Structural and Biochemical Characterization of a Quinol Binding Site of Escherichia coli Nitrate Reductase A*

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The crystal structure of Escherichia coli nitrate reductase A (NarGHI) in complex with pentachlorophenol has been determined to 2.0 Å of resolution. We have shown that pentachlorophenol 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.

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 and an [Fe-S] cluster (FSO); 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 bD, and bD2, to indicate their proximal (bD) and distal (bD2) positions to the catalytic site. NarG and NarH form a soluble cytoplasmically localized catalytic domain anchored to the membrane by NarI. NarGHI catalyzes electron transfer from a quinol binding site located in NarI through the redox cofactors aligned as an “electric wire” through the complex (bD to bD2 to FS4 to FS3 to FS2) to the molybdo-bis(molybdopterin guanine dinucleotide) 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 toward 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 2.0 Å of 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 bD2), 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 (aroD139 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 5 liter batches with a 1% inoculum in a B. Braun Biostat B fermenter at 30 °C in the presence of 100 μg ml−1 ampicillin and 100 μg ml−1 streptomycin. The growth medium contained 12 g liter−1 Tryptone, 24 g liter−1 yeast extract, 5 g liter−1 NaCl, and 4 ml liter−1 glycerol. NarGHI overexpression was induced at A600 = 2.0, after which the cultures were grown for 10–11 h. Cells were harvested by centrifugation, washed in a buffer containing 100 mM MOPS and 5 mM EDTA, and frozen in liquid nitrogen at −80 °C.

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**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 described previously (6). All membrane preparation steps were carried out in a buffer containing 100 mM MOPS and 5 mM EDTA (pH 7.0). Excess sucrose was removed by resuspension and recentrifugation in this buffer. Purified NarGHI was prepared by anion exchange chromatography on a DEAE-FF column (Amersham Biosciences) as described by Bertero et al. (1). Membrane and purified samples were flash-frozen in liquid nitrogen and subsequently stored at −70 °C before 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 20 mM stock ethanolic solution was reduced by metallic zinc in acidified ethanol as described previously (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 419 nm using an extinction coefficient of 3.95 mM⁻¹ cm⁻¹ (15). Enzyme assays were carried out in a degassed buffer containing 100 mM MOPS/KOH, 4 mM KNO₃ and 5 mM EDTA (pH 7.0). A concentration range of 0.140–1.4 mM PBH₂ was used to determine kinetic parameters, and reactions were initiated by the addition of protein (either enriched membranes or purified protein). The competitive inhibitor constant for PCP (*Kᵢ*) was estimated from plots of *Kᵢ* versus PCP concentration, and the competitive inhibitor constant (*Kᵢ*) was estimated from plots of 1/νₚ versus PCP concentration (16).

