Structural and biochemical characterization of a quinol binding site of *Escherichia coli* Nitrate Reductase A (NarGHI)

Michela G. Bertero◊, Richard A. Rothery‡, Nasim Boroumand‡, Monica Palak‡, Francis Blasco¶, Nicolas Ginet+, Joel H. Weiner‡ and Natalie C.J. Strynadka◊*

◊ Department of Biochemistry, University of British Columbia, 2146 Health Science Mall, Vancouver, British Columbia, V6T 1Z3
‡ CIHR Membrane Protein Research Group, Department of Biochemistry, 474 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2H7.
¶ Laboratoire de Chimie Bactérienne, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 9, France
+ UMR6191, LBC, CEA Cadarache, 13108 Saint-Paul-lez-Durance Cedex, France

* To whom correspondence should be addressed: email: natalie@byron.biochem.ubc.ca, Tel. 604-8220789, Fax. 604-8225227

*RUNNING TITLE:* Electron-transfer and proton translocation by *E. coli* nitrate reductase
Abbreviations. PCP, pentachlorophenol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Mo-bisMGD, molybdo-bis(molybdopterin guanine dinucleotide); NarGHI, nitrate reductase holoenzyme.

ABSTRACT
The crystal structure of *Escherichia coli* nitrate reductase A (NarGHI) in complex with pentachlorophenol (PCP) has been determined to 2.0Å resolution. We have shown that PCP is a potent inhibitor of quinol:nitrate oxidoreductase activity, and that it also perturbs the EPR spectrum of one of the hemes located in the membrane anchoring subunit (NarI). This new structural information, together with site-directed mutagenesis data, biochemical analyses and molecular modeling, provide the first molecular characterization of a quinol binding and oxidation site (Q-site) in NarGHI. A possible proton conduction pathway linked to electron transfer reactions has also been defined providing fundamental atomic details of ubiquinol oxidation by NarGHI at the bacterial membrane.

INTRODUCTION
*Escherichia coli*, when grown anaerobically in the presence of nitrate, synthesizes the membrane-bound quinol:nitrate oxidoreductase NarGHI (1,2). This enzyme has been the subject of intense biochemical, biophysical and structural studies. The protein complex contains three subunits with characteristic redox prosthetic groups: NarG (140 kDa), the catalytic subunit with a molybdo-bis(molybdopterin guanine dinucleotide) cofactor (Mo-bisMGD) and an [Fe-S] cluster (FS0); NarH (58 kDa), the electron-transfer subunit with four [Fe-S] clusters (FS1, FS2, FS3 and FS4); NarI (26 kDa), the integral membrane subunit with two b-type hemes, termed bP and bD to indicate their proximal (bP) and distal (bD) positions to the catalytic site. NarG and NarH form a soluble cytoplasmically-localized catalytic domain anchored to the membrane by NarI. NarGHI catalyses electron transfer from a quinol binding site located in NarI through the redox cofactors aligned as an “electric wire” through the complex (bD → bP → FS4 → FS3 → FS2 → FS1 → FS0) to the Mo-bisMGD cofactor in NarG, where nitrate is reduced to nitrite.

NarGHI often forms a respiratory chain with the formate dehydrogenase FdnGHI via the lipid soluble quinol pool. Electron transfer from formate to nitrate is coupled to proton translocation across the cytoplasmic membrane generating proton motive force by a redox loop mechanism (3). In the redox loop mechanism proton translocation is the net result of the topographically segregated reduction of quinone and oxidation of quinol on opposite sites of the membrane. The high-resolution structures of both respiratory complexes, FdnGHI and NarGHI, have been recently solved (1,4). Crystallographic analysis of FdnGHI has shown the presence of a quinone reduction site oriented towards the cytoplasm (4). Existing biochemical and biophysical evidence indicates that the quinol binding and oxidation functionality of NarGHI is provided by the NarI subunit (5-7) but no high resolution structural information has been made available to date.

