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

Colin W.J. Lockwood, Thomas A. Clarke[1], Julea N. Butt, Andrew M. Hemmings and David J. Richardson[1]
Centre for Molecular and Structural Biochemistry, School of Chemistry and School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

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 Ca^{2+} 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 Tyr^{331} and Cys^{333} [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
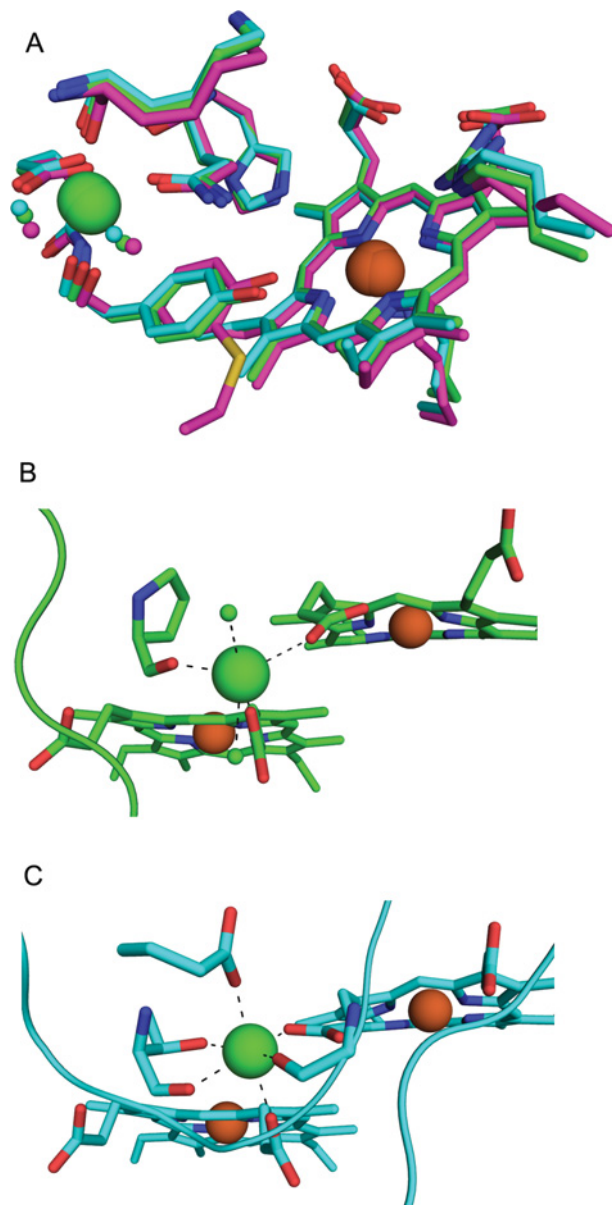
Figure 1 | Comparison of Ca^{2+} (green sphere)-binding sites of NrfA from *E. coli* PDB code 2RDZ (green), *D. desulfuricans* PDB code 1OAH (cyan) and TvNir from *T. nitratireducens* PDB code 3MMO (pink)

Small spheres represent conserved water molecules. (**A**) Active-site Ca^{2+} ion, showing active-site haem and Lys[126], Arg[106], Tyr[216], His[264], Gln[263], Glu[215] and Lys[261] (*E. coli* numbering). (**B**) Distal Ca^{2+} of *E. coli* showing haems 3 and 4 and Pro[91]. (**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 Glu[114], Thr[115] and Gly[75] (*D. desulfuricans* numbering).

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. Tyr[216] 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 Gln[263] 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

