2008

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The role of multihaem cytochromes in the respiration of nitrite in *Escherichia coli* and Fe(III) in *Shewanella oneidensis*

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Abstract

The periplasmic nitrite reductase system from *Escherichia coli* and the extracellular Fe(III) reductase system from *Shewanella oneidensis* contain multihaem *c*-type cytochromes as electron carriers and terminal reductases. The position and orientation of the haem cofactors in multihaem cytochromes from different bacteria often show significant conservation despite different arrangements of the polypeptide chain. We propose that the decahaem cytochromes of the iron reductase system MtrA, MtrC and OmcA comprise pentahaem 'modules' similar to the electron donor protein, NrfB, from *E. coli*. To demonstrate this, we have isolated and characterized the N-terminal pentahaem module of MtrA by preparing a truncated form containing five covalently attached haems. UV–visible spectroscopy indicated that all five haems were low-spin, consistent with the presence of bis-His ligand co-ordination as found in full-length MtrA.

Introduction

Escherichia coli can catalyse the reduction of nitrate to ammonium in the periplasmic cellular compartment and couple this with energy-conserving respiratory electron transport. The process involves two enzymes: a periplasmic nitrate reductase (NapA) that converts nitrate into nitrite and a periplasmic cytochrome-*c* nitrite reductase (NrfA) that converts nitrite into ammonia. These anaerobic respiratory reactions are important for host invasion and colonization. They provide energy for ATP synthesis and also allow growth in micro-oxic environments, such as biofilms in lungs and in the intestinal-colonic tract, as well as reductive detoxification of reactive nitrogen species such as nitric oxide. While separate respiratory systems can be expressed for either anaerobic respiration or NO detoxification, the Nrf system has been shown to be involved in both. The Nrf system was originally shown to be involved in nitrite reduction, but more recently, the deletion of the *nrf* operon was shown to result in a susceptibility to NO in both *E. coli* [1] and *Salmonella enterica* serotype Typhimurium [2], resulting in a proposed physiological NO detoxification role. The importance of NapA and NrfA in anaerobic metabolism in enteric bacteria has led to structure–function studies of these enzymes that have yielded their crystal structures and a spectropotentiometric description of their redox centres [3,4]. This has then allowed for the development of mechanistic models of electron transfer and catalysis using protein film voltammetry [5–7].

Shewanella species are renowned for their incredible respiratory versatility and are reportedly able to use over 20 terminal electron acceptors for respiration [8]. These include metal oxide minerals, particularly those of Fe(III) and Mn(III/IV). Fe(III) mineral oxide (i.e. ferrihydrite and goethite) reduction is one of the most widespread respiratory processes in anoxic zones and has environmental significance, influencing several biogeochemical cycles. Fe(III) is highly insoluble in most environments at circumneutral pH and in the absence of strong complexing ligands and, unlike other terminal electron acceptors such as oxygen, nitrate and sulfate, it cannot freely diffuse into cells. For respiration of insoluble substrates, the bacterium must transfer electrons from the central cell metabolism to the Fe(III) oxide surface.

There are parallels in nitrite and Fe(III) respiration. Nitrite respiration in γ-proteobacteria such as *E. coli* involves two proteins, NrfA and NrfB, which together make up a 20-haem electron transfer periplasmic complex. In Fe(III) respiration, it is generally considered that electrons are transferred from hydroquinones in the inner membrane to a series of multihaem *c*-type cytochromes. These cytochromes transfer electrons from the periplasm to the extracellular side of the outer cell membrane and across the microbe/mineral oxidant interface [9]. Here, we explore the multihaem cytochromes in both processes and the evolutionary relationship between them.