We have solved the crystal structure at 2.0Å resolution of NarGHI in complex with the quinol-binding inhibitor pentachlorophenol, PCP, which is structurally related to the physiological quinol substrates of the enzyme. This structure shows the existence of a periplasmically oriented Q-site in NarI (termed QD in reference to its close proximity to the heme bD), confirming previous spectrophotometric and kinetic results (6-9). Further structural and functional studies within the QD-site are also presented using site-directed mutants of conserved residues in its vicinity. Collectively, this work provides significant new insight into the electron transfer and proton conduction pathways through the transmembrane subunit NarI.

MATERIALS AND METHODS
Bacterial strains and plasmids. *E. coli* LCB79 *(araD139 Δ(lacIPOZYA-argF) rpsL, thi φ79(nar-lac))* (10) was used as the host for all the experiments described herein. NarGHI was expressed from plasmid pVA700 (11) carrying a single point mutation, C26A in NarH.

Growth of cells. *E. coli* LCB79/pVA700 was grown overnight in 5L batches with a 1% inoculum in a B. Braun Biostat B fermenter at 30°C in the presence of 100µg mL⁻¹ ampicillin and 100µg mL⁻¹ streptomycin. The growth medium contained 12g L⁻¹ tryptone, 24g L⁻¹ yeast extract, 5g L⁻¹ NaCl, and 4mL L⁻¹ glycerol. NarGHI overexpression was induced at OD₆₀₀=2.0, after which the cultures were grown for 10-11 hours.
Cells were harvested by centrifugation, washed in a buffer containing 100mM MOPS and 5mM EDTA, and frozen in liquid nitrogen for subsequent storage at -70°C.

**Isolation of membrane fractions and purified preparations.** Crude membrane vesicles were prepared from *E. coli* cells by French pressure cell lysis and differential centrifugation (12). Enriched inner membrane vesicles were isolated from these crude membranes by sucrose step centrifugation as previously described (6). All membrane preparation steps were carried out in a buffer containing 100mM MOPS and 5mM EDTA (pH7.0). Excess sucrose was removed by resuspension and recentrifugation in this buffer. Purified NarGHI was prepared by anion exchange chromatography on a DEAE-FF column (Pharmacia) as described by Bertero et al. (1).

**Membrane and purified samples were flash frozen in liquid nitrogen and subsequently stored at -70°C prior to use.**

**Protein assays.** Protein concentrations were assayed by the Lowry method (13), modified by the inclusion of 1% (w/v) sodium dodecyl sulfate in the incubation mixture to solubilize membrane proteins (14).

**Enzyme assays.** Quinol:nitrate oxidoreductase activities were determined using reduced plumbagin (PBH₂). A 20mM stock ethanolic solution was reduced by metallic zinc in acidified ethanol as previously described (15). Optical data were recorded using a Hewlett Packard (Agilent) HP8453 spectrophotometer. Nitrate-dependent oxidation of PBH₂ was followed by the appearance of absorption maxima at 419nm using an extinction coefficient of 3.95mM⁻¹ cm⁻¹ (15).

Enzyme assays were carried out in a degassed buffer containing 100mM MOPS/KOH, 4mM KNO₃, and 5mM EDTA (pH7.0). A concentration range of 0.140-1.4mM PBH₂ was used to determine kinetic parameters, and reactions were initiated by addition of protein (either enriched membranes or purified protein). The competitive inhibitor constant for PCP (Ki) was estimated from plots of Km/kcat versus PCP concentration, and the uncompetitive inhibitor constant (Kiu) was estimated from plots of 1/kcat versus PCP concentration (16).

**EPR spectroscopy.** EPR spectra were recorded using a Bruker Bruker Elexsys E500 spectrometer equipped with an ESR-900 flowing helium cryostat. EPR conditions were as described in the legend to Figure 4.

**FQ titrations with HOQNO and estimation of NarGHI content in membrane samples.** The affinity of NarGHI for HOQNO was determined by performing FQ titrations using a Perkin Elmer LS-50B luminescence spectrometer (6,17,18). Fluorescence intensities were measured using an excitation wavelength of 341nm and an emission wavelength of 479nm. All experiments were carried out at room temperature and pH=7.0 in 100mM MOPS/KOH and 5mM EDTA. HOQNO was added to the fluorescence cuvette from a 0.25mM stock ethanolic solution. A range of protein concentrations was used as indicated in the individual figure legends. The observed fluorescence (Fobs) was fitted to an equation describing ligand binding to a single site as described by Okun et al. (18). Enzyme concentration was deemed to be equivalent to the estimated concentration of HOQNO binding sites. In the case of the NarI-K86A mutant enzyme which is not amenable to concentration determination by FQ titration, enzyme content was estimated by comparing the fluorescence intensities of an extract containing the form A derivative of the Mo-bisMGD cofactor with those of a wild-type preparation of known NarGHI content (19-21).

