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Structures of Protein-Protein Complexes involved in electron transfer

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Abstract

Electron transfer (ET) reactions are essential for life since they underpin oxidative phosphorylation and photosynthesis, processes leading to the generation of ATP, and are involved in many reactions of intermediary metabolism[1]. Key to these roles is the formation of transient inter-protein ET complexes. The structural basis for the control of specificity between partner proteins is lacking since these weak transient complexes have remained largely intractable for crystallographic studies[2,3]. Inter-protein ET processes are central to all of the key steps of denitrification, an alternative form of respiration in which bacteria reduce nitrate or nitrite to N_2 via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O) when oxygen concentrations are limiting. The one electron reduction of nitrite to NO, a precursor to N_2O, is performed by either a heme- or copper-containing nitrite reductase (CuNiR) where they receive an electron from redox partner proteins a cupredoxin or a c-type cytochrome[4,5]. Here we report the structures of the newly characterized three-domain heme*c*-Cu nitrite reductase from *Ralstonia pickettii* (*Rp*NiR) at 1.01Å resolution and its M92A and P93A mutains. Very high resolution provides the first view of the atomic detail of the interface between the core trimeric cupredoxin structure of CuNiR and the tethered cytochrome *c* domain that allows the enzyme to function as an effective self-electron transfer system i.e. where the donor and acceptor proteins are fused together by genomic acquisition for functional advantage. Comparison of *Rp*NiR with the binary complex of a CuNiR with a donor protein, *Ax*NiR-cytc551[6], and mutagenesis studies provide direct evidence for the importance of a hydrogen bonded water at the interface in ET. The structure also provides an explanation for the preferential binding of nitrite to the reduced copper ion at the active site in *Rp*NiR, in contrast to other CuNiRs where reductive inactivation occurs, preventing substrate binding.

Conversion of nitrate to N_2 known as the denitrification process is performed by a number of distinct enzymes[5,6]. The first committed step, $NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$ is catalyzed by NiR. The well-studied two-domain CuNiRs are trimers of 106 kDa with each monomer having two domains with a characteristic β-sandwich curpredoxin motif. Each monomer has a type-1 Cu (T1Cu) center with (Cys–Met–His_2) ligation and a type-2 Cu (T2Cu) site with (His_3–H_2O) ligation at the interface between two monomers, both of which

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Author Contributions S.V.A. and H.C. contributed equally. S.V.A., R.R.E. and S.S.H. conceived and designed the project; H.C. cloned, expressed and purified proteins; S.V.A. and H.C. crystallized; S.V.A. did data processing, structure determination and refinement; S.V.A., R.R.E. and S.S.H. wrote the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 3ziy(r3ziysf), 4ax3(r4ax3sf), 2yqb(r2yqbsf) and 3zbm(r3zbmsf). Reprints and permissions information is available at www.nature.com/reprints.

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provide its ligands. During catalysis nitrite binds to the T2Cu displacing the liganded H_2O. Electrons produced by respiration are transferred to the T1Cu site by a partner redox protein. Subsequently electrons are transferred via a 12.6Å internal His-Cys bridge in a proton-gated reaction, to the T2Cu site, where nitrite is reduced to NO.[7,8,9,10,11,12] The timing of electron and substrate delivery to the active site of CuNiRs is critical to catalysis, since if the water coordinated at the T2Cu site of the oxidized enzyme dissociates before nitrite binds to displace this water, the enzyme is inactivated .[13,14]

Recently, two new sub-classes of CuNiRs have been recognised that retain the core structure of two-domain enzymes but contain an additional cupredoxin or heme *c*-containing domain, fused at the N- and C- terminals respectively.[15,16] Genome analysis has shown that these variant enzymes are quite widely distributed in Nature. The cupredoxin N-terminal fused three-domain NiR from *Hyphomicrobium denitrificans* (*Hd*NiR) has been biochemically and structurally defined[17,18]. Surprisingly the observed hexameric structure, against all expectations, revealed that the Cu site in the additional cupredoxin domain was located too far away from the catalytic core for effective ET.[17]

