LETTER

Structures of protein–protein complexes involved in electron transfer

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Electron transfer reactions are essential for life because they underpin oxidative phosphorylation and photosynthesis, processes leading to the generation of ATP, and are involved in many reactions of intermediary metabolism. Key to these roles is the formation of transient inter-protein electron transfer complexes. The structural basis for the control of specificity between partner proteins is lacking because these weak transient complexes have remained largely intractable for crystallographic studies2,3. Inter-protein electron transfer processes are central to all of the key steps of denitriﬁcation, an alternative form of respiration in which bacteria reduce nitrate or nitrite to N2 through the gaseous intermediates nitric oxide (NO) and nitrous oxide (N2O) when oxygen concentrations are limiting. The one-electron reduction of nitrite to NO, a precursor to N2O, is performed by either a haem- or copper-containing nitrite reductase (CuNiR) where they receive an electron from a redox partner proteins a cupredoxin or a c-type cytochrome4–6. Here we report the structures of the newly characterized three-domain haem-c-Cu nitrite reductase from *Ralstonia pickettii* (*RpNiR*) at 1.01 Å resolution and its M92A and P93A mutants. 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 where the donor and acceptor proteins are fused together by genomic acquisition for functional advantage. Comparison of *RpNiR* with the binary complex of a CuNiR with a donor protein, *A. Xyloferax*–cytc551 (ref. 6), and mutagenesis studies provide direct evidence for the importance of a hydrogen-bonded water at the interface in electron transfer. The structure also provides an explanation for the preferential binding of nitrite to the reduced copper ion at the active site in *RpNiR*, in contrast to other CuNiRs where reductive inactivation occurs, preventing substrate binding.

Conversion of nitrate to N2, known as the denitriﬁcation process, is performed by several distinct enzymes4–9. The ﬁrst committed step, NO2− + H2O → NO + H2O is catalysed by NiR. The well-studied two-domain CuNiRs are trimers of 106 kDa, with each monomer having two domains with a characteristic β-sandwich cupredoxin motif. Each monomer has a type-1 Cu (T1Cu) centre with (Cys–Met–b)3-sandwich cupredoxin N-terminal-fused three-domain NiR, the enzyme from *R. pickettii* (*RpNiR*) (Protein Data Bank entry 2zoo) showed differences in the overall organization of the subunits. The pseudoalteromonas haloplanktis (*PhNiR*) (1.7 Å resolution)10 and the integral membrane complex *c*iaa5–type cytochrome c oxidase (2.36 Å resolution)11, for example. Our structure of *RpNiR* clearly deﬁnes electron transfer routes between a haem c domain and a catalytic cupredoxin core. The fold of the trimeric CuNiR core domain is preserved and the haem c domain of one monomer is in close proximity to the T1Cu site of another monomer at an electron transfer compatible distance of 10.1 Å between the T1Cu ion and the closest haem edge (CBC methyl carbon) of the porphyrin (Fig. 2). The linker between haem c and core NiR domain forms ten salt bridges, eleven hydrogen bonds with the core and nine hydrogen bonds with the haem domain contributing to the stability of the haem c domain. These interactions would preclude any signiﬁcant movement of the haem domain from its position that favours electron transfer to the T1Cu centre. Comparison of *RpNiR* with the structure of the related but biochemically uncharacterized enzyme from *Pseudalteromonas haloplanktis* (*PhNiR*) (Protein Data Bank entry 2zoo) showed differences in the overall organization of the subunits. The *RpNiR* monomer is more compact, the linker between haem 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 haem and cupredoxin.

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The significance of these differences awaits the biochemical characterization of cytochrome and catalytic domain interface (Supplementary Fig. 1). In contrast, in PhNiR the linker wraps around the neighbouring monomer and reaches the distant third monomer, creating the cytochrome domain from the adjacent monomer at a distance commensurate with efficient electron transfer. 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 (c) and details of the water (red) channel linking it to W1 coordinating to T2Cu (d). The stability of the haem c domain in RpNiR is also reinforced by the extended interface area of 2267 Å² between the two monomers in the trimer, of which 452 Å² involves the haem c domain.

