em>$^{306}$</td>
<td>160 to 1450</td>
<td>0.3 to 7.9</td>
<td>−2.3 to −3.6</td>
<td>20</td>
<td>4 to 75</td>
<td>2.7 to 4.7</td>
<td>Ca$^{2+}$, Cl$^−$, H$^+$</td>
<td>TS$^c$</td>
</tr>
<tr>
<td><em>Cancer magister</em>$^{210}$</td>
<td>320 to 14</td>
<td>1.2 to 1.8</td>
<td>−1.2 to −2.2</td>
<td>15</td>
<td>5 to 57</td>
<td>1.1 to 3.4</td>
<td>Dopamine</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Carcinus aestuarii</em>$^{208}$</td>
<td>25 to 170</td>
<td>0.5 to 4.0</td>
<td>−2.2</td>
<td>20</td>
<td>6.8 to 44</td>
<td>3.1 to 3.9</td>
<td>Ca$^{2+}$, Cl$^−$, H$^+$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Carcinus maenas</em>$^{209}$</td>
<td>100</td>
<td>2</td>
<td>−1.3</td>
<td>20</td>
<td>1</td>
<td>2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><em>Ocyopa cursor</em>$^{210}$</td>
<td>280</td>
<td>74</td>
<td>−0.8</td>
<td>20</td>
<td>130</td>
<td>2.0</td>
<td>---</td>
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<tr>
<td><em>Pagurus bernhardus</em>$^{73}$</td>
<td>410</td>
<td>3</td>
<td>−2.8</td>
<td>15</td>
<td>22 to 230</td>
<td>2.5−3.7</td>
<td>whole blood, H$^+$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Scylla serrata</em>$^{211}$</td>
<td>33</td>
<td>0.27</td>
<td>−2.8</td>
<td>25</td>
<td>11</td>
<td>---</td>
<td>Ca$^{2+}$, Cl$^−$</td>
<td>TS$^c$</td>
</tr>
<tr>
<td><em>Telphusa flaviatilis</em>$^{210}$</td>
<td>100</td>
<td>14</td>
<td>−1.2</td>
<td>20</td>
<td>49</td>
<td>2.6</td>
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</tr>
<tr>
<td>Insect</td>
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<tr>
<td><em>Pachnoda marginata</em>$^{151}$</td>
<td>16</td>
<td>1.6</td>
<td>−1.3</td>
<td>20</td>
<td>8</td>
<td>2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lobster</td>
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<td></td>
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<tr>
<td><em>Homarus americanus</em>$^{211}$</td>
<td>160</td>
<td>1.3</td>
<td>−2.9</td>
<td>25</td>
<td>91</td>
<td>---</td>
<td>Ca$^{2+}$, Cl$^−$</td>
<td>TS$^c$</td>
</tr>
<tr>
<td><em>Homarus vulgaris</em>$^{212}$</td>
<td>125</td>
<td>0.6</td>
<td>−3.2</td>
<td>25</td>
<td>6 to 95</td>
<td>3.7 to 4.4</td>
<td>urate, H$^+$</td>
<td>N$^d$</td>
</tr>
<tr>
<td><em>Panulirus interruptus</em>$^{399}$</td>
<td>9.6</td>
<td>1.25</td>
<td>−1.2</td>
<td>20</td>
<td>3.5</td>
<td>3.2</td>
<td>Ca$^{2+}$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Panulirus japonicas</em>$^{213}$</td>
<td>13</td>
<td>2.3</td>
<td>−1.0</td>
<td>25</td>
<td>3</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Scorpion</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>Leirus quinquestriatus</em>$^{210}$</td>
<td>100</td>
<td>1.3</td>
<td>−2.5</td>
<td>20</td>
<td>14</td>
<td>6.8</td>
<td>---</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Pandinus imperator</em>$^{214}$</td>
<td>72</td>
<td>0.3</td>
<td>−3.3</td>
<td>20</td>
<td>4 to 13</td>
<td>5.4 to 6.5</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^−$, H$^+$</td>
<td>N$^d$</td>
</tr>
<tr>
<td>Shrimp</td>
<td></td>
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</tr>
<tr>
<td><em>Callianassa californiensis</em>$^{98}$</td>
<td>170</td>
<td>0.6</td>
<td>−3.4</td>
<td>25</td>
<td>2.4 to 132</td>
<td>1.1 to 3.6</td>
<td>Mg$^{2+}$, Ca$^{2+}$, H$^+$, Cl$^−$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Penaeus setiferus</em>$^{15}$</td>
<td>500</td>
<td>0.1</td>
<td>−5.0</td>
<td>20</td>
<td>0.3 to 112</td>
<td>2.2 to 4.2</td>
<td>Ca$^{2+}$, H$^+$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Upogebia pusilla</em>$^{316}$</td>
<td>29</td>
<td>0.1</td>
<td>−3.3</td>
<td>20</td>
<td>0.8 to 19</td>
<td>1.8 to 4.2</td>
<td>H$^+$, L-lactate</td>
<td>TS$^c$</td>
</tr>
</tbody>
</table>
### Arthropod

<table>
<thead>
<tr>
<th>Organism</th>
<th>$P_{1/2}^T$ (torr) $^a$</th>
<th>$P_{1/2}^R$ (torr) $^a$</th>
<th>$\delta (\Delta G)$ (kcal/mol)</th>
<th>$T$ (°C)</th>
<th>$P_{1/2}$ (torr)</th>
<th>$n_H$</th>
<th>Heterotrophic Affectors</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spider</strong></td>
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<tr>
<td><em>Eurypelma californicum</em></td>
<td>278</td>
<td>0.4</td>
<td>$-3.9$</td>
<td>25</td>
<td>3.6 to 33</td>
<td>2 to 7</td>
<td>H$^+$</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><strong>Molluse</strong></td>
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</tr>
<tr>
<td><strong>Octopus</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Octopus doflein</em> $^{188}$</td>
<td>4 to 280</td>
<td>1 to 11</td>
<td>$-0.6$ to $-2.2$</td>
<td>20</td>
<td>5 to 360</td>
<td>1.9 to 3.3</td>
<td>saline, H$^+$</td>
<td>TS$^c$</td>
</tr>
<tr>
<td><strong>Snail</strong></td>
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</tr>
<tr>
<td><em>Haliotis iris</em> $^{93}$</td>
<td>13</td>
<td>2.2</td>
<td>$-1.0$</td>
<td>15</td>
<td>8.9</td>
<td>3.3</td>
<td>Mg$^{2+}$, Cl</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Hexaplex trunculus</em> $^{217}$</td>
<td>42</td>
<td>2</td>
<td>$-1.8$</td>
<td>20</td>
<td>2.8 to 28</td>
<td>1.3 to 4.0</td>
<td>Ca$^{2+}$, Cl</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Levantina hierosolima</em> $^{218}$</td>
<td>11–25</td>
<td>1.9 to 5.0</td>
<td>$-0.5$ to $-1.5$</td>
<td>25</td>
<td>7 to 12</td>
<td>&gt;1</td>
<td>Ca$^{2+}$, Cl</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><em>Lymnaea stagnalis</em> $^{203}$</td>
<td>7.8 to 15.5</td>
<td>0.8 to 5.1</td>
<td>$-0.6$ to $-1.8$</td>
<td>20</td>
<td>2.0 to 7.2</td>
<td>2.0 to 8.6</td>
<td>Ca$^{2+}$, Cl</td>
<td>MWC$^b$</td>
</tr>
<tr>
<td><strong>Squad</strong></td>
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<tr>
<td><em>Sepioteuthis lessoniana</em> $^{19}$</td>
<td>65</td>
<td>4.1</td>
<td>$-1.6$</td>
<td>20</td>
<td>15</td>
<td>2.4</td>
<td>Mg$^{2+}$</td>
<td>MWC$^b$</td>
</tr>
</tbody>
</table>

$^a$For models with more than one state, the highest affinity state is $P_{1/2}^T$ and the lowest affinity state is $P_{1/2}^R$.

$^b$Monod, Wyman, Changeux Model

$^c$Three State MWC Model

$^d$Nested MWC Model
Thermodynamic and Bohr effects for Hc from various organisms.

