The Diheme Cytochrome b Subunit (Narl) of *Escherichia coli* Nitrate Reductase A (NarGHI): Structure, Function, and Interaction with Quinols

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**Abstract**

Significant recent advances have been made in studies of the major dissipatory nitrate reductase (NarGHI) of *Escherichia coli*. This enzyme is a complex iron-sulfur ([Fe-S]) molybdoenzyme that oxidizes menaquinol or ubiquinol at a periplasmically oriented Q-site (Qp site), and reduces nitrate at a cytoplasmically-oriented molybdo-(bis)molybdopterin guanine dinucleotide (Mo-bisMGD) cofactor. The Qp site, as well as two hemes, termed bh and bL, are localized in a hydrophobic diheme cytochrome b (Narl) that: (i) provides a conduit for electron-transfer from the periplasmically-oriented Qp-site; (ii) provides a membrane anchoring functionality for the membrane-extrinsic subunits (NarGH) that coordinate the Mo-bisMGD (NarG) and four [Fe-S] clusters (NarH); and (iii) helps ensure the separation of sites of H+-yielding and H+--consuming reactions such that enzyme turnover leads to the generation of a proton-electrochemical potential across the cytoplasmic membrane. This minireview focuses on recent advances and future prospects for the diheme cytochrome b subunit (Narl) of NarGHI.

**Introduction**

Facultative anaerobes such as *Escherichia coli* are able to grow anaerobically on nitrate by inducing a combination of two respiratory nitrate reductases (Berks et al., 1995a; Moreno-Vivían et al., 1999; Philippet and Højberg, 1999; Rothery and Watmough, 1999). These comprise the soluble periplasmic NapAB and the membrane-bound NarGHI (nitrate reductase A). In combination, or individually, these enzymes are able to support anaerobic growth on nitrate with a non-fermentable carbon source, such as glycerol (Potter et al., 1989; Potter et al., 2000). A third enzyme, nitrate reductase Z (NarZV), appears to have very similar enzymatic activity to NarGHI (Blasco et al., 1990; Guigliarelli et al., 1992), but is expressed at low levels and is unable to support respiratory growth (Potter et al., 1999). Significant recent progress has been made in our understanding of NarGHI, enabling delineation of its prosthetic group composition and redox chemistry (Augier et al., 1993a; Augier et al., 1993b; Guigliarelli et al., 1992; Guigliarelli et al., 1996; Magalon et al., 1997a; Rothery et al., 1998b), as well as allowing a preliminary view of its electron-transfer pathway and catalytic mechanism (Magalon et al., 1998a; Magalon et al., 1997b; Rothery et al., 1999a). This review focuses on the structure and function of the diheme cytochrome b subunit (Narl) of NarGHI which is emerging as an excellent model system for the study of membrane-intrinsic quinol-binding hemoproteins (Magalon et al., 1997a; Magalon et al., 1998b; Rothery et al., 1999a).

NarGHI is a complex iron-sulfur molybdoenzyme comprising three subunits that coordinate a total of seven prosthetic groups (Guigliarelli et al., 1992; Guigliarelli et al., 1996; Rothery et al., 1999a; Rothery et al., 1998b). These prosthetic groups delineate an electron-transfer conduit from a site of quinol oxidation to a site of nitrate reduction. In addition, they ensure separation of the sites of proton-yielding (quinol oxidation) and proton-consuming (nitrate reduction) reactions such that enzyme turnover is coupled to the generation of a proton electrochemical potential (Berks et al., 1995b; Jones et al., 1980). The three subunits are: (i) a catalytic subunit (NarG, 1246 amino acid residues, 140kDa) which contains a molybdenum cofactor (molybdo-bis(molybdopterin guanine dinucleotide), Mo-bisMGD) at its active site (Magalon et al., 1998a; Rothery et al., 1998b); (ii) an electron-transfer subunit (NarH, 512 amino acid residues, 58kDa) which contains three [4Fe-4S] clusters and one [3Fe-4S] cluster (Augier et al., 1993a; Augier et al., 1993b; Guigliarelli et al., 1992; Guigliarelli et al., 1996; Rothery et al., 1998b); and (iii) a membrane anchor subunit (Narl, 225 amino acid residues, 26kDa) which contains two hemes b (bh and bL) (Berks et
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However, the Mo-bisMGD (Guigliarelli et al., 1996) for the [3Fe-4S] cluster and the Mo(V/VI) couple of the NarGHI, in particular of NarH. It has been proposed that there is an eighth prosthetic group in NarGHI, a [4Fe-4S] cluster coordinated by an N-terminal Cys motif in NarG (Magalon et al., 1995b; Breton et al., 1994), but existing experimental evidence does not support this hypothesis (Magalon et al., 1998a; Rothery et al., 1998b; Trieber et al., 1996).

