PROTON TRANSLOCATION IN PROTEINS

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INTRODUCTION

The active transport of protons across the low dielectric barrier imposed by biological membranes is accomplished by a plethora of proteins that span the ca. 40 Å of the phospholipid bilayer. The free energy derived from the proton electrochemical potential established by the translocation of these protons can subsequently be used to drive vital chemical reactions of the cell, such as ATP synthesis and cell locomotion. Membrane-bound proton translocating proteins have now been found for a variety of organisms and tissues (1). The driving force for proton pumping in these proteins is supplied by numerous mechanisms, including light absorption (e.g. bacteriorhodopsin) (2a,b), ligand binding (e.g. ATPase) (3), and electrochemistry (e.g. electron transfer through cytochrome c oxidase) (4). Thus nature has devised a variety of methods for supplying the energy required for proton pumping by these proteins. Such diversity notwithstanding, the proteins most likely share some common elements of structure and mechanism that allow them to function as proton pumps. A number of theoretical mechanisms have been put forth for both general proton translocation (5–7) and for energy coupling in specific proton pumps. However, despite almost three decades of intensive research, the details of the mechanism(s) and structural requirements for proton pumping remain largely unresolved. To some extent this is the result of the paucity of structural information available for integral membrane proteins. This situation may soon improve as a result of advances in protein methodologies

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that have allowed several integral membrane proteins to be successfully crystalized (8), and the increased use of genetic engineering to obtain recombinant proton translocating proteins that will offer an opportunity to assess the importance of specific amino acids for the proton translocation process (9).

A number of reviews of protein-mediated proton translocation have appeared, mostly dealing with various of theoretical aspects of proton pumping (5–7, 10–12). However, the subject of the structural requirements for integral membrane proton translocators has not been reviewed in the recent literature. In this chapter we thus concentrate on the structural aspects of proton translocation by proteins. Rather than attempting a comprehensive review of all proton translocating proteins, we first focus on some of the more important theoretical mechanisms for protein-facilitated movement of protons across membranes and, where possible, show how well these theoretical mechanisms fit with experimental data for particular proteins: bacteriorhodopsin and F_o/F_1 ATP synthase. In the remainder of the chapter we provide a detailed account of the current state of knowledge for a particular proton-translocating protein, cytochrome c oxidase, which has been the focus of research in our laboratory for a number of years.

MECHANISMS FOR PROTON MOTION THROUGH PROTEINS

One can envisage a variety of mechanisms by which a protein could mediate transport of protons across a biological membrane. Several such mechanisms are considered in Figure 1 (10).

Figure 1A illustrates what might be referred to as the water wheel mechanism for proton pumping. Here a particular group within the protein picks up a proton while in contact with one side of the membrane. A conformational transition of the protein then ensues that moves the protonated group into contact with the other side of the membrane where the proton is released. Reversal of the protein conformational change completes the cycle and primes the protein for the next proton-pumping event. A mechanism of this type would require major rearrangements of a large portion of the protein matrix, and would thus require a significant free energy dissipation to drive the pumping cycle. Although conformational transitions clearly occur in proton-translocating proteins, we know of no experimental evidence at the moment for alternating access of portions of the protein matrix to the two sides of the membrane, as would be required for the water wheel mechanism.

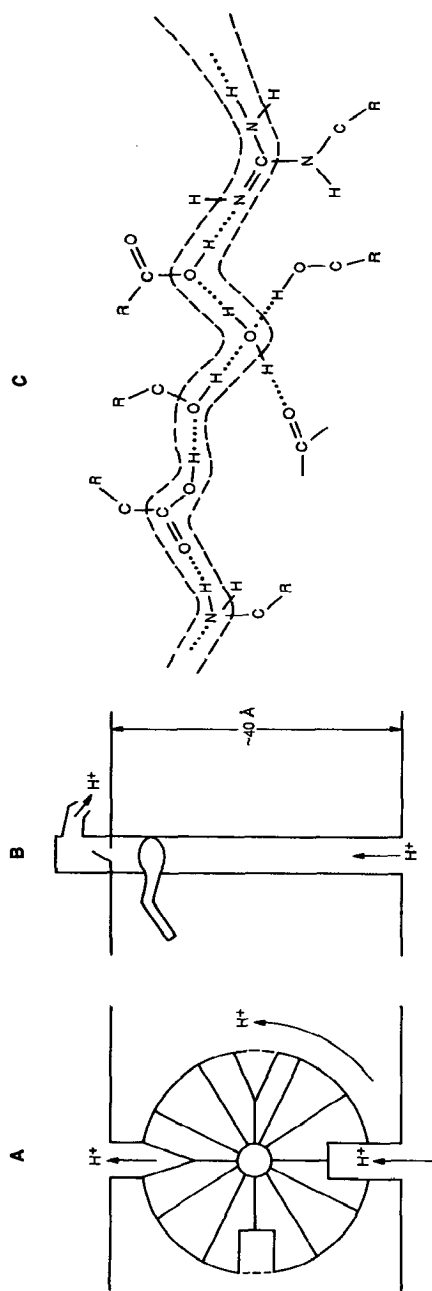Figure 1B illustrates the "gated channel" mechanism for proton pump-

Figure 1 Three possible mechanisms for transmembrane proton translocation. (A) The "water wheel" mechanism, which requires alternating access of a particular portion of the protein matrix to the two sides of the membrane. (B) The "gated channel" mechanism in which some group or groups within the protein provide a physical barrier to proton movement across the bilayer. In this mechanism the barrier is displaced in response to some conformational transition of the protein. (C) The "hydrogen-bonded chain" mechanism in which a chain of hydrogen bonds is set up among amino acid side chains that spans the membrane bilayer. This figure was adapted from Refs. (10, 11).

ing in which protons passively travel through a pore in the protein until they encounter a group or groups that block their further movement to the other side of the membrane. This proton gate acts as a turnstile, allowing the proton alternate access to the two sides of the membrane in response to some signal from the protein matrix (i.e. a conformational change). As first pointed out by Nagle & Tristam-Nagle (10), pores through integral membrane proteins cannot be simple water channels because the electric field in such a case would exceed the dielectric break-down field of most materials (ca. 10^6 V/cm). Thus, in order to avoid dielectric breakdown, one must restrict attention to *narrow* channels.

Figure 1C illustrates the hydrogen-bonded chain (HBC) mechanism for proton translocation that has been championed by Nagle and co-workers (10, 11). Here the side chains of certain amino acids are used to construct a hydrogen-bonding network that traverses the bilayer. The HBC hypoth-esis has been well received by the biophysical community and we thus describe it in some detail. Figure 2 illustrates the mechanism of proton pumping for a HBC constructed of hydroxyl side chains (10). In the resting state of the protein, the hydrogen bonds along the HBC occur in a particular configuration that minimizes their collective potential energy; this is represented in Figure 2 by having all of the protons on the left side of the hydrogen bonds (a). A conformational change then occurs that alters the relative energies of this resting state and some alternative con-figuration (c; represented by having all of the protons now on the right side of the bonds) so that the alternative configuration is now favored, and the protons along the chain move to the other side of the bonds in response to the configurational energy differential. The protein, which has up to now assumed an intermediate excited state, must ultimately relax back to its original conformation, as the input impulse is dissipated. This decay can be coupled energetically to endergonic translocation of protons across the membrane: An ion can enter the chain on the right side and initiate the tandem propagation of a charge, from left to right, along the chain. In this manner the protein reverts back to the original "resting" configuration, completing the cycle. An in-depth discussion of the HBC mechanism can be found in the recent review by Nagle & Tristam-Nagle (10).