<table>
<thead>
<tr>
<th>Arthropod Organism</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (cal mol$^{-1}$ K$^{-1}$)</th>
<th>$P_{1/2}$ (torr)</th>
<th>Bohr Effect</th>
<th>Heterotrophic Affectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alvinocaris lusca</td>
<td>5.0 ± 7.0</td>
<td>43 ± 25</td>
<td>1.3 to 2.8</td>
<td>-0.77</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$.</td>
</tr>
<tr>
<td>Birgus latro</td>
<td>-6.9 ± 0.2 to -6.3 ± 0.6</td>
<td>-0.7 ± 0.7 to 0.8 ± 2.0</td>
<td>5.6 to 20</td>
<td>-0.60</td>
<td>whole blood$^a$</td>
</tr>
<tr>
<td>Bythograea thermydron</td>
<td>-2.3 ± 2.7 to 3.0 ± 2.2</td>
<td>16 ± 9 to 34 ± 8</td>
<td>1.6 to 7.1</td>
<td>-0.67 to -0.34</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$, H$^+$.</td>
</tr>
<tr>
<td>Calappa granulate</td>
<td>-20 ± 1 to -16 ± 1</td>
<td>-50 ± 4 to -36 ± 3</td>
<td>11 to 106</td>
<td>-0.95</td>
<td>Ca$^{2+}$, Cl$^-$. lactate</td>
</tr>
<tr>
<td>Calcinectes sapidus</td>
<td>-5.2 ± 3.5 to 9.5 ± 2.0</td>
<td>3.8 ± 12.0 to 52 ± 7.7</td>
<td>5 to 75</td>
<td>-1.7</td>
<td>seawater</td>
</tr>
<tr>
<td>Cancer anthonyi</td>
<td>-4.8 ± 3.0 to 0.3 ± 1.8</td>
<td>3 ± 10 to 23 ± 6</td>
<td>8 to 54</td>
<td>-1.11 to -0.49</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$, H$^+$.</td>
</tr>
<tr>
<td>Cancer borealis</td>
<td>-31.7</td>
<td>-89.7</td>
<td>2.5 to 23</td>
<td>-1.2 to -0.6</td>
<td>seawater</td>
</tr>
<tr>
<td>Cancer gracilis</td>
<td>0.4 ± 1.8 to 1.8 ± 2.2</td>
<td>23 ± 6 to 26 ± 8</td>
<td>11 to 57</td>
<td>-0.82 to -0.46</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$. H$^+$.</td>
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<tr>
<td>Cancer magister</td>
<td>-7.1 ± 0.3</td>
<td>-5.1 ± 0.9</td>
<td>19–68</td>
<td>-0.27</td>
<td>whole blood$^a$</td>
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<tr>
<td>Coenobita clypeatus</td>
<td>-3.3 ± 1.9</td>
<td>11 ± 6</td>
<td>6.7 to 13.5</td>
<td>-0.47</td>
<td>whole blood$^a$</td>
</tr>
<tr>
<td>Cyamusagrae praedator</td>
<td>2.5 ± 3.2 to 5.5 ± 3.0</td>
<td>34 ± 11 to 42 ± 10</td>
<td>0.8 to 8</td>
<td>-1.8</td>
<td>whole blood$^a$, H$^+$.</td>
</tr>
<tr>
<td>Dardanus calidus</td>
<td>-8.7 ± 0.8</td>
<td>-7.3 ± 2.7</td>
<td>5 to 10</td>
<td>-0.8 to -0.6</td>
<td>Ca$^{2+}$, Cl$^-$. L-lactate</td>
</tr>
<tr>
<td>Eurythium albidigitum</td>
<td>-3.5 ± 1.2 to -2.2 ± 1.4</td>
<td>12 ± 4 to 16 ± 5</td>
<td>1.8 to 7.9</td>
<td>-0.48 to -0.32</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$. H$^+$.</td>
</tr>
<tr>
<td>Geocarcina natatilis</td>
<td>-10 ± 1</td>
<td>-10 ± 4</td>
<td>0.9 to 3.9</td>
<td>-0.26</td>
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<tr>
<td>Holothuria transversa</td>
<td>-9.8 ± 0.5</td>
<td>-9.7 ± 1.8</td>
<td>2 to 11</td>
<td>-0.90 to -0.44</td>
<td>whole blood$^a$</td>
</tr>
<tr>
<td>Leptograpsus variegatus</td>
<td>-13.1 to -11.7</td>
<td>-25.0 to -20.0</td>
<td>15 to 41</td>
<td>-0.78</td>
<td>whole blood, H$^+$.</td>
</tr>
<tr>
<td>Lopholithodes fominitus</td>
<td>1.2 ± 1.9 to 1.9 ± 1.9</td>
<td>24 ± 7 to 25 ± 7</td>
<td>21 to 91</td>
<td>-0.70 to -0.60</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$. H$^+$.</td>
</tr>
<tr>
<td>Menippe mercenaria</td>
<td>-13.3 to -1.4</td>
<td>-24.4 ± 18.0</td>
<td>6 to 28</td>
<td>-1.5 to -0.9</td>
<td>H$^+$, seawater</td>
</tr>
<tr>
<td>Metopograpsus messor</td>
<td>-10.4 ± 0.4</td>
<td>-13 ± 1</td>
<td>6 to 28</td>
<td>-0.96</td>
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<tr>
<td>Ocypode sanatan</td>
<td>-0.6 ± 1.3 to 0.0 ± 2.6</td>
<td>20 ± 4 to 23 ± 9</td>
<td>5.6 to 12</td>
<td>-0.67</td>
<td>whole blood$^a$, H$^+$.</td>
</tr>
<tr>
<td>Paralithodes camtschaticus</td>
<td>2.3 ± 1.7 to 4.5 ± 1.2</td>
<td>27 ± 3 to 36 ± 4</td>
<td>8 to 83</td>
<td>-0.7</td>
<td>Ca$^{2+}$, Mg$^{2+}$, Cl$^-$. H$^+$.</td>
</tr>
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<td>Segonzacia mesatlantica</td>
<td>8.4 ± 1.8</td>
<td>52 ± 6</td>
<td>3 to 5</td>
<td>-2.7</td>
<td>whole blood$^a$</td>
</tr>
<tr>
<td>Uca inversa</td>
<td>-13 ± 1</td>
<td>-22 ± 3</td>
<td>3 to 21</td>
<td>-1.07</td>
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</tr>
<tr>
<td>Arthropod</td>
<td>Organism</td>
<td>ΔH (kcal/mol)</td>
<td>ΔS (cal mol⁻¹ K⁻¹)</td>
<td>P₁/₂ (torr)</td>
<td>Bohr Effect</td>
</tr>
<tr>
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</tr>
<tr>
<td>Crayfish</td>
<td>Procambarus clarkii</td>
<td>−9.3 to −8.7</td>
<td>−5.5 to −4.0</td>
<td>0.5 to 2.9</td>
<td>−0.42</td>
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<tr>
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<td>Procambarus similans</td>
<td>−6.8 ± 0.3</td>
<td>1.5 ± 0.9</td>
<td>1.6 to 2.8</td>
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<tr>
<td></td>
<td>Procambarus zonangulus</td>
<td>−5.9 to −3.8</td>
<td>5.3 ± 1.17</td>
<td>0.9 to 3.5</td>
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</tr>
<tr>
<td>Horseshoe Crab</td>
<td>Limulus Polyphemus</td>
<td>−4.5 ± 1.2</td>
<td>6.1 ± 4.2</td>
<td>4.1 to 25</td>
<td>0.53 to 0.67</td>
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<tr>
<td>Isopod</td>
<td>Glyptonotus antarcticus</td>
<td>−4.0 to −32.4</td>
<td>3.0 to −97</td>
<td>48 to 63</td>
<td>−1.4 to −0.7</td>
</tr>
<tr>
<td>Krill</td>
<td>Meganyctiphanes norvegica</td>
<td>12.5 to 35.5</td>
<td>59.6 to 146</td>
<td>18 to 249</td>
<td>−1.99 to −1.85</td>
</tr>
<tr>
<td>Lobster</td>
<td>Jasus edwardsii</td>
<td>−3.88</td>
<td>11.2</td>
<td>1.6 to 3.8</td>
<td>−0.2 to −0.09</td>
</tr>
<tr>
<td></td>
<td>Munida rugosa</td>
<td>−3.6 ± 0.2</td>
<td>6.8 ± 0.7</td>
<td>18 to 37</td>
<td>−0.39</td>
</tr>
<tr>
<td></td>
<td>Pandaras Interruptus</td>
<td>−10.32 ± 0.07</td>
<td>−11.4 ± 0.2</td>
<td>1.5 to 6</td>
<td>−0.4</td>
</tr>
<tr>
<td></td>
<td>Scyllarides latuś</td>
<td>−14 ± 1 to −7.2 ± 2.1</td>
<td>−26 ± 5 to −4.8 ± 7.2</td>
<td>3 to 33</td>
<td>−0.17 to −0.15</td>
</tr>
<tr>
<td>Shrimp</td>
<td>Callianassa californiensis</td>
<td>−6.8 ± 0.7</td>
<td>−1.0 ± 2.6</td>
<td>5.7 to 10.5</td>
<td>−1.59</td>
</tr>
<tr>
<td></td>
<td>Gnathophausia ingens</td>
<td>−2.9 ± 1.7 to 0.8 ± 0.35</td>
<td>14 ± 6 to 28 ± 1</td>
<td>1.6 to 4.0</td>
<td>−0.81 to −0.80</td>
</tr>
<tr>
<td></td>
<td>Macrobrachium rosenbergii</td>
<td>−18 ± 2 to −18 ± 2</td>
<td>−41 ± 6 to −40 ± 5</td>
<td>6.7 to 35</td>
<td>−0.93 to −0.96</td>
</tr>
<tr>
<td></td>
<td>Palaemon elegans</td>
<td>3.1 ± 0.9 to 4.0 ± 5.3</td>
<td>32 ± 3 to 36 ± 19</td>
<td>4 to 12</td>
<td>−2.00 to −1.01</td>
</tr>
<tr>
<td></td>
<td>Rimicaris exoculata</td>
<td>1.1 ± 0.3 to 2.26 ± 0.02</td>
<td>26.8 ± 0.8 to 32.12 ± 0.06</td>
<td>2.9 to 6.8</td>
<td>−2.26 to −1.98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mollusc</th>
<th>Organism</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (cal mol⁻¹ K⁻¹)</th>
<th>P₁/₂ (torr)</th>
<th>Bohr Effect</th>
<th>Heterotrophic Affectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiton</td>
<td>Katharina tunicata</td>
<td>−5.6 ± 1.0</td>
<td>1.9 ± 3.5</td>
<td>10 to 23</td>
<td>−0.20 to −0.08</td>
<td>Ca²⁺, Mg²⁺, Cl⁻</td>
</tr>
<tr>
<td>Cuddlefish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mollusc</td>
<td>Organism</td>
<td>ΔH (kcal/mol)</td>
<td>ΔS (cal mol(^{-1}) K(^{-1}))</td>
<td>P(_{1/2}) (torr)</td>
<td>Bohr Effect</td>
<td>Heterotrophic Affectors</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>--------------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td>-------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Sepia officinalis(^{136})</td>
<td>0.20</td>
<td>23.3</td>
<td>5.3 to 6.5</td>
<td>-1.33 to -0.99</td>
<td>whole blood(^a)</td>
<td></td>
</tr>
<tr>
<td>Nautilus</td>
<td>Nautilus pompilius(^{187})</td>
<td>-6.06</td>
<td>-0.25</td>
<td>17 to 25</td>
<td>-0.20</td>
<td>whole blood(^a)</td>
</tr>
<tr>
<td>Octopus</td>
<td>Octopus dolefin(^{188,246})</td>
<td>-10.9</td>
<td>-17.0</td>
<td>10 to 37</td>
<td>-1.7</td>
<td>whole blood(^a)</td>
</tr>
<tr>
<td>Octopus vulgaris(^{223})</td>
<td>-6.82</td>
<td>-3.05</td>
<td>18 to 33</td>
<td>-1.34 to -1.10</td>
<td>Ca(^{2+}), Mg(^{2+}), Cl(^{-})</td>
<td></td>
</tr>
<tr>
<td>Megaleledone senoi(^{186})</td>
<td>2.20</td>
<td>33.4</td>
<td>0.98 to 1.1</td>
<td>-2.33 to -0.9</td>
<td>whole blood(^a)</td>
<td></td>
</tr>
<tr>
<td>Snail</td>
<td>Busycon canaliculatum(^{192})</td>
<td>-16.2 to -6.9</td>
<td>-33.5 to -1.9</td>
<td>3 to 12</td>
<td>NL(^b)</td>
<td>whole blood(^a), H(^+)</td>
</tr>
<tr>
<td>Haliotis australis(^{247})</td>
<td>-13.2 to -4.3</td>
<td>-23.5 to 4.8</td>
<td>3.8 to 34</td>
<td>NL(^b)</td>
<td>whole blood(^a), H(^+), saline</td>
<td></td>
</tr>
<tr>
<td>Haliotis iris(^{247})</td>
<td>-10.6 to -4.9</td>
<td>-15.1 to 2.5</td>
<td>5.5 to 38</td>
<td>NL(^b)</td>
<td>whole blood(^a), H(^+), saline</td>
<td></td>
</tr>
<tr>
<td>Helix pomatia(^{248})</td>
<td>-14.6 to -3.8</td>
<td>-29.2 to 7.8</td>
<td>4.4 to 16</td>
<td>NL(^b)</td>
<td>whole blood(^a), H(^+)</td>
<td></td>
</tr>
<tr>
<td>Levanina hierosolima(^{228})</td>
<td>0.58 ± 1.13</td>
<td>24 ± 4</td>
<td>9.4 to 11.9</td>
<td>NL(^b)</td>
<td>Ca(^{2+}), Cl(^{-})</td>
<td></td>
</tr>
<tr>
<td>Neptunea antiqua(^{249})</td>
<td>-16.4 to 4.6</td>
<td>-35.9 to 33.9</td>
<td>11 to 73</td>
<td>NL(^b)</td>
<td>whole blood(^a)</td>
<td></td>
</tr>
<tr>
<td>Squid</td>
<td>Architeuthis monachus(^{223})</td>
<td>-36 ± 14</td>
<td>-110 ± 50</td>
<td>14 to 126</td>
<td>-0.85 to -0.83</td>
<td>Ca(^{2+}), Mg(^{2+}), Cl(^{-})</td>
</tr>
<tr>
<td>Loligo pealei(^{223,241})</td>
<td>-7.3 ± 0.1</td>
<td>-4.9 ± 0.5</td>
<td>8 to 31</td>
<td>-2.18 to -1.56</td>
<td>whole blood(^a)</td>
<td></td>
</tr>
<tr>
<td>Loligo vulgaris(^{223})</td>
<td>-11.2</td>
<td>-20.0</td>
<td>31 to 74</td>
<td>-0.81 to -0.38</td>
<td>Ca(^{2+}), Mg(^{2+}), Cl(^{-})</td>
<td></td>
</tr>
<tr>
<td>Todarodes sagittatus(^{223})</td>
<td>-6.6 ± 0.4</td>
<td>-4.5 ± 1.5</td>
<td>32 to 105</td>
<td>-1.17 to -0.92</td>
<td>Ca(^{2+}), Mg(^{2+}), Cl(^{-})</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Salt concentration assumed to be 0.0 g/L
Table 10
Thermodynamic parameters for the T and R-states of several hemocyanins.

<table>
<thead>
<tr>
<th>Organism</th>
<th>ΔH  (kcal/mol)</th>
<th>ΔS  (cal mol⁻¹ K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Panulirus interruptus</em>¹⁹⁹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-State</td>
<td>−10.6</td>
<td>−14.4</td>
</tr>
<tr>
<td>R-State</td>
<td>−6.9</td>
<td>2.4</td>
</tr>
<tr>
<td><em>Helix pomatia</em>²²⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-State</td>
<td>−15.4</td>
<td>−31.1</td>
</tr>
<tr>
<td>R-State</td>
<td>−11.5</td>
<td>−12.6</td>
</tr>
<tr>
<td><em>Levantina hierosolima</em>²²⁸</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-State</td>
<td>0.31</td>
<td>30.5</td>
</tr>
<tr>
<td>R-State</td>
<td>−0.75</td>
<td>−1.8</td>
</tr>
</tbody>
</table>

¹ Thermodynamic parameters were calculated as described for Table 9.
Table 11
Thermodynamic parameters for ligands that form side-on Cu$_2$O$_2$ complexes.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binucleating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeL66$^{253}$</td>
<td>$-7.7$</td>
<td>$-21$</td>
</tr>
<tr>
<td>N$^3$$^{254}$</td>
<td>$-20$</td>
<td>$-71$</td>
</tr>
<tr>
<td>N$_4$$^{254}$</td>
<td>$-20$ to $-14$</td>
<td>$-65$ to $-39$</td>
</tr>
<tr>
<td>N$^5$$^{254}$</td>
<td>$-19$</td>
<td>$-57$</td>
</tr>
<tr>
<td>XYL$^b$$^{254}$</td>
<td>$-15$ to $-10$</td>
<td>$-47$ to $-42$</td>
</tr>
<tr>
<td>Mononucleating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeAN$^{255}$</td>
<td>$-28$</td>
<td>$-61$</td>
</tr>
<tr>
<td>MePY$^{256}$</td>
<td>$-21$</td>
<td>$-57$</td>
</tr>
<tr>
<td>PYAN$^b$$^{257}$</td>
<td>$-19$ to $-14$</td>
<td>$-50$ to $-34$</td>
</tr>
</tbody>
</table>

$^a$With different coordinating ligands

$^b$In different solvents
### Table 11

Kinetic parameters for reversible O$_2$ binding.