**Crystallization, data collection and structure determination of the NarGHI-PCP complex.** Highly ordered crystals of three single point mutants of NarGHI, C26A in NarH, K86A and H66Y in NarI, were obtained in conditions similar to that reported earlier for the native enzyme (1). NarGHI-C26A crystals were soaked in 50µl cryoprotectant solution (35% PEG 3K, 350 mM Sodium Acetate, 200 mM KCl, 100 mM Hepes pH=7.0, 5 mM EDTA and 0.7 mM Thesit) in the presence of 0.5mM PCP. This mutant retains the activity of the native enzyme (11) and allowed us to obtain crystals yielding higher resolution data than that obtained with crystals of the native enzyme after soaking with PCP. Single datasets for each mutant were collected at 100K at the Advanced Light Source (beamlines 8.2.1 and 8.2.2). Crystals were isomorphous with the native crystals. Data were integrated and scaled with the HKL suite of programs (22). The structures were determined using difference Fourier techniques followed by rigid body refinement of the 1.9Å
resolution native model (1) with the CNS program (23). Further cycles of manual rebuilding with Xfit (24) and refinement with CNS allowed us to obtain the final models (R/Rfree values of 0.200/0.234 for NarGHI-PCP, R/Rfree values of 0.186/0.212 for NarGHI-K86A, R/Rfree values of 0.188/0.237 for NarGHI-H66Y). Figures with ribbon representations were created with MolScript (25) and Raster3D (26).

A summary of data collection and refinement statistics is shown in Table 1. Coordinates and structure factors of the structures presented in this work have been deposited in the Protein Data Bank with the following accession codes: 1Y4Z for the complex NarGHI-PCP, 1Y5I for NarGHI-K86A, 1Y5N for the complex NarGHI-K86A-PCP, and 1Y5L for NarGHI-H66Y.

RESULTS

PCP is a potent inhibitor of NarGHI.

NarGHI is able to use both menaquinol (MQH2) and ubiquinol (UQH2) as physiological electron donors (Figures 1a, b) (27,28). Studies of quinol binding to NarGHI have been expedited by the use of the menaquinone analog 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO, Figure 1d) which has been demonstrated to inhibit enzyme activity by binding to a single site in close proximity to heme bD within NarI (6,7). We have so far been unable to produce crystals of the NarGHI-HOQNO complex, so we pursued the characterization of other potential inhibitors of the enzyme. Pentachlorophenol (PCP, Figure 1c), has been shown to be a potent inhibitor of both succinate dehydrogenase and fumarate reductase (29). We have characterized the effects of this inhibitor on the activity of the native NarGHI and NarGHI bearing a specific mutation of a highly conserved residue in NarI (NarI-K86A).

The NarI-K86A mutant has a lower plumbagin:nitrate oxidoreductase activity than the wild-type enzyme, 10 sec\(^{-1}\) compared to 68 sec\(^{-1}\), respectively. The decreased activity of the mutant enzyme suggests that the NarI-K86 residue is probably located in the vicinity of a functional Q-site within the NarGHI complex. Figure 2 shows the effect of HOQNO and PCP on the plumbagin:nitrate (PBH2:NO\(_3^-\)) oxidoreductase activity of the native enzyme and of the NarI-K86A mutant. HOQNO inhibits NarGHI with an I\(_{50}\) of approximately 1.5µM, but has little inhibitory effect on the NarI-K86A mutant (Figure 2a). PCP is a significantly more potent inhibitor than HOQNO, with an I\(_{50}\) of approximately 0.4µM. The NarI-K86A mutant also attenuates the inhibitory effect of PCP, but not to the same extent as is observed with HOQNO, with the I\(_{50}\) increasing to 2.5µM (Figure 2b).

