

Characterization of the active site and calcium binding in cytochrome *c* nitrite reductases

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Abstract

The decahaem homodimeric cytochrome *c* nitrite reductase (NrfA) is expressed within the periplasm of a wide range of Gamma-, Delta- and Epsilon-proteobacteria and is responsible for the six-electron reduction of nitrite to ammonia. This allows nitrite to be used as a terminal electron acceptor, facilitating anaerobic respiration while allowing nitrogen to remain in a biologically available form. NrfA has also been reported to reduce nitric oxide (a reaction intermediate) and sulfite to ammonia and sulfide respectively, suggesting a potential secondary role as a detoxification enzyme. The protein sequences and crystal structures of NrfA from different bacteria and the closely related octahaem nitrite reductase from *Thioalkalivibrio nitratireducens* (TvNir) reveal that these enzymes are homologous. The NrfA proteins contain five covalently attached haem groups, four of which are bis-histidine-co-ordinated, with the proximal histidine being provided by the highly conserved CXXCH motif. These haems are responsible for intraprotein electron transfer. The remaining haem is the site for nitrite reduction, which is ligated by a novel lysine residue provided by a CXXCK haem-binding motif. The TvNir nitrite reductase has five haems that are structurally similar to those of NrfA and three extra bis-histidine-coordinated haems that precede the NrfA conserved region. The present review compares the protein sequences and structures of NrfA and TvNir and discusses the subtle differences related to active-site architecture and Ca²⁺ binding that may have an impact on substrate reduction.

Active-site conservation among cytochrome *c* nitrite reductases

The active-site cavity architectures of the decahaem homodimeric cytochrome *c* nitrite reductase NrfA and octahaem nitrite reductase from *Thioalkalivibrio nitratireducens* (TvNir) are dominated by a catalytic triad of arginine, tyrosine and histidine residues (Figure 1A). These residues are highly conserved in all NrfA sequences and are believed to be important in the successful binding and reduction of substrate [1–6]. A key variation of the NrfA and TvNir active sites was revealed in the crystal structure; TvNir contains a unique covalent modification between Tyr³³¹ and Cys³³³ [6] (Figure 1A). This modification cannot occur within NrfA enzymes as the cysteine residue is not present, and it is still unclear how this modification affects substrate reduction.

The structures of NrfA solved so far only represent a small fraction of the total number of different NrfA enzymes expressed in different proteobacteria. The variation of NrfA in different proteobacteria can be seen through the comparison of protein sequences. This has previously revealed that some Campylobacteraceae such as *Campylobacter jejuni* encode an NrfA protein that varies from the archetypal sequence [7]. These NrfA enzymes lack the novel CXXCK motif and

instead maintain the common CXXCH motif. The other four haem motifs and active-site residues are conserved. It is, however, not clear whether the NrfA protein from these *Campylobacter* species function with an active-site haem ligated with a histidine residue or whether, during protein folding, the histidine ligation is prevented and a lysine residue from another region of the protein is provided as the ligand. This proposed advantageous lysine ligation can be seen in the crystal structure of an octahaem tetrathionate/nitrite reductase from *Shewanella oneidensis* [7,8].

The NrfA sequence from one of the *Campylobacter* species, *Campylobacter rectus* (draft genome 2009, NZ_ACFU00000000) identified the *nrfA* gene as lacking the novel CXXCK motif and the other conserved active-site residues while maintaining the other haem-binding motifs (Figure 2). This would indicate that the overall structure of NrfA is maintained, but that the active site is likely to be very different. Early characterization studies looking at the growth of *C. rectus* suggest that it is a microaerobic bacterium unable to grow on nitrite [9]. These data could suggest that the NrfA is not functioning as a nitrite reductase, but instead has adapted to perform a different role. The most likely role would be that of a nitric oxide detoxifier, as native NrfA has been reported to reduce nitric oxide as a substrate and to provide some tolerance to nitric oxide exposure *in vivo* [10,11]. This adaptation could help *C. rectus* to survive in its natural environment, which includes the oral cavity, where it contributes to periodontal disease, and within the circulatory system, where it has been reported to be present in arterial