A striking feature of the RpNiR structure is the extensive water network at the interface of the haem c domain and the surface above the T1Cu of the core of the enzyme (Figs 2 and 3a). Several computational studies have identified the importance of water molecules hydrogen bonded between the donor and acceptor sites in providing superior electronic coupling. This results in substantially enhanced rates of electron transfer, as between methylene dehydrogenase and the cupredoxin amicyanin. In the case of RpNiR, the interactions between the cytochrome and the catalytic-core domains are either C–C interactions of haem with Met 92 and Pro 93 or are mediated by water hydrogen bonds between the two domains. Similar interactions are seen in PhNiR (Supplementary Fig. 2). In contrast to these hydrated interfaces, the structure of the transient binary AxNiR–Cytc551 complex shows that the protein–protein interface is a primarily hydrophobic patch with a non-polar core sealed off from the aqueous environment, with no water molecules that may be involved in electron transfer. In this complex, and in native AxNiR, His 135 (His143 in RpNiR) is protected from hydrophilic environment by Met 135 and Trp 138. A similar situation is found in the tethered membrane complex cytc oxidase of Thermus thermophilus, where the electron transfer pathway from haem c to the Cu₃ centre does not involve water, but rather the π-pyrrole and π-propionate of haem c to the cis amide N and α C of Phe 126 of the cupredoxin domain.

The potential involvement of Met 92 and Pro 93 in electron transfer of RpNiR was tested by individual substitution by Ala. Structures of these variants determined at 1.9 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 RpNiR was reoxidized by nitrite, the electron transfer efficiency from haem to T2Cu was similar to the wild-type enzyme, with M92A variant showing a small decrease in rate (see Supplementary Information). These small effects suggest that the conserved water molecule hydrogen bonded to the solvent-exposed T1Cu histidine ligand and that the carbonyl of the peptide bond of Ala 138 plays a dominant role in
between the cytochrome domain and the T1Cu site has two conserved water structures, the distances between Fe and T1 Cu is 10.1–10.3 Å. The interface between the cytochrome domain and the T1Cu site has two conserved water molecules (W1 and W2) located in very close proximity to the CBC haem atom (3.5–3.6 and 3.3–3.4 Å respectively); the waters are hydrogen bonded to each other. The distances from the W1 molecule to His 143(Ne2) and Ala 138(O) are approximately 2.8 Å in three structures. Extra water W3 is seen in P93A close to the mutation site.

Figure 3 | Comparison of interactions between cytochrome and Cu binding domains in wild type RpNiR, P93A and M92A mutants. a–c, RpNiR (a), P93A (b) and M92A (c) in H3 crystal form. A 2Fo – Fo map contoured at 1.0 at 1.01/1.4/1.8 Å resolution for wild type/P93A/M92A. For all three structures, the distances between Fe and T1 Cu is 10.1–10.3 Å. The interface between the cytochrome domain and the T1Cu site has two conserved water electron transfer in this tethered complex. The nature of this interaction precludes further testing of its involvement in electron transfer by mutation. This role for hydrogen-bonded water in electron transfer between the haem and T1Cu site contrasts with the transient binary AxNiR–Cytc551 complex, where the close contact between the two proteins results in use of C–C interactions between the CBC methyl group and Pro 88 of the cupredoxin domain of the core NiR. These two electron transfer systems illustrate the two classes of predicted electron tunnelling processes: protein mediated and structured water mediated.

The structure also provides insights into the mechanism of this recently characterized class of CuNiRs. A surprising feature of the RpNiR 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 shown the detail of two proton pathways to the active site and the hydrophobic substrate access channel to the protein surface. Mutagenesis studies of AxNiR established only one proton pathway to be functionally relevant, namely the highly ordered (Asp 92–water–water–Ala 131–Asn 90–Asn 107) network from the T2Cu site (Supplementary Fig. 3) to the protein surface. This hydrophobic channel, approximately 6 Å wide formed by residues belonging to adjacent monomers, was also assigned as the route of substrate access to the catalytic centre. In RpNiR this channel extends towards the haem domain, but lacks water molecules as it is blocked by residue Tyr 323 that forms part of the linker between cytochrome and cupredoxin domains. Instead, the non-functional proton pathway of two domain NiRs predominates (Fig. 4 and Supplementary Fig. 1d). This channel is present in the structure of PhNiR also. However, in PhNiR, Ile 235 adopts a different orientation to Ile 245 of RpNiR 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 RpNiR have a very low affinity for nitrite. This does not arise from constraints imposed by the altered substrate access channel described above because the apparent Michaelis constant 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 H2O ligand to form a catalytically inactive Cu2+ (His1) coordination at the active site. The water ligated to the T2Cu of NiR in all other structures hydrogen bonds to the carboxylate group of the active pocket Asp at 2.54–2.83 Å and a histidyl nitrogen atom. In RpNiR the coordinated water W1 has two extra hydrogen bonds (Supplementary Fig. 4), being connected to a second water molecule (labelled W2) as well as O61 of Asp97 that is also hydrogen bonded to the OH of Tyr 323. Water W2 also has a hydrogen bond to the OH of Tyr 323 that blocks the putative substrate entry channel identified in two-domain NiRs. This would be expected to provide more 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 Cu2+ (His1) coordination species is formed.