The periplasmic nitrite reductase, NrfA

The *E. coli* NrfA protein is a 50 kDa pentahaem *c*-type cytochrome. It catalyses the six-electron reduction of nitrite to ammonium, but the putative reaction intermediates

Key words: *Escherichia coli*, MtrA, multihaem cytochrome, NrfA, NrfB, *Shewanella oneidensis*.
Abbreviations used: HAO, hydroxylamine oxidoreductase; STC, small tetrahaem cytochrome *c*.
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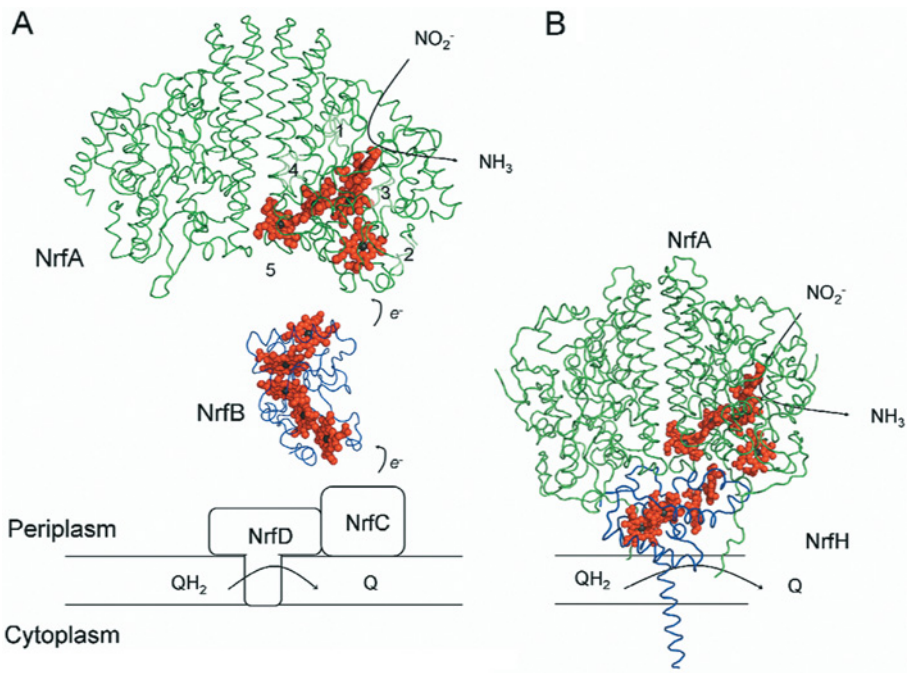
Figure 1 | A model of electron transport through the periplasmic nitrite reductases

(**A**) The electron transport chain of the Nrf system from *E. coli*. The structures of NrfA (green) and NrfB (blue) are shown as ribbons, with the haem cofactors from NrfB and one subunit of NrfA displayed as red spheres. The haems of NrfA are numbered 1–5 as described in the text. (**B**) One-half of the $NrfA_4$–$NrfH_2$ electron transport chain from *D. vulgaris*. The structure of NrfH (blue) and NrfA (green) are shown as ribbons with the haem cofactors of NrfH and one subunit of NrfA displayed as red spheres. Structures were prepared from PDB entries 2OZY, 2RDZ and 2J7A.



[nitric oxide ($NO^{\bullet}$) and hydroxylamine (NH_2OH)] in addition to sulfite, are alternative substrates, with ammonia and sulfide as products respectively. NrfA crystallizes as a homodimer, with each monomer containing five haems (Figure 1A). All haems have bis-His ligands, except the active site haem (haem 1), which is unusual as it is a high-spin species co-ordinated with a lysine residue on the proximal side and either water or nitrite on the distal side. The active site haem is attached to a novel CXXCK motif, whereas the other four haems are attached to the conventional CXXCH motifs [3].

Nearest-neighbour Fe–Fe distances throughout the NrfA monomer are below 13 Å (1 Å = 0.1 nm), close enough to facilitate transfer of electrons (Figure 1A). Structural analysis reveals that the only plausible site for NrfA–NrfB complex formation that would allow electron transfer is at the solvent-exposed edge of NrfA haem 2 [10]. A logical, natural, flow of electrons in NrfA would be along a decreasing E_m gradient, but haem 2 has the highest midpoint potential (−37 mV); it is thus being forced to transfer electrons to a lower potential haem (−107 mV). Electron transfer steps along increasing redox gradients are of course slower, but given that the distance between the two redox centres is small enough, transfer should still be faster than catalytic turnover, and not be a rate-limiting step. It has also been postulated that there can be intermolecular electron transfer in the NrfA homodimer. Haem 5 is only 5.7 Å away from its counterpart in the other monomer, potentially allowing transfer of electrons between monomers of NrfA [5].