In Figure 2 the HBC is constructed by using only hydroxyl side chains as the proton conductors. In principle, any groups capable of simultaneously serving as a proton donor and acceptor could form part of a HCB, including amide backbone protons. Nagle and co-workers however, argue that backbone protons are unlikely to participate in the HCB because of the requirement for bond "turning" during pumping (10). "Turning" of amide groups, particularly in regions of defined secondary structure, would
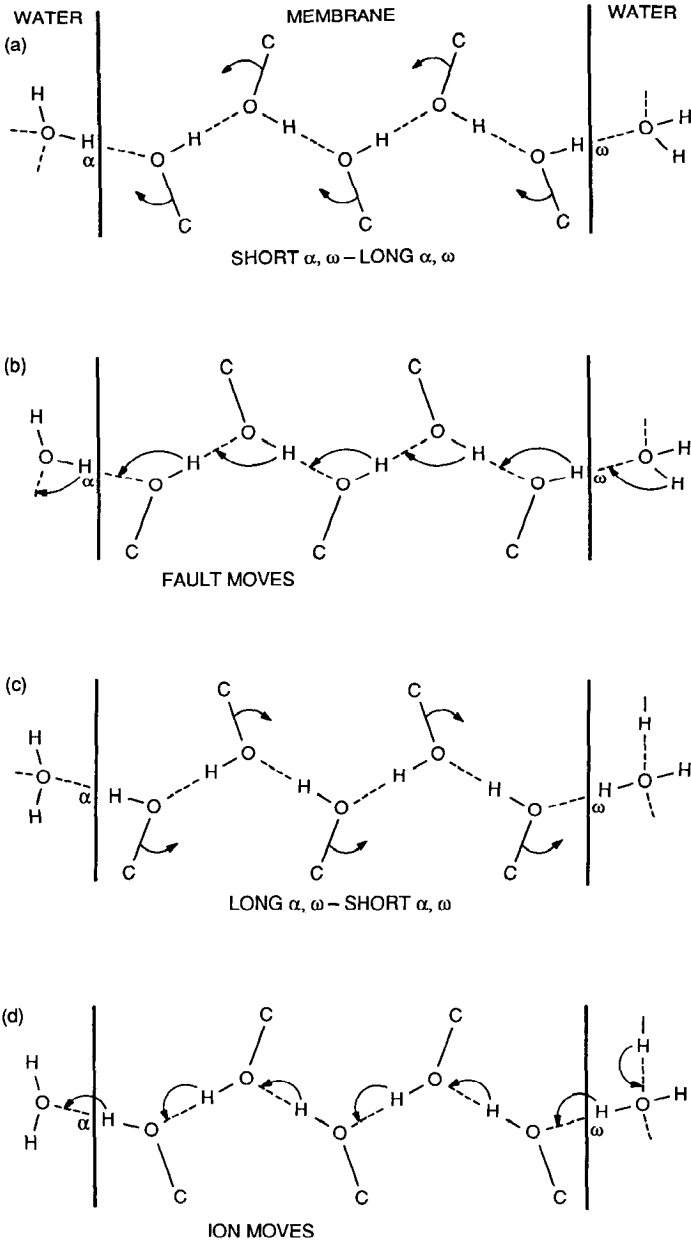
Figure 2 Mechanism for ion movement across the membrane bilayer facilitated by a hydrogen-bonded chain of amino acids. Adapted from Ref. (10).

involve a prohibitively large activation energy. Thus HCB are most likely restricted to the side chains of certain amino acids. The amino acid side chains capable of HBC involvement are illustrated in Figure 3. Assuming an average length of 2.5–3.5 Å for each hydrogen bond, and making allowance that not all hydrogen bonds will be parallel to the membrane normal, Nagle & Morowitz conclude that a HBC would require 20 amino acid residues to traverse the 40 Å distance of a typical biological membrane (11). Such a HBC should be quite stable: Nagle & Morowitz estimate an enthalpy of formation of ca. 120 kcal/mol.

One attractive aspect of the HBC hypothesis is that it is amenable to testing. The hypothesis provides a rationale for locating the pumping machinery of a proton-translocating protein from a knowledge of the protein's primary and secondary structure. We now briefly review how well the HBC hypothesis is standing up against the structural data available for two proton-translocating proteins, namely bacteriorhodopsin (BR) and F_o/F_1 ATP synthase.

BACTERIORHODOPSIN

Bacteriorhodopsin is a proton-translocating protein for which a great deal of structural information is available, and thus it provides a good test of the HBC hypothesis (2a). BR is the major protein component of the purple membranes of *Halobacterium halobium*. The protein consists of a single polypeptide chain of 248 amino acids whose complete primary structure has been determined (13). The protein contains a single cofactor, all-*trans* retinal covalently linked to the protein via a Schiff base to lys 216, which serves as a photon sink to provide the driving force for proton pumping (2a). An electron diffraction map has been obtained to 7 Å resolution for two-dimensional ordered arrays of BR within purple membranes. This map suggests seven transmembrane helical segments (14), whose location along the protein's primary sequence has been inferred from hydropathy analysis and chemical modification experiments (2a). Figure 4 summarizes the most widely accepted structural model for BR. HBC-forming amino acid residues located within the seven transmembrane helices of BR are circled in Figure 4. There is clearly an ample number of these residues for the formation of a membrane-spanning HBC, particularly when one recalls that a single HBC may be composed of residues from more than one transmembrane helix in the folded protein. Spectroscopic data have suggested that during the proton pumping photocycle of BR, protonation/deprotonation events occur at several potential HBC residues, tyrosine and aspartate (15); environmental change also occurs about tryptophan residues (16). Most recently, Khorana and co-workers have expressed the
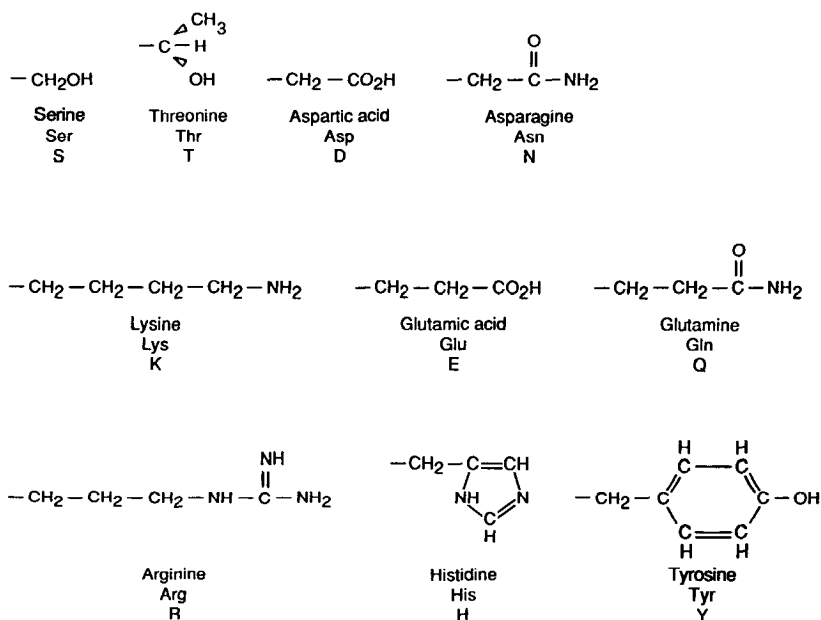
Figure 3   The side-chain structures for hydrogen-bond-chain-forming amino acids. Adapted from Ref. (76).

gene for BR in *E. coli* and have begun systematic site-directed mutagenesis studies to identify the key amino acid residues required for proton pumping. Among the mutants studied so far, several involve residues at potential HBC sites, within the transmembrane helices: Tyr 185 → Phe; Asp 85 → Asn; Asp 96 → Asn; and Asp 212 → Glu, Asn, or Ala. These residues are highlighted in Figure 4 by *darker circles* (2a,b). As expected, the substitutions made here lead to dramatic effects on the proton pumping efficiency of the mutant BRs. In particular, mutants affecting Asp 85 or 96 completely abolish proton pumping activity!