NarI anchors NarGH to the inner surface of the cytoplasmic membrane (Blasco et al., 1989; Chaudhry and MacGregor, 1983; Jones et al., 1980; Magalon et al., 1997a). It is also the site of quinol binding and oxidation, and provides an electron-transfer conduit from this functionality to the [Fe-S] clusters of NarH. Electron-transfer out of NarI is mediated by two hemes, one of relatively low \( E_m = +20 \text{mV} \), heme \( b_h \), and one of relatively high \( E_m = +120 \text{mV} \), heme \( b_l \) (Hacket and Bragg, 1982; Magalon et al., 1997a; Rothery et al., 1999a). NarI is of interest because: (i) it can be readily overexpressed to high levels in the cytoplasmic membrane, either as part of the NarGHI holoenzyme or by itself as NarI; (ii) it can readily be mutagenized.

**Sequence, Structure, and Heme Composition**

A NarGHI-type enzyme has been identified in a range of bacterial species. Figure 2 shows ten NarI sequences aligned using the ClustalW alignment algorithm (Thompson et al., 1994). The diversity of the sequences provides a convenient filter for the identification of important amino acid residues. Hydrophytis analyses suggest the presence of five transmembrane helices (TM1-TM5), and the distribution of changes within the membrane-extrinsic loops (the “positive inside rule” (von Heijne, 1989)) suggests that there is a periplasmic amino-terminus and a cytoplasmic carboxy-terminus (Berks et al., 1995b; Hacket and Bragg, 1982; Magalon et al., 1997a; Magalon et al., 1998b; Rothery et al., 1999a). The NarG and NarH subunits (NarGH) comprise a membrane-extrinsic catalytic dimer that is anchored to the inner surface of the cytoplasmic membrane by NarI. Figure 1 summarizes the overall topology and thermodynamics of NarGHI.

Potentiometric analyses of NarGHI reveal that its prosthetic groups range in midpoint potential \( E_m \) from -400 mV for the lowest potential [4Fe-4S] cluster to +180 mV for the [3Fe-4S] cluster and the Mo(V/VI) couple of the Mo-bisMGD (Guigliarelli et al., 1996; Rothery et al., 1998b; Rothery et al., 1999b; Vincent and Bray, 1978) (Figure 1). However, the \( E_m = -400 \text{mV} \) [4Fe-4S] cluster is the only prosthetic group with a potential apparently too low to undergo formal oxidation-reduction in an electron-transfer pathway from menaquinol (MQH\(_2\), \( E_m = -74 \text{mV} \)) to nitrate (\( E_m = +420 \text{mV} \)). In other enzyme systems, for example in the E. coli and Wolinella succinogenes fumarate reductases (FrdABCD and FrdCAB), a low potential cluster has been identified (a [4Fe-4S] cluster, FR2) that appears to be directly on the electron-transfer conduit (Iverson et al., 1999). Theoretical analyses of inter-centre electron tunnelling suggest that electron-transfer involving such low potential clusters can be high enough to support observed rates of catalytic turnover (Ohnishi et al., 2000; Page et al., 1999). It is therefore possible that the \( E_m = -400 \text{mV} \) [4Fe-4S] cluster of NarGHI is part of the electron-transfer pathway through NarGHI. However, experimental evidence suggests that a mutant lacking this cluster is able to support respiratory growth on nitrate and retains significant quinol:nitrate oxidoreductase activity (Guigliarelli et al., 1996). It is therefore possible that it plays an alternative role, for example in helping to define the structure of NarGHI, in particular of NarH. It has been proposed that there is an eighth prosthetic group in NarGHI, a [4Fe-4S] cluster coordinated by an N-terminal Cys motif in NarG (Magalon et al., 1995a; Breton et al., 1994), but existing experimental evidence does not support this hypothesis (Magalon et al., 1998a; Rothery et al., 1998b; Trieber et al., 1996).