METHODS SUMMARY

RpNiR was expressed in Escherichia coli BL21(DE3) and purified as previously described. For M92A and P92A, site-directed mutagenesis was performed following the QuickChange site-directed mutagenesis protocol (Agilent), using KOD Hot Start DNA polymerase (Merck). The plasmid pET26b-RpNiR was used as template for introducing the following single amino-acid changes in RpNiR by site-directed mutagenesis: M92A and P92A. The primers for the M92A mutant were (sense) 5′-CACCGGAGCAAGGAGCGGCGACAACTCAGA-3′ and
E. coli transformation into mers for the P93A mutant were (sense) 5'-TGAGTCTTTGCGGCGCCCTTGTGTCCGGTTG-3'. The primers for the P93A mutant were (sense) 5'-GAGGCGACAGGATGCCGAC AACATCGAC-3' and (antisense) 5'-GTGAGTCTTTGCGGCGCCCTTGTGTCCGGTTG CTTG-3'.

The mutant plasmids were confirmed by DNA sequencing before transformation into E. coli strain BL21(DE3). Both mutants were purified following native protein protocols. Crystals for RpNiR and its mutants (H3 crystal form) were grown over a month using the hanging drop vapour diffusion method at 4 °C. Wild-type RpNiR P2,3 crystal form grew in 4 days. Diffraction data were collected from the single crystal at 100 K using a PILATUS-6M detector at Proxima1 (Soleil), at ID24 and IO2 (Diamond). For native protein crystals (RpNiR-H3/RpNiR-P2,3), four data sets were collected at 0.98, 1.22, 1.33 and 1.7 Å X-ray wavelengths to confirm correct metal incorporation. All data were processed and scaled by HKL2000 software. Only data sets collected at the 0.98 Å wavelength were used for structural determination and refinement. Native RpNiR-H3 structure was solved by molecular replacement using MOLREP in the CCP4 (ref. 27) program suite and PHINiR (Protein Data Bank entry 2xoo) as the search model. H3 and P2,3 forms have one and four monomers, respectively, in an asymmetric unit. RpNiR-P2,3 was solved by molecular replacement using an RpNiR-H3 model. All structures were refined using REFMAC5 (ref. 28) in the CCP4 program suite. Rebuilding of the model between refinement cycles and adding waters was performed in Coot (ref. 29). Data collection and refinement statistics are summarized in Supplementary Table 1.

Full Methods and any associated references are available in the online version of the paper.

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29. Supplementary Information is available in the online version of the paper.

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Author Contributions S.V.A., R.R.E. and S.S.H. conceived and designed the project; C.H. 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 crystal structures have been deposited in Protein Data Bank under accession numbers 3zyi, 3zyf, 4ax3, 4ax3s, 2xpy (2xpybf) and 3zbm (3zbmfs). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.S.H. (s.s.hasnain@liverpool.ac.uk) or S.V.A. (antoynyuk@liverpool.ac.uk).
METHODS

RpNiR was purified by DEAE ion-exchange chromatography and size-exclusion chromatography. The size-exclusion chromatography buffer was 20 mM Tris-HCl, pH 8.0, 200 mM NaCl. The RpNiR protein was dialysed against size-exclusion chromatography buffer plus 0.1 mM CuSO\(_4\) for reconstitution of type 2 copper sites. The fraction was then dialysed 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 up to 15 mg ml\(^{-1}\).

Crystals for RpNiR and its mutants (H3 crystal form) were grown using the hanging-drop vapour-diffusion method at 4 °C temperature from similar crystallization conditions: 2 ml of protein solution in 20 mM Tris-HCl, 200 mM 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 20 mM MES pH 6.5 for the mutants. Protein concentration was approximately 15 mg ml\(^{-1}\) H3 crystal form. Wild-type RpNiR P2\(_{1}\)\(_{3}\) crystal form was grown from 20% PEG3350, 0.2 sodium citrate, 200 mM NaCl and protein concentration 7.5 mg ml\(^{-1}\). All crystals were flashed cooled in the mother liquor plus 10% glycerol solution.

For RpNiR-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 restraints were used for Cu atoms refinement. The quality of the models was assessed using Coot\(^{30}\), Procheck\(^{30}\) and the Molprobity\(^{31}\) server. Comparison of the Cα atom positions in all RpNiR structures (RpNiR-H3, RpNiR-P2\(_{1}\)\(_{3}\)) showed average root mean squared deviation between the Cα atoms of approximately 0.2 Å, with higher flexibility in the N terminus (residues 1–16) and part of cytochrome domain (residues 383–393) atoms. The first three residues of the N terminus and the last six residues of the C terminus were not visible in the electron density map. Within five monomers in two structures (RpNiR-H3, RpNiR-P2\(_{1}\)\(_{3}\)), the position of the cytochrome domain was identical and crystal contacts independent. Analyses of anomalous signal in the Cu and Fe sites confirmed correct Cu and Fe incorporation.

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