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

**FQ Titrations with 2-n-Heptyl-4-hydroxyquinoline-N-oxide (HQNO) and Estimation of NarGHI Content in Membrane Samples—**The affinity of NarGHI for HQNO was determined by performing FQ titrations using a PerkinElmer Life Sciences LS-50B luminescence spectrometer (6, 17, 18). Fluorescence intensities were measured using an excitation wavelength of 341 nm and an emission wavelength of 479 nm. All experiments were carried out at room temperature and pH 7.0 in 100 mM MOPS/KOH and 5 mM EDTA. HQNO was added to the fluorescence cuvette from a 0.25 mM stock ethanolic solution. A range of protein concentrations was used as indicated in the individual figure legends. The observed fluorescence (*Fₜₜ*) 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 HQNO 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 molybdo-bis(molybdopterin guanine dinucleotide) 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 and K86A and H66Y in NarI, were obtained in conditions similar to those reported earlier for the native enzyme (1). NarGHI-C26A crystals were soaked in 50 μl of cryoprotectant solution (35% polyethylene glycol 3000, 350 mM sodium acetate, 200 mM KCl, 100 mM Hepes (pH 7.0), 5 mM EDTA, and 0.7 mM Thesit (Fluka)) in the presence of 0.5 mM 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 100 K at the Advanced Light Source (beamslines 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 1.9 Å 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 I. 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, 1Y5L 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 and ubiquinol (UQH₂) as physiological electron donors (Figs. 1, a and b) (27, 28). Studies of quinol binding to NarGHI have been expedited by the use of the menaquinone analog HQNO (Fig. 1d), which has been demonstrated to inhibit enzyme activity by binding to a single site in close proximity to heme b₅ within NarI (6, 7). We have so far been unable to produce crystals of the NarGHI-HQNO complex, so we pursued the characterization of other potential inhibitors of the enzyme. PCP (Fig. 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 spe-
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 s⁻¹ compared with 68 s⁻¹, respectively. The decreased activity of the mutant enzyme suggests that the NarI Lys-86 residue is probably located in the vicinity of a functional Q-site within the NarGHI complex. Fig. 2 shows the effect of HOQNO and PCP on the plumbagin:nitrate (PBH₂NO₃⁻) oxidoreductase activity of the native enzyme and of the NarI-K86A mutant. HOQNO inhibits NarGHI with an I₅₀ of ~1.5 μM but has little inhibitory effect on the NarI-K86A mutant (Fig. 2A). PCP is a significantly more potent inhibitor than HOQNO, with an I₅₀ of ~0.4 μM. The NarI-K86A mutant also attenuates the inhibitory effect of PCP but not to the same extent as is observed with HOQNO, with the I₅₀ increasing to ~2.5 μM (Fig. 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 Kₘ of 147 ± 17 μM. Interestingly, PCP exhibits mixed inhibition with a competitive inhibitor binding constant (Kᵢ) of 57 ± 14 nM and an uncompetitive inhibitor binding constant of 490 ± 9 nM. The relative magnitudes of these constants suggest that the inhibition elicited by PCP is mixed, but primarily competitive in nature.

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 an affinity of ~250 nM, but it does not bind with high affinity to the NarI-K86A mutant (Fig. 3A). This result is in agreement with the inhibition data presented in Fig. 2A. Furthermore, competition between HOQNO and PCP occurs at an equivalent site within the NarGHI complex (Fig. 3B). The addition of 5 μM PCP to the fluorescence cuvette before 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 2.7 μM HOQNO and titrated with PCP (Fig. 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 effect, we recorded EPR spectra of the hemes of oxidized NarGHI and NarI-K86A mutant (Fig. 4). As previously reported (6, 7), HOQNO elicits a shift in the gₛ of heme b₅ from 3.36 to 3.50 (Fig. 4, A and B). PCP produces a similar shift on b₅ from 3.36 to 3.45 (Fig. 4, A and C). Neither inhibitor alters the gₛ feature of the heme b₇ spectrum. The NarI-K86A mutation has no significant effect on the gₛ of heme b₅ (Fig. 4D). HOQNO and PCP elicit shifts in the heme b₇ gₛ of the NarI-K86A mutant from 3.36 to 3.39 and from 3.36 to 3.48, respectively (Fig. 4, E and F). As for the wild-type enzyme, no PCP or HOQNO effects are observed on heme b₅ gₛ. Overall, these results are consistent with the kinetic and FQ data (Figs. 2 and 3). 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 toward the periplasmic and the cytoplasmic side of the membrane, respectively (Fig. 5a). The four helices II to IV are arranged in a bundle which accommodates the two hemes, bP and bD. Each heme is coordinated by two conserved histidine residues, His-56 and His-205 for bP and His-66 and His-187 for bD. Helices IV and V are connected by two extra helices, IV/H11032 and IV/H11033, 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 (Fig. 5b), an elongated cavity (A) located between helices I, II, and IV’ that exposes the edges of both hemes to the membrane and a smaller pocket (B) that is delimited by helices II, III, and IV’ and leads to heme bD. Both clefts, with the exclusion of bulk solvent, represent plausible locations for Q-sites.