![Figure 1. Overall topology and redox chemistry of NarGHI.](image-url)

**Figure 1.** Overall topology and redox chemistry of NarGHI. A. Topological model of the enzyme showing its prosthetic group composition, overall electron-transfer pathway, and biocatalytic function (Breton et al., 1994) that there is a fifth [Fe-S] cluster in NarG (Magalon et al., 1998a; Rothery et al., 1998b). B. Overall electrochemistry of the NarGHI prosthetic groups. Arrows indicate a plausible electron-transfer pathway based on experimental data (Magalon et al., 1998a; Magalon et al., 1997b; Rothery et al., 1998b; Vincent and Bray, 1978).

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positions of the two putative Q-sites of NarGHI are indicated. These are the Q\(_P\) (Periplasmic) and Q\(_{nr}\) (nitrate reductase) sites (Brito et al., 1995; Magalon et al., 1998b; Rothery et al., 1999a) (see below for a description of an alternative nomenclature). In considering the proposed transmembrane topology of NarI presented in Figure 3A, it should be noted that the absolute length of the individual TM segments and the relative depth of individual residues within the membrane may differ significantly from the predictions of hydropathy analyses, as has been observed in the structure of \(E.\) coli FrdABCD determined by X-ray crystallography (Iverson et al., 1999).

Among hydrophobic diheme cytochromes b, much confusion reigns in the terminology used for both the hemes and the Q-sites. An emerging terminology for those enzymes with membrane-extrinsic catalytic subunits names the sites and hemes based on their location relative to the membrane-extrinsic dimers (Iverson et al., 1999; Lancaster et al., 1999; Ohnishi et al., 2000). In this system, heme \(b_H\) of NarGHI would become heme \(b_{P}\) (Proximal) and heme \(b_L\) would become heme \(b_{D}\) (Distal). Likewise the putative Q\(_{nr}\) site would become the Q\(_P\) site and the Q\(_P\) site would become the Q\(_D\) site. However, in contrast to other systems, a consistent terminology (Q\(_P\)/Q\(_{nr}\)/b\(_L\)/b\(_H\)) has been in use for a number of years for the NarGHI system, and we therefore favour retaining it (Magalon et al., 1997a; Magalon et al., 1998b; Rothery et al., 1999a). For clarity in comparisons between different diheme cytochrome b systems, we have indicated the alternative terminology (Q\(_D\)/Q\(_P\)/b\(_D\)/b\(_P\)) in the appropriate Figures (Figure 3A and Figure 7).

Figure 3B shows a plot of the overall sequence similarity in the alignment of Figure 2 versus residue position in combination with the proposed transmembrane topology of NarI (Figure 3A). Within the family of NarI sequences, above average similarity is observed in loop TM1-TM2 (putative cytoplasmic) and after the end of TM5 (putative cytoplasmic). Below average similarity is observed in loops...
Figure 3. Transmembrane topology and plotsimilarity of the sequence of NarI. A. Amino acid sequence of NarI showing absolutely conserved residues (black circles) within the group of sequences presented in Figure 2. $Q_{nr}/Q_P$ - Periplasmically oriented or Distal Q-site that has been identified as being dissociable in inhibitor-binding experiments (Magalon et al., 1998b; Rothery et al., 1999a). $Q_{nr}$ - putative Q-site localized between heme $b_6$ and the $[3Fe-4S]$ cluster of NarH (Q$_{nr}$ - nitrate reductase; $Q_P$ - Proximal) (Brito et al., 1995; Magalon et al., 1997b). B. Plot of sequence similarity versus residue position of the sequence alignment of Figure 2 using a window average of 10. The positions of transmembrane segments 1-5 are indicated, as are the proposed locations of the extramembrane loops. The plot was generated using the PLANTSIMILARITY program of the Wisconsin Sequence Analysis Package in combination with the alignment of Figure 2. Note that residue numbering corresponds to that of the alignment of Figure 2.
TM2-TM3 (putative periplasmic), TM3-TM4 (putative cytoplasmic), and TM4-TM5 (putative periplasmic). However, in the latter case there is a significant spike of similarity within this rather long periplasmic loop. These observations are consistent with the cytoplasmically localized similarity defining NarGH-NarI subunit interactions and an electron-transfer conduit. The spike within loop TM4-TM5 may define some other important functionality within NarI such as a Q-site.

Conserved His residues in the NarI sequence correspond to H56/H66 in TM2 and H187/H205 in TM5 and their role in heme coordination has been demonstrated by site-directed mutagenesis (Magalon, 1997; Magalon et al., 1997a; Rothery et al., 1999a). In the case of TM5, the two His residues are located on the same segment of the helix in a helical wheel projection (Figure 4). In TM2, the His residues fall in separate segments with approximately 80° between them on the vertical axis, implying that there is either: (a) a kink in one of the two heme-coordinating helices (most likely TM5), or; (b) a significant angle between the axis of the longer helix (TM5) and the membrane normal (and the axis of TM2). In this context, it should be noted that in proteins of known structure it is common for the helices to be tilted relative to the membrane normal (Iverson et al., 1999; Lancaster, 1998; Xia et al., 1997; Zhang et al., 1998).