<table>
<thead>
<tr>
<th>Organism</th>
<th>State$^a$</th>
<th>$k_{on}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Panulirus interruptus</em>$^{109}$</td>
<td>R</td>
<td>30 to 44</td>
<td>13 to 80</td>
</tr>
<tr>
<td><em>Panulirus interruptus</em>$^{109}$</td>
<td>T</td>
<td>20 to 44</td>
<td>280 to 2750</td>
</tr>
<tr>
<td><em>Limulus polyphemus</em>$^{268}$</td>
<td>C</td>
<td>1.4 to 2.5</td>
<td>9 to 55</td>
</tr>
<tr>
<td><em>Buccinum undatum</em>$^{105}$</td>
<td>R</td>
<td>8.5</td>
<td>80</td>
</tr>
<tr>
<td><em>Helix pomatia</em>$^{303}$</td>
<td>R</td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td><em>Helix pomatia</em>$^{303}$</td>
<td>T</td>
<td>1.3</td>
<td>300</td>
</tr>
<tr>
<td><em>Lymnaea stagnalis</em>$^{203}$</td>
<td>C</td>
<td>25 to 31</td>
<td>44 to 188</td>
</tr>
</tbody>
</table>

$^a$ R-state (R), T-state (T), or cooperative (C)
### Table 12

Atomic structures of arthropod and mollusc Hc.

<table>
<thead>
<tr>
<th>species</th>
<th>state</th>
<th>resolution</th>
<th>$O_2$ affinity</th>
<th>PDB code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthropod</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. polyphemus</em>&lt;sup&gt;306,309&lt;/sup&gt;</td>
<td>deoxy-Hc</td>
<td>2.18 Å</td>
<td>T-state</td>
<td>1LLA</td>
</tr>
<tr>
<td></td>
<td>oxy-Hc (hexameric)</td>
<td>2.40 Å</td>
<td>T-state</td>
<td>1OXY</td>
</tr>
<tr>
<td></td>
<td>oxy-Hc (subunit II)</td>
<td>2.18 Å</td>
<td>T-state</td>
<td>1NOL</td>
</tr>
<tr>
<td><em>P. interruptus</em>&lt;sup&gt;143&lt;/sup&gt;</td>
<td>deoxy-Hc</td>
<td>3.20 Å</td>
<td>none</td>
<td>1HCY</td>
</tr>
<tr>
<td>Mollusc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. dofleini</em>&lt;sup&gt;137&lt;/sup&gt;</td>
<td>oxy-Hc</td>
<td>2.30 Å</td>
<td>unknown</td>
<td>1JS8</td>
</tr>
<tr>
<td><em>R. thomasiana</em>&lt;sup&gt;110&lt;/sup&gt;</td>
<td>deoxy-Hc</td>
<td>3.30 Å</td>
<td>unknown</td>
<td>1LNL</td>
</tr>
</tbody>
</table>
### Table 13

Selected geometric parameters from deoxy and oxy-Hc in Å.

<table>
<thead>
<tr>
<th></th>
<th>deoxy-Hc</th>
<th>oxy-Hc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>atoms</strong></td>
<td>L. polyphemus</td>
<td>P. interruptus</td>
</tr>
<tr>
<td>NHis173•CuA</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>NHis177•CuA</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>NHis204•CuA</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>NHis124•CuB</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>NHis128•CuB</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>NHis164•CuB</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>CuA•••CuB</td>
<td>4.6</td>
<td>2.9</td>
</tr>
<tr>
<td>τCuA</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>τCuB</td>
<td>0.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>atoms</th>
<th>L. polyphemus</th>
<th>E. dolfin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHis173•CuA</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>NHis177•CuA</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>NHis204•CuA</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>NHis124•CuB</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>NHis128•CuB</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>NHis164•CuB</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>CuA•••CuB</td>
<td>3.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*a* Residue numbering from *L. polyphemus*

*b* Subunit 1

*c* The shortest distance from the Cu to the N,N,N plane.
Table 14

Normal coordinate analysis of \{[[\text{Tp}]\text{Cu}]-_{2}(\mu-\eta^{2}\eta^{2}-\text{O}_{2})\} based on a four atom diamond core model in the $D_{2h}$ point group.$^{128}$

<table>
<thead>
<tr>
<th>mode</th>
<th>obsvd frequencies (cm$^{-1}$)</th>
<th>calcd frequencies (cm$^{-1}$)</th>
<th>force const. (mdyn/Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{16}\text{O}_2$</td>
<td>$^{18}\text{O}_2$</td>
<td>$^{16}\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A$_g$</td>
<td>763</td>
<td>723</td>
<td>764.7</td>
</tr>
<tr>
<td>B$_{1g}$</td>
<td>572</td>
<td>549</td>
<td>573.8</td>
</tr>
<tr>
<td>B$_{3u}$</td>
<td>523.7</td>
<td>494.9</td>
<td></td>
</tr>
<tr>
<td>B$_{2u}$</td>
<td>331</td>
<td>321</td>
<td>333.4</td>
</tr>
<tr>
<td>A$_g$</td>
<td>284</td>
<td>284</td>
<td>284.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B$_{3u}$</td>
<td>629.9</td>
<td>601.3</td>
<td>575.3</td>
</tr>
<tr>
<td>B$_{2u}$</td>
<td>331</td>
<td>321</td>
<td>333.5</td>
</tr>
<tr>
<td>A$_g$</td>
<td>284</td>
<td>284</td>
<td>284.2</td>
</tr>
</tbody>
</table>
Table 15

Kinetic parameters for tyrosinase variants in the vicinity of Cu\textsubscript{A} and Cu\textsubscript{B}.

<table>
<thead>
<tr>
<th>Cu\textsubscript{A} Variants</th>
<th>Organism</th>
<th>Mutation</th>
<th>Substrate</th>
<th>wt Specific Activity\textsuperscript{a}</th>
<th>Variant Specific Activity\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human\textsuperscript{a53}</td>
<td>A206T</td>
<td>L-Dopa</td>
<td>7.6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>A206T</td>
<td>L-Tyrosine</td>
<td>9.7</td>
<td>19.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cu\textsubscript{B} Variants</th>
<th>Organism</th>
<th>Mutation</th>
<th>Substrate</th>
<th>(K_m) (mM)</th>
<th>(V_{max})</th>
<th>(K_m) (mM)</th>
<th>(V_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse\textsuperscript{a82}</td>
<td>H378Q</td>
<td>L-Dopa</td>
<td>0.46</td>
<td>38.8\textsuperscript{b}</td>
<td>22.2</td>
<td>32.67\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>H378Q</td>
<td>L-Tyrosine</td>
<td>0.079</td>
<td>0.9\textsuperscript{b}</td>
<td>0.076</td>
<td>1.47\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>B. megaterium\textsuperscript{a83}</td>
<td>R209H</td>
<td>L-Dopa</td>
<td>0.40</td>
<td>18.0\textsuperscript{c}</td>
<td>0.62</td>
<td>12.2\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>B. megaterium</td>
<td>R209H</td>
<td>L-Tyrosine</td>
<td>0.038</td>
<td>2.2\textsuperscript{c}</td>
<td>1.3</td>
<td>3.8\textsuperscript{c}</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} picomol per µg per hour  
\textsuperscript{b} milliunits mg\textsuperscript{-1}  
\textsuperscript{c} µmol min\textsuperscript{-1} mg\textsuperscript{-1}
**Table 16**

Crystal structures of the coupled binuclear polyphenol oxidases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>State (modifier)</th>
<th>Resolution</th>
<th>PDB code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant CO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>I. batatas</em></td>
<td>deoxy-CaOx</td>
<td>2.7</td>
<td>1BT2</td>
</tr>
<tr>
<td></td>
<td>met-CaOx (I)</td>
<td>2.7</td>
<td>1BT1</td>
</tr>
<tr>
<td></td>
<td>met-CaOx (II)</td>
<td>2.5</td>
<td>1BT3</td>
</tr>
<tr>
<td></td>
<td>PTU bound</td>
<td>2.7</td>
<td>1BUG</td>
</tr>
<tr>
<td><em>V. vinifera</em></td>
<td>met-CaOx</td>
<td>2.2</td>
<td>2P3X</td>
</tr>
<tr>
<td>Bacterial Ty</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. castaneoglobisporus</em></td>
<td>apo-Ty</td>
<td>2.02</td>
<td>1WX5</td>
</tr>
<tr>
<td></td>
<td>apo-Ty</td>
<td>1.20</td>
<td>1WXC</td>
</tr>
<tr>
<td></td>
<td>deoxy-Ty</td>
<td>1.37</td>
<td>2ZMZ</td>
</tr>
<tr>
<td></td>
<td>met-Ty (I)</td>
<td>1.33</td>
<td>2ZMX</td>
</tr>
<tr>
<td></td>
<td>met-Ty (II)</td>
<td>1.45</td>
<td>2ZMY</td>
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<td></td>
<td>oxy-Ty</td>
<td>1.80</td>
<td>1WX2</td>
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<tr>
<td><em>B. megaterium</em></td>
<td>half apo-Ty</td>
<td>2.2</td>
<td>3NQ0</td>
</tr>
<tr>
<td></td>
<td>met-Ty (I)</td>
<td>2.0</td>
<td>3NM8</td>
</tr>
<tr>
<td></td>
<td>met-Ty (II)</td>
<td>2.3</td>
<td>3NTM</td>
</tr>
<tr>
<td></td>
<td>met-Ty (III)</td>
<td>2.2</td>
<td>3NPY</td>
</tr>
<tr>
<td></td>
<td>met-Ty + kojic</td>
<td>2.3</td>
<td>3NQ1</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td>2.3</td>
<td>3NQ5</td>
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<tr>
<td></td>
<td>met-Ty R209H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal Ty</td>
<td>met-Ty</td>
<td>2.30</td>
<td>2Y9W</td>
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<tr>
<td></td>
<td>met-Ty + tropolone</td>
<td>2.78</td>
<td>2Y9X</td>
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<tr>
<td>Insect Ty</td>
<td>met-Ty PPO1</td>
<td>1.97</td>
<td>3HHS</td>
</tr>
<tr>
<td><em>M. sexta</em></td>
<td>met-Ty PPO2</td>
<td>1.97</td>
<td>3HHS</td>
</tr>
</tbody>
</table>

Chem Rev. Author manuscript; available in PMC 2015 April 09.
Table 17
Selected geometric parameters for deoxy and met tyrosinase and catechol oxidase.