Electron transport from hydroquinone dehydrogenases to NrfA

Despite the importance of periplasmic nitrite reduction to ammonium in enteric bacteria and the developing structure-informed biochemical understanding of the enzyme that catalyses this process, the nature of electron delivery from the hydroquinone pool has never been addressed structurally or spectroscopically. The key to coupling NrfA with energy conservation lies in the communication with the MQH_2 (menaquinol) pool and thereby electrogenic quinone reductases, such as formate dehydrogenase. Genetic studies suggest that hydroquinone oxidation is catalysed by an NrfC–NrfD complex [11] (Figure 1A), but there is no biochemical information on what would be a structurally novel iron–sulfur-dependent hydroquinone dehydrogenase complex available from any organism.

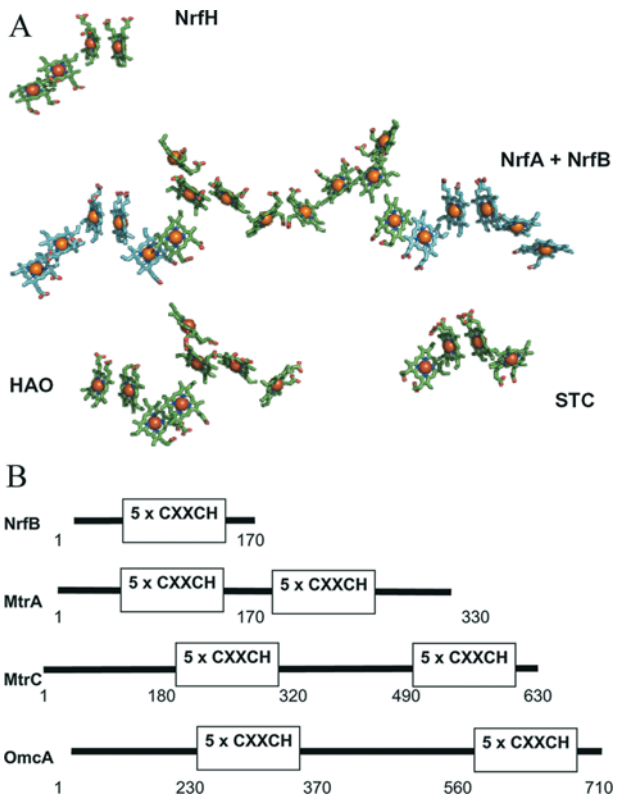
The mediator between the NrfC–NrfD complex and NrfA is the pentahaem NrfB protein, the structure of which has been resolved [10] and is unique among structurally solved multihaem *c*-type cytochromes. The haem groups are covalently attached to the polypeptide chain approximately every 25 amino acids, which results in a compact structure with a distance of less than 6 Å between neighbouring

haems. The arrangement of haems within the structure is such that the maximum edge-to-edge distance of the haem porphyrin rings across the NrfB molecule is 40 Å (Figure 1A). NrfA and NrfB form a reversible complex with an approximate dissociation constant of 10 nM [10], low enough to speculate that they exist as a heterodimer *in vivo*. Indeed, 500 nM dithionite-reduced NrfB rapidly oxidizes in the presence of nitrite and 5 nM NrfA, a concentration at which most of NrfA is monomeric ($K_d = 4\ \mu M$), but can nevertheless perform the reduction of nitrite in this reaction mixture. This suggests that although electrons may be able to flow through the NrfA homodimer interface, this may not be the primary electron path for nitrite reduction *in vivo*. Instead, electrons may primarily flow through a decahaem NrfA–NrfB heterodimer. In support of this hypothesis is the finding that nitrite reduction is catalysed at potentials reducing haem 1, 2 and 3 of NrfA [5], potentials where haem 4 and 5 would not formally be reduced.