In NarGHI, the primary location of quinol binding and
oxidation appears to be located towards the periplasmic side of NarI (the QP site) in close association with heme bL (Magalon et al., 1997a; Magalon et al., 1998b; Rothery et al., 1999a). However, almost all of the sequence conservation identified in Figures 2, 3, and 4 lies towards the cytoplasmic side of the membrane. The exception to this is in the rather long loop between TM4 and TM5. In particular, there is a conserved Trp residue (W162) that appears in all the sequences of Figure 2 with the exception of the NarI subunit of Thermus thermophilus, where it is replaced with a Tyr residue. Such a highly conserved Trp residue plays an important role in the proximal (QPFRD) site of E. coli FrdABCD (Iverson et al., 1999; Westenberg et al., 1993) and a number of other Q-sites (Murray et al., 1999). Given the generally very weak sequence conservation associated with Q-sites in different enzyme systems (Fisher and Rich, 2000), it is possible that the spike in sequence similarity in the TM4-TM5 loop may play a role in defining the QP site.

Within the TM segments, a number of additional residues warrant mention. There are five Gly residues that are absolutely conserved, comprising pairs in TM2 and TM3, and a single Gly in TM4 (Figures 2 and 3A). In complex III at least one of the conserved Gly residues is involved in the packing of heme bL into the cytochrome b subunit (Saribas et al., 1997). In the helical wheel projection of Figure 4, the two conserved Gly residues in TM2 are almost on the opposite side of the helix from the heme-coordinating His residues, so are unlikely to have such a role. But the two Gly residues in TM3 and the conserved Gly in TM4 might be involved in accommodating the hemes. In TM5 of NarI, in addition to the two conserved heme-coordinating His residues, there is a sequence towards the cytoplasmic end of the segment (PFSRLIHxxSxPxYxxR) that is highly conserved that includes one of the heme ligands (H205). Thus, consideration of the sequence identity within the family of NarI proteins identifies a number of residues beyond the heme-coordinating His residues as targets for mutagenesis.

EPR Spectroscopy of the Two Hemes

Although the hemes of NarGH1 have optical spectra typical of low-spin cytochromes b (Hacket and Bragg, 1982; Magalon et al., 1997a), it has been through the application of EPR spectroscopy that significant advances have been made in understanding their structure and function. This is because low-spin hemes b have overlapping optical spectra that can be difficult to deconvolute, whereas they typically have clearly resolved EPR spectra. This advantage has also been exploited in other proteins having multiple low-spin hemes b such as the SdhC subunit of Bacillus subtilis succinate dehydrogenase (Hägerhäll et al., 1992; Hägerhäll et al., 1995a) and the cytochrome b subunit of complex III.
(Salerno, 1984; Saribas et al., 1997; Valkova-Valchanova et al., 1998).

High level protein overexpression (to ~50% of the inner membrane protein) has allowed intensive scrutiny of the hemes of NarGHI in situ (Magalon, 1997; Magalon et al., 1997a; Magalon et al., 1998b; Rothery et al., 1999a). The NarGHI hemes are low-spin (six-coordinate iron) and exhibit EPR spectra with features typical of HALS (highly anisotropic low-spin) hemes (Walker et al., 1986), in which there is a large imidazole ligand interplanal angle (Dou et al., 1995). In this type of heme, only one of the three g-tensors (g3) is observed by EPR (Salerno, 1984). Figure 5a shows the deconvoluted EPR spectrum of the two hemes, showing features at g3=3.36 and g3=3.76. This spectrum is reminiscent of that of the hemes b of complex III (Salerno, 1984; Valkova-Valchanova et al., 1998), and the proposed model for NarI heme coordination (Figure 3A) is superficially similar to that seen in the structure of complex III (Xia et al., 1997; Zhang et al., 1998), with the two hemes being coordinated between two transmembrane helices.