The first biochemical and functional characterization of a fused three-domain cytochrome *c* CuNiR, the enzyme from *Ralstonia pickettii* (*Rp*NiR)[19] showed it to be trimeric similar to two-domain CuNiRs such as *Ax*NiR. However, *Rp*NiR revealed a major difference in that the oxidized resting state had a significantly lower nitrite binding affinity. Our structure of *Rp*NiR at 1.01Å resolution reported here (Fig.1) provides the structural basis of the functional importance of the heme *c* in *Rp*NiR, where ET from the reduced heme occurs during turnover. It also provides an explanation as to how the coordinated water at the T2Cu site is stabilized preventing its displacement by nitrite rationalizing the low binding affinity of nitrite to the oxidized enzyme. A comparison of the *Rp*NiR structure with the cupredoxin N-terminal fused NiR, *Hd*NiR, reveals a remarkable conservation of the 3D structural organization for these two new classes of extended three domains NiRs (Fig.S1). T1Cu of the cupredoxin domain of *Hd*NiR can be brought to an appropriate distance for ET to the catalytic T1Cu-T2Cu core *via* a simple rotation of the cupredoxin domain.

Potential ET routes between cupredoxin and heme *c*- domains have been clarified to some extent by structural studies of a number of other ET complexes. For example, the transient binary *Ax*NiR-*Cytc*$_{551}$complex (1.7 Å resolution)[6] and the integral membrane complex *caa*$_3$-type cytochrome *c* oxidase (2.36 Å resolution)[20]. Our structure of *Rp*NiR clearly defines ET routes between a heme *c*- domain and a catalytic cupredoxin core. The fold of the trimeric CuNiR core domain is preserved and the heme *c* domain of one monomer is in close proximity to the T1Cu site of another monomer at an ET compatible distance of 10.1 Å between the T1Cu ion and the closest heme edge (CBC methyl carbon) of the porphyrin (Fig.2). The linker between heme *c* and core NiR domain forms ten salt bridges and eleven H-bonds with the core and nine H-bonds with the heme domain contributing to the stability of the heme *c* domain. These interactions would preclude any significant movement of the heme domain from its position that favours ET to the T1Cu centre. Comparison of *Rp*NiR with the structure of the related but biochemically uncharacterised enzyme from *Pseudoalteromonas haloplanktis* (*Ph*NiR) (PDB entry 2ZOO) revealed differences in the overall organization of the subunits. The *Rp*NiR monomer is more compact, the linker between heme and Cu binding domains (residues 315-333) is arranged in a helical structure and only two monomers are involved in forming the T2Cu site and the interface between the heme and cupredoxin domains. In contrast, in *Ph*NiR the linker wraps around the neighbouring monomer and reaches the distant third monomer creating the cytochrome and catalytic domain interface (Fig.S1). The significance of these differences awaits the biochemical characterization of *Ph*NiR.

A striking feature of the *Rp*NiR structure is the extensive water network at the interface of the heme *c* domain and the surface above the T1Cu of the core of the enzyme (Fig.2). Several computational studies have identified the importance of water molecules H-bonded between the donor and acceptor sites in providing superior electronic coupling resulting in substantially enhanced rates of ET, as between methylamine dehydrogenase and the cupredoxin amicyanin.[21] In the case of *Rp*NiR, the interactions between the cytochrome and the catalytic-core domains are either C-C interactions of heme with Met92 and Pro93 or are mediated by water H-bonds between the two domains. Similar interactions are seen in *Ph*NiR (Fig.S2). In contrast to these hydrated interfaces, the structure of the transient binary *Ax*NiR-*Cytc*$_{551}$complex shows that the protein-protein interface is primarily hydrophobic patch with a non-polar core sealed off from the aqueous environment[6], with no water molecules that may involve in ET. In this complex, and in native *Ax*NiR His 135 (His143 in *Rp*NiR) is protected from hydrophilic environment by Met 135 and Trp138. A similar situation is found in the tethered membrane complex *caa*$_3$-type cytochrome *c* oxidase of *Thermus thermophilus* where the ET pathway from heme *c* to the Cu_A centre does not involve water, but rather the D-pyrrole and D-propionate of heme *c* to the *cis* amide N and α C of Phe126 of the cupredoxin domain.[20]