ATP SYNTHASE

The ATP synthases of mitochondria, chloroplasts, and bacteria represent a second family of proton-translocating proteins for which significant structural information is available (17). These proteins are multi-subunit complexes that share the common structural motif illustrated in Figure 5. The proteins can be divided into two functional domains: a transmembrane proton-conducting domain, F_o, and an extramembrane nucleotide-binding domain, F_1. In the intact complex proton translocation by the F_o domain
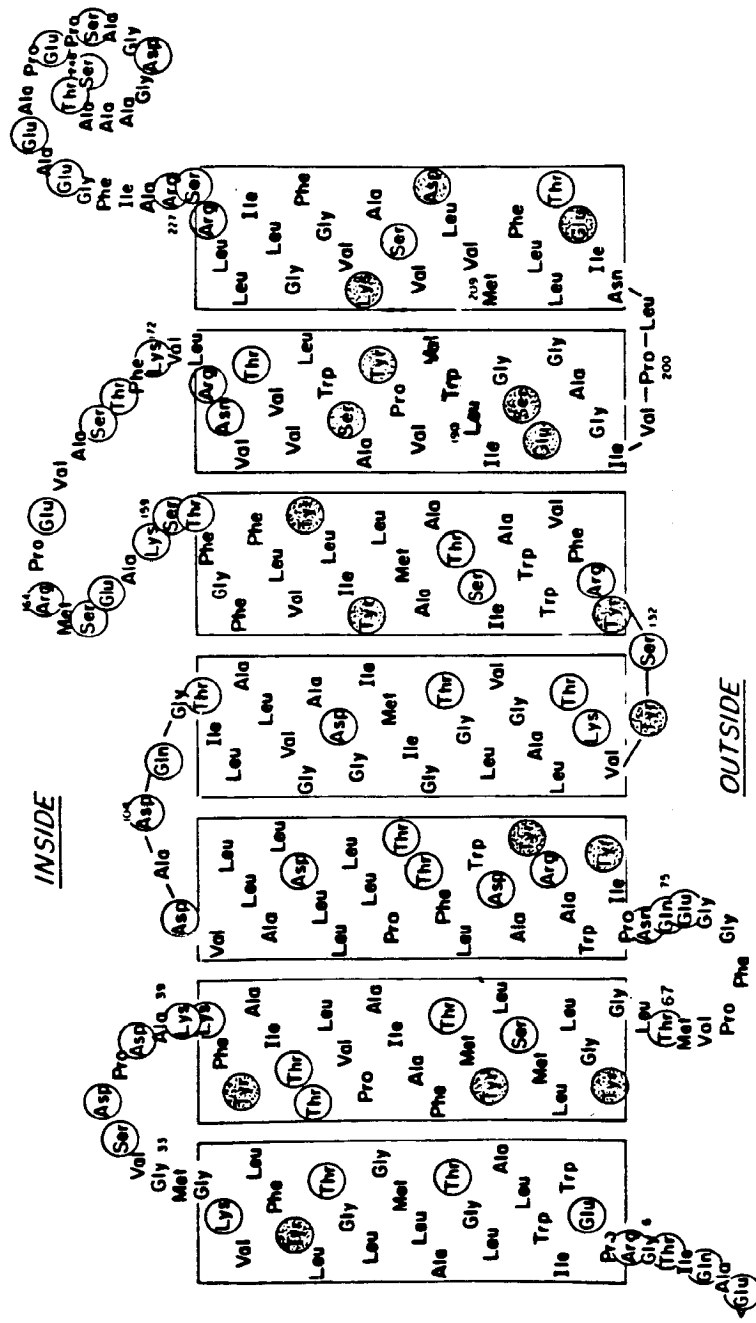
Figure 4   Structural model of bacteriorhodopsin showing the location of HCB forming amino acids (*circled*). HBC-forming amino acid residues that have been altered via site directed mutagenesis are highlighted by *shaded circles*. Adapted from Ref. (2a).
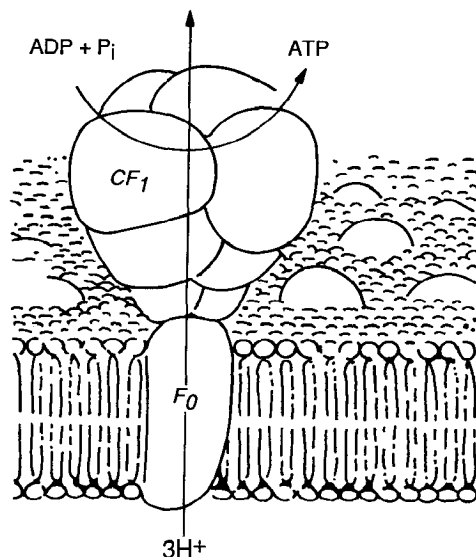
Figure 5   Structural model for the F_o/F_1 ATP synthase within a membrane bilayer.

is ATP-dependent, suggesting some type of allosteric communication between the F_o and F_1 domains (17). The F_o domain can be isolated free of the F_1 domain and reconstituted into artificial phospholipid vesicles. Under these conditions, the isolated F_o domain can also translocate protons in response to an ionophore-induced potassium diffusion potential.

The proton-translocating F_o domain consists of multiple copies of three subunits designated a, b, and c. Complete amino acid sequences have been reported for the a, b, and c, subunits of the F_o domain of the *E. coli* protein (18). Recently, isolated subunit c from the F_o domain of lettuce chloroplasts and yeast mitochondria has been reconstituted into phospholipid vesicles and shown to conduct protons. Thus there is good reason to suspect that subunit c constitutes at least part of the proton-translocating apparatus of the F_o domain (18). Unfortunately, there are only six conserved HBC-forming amino acid residues in subunit c. Sebald & Wachter accordingly have argued against the HBC hypothesis in the case of the ATP synthase (19). However, as first noted by Nagle & Tristam-Nagle, one must bear in mind that multiple copies of subunit c occur in the F_o domain and together these would provide more than the requisite 20 HBC-forming residues (10). Recently, Cox et al reported that subunit a from a variety of sources also contains a membrane-spanning amphipathic helix with a number of conserved polar residues (20). These workers suggest that these polar residues on subunit a combine with Asp 61 of

subunit c to form a transmembrane proton-conducting network, as illustrated in Figure 6. This model is consistent with predictions of the secondary and tertiary structure for the F_o domain of ATP synthase.

It should be clear from the above discussion of the structural data for bacteriorhodopsin and ATP synthase that no direct experimental support for the HBC-hypothesis has yet been reported. The best one can say at this point is that the available structural information is not inconsistent with this hypothesis.

Protons are not unique in their role as the coupling ions in energy transducing systems. Recently, Skulachev reviewed the evidence for sodium
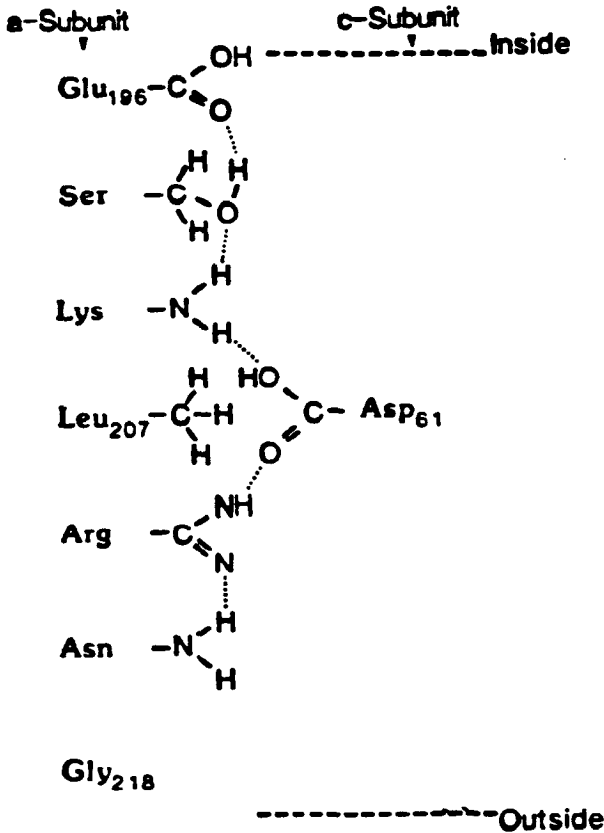
Figure 6   Model of intersubunit HBC formed in the F_o domain of the F_o/F_1 ATP synthase. Based on the model of Cox et al (20).