Site-Directed Mutagenesis of Heme-Coordinating His Residues

Assignment of the His ligands of hemes b4 and b5 in NarGHI is based on EPR/optical studies of four site-directed mutants, NarGHIH56R, NarGHIH66Y, NarGHIH187Y, and NarGHIH205Y (Magalon, 1997; Magalon et al., 1997a). The ligands for hemes b4 and b5 are H66/H187 and H56/H205, respectively, in agreement with the sequence conservation of these residues (Figure 2) (Berkers et al., 1995b). This strongly supports the model for the overall topology of NarI presented in Figure 3.

Loss of heme b4 causes readily interpretable changes in the properties of NarGHI (loss of the corresponding EPR signal and loss of quinol binding and oxidation) with few consequences for the remaining prosthetic groups of NarGHI (Magalon et al., 1997a; Rothery et al., 1999a). In contrast, loss of heme b5 in NarGHIH187Y causes: (i) a decrease in the anisotropy (g3) of heme b5 (Magalon et al., 1997a); (ii) an increase in the E_m of heme b5 (Rothery et al., 1999a); and (iii) an alteration in the EPR spectrum of the [4Fe-4S] cluster of NarH. The first two observations can be rationalized on the basis that both hemes are coordinated between TM2 and TM5 of NarI, and any conformational effects could easily be transferred via these two transmembrane helices. For example, in complex III, loss of heme b5 also increases the E_m of heme b5 (Yun et al., 1991). The effect of the loss of heme b5 on the NarH [3Fe-4S] cluster suggests that this cluster and the heme are located close to the NarH-NarI interface. This is consistent with experimental and structural data on three other enzyme systems. (i) In the crystal structure of E. coli FrdABCD (Iversen et al., 1999), the [3Fe-4S] cluster (FR3) of FrdB is located close to the FrdCD-FrdB interface. A number of residues of FrdB are located within 5 Å of the proximal MQ binding site (Q_b,FR3), and FR3 is located ~11 Å from this site. (ii) In W. succinogenes fumarate reductase (FrdCAB), the distance between FR3 and the edge of the proximal heme (heme b5) is also ~11 Å (Lancaster et al., 1999). (iii) In E. coli DMSO reductase (DmsABC), a [4Fe-4S] cluster of DmsB that is essential for MOH2 oxidation is located close to a Q-site in DmsC (Q_(DmsC)) and has also been localized close to the DmsB-DmsC interface (Rothery and Weiner, 1991; Rothery and Weiner, 1993; Rothery and Weiner, 1996). Thus, in NarGHI, it is possible that heme b5 is in a location similar, relative to the electron-transfer subunit, to the Q_(FR3)/Q_(DmsC) site of FrdABCD/DmsABC and the heme b5 of W. succinogenes FrdCAB.

When NarI is expressed and assembled into the cytoplasmic membrane in the absence of NarGH (NarI(∆GH)), its low-spin heme EPR spectrum is dramatically altered compared to that of the holoenzyme (Magalon et al., 1997b; Magalon et al., 1998b). The EPR spectrum of NarI(∆GH) (Figure 5b) exhibits two g features attributable to low-spin ferric heme, one at g3=3.15, and another with a g3=2.92. On the basis of its inhibitor-sensitivity, the g3=3.15 feature can be assigned to heme b4 (Magalon et al., 1998b). The observation of a g3=2.92 feature may correlate with an almost parallel imidazole ligand orientation (Dou et al., 1995; Walker et al., 1986), indicating that a “relaxation” in the environment of this heme occurs in the absence of NarGH, as has been observed in the membrane anchors of mitochondrial and E. coli succinate dehydrogenase (Yang et al., 1997; Yu et al., 1987). The g3=2.92 feature of the spectrum is eliminated in NarI(∆GH)H56R, unequivocally assigning it to a “relaxed” form of heme b5 (Rothery et al., 1999a). The E_m of heme b5 in NarI(∆GH) is +37mV, which is a modest increase from the value of +20 observed in NarGHI (Rothery et al., 1999a). Surprisingly, the E_m of heme b5 drops from +120mV in NarGHI to -178mV in NarI(∆GH), a ΔE_m of almost -300mV. In chloroplast cytochrome b599 exposure of the heme to the aqueous milieu results in a large negative ΔE_m (Krishtalik et al., 1993), suggesting that the absence of the NarGH results in a similar exposure of heme b5 in NarI(∆GH). This is further evidence for the localization of heme b5 to the NarH-NarI interface region of NarGHI.