The potential involvement of Met92 and Pro93 in ET of *Rp*NiR was tested by individual substitution by Ala. Structures of these variants determined at 1.9 Å and 1.4 Å respectively show that the conserved water was not perturbed by these substitutions (Fig.3). The mutations had no significant effect on the specific activity of the enzyme and in single turnover experiments where reduced *Rp*NiR was reoxidised by nitrite, the ET efficiency from heme to T2Cu was similar to the wild-type enzyme, with M92A variant showing a small decrease in rate (see supplementary material). These small effects suggests that the conserved water molecule H-bonded to the solvent-exposed T1Cu histidine ligand and the carbonyl of the peptide bond of Ala138 plays a dominant role in ET in this tethered complex. The nature of this interaction precludes further testing of its involvement in ET by mutation. This role for H-bonded water in ET between the heme and T1Cu site contrasts with the transient binary *Ax*NiR-*Cytc*$_{551}$complex where the close contact between the two proteins results in utilization of C-C interactions between the CBC methyl group and Pro88 of the cupredoxin domain of the core NiR. These two ET systems nicely illustrate the two classes of electron tunneling processes predicted ie protein-mediated and structured water–mediated.[22]

The structure also provides insights into the mechanism of this recently characterized class of CuNiRs. A surprising feature of the *Rp*NiR structure was that despite the similarity to two domain NiRs, the usual substrate access channel is closed. Atomic resolution structures of two-domain NiRs have revealed the detail of two proton pathways to the active site and also the hydrophobic substrate access channel to the protein surface.[8,9] Mutagenesis studies of *Ax*NiR established only one proton pathway to be functionally relevant, namely the highly ordered (Asp92-water-water-Ala131-Asn90-Asn107) network from the T2Cu site (Fig.S3) to the protein surface.[23] This hydrophobic channel ~6Å wide formed by residues belonging to adjacent monomers was also assigned as the route of substrate access to the catalytic centre. In *Rp*NiR this channel extends towards the heme domain, but lacks water molecules as it is blocked by residue Tyr323 that forms part of the linker between cytochrome and cupredoxin domains. Instead, the non-functional proton pathway of two domain NiRs predominates (Figs. 1d and 4). This channel is present in the structure of *Ph*NiR also. However, in *Ph*NiR Ile235 adopts a different orientation to Ile245 of *Rp*NiR and interrupts it. This residue may thus be a channel activator and the two structures may represent two alternative protein states.

In contrast to other CuNiRs, the oxidized T2Cu catalytic sites of *Rp*NiR have a very low affinity for nitrite.[19] This does not arise from constraints imposed by the altered substrate access channel described above since the apparent K_m for nitrite is 26 μM, comparable to other CuNiRs. Structural and kinetic studies of CuNiRs indicate that reduction of the T2Cu can result in the loss of the coordinated H_2O ligand to form a catalytically inactive $Cu^+(His)_3$ coordination at the active site.[13,14] The water ligated to the T2Cu of NiR in all other structures H-bonds to the carboxylate group of the active pocket Asp at 2.54-2.83Å and also a histidyl nitrogen atom [8,9,24]. In *Rp*NiR the coordinated water W1 has two additional H-bonds (Fig.S4) being connected to a second water molecule (labeled W2) as well as Oδ1 of Asp97 that is also hydrogen bonded to the OH of Tyr323. Water W2 also has a hydrogen bond to the OH of Tyr323 that blocks the putative substrate entry channel identified in two-domain NiRs. This would be expected to provide additional stability to the coordinated water molecule, hindering nitrite binding to the oxidized site but stabilizing it on reduction to allow nitrite to bind by displacement of the water ligand before the catalytically inactive $Cu^+(His)_3$ coordination species is formed.