ion-based bioenergetics (21). Onsager originally proposed a form of the HBC-hypothesis to account for sodium and potassium ion transport in nerve axons, but quickly realized that such ions could not be efficiently transported in this fashion (22). Thus if one assumes that similar structural elements are needed for proton and sodium ion translocation, an alternative to the HBC hypothesis is required. That we consider mechanisms suitable for either proton or sodium ion translocation is imperative in light of the fact that several systems are now known to use either protons or Na^+ translocation for energy coupling. These observations have led Boyer to propose recently that the hydronium ion (H_3O^+) is the actual translocated species in what have traditionally been considered as *proton*-translocating proteins (23). No specific molecular mechanisms have been offered for how hydronium ion transport might occur; however, the concept of protein-mediated hydronium ion transport certainly deserves greater attention.

CYTOCHROME c OXIDASE

The remainder of this chapter is devoted to an in-depth review of a third proton-translocating protein, cytochrome c oxidase. The choice to highlight this particular protein is in part due to our personal biases, since a significant amount of our research efforts have been focused on this enzyme. Recently we have begun to investigate the structural elements required for proton pumping in cytochrome c oxidase. An excellent review of the relationship between structure and function in cytochrome c oxidase has recently been provided by Wikström et al (24).

Cytochrome c oxidase is the terminal enzyme in the respiratory electron transport chain of mitochondria and many aerobic prokaryotes (4). In mammals the enzyme is composed of 12–15 subunits that assemble to form a Y-shaped complex spanning the inner mitochondrial membrane, as illustrated in Figure 7. The enzyme accepts four electrons from ferrocytochrome c and transfers them, via its four redox active metal centers, to molecular oxygen. During the course of dioxygen reduction, four protons are consumed from the mitochondrial matrix to form two molecules of water. This scalar proton consumption results in an electrochemical gradient across the inner mitochondrial membrane. Additionally, the enzyme can actively pump up to one proton for each electron that traverses the membrane from ferrocytochrome c to molecular oxygen. This proton pumping is intimately coupled to the electron transfer activity of the oxidase, and the enzyme is thus referred to as a *redox-linked proton pump* (24).
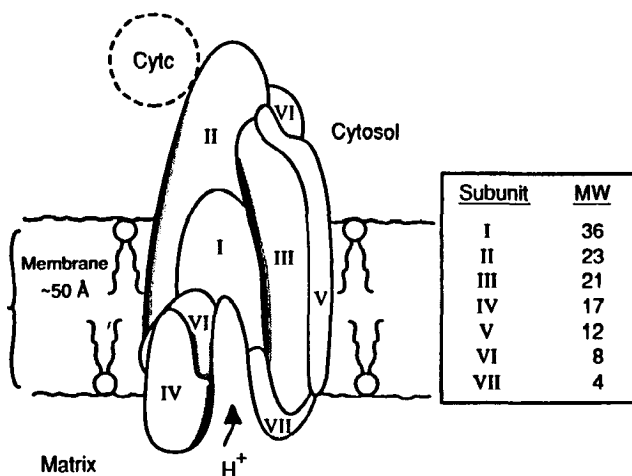
Figure 7   Structural model of mammalian cytochrome c oxidase. Adapted from Ref. (4).

Structure of the Redox-Active Metal Centers

Cytochrome c oxidase is a metallo-enzyme that contains two iron, three copper, one zinc, and one magnesium ion. The zinc, magnesium, and one of the copper ions (Cu_X) do not appear to participate in the electron transfer mechanism of the enzyme, and it is doubtful that they play any direct role in the redox-linked proton translocation. The four remaining metal centers consist of two iron-containing heme A chromophores (referred to as heme a and heme a_3) and two copper ions (referred to as Cu_A and Cu_B). Two of these metal centers, heme a and Cu_A, serve as the initial electron acceptors from ferrocytochrome c. The other two metals, heme a_3 and Cu_B, form a binuclear site for dioxygen binding and reduction. The chemistry of dioxygen reduction by cytochrome c oxidase has been worked out in some detail, and offers a fascinating view of the complexity of enzyme catalysis. This aspect of cytochrome c oxidase's enzymatic activity has been reviewed several times in recent years, and the reader is referred to these excellent accounts for further details (25–27). It is now widely accepted that one or both of the low-potential metal centers provide the link between redox activity and proton pumping. The most compelling evidence for this has come from the recent work of Wikström & Casey on proton pumping in inhibited submitochondrial particles (28).

Heme a is a six-coordinate, low-spin heme in both its ferric and ferrous states. Comparative ENDOR measurements on the yeast oxidase and the enzyme in which all the imidazole side chains of the histidine residues have been replaced by ^{15}N-substituted imidazole have provided unequivocal

evidence for bis-imidazole coordination in this heme center (29), at least in the ferric state. Comparison of optical and resonance Raman spectra of heme a with those of heme A model compounds has also led Babcock and co-workers to suggest that heme a is bis-imidazole coordinated in both the ferric and ferrous oxidation states of the iron (30). Studies of fluorescence energy transfer from bound Zn-substituted cytochrome c suggest that heme a is within 25 Å of the heme of cytochrome c (31). The standard entropy of reduction for heme a is quite large, -50.8 eu, and suggests significant structural alterations about this metal or in the protein upon reduction (32). Since no ligation changes occur upon reduction, one possibility is that these structural changes involve relative motions of the entire heme group, or its peripheral substituents, with respect to the surrounding polypeptide. Evidence for structural perturbations of heme a is provided by optical spectroscopy. Both hemes contribute to the enzyme's characteristic absorption spectrum in the 350–700 nm region. The allowed (Soret) π–π^* transition of heme a is red shifted relative to that of heme a_3 and heme A model compounds. Babcock's group has suggested that this red shift is the result of hydrogen bonding between the formyl oxygen of heme a and a protonated amino acid side chain, most probably tyrosine, from the protein matrix (33a,b). Using resonance Raman spectroscopy, this group has shown that formyl-stretching frequency for heme a is significantly downshifted relative to that of heme a_3, as expected if the former were involved in a strong hydrogen bond as a hydrogen acceptor. Babcock & Callahan (33a,b) have estimated the strength of this hydrogen-bonding for ferric and ferrous heme a as 3.0 and 5.3 kcal/mol, respectively. This change in the formyl group's hydrogen-bond strength upon reduction of heme a has formed the basis of a mechanism for redox-linked proton translocation by cytochrome c oxidase, based on heme a, as discussed below. The suggestion that the heme a formyl moiety is involved in hydrogen-bonding to an amino acid side chain is supported by the observation of Copeland & Spiro (34) that the frequency of the Raman band assigned to the heme a formyl stretch is shifted when the enzyme is incubated in ^2H_2O buffer. Copeland & Spiro reported that the rate of hydrogen/deuterium exchange at the heme a formyl group's proton donor was unaffected by enzyme turnover (34); however, Babcock's group has recently shown that this result is an artifact of the reductant used by Copeland & Spiro, and that in fact the rate of exchange is accelerated by turnover (G. T. Babcock et al, personal communication).

Thus the heme a binding pocket appears to be at least transiently in contact with the aqueous phase. Artzatbanov et al have shown that the midpoint reduction potential of heme a displays a ca. 30 mv/pH unit dependence, which is specifically associated with the matrix pH (35). Heme

a can also be perturbed by addition of Ca^{2+} or protons (36). The heme *a* group also appears to undergo some structural perturbation upon membrane energization. Wikström and co-workers have shown that in reduced, well-coupled submitochondrial particles, addition of ATP results in a red shift of the enzyme's heme absorption bands (37). Based on studies of isolated cytochrome *c* oxidase in detergent solution and on heme A model compounds, Wikström and co-workers concluded that the energy-linked absorption change of heme *a* is due to ATP-induced protonation of the heme *a* propionate group (38).