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

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 proceed the NrfA conserved sequence are labelled HemeA–HemeC and distal haem ligand HemA–HemC.

```
                                  HemA            HemC HemeB    HemA         Ca²⁺ binding loop HemC
D.vulgaris       ----------MNNQKTFKGIRLAALGLVAVAAFT------AGCSDVSTELKTPVYKTKLTAEEIRNSAFKPEFFKQYASYERNDETTVMTEYKGSVPFNKDNDNVNPLPEGYR-----------HAQPYLKNLWLGY-PFMYEYRE 117
D.desulfuricans  ----------MNKRIVTTAIALATL---LGVALL------SGCQDVSTELKAPKYKTGIAETETKMSAFKGQFPQQYASYMKNNEDRIMTDYKGSVPYHKDNDNVNPLPKGFK-----------HAQPYLKNLWLGY-PFMYEYNE 114
W.succinogenes   ----------MTKFFKLLLAGSLVAIVSMGLLASN-----INEREKERVALNKTAHSQGIKGKAMSEEWARYYFRQFDSWKKTKE-------------SDNITDMLKE------------------KPALVVAWAGY-PFSKDYNA 95
S.deleyianum     ----------MMMKFKLLLAGSLVAVGAMALLASN-----INEKEKQRVELAKAPSEAGIAGKEKSEEWAKYYFRQFDSWKKTKE-------------YDSFTDMLAK-------------------DPALVLAWAGY-AFSKDYNS 99
E.coli           ----------MTRIKINARRIFSLLIPFFFFTSV------HAEQT-------AAPAKPVTVEAKNETFAPQHPDQYLSWKATSE---------------QSERVDALAE------------------DPRLVILWAGY-PFSHDYNK 90
C.rectus         ----------MKRILVLACIVVAILAMIFIGFD-------ITKKEEQRIAINTPRGVPQMP--SKSSDWKQFYINQYESFMHSKD----------------SDKSIDMLDV------------------RPNVTIVRAGESAMGVKWRH 96
C.jejuni         ----------MKKNILRLGIVVLVLLIAGVLWLNNDINQKKEDEANKNAIAANADFSLLSDDDPNFEKWGKVFPEQLKMYLTVEKEEP---------KATEFGGNLAYSKL------------IRFPQLTILWAGY-PFSLDFNE 113
T.nitratireducens MNDLNRLGRVGRWIAGAACLFLASAAHAEPGENIKPVDAMQCFICHTQIEDMHTVGKHATVNCVHCHDATEHVETASRRMGERPVTRMDLEACATCHTAQFNSFVEVRHESHPRLEKATPTSRSPMFDKLIAGH-GFAFEHAE 143

                 Hem3      R106                                                          Heme1                                                            Heme2 HemB
D.vulgaris       ARGHTYAIQDFLHIDRIN------------------RYAE--KGG----------LPATCWNQTPKMMEWVKESGD-GFWAKDVNEFR--DKIDMKDHTIG----------------------------------PAD 261
D.desulfuricans  TRGHTYAIDDFLNIDRIN------------------RFAADGKGN---------LPATCWNQTPKMMEWVSQYGD-KFWSMDVNEFRAKDKINAHDETIG---------------------------------PED 262
W.succinogenes   PRGHYYALQDINITLRTG------------------APVDGKTGP---------LPSACWTCSPDVFRIIEQDGELEYFTGKWAKYG-----DEIVNTIG------------------------------------TEQ 250
S.deleyianum     PRGHYYALQDVNNSLRTG------------------APVDAKTGP---------LPTACWTCSPDVFRLIEEDGELEYFTGKWAKYG----SQIVNNVIG---------------------------------DGNAGVEG 257
E.coli           PRGHAFAVTDVRETLRTG------------------APKNAEDGP---------LPMACWSCKSPDVARLIQKDGEDGYFHGKWARGG------PEIVNNLG---------------------------------VEN 237
C.rectus         NRGKYYSTIDAMFSMNSE------------------FPSDPKNENE--------YPYAKCIVCSSDTIRLIERDGRNEFFSRPLAAYG----HEAQNPIG------------------------------------VEE 250
C.jejuni         ERGHHWVQVDQMKTARNNK-----------------DFLNAHGLAAFK------GQPAACMNCSGWTFWLIKNVAKGDFTAFNSTNYWTMIKNIPAVDGIVENSPEHAGPHGGKRMGVTCADCNPND------------RRA 336
T.nitratireducens PRSHAFMLVDHFVVDRAYGGRFQFKNWQKVTDGMGAVRGAWTVLTDADPESSDQRRFLSQTATAANPVCLNCHQDHILDWAYMGDEHEAAKWSRTSEVVEFARDLNHPLN------------------CFMICDPPBS----- 263

                                              Heme3  Y216
D.vulgaris       -MELRITSVPLTDYLVSQG-----KDPKKLPRNEMRA-----------------LVSGCEVEYYFN------GPTMGVNKKPVFPWAEGFD-----------------------------------PAD 261
D.desulfuricans  -MELRLYSEPLKDWLKRSG-----KDWQKMSRNEKRT----------------LVTGCEVEYYFT------HKDNGPAAKPVFPWINGFN----------------------------------PED 262