To gain a more detailed understanding of the inhibitory effect of PCP, we subjected the wild-type enzyme to steady-state kinetic analyses in the presence of PCP. PBH2 is oxidized by NarGHI with an estimated K\(_m\) of 147 ± 17µM. Interestingly, PCP exhibits mixed inhibition with a competitive inhibitor binding constant (K\(_{ic}\)) of 57 ± 14nM and an uncompetitive inhibitor binding constant of 490 ± 9nM. The relative magnitudes of these constants suggest that the inhibition elicited by PCP is mixed, but primarily competitive in nature.

In order to determine whether the PCP binding site is equivalent to the HOQNO binding site identified by biophysical means, we performed fluorescence (FQ) titrations which exploit the fluorescent properties of the latter inhibitor. HOQNO binds to the wild-type enzyme with affinity of approximately 250nM but it does not bind with high affinity to the NarI-K86A mutant (Figure 3a). This result is in agreement with the inhibition data presented in Figure 2a. Furthermore, competition between HOQNO and PCP occurs at an equivalent site within the NarGHI complex (Figure 3b). Addition of 5µM PCP to the fluorescence cuvette prior to performing a FQ titration with HOQNO essentially eliminates detectable HOQNO binding. A fluorescence increase is observed when the enzyme is incubated in the presence of 2.7µM HOQNO and titrated with PCP (Figure 3b), consistent with PCP being able to displace HOQNO from its binding site. Overall, the FQ data demonstrate the presence of a common binding site for PCP and HOQNO within the NarGHI complex.

To further rationalize the PCP inhibitory activity, we recorded EPR spectra of the hemes of oxidized NarGHI and NarI-K86A mutant (Figure 4). As previously reported (6,7), HOQNO elicits a shift in the g\(_z\) of heme bD from 3.36 to 3.50 (Figures 4a, b). PCP produces a similar shift on bD from 3.36 to 3.45 (Figure 4a, c). Neither inhibitor alters the g\(_z\) feature of the heme bD spectrum. The NarI-K86A mutation has no significant effect on the g\(_z\) of heme bD (Figure 4d). HOQNO and PCP elicit
shifts in the heme \( b_D \) of the NarI-K86A mutant from 3.36 to 3.39 and from 3.36 to 3.48, respectively (Figure 4e, f). As for the wild-type enzyme, no PCP or HOQNO effects are observed on heme \( b_P \). Overall, these results are consistent with the kinetic and FQ data (Figures 2 and 3). In order to explain the inhibitory effects of PCP and the behavior of the NarI-K86A mutant, we have determined the crystal structure of the wild-type and mutant enzymes in the presence and absence of inhibitor.

**The structural environment of the PCP binding site.**

The NarI subunit (225 aa) consists of five tilted transmembrane helices numbered sequentially from I to V with the N and C terminus located towards the periplasmic and the cytoplasmic side of the membrane, respectively (Figure 5a). The four helices II to IV are arranged in a bundle which accommodates the two hemes \( b_P \) and \( b_D \). Each heme is coordinated by two conserved histidine residues, H56 and H205 for \( b_P \) and H66 and H187 for \( b_D \). Helices IV and V are connected by two extra helices, IV' and IV'', which are in a hairpin-like arrangement and located on the periplasmic side. The C terminus of NarI extends into the cytoplasmic domain of the enzyme establishing extensive interactions with both NarG and NarH. The structure of NarI reveals two hydrophobic clefts (Figure 5b): an elongated cavity (A) located between helices I, II and IV' which exposes the edges of both hemes to the membrane, and a smaller pocket (B) which is delimited by helices II, III and IV' and leads to heme \( b_D \). Both clefts, with the exclusion of bulk solvent, represent plausible locations for Q-sites.