Methods

*Rp*NiR was purified by DEAE ion-exchange chromatography and size exclusion chromatography (SEC). The SEC buffer was 20 mM Tris-HCl, pH 8.0, 200 mM NaCl. The *Rp*NiR protein was dialyzed against SEC buffer plus 0.1 mM $CuSO_4$ for reconstitution of type 2 copper sites. The fraction was then dialyzed exhaustively against three changes of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl with a minimum equilibration period of 5 h between buffer changes. The protein samples were pooled and concentrated upto 15 mg ml^{-1}.

Crystals for *Rp*NiR and its mutants (H3 crystal form) were grown using the hanging drop vapor diffusion method at 4°C temperature from similar crystallisation condition, 2 ml of protein solution in 20 mM Tris-HCl, 200mM NaCl (pH 8.0) was mixed with an equal volume of reservoir solution containing 20% PEG3350, 0.2 M Sodium citrate. The buffer for reservoir solution was 20 mM Tris-HCl, pH 7.5 for wild type protein and 20mM MES pH 6.5 for the mutains. Protein concentration was ~15 mg/ml H3 crystal form. Wild type *Rp*NiR $P2_13$ crystal form has been grown from 20% PEG3350, 0.2 Sodium citrate, 200 mM NaCl and protein concentration 7.5 mg/ml. All crystals were flashed cooled in the mother liquor plus 10% glycerol solution.

For *Rp*NiR-H3 and P93A-H3 structures, anisotropic temperature factors were refined and riding hydrogen atoms were added to the model. The lower resolution M93A-H3 structure was refined with isotropic B-factors and riding hydrogen atoms in the calculated positions. No restrains were used for Cu atoms refinement. The quality of the models was assessed using Coot[29], Procheck[30] and the Molprobity[31] server. Comparison of the Cα atom positions in all *Rp*NiR structures *Rp*NiR-H3, *Rp*NiR-p213 show average R.M.S. deviation between the CA atoms ~ 0.2 Å with higher flexibility within the N-terminus (residues 1-16) and part of cytochrome domain (residues 383-393) atoms. First 3 residues of the N-terminus and last 6 residues of the C-terminus are not visible in the electron density. Within five monomers in 2 structures (*Rp*NiR-H3, *Rp*NiR-$P2_13$) the position of the cytochrome domain is identical and crystal contacts independent. Analyses of anomalous signal in the Cu and Fe sites confirmed correct Cu and Fe incorporation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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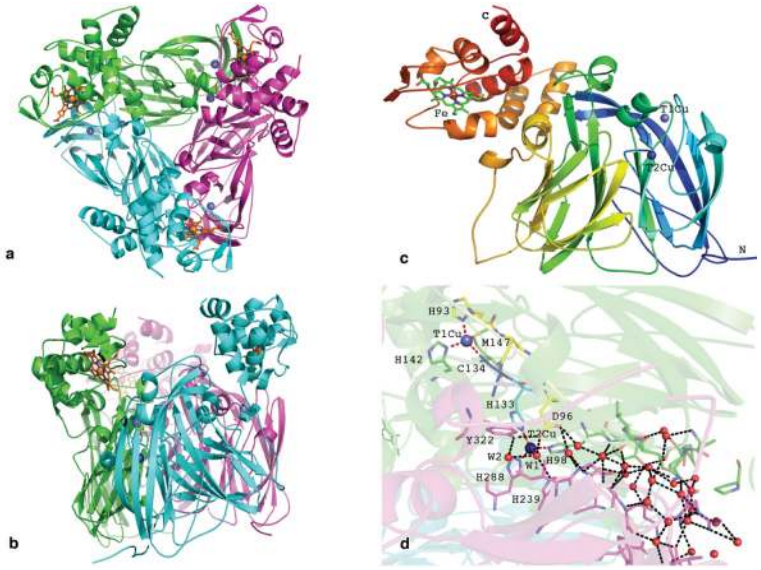

Fig.1.