W.succinogenes   -AELKSKVPLYLDRGLSAAGF----KTFAESTHQEKRS---------------LPSACEVEYFKKTEWKDIKGVDKTAMVVTLPWSKGIS----------------------------------TEQ 250
S.deleyianum     -AELKRVVPHLNRGLQAAGL----KTFEESTHQDKRT----------------LPTACEVEYFKKTEWKDAKGADKTAMVVTLPWANGVGK-----------------------------DGNAGVEG 257
E.coli           KPELTLSRPYAARAMEAIG------KPFEKAGRFDQQS---------------MVTGCEVEYYFD------GKNK---AVKFPWDDGMK-----------------------------------VEN 237
C.rectus         -TELSVKRDHLNKALVSAGL----KTFEESTHQQKRT---------------FLTAACEVEYYAKCIVCSSDTLSFTSRYEWTDTNGAKHIAKDIANPWGNGIG-------------------VEE 250
C.jejuni         -MSLRLTRPAAINALVSRGYEKDPVQGVKATREEMRT----------------LVPGCEVEYYFKPTGEKVKVMGETIVDDSSKKWWMGTQKNYDEYEFWRDGNKVKEIETDGIVLTFPWSEWKKGQPFRIEM 336
T.nitratireducens -AGPRVVRDGLINAVVDRGL-----GTYPHDPVKSEQQGMKVTFQRGREDFRAIGLLDTADSNVMAACEVEYNGNPGYQLSDGSRVG------------------------------------MDDRRA 352

                      H264        Hem5  Heme4                               Heme2              Heme5
D.vulgaris       MYRYYDKHGDLQVKGFEGKFADWTHPASKTPMLKAQHPEYETWINGTHGAAGVTTADCHMSYTRSDDKKKISSHWWTSPMKDPEMRARCRQCHSDKTPDYLKSRVLFTQKRTFDLLLAAQEVSVKAHEAVRLANEYQGAKAAGYD 405
D.desulfuricans  MYQYYKGHGAKGPDGKPGPFVDWVHAASKVPMIRMQHPEYETFQDGIHGVGVR-EDGKKISSHWMTSPMKDPEMRARCRDCHADKTGEYLRQRVLYTQQKTFDQLLKAQEMSVKAHEAVRLANAYEGHRAANYE 405
W.succinogenes   MEAYYDEIN----------FADWTHGISKTPMLKAQHPIDWELYKTGIHGQKGVSFADCHMPYTQEGA-VKYSDIKVG-NPLDNMDKSTMNCHRESE-QKLKDIVKQKFERKEFLQDIAFDNIGKAHIETGKAMELGDATDAE--- 378
S.deleyianum     MIKYYDEIN----------FSDWINISKTPMLKAQHPGFEFWKSGIHGQKGVSFADCHMPYTQEGS-VKYSDIQVKNENPLDSMDQSTMNCHRESE-SKLRGIVHQKYERKEFLNKVAFDNIGKAHIETGKAIEAGASDEE--- 386
E.coli           MEQYYDKIA----------FSDWINSLSKTPMLKAQHPEYETWTAGIHGKNNVTTIDCHMPKVQNNAEGKLYTIHKIG-NPFDNFAQTTANCHTQDK-AALQKVAERKQSINDLKIKVEDQLVHAHFEAKAALDAGATEAE--- 366
C.rectus         IEAFYDDGANFPDGK---PAVHITNMLSKAPTIIGDNTDFEIFVKGTHAKNGVSTADCHLPYTAEGG-VKYSDIKIS-KPLENMDKSTLTCHELMQILKDKSKYDQIGSIALDNLAAANIEAAKAWEAGANAEE--- 385
C.jejuni         LDDYYDKVRGVFG------ADFTHKLTGAQIIKIQHPESELYSGGVHAANGVSSVDCHMPYVREGA-VKYTQHNIT-SPLRDINSACSCHQSE-DYLKAQVLDIQNSVAHDQRTAEYAIVSLIMDTKKIRDELGNMEKFQS 470
T.nitratireducens NHFFWANVFDYKEAAQEIDFFDFHATTGAALPKLQHPEAETFWGSVHERNGVATADCHMPKVQLENGKVYTSQR-TPRDMMGQATLNCHAEWTEDQALYAIDYIKNYTHGKIVKSEYWLAKMIDLFPVAKRAGVSEDV--- 492

                                                Heme4
D.vulgaris       D----------LMIQAREMVRKGQFFWDYVSAENSVGFFNPAKALDTLAQSQQFSQKA-IDLAMEATQYGIGKDLS---GDIKTIVPPILKMN---RKLQQDPEFMKTHKWFQYLPVLPKADQVWDGQKRLVSAKQ--------- 524
D.desulfuricans  A----------LMAEAREMVRKGQLFWDYVSAENSVGFFNPAKALDTLMTSMECSQKA-VDLATEATDFGIAPALA---GDIKKLVPPILTLS---RKLQQDPEFLKQNPWTRLLPALPKAEQVWE------------------- 514
W.succinogenes   ----------LKEIRTHIRHAQWRADMAIAGHGSFFIAPEEVLRLLASGNEEAQKARIKLVKVLAKYGAIDYVA-PDFETKEKAQKLAKVD-MEAFIAEKLKFKQTLQEEWKKQAIAKGRLNPESLKGVDEKSSYYDKTKK--- 507
S.deleyianum     ----------LKEVRKLIRHGQFKADMAIAAHGNYFIAPEETLRLLAAGSDDAQKARIKLVKIENGK-VKD-IAALAAEKMKFKQTLQEEWKKKEAKAKGRANPELYKDVDTINDGKSSWNKK--- 516
E.coli           ----------MKPIQDDIRHAQRWRWDIAIASHGIHMFIAPEEGLRMLGTAMDKAADARTKLARLLATKGITHEIQIFDISTKEKAQQAIGLN-MEQIKAEKQDFIKTVIPQNEEQARKNGLLSQ---------- 478
C.rectus         ----------MKDVMQYLRKATWRYTYAGTSHGAYFBATEEALRIFADGNNYAMKARIKKKCEWIDTGRRWLKEAKENGTLSEDLDFSNTKTWYDSECKK--- 514
C.jejuni         DGKADAKKISEELKEVLELHRKAQMRADFVNAENSTGFFNPEASRMLLQAVDMARMGGTKLVEIAAANGIKDFKT---SNLGFEDIQKFNPGLYYKVDVNNHKAGERYADEKDVNGNPPKELLEHDKELAPYNYQVIDKK 610
T.nitratireducens -----------LNQARELHYDAHLYWEWWTAENSVGFFNPDQARESLMTSISKSKEAVSLLNDAIDAQVASR------------------- 553
```