<table>
<thead>
<tr>
<th>deoxy-Ty and CaOx</th>
<th>Ty</th>
<th>CaOx</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>I. batatas</em>&lt;sup&gt;98&lt;/sup&gt;</td>
<td><em>I. batatas</em>&lt;sup&gt;98&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu&lt;sub&gt;A&lt;/sub&gt;•••Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>4.1</td>
<td>4.4</td>
</tr>
<tr>
<td>∠ Cu&lt;sub&gt;A&lt;/sub&gt;, O, Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>136°</td>
<td>131°</td>
</tr>
<tr>
<td>τ Cu&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>τ Cu&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>PDB</td>
<td>2ZMZ</td>
<td>1BT2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>met-CaOx</th>
<th><em>I. batatas</em>&lt;sup&gt;98&lt;/sup&gt;</th>
<th><em>I. batatas</em>&lt;sup&gt;98&lt;/sup&gt;</th>
<th><em>V. vinifera</em>&lt;sup&gt;99&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu&lt;sub&gt;A&lt;/sub&gt;•••Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>3.0, 3.0</td>
<td>2.9</td>
<td>4.2</td>
</tr>
<tr>
<td>∠ Cu&lt;sub&gt;A&lt;/sub&gt;, O, Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>104°, 105°&lt;sup&gt;f&lt;/sup&gt;</td>
<td>104°</td>
<td>92°&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>PDB</td>
<td>1BT1</td>
<td>1BT3</td>
<td>2P3X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>met-Ty</th>
<th><em>Streptomyces</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Streptomyces</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Bacillus</em>&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met I&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Met II&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Met I&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Met I&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu&lt;sub&gt;A&lt;/sub&gt;•••Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>3.9</td>
<td>3.3</td>
<td>3.6, 3.6</td>
</tr>
<tr>
<td>∠ Cu&lt;sub&gt;A&lt;/sub&gt;, O, Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>128°</td>
<td>92°, 107°&lt;sup&gt;f&lt;/sup&gt;</td>
<td>116, 106°&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>PDB</td>
<td>2ZMX</td>
<td>2ZMY</td>
<td>3NM8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Bacillus</em>&lt;sup&gt;d&lt;/sup&gt;</th>
<th><em>A. bisporus</em>&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;502&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met II&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Met I&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;501&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu&lt;sub&gt;A&lt;/sub&gt;•••Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>3.2</td>
</tr>
<tr>
<td>∠ Cu&lt;sub&gt;A&lt;/sub&gt;, O, Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>106°</td>
</tr>
<tr>
<td>PDB</td>
<td>3NTM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>atoms</th>
<th><em>M. Sexta</em> PPO&lt;sup&gt;1&lt;/sup&gt;</th>
<th><em>M. Sexta</em> PPO2&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu&lt;sub&gt;A&lt;/sub&gt;•••Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>4.5</td>
<td>4.9</td>
</tr>
<tr>
<td>∠ Cu&lt;sub&gt;A&lt;/sub&gt;, O, Cu&lt;sub&gt;B&lt;/sub&gt;</td>
<td>---</td>
<td>142°</td>
</tr>
<tr>
<td>PDB</td>
<td>3HHS</td>
<td>3HHS</td>
</tr>
</tbody>
</table>

<sup>a</sup>*Streptomyces castaneoglobisporus.*

<sup>b</sup>The shortest distance from the Cu to the N,N,N plane.

<sup>c</sup>Two unique molecules in the asymmetric cell.
Only one subunit was fully loaded with Cu.

Contains two water derived ligands bridging the active site.
**Table 18**

Representative Steady-State parameters for the non-coupled binuclear copper enzymes.

<table>
<thead>
<tr>
<th>Enzyme (Substrate)</th>
<th>$k_{cat}$/s$^{-1}$</th>
<th>$K_m$/mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHM (hippuric acid)\textsuperscript{564,578,579}</td>
<td>39.1</td>
<td>1.7</td>
</tr>
<tr>
<td>D\textsubscript{3}M (dopamine)\textsuperscript{580}</td>
<td>12.7</td>
<td>0.49</td>
</tr>
<tr>
<td>T\textsubscript{3}M (tyramine)\textsuperscript{534}</td>
<td>11.9</td>
<td>0.088</td>
</tr>
</tbody>
</table>
Table 19
Primary Deuterium isotope effects on steady-state parameters

<table>
<thead>
<tr>
<th>Enzyme (Substrate)</th>
<th>Δ((V_{\text{max}})) or (\Delta k_{\text{cat}})</th>
<th>Δ((V_{\text{max}}/K_m)) substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHM (hippuric acid)(^580,586)</td>
<td>1.56</td>
<td>~ 3.9</td>
</tr>
<tr>
<td>DJM (dopamine)(^587)</td>
<td>1.00</td>
<td>~ 3.7</td>
</tr>
<tr>
<td>TjM (tyramine)(^534)</td>
<td>2.7</td>
<td>~ 2.0</td>
</tr>
</tbody>
</table>
Table 20

Dioxygen $^{18}$O isotope effects with primary proteo- and deterio- substrate.

<table>
<thead>
<tr>
<th>Enzyme (Substrate)</th>
<th>$^{18}$O/(V/K)$<em>{H}$ ($k</em>{cat}/K_{m}(O_{2})$)</th>
<th>$^{18}$O/(V/K)$<em>{D}$ ($k</em>{cat}/K_{m}(O_{2})$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHM (hippuric acid)$^{578}$</td>
<td>1.0173</td>
<td>1.0212</td>
</tr>
<tr>
<td>DjM (dopamine)$^{587}$</td>
<td>1.0197</td>
<td>1.0256</td>
</tr>
</tbody>
</table>
Table 21

Intrinsic isotope effects for the H-atom abstraction step.

<table>
<thead>
<tr>
<th>Enzyme (Substrate)</th>
<th>D_k (intrinsic) Primary</th>
<th>D_k (intrinsic) Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHM (hippuric acid$^{57}$)</td>
<td>10.6</td>
<td>1.20</td>
</tr>
<tr>
<td>DβM (dopamine$^{580,592}$)</td>
<td>10.9</td>
<td>1.19</td>
</tr>
</tbody>
</table>
### Table 22

Crystal structures of PHM.

<table>
<thead>
<tr>
<th>Oxidation State</th>
<th>Bound Ligand</th>
<th>Resolution (Å)</th>
<th>PDB ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.35</td>
<td>3MIB</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.42</td>
<td>3MIC</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.06</td>
<td>3MID</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.26</td>
<td>3MIE</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>CO</td>
<td>2.00</td>
<td>3MIF</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.70</td>
<td>3MIG</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.74</td>
<td>3MIH</td>
<td>601</td>
</tr>
<tr>
<td>No CuM, Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td>1.70</td>
<td>1YIP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>583</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>c</td>
<td>2.20</td>
<td>1YIP</td>
<td>583</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>d</td>
<td>2.00</td>
<td>1YJK</td>
<td>583</td>
</tr>
<tr>
<td>No copper</td>
<td></td>
<td>2.40</td>
<td>1YJL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>583</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Substrate&lt;sup&gt;e&lt;/sup&gt;, dioxygen</td>
<td>1.85</td>
<td>1SDW</td>
<td>125</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Substrate&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.10</td>
<td>1OPM</td>
<td>584</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>d</td>
<td>2.10</td>
<td>3PHM</td>
<td>584</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>d</td>
<td>1.90</td>
<td>1PHM</td>
<td>538</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CO</td>
<td>2.15</td>
<td>3MLJ&lt;sup&gt;g&lt;/sup&gt;</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.10</td>
<td>3MLK&lt;sup&gt;g&lt;/sup&gt;</td>
<td>601</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.25</td>
<td>3MLL&lt;sup&gt;g&lt;/sup&gt;</td>
<td>601</td>
</tr>
</tbody>
</table>

<sup>a</sup>Substrate N-R-acetyl-3,5-diiodotyrosylglycine (Ac-DiI-YG, IYG) is present but showed not to bind to copper.

<sup>b</sup>With mutation M314I.

<sup>c</sup>Two solvent derived aquo ligands

<sup>d</sup>A solvent derived aquo ligand

<sup>e</sup>Substrate N-acetyl-diiodotyrosyl-D-threonine (IYT)

<sup>f</sup>N-α-acetyl-3,5-diiodotyrosylglycine (IYG)
Table 23

Structures and vibrational properties of Cu(II)-superoxo model complexes

<table>
<thead>
<tr>
<th>Supporting Ligand</th>
<th>Superoxide Binding Mode</th>
<th>ν(16O−16O)/cm⁻¹ (Δ(18O)/cm⁻¹)</th>
<th>ν(Cu–16O)/cm⁻¹ (Δ(18O)/cm⁻¹)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA 6'-NHPv</td>
<td>η¹</td>
<td>1130 (63)</td>
<td>482 (20)</td>
<td>606</td>
</tr>
<tr>
<td>TPA NMe₂</td>
<td>η¹</td>
<td>1121 (63)</td>
<td>472 (20)</td>
<td>607</td>
</tr>
<tr>
<td>Tren Me·Bn</td>
<td>η¹</td>
<td>1120 (61)</td>
<td>474 (20)</td>
<td>608</td>
</tr>
<tr>
<td>Tren TMG</td>
<td>η¹</td>
<td>1120 (63)</td>
<td>435 (20)</td>
<td>123</td>
</tr>
<tr>
<td>Pyridyl-diazacyclooctane tridentate</td>
<td>η¹</td>
<td>1033 (65)</td>
<td>457 (15)</td>
<td>609</td>
</tr>
<tr>
<td>DPH₃</td>
<td>η¹</td>
<td>964 (55)</td>
<td>*</td>
<td>610</td>
</tr>
<tr>
<td>Pyridyl diamido dianionic tridentate</td>
<td>η¹</td>
<td>1104(60)</td>
<td>*</td>
<td>611</td>
</tr>
<tr>
<td>Me₇</td>
<td>1122(52)</td>
<td></td>
<td></td>
<td>612</td>
</tr>
<tr>
<td>HB(3-Ad-5-Prpz)₃</td>
<td>η²</td>
<td>1043 (59)</td>
<td>*</td>
<td>613</td>
</tr>
<tr>
<td>HB(3-tBu-5-Prpz)₃</td>
<td>η²</td>
<td>1112</td>
<td>554 (20)</td>
<td>121</td>
</tr>
<tr>
<td>TACN PhOH</td>
<td>η²</td>
<td>1120 (62)</td>
<td>450 (8), 422(5)</td>
<td>121</td>
</tr>
</tbody>
</table>

* IR Data
Table 24

Crystal structures for Galactose Oxidase and derivatives.

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Resolution/Å</th>
<th>Enzyme Variant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2VZ1</td>
<td>1.91</td>
<td>apo preprocessed GO</td>
<td>636</td>
</tr>
<tr>
<td>2VZ3</td>
<td>1.90</td>
<td>Bleached GO</td>
<td>636</td>
</tr>
<tr>
<td>2JKX</td>
<td>1.84</td>
<td>apo processed</td>
<td>642</td>
</tr>
<tr>
<td>2EIB</td>
<td>2.10</td>
<td>W290H variant</td>
<td>641</td>
</tr>
<tr>
<td>2EIC</td>
<td>2.80</td>
<td>W290F variant</td>
<td>641</td>
</tr>
<tr>
<td>2EID</td>
<td>2.20</td>
<td>W290G variant</td>
<td>641</td>
</tr>
<tr>
<td>2EIE</td>
<td>1.80</td>
<td>N$_3^-$ coordinated to Cu(II)</td>
<td>641</td>
</tr>
<tr>
<td>1T2X</td>
<td>2.30</td>
<td>C383S variant$^{643}$</td>
<td></td>
</tr>
<tr>
<td>1K3I</td>
<td>1.40</td>
<td>Precursor GO$^{638}$</td>
<td></td>
</tr>
<tr>
<td>1GOF</td>
<td>1.70</td>
<td>First report of the thioether bond pH = 4.5</td>
<td>644</td>
</tr>
<tr>
<td>1GOG</td>
<td>1.90</td>
<td>First report of the thioether bond pH = 7.0</td>
<td>644</td>
</tr>
<tr>
<td>1GOH</td>
<td>2.20</td>
<td>Apo postprocessed</td>
<td>644</td>
</tr>
</tbody>
</table>
### Table 25

Summary of DFT studies related to several putative Cu$_x$O$_y$ intermediate species for methane oxidation in pMMO.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$E_a$ (kcal/mol)</th>
<th>Mechanism</th>
<th>C-H(O-H) Bond Distance (Å)</th>
<th>$i$ (cm$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cu$^{III}$—O; Cu$^{II}$—O$^*$</td>
<td>18.8</td>
<td>H-atom</td>
<td>1.321(1.184)</td>
<td>1445</td>
<td>591</td>
</tr>
<tr>
<td>2 Cu$^{III}$ O Cu$^{III}$</td>
<td>44.0</td>
<td>Concerted</td>
<td>1.731(1.014)</td>
<td>703</td>
<td>719</td>
</tr>
<tr>
<td></td>
<td>21.0</td>
<td>Concerted</td>
<td>1.631(1.039)</td>
<td>799</td>
<td>720</td>
</tr>
<tr>
<td>3 Cu$^{II}$ O Cu$^{III}$</td>
<td>14.2</td>
<td>H-atom</td>
<td>1.396(1.132)</td>
<td>1226</td>
<td>719</td>
</tr>
<tr>
<td></td>
<td>19.1</td>
<td>H-atom</td>
<td>1.394(1.144)</td>
<td>1298</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>40.5</td>
<td>Concerted</td>
<td>1.382(1.255)</td>
<td>1487</td>
<td>721</td>
</tr>
<tr>
<td></td>
<td>21.7</td>
<td>H-atom</td>
<td>1.455(1.113)</td>
<td>1052</td>
<td>721</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Concerted</td>
<td>1.384(1.261)</td>
<td>1488</td>
<td>721</td>
</tr>
<tr>
<td>4 Cu$^{III}$ O Cu$^{III}$</td>
<td>9.5</td>
<td>H-atom</td>
<td>1.325(1.176)</td>
<td>1350</td>
<td>719</td>
</tr>
<tr>
<td>5 Cu$^{II}$ O Cu$^{III}$</td>
<td>15.0</td>
<td>Concerted</td>
<td>1.654(1.095)</td>
<td>593</td>
<td>720</td>
</tr>
</tbody>
</table>
Table 26

Spectroscopically characterized Cu\textsubscript{x}/O\textsubscript{2} species. Adapted from Reference 39.