Q-Site Inhibitors

NarGHI is able to use both menaquinol (MOH2) and ubiquinol (UQH2) as physiological reductants (Giordani et al., 1997; Guigliarelli et al., 1996; Wallace and Young, 1977). Significant progress in studies of quinol binding has been made using quinol analog substrates and inhibitors in combination with optical, fluorescence and EPR spectroscopies. Of a range of Q-site inhibitors of complex III tested (Magalon et al., 1998b), only HOQNO and stigmatellin inhibit NarGHI with a sufficiently low b5 for them to be useful for further biochemical/biophysical studies. HOQNO binding to NarGHI renders heme b5 more anisotropic (g3 increases to ~3.50), suggesting that it increases the imidazole ligand interplanal angle (this is the angle between the planes of the imidazole rings measured along the imidazole nitrogen-iron-imidazole nitrogen axis) (Rothery et al., 1999a; Walker et al., 1986). Stigmatellin also binds in the vicinity of heme b5, but in this case there is a decrease in anisotropy, suggesting a decrease in the
imidazole ligand interplanal angle. These contrasting effects on the possible imidazole interplanal angle may explain the different effects of these inhibitors on the $E_m$ values of the two hemes. HOQNO causes a near reversal of the $E_m$ values (a positive $\Delta E_m$ for heme $b_1$ and a negative $\Delta E_m$ for heme $b_2$), whereas stigmatellin elicits a modest positive $\Delta E_m$ on heme $b_1$ and has no effect on heme $b_2$ (Rothery et al., 1999a). Since neither inhibitor has an effect on the EPR spectrum of heme $b_1$ and both inhibitors affect the $E_m$ of heme $b_2$, it is clear that there is at least one Q-site (the Q$_b$ site) located in the vicinity of the latter heme. The large negative $\Delta E_m$ elicited by HOQNO on heme $b_1$, suggests either that a conformational change is propagated to this heme via the heme $b_1$ and TM2/TM5, or that there is another site of HOQNO binding in the vicinity of heme $b_1$.

The determination of the number of dissociable HOQNO binding sites (and presumably MQH$_2$/UQH$_2$ binding sites) was achieved through the use of fluorescence quench titrations (Brandt and von Jagow, 1991; Okun et al., 1999; van Ark and Berden, 1977). HOQNO binding to NarGHI occurs with a $K_b$ of ~0.2 mM at a single site that is sensitive to the absence of heme $b_2$, but not to the absence of heme $b_1$ (Rothery et al., 1999a). This site appears to overlap with, or be sterically hindered by, the site for stigmatellin binding. The absence of NarG in NarI(AGH) does not appear to affect HOQNO binding. Thus, a combination of EPR and fluorescence spectroscopies point to a model for quinol binding in which there is a single dissociable high-affinity site (the Q$_b$ site) located towards the periplasmic side of NarI in close proximity to heme $b_1$. This model is consistent with the proposed bioenergetics of NarGHI in which scalar protons from MQH$_2$ oxidation are released into the periplasm during enzyme turnover (Berks et al., 1995b; Jones et al., 1980).

There is evidence suggesting that a second Q-site exists in NarGHI (the Q$_{nr}$ site). During nitrate-induced enzyme turnover, an HOQNO-sensitive radical species is observed that is likely to be located in the vicinity of the NarH[3Fe-4S] cluster (Magalon et al., 1997b). It has been proposed that this species is a menasemiquinone anion that arises from a tightly bound MQ-9 that copurifies with the NarG catalytic dimer (Brito et al., 1995; Magalon et al., 1997b). Also, HOQNO and stigmatellin inhibit nitrate-dependent heme reoxidation (Magalon et al., 1998b) (Figure 6), suggesting the presence of a second dissociable Q-site between heme $b_1$ and the [3Fe-4S] cluster (the Q$_{nr}$ site). This site would perhaps be similar to the Q$_b$F$_{RD}$ (Iverson et al., 1999) and Q$_b$DMS sites (Rothery and Weiner, 1991; Rothery and Weiner, 1993; Rothery and Weiner, 1996). However, in contrast to what is observed with FrdABCD (Hägerhäll et al., 1999; Rothery and Weiner, 1998), HOQNO elicits no effect on EPR properties of the [3Fe-4S] cluster of NarH (Rothery et al., 1999a). It is possible that the observed inhibition of heme reoxidation in NarGHI is due to bound inhibitor (at the Q$_b$ site) preventing oxidation of heme $b_1$ (in the case of HOQNO, the $\Delta E_m$ elicited on heme $b_2$ is entirely consistent with this possibility). It should also be noted that the presence of a dissociable Q$_{nr}$ site would be difficult to reconcile with the proposed bioenergetics of NarGHI (Berks et al., 1995b; Jones et al., 1980), as it would presumably result in the release of protons on both the periplasmic and cytoplasmic faces of NarI, reducing the $H^+/e^-$ ratio for enzyme turnover. In fact, the proton gradient across the cytoplasmic membrane would favor quinol oxidation and proton release at the putative Q$_{nr}$ site over the Q$_b$ site.