In other bacteria, such as *Wolinella succinogenes* and the sulfate-respiring bacteria *Desulfovibrio desulfuricans*, the electron donor is NrfH, a membrane-associated tetrahaem cytochrome that directly mediates electron transfer from the hydroquinone pool to NrfA (Figure 1B). A recent solved structure of a *Desulfovibrio vulgaris* NrfA–NrfH complex showed an $\alpha_4\beta_2$ arrangement with two NrfA dimers bound to one NrfH dimer [12]. In contrast with the NrfA–NrfB complex, NrfH and NrfA form a very tight 'hard-wired' interaction. The crystal structure provides insights into this tight interaction as a monomer from each NrfA dimer provides a lysine ligand to a haem on the corresponding NrfH protein in the $(NrfA_2–NrfH)_2$ complex. Solution-state protein interaction studies strongly suggest that NrfA and NrfB form a 2:2 icosa-haem complex that will be structurally distinct from the $(NrfA_2–NrfH)_2$ complex [10].

The polypeptide chains of NrfB and the soluble domain of NrfH cannot be superimposed. Despite this, four of the NrfB haems adopt similar haem–haem packing motifs to the four NrfH haems (Figure 2A). However, two of these four superimposable haems have different haem ligations. In NrfB, all five haem irons are low-spin hexa-co-ordinate with bis-histinyl axial ligation, whereas in the $(NrfA_2–NrfH)_2$ complex one of the NrfH haem irons is penta-co-ordinate (and therefore likely to be high-spin) with a methionine distal ligand and an aspartate residue occupying the proximal position, but not within bonding distance. In addition, a fifth haem is present in the NrfB structure that is absent from NrfH. This haem is the most likely electron output site for NrfB and its presence makes the construction of an NrfAB complex model using an NrfAH structural template impossible. However, it is notable that the fifth NrfB haem position has equivalents in some other multihaem cytochromes. Most intriguingly, haems 1–5 of NrfA can be superimposed on to haems 4–8 of the octahaem HAO (hydroxylamine oxidoreductase) subunit and the remaining haems of HAO can be superimposed on to the haems of NrfB [7]. Thus the octahaem HAO protein, rather than the NrfH–NrfA complex, may provide a better conceptual template for a model for the arrangement of haems in the NrfA–NrfB complex. The first two haems of NrfB have no corresponding haems in the HAO structure and these two additional haems may provide the link to the hydroquinone-oxidizing NrfC–NrfD complex.

Figure 2 | Arrangement of haem motifs in multihaem cytochromes
(**A**) Comparison of the haem packing motifs of NrfB (PDB entry 2OZY) and NrfA (2RDZ) with NrfH (PDB entry 2J7A), HAO (1FGJ) and STC (1M1Q). The putative 20-haem NrfA–NrfB complex is shown in full, with the NrfB haems shown in blue and NrfA haems shown in green. (**B**) Cartoon of the polypeptides of *E. coli* NrfB and the *S. oneidensis* decahaem cytochromes, MtrA, MtrC and OmcA. The approximate start and stop positions of regions containing the CXXCH haem-binding motifs are shown.