<table>
<thead>
<tr>
<th>Cu/O\textsubscript{2} species</th>
<th>rR Vibrations (\textdelta\textsuperscript{18}O\textsubscript{2}) / cm\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>O\textsubscript{2}-activated Cu-ZSM-5</td>
<td>456 (8) 870 (40)</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{Cu}^{III})</td>
<td>(\nu(\text{Cu-O}) = 606 (23))</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{Cu}^{II})</td>
<td>(\nu(\text{Cu-Cu}) = 284 (0)) (\nu(\text{O-O}) = 763 (40))</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{O})</td>
<td>(\nu(\text{Cu-O}) = 472 (20)) (\nu(\text{O-O}) = 1121 (63))</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{O})</td>
<td>(\nu(\text{Cu-O}) = 554 (20)) (\nu(\text{O-O}) = 1043 (59))</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{OH})</td>
<td>(\nu(\text{Cu-O}) = 624 (17)) (\nu(\text{O-O}) = 843 (44))</td>
</tr>
<tr>
<td>(\text{Cu}^{II}\cdot\text{O}\cdot\text{Cu}^{II})</td>
<td>(\nu(\text{Cu-O}) = 561 (26)) (\nu(\text{O-O}) = 832 (44))</td>
</tr>
<tr>
<td>(\text{OH}\cdot\text{Cu}^{II}\cdot\text{O}\cdot\text{Cu}^{II})</td>
<td>(\nu(\text{Cu-O}) = 322 (10)) (\nu(\text{O-O}) = 892 (52))</td>
</tr>
</tbody>
</table>
Table 27
The two classes of multicopper oxidases.

<table>
<thead>
<tr>
<th>Phenolic substrates (broad)</th>
<th>Metal ion substrate (specific)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccases (plant, fungal, bacterial, insect)</td>
<td>Ceruloplasmin/ Hephaestin</td>
</tr>
<tr>
<td>Ascorbate Oxidase</td>
<td>CueO</td>
</tr>
<tr>
<td>Bilirubin Oxidase (including CotA)</td>
<td>Fet3p</td>
</tr>
<tr>
<td></td>
<td>MnxG</td>
</tr>
</tbody>
</table>
Table 28
Multicopper oxidase subgroups and their proposed functionalities.

<table>
<thead>
<tr>
<th>Multicopper Oxidase subgroups</th>
<th>Proposed Functionalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant laccases</td>
<td>wound healing\textsuperscript{738}</td>
</tr>
<tr>
<td></td>
<td>lignin formation\textsuperscript{739}</td>
</tr>
<tr>
<td></td>
<td>flavonoid oxidation\textsuperscript{740}</td>
</tr>
<tr>
<td></td>
<td>cell wall formation\textsuperscript{741}</td>
</tr>
<tr>
<td>Fungal laccases</td>
<td>lignin degradation\textsuperscript{742}</td>
</tr>
<tr>
<td></td>
<td>pathogenesis\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>detoxification\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>fungal development and morphogenesis\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>lignin degradation\textsuperscript{742}</td>
</tr>
<tr>
<td></td>
<td>pathogenesis\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>detoxification\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>fungal development and morphogenesis\textsuperscript{743}</td>
</tr>
<tr>
<td></td>
<td>melanin formation\textsuperscript{744,745}</td>
</tr>
<tr>
<td></td>
<td>resistance against UV-light\textsuperscript{744,745}</td>
</tr>
<tr>
<td></td>
<td>resistance against hydrogen peroxide\textsuperscript{744,745}</td>
</tr>
<tr>
<td>Prokaryotic laccases</td>
<td>cuticle formation\textsuperscript{746}</td>
</tr>
<tr>
<td>Insect laccases</td>
<td>plant growth\textsuperscript{747}</td>
</tr>
<tr>
<td>Ascorbate Oxidase</td>
<td>terminal oxidase in electron transport chain\textsuperscript{748}</td>
</tr>
<tr>
<td></td>
<td>plant defense against insects\textsuperscript{749}</td>
</tr>
<tr>
<td></td>
<td>hyperoxia prevention\textsuperscript{750}</td>
</tr>
<tr>
<td>Bilirubin Oxidase</td>
<td>lignin degradation\textsuperscript{751}</td>
</tr>
<tr>
<td>Ferroxidases (Fet3p, ceruloplasmin)</td>
<td>iron metabolism-including iron transport\textsuperscript{752}</td>
</tr>
<tr>
<td>Cuprous Oxidase (CueO)</td>
<td>copper homeostasis\textsuperscript{753}</td>
</tr>
<tr>
<td>Manganese Oxidase</td>
<td>copper homeostasis\textsuperscript{754}</td>
</tr>
<tr>
<td></td>
<td>antioxidant activity\textsuperscript{755}</td>
</tr>
<tr>
<td></td>
<td>manganese oxidation\textsuperscript{756}</td>
</tr>
</tbody>
</table>
### Table 29

Steady-State Kinetic properties for multicopper oxidases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic Oxidases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laccase (Tree)</td>
<td>Hydroquinone</td>
<td>7.5</td>
<td>852</td>
</tr>
<tr>
<td>R. vernicifera</td>
<td>200</td>
<td>560</td>
<td></td>
</tr>
<tr>
<td>Laccase (SLAC)</td>
<td>2,6-DMP</td>
<td>7.0</td>
<td>858</td>
</tr>
<tr>
<td>S. coelicolor</td>
<td>3.6</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Laccase (Fungal)</td>
<td>Hydroquinone</td>
<td>5.0</td>
<td>859</td>
</tr>
<tr>
<td>T. villosa</td>
<td>0.13</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>M. thermophila</td>
<td>0.73</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>Cota</td>
<td>Hydroquinone</td>
<td>5.0</td>
<td>859</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>0.0567</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>CotA</td>
<td>2,6-DMP</td>
<td>7.0</td>
<td>854</td>
</tr>
<tr>
<td>B subtilis</td>
<td>0.216</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Ascorbate Oxidase</td>
<td>Ascorbate</td>
<td>6.0 &amp; 7.5</td>
<td>856</td>
</tr>
<tr>
<td>C. Pepo medullosa</td>
<td>0.200</td>
<td>7.50 × 10³</td>
<td></td>
</tr>
<tr>
<td><strong>Metaloxidases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe5p</td>
<td>Fe(II)</td>
<td>5.0</td>
<td>843</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.0054</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Fe(II)</td>
<td>5.0</td>
<td>843</td>
</tr>
<tr>
<td>H. sapien</td>
<td>0.0083</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>CuoO</td>
<td>Cu(I)</td>
<td>5.0</td>
<td>853</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.165</td>
<td>15.2</td>
<td></td>
</tr>
</tbody>
</table>

*2,6-dimethoxy-phenol;*
### Table 30

Decay rates and SKIE’s of T1D WT and variants in Fet3p

<table>
<thead>
<tr>
<th></th>
<th>T1D</th>
<th>T1DE487D</th>
<th>T1DE487A</th>
<th>T1DD94E</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH=5.0 rate of PI decay</td>
<td>1.8x10^-3 s^-1</td>
<td>(1.6±0.1)x10^-3 s^-1</td>
<td>(7.2±0.5)x10^-5 s^-1</td>
<td>(3.7±0.4)x10^-5 s^-1</td>
</tr>
<tr>
<td>pH=7.5 rate of PI decay</td>
<td>2.9x10^-4 s^-1</td>
<td>(5.2±0.9)x10^-5 s^-1</td>
<td>(8±1)x10^-5 s^-1</td>
<td>(4±1)x10^-5 s^-1</td>
</tr>
<tr>
<td>Rate enhancement</td>
<td>6.2×</td>
<td>3.1×</td>
<td>1×</td>
<td>8.0×</td>
</tr>
<tr>
<td>SKIE@low pH</td>
<td>0.89</td>
<td>2.0</td>
<td>–1</td>
<td>2.3</td>
</tr>
<tr>
<td>pH effect on resting T2 Cu</td>
<td>yes</td>
<td>Yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

*Chem Rev*. Author manuscript; available in PMC 2015 April 09.
Table 31

Midpoint Potentials of Cyt c and the Metal Sites of CcO (Eₘ values in mV).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyt c</th>
<th>Cuₐ</th>
<th>heme a</th>
<th>heme a₃</th>
<th>Cu₃</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated</td>
<td>283</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>971</td>
</tr>
<tr>
<td>In mitochondria</td>
<td>235</td>
<td>205</td>
<td>390'</td>
<td></td>
<td></td>
<td>971</td>
</tr>
<tr>
<td>Sub-mitochondria</td>
<td>235</td>
<td>205</td>
<td>365'</td>
<td></td>
<td></td>
<td>971</td>
</tr>
<tr>
<td>Sub-mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>340</td>
<td>973</td>
</tr>
<tr>
<td>In mitochondria</td>
<td>195</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>975</td>
</tr>
<tr>
<td>Isolated, Cyt c present</td>
<td>280</td>
<td>230</td>
<td>370'</td>
<td></td>
<td></td>
<td>974</td>
</tr>
<tr>
<td>Isolated, Cyt c present</td>
<td>210</td>
<td></td>
<td>340–350'</td>
<td></td>
<td></td>
<td>977</td>
</tr>
<tr>
<td>Isolated, TMPD present</td>
<td>215</td>
<td></td>
<td>340'</td>
<td></td>
<td></td>
<td>969</td>
</tr>
<tr>
<td>In mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>high 362, low 238'</td>
<td>978</td>
</tr>
</tbody>
</table>

* When both heme sites are oxidized the Eₘ values of Cyt a and a₃ are similar (362 mV). Reduction of one heme lowers the Eₘ of the other to 238 mV. Assuming heme a₃ gets reduced before heme a.
### Table 32

X-ray crystallographic structures of HCO’s.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type</th>
<th>Year</th>
<th>PDB ID</th>
<th>Resolution/Å</th>
<th>Subunits</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>αβ3</td>
<td>1996</td>
<td>1OCC</td>
<td>2.80</td>
<td>13</td>
<td>A1</td>
</tr>
<tr>
<td><em>P. denitrificans</em></td>
<td>αβ3</td>
<td>1999</td>
<td>1QLE</td>
<td>3.00</td>
<td>4</td>
<td>A1</td>
</tr>
<tr>
<td>E. coli</td>
<td>bα3</td>
<td>2000</td>
<td>1FFT</td>
<td>3.5</td>
<td>4</td>
<td>A1</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>αβ3</td>
<td>2002</td>
<td>1M56</td>
<td>2.30</td>
<td>4</td>
<td>A1</td>
</tr>
<tr>
<td><em>T. thermophilus</em></td>
<td>bα3</td>
<td>2001</td>
<td>1EHK</td>
<td>2.4</td>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>cbb3</td>
<td>2010</td>
<td>3MK7</td>
<td>3.2</td>
<td>4</td>
<td>C</td>
</tr>
</tbody>
</table>
Table 33

X-ray Crystallographic Structures of Bovine $aa_3$ CeO.