In order to structurally characterize the PCP binding site, NarGHI crystals were soaked in the presence of this inhibitor and the structure of the NarGHI-PCP complex was determined to 2.0Å resolution (Table 1, Figure 5). The C\( \alpha \) backbone of the NarI subunit from the NarGHI-PCP complex superposes with NarI of the native enzyme with a root mean square deviation (rmsd) of only 0.14Å for 217 C-\( \alpha \) atoms (as determined with Protein Structure Comparison Service SSM at the European Bioinformatics Institute (30)). The initial \( \text{Fo}-\text{Fc} \) difference electron density map, calculated prior to inclusion of the inhibitor molecule into the model, clearly reveals the presence of one PCP molecule intimately bound to NarI. Refinement statistics indicate the PCP is likely bound with full occupancy to NarI and temperature factor analysis supports the strong binding of the inhibitor with average thermal parameters of the PCP very similar to that of the surrounding NarI residues, (38 and 33Å\(^2\), respectively). The PCP binding pocket (pocket B in Figure 5b) is located between the transmembrane helices II and III in close proximity to heme \( b_D \) towards the periplasmic side of the membrane (Figure 5a). The PCP aromatic ring binds in a cavity formed by primarily nonpolar residues: three glycine residues (G65, G69 and G94) and M70 from helix II, M89 and A90 from helix III and M156 and V160 from a short helix connecting helices IV and V (Figure 6a). The majority of these residues (G65, G69, A90 and G94) are highly conserved in the NarI proteins from different bacterial species (31). Although the nature of the pocket is primarily hydrophobic (Figure 5b), a few significant hydrogen-bonds between PCP and NarI are also observed. The hydroxyl group of PCP is hydrogen-bonded to one of the propionate groups of \( b_D \) and to N\( \varepsilon \)-H66 (2.8Å distance from both ligands, Figure 6a). H66 simultaneously coordinates the heme \( b_D \) iron atom via the side chain N\( \delta \) atom. The edge-to-edge distance between the heme \( b_D \) and PCP is 2.8Å which is well within the physiological limits for electron transfer (32). In light of these observations, we propose that the observed PCP pocket mimics the physiological Q\( D \)-site. We have modeled an ubiquinol molecule in this cavity with the head group overlapping with the PCP aromatic ring (Figures 6a, b). One hydroxyl group (position 1) of UQH\(_2\) points in the same direction as the hydroxyl group of the inhibitor suggesting a pathway for the electron transfer to the heme cofactor. The hydroxyl group on the opposite site of the quinol molecule (position 4) is oriented as Cl4 and the long alkyl chain extends gradually towards the opening of the hydrophobic cleft stabilized by M89, A93, V64 and G65. The PCP binding pocket can also accommodate a menaquinol molecule (Figure 6c) suggesting a potential multifunctional Q-site for both napthoquinol and benzoquinol substrates.

**Structural characterization of the mutant K86 in NarI.**

The conserved residue K86 in NarI is located at one end of the PCP binding pocket (Figure 6a). The positively charged N\( \varepsilon \) of K86 has no logical
hydrogen bond donor with the chlorinated PCP and lies ~6Å from the Cl4 atom in the inhibitor. However, our molecular modeling of the physiological substrate UQH2 based on the PCP position suggests that relatively small conformational movements of the K86 side chain could allow it to play a role in substrate stabilization, providing a hydrogen bond donor for the hydroxyl oxygen at position 4 (analogous to Cl4 in PCP) (Figure 6b). In order to prove our hypothesis, we have determined the crystal structure of NarGHI carrying the single-point mutation K86A in NarI, previously characterized by kinetic, FQ and EPR experiments (Figures 2-4). The structure of NarGHI-K86A (1.9 Å resolution; Table 1) is highly similar to the native structure (rmsd of 0.12Å for 217 superposed Cα atoms in NarI (30)) indicating that the structural integrity of the PCP binding pocket has been largely preserved in this mutant form. In addition, after soaking the NarGHI-K86A crystals with PCP, the inhibitor can still bind to the QD-site (Supplementary Material). On the other hand, our fluorescence quench titration experiments showed that NarGHI-K86A has much lower affinity for the menaquinol analog HOQNO compared to the native enzyme (Figure 3a) (6) and has a lower plumbagin:nitrate oxidoreductase activity (10 sec⁻¹) than the wild-type NarGHI (68 sec⁻¹). Overall, the experimental data strengthen our working model that K86 is an essential residue in defining the Q-site and that the binding of ubiquinol as well as menaquinol electron donors can occur at our structurally defined pocket.

Structural characterization of the mutant H66Y in NarI.