Cytochrome *c* oxidase forms a tight 1:1 complex with its physiological electron transfer partner, cytochrome *c*, in low ionic strength solution. Recently Weber et al studied this 1:1 complex by circular dichroism (CD) and magnetic circular dichroism (MCD) in both the fully oxidized and fully reduced forms of the complex (39). Significant spectroscopic changes in the *c* heme were observed upon complexation of either the oxidized or reduced proteins, and an additional change was seen in the oxidized complex for heme *a*. The origin of the spectroscopic change in heme *a* could not be elucidated from the data of Weber et al; it could be due to an electronic rearrangement at the heme or a structural change in relative heme orientation with respect to the surrounding polypeptide. Whatever the nature of this heme *a* perturbation, it seems to be unique to cytochrome *c* binding as it could not be mimicked by molecules known to inhibit binding of cytochrome *c* to the oxidase: apocytochrome *c*, porphyrin cytochrome *c*, or spermine (39). Thus although heme *a* does not undergo any redox-induced ligation changes, this chromophore does seem to show some structural flexibility, with respect to the surrounding polypeptide, under a variety of conditions relevant to the enzyme's catalytic activities.

Cu_A is structurally and spectroscopically unique among biological copper centers (40). The weak ($\varepsilon = 2$ mM^{-1} cm^{-1}) near IR band seen at ca. 830 nm in the oxidized enzyme's spectrum has been suggested to arise exclusively from Cu_A, and has been assigned to a ligand-to-metal charge transfer transition involving Cu_A and a sulfur ligand (41). This band disappears in the fully reduced and CN-bound, mixed-valence enzyme, consistent with the above assignment. MCD and optically detected magnetic resonance spectroscopy reveal additional absorption bands of Cu_A at 455, 470, 520, 560, 580, and 790 nm. All of these bands are *x, y* polarized, suggesting a high degree of axial symmetry for the metal center, and are assigned as sulfur (cysteine) to copper charge transfer transitions (42). ENDOR studies of isotopically enriched yeast cytochrome *c* oxidase have provided definitive evidence that Cu_A is ligated by at least one cysteine sulfur and one histidine nitrogen in its oxidized state (43a,b).

The EPR spectrum of Cu_A is also atypical for biological copper ions in

that it shows virtually no hyperfine coupling, and its g-values (1.99, 2.03, and 2.18) are unusually small. These unusual EPR characteristics have been interpreted as arising from a tetragonally distorted ligand geometry about the Cu_A center (44). Chan and co-workers have further suggested that the Cu_A EPR spectrum indicates significant delocalization of a sulfur σ-electron onto the copper ion so that this center might more correctly be viewed as a Cu(I)-sulfur radical complex rather than a simple Cu(II) center (44).

As with heme a, reduction of Cu_A is associated with a large negative entropy change ($\Delta S^{o'} = -49.7$ eu), suggesting a conformational change associated with the redox activity at this metal center (45). Recently, preliminary evidence for such a conformational transition has been provided by this laboratory from Cu EXAFS studies of native and Cu_A-depleted cytochrome c oxidase in both the fully oxidized and fully reduced states. These data have suggested a bis-dithiolate coordination for the Cu_A site, and that one of the Cu–S bonds becomes elongated when the site is reduced (P. M. Li, personal communication).

Structural Aspects of the Protein

As mentioned above, mammalian cytochrome c oxidase is composed of 12–15 subunits, whereas the enzyme from prokaryotic sources usually contains far fewer subunits. It is now generally agreed that the catalytic core of the enzyme, in terms of both its electron transfer and proton pumping activity, is made up of two or three subunits (subunits I, II, and III); the other subunits in the enzyme from higher organisms most likely play some type of regulatory function. All of the redox-active metal ions are located in subunits I and II. Based on the spectroscopic evidence (vide supra), the Cu_A binding site requires two cysteine residues and at least one histidine. Comparison of the amino acid sequences for subunits I–III of various organisms suggests that the Cu_A binding site is located within subunit II, since this is the only subunit with two highly conserved cysteine residues (46a,b). Supporting evidence has come from recent chemical modification labeling experiments (47). Photoaffinity cross-linking studies have shown that subunit II also contains the high-affinity binding site for cytochrome c (48). The binding of cytochrome c to the enzyme is electrostatic and involves a cluster of lysine residues on one surface of cytochrome c and carboxylate residues on the oxidase. Two highly conserved carboxylate residues within subunit II have been implicated in the electrostatic interaction of the enzyme with cytochrome c, and these are located close to the suggested Cu_A binding domain (49). Since the cytochrome c binding site must be in contact with the cytosolic aqueous phase, these results indicate that Cu_A must also be close to the cytosol. Only two

histidine residues are highly conserved within subunit II, and both of these are within the proposed Cu_A binding site. Since both heme A chromophores require histidine residues as axial ligands, it is unlikely that either heme is located within subunit II. Thus both hemes are suggested to be located within subunit I, which does contain enough conserved histidines to accommodate both heme chromophores. Since Cu_B is known to be within 5 Å of heme a_3, it is believed that this metal center is also contained within subunit I (46a).

Subunits I and II are clearly necessary for the proper functioning of cytochrome c oxidase. Previously, it was believed that subunit III was also required for the proton-pumping activity of the enzyme. This inference was based on the observation that the proton pumping activity could be impaired by binding DCCD to a specific glutamate residue within subunit III (50). However, it has more recently been demonstrated that subunit III-depleted bovine cytochrome c oxidase retains its proton pumping activity when reconstituted into phospholipid vesicles, albeit with a reduced proton/electron stoichiometry (51). Likewise the two-subunit enzyme isolated from *Paracoccus denitrificans* pumps protons in reconstituted vesicles (52). Thus it now seems that subunit III plays an ancillary role, if any, in proton pumping; the necessary molecular machinery for proton translocation must therefore reside within subunits I and II.

The method of Kyte & Doolittle has been used to locate transmembrane segments within the primary sequences of subunits I and II; Figure 8 depicts the proposed structures for these two subunits that result from such analysis (46a). Only strictly conserved residues are shown in this figure. The proposed metal-ligating residues are highlighted, and those residues capable of participating in HBC formation are circled. In Figure 8 a large portion of the subunit I polypeptide is buried within the lipid bilayer, in agreement with the experimental observation that this subunit does not react with water-soluble chemical or immunological labels presented to either the matrix (inner) or cytosolic (outer) surfaces (4). There are 12 transmembrane helices within subunit I, ten of which contain HBC-forming amino acids. Segments VI and X are particularly rich in HBC-competent residues and also invariant histidines that could serve as the axial ligands to heme a. Wikström and co-workers have proposed a model for the heme a binding site that uses one histidine from segment VI and one from segment X as the axial ligands to the iron, and an invariant tyrosine residue within segment X as the hydrogen-bond partner of the heme a formyl oxygen (46a). This model provides a structural basis for a heme a based mechanism of redox-linked proton translocation that utilizes intersubunit hydrogen-bonds to make up a HBC network that includes the tyrosine-heme a formyl hydrogen bond. The idea that the heme a
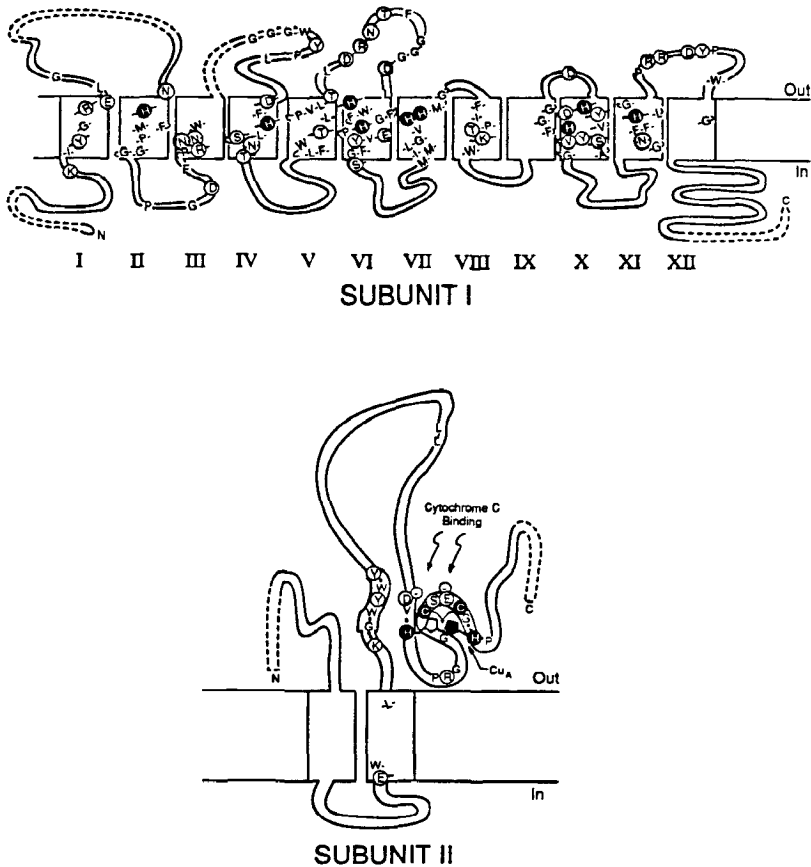
SUBUNIT I