A more compelling explanation for the data that has been used to suggest the presence of a dissociable Q$_{nr}$ site is that inhibitors such as HOQNO and stigmatellin bind with much higher affinity to the reduced form of the enzyme than to the oxidized form. Thus, when the reduced enzyme is subjected to nitrate-induced oxidation, oxidation of heme $b_1$ has to await dissociation of inhibitor from the Q$_b$ site. This would explain the apparent inhibition of heme reoxidation shown in Figure 6 and elsewhere (Magalon et al., 1998b). In the data presented in Figure 6, the rapid first phase of nitrate-dependent reoxidation in the presence of HOQNO is likely to correspond to heme $b_1$ oxidation, whereas the second slower phase is likely to correspond to heme $b_2$ oxidation. This second phase appears to occur following exhaustion of reduced quinol substrate (plumbagin).

Quinol binding and oxidation by NarGHI has also been addressed by steady-state enzymology. When the hydroxylated naphthoquinols reduced lapachol and plumbagin are used as substrates, kinetics are observed that are consistent with binding to a single site within the NarGHI complex (Rothery et al., 1999a). However, a more complex analysis (Giordani et al., 1997) suggests the presence of two quinol binding sites, one which preferentially binds a MQH$_2$ analog and another which preferentially binds a UQH$_2$ analog (i.e. the analyses suggests two sites for both MQH$_2$ and UQH$_2$ analogs). However, in another steady-state kinetics study, (Morpeth and Boxer, 1985) the data was consistent with only a single dissociable Q-site (for UQH$_2$). Thus, at the present time, not all the kinetic data agrees with the EPR/fluorescence data suggesting only a single dissociable Q$_b$ site in the vicinity of heme $b_1$.

In the context of identifying the number of Q-sites in NarGHI, it is important to distinguish between dissociable and non-dissociable sites. For example, the MQ-9 identified in purified NarGHI dimer (Brito et al., 1995) could reside at a non-dissociable Q-site in a position equivalent to the proposed Q$_{nr}$ site. Such a non-dissociable quinol species has been identified in E. coli cytochrome bo (Ingledew et al., 1995; Sato-Watanabe et al., 1995; Sato-Watanabe et al., 1994), as well as in the well-characterized bacterial photoreaction centre (the so-called Q$_b$ site) (Allen and Williams, 1998; Ermler et al., 1994).

Comparison with Other Diheme Membrane-Bound Cytochromes $b$

Figure 7 illustrates the overall topology of NarI in comparison with two hydrophobic diheme cytochromes $b$ of known structure: the cytochrome $b$ subunit of mitochondrial complex III (Xia et al., 1997; Zhang et al., 1998) and the FrdC subunit of W. succinogenes fumarate reductase (FrdCAB) (Lancaster et al., 1999). The latter enzyme has considerable sequence, and inferred structural similarity with B. subtilis succinate dehydrogenase (SdCab) (Hägerhäll, 1997; Hägerhäll and Herderstedt, 1981).
1996). Also shown is the correlation between the hemes of the proteins and their inferred EPR anisotropy. In the models presented in Figure 7, the cytoplasmic side of the membrane and the membrane-extrinsic catalytic dimers of NarGHI/FrdCAB are located above each protein model. In the case of the cytochrome b subunit of complex III, electrons from quinol oxidation are passed to a Rieske [Fe-S] protein located on the periplasmic side of the complex (below the cytochrome b model of Figure 7).

The structure of the cytochrome b subunit of complex III provides a good model for the coordination of two hemes b by four His residues in two transmembrane helices (Xia et al., 1997; Zhang et al., 1998). Both hemes b have architypal HALS EPR lineshapes (Salerno, 1984; Walker et al., 1986), are located approximately 20Å apart (Fe-Fe distance) (Xia et al., 1997), and appear to undergo the same type of long-range redox interactions as are observed between the hemes of Narl (Howell and Robertson, 1993; Rothery et al., 1999a). However, there are significant differences between the hemes b of Narl and those of complex III:

(i) In complex III, the more anisotropic gE has been assigned to heme bP, whereas the less anisotropic gM has been assigned to heme bL. In mouse complex III, these hemes have Eps values of approximately -31 (bM) and 92mV (bP), respectively (Howell and Robertson, 1993). In NarGHI, the more anisotropic heme (gM=3.76; bL) has an Eps of approximately 120mV and the less anisotropic heme (gE=3.36; bP) has an Eps of approximately 20mV (Hacket and Bragg, 1982; Magalon et al., 1997a; Rothery et al., 1999a). Thus, in NarGHI, it is the higher potential heme that is most anisotropic, whereas in complex III it is the lower potential heme.