In native NarGHI, the Nδ side-chain atoms of the highly conserved H66 (helix II) and H187 (helix V) in NarI provide direct coordination to the iron atom of the heme bD. It has been shown that the single point mutation of H66 to tyrosine (H66Y) prevents the insertion of the heme bD as well as the binding of quinol analogs and inhibitors. Consequently, no quinol-dependent heme reduction is detected (7,9). However, in the NarGHI-PCP complex structure we present here, the 13.2Å edge-to-edge distance between the heme bD and the bound PCP could still support electron transfer even in the absence of the heme bD (32). In order to understand the molecular basis for the effect of the H66Y mutation on quinol-dependent activity we have determined the crystal structure of NarGHI-H66Y to 2.5Å resolution (Figure 7). The structure is very similar to the native NarGHI with a rmsd of only 0.27Å for 211 common Cα atoms between the NarI-H66Y subunit and the native NarI (30). As expected, NarI-H66Y shows only one redox center, the heme bD. No electron density for the distal heme bD is observed at any level in our maps. It appears that in the absence of the heme bD, the structure is stabilized in a native-like conformation via a hydrogen bond between Y66-OH and H187-Nδ (~3.0Å). A localized but major change is also observed for the transmembrane helix II where no electron density is observed for a stretch of residues from the mutated Y66 to L81 (T72 to L81 is also highly disordered in the native enzyme). This disordered region contains two residues, G69 and M70, which directly form one side of the PCP binding pocket. The less rigid protein environment around the potential QD site explains the loss of PCP and quinol binding in the mutant enzyme.

Proton pathway from the PCP binding site.

Experimental evidence shows that NarI allows for the coupling of quinol oxidation and proton translocation towards the periplasm (31). The two propionate groups of bD in NarI point towards the periplasmic face of the membrane. Between the propionates and the bulk solvent (~9Å distance from the surface), there exists a network of water molecules formed by W580, W595, W676 and W677 (also present in the native structure, PDB code 1Q16). The observed water molecules (temperature factors range from 28-48Å² at full occupancy) are buried within NarI and are the only water molecules observed within the transmembrane helices. These waters form several hydrogen bonds amongst themselves and with both the propionate groups of heme bD, the main chain nitrogen atoms of S154, E155, M156 and the side chains of the highly conserved S143, S147 and Q87 (Figure 6a). This water channel suggests a possible proton pathway from the QD-site to the periplasm.

DISCUSSION

We have presented herein the crystal structure of Nitrate Reductase A (NarGHI) in complex with the specific inhibitor PCP at 2.0Å resolution. The high quality electron density map shows that the bound PCP is well ordered in the structure corroborating the measured affinity of this compound for NarI.
The close proximity of PCP to heme \( b_D \) (edge-to-edge distance of only 2.8 Å) and the significant change in the EPR line shape of \( b_D \) elicited by this compound are highly suggestive that PCP mimics the physiological quinol electron donors. Furthermore, the location of the PCP binding pocket is consistent with the involvement of both hemes in electron transfer from quinol to nitrate (1, 6, 12).

The PCP binding pocket is located in the narrow hydrophobic cleft B (Figure 5b) which provides a more ideal spatial constriction for the correct quinol orientation compared to the wider cleft A. Molecular modeling shows that both physiological electron donors, ubiquinol and menaquinol, can occupy the PCP binding pocket supporting the proposal of a multifunctional site in NarI. This hypothesis is also strengthened by the mainly competitive inhibitory effect of PCP (an UQH\(_2\) analog) on NarGHI activity in the presence of plumbagin (a MQH\(_2\) analog). In addition, mutation of the amino acid K86, which lines the proposed Q-site, to a neutral residue affects HOQNO (a MQH\(_2\) analog) binding. The Q\(_b\) site in \textit{E.coli} Fumarate Reductase (QFR) represents a similar example of multifunctional site. In fact, crystallographic analysis has shown that the two inhibitors, DNP-19 and HOQNO, can both bind at Q\(_b\). It has to be noted that these molecules adopt different locations within the same pocket (33). It is conceivable that also within Q\(_b\) of NarI menaquinol and ubiquinol occupy different positions and further structural studies will be pursued with diverse analogs and inhibitors.