SUBUNIT II

Figure 8   Structural models for subunits I and II of cytochrome *c* oxidase highlighting the locations of conserved HBC-forming amino acid residues (*open circles*) and the proposed metal-ligating amino acid residues (*closed circles*). Adapted from Ref. (46a).

formyl hydrogen bond might be involved in proton translocation was originally proposed by Babcock & Callahan (33a,b); the details of such a mechanism are discussed in the next section.

Subunit II is thought to contain two transmembrane helices. The majority of the polypeptide of this subunit is exposed to the cytosol, again in agreement with both chemical and immunological labeling studies (4). Only one HBC-forming residue, a glutamate at the matrix terminus of the second transmembrane helix, is strictly conserved in this subunit. However, the oxidase from every species thus far sequenced shows between four

and six HBC-forming residues within the second transmembrane helix of subunit II. Even accounting for the nonconserved HBC-forming residues, a membrane-spanning HBC network cannot be constructed from subunit II alone. This does not, by any means, exclude a role for subunit II in proton translocation. Intersubunit hydrogen bonding between subunits I and II could be involved in proton pumping. Since the functional unit of cytochrome c oxidase appears to be a dimer (4), one also cannot exclude a HBC involving subunit II–subunit II intersubunit hydrogen bonds.

Mechanisms of Redox-Linked Proton Translocation

A redox-linked proton pump has specific requirements beyond those so far discussed for a generalized proton pump. To illustrate this, we consider simple models in which oxido-reduction of a single metal center (either heme a or Cu_A) provides the energetic link between electron transfer and proton translocation. In these models, a proton is taken up or ejected as the metal ion alternates between two valence states, oxidized and reduced. Clearly one also needs to specify the protonation state of an acidic group linked to the proton pumping redox center (4, 53). In addition, two protein conformations must exist for each of the valence states; one providing access for the proton pumped to the cytosol (proton output), and one providing access of the proton from the matrix side of the membrane (proton input). Thus one requires a total of eight states to describe fully the proton pumping cycle. These eight states are represented in the now familiar cubic scheme of Wikström et al (4) in Figure 9.
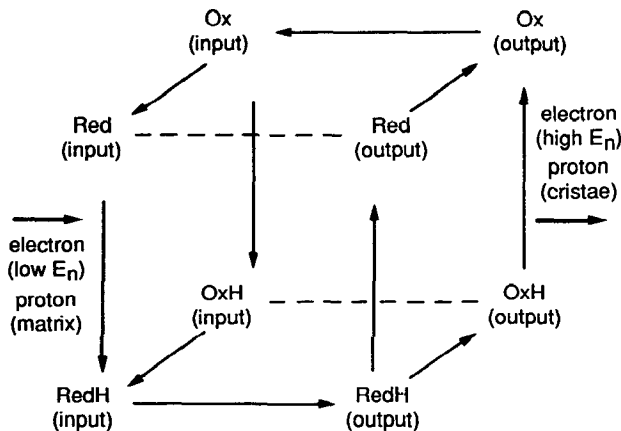

Figure 9   The eight-state "cubic" model for redox-linked proton translocation by cytochrome c oxidase as first proposed by Wikström et al (4).

Any of the vectorial transitions from the top face of the cube in Figure 9 to the bottom face must proceed via protonation of the linked acidic group. It should be emphasized that this acidic group need not be in close proximity to the redox center. If the acidic group were in direct spatial contact with the metal center, this would correspond to the most direct form of coupling that one can imagine. On the other hand, the acidic group could be far removed from the metal center. The coupling between redox activity and protonation in this extreme would be indirect and mediated by conformational transition of the protein. The simplicity of the direct coupling mechanism is quite appealing, but we must point out that increasing evidence favors the indirect mechanism for two other proton pumps, ATP synthase (3) and bacteriorhodopsin (54). As far as cytochrome c oxidase is concerned, no compelling evidence favours either coupling mechanism at this juncture (but see the next section on redox-linked conformational transitions). At first glance one might expect the model in Figure 9 to impose a strong pH dependence on the redox potential of the pump site. In fact, it has been argued that such a requirement favors heme a as the site of redox linkage over Cu_A, since the former metal center shows a pH-dependent midpoint potential. Blair et al have specifically addressed this issue and have shown on theoretical grounds that a pH-mediated midpoint potential is not obligatory for a redox center linked to proton translocation (55). The recent spectroelectrochemical studies of Ellis et al (32) and Blair et al (56) have shown that neither heme a nor Cu_A exhibits a strong pH dependence of their redox potentials. Thus, at present, no compelling theoretical or experimental data exist to distinguish between heme a and Cu_A as the more likely site of linkage on these grounds. Despite the paucity of data, however, two structurally detailed models for redox-linked proton translocation in cytochrome c oxidase have emerged, one involving heme a (33a,b), and the other involving Cu_A (57).

The heme a-based model was originally proposed by Babcock & Callahan, and is based on the observation that the strength of hydrogen bonding between the formyl oxygen of heme a and some proton donor(s) in the protein varies between the oxidized and reduced states of the heme iron (33a,b). Measurement of the heme a formyl's $C{=}O$ stretching frequency from resonance Raman spectroscopy suggests that the hydrogen-bond strength differs by ca. 110 mV (2.5 kcal/mol) between the ferric and ferrous states of heme a. In the model of Babcock & Callahan this energy difference is used to provide part of the driving force for proton translocation against the ca. 200 mV electrochemical gradient of the inner mitochondrial membrane. Figure 10 outlines the proton pumping mechanism proposed by Babcock & Callahan based on these ideas (33a,b). In the stable oxidized state, the formyl oxygen is hydrogen-bonded to a proton donor group
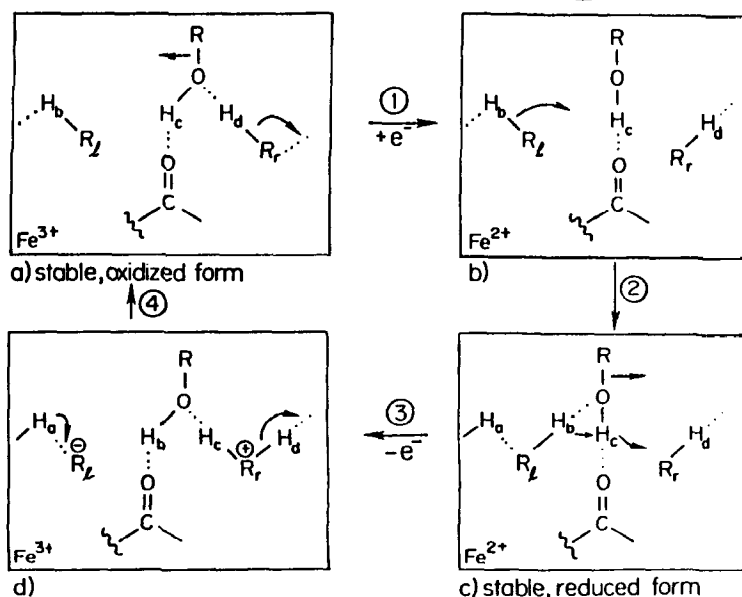
A Proton Pump Mechanism for Cytochrome $\underline{a}$

Figure 10   A heme a-based mechanism for redox-linked proton translocation by cytochrome c oxidase, as first proposed by Babcock & Callahan (33a,b).