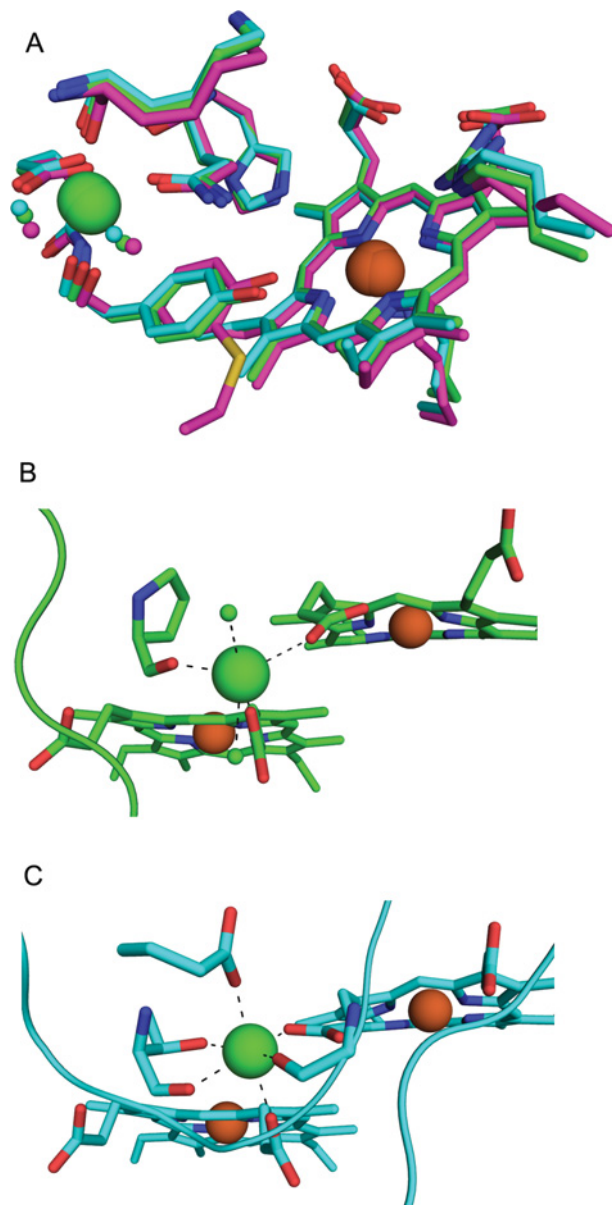
Key words: calcium binding, cytochrome *c* nitrite reductase, multihaem cytochrome.

Abbreviations used: NrfA, decahaem cytochrome *c* nitrite reductase; TvNir, octahaem nitrite reductase from *Thioalkalivibrio nitratireducens*; PFV, protein film voltammetry.

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Figure 1 | Comparison of Ca^{2+} (green sphere)-binding sites of NrfA from *E. coli* PDB code 2RDZ (green), *D. desulfuricans* PDB code 10AH (cyan) and TvNir from *T. nitratireducens* PDB code 3MMO (pink)

Small spheres represent conserved water molecules. (A) Active-site Ca^{2+} ion, showing active-site haem and Lys¹²⁶, Arg¹⁰⁶, Tyr²¹⁶, His²⁶⁴, Gln²⁶³, Glu²¹⁵ and Lys²⁶¹ (*E. coli* numbering). (B) Distal Ca^{2+} of *E. coli* showing haems 3 and 4 and Pro⁹¹. (C) Distal Ca^{2+} of *D. desulfuricans* showing haems 3 and 4, the presence of a protein loop which is absent from *E. coli* NrfA and TvNir (see the text) and Glu¹¹⁴, Thr¹¹⁵ and Gly⁷⁵ (*D. desulfuricans* numbering).



wall plaques [12,13]. Within the mouth, it is likely to come into contact with differing concentrations of free NO released to combat infection and from dietary nitrite, whereas, on systemic invasion, the bacterium is likely to be targeted by phagocytes. This would expose the bacteria to high levels of NO and other reactive species. This hypothesis of the

function of *C. rectus* NrfA is based only on a small number of observations and more work is needed to characterize these novel NrfA proteins.