The available information for many quinone reactive sites in different respiratory and photosynthetic complexes shows very weak sequence and structural similarities (34). However, the Q-site identified in our study contains some critical residues which have been observed in other respiratory proteins. For example, H66, which coordinates heme \( b_D \), is positioned within hydrogen bonding distance from the hydroxyl group of PCP. Histidine residues are emerging as a common theme in quinol/quinone binding and in particular a heme-ligand histidine functioning as a quinone-ligand has been described in \textit{E.coli} FdnI, the redox partner of NarGHI during nitrate respiration (4). Furthermore, our mutagenesis studies have shown the importance of another residue, K86, for NarI functionality. Similarly, in \textit{E.coli} QFR a lysine residue in close proximity to the quinone molecule at the Q\(_b\) site plays a potential role in proton shuttling (33).

A second Q-site in NarI has been recently proposed according to the available kinetic data (9). The elongated hydrophobic cavity A (Figure 5b) where both hemes are exposed is a good candidate for a secondary quinone reactive site. On the other hand, the apolar pocket A does not contain any ionizable group as found in a number of Q-sites, including Q\(_b\) in \textit{E.coli} QFR (35), Q\(_b\) in \textit{Wolinella succinogenes} QFR (36) and Q\(_b\) in \textit{E.coli} FdnGHI (4). This observation suggests that the hypothetical secondary Q-site may be important for structural integrity of NarI with possible electron transfer but not proton translocation activities. The lack of density for any quinone molecule at this site in the native and NarGHI-PCP complex structures could be due to the protein purification protocol in the presence of the detergent Thesit. In fact, tubular-shaped densities which can be modeled as the aliphatic chains of detergent or lipid molecules were observed in this cavity in the native NarGHI structure (1).

Quinol oxidation by NarI leads to electron transfer through the prosthetic groups of the enzyme as well as proton release into the periplasm. The chain of water molecules located between the propionates of heme \( b_D \) and the bulk solvent recalls a “proton wire” which has been proposed in other respiratory enzymes, such as \textit{E.coli} FdnGHI (4) and the yeast cytochrome bc\(_1\) complex (37). Assuming that the PCP binding site functions as a Q-site, we suggest a potential quinol oxidation mechanism. (i) A fully reduced ubiquinol molecule binds to the site in NarI. The hydroxyl group in position 1 of UQH\(_2\) forms hydrogen bonds to one of the \( b_D \) propionates and to H66. (ii) One electron is transferred to \( b_D \), the first redox cofactor in the Nar chain and one proton is shuttled towards the periplasm via the propionate group and the water channel illustrated in the structure. A semiquinone intermediate species is formed. (iii) The second electron is transferred with complete oxidation of quinol to quinone. A second proton has to be released and K86 could provide the pathway for the proton to the aqueous milieu. The fully oxidized ubiquinone must now dissociate to allow further enzyme turnover.

**ACKNOWLEDGMENTS**
The authors wish to thank Delilah Mroczko for her assistance with the B. Braun Biostat B fermentation system. We also thank the Department of Energy and staff at the Advanced Light Source - beamlines 8.2.1/8.2.2 for access to synchrotron radiation. This work was funded by grants from NIH, CIHR and HHMI. MB is a CIHR fellow, JHW a Canada Research Chair in Membrane Biochemistry, NCJS a CIHR Investigator and HHMI International Scholar and JW is a CRC I Chair.

REFERENCES


FIGURE LEGENDS

Figure 1: Chemical structures for oxidized menaquinol (a), ubiquinol (b), pentachlorophenol (c), 2-n-heptyl-4-hydroxyquinoline-N-oxide (d) and plumbagin (e).

Figure 2: Inhibitory effect of HOQNO and PCP on the PBH$_2$NO$_3$ oxidoreductase activity of NarGHI. (a) Effect of increasing HOQNO concentration on enzyme activity. (b) Effect of increasing PCP concentration on enzyme activity. Diamonds, wild-type. Squares, NarGHI-K86A mutant. Assays were carried out as described in Materials and Methods. PBH$_2$ was used at a concentration of 300µM, and the activities were normalized so that the activity at zero inhibitor concentration was 1.0.