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 2.0 Å of resolution (Table I, Fig. 5). The Cα backbone of the NarI subunit from the NarGHI-PCP complex superposes with NarI of the native enzyme with a root mean square deviation (r.m.s.d.) of only 0.14 Å for 217 Cα atoms (as determined with Protein Structure Comparison Service SSM at the European Bioinformatics Institute (30)). The initial $F_o - F_c$ difference electron density map, calculated before 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 33 Å², respectively). The PCP binding pocket (pocket B in Fig. 5b) is located between transmembrane helices II and III, in close proximity to heme bD toward the periplasmic side of the mem-
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Table 1
Data collection and refinement statistics

|       | NarGHI/PCP | K86A   | H66Y   |
|-------|------------|--------|--------|
| λ (Å) | 1.0781     | 1.0781 | 1.0000 |
| Resolution (Å) | 30.0–2.0 | 30.0–1.9 | 25.0–2.5 |
| Unique reflections | 161,680 | 195,380 | 90,666 |
| Completeness (%)<sup>a</sup> | 92.2 (68.0) | 96.5 (78.6) | 99.5 (97.7) |
| Redundancy | 4.9 | 4.8 | 6.4 |
| R<sub>merge</sub> (%)<sup>b</sup> | 7.3 (39.2) | 9.0 (42.4) | 11.1 (48.2) |
| I/σ<sub>I</sub> | 14.6 (2.2) | 14.8 (2.3) | 15.2 (3.5) |
| Refinement | | | |
| Resolution (Å) | 30.0–2.0 | 30.0–1.9 | 25.0–2.5 |
| R<sub>work</sub> (%)<sup>c</sup> | 19.9 | 18.6 | 18.8 |
| R<sub>free</sub> (%)<sup>d</sup> | 23.6 | 21.2 | 23.7 |
| Average B factor (Å<sup>2</sup>) | | | |
| Main chain | 26.1 | 21.9 | 27.1 |
| Side chain | 28.2 | 24.5 | 27.3 |
| r.m.s.d. bond length (Å) | 0.006 | 0.006 | 0.006 |
| r.m.s.d. bond angles (%) | 1.4 | 1.4 | 1.4 |

<sup>a</sup> Values in parentheses are for the highest resolution shell (2.07–2.0 Å).

<sup>b</sup> R<sub>merge</sub> = Σ|I<sub>obs</sub>−(I/ΣI<sub>obs</sub>), where I<sub>obs</sub> is the integrated intensity of a given reflection.

<sup>c</sup> R<sub>work</sub> = (Σ||F<sub>c</sub>−|F<sub>s</sub>||/Σ|F<sub>c</sub>|), where F<sub>c</sub> and F<sub>s</sub> are observed and calculated structure factors.

<sup>d</sup> 5% of reflections were excluded from the refinement.