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 Ca^{2+} ions are green, three Y^{3+} ions are in purple and four Eu^{2+} ions are in blue.

co-ordinated with the Ca^{2+} ion and the Ca^{2+}–iron distances are only ~10 and ~11 Å respectively. In the crystal structures, the distal Ca^{2+} 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 Y^{3+} and the divalent Eu^{2+} ions respectively; in both structures, the active-site Ca^{2+} ion is maintained, suggesting that the distal Ca^{2+} ion is more mobile than the active site Ca^{2+} ion [1,15] (Figure 3). The *W. succinogenes* and *E. coli* structures also reveal binding of Y^{3+} and Eu^{2+} 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 Ca^{2+} 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 Ca^{2+}, inhibition of nitrite reduction occurred at high substrate concentration (>380 μM) and low potentials. The inhibition seen at low Ca^{2+} concentration was not relieved by the addition of Mg^{2+} ions, suggesting that maximal activity can only be reached in the presence of Ca^{2+} (>0.5 mM) [18]. It has also been reported that NrfA activity is inhibited by the presence of Zn^{2+} ions, with a review by Clarke et al. [19] reporting that a concentration of 50 μM $ZnCl_2$ caused a ~90% decrease in nitrite reductase activity. These data suggest that the solvent-exposed Ca^{2+} 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 Ca^{2+}-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 Ca^{2+} 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.

Funding

References

1 Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G.P., Bartunik, H.D., Huber, R. and Kroneck, P.M.H. (1999) Structure of cytochrome c nitrite reductase. Nature **400**, 476–480

2 Bamford, V.A., Angove, H.C., Seward, H.E., Thomson, A.J., Cole, J.A., Butt, J.N., Hemmings, A.M. and Richardson, D.J. (2002) Structure and spectroscopy of the periplasmic cytochrome c nitrite reductase from *Escherichia coli*. Biochemistry **41**, 2921–2931

3 Cunha, C.A., Macieira, S., Dias, J.M., Almeida, G., Goncalves, L.L., Costa, C., Lampreia, J., Huber, R., Moura, J.J.G., Moura, I. and Romão, M.J. (2003) Cytochrome c nitrite reductase from *Desulfovibrio desulfuricans* ATCC 27774: the relevance of the two calcium sites in the structure of the catalytic subunit (NrfA). J. Biol. Chem. **278**, 17455–17465

4 Rodrigues, M.L., Oliveira, T.F., Pereira, I.A.C. and Archer, M. (2006) X-ray structure of the membrane-bound cytochrome c quinol dehydrogenase NrfH reveals novel haem coordination. EMBO J. **25**, 5951–5960