Figure 3: (a) HOQNO does not bind to the NarGHI-K86A mutant. Open symbols, HOQNO binding determined by FQ titration to wild-type NarGHI in situ in E. coli LCB79/pVA700 membranes at 0.2mg mL$^{-1}$ (circles), 0.4mg mL$^{-1}$ (triangles), 0.6mg mL$^{-1}$ (diamonds), and 0.8mg mL$^{-1}$ (squares). Data were fitted to a $K_d$ of 0.25µM and a specific Q-site concentration of 0.88nmol (mg protein)$^{-1}$. No HOQNO binding was detected in membranes enriched with NarGHI-K86A (filled squares). (b) PCP competes at the HOQNO binding site of NarGHI. Titrations were carried out at a membrane protein concentration of 1mg mL$^{-1}$. Squares, titration of NaGHI-enriched membranes with HOQNO. Data were fitted to a $K_d$ of 270nM and an enzyme (binding site) concentration of 1.1nmol mg$^{-1}$. Triangles, titration of NarGHI enriched membranes with HOQNO in the presence of a saturating amount of PCP (5µM). Data were fit to a nominal $K_d$ of 2µM. Triangles, displacement of HOQNO by PCP. Prior to titration with PCP, HOQNO was added to a final concentration (total, bound plus unbound) of 2.7µM. HOQNO released from its binding site by PCP was detected by the increased HOQNO-specific fluorescence.

Figure 4: Effects of HOQNO and PCP on the EPR spectrum of the oxidized hemes of NarI. (a) NarGHI, (b) NarGHI plus 0.5mM HOQNO, (c) NarGHI plus 0.5mM PCP, (d) NarI-K86A mutant, (e) NarGHI-K86A plus 0.5mM HOQNO and (f) NarGHI-K86A plus 0.5mM PCP. Air oxidized samples were prepared with no additions, with 0.5mM HOQNO, or with 0.5mM PCP. Spectra were recorded at 12K using a microwave power of 20mW at 9.380GHz with a modulation amplitude of 20Gpp at 100KHz. 10 scans were averaged for each spectrum. Purified protein was used at a concentration of approximately 10mg mL$^{-1}$, prepared as previously described (1).

Figure 5: The transmembrane subunit NarI and the PCP binding site. (a) Ribbon representation of NarI viewed parallel to the membrane. Transmembrane helices are numbered from I to V. The two hemes (b$P$ and bD) and the PCP molecule are shown in stick rendering. The 2Fo-Fc difference electron density map is shown for PCP (contoured at 1σ to a resolution of 2.0Å). (b) GRASP (38) surface representation of NarI viewed from a similar direction as in a. Polar and hydrophobic areas are in grey and green, respectively. The region above the dotted line is buried by interactions with NarG and NarH.

Figure 6: Detailed view of the PCP binding pocket (a), models of the ubiquinol (b) and menaquinol (c) binding sites. The heme b$_D$, PCP, UQH$_2$ and MQH$_2$ are shown in stick rendering. Side-chains of residues forming the PCP binding pocket and side-chains of residues involved in hydrogen-bond interactions are also shown.

Figure 7: Structure of the mutant NarGHI-H66Y. Ribbon representation of NarI bearing the mutation H66 to Y viewed parallel to the membrane.
Table 1. Data collection and refinement statistics.

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<th>NarGHI/PCP</th>
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<td>30.0-1.9</td>
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1. Values in parentheses are for the highest resolution shell (2.07-2.0 Å).
2. $R_{merge}=\frac{\Sigma (I_{hk}\text{kl})-<I>}{\Sigma I_{hk\text{kl}}}$, where $I_{hk\text{kl}}$ is the integrated intensity of a given reflection.
3. $R_{work}=\frac{\Sigma |F_o-F_c|}{\Sigma |F_o|}$, where $F_o$ and $F_c$ are observed and calculated structure factors.
4. 5% of reflections were excluded from the refinement to calculate $R_{free}$. 
Structural and biochemical characterization of a quinol binding site of escherichia coli nitrate reductase A (NarGHI)
Michela G. Bertero, Richard R. A. Rothery, Nasim Boroumand, Monica Palak, Francis Blasco, Nicolas Ginet, Joel H. Weiner and Natalie C. J. Strynadka

J. Biol. Chem. published online December 22, 2004

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