(ROH) that is intermediate between two HBCs, one connected to the matrix side of the membrane and the other to the cytosol. Upon reduction of the heme iron, the hydrogen-bond strength increases between the now electron-rich formyl oxygen and the proton of the donor group (H_c). Babcock & Callahan proposed that this change in hydrogen-bond strength causes a geometry change in the donor group, allowing it to interact with the hydrogen of another acidic residue in close juxtaposition at the end of the matrix side HBC (H_b). As the cycle continues, the hydrogen-bond strength between the conjugate base RO^- and H_b increases at the expense of the formyl-H_c bond. Eventually H_c is replaced by H_b as the proton hydrogen-bonded to the heme a formyl, as H_c is transferred to the cytosolic hydrogen-bonded chain. To complete the cycle, there must be tandem proton migration within the HBC on the matrix side to replenish the proton hole originally occupied by H_b, followed by subsequent uptake of a proton at the opening of the channel from the matrix aqueous phase. This scheme offers a simple mechanism for providing alternating access of the pump site to the two sides of the membrane. That is, the heme a formyl hydrogen bond acts as a redox-linked proton gate. Unfortunately, the

scheme makes no provisions for the gating of electron flow to obviate futile cycles. A number of treatments of the enzyme have also been recently reported that disrupt the proton-pumping activity of the enzyme without perturbation of the heme *a* environment. In this connection, Babcock & Callahan have offered a modified version of their model in which the proton pumping machinery is not in close proximity to heme *a*. Here the change in hydrogen-bond strength between the formyl and the proton donor upon reduction of heme *a* is proposed to result in a global conformational change in the protein that is transmitted to the proton-translocating element of the enzyme.

An alternative model for proton pumping in cytochrome *c* oxidase has been put forth by Gelles et al (57) based on Cu_A as the site of redox coupling. As in the model of Babcock & Callahan (33a,b), the site is hypothesized to be an electron-driven proton gate. In contrast to Babcock & Callahan (33a,b), however, Gelles et al (57) allowed for the gating of electron flow. Because of electron leaks, Gelles et al (57) argued that electron gating should be an essential element of any model of a redox-linked proton pump. The protein must be able to tune the rate constants of the relevant electron transfer processes to enhance the coupled process and suppress the uncoupled pathway. Gelles et al (57) proposed conformational switching as a means of achieving this. In their proposal, the electron enters the Cu_A site in one conformation of the enzyme, and is transferred out of the site in a different conformation during the coupled reaction. When the electron transfer is not coupled to proton pumping, the electrons can leak from the Cu_A center to the dioxygen reduction site in the same protein conformation. Clearly, to obviate the futile cycle, the conformational switching must be kinetically more facile than the electron leak. Gelles et al (57) pointed out that the electron gating itself need not involve a global conformational change. A local structural change at the site of redox-linkage might suffice so long as the necessary gating ratios are achieved. On the other hand, more global structural changes probably accompany the proton translocation steps of the proton-pumping cycle (the gating of proton flow).

The details of the Gelles et al model are outlined in Figure 11 (57). Two proton conducting channels are implicated, one leading to the cytosol from the Cu_A site and the other in communication with the matrix. In the oxidized state of Cu_A, the copper ion is ligated by two histidine nitrogens and two cysteine sulfurs arranged in a distorted tetrahedral geometry. This is the electron input state. Upon electron reduction, the bis-dithiolate cysteine coordination is expected to become asymmetric; i.e. one of the Cu–S bonds becomes more elongated relative to the other (43b). Gelles et al (57) proposed that a tyrosine (or a residue with a similar pK_a) is in close
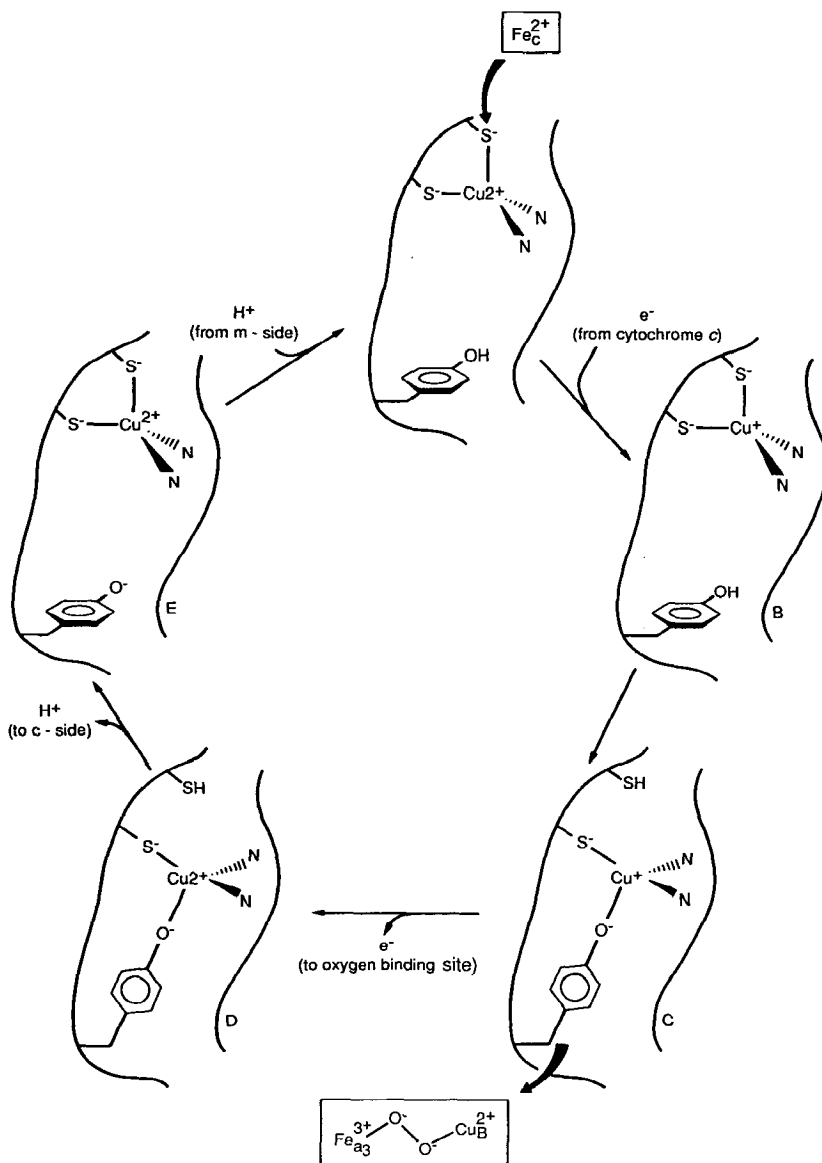
Figure 11 A Cu_A-based mechanism for redox-linked proton translocation by cytochrome *c* oxidase, as first proposed by Gelles et al (57).