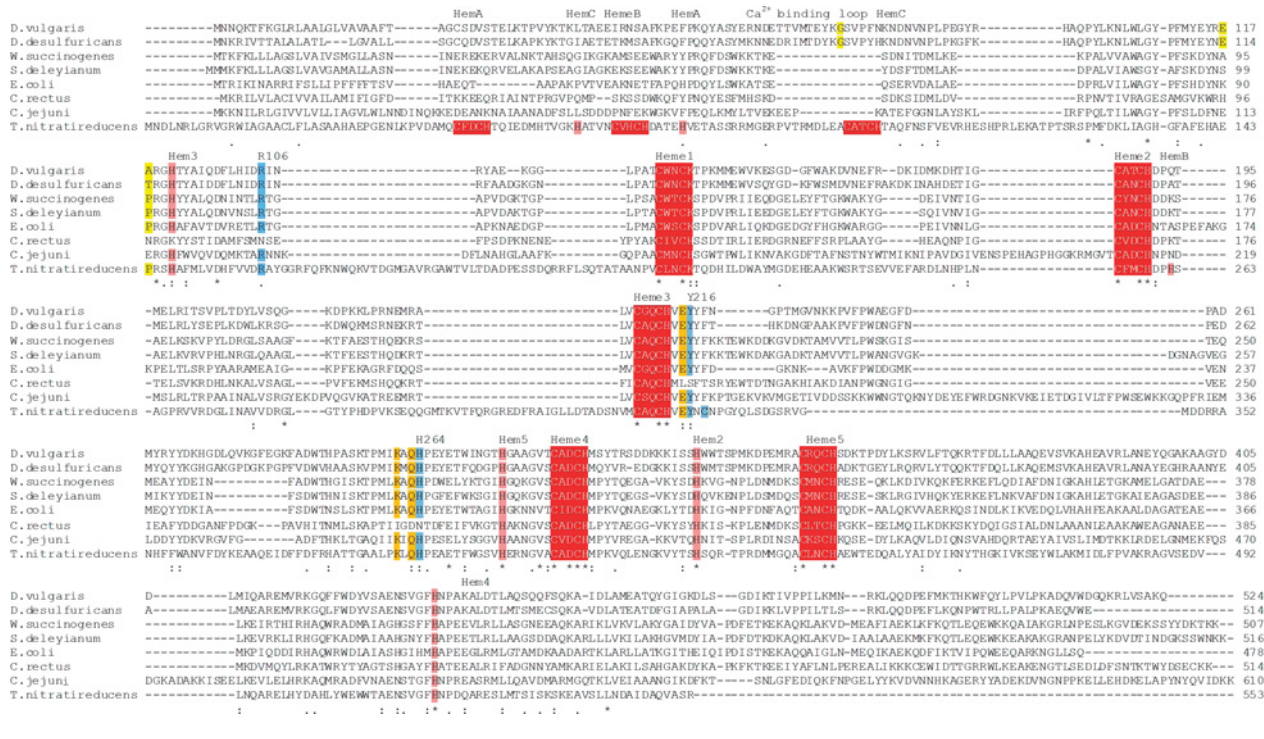
The conserved residues that maintain the active-site cavity have been well documented for their importance in NrfA-mediated nitrite reduction; however, their exact functions are still not clear [14]. A number of studies have used mutagenesis to begin to elucidate the roles of some of these residues. Tyr²¹⁶ was characterized by Lukat et al. [15] in *Wolinella succinogenes* NrfA. The hydroxy group of the tyrosine interacts with a network of conserved active-site water molecules. Mutation of the tyrosine residue to phenylalanine removed the hydroxy group while maintaining the aromatic ring, resulting in minimal disruption of the protein structure. The changes within the active site resulted in a decrease in activity, with the mutated enzyme maintaining only 0.7% activity when compared with that of the native protein. The study used crystallography to identify the structural implication of the mutation and measured the production of ammonia to determine the activity [15]. The structure revealed that the removal of the hydroxy group caused a change in the water network within NrfA, but had a limited impact on the orientation of the bound nitrite substrate. The findings of the study concluded that the mutation had removed the ability of the enzyme to reduce nitrite to ammonia [15]. However, it is unknown whether nitrite could be reduced to another compound such as NO, hydroxylamine or nitrous oxide.