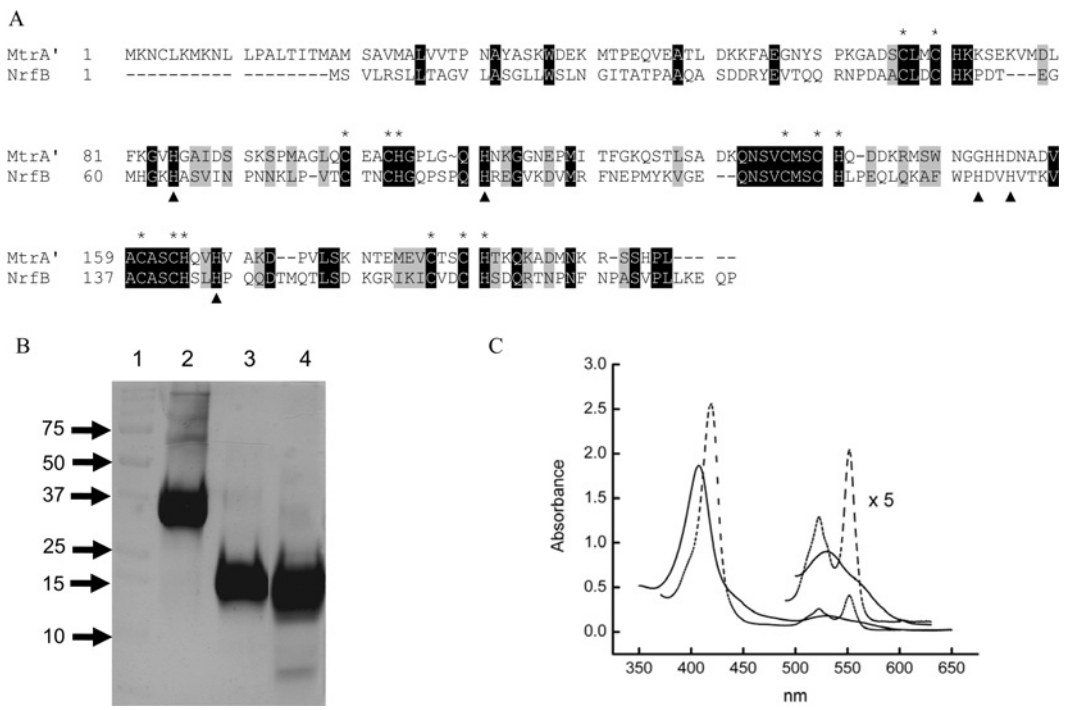


Multihaem cytochromes and Fe(III) respiration in *Shewanella oneidensis*

The *S. oneidensis* MR-1 genome encodes up to 42 putative *c*-type cytochromes [8], many of which are predicted to be multihaem cytochromes owing to the presence of multiple CXXCH motifs within their primary sequences. Haem groups are covalently attached to the cysteine residues in the mature protein and the histidine becomes a ligand to the haem iron. These 42 multihaem *c*-type cytochromes form a cytochrome *c* 'ome' that has attracted a great deal of interest recently, and the functions and structures of a number of the cytochromes have emerged. These include the tetrahaem hydroquinone dehydrogenase CymA [13,14]; the 'metal respiration' system OmcA–MtrCAB, which includes two outer membrane decahaem cytochromes (OmcA and MtrC), a periplasmic decahaem cytochrome (MtrA) and a putative

Figure 3 | The first 202 residues of MtrA from a soluble pentahaem domain

(**A**) Amino acid alignment of the first 202 amino acids of *S. oneidensis* MtrA with the pentahaem NrfB from *E. coli*. Identical residues are box-shaded black. Similar residues are box-shaded grey. The CXXCH motifs are indicated with asterisks (*), and histidine residues identified as distal haem ligands in NrfB are indicated with arrowheads. (**B**) Haem-stained SDS/polyacrylamide gel of multihaem cytochrome proteins. Lane 1, molecular mass markers; lane 2, the purified decahaem MtrA with a molecular mass of 38 kDa; lane 3, the purified pentahaem protein NrfB with a molecular mass of 18 kDa; lane 4, the purified pentahaem MtrA′. (**C**) Absorbance spectrum of MtrA′ in the oxidized (solid line) and sodium dithionite reduced (broken line) state.



outer membrane β-barrel protein (MtrB) [15–19]; the tetrahaem cytochrome domains of the structurally defined flavocytochrome *c* fumarate reductases (Fcc) [20]; multihaem nitrite reductases of the Nrf type that have already been described for *E. coli*; and a number of small periplasmic cytochromes such as the structurally defined STC (small tetrahaem cytochrome *c*) [21,22]. All of these multihaem cytochromes have in common that, where characterized, they operate in a rather low potential domain (~0 to −350 mV).

It is notable that in many *Shewanella* species the respirome is distributed on both the inner and outer membranes. The outer membrane cytochromes OmcA and MtrC are believed to be the terminal contact point of the 'multihaem electron-conducting wire' connecting the bacterial inner membrane to the insoluble terminal electron accepting minerals of Fe(III) and Mn(IV). This view is supported by the experimental observation that mutation of OmcA or MtrC has no effect on the ability of *S. oneidensis* MR-1 to reduce soluble respiratory substrates such as nitrate, nitrite or anthraquinone-2,6-disulfonate, although a Δ*mtrC* strain of *S. oneidensis* MR-1 had a decreased ability to reduce insoluble Fe(III)-oxides [17].