<table>
<thead>
<tr>
<th>Redox State</th>
<th>PDB ID</th>
<th>Year</th>
<th>Resolution (Å)</th>
<th>Fe--Cu (Å)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidized</td>
<td>3ASN</td>
<td>2011</td>
<td>3.00</td>
<td>4.67, 4.69</td>
<td>1005</td>
</tr>
<tr>
<td>oxidized</td>
<td>3ASO</td>
<td>2011</td>
<td>2.30</td>
<td>4.91–4.93</td>
<td>1005</td>
</tr>
<tr>
<td>oxidized</td>
<td>2Y69</td>
<td>2011</td>
<td>1.95</td>
<td>4.86–4.89</td>
<td>1006</td>
</tr>
<tr>
<td>oxidized</td>
<td>3ABL</td>
<td>2009</td>
<td>2.10</td>
<td>4.83–4.87</td>
<td>1000</td>
</tr>
<tr>
<td>oxidized</td>
<td>3ABM</td>
<td>2009</td>
<td>1.95</td>
<td>4.86–4.89</td>
<td>1000</td>
</tr>
<tr>
<td>oxidized</td>
<td>2ZXW</td>
<td>2009</td>
<td>2.50</td>
<td>4.814.88</td>
<td>1000</td>
</tr>
<tr>
<td>reduced</td>
<td>2EIJ</td>
<td>2007</td>
<td>1.90</td>
<td>5.12–5.16</td>
<td>1007</td>
</tr>
<tr>
<td>reduced</td>
<td>2EIK</td>
<td>2007</td>
<td>2.10</td>
<td>5.11–5.12</td>
<td>1007</td>
</tr>
<tr>
<td>oxidized</td>
<td>2EIL</td>
<td>2007</td>
<td>2.10</td>
<td>4.82–4.90</td>
<td>1007</td>
</tr>
<tr>
<td>reduced</td>
<td>2EIM</td>
<td>2007</td>
<td>2.60</td>
<td>5.00–5.07</td>
<td>1007</td>
</tr>
<tr>
<td>oxidized</td>
<td>2EIN</td>
<td>2007</td>
<td>2.70</td>
<td>4.34–4.60</td>
<td>1007</td>
</tr>
<tr>
<td>oxidized</td>
<td>2DYR</td>
<td>2007</td>
<td>1.80</td>
<td>4.99</td>
<td>1008</td>
</tr>
<tr>
<td>oxidized</td>
<td>2DYS</td>
<td>2007</td>
<td>2.20</td>
<td>4.59</td>
<td>1008</td>
</tr>
<tr>
<td>oxidized</td>
<td>1VS4</td>
<td>2003</td>
<td>1.80</td>
<td>4.99–5.02</td>
<td>998</td>
</tr>
<tr>
<td>reduced</td>
<td>1VS5</td>
<td>2003</td>
<td>1.90</td>
<td>5.13–5.16</td>
<td>998</td>
</tr>
<tr>
<td>reduced</td>
<td>1OCR</td>
<td>1998</td>
<td>2.35</td>
<td>5.13–5.19</td>
<td>1002</td>
</tr>
<tr>
<td>oxidized</td>
<td>2OCC</td>
<td>1998</td>
<td>2.30</td>
<td>4.85–4.86</td>
<td>1002</td>
</tr>
<tr>
<td>oxidized</td>
<td>1OCC</td>
<td>1996</td>
<td>2.80</td>
<td>4.70</td>
<td>989</td>
</tr>
</tbody>
</table>
Table 34

X-ray Crystallographic Structures of Bovine \( \alpha_3 \)CcO of the O\(_2\) Reduction Site in Different States.

<table>
<thead>
<tr>
<th>Protein State</th>
<th>PDB ID</th>
<th>Resolution/Å</th>
<th>Bridging Ligand</th>
<th>( \text{Fe}_{\alpha 3}\text{-Cu}_B ) Å</th>
<th>( \text{Fe}_{\alpha 3}\text{-X} ) Å</th>
<th>( \text{Cu}_B\text{-Y} ) Å</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully oxidized</td>
<td>2ZXW</td>
<td>2.5</td>
<td>( \text{O}_2^{2-} )</td>
<td>4.87</td>
<td>2.24</td>
<td>2.17</td>
<td>1000</td>
</tr>
<tr>
<td>Fully reduced</td>
<td>1OCR</td>
<td>2.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1002</td>
</tr>
<tr>
<td>Fully oxidized Azide bound</td>
<td>1OCO</td>
<td>2.9</td>
<td>( \text{N}_3^- )</td>
<td>5.3</td>
<td>1.97</td>
<td>1.9</td>
<td>1002</td>
</tr>
<tr>
<td>Fully reduced CO bound</td>
<td>3AG1</td>
<td>2.2</td>
<td>CO</td>
<td>5.35</td>
<td>1.8</td>
<td>2.7</td>
<td>999</td>
</tr>
<tr>
<td>Fully reduced CO bound</td>
<td>3AG2</td>
<td>1.8</td>
<td>CO</td>
<td>4.97</td>
<td>3.0</td>
<td>2.4</td>
<td>999</td>
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<tr>
<td>Fully reduced NO bound</td>
<td>3AG3</td>
<td>1.8</td>
<td>NO</td>
<td>4.91</td>
<td>1.8</td>
<td>2.5</td>
<td>999</td>
</tr>
<tr>
<td>Fully reduced CN-bound</td>
<td>3AG4</td>
<td>2.05</td>
<td>CN(^-)</td>
<td>4.99</td>
<td>2.4</td>
<td>2.3</td>
<td>999</td>
</tr>
</tbody>
</table>
Table 35

Key Residues in the K-, D- and H-Channels in Bovine CeO (Values in Parentheses are the Corresponding Residues in *R. sphaeroides*).

<table>
<thead>
<tr>
<th></th>
<th>K</th>
<th>H</th>
<th></th>
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<tbody>
<tr>
<td>D</td>
<td>91</td>
<td>(D132)</td>
<td>51</td>
</tr>
<tr>
<td>H</td>
<td>503</td>
<td>(N121)</td>
<td>205</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>(N139)</td>
<td>440</td>
</tr>
<tr>
<td>N</td>
<td>98</td>
<td>(N207)</td>
<td>441</td>
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<td></td>
<td>(S142)</td>
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<tr>
<td>Y</td>
<td>19</td>
<td>(Y33)</td>
<td>371</td>
</tr>
<tr>
<td>S</td>
<td>101</td>
<td>(S201)</td>
<td>38</td>
</tr>
<tr>
<td>S</td>
<td>156</td>
<td>(S200)</td>
<td>382</td>
</tr>
<tr>
<td>S</td>
<td>157</td>
<td>(S197)</td>
<td>386</td>
</tr>
<tr>
<td>E</td>
<td>242</td>
<td>(E286)</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>413</td>
</tr>
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Table 36
Various 2,4-QD substrates and steady-state kinetics parameters for 2,4-dioxygenation by *P. olsonii* 2,4-QD (obtained with 20% DMSO assay content).\textsuperscript{1112}

<table>
<thead>
<tr>
<th>Substrate structure</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(K_M) ((\mu)M)</th>
<th>(k_{cat}/K_M) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>167±3</td>
<td>19±1</td>
<td>8.8×10(^6)</td>
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<tr>
<td>kaempferol</td>
<td>467±17</td>
<td>13±2</td>
<td>3.6×10(^7)</td>
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<tr>
<td>galangin</td>
<td>33±5</td>
<td>20±4</td>
<td>1.7×10(^6)</td>
</tr>
<tr>
<td>fisetin</td>
<td>57±7</td>
<td>85±16</td>
<td>6.7×10(^5)</td>
</tr>
</tbody>
</table>
Table 37

Representative kinetic parameters for the stoichiometric reduction of various flavonolate model complexes with \( \text{O}_2 \)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( k_2 ) (M(^{-1}) s(^{-1}))</th>
<th>( \Delta H^\ddagger ) kcal/mol</th>
<th>( \Delta S^\ddagger ) cal/mol•K</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>potassium flavonolate</td>
<td>80°C, DMF 0.33±0.01</td>
<td>6.9±0.5</td>
<td>−38±1</td>
<td>1128</td>
</tr>
<tr>
<td>( \text{Zn}^{II}(idpa)(fla)\text{ClO}_4 )</td>
<td>100°C, DMF (3.1±0.1)×10(^{-3})</td>
<td>18±1</td>
<td>−23±3</td>
<td>1131</td>
</tr>
<tr>
<td>( \text{Cu}^{I}(PPh_3)_2(fla) )</td>
<td>90°C, DMF (4.2±0.5)×10(^{-3})</td>
<td>24</td>
<td>−3</td>
<td>1136</td>
</tr>
<tr>
<td>( \text{Cu}^{II}(idpa)(fla)\text{ClO}_4 )</td>
<td>100°C, DMF (6.1±0.2)×10(^{-3})</td>
<td>15±1</td>
<td>−29±4</td>
<td>1133</td>
</tr>
</tbody>
</table>
Table 38

Crystal structures of processed copper amine oxidases deposited in the Protein Data Bank as of October 10, 2012.

<table>
<thead>
<tr>
<th>Perturbation</th>
<th>Source Organism</th>
<th>Coordination Sphere</th>
<th>TPQ State</th>
<th>Resolution (Å)</th>
<th>PDB ID</th>
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<td>_..._193</td>
<td><em>E. coli</em></td>
<td>3His, H₂O</td>
<td>quinone</td>
<td>2.0</td>
<td>1dyu</td>
</tr>
<tr>
<td>_..._194</td>
<td><em>A. globiformis</em></td>
<td>3His, H₂O</td>
<td>quinone</td>
<td>2.3</td>
<td>1av4</td>
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<tr>
<td>anaerobically Cu loaded exposed to O₂¹⁹⁵</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>_..._196</td>
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<td>3His, H₂O</td>
<td>quinone</td>
<td>2.2</td>
<td>1ivx</td>
</tr>
<tr>
<td>_..._197</td>
<td><em>H. polymorpha</em></td>
<td>3His, 2H₂O</td>
<td>quinone</td>
<td>2.4</td>
<td>1a2v</td>
</tr>
<tr>
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<td>quinone</td>
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<td>3koy</td>
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<tr>
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<td>1ksl</td>
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<td>quinone</td>
<td>2.5</td>
<td>2c10</td>
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<tr>
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<td>quinone</td>
<td>2.95</td>
<td>2y74</td>
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<td>quinone</td>
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<td>3ala</td>
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<tr>
<td>_..._203</td>
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<td>quinone</td>
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<td>3ggb</td>
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<td>1oac</td>
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<tr>
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<td>quinone</td>
<td>1.9</td>
<td>1ivw</td>
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<td>_..._197</td>
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<td>quinone</td>
<td>1.55</td>
<td>1w6g</td>
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<td>1w6v</td>
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<td>3His, TPQ</td>
<td>quinone</td>
<td>1.65</td>
<td>1n9e</td>
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<td>_..._201</td>
<td><em>H. sapiens</em></td>
<td>3His, TPQ</td>
<td>quinone</td>
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<td>1us1</td>
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<td>Resolution (Å)</td>
<td>PDB ID</td>
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<tr>
<td>...1209</td>
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<td>quinone</td>
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<td>1pu4</td>
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<td>2y73</td>
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<td>3hi7</td>
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### Variants

<table>
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<tr>
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<td>2His, Tyr, H₂O</td>
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<td>quinone</td>
<td>2.2</td>
<td>1qaf</td>
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<td>quinone</td>
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<td>1qak</td>
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<td>3His, H₂O</td>
<td>quinone</td>
<td>1.82</td>
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### Substrate-reduced

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<th>Resolution (Å)</th>
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<td>anaerobically substrate reduced1214</td>
<td><em>E. coli</em></td>
<td>3His, H₂O</td>
<td>aminooquinol</td>
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<td>1d6a</td>
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<tr>
<td>anaerobically substrate reduced + NO1214</td>
<td><em>E. coli</em></td>
<td>3His, NO</td>
<td>aminooquinol</td>
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### Substrate-bound

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<th>Resolution (Å)</th>
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<td><em>E. coli</em></td>
<td>3His, H₂O</td>
<td>Schiff base</td>
<td>2.0</td>
<td>1spu</td>
</tr>
<tr>
<td>ethylamine1215</td>
<td><em>A. globiformis</em></td>
<td>3His, H₂O</td>
<td>Schiff base</td>
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<td>5-phenoxy-2,3-pentadienylamine1216</td>
<td><em>A. globiformis</em></td>
<td>3His, H₂O</td>
<td>Schiff base</td>
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<td>3kii</td>
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<td>6-phenyl-2,3-hexadienylamine1216</td>
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<td>3His, H₂O</td>
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<td>D298A + tyramine1217</td>
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### Inhibitor Complexes