Clarke et al. [16] carried out a study looking at the importance of Gln²⁶³ in *Escherichia coli*. This residue forms a ligand via its amine oxygen to the active-site Ca^{2+} ion. To understand the role of the glutamine residue, it was mutated to glutamate. These amino acids are similar in the length of side chain, but glutamine has an uncharged amide group which can form monodentate ligation, whereas glutamate has a carboxylic acid functional group which is negatively charged at neutral pH and can potentially form bidentate ligation. The crystal structure of the Q263E mutant revealed that the overall structure of NrfA was unaffected by the mutation, with the glutamate residue maintaining the same orientation and monodentate ligation to the Ca^{2+} ion as the native glutamine residue. The mutation did, however, cause a disruption to the conserved water residues. The study found that the mutated enzyme was still able to carry out the ammonification of nitrite at a similar rate to wild-type NrfA. This was reflected by the same V_{max} for wild-type NrfA and the Q263E mutant, but a 10-fold increase in K_m in the Q263E mutant, suggesting that the active site has lower affinity for nitrite. The PFV (protein film voltammetry) data also revealed that catalysis occurs at lower potentials in the mutated NrfA compared with the wild-type. These observations suggests that the addition of a negative charge near the vicinity of the nitrite anion reduces substrate-binding affinity and may also cause a lowering of the active-site haem reduction potential such that a greater driving force is required to observe catalysis.

The two studies of NrfA variants reported to date would indicate that not only are the conserved residues important

Figure 2 | An alignment of NrfA protein sequences from solved NrfA structures and NrfA sequences which are not archetypal (structures are unknown) from *C. jejuni* and *C. rectus*

CXXCH, cytochrome *c*-binding motif; H, distal histidine haem ligand; Q, residue involved in active-site Ca²⁺ ligation, E, residue involved in distal haem ligation; R, active-site mutated residue (annotated with *E. coli* residue numbering). Haem-binding motifs numbered Heme1-Heme5 as they appear in the NrfA sequence and distal haem ligands are numbered Hem1-Hem5. The three TvNir haems that precede the NrfA conserved sequence are labelled HemeA-HemeC and distal haem ligand HemA-HemC.



in enzyme function, but also so are the conserved water molecules within the active site.

Calcium and the nitrite reductases

The crystal structure of NrfA purified from a number of bacteria and that of TvNir has revealed that each monomer contains two Ca²⁺ ions [3]. One of these Ca²⁺ ions has conserved co-ordination in all NrfA and TvNir structures to date. The Ca²⁺ ion is positioned ~11 Å (1 Å = 0.1 nm) away from the active-site iron, buried within the protein. It is octahedrally co-ordinated by a glutamine and a glutamate side chain and carbonyl groups provided by a lysine residue and a tyrosine residue, as well as two water molecules [16] (Figure 1A). The Ca²⁺ ion is too distant from the active site to play a direct role in catalysis. It is believed to have a number of important functions, including the stabilization of the active-site cavity and the tuning of the reduction potential of the active-site haem by the introduction of a positive charge.

The second Ca²⁺ ion is more distant from the active site, being positioned ~14 Å away. The ligation of this Ca²⁺ ion varies in the known NrfA and TvNir structures (Figures 1B and 1C). The NrfA structures from Gamma- and Epsilon-proteobacteria exemplified by the *E. coli* and *W. succinogenes*

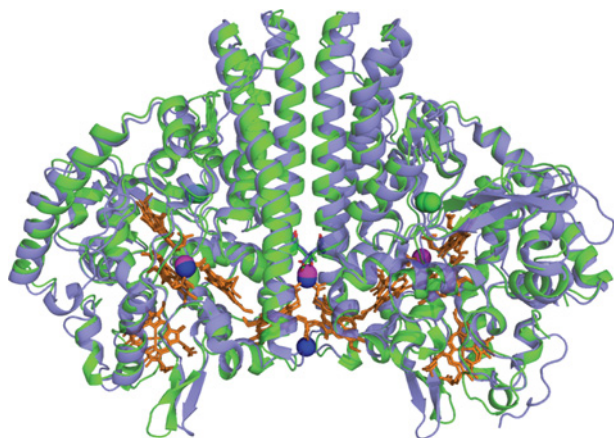
show that the Ca²⁺ ion is ligated by a propionate group from haems 3 and 4, a carbonyl group from Pro⁹¹ and between two and three water molecules, suggesting that this Ca²⁺ ion is most likely to be octahedrally co-ordinated [5,16]. This ligation pattern is mirrored in the structure of TvNir, with Pro¹⁴⁴ providing the carbonyl oxygen ligand [8].