5 Einsle, O., Stach, P., Messerschmidt, A., Simon, J., Kröger, A., Huber, R. and Kroneck, P.M.H. (2000) Cytochrome c nitrite reductase from *Wolinella succinogenes*: structure at 1.6 Å resolution, inhibitor binding, and heme-packing motifs. J. Biol. Chem. **275**, 39608–39616

6 Polyakov, K.M., Boyko, K.M., Tikhonova, T.V., Slutsky, A., Antipov, A.N., Zvyagilskaya, R.A., Popov, A.N., Bourenkov, G.P., Lamzin, V.S. and Popov, V.O. (2009) High-resolution structural analysis of a novel octaheme cytochrome c nitrite reductase from the haloalkaliphilic bacterium *Thioalkalivibrio nitratireducens*. J. Mol. Biol. **389**, 846–862

7 Klotz, M.G., Schmid, M.C., Strous, M., Op den Camp, H.J.M., Jetten, M.S.M. and Hooper, A.B. (2008) Evolution of an octahaem cytochrome c protein family that is key to aerobic and anaerobic ammonia oxidation by bacteria. Environ. Microbiol. **10**, 3150–3163

8 Mowat, C.G., Rothery, E., Miles, C.S., McIver, L., Doherty, M.K., Drewette, K., Taylor, P., Walkinshaw, M.D., Chapman, S.K. and Reid, G.A. (2004) Octaheme tetrathionate reductase is a respiratory enzyme with novel heme ligation. Nat. Struct. Mol. Biol. **11**, 1023–1024

9 Han, Y.H., Smibert, R.M. and Krieg, N.R. (1991) *Wolinella recta, Wolinella curva, Bacteroides ureolyticus*, and *Bacteroides gracilis* are microaerophiles, not anaerobes. Int. J. Syst. Bacteriol. **41**, 218–222

10 Poock, S.R., Leach, E.R., Moir, J.W.B., Cole, J.A. and Richardson, D.J. (2002) Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of *Escherichia coli*. J. Biol. Chem. **277**, 23664–23669

11 Pittman, M.S., Elvers, K.T., Lee, L., Jones, M.A., Poole, R.K., Park, S.F. and Kelly, D.J. (2007) Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. Mol. Microbiol. **63**, 575–590

12 Macuch, P.J. and Tanner, A.C.R. (2000) *Campylobacter* species in health, gingivitis, and periodontitis. J. Dent. Res. **79**, 785–792

13 Marcelino, S.L., Gaetti-Jardim, E., Nakano, V., Canônico, L.A.D., Nunes, F.D., Lotufo, R.F.M., Pustiglioni, F.E., Romito, G.A. and Avila-Campos, M.J. (2010) Presence of periodontopathic bacteria in coronary arteries from patients with chronic periodontitis. Anaerobe **16**, 629–632

14 Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P.M.H. and Neese, F. (2002) Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase. J. Am. Chem. Soc. **124**, 11737–11745

15 Lukat, P., Rudolf, M., Stach, P., Messerschmidt, A., Kroneck, P.M.H., Simon, J. and Einsle, O. (2008) Binding and reduction of sulfite by cytochrome *c* nitrite reductase. Biochemistry **47**, 2080–2086

16 Clarke, T.A., Kemp, G.L., Van Wonderen, J.H., Doyle, R.M.A.S., Cole, J.A., Tovell, N., Cheesman, M.R., Butt, J.N., Richardson, D.J. and Hemmings, A.M. (2008) Role of a conserved glutamine residue in tuning the catalytic activity of *Escherichia coli* cytochrome *c* nitrite reductase. Biochemistry **47**, 3789–3799

17 Pereira, I.A.C., Legall, J., Xavier, A.V. and Teixeira, M. (2000) Characterization of a heme *c* nitrite reductase from a non-ammonifying microorganism, *Desulfovibrio vulgaris* Hildenborough. Biochim. Biophys. Acta **1481**, 119–130

18 Burlat, B., Gwyer, J.D., Poock, S., Clarke, T., Cole, J.A., Hemmings, A.M., Cheesman, M.R., Butt, J.N. and Richardson, D.J. (2005) Cytochrome *c* nitrite reductase: from structural to physicochemical analysis. Biochem. Soc. Trans. **33**, 137–140

19 Clarke, T.A., Mills, P.C., Poock, S.R., Butt, J.N., Cheesman, M.R., Cole, J.A., Hinton, J.C.D., Hemmings, A.M., Kemp, G., Söderberg, C.A.G. et al. (2008) *Escherichia coli* cytochrome *c* nitrite reductase NrfA. Methods Enzymol. **437**, 63–77