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<th>PDB ID</th>
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<td>tranylcypromine1218</td>
<td><em>E. coli</em></td>
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<td>Schiff base</td>
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<td>1hvn</td>
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<td>Perturbation</td>
<td>Source Organism</td>
<td>Coordination Sphere</td>
<td>TPQ State</td>
<td>Resolution (Å)</td>
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<tr>
<td>tranylcypromine\textsuperscript{219}</td>
<td>A. globiformis</td>
<td>3His, 2H\textsubscript{2}O</td>
<td>Schiff base</td>
<td>1.65</td>
<td>1w4n</td>
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<tr>
<td>phenylhydrazine\textsuperscript{1220}</td>
<td>A. globiformis</td>
<td>3His, 2H\textsubscript{2}O</td>
<td>hydrazone</td>
<td>2.05</td>
<td>2e2t</td>
</tr>
<tr>
<td>benzylhydrazine\textsuperscript{219}</td>
<td>A. globiformis</td>
<td>3His, 2H\textsubscript{2}O</td>
<td>hydrazone</td>
<td>1.86</td>
<td>1w5z</td>
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<tr>
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<td>hydrazone</td>
<td>1.80</td>
<td>2e2v</td>
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<td>4-hydroxybenzylhydrazine\textsuperscript{1220}</td>
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<td>3His, H\textsubscript{2}O</td>
<td>hydrazone</td>
<td>1.68</td>
<td>2e2a</td>
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<tr>
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<td>3His, H\textsubscript{2}O</td>
<td>Schiff base</td>
<td>1.73</td>
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<td>3His, H\textsubscript{2}O</td>
<td>Schiff base</td>
<td>1.70</td>
<td>1sii</td>
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<tr>
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<td>3His, 2H\textsubscript{2}O</td>
<td>quinone</td>
<td>1.73</td>
<td>2bt3</td>
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<td>quinone</td>
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<td>3His, 2H\textsubscript{2}O</td>
<td>quinone</td>
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<td>2-hydrazinopyradine\textsuperscript{1200}</td>
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<td>3His, H\textsubscript{2}O</td>
<td>hydrazone</td>
<td>2.9</td>
<td>2cl1</td>
</tr>
<tr>
<td>aminoguanidine\textsuperscript{1211}</td>
<td>H. sapiens</td>
<td>3His, H\textsubscript{2}O</td>
<td>Schiff base</td>
<td>2.05</td>
<td>3mph</td>
</tr>
<tr>
<td>berenil\textsuperscript{211}</td>
<td>H. sapiens</td>
<td>3His, TPQ</td>
<td>quinone</td>
<td>2.09</td>
<td>3hig</td>
</tr>
<tr>
<td>pentamidine\textsuperscript{211}</td>
<td>H. sapiens</td>
<td>3His, TPQ</td>
<td>quinone</td>
<td>2.15</td>
<td>3hii</td>
</tr>
<tr>
<td>clonidine\textsuperscript{1224}</td>
<td>B. taurus</td>
<td>3His, TPQ</td>
<td>quinone</td>
<td>2.4</td>
<td>2pnc</td>
</tr>
</tbody>
</table>

**Trapped Catalytic Intermediates**

<table>
<thead>
<tr>
<th>Trapped Species</th>
<th>Source Organism</th>
<th>Coordination Sphere</th>
<th>TPQ State</th>
<th>Resolution (Å)</th>
<th>PDB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>aerobically trapped O\textsubscript{2} equilibrium species\textsuperscript{1223}</td>
<td>E. coli</td>
<td>3His</td>
<td>aminquinol</td>
<td>2.1</td>
<td>1d6e</td>
</tr>
<tr>
<td>time-resolved X-ray structure\textsuperscript{1226}</td>
<td>A. globiformis</td>
<td>3His, H\textsubscript{2}O</td>
<td>Schiff base</td>
<td>2.1</td>
<td>3amo</td>
</tr>
</tbody>
</table>

**Alternative Metal Substituted**

<table>
<thead>
<tr>
<th>Substituted</th>
<th>Source Organism</th>
<th>Coordination Sphere</th>
<th>TPQ State</th>
<th>Resolution (Å)</th>
<th>PDB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-substituted\textsuperscript{1191}</td>
<td>A. globiformis</td>
<td>3His, H\textsubscript{2}O</td>
<td>quinone</td>
<td>1.8</td>
<td>1wmn</td>
</tr>
<tr>
<td>Co-substituted\textsuperscript{1227}</td>
<td>A. globiformis</td>
<td>3His, H\textsubscript{2}O</td>
<td>quinone</td>
<td>2.0</td>
<td>1qix</td>
</tr>
<tr>
<td>Ni-substituted\textsuperscript{1191}</td>
<td>A. globiformis</td>
<td>3His, H\textsubscript{2}O</td>
<td>quinone</td>
<td>1.8</td>
<td>1ky</td>
</tr>
<tr>
<td>Perturbation</td>
<td>Source Organism</td>
<td>Coordination Sphere</td>
<td>TPQ State</td>
<td>Resolution (Å)</td>
<td>PDB ID</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>---------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>Ni-substituted (^{1227})</td>
<td><em>A. globiformis</em></td>
<td>3His, 3H(_2)O</td>
<td>quinone</td>
<td>1.8</td>
<td>1wmo</td>
</tr>
<tr>
<td>Sr soaked (^{1205})</td>
<td><em>E. coli</em></td>
<td>3His, H(_2)O</td>
<td>quinone</td>
<td>2.7</td>
<td>2woh</td>
</tr>
</tbody>
</table>

**Xe Complexes**

<table>
<thead>
<tr>
<th>Xe complex (^{1206})</th>
<th><em>H. polymorpha</em></th>
<th>3His</th>
<th>quinone</th>
<th>1.6</th>
<th>2oqe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xe complex (^{1228})</td>
<td><em>A. globiformis</em></td>
<td>3His, H(_2)O</td>
<td>quinone</td>
<td>1.67</td>
<td>1rjo</td>
</tr>
<tr>
<td>Xe complex (^{1228})</td>
<td><em>P. sativum</em></td>
<td>3His, H(_2)O</td>
<td>quinone</td>
<td>2.24</td>
<td>1w2z</td>
</tr>
<tr>
<td>Xe complex (^{1228})</td>
<td><em>P. pastoris</em></td>
<td>3His, TPQ</td>
<td>quinone</td>
<td>1.68</td>
<td>1rky</td>
</tr>
<tr>
<td>Xe complex (^{1229})</td>
<td><em>E. coli</em></td>
<td>3His, H(_2)O</td>
<td>quinone</td>
<td>2.48</td>
<td>2w0q</td>
</tr>
</tbody>
</table>
Table 39

X-ray crystal structures of apo, preprocessed, metal-substituted and mutant copper amine oxidase variants relevant to TPQ biogenesis.

<table>
<thead>
<tr>
<th>Perturbation</th>
<th>Source Organism</th>
<th>Coordination Sphere</th>
<th>TPQ State</th>
<th>Resolution (Å)</th>
<th>PDB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo&lt;sup&gt;194&lt;/sup&gt;</td>
<td>A. globiformis</td>
<td>---</td>
<td>Tyr</td>
<td>2.2</td>
<td>1avk</td>
</tr>
<tr>
<td>apo&lt;sup&gt;230&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>---</td>
<td>Tyr</td>
<td>1.73</td>
<td>3sx1</td>
</tr>
<tr>
<td>anaerobically Cu loaded&lt;sup&gt;1195&lt;/sup&gt;</td>
<td>A. globiformis</td>
<td>3His, Tyr at 2.58 Å</td>
<td>Tyr</td>
<td>1.9</td>
<td>1ivu</td>
</tr>
<tr>
<td>anaerobically Cu loaded + O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;1195&lt;/sup&gt;</td>
<td>A. globiformis</td>
<td>3His, H&lt;sub&gt;2&lt;/sub&gt;O, DPQ</td>
<td>DPQ</td>
<td>2.1</td>
<td>1ivv</td>
</tr>
<tr>
<td>Zn-substituted&lt;sup&gt;231&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>3His, Tyr at 2.02 Å</td>
<td>Tyr</td>
<td>2.5</td>
<td>1ekm</td>
</tr>
<tr>
<td>Zn-substituted&lt;sup&gt;232&lt;/sup&gt;</td>
<td>E. coli</td>
<td>3His, Tyr at 1.87 Å</td>
<td>Tyr</td>
<td>2.5</td>
<td>2wgq</td>
</tr>
<tr>
<td>Co-substituted&lt;sup&gt;1230&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>3His, Tyr at 2.19 Å</td>
<td>Tyr</td>
<td>1.27</td>
<td>3sxx</td>
</tr>
<tr>
<td>Co-substituted&lt;sup&gt;1191&lt;/sup&gt;</td>
<td>A. globiformis</td>
<td>3His</td>
<td>Tyr</td>
<td>2.0</td>
<td>1wmp</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;1&lt;/sup&gt;-substituted&lt;sup&gt;1230&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>3His, Tyr at 2.8 Å</td>
<td>Tyr</td>
<td>1.90</td>
<td>3tou</td>
</tr>
<tr>
<td>Y305F&lt;sup&gt;1090&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>3His, quinone</td>
<td>peroxy-quinone</td>
<td>2.05</td>
<td>3nbb</td>
</tr>
<tr>
<td>Y305F&lt;sup&gt;1090&lt;/sup&gt;</td>
<td>H. polymorpha</td>
<td>3His</td>
<td>peroxy-quinone</td>
<td>1.9</td>
<td>3nbj</td>
</tr>
<tr>
<td>D298K&lt;sup&gt;1233&lt;/sup&gt;</td>
<td>A. globiformis</td>
<td>3His, H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Schiff base</td>
<td>1.68</td>
<td>2yx9</td>
</tr>
</tbody>
</table>

Summary of the spectral features of tetrahedral CuL(OPh-4-F) model complexes, including gaussian resolved peak positions and band assignments from absorption (5 K), MCD (5 K, 7T) and TD-DFT, Cu-O stretching frequencies from resonance Raman excited at 647 nm (77 K), and EPR parameters from 77 K X-band EPR.$^{1239}$

<table>
<thead>
<tr>
<th></th>
<th>Bands from LT Abs/MCD</th>
<th>Resonance Raman</th>
<th>EPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm$^{-1}$</td>
<td>ε M$^{-1}$ cm$^{-1}$</td>
<td>Assignment</td>
</tr>
<tr>
<td>CuL1(OPh-4-F)</td>
<td>4869</td>
<td>---</td>
<td>dz$^2$</td>
</tr>
<tr>
<td></td>
<td>6745</td>
<td>---</td>
<td>d$<em>{xy}$-d$</em>{xz}$</td>
</tr>
<tr>
<td></td>
<td>11019</td>
<td>493</td>
<td>d$<em>{xy}$+d$</em>{xz}$</td>
</tr>
<tr>
<td></td>
<td>12409</td>
<td>575</td>
<td>d$_{y}$</td>
</tr>
<tr>
<td></td>
<td>14727</td>
<td>2363</td>
<td>oop CT</td>
</tr>
<tr>
<td></td>
<td>26147</td>
<td>842</td>
<td>ip CT</td>
</tr>
<tr>
<td>CuL3(OPh-4-F)</td>
<td>5100</td>
<td>---</td>
<td>oop CT</td>
</tr>
<tr>
<td></td>
<td>7499</td>
<td>---</td>
<td>d$_{xy}$</td>
</tr>
<tr>
<td></td>
<td>9483</td>
<td>162</td>
<td>d$_{yz}$</td>
</tr>
<tr>
<td></td>
<td>10860</td>
<td>321</td>
<td>dz$^2$</td>
</tr>
<tr>
<td></td>
<td>14576</td>
<td>890</td>
<td>d$_{xy}$</td>
</tr>
<tr>
<td></td>
<td>28216</td>
<td>1764</td>
<td>ip CT</td>
</tr>
</tbody>
</table>
### Table 41

Selected parameters for crystallographically characterized nitrite-bound species in the Cu dependent nitrite reductases.

<table>
<thead>
<tr>
<th>Source Organism</th>
<th>Mutations</th>
<th>Resolution (Å)</th>
<th>pH</th>
<th>Cu-N Distance (Å)</th>
<th>Cu-O Bond Lengths (Å)</th>
<th>PDB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidized CuNiR + Nitrite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td>WT</td>
<td>2.80</td>
<td>6.0</td>
<td>2.484</td>
<td>1.732, 2.376</td>
<td>1nds</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td>N90S</td>
<td>3.00</td>
<td>7.4</td>
<td>2.488</td>
<td>2.000, &gt;2.367</td>
<td>2xx1</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td>H313Q</td>
<td>1.72</td>
<td>7.1</td>
<td>2.406</td>
<td>1.861, &gt;2.419</td>
<td>1wa2</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>WT</td>
<td>1.40</td>
<td>7.0</td>
<td>2.360</td>
<td>2.039, &gt;2.290</td>
<td>1sji</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>WT</td>
<td>1.80</td>
<td>7.0</td>
<td>2.351</td>
<td>2.165, &gt;2.404</td>
<td>1as6</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>WT</td>
<td>1.85</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>WT</td>
<td>1.90</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>WT</td>
<td>1.95</td>
<td>7.0</td>
<td>2.313</td>
<td>2.022, &gt;2.678</td>
<td>1kbv</td>
</tr>
<tr>
<td>A. cycloclastes</td>
<td>WT</td>
<td>2.20</td>
<td>5.4</td>
<td>2.697</td>
<td>2.157, &gt;2.530</td>
<td>1nd1</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>D98N</td>
<td>1.65</td>
<td>7.0</td>
<td>2.498</td>
<td>2.289, &gt;2.475</td>
<td>1j9l</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>H255N</td>
<td>1.90</td>
<td>7.0</td>
<td>3.2</td>
<td>2.111</td>
<td>1j9s</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>I257A</td>
<td>1.70</td>
<td>7.0</td>
<td>3.3</td>
<td>1.935</td>
<td>1j9l</td>
</tr>
<tr>
<td>A. faecalis</td>
<td>I257G</td>
<td>1.75</td>
<td>7.0</td>
<td>2.285</td>
<td>&gt;2.144, 2.096</td>
<td>1j9p</td>
</tr>
<tr>
<td><strong>Reduced CuNiR + Nitrite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. faecalis</td>
<td>WT</td>
<td>1.85</td>
<td>7.0</td>
<td>2.458</td>
<td>2.229, &gt;2.674</td>
<td>1as8</td>
</tr>
<tr>
<td><strong>Reduced CuNiR + NO</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. faecalis</td>
<td>WT</td>
<td>1.58</td>
<td>7.0</td>
<td>2.4</td>
<td>1.982, &gt;2.4</td>
<td>2ppc</td>
</tr>
<tr>
<td><strong>Endogenous Nitrite</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>A. cycloclastes</td>
<td>WT</td>
<td>1.15</td>
<td>6.5</td>
<td>2.154</td>
<td>1.978, &gt;2.190</td>
<td>2bwl</td>
</tr>
</tbody>
</table>
Table 42

Biochemical properties of nitrous oxide reductase purified from different organisms and their dependence on O$_2$ during purification.