The second example of Ca²⁺ ligation is seen in Deltaproteobacteria. The structure of NrfA from *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* reveals that the Ca²⁺ ion is positioned in the same location and remains ligated by propionate groups of haems 3 and 4, as seen in *E. coli* and *W. succinogenes*. However, unlike *E. coli* and *W. succinogenes*, the *D. vulgaris* and *D. desulfuricans* Ca²⁺ ion is not ligated by any water molecules, instead the Ca²⁺ ion has a co-ordination sphere that is entirely protein-derived. These extra ligands are provided by a single carboxy group from glutamate, hydroxy and carbonyl groups of a threonine residue (in *D. vulgaris*, only the carbonyl is present from an alanine residue) and the carbonyl from a glycine residue which is found within a protein loop that is absent from *E. coli* and *W. succinogenes* NrfA, and TvNir [3,17] (Figures 1A and 1B).

The function of the second Ca²⁺ ion is hypothesized to be related to the tuning of the reduction potentials of haems 3 and 4. This seems the most likely function as the haems are

Figure 3 | Overlay of NrfA from *W. succinogenes* (PDB code 3BNG, light blue) and *E. coli* (PDB code not yet available, light green)

Haem cofactors are shown in orange, four Ca²⁺ ions are green, three Y³⁺ ions are in purple and four Eu²⁺ ions are in blue.



co-ordinated with the Ca²⁺ ion and the Ca²⁺–iron distances are only ~10 and ~11 Å respectively. In the crystal structures, the distal Ca²⁺ site is not always fully occupied. It is absent from some structures, and in others, such as *W. succinogenes* (PDB code 3BNG) and *E. coli* (PDB code 3TOR), it has been replaced by the trivalent Y³⁺ and the divalent Eu²⁺ ions respectively; in both structures, the active-site Ca²⁺ ion is maintained, suggesting that the distal Ca²⁺ ion is more mobile than the active site Ca²⁺ ion [1,15] (Figure 3). The *W. succinogenes* and *E. coli* structures also reveal binding of Y³⁺ and Eu²⁺ ions at the interface of the NrfA dimer where they are co-ordinated by the propionates of haem 5. These observations could suggest that, *in vivo*, the transient binding of cations could stabilize the negative charge of the solvent-exposed propionates.

The effect of low Ca²⁺ concentrations on the catalytic activity of *E. coli* NrfA has been probed by the use of PFV. This technique revealed that, under conditions of limited Ca²⁺, inhibition of nitrite reduction occurred at high substrate concentration (>380 μM) and low potentials. The inhibition seen at low Ca²⁺ concentration was not relieved by the addition of Mg²⁺ ions, suggesting that maximal activity can only be reached in the presence of Ca²⁺ (>0.5 mM) [18]. It has also been reported that NrfA activity is inhibited by the presence of Zn²⁺ ions, with a review by Clarke et al. [19] reporting that a concentration of 50 μM ZnCl₂ caused a ~90% decrease in nitrite reductase activity. These data suggest that the solvent-exposed Ca²⁺ ion may be replaced by other divalent cations, causing a decrease in activity. The sensitivity of NrfA activity to ion replacement could be the reason the Deltaproteobacteria such as *Desulfovibrio* sp. have maintained or evolved the protein loop structure in these enzymes. The loop allows the Ca²⁺–co-ordination sphere to be filled by protein-derived ligands, rather than a combination of protein residues and water molecules as seen for the *E. coli* NrfA. This co-ordination may have higher

affinity for a Ca²⁺ ion over other divalent cations and thus protect the enzyme from inactivation.

Conclusions

The comparison of different NrfA and TvNir sequences and structures reveal enzymes that contain highly conserved residues such as those that form the catalytic triad of the active site. These residues seem pivotal to the efficient functioning of these enzymes. The continual study of these residues will allow greater understanding of the role they play in nitrite reduction. The comparison has also shown divergence in the protein sequence and structure of the NrfA enzymes that may indicate different characteristics or functions for those enzymes.

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