(ii) Present evidence suggests the presence of only a single dissociable Q-site in Narl (the Qb site), whereas in complex III there are regions of dissociable quinone/quinol binding oriented towards opposite sides of the cytochrome b subunit (Xia et al., 1997).

(iii) In the structure of complex III, two of the TM segments (B and D, of a total of eight) provide heme ligands, but these run in the same direction (inward, towards the mitochondrial matrix/bacterial cytoplasm) (Zhao et al., 1998), whereas in Narl the equivalent helices (TM2 and TM5) are antiparallel.

The membrane anchor subunit of W. succinogenes FrdCAB (Lancaster et al., 1999), although being a five TM segment diheme cytochrome b, is clearly structurally distinct from Narl. In this case, four rather than two of the helices coordinate the hemes via His residues. The proximal heme of FrdCAB (heme bP) is coordinated between TM2 and TM4, whilst the distal heme (heme bL) is coordinated between TM1 and TM3 (Lancaster et al., 1999). This results in an interplanal angle for the two hemes of 95°. The membrane anchor of W. succinogenes FrdCAB is likely to be very similar in overall structure to that of the succinate dehydrogenase of B. subtilis succinate dehydrogenase (SdhCAB) (Hägerhäll, 1997). In the latter case, it has been shown that HOQNO elicits a large negative ΔEps on heme b1 (equivalent to heme bL of W. succinogenes FrdC), but has no effect on the Eps of heme b2 (equivalent to heme bP of W. succinogenes FrdC) (Smirnova et al., 1995) or the [3Fe-4S] cluster of SdhB (Hägerhäll et al., 1995b). These observations are consistent with the long-range redox interactions observed between the hemes of Narl being propagated via the two heme-coordinating TM segments.

In NarGHI and B. subtilis SdhCAB, it is the more anisotropic heme (bP=NarGHI; bL=SdhCAB) that is close to the interface between the membrane anchor (Narl/SdhC) subunit and the electron-transfer subunit (Hägerhäll et al., 1992; Magalon et al., 1997a; Rothery et al., 1999a). It is very likely that in W. succinogenes FrdCAB, heme bP is the more anisotropic heme. As described above, the environment of heme bP of Narl is significantly altered in the absence of the membrane-extrinsic dimer. In B. subtilis SdhC in the absence of SdhAB, the EPR signal corresponding to heme bP is not observed, suggesting that this heme is absent in the absence of the membrane-extrinsic subunits (Fridén et al., 1990; Hederstedt and Andersson, 1986). Given these observations, it is likely that the interaction between the membrane anchor subunit and the electron-transfer subunit plays a large role in defining the environment of heme bP (Narl) and heme bP (FrdCAB/SdhCAB). It is also of note that in complex III, the most anisotropic heme is that one that is closest to the membrane-extrinsic Rieske [Fe-S] protein subunit. However, due to the overall complexity of complex III, in this case the correlation may be simply coincidental.

Conclusions and Outlook

Much recent progress has been made towards an understanding of the structure and function of Narl. At present, the majority of the data can only be interpreted in terms of a two dimensional model for its overall structure in which the highest resolution information relates to the overall number and orientation of the TM segments and estimates of the overall positions of the two hemes and putative Q-sites with respect to the membrane and the other prosthetic groups of NarGHI. What is now clearly necessary is an intensive effort to determine the structure of NarGHI or Narl(ΔGH) at atomic resolution. Recent successes in determining the structures of a number of membrane-bound, multisubunit, multifactor enzymes (Verson et al., 1999; Lancaster et al., 1999; Xia et al., 1997; Zhang et al., 1998) suggests that success with NarGHI will soon be forthcoming. In addition, careful analyses of the sequence of E. coli Narl in comparison with other members of the Narl family of proteins suggest a number of residues suitable for site-directed mutagenesis and subsequent study. Both the structure-solving and mutagenesis approaches are in progress in our laboratories.

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