<table>
<thead>
<tr>
<th></th>
<th>Molecular Weight</th>
<th>Structure</th>
<th>Cu Content per Dimer</th>
<th>S$^{2-}$ Content per Dimer</th>
<th>Sequence Similarity with PsN$_2$OR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic Purifications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. stutzeri</em>1280</td>
<td>120 kDa</td>
<td>homodimer</td>
<td>6.6±0.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><em>P. pantotrophus</em>1290</td>
<td>140 kDa</td>
<td>homodimer</td>
<td>7.2±1.2</td>
<td>---</td>
<td>92%</td>
</tr>
<tr>
<td>*A. cycloclastes (recombinant)*201</td>
<td>140 kDa</td>
<td>homodimer</td>
<td>4.2±1.2</td>
<td>---</td>
<td>70%</td>
</tr>
<tr>
<td><em>A. cycloclastes</em>1282</td>
<td>72 kDa</td>
<td>monomer</td>
<td>7.6±0.2</td>
<td>---</td>
<td>70%</td>
</tr>
<tr>
<td><em>P. denitrificans</em>1292</td>
<td>144 kDa</td>
<td>homodimer</td>
<td>11.0</td>
<td>1.4</td>
<td>71%</td>
</tr>
<tr>
<td><em>P. nautica</em>1281</td>
<td>130 kDa</td>
<td>homodimer</td>
<td>10.7±1.7</td>
<td>2.1±0.1</td>
<td>86%</td>
</tr>
<tr>
<td><em>R. sphaeroides</em>1279</td>
<td>95 kDa</td>
<td>homodimer</td>
<td>8.0±0.2</td>
<td>---</td>
<td>75%</td>
</tr>
<tr>
<td><em>H. denitrificans</em>1287</td>
<td>130 kDa</td>
<td>homodimer</td>
<td>9.0±0.2</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><em>T. denitrificans</em>1285</td>
<td>160 kDa</td>
<td>homodimer</td>
<td>1.6</td>
<td>---</td>
<td>66%</td>
</tr>
<tr>
<td><em>W. succinogenes</em>1281</td>
<td>162 kDa</td>
<td>homodimer</td>
<td>5.8±0.6</td>
<td>---</td>
<td>51%</td>
</tr>
<tr>
<td><strong>Anaerobic Purifications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. stutzeri</em>1280</td>
<td>120 kDa</td>
<td>homodimer</td>
<td>7.3±0.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><em>P. pantotrophus</em>1290</td>
<td>140 kDa</td>
<td>homodimer</td>
<td>10.5±2.6</td>
<td>1.7±0.4</td>
<td>92%</td>
</tr>
<tr>
<td>*A. cycloclastes (recombinant)*201</td>
<td>140 kDa</td>
<td>homodimer</td>
<td>9.0±1.2</td>
<td>2.4±0.4</td>
<td>70%</td>
</tr>
<tr>
<td><em>P. stutzeri</em>1280</td>
<td>120 kDa</td>
<td>homodimer</td>
<td>9.9±1.7</td>
<td>1.6±0.2</td>
<td>---</td>
</tr>
<tr>
<td><em>P. denitrificans</em>1280</td>
<td>144 kDa</td>
<td>homodimer</td>
<td>7.6±0.4</td>
<td>---</td>
<td>71%</td>
</tr>
<tr>
<td><em>P. aeruginosa</em>1280</td>
<td>120 kDa</td>
<td>homodimer</td>
<td>8.2±0.4</td>
<td>---</td>
<td>94%</td>
</tr>
<tr>
<td><em>A. xylosoxidans</em>1286</td>
<td>130 kDa</td>
<td>homodimer</td>
<td>7.1±0.4</td>
<td>---</td>
<td>73%</td>
</tr>
</tbody>
</table>
Specific activities of N\textsubscript{2}ORs from different source organisms and a comparison between as isolated values and activities after base activation or reductive activation (results from anaerobic purifications shaded).

<table>
<thead>
<tr>
<th>Source Organism</th>
<th>Purification Conditions</th>
<th>Specific Activity (µmol N\textsubscript{2}O min\textsuperscript{-1} mg\textsuperscript{-1})</th>
<th>as isolated</th>
<th>methyl viologen activation</th>
<th>base activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>as isolated</td>
<td>methyl viologen activation</td>
<td>base activation</td>
</tr>
<tr>
<td>P. stutzeri</td>
<td>aerobic</td>
<td>1.8 (30°C, pH 7.1)\textsuperscript{1,278}</td>
<td>11 (30°C, pH 10)\textsuperscript{1,278}</td>
<td>1278</td>
<td>1278</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>4.3 (30°C, pH 7.1)\textsuperscript{1,278}</td>
<td>60 (30°C, pH 10)\textsuperscript{1,278}</td>
<td>1278</td>
<td>1278</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>aerobic</td>
<td>25 (pH 9)\textsuperscript{1,279}</td>
<td>63 (pH 9)\textsuperscript{1,279}</td>
<td>1279</td>
<td>1279</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>anaerobic</td>
<td>122 (30°C, pH 7.1)\textsuperscript{1,280}</td>
<td></td>
<td></td>
<td>1280</td>
</tr>
<tr>
<td>W. succinogenes</td>
<td>aerobic</td>
<td>160 (25°C)\textsuperscript{1,281}</td>
<td></td>
<td></td>
<td>1281</td>
</tr>
<tr>
<td>A. cycloclastes</td>
<td>aerobic</td>
<td>86.4 (20°C, pH 7.1)\textsuperscript{1,282}</td>
<td></td>
<td></td>
<td>1282</td>
</tr>
<tr>
<td></td>
<td>aerobic</td>
<td>13 (25°C, pH 8)\textsuperscript{1,291}</td>
<td></td>
<td></td>
<td>1291</td>
</tr>
<tr>
<td>A. cycloclastes (recombinant)</td>
<td></td>
<td>7 (25°C, pH 8)\textsuperscript{1,291}</td>
<td>124 (25°C, pH 8)\textsuperscript{1,291}</td>
<td>1283</td>
<td>1283</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>anaerobic</td>
<td>0.5\textsuperscript{1,280}</td>
<td>27 (pH 10)\textsuperscript{1,283}</td>
<td>1283</td>
<td>1283</td>
</tr>
<tr>
<td>P. pantotrophus</td>
<td>anaerobic</td>
<td>8\textsuperscript{1,280}</td>
<td>16 (pH 9)\textsuperscript{1,284}</td>
<td>3.7 (pH 9)\textsuperscript{1,284}</td>
<td>1284</td>
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<tr>
<td>A. xylosoxidans</td>
<td>anaerobic</td>
<td>3\textsuperscript{1,280}</td>
<td></td>
<td></td>
<td>1286</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>6 (25°C, pH 7.1)\textsuperscript{1,286}</td>
<td>8 (pH 10)\textsuperscript{1,286}</td>
<td>1286</td>
<td>1286</td>
</tr>
<tr>
<td>P. nautica</td>
<td>aerobic</td>
<td>55 (25°C, pH 7.1)\textsuperscript{1,288}</td>
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<td></td>
<td>1288</td>
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<tr>
<td></td>
<td></td>
<td>157 (pH 7.6)\textsuperscript{1,309}</td>
<td></td>
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<td>1309</td>
</tr>
</tbody>
</table>
Table 44

Bond lengths and angles for Cu_{Z}^{*} for aerobically crystallized PdN_{2}OR\textsuperscript{1327} and Cu_{Z} from anaerobically crystallized PsN_{2}OR\textsuperscript{1296}

<table>
<thead>
<tr>
<th></th>
<th>PdN_{2}OR\textsuperscript{1327}</th>
<th>PsN_{2}OR\textsuperscript{1296}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>1.6 Å</td>
<td>1.7 Å</td>
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<td>Bond Lengths (Å)</td>
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<tr>
<td>Cu_{I}-Cu_{IV}</td>
<td>3.40</td>
<td>3.6</td>
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<tr>
<td>Cu_{I}-Cu_{III}</td>
<td>3.39</td>
<td>3.0</td>
</tr>
<tr>
<td>Cu_{II}-Cu_{IV}</td>
<td>2.58</td>
<td>2.9</td>
</tr>
<tr>
<td>Cu_{III}-Cu_{IV}</td>
<td>2.58</td>
<td>2.6</td>
</tr>
<tr>
<td>Cu_{III}-Cu_{IV}</td>
<td>3.04</td>
<td>3.4</td>
</tr>
<tr>
<td>Cu_{I}-S_{1}</td>
<td>2.30</td>
<td>2.4</td>
</tr>
<tr>
<td>Cu_{III}-S_{1}</td>
<td>2.24</td>
<td>1.9</td>
</tr>
<tr>
<td>Cu_{III}-S_{1}</td>
<td>2.28</td>
<td>2.1</td>
</tr>
<tr>
<td>Cu_{IV}-S_{1}</td>
<td>2.36</td>
<td>2.4</td>
</tr>
<tr>
<td>Cu_{I}-O</td>
<td>2.62</td>
<td>-</td>
</tr>
<tr>
<td>Cu_{IV}-O</td>
<td>2.80</td>
<td>-</td>
</tr>
<tr>
<td>Cu_{I}-S_{2}</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Cu_{IV}-S_{2}</td>
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<td>2.3</td>
</tr>
</tbody>
</table>
Table 45

Energies and assignments of the electronic transitions of 1-hole Cu$^{+}$ from simultaneous fit of its absorption, CD, and MCD spectra.\textsuperscript{1330}

<table>
<thead>
<tr>
<th>band</th>
<th>assignment</th>
<th>$\nu_{\text{max}}$ (cm$^{-1}$)</th>
<th>$\varepsilon$ (abs) (M$^{-1}$ cm$^{-1}$)</th>
<th>$\Delta\varepsilon$ (MCD) (mM$^{-1}$ cm$^{-1}$)</th>
<th>$C_{z}/D_{z}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>z$^{2}$</td>
<td>8015</td>
<td>1320</td>
<td>$-20$</td>
<td>$-0.016$</td>
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<td>IT</td>
<td>10000</td>
<td>1760</td>
<td>$-$</td>
<td>$-$</td>
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<tr>
<td>3</td>
<td>xz</td>
<td>11140</td>
<td>1100</td>
<td>$-225$</td>
<td>$-0.218$</td>
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<tr>
<td>4</td>
<td>yz</td>
<td>12900</td>
<td>1075</td>
<td>$-196$</td>
<td>$-0.194$</td>
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<tr>
<td>5</td>
<td>S p$_{z}$</td>
<td>14300</td>
<td>1455</td>
<td>$-446$</td>
<td>$-0.327$</td>
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<tr>
<td>6</td>
<td>S p$_{y}$</td>
<td>15675</td>
<td>3470</td>
<td>640</td>
<td>0.196</td>
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<tr>
<td>7</td>
<td>S p$_{y}$</td>
<td>16520</td>
<td>2135</td>
<td>182</td>
<td>0.091</td>
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<tr>
<td>8</td>
<td>xy</td>
<td>17980</td>
<td>740</td>
<td>119</td>
<td>0.170</td>
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<tr>
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<td>$\pi_{1}$</td>
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<td>725</td>
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<td>$-0.029$</td>
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<td>22270</td>
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<tr>
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<td>1590</td>
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<tr>
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<td>$\pi_{2}$</td>
<td>28055</td>
<td>3295</td>
<td>$-8$</td>
<td>$-0.002$</td>
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