Structural Organization of *Ralstonia pickettii* NiR (a) along and (b) perpendicular to the three-fold axis. T2Cu (darker blue sphere) binds at the interface between two monomers. T1Cu (lighter blue sphere) is located closer to the surface of each monomer in the proximity of the cytochrome domain from the adjacent monomer at a distance commensurate with efficient ET, (c) Ribbon diagram of a monomer with cytochrome domain (orange-red), cupredoxin domains (green-blue) containing T1Cu and T2Cu and the helical linker structure formed by residues 315-333 (yellow) between the two and (d) details of the water (red) channel linking it to W1 coordinating to T2Cu. The stability of the heme *c* domain in *Rp*NiR, is also reinforced by the extended interface area of 2267Å^2 between the two monomers in the trimer of which 452 Å^2 involves the heme *c* domain.

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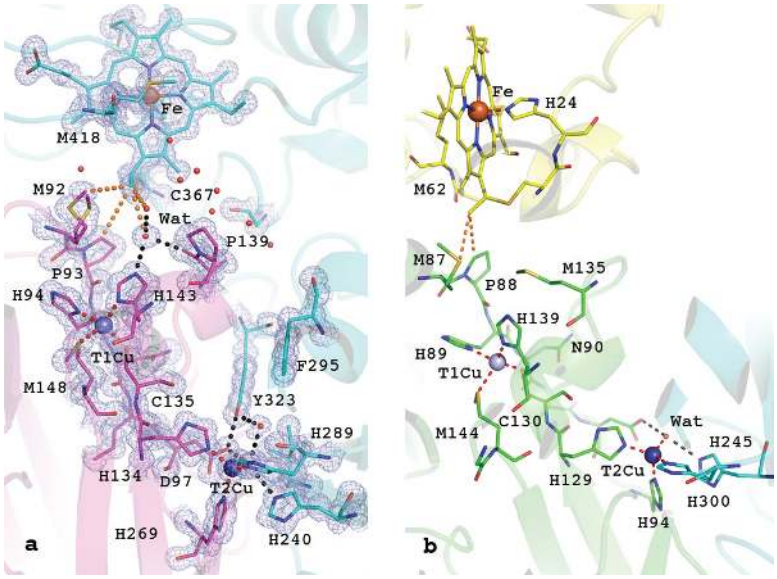

Fig.2.

Details of interaction between cytochrome and Cu binding domains for (a) *Rp*NiR and (b) *Ax*NIR-*Cytc*$_{551}$ complex[6] with shortest distances between heme and Cu binding domain shown as dotted orange lines. An ET route can be traced from the porphyrin CBC atom via a water molecule, the Nε2 atom of the His143 ligand of the T1Cu centre. For *Rp*NiR-P2$_1$3 monomer-A, 2Fo-Fc map contoured at 1.0 σ for the T1Cu and T2Cu Sites for. Fe-T1Cu distance is 10.4Å, T1-T2Cu is 12.7Å. For simplicity only one conserved water molecule on the shortest route to T1Cu is shown at the interface between cytochrome domain and T1Cu. This water is located in very close proximity to both CBC heme atom (3.6Å) and Nε2 atom of Cu ligating His143 (2.8Å). The binary complex, *Ax*NIR-*Cytc*$_{551}$, has a hydrophobic interface between Cytochromec_{551} and T1Cu site.