The *Shewanella* respiratory chains provide examples of electrons being moved long distances through electron wires made up of haems. At present the structure of the outer membrane decahaem cytochromes are not known; however, primary structure analysis suggests that they, and also the periplasmic decahaem MtrA, comprise two ~150-amino-acid pentahaem modules (Figure 2B). These modules may be structurally organized in a similar manner to the *E. coli* pentahaem NrfB protein discussed above that is involved in electron transfer to the nitrite reductase NrfA and for which a structure-based model for a 20-haem NrfAB complex has been proposed [3,10] (Figure 2A). The NrfB protein is essentially a small pentahaem electron wire with haems at each end sufficiently solvent-exposed to introduce the possibility of electron exchange between each haem and an external electron acceptor/donor [10]. Packing many such haem wires together in the periplasm or on the cell surface could, in principle, provide for rapid electron transport across the periplasm and multiple sites of electron transfer to an amorphous substrate such as mineral iron.

Experimental homologies between NrfB and MtrA

MtrA and NrfB share a significant level of sequence homology (Figure 3A). The N-terminal half of the MtrA protein has

five CXXCH motifs that align with those in NrfB in pairwise alignments. In addition, many of the histidine ligands are also conserved. To explore the possibility that the *Shewanella* multihaem decahaem cytochromes comprise pentahaem modules, we truncated the decahaem cytochrome MtrA to a cytochrome containing the first five CXXCH motifs.

A truncation MtrA protein (MtrA′) was prepared using a recombinant MtrA expression plasmid, pKP1, which has been developed previously [15]. Using pKP1, the ATG stop codon was inserted after position 202 in the predicted amino acid sequence to give the plasmid pKP1Ter203. The truncated protein was expressed in *E. coli* JM109 cells containing plasmids pKP1Ter203 and pEC86. The expressed truncated MtrA′ protein was purified according to published procedures for MtrA [15] and observed to run at the same position as NrfB on SDS/polyacrylamide gels (Figure 3B), indicating that MtrA′ was approximately the same size as NrfB. Samples of the purified MtrA′ were analysed using MALDI-MS (matrix-assisted laser-desorption ionization MS). The predicted molecular mass of the processed protein with the signal peptide removed and five haems covalently attached was 21456 Da, whereas the experimental value was 21460 Da, indicating that the purified MtrA′ protein has been fully processed and the five haems are attached. Analytical gel filtration was used to gain information about the size of the protein and confirm whether the truncated protein was folded. An unfolded protein would be expected to have a larger hydrodynamic radius and elute at a lower volume than a compact, folded protein. When eluted from a Superdex 200 HR 10/30 gel filtration column, MtrA′ had an elution volume of 11.1 ml, whereas NrfB eluted at 10.8 ml. This indicates that the MtrA′ enzyme is slightly smaller than NrfB despite having a similar molecular mass, and is indicative of a folded protein.

The absorbance spectra of the oxidized and reduced forms of the protein (Figure 3C) were typical of low-spin *c*-type cytochromes. Spectroscopic evidence has revealed that the ten haems of the decahaem MtrA protein are all bis-His co-ordinated [15]. The amino acid sequence of the truncated protein contains six histidine residues that could act as proximal ligands to the five haems, but two of these are sequential, which would severely restrict the positioning of the two haems were they both to act as ligands (Figure 3A). It is, however, clear that a stable pentahaem 'module' similar to NrfB can be constructed by truncation of MtrA. In the future, similar studies will establish whether the pentahaem modules of the other outer membrane decahaem cytochromes can be stably synthesized.

T.A.C. is supported by a fellowship from the Research Councils UK. T.A.C and D.J.R. are partially supported by grants from the Royal Society. This work was partially supported by the EMSL (Environmental Molecular Sciences Laboratory) Scientific Grand Challenge project that is being performed in part at the W.R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy's Office of Biological and Environmental Research and located at the PNNL (Pacific Northwest National Laboratory). PNNL is operated for the Department of Energy by Battelle. We thank Julea Butt for a helpful discussion and advice on manuscript preparation.

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Received 3 May 2008
doi:10.1042/BST0361005