proximity to the copper ion in the matrix HBC, and it can interact with the copper ion when it becomes reduced. If this interaction becomes sufficiently strong, the tyrosine oxygen can displace the cysteine sulfur in the already elongated Cu–S bond away from the copper ion toward the cytosolic HBC. The change in pK_as of the incoming tyrosine oxygen and outgoing cysteine sulfur accompanying this ligand rearrangement can lead to ionization of the tyrosine and protonation of the displaced cysteine. In this manner, part of the redox energy of the Cu_A site is expended in transferring the proton from the matrix HBC to the cystosolic side of the membrane, i.e. in gating the proton flow. The Cu_A site is now in the electron output state and poised for facile electron transfer to the dioxygen reduction site. To restore the original ligand arrangement following reoxidation of the copper, the displaced cysteine must become coordinated to the copper ion and give up its proton to the cystosol, and the ionized tyrosine must be re-protonated from the matrix HBC. Protein conformational changes must play a role here, to ensure tandem proton migrations within the matrix HBC to neutralize the tyrosinate anion, as does the positive charge at the Cu_A site, which provides a barrier for proton slippage from the cytosol to the matrix. The model proposed by Gelles et al is consistent with the data available on the ligand structure about Cu_A. As discussed above, the coordinated ligands for cupric Cu_A are almost certain to include two cysteine sulfurs and at least one histidine nitrogen. That a conformational transition occurs upon electron input to Cu_A is suggested by the large entropic change associated with reduction of this metal center (45), as well as some recent preliminary Cu EXAFS results on the reduced enzyme (P. M. Li, personal communication).

Although no direct experimental evidence supports the model of Gelles et al, some circumstantial data suggest a role for the Cu_A center in proton pumping by the enzyme. Several methods for perturbing the local environment of Cu_A, and their effects on the enzyme's proton pumping activity, have recently been reported. Gelles & Chan have shown that treatment of the oxidase with the sulfhydl reagent p-hydroxymercuri benzoate (pHMB) disrupts the coordination sphere of Cu_A and converts this metal center into a form resembling a type 2 copper center in terms of its EPR spectrum and redox potential (58). Nilsson et al found that a similar $Cu_A \rightarrow$ type 2 Cu conversion could be induced by heating the enzyme to ca. 43°C in the presence of a zwitterionic detergent (59). In both of these modified Cu_A enzymes, low temperature EPR and resonance Raman spectroscopies indicate little, if any, perturbation of heme a (59, 60). Heating the enzyme in a similar fashion in the presence of nonionic detergents also leads to a disruption of the Cu_A coordination sphere, but in this case one obtains a mixture of Cu_A forms (61). When these modified forms of cytochrome c

oxidase were reconstituted into phospholipid vesicles, they were found to be no longer competent in terms of proton pumping (61) or capable of sustaining a transmembrane proton gradient when compared to vesicles containing the native enzyme (59, 62, 63). In the latter, the modified enzyme vesicles showed a significantly increased permeability toward protons (i.e. they were leaky). This proton permeability could arise from the fusion of the matrix and cystosol HBCs into one continuous transmembrane proton-conducting channel due to disruption of the proton gating machinery at the Cu_A center (63). Although a variety of control experiments have been conducted in the studies of the Cu_A-modified enzymes, one cannot completely rule out the possibility that disruption of the Cu_A site has led to creation of a transmembrane aqueous or proton channel in a region of the protein that is only remotely connected to the perturbed metal center. Such a scenario is likely in indirect coupling models in which the coupling between the electron and proton flow must be transmitted over some distance between the redox center and proton-translocating machinery. Nevertheless, taken together these results do provide some indication that the Cu_A center of cytochrome c oxidase plays a role in proton translocation.

Redox-Linked Conformational Transitions of Cytochrome c Oxidase

One common feature of all the hypothetical mechanisms for redox-linked proton translocation in cytochrome c oxidase is an obligatory conformational transition that provides the switch between proton accessibility from one side of the membrane to the other; i.e. a proton-input state to proton-output state conversion. In the direct coupling models this conformational change may be restricted to the immediate vicinity of the involved redox center, and may involve only slight motions of the amino acid side chains associated with the metal center. In the indirect coupling models the conformational change must be more global in nature, since here the driving force for proton translocation must be communicated over some distance between the redox center and the proton-translocating machinery. One might therefore expect that cytochrome c oxidase would display some type of redox-linked conformational change associated with the low potential metal centers of the enzyme. Indeed a number of results now suggest that such a conformational transition does occur in this enzyme.

In the early 1970s Yamamoto & Okunuki showed that reduction of the metal centers of cytochrome c oxidase results in a significant stabilization of the enzyme toward proteolytic digestion (64). Cabrel & Love also showed via sedimentation studies that reduction of the enzyme resulted in a ca. 3% increase in the protein's volume (65). The majority of this volume

change was attributed to reduction of the low potential metal centers by comparison of the relative volumes of the fully reduced and CO mixed-valence forms of the enzyme. Since reduction of both heme a and Cu_A are associated with large, negative entropy changes, such redox-linked changes of the enzyme are reasonable. These conformational changes are apparently not limited to the immediate vicinity of the metal centers. Several groups have shown that reduction of the low-potential metal centers greatly accelerates the rate of inhibitory cyanide binding at the oxygen binding site (66–68). This suggests that cytochrome c oxidase is an allosteric enzyme capable of transmitting redox-induced structural changes at the low potential metal centers between subunits and over a large distance to the oxygen-binding site. There is also evidence that intramolecular electron transfer from the low-potential metal centers to the oxygen-binding site is mediated by a conformational transition of the enzyme. Malmström and co-workers have reported data that suggest that such intramolecular electron transfer does not occur until two electrons have entered the enzyme and a conformational transition occurs (69).

It should be pointed out, however, that this conformational transition was introduced here for the purpose of electron gating. Electron gating is a necessary but not sufficient condition for a redox-linked proton pump. Additional structural and/or conformational rearrangements need to be invoked to provide for energetic linkage between the electrons and the protons and to achieve the gating of proton flow. When all these requirements are incorporated into the model of Malmström et al (70, 71), their scheme becomes formally equivalent to the mechanism advocated by Gelles et al (57).

Unfortunately, at this juncture, we do not have more direct handles on the conformational transitions that occur during turnover of the enzyme. Copeland et al have recently reported evidence for a redox-linked conformational transition in cytochrome c oxidase based on steady-state and stopped-flow tryptophan fluorescence spectroscopy (71, 72). Inasmuch as we, as well as others (B. Hill, personal communication; D. Rousseau, personal communication) have obtained evidence that the steady-state fluorescence results are artifactual, these findings must now be discounted.

Future Prospects for Cytochrome c Oxidase

The prospects for further elucidation of the mechanism of proton translocation in cytochrome c oxidase are quite promising, in view of several recent advances that should provide more detailed structural information on the enzyme as a whole, and the proton pumping machinery in particular. New spectroscopic handles, such as picosecond laser spectroscopy, may provide new insights into the nature and extent of structural perturbations

of the protein matrix associated with proton pumping. The recent cloning of the three subunits of the *P. denitrificans* enzyme holds the promise of identifying key amino acid residues via site directed mutagenesis (73). There has also been a recent report that the bacterium *Thermus thermophilus* can express a single subunit terminal oxidase in which heme *a* is replaced with a *b*-type heme (74). Whether this enzyme pumps protons when reconstituted into phospholipid vesicles is not yet known, but experiments to address this issue can potentially provide a means of determining the low-potential metal center involved in proton pumping. Finally, we note with great interest the report by Caughey and co-workers on the preparation of crystals of bovine cytochrome *c* oxidase that diffract to 8 Å resolution (75). If the quality and size of these crystals can be improved in the near future, the possibility for a three-dimensional structure for at least the oxidized enzyme will soon be within reach.

ACKNOWLEDGMENTS

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