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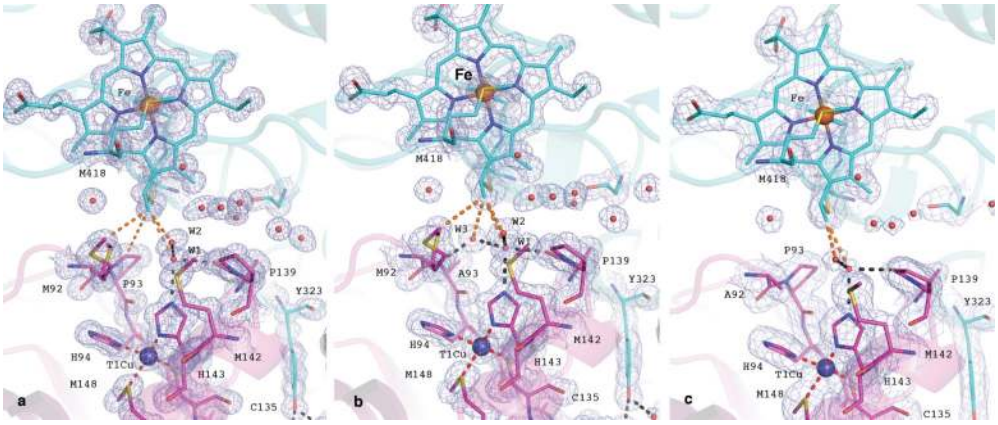

Fig.3.

Details of interactions between cytochrome and Cu binding domains in (a) *Rp*NiR, (b) P93A and (c) M92A in H3 crystal. A 2Fo-Fc map contoured at 1.0 σ at 1.01/1.4/1.8Å resolution for wt/P93A/M92A. For all three structures the distances between Fe and T1 Cu is 10.1-10.3 Å. The interface between cytochrome domain and T1Cu site has 2 conserved water molecules (W1 and W2) located in very close proximity to CBC heme atom (3.5-3.6 and 3.3-3.4Å respectively), the waters are hydrogen bonded to each other. Distances from W1 molecule to His143($N\varepsilon_2$) and Ala138(O) are 2.8Å. Additional water W3 is seen in P93A close to the mutation site.

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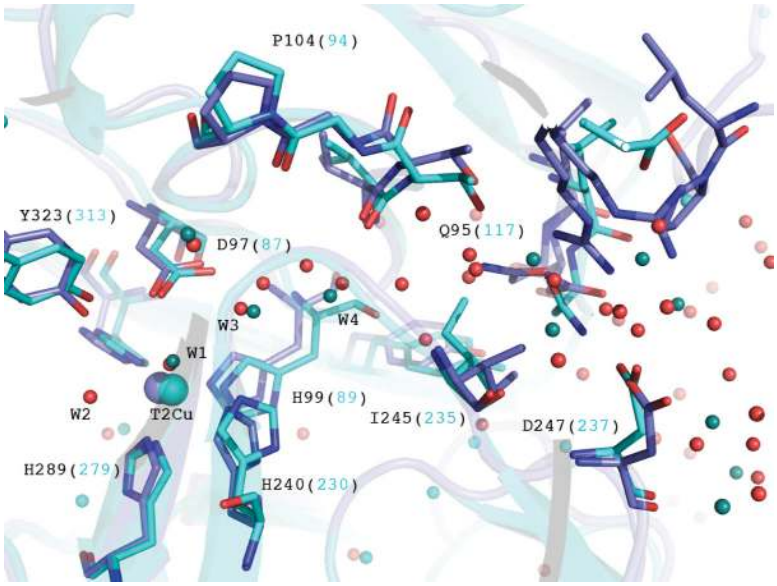

Fig.4.

Details of the proton pathway in *Rp*NiR (dark blue with water as red sphere) and its comparison with *Ph*NiR (light blue) (PDB, 2zoo). This channel is comprised of residues on the interface between two monomers that extends from the T2Cu catalytic centre to the protein surface and is filled with an extensive water network. In the case of *Ph*NiR the channel is interrupted by Ile 235 that has a different orientation to the corresponding Ile245 of *Rp*NiR. Ile235/245 in these extended NiRs correspond to conserved His254 residue in two domain *Ax*NiR[23].