The edge-to-edge distance between the heme bD and PCP is 2.8 Å (2.8-Å distance from both ligands, Fig. 6). 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 (Figs. 2–4). The structure of NarGHI-K86A (1.9-Å resolution; Table I) is highly similar to the native structure (r.m.s.d. of 0.12 Å for 217 superposed Ca 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 Q<sub>d</sub> site. On the other hand, our fluorescence quench titration experiments showed that NarGHI-K86A has much lower affinity for the menaquinol analog HOQNO compared with the native enzyme (Fig. 3A) (6) and has a lower plumbagin:nitrate oxidoreductase activity (10 s<sup>−1</sup>) than the wild-type NarGHI (68 s<sup>−1</sup>). Overall, the experimental data strengthen our working model that Lys-86 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 N<sub>δ</sub> side-chain atoms of the highly conserved His-66 (helix II) and His-187 (helix V) in NarI provide direct coordination to the iron atom of the heme b<sub>δ</sub>. It has been shown that the single point mutation of His-66 to tyrosine (H66Y) prevents the insertion of the heme b<sub>δ</sub> 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 13.2-Å edge-to-edge distance between the heme b<sub>δ</sub> and the bound PCP could still support electron transfer even in the absence of heme b<sub>δ</sub> (32). 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 2.5-Å resolution (Fig. 7). The structure is very similar to the native NarGHI, with a r.m.s.d. of only 0.27 Å for 211 common Ca atoms between the NarI-H66Y subunit and the native NarI (30). As expected, NarI-H66Y shows only one redox center, the heme b<sub>δ</sub>. No electron density for the distal heme b<sub>δ</sub> is observed at any level in our maps. It appears that in the absence of the heme b<sub>δ</sub>, the structure is stabilized in a native-like conformation via a hydrogen bond between Tyr-66-OH and His-187-N<sub>δ</sub> (3.0 Å). 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 Tyr-66 to Leu-81 (Thr-72 to Leu-81 is also highly disordered in the native enzyme). This disordered region contains two residues, Gly-69 and Met-70, which directly form one side of the PCP binding pocket. The less rigid protein environment around the potential Q<sub>d</sub> site explains the loss of PCP, HOQNO, 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 toward the periplasm (31). The two propionate groups of bD in NarI point toward the periplasmic face of the membrane. Between the propionates and the bulk solvent (∼9 Å distance from the surface) there exists a network of water molecules formed by Trp-580, Trp-595, Trp-676, and Trp-677 (also present in the native structure, Protein Data Bank code 1Q16). The observed water molecules (temperature factors range from 28 to 48 Å<sup>2</sup> at full occupancy) are buried within NarI and are the only water molecules observed within the transmembrane helices. These waters form several hydrogen bonds among themselves and with both the propionate groups of heme b<sub>δ</sub>, the main chain nitrogen atoms of Ser-154, Glu-155, Met-156, and the side chains of the highly conserved Ser-143, Ser-147, and Gln-87 (Fig. 6a). This water channel suggests a possible proton pathway from the Q<sub>d</sub>-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 2.0 Å resolution. The high quality electron density map shows that the bound PCP is well ordered in the structure corroborating the measured affinity of NarI for this compound ($K_{i,c} = 57$ nM). 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

![Diagram](image)

**Fig. 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 menaquinol (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.
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cleft B (Fig. 5b), which provides a more ideal spatial constription for the correct quinol orientation compared with 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 UQH2 analog) on NarGHI activity in the presence of plumbagin (a menaquinol analog). In addition, mutation of the amino acid Lys-86, which lines the proposed Q-site, to a neutral residue affects HOQNO (a menaquinol analog) binding. The Qp-site in E. coli fumarate reductase represents a similar example of a multifunctional site. In fact, crystallographic analysis has shown that the two inhibitors, DNP-19 and HOQNO, can both bind at Qp. It has to be noted that these molecules adopt different locations within the same pocket (33). It is conceivable that also within Qp 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 that have been observed in other respiratory proteins. For example, His-66, which coordinates heme b2, 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-ligated histidine functioning as a quinone-ligand has been described in E. coli FdhGHI, the redox partner of NarGHI during nitrate respiration (4). Furthermore, our mutagenesis studies have shown the importance of another residue, Lys-86, for NarI functionality. Similarly, in E. coli fumarate reductase a lysine residue in close proximity to the quinone molecule at the Qp-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 (Fig. 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 Qp in E. coli fumarate reductase (35), Qp in Wolinella succinogenes fumarate reductase (36), and Qp in E. coli FdhGHI (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 presence of Theis during the purification of the complex. 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 b2 and the bulk solvent recalls a “proton wire,” which has been proposed in other respiratory enzymes such as E. coli FdhGHI (4) and the yeast cytochrome bc1 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 UQH2 forms hydrogen bonds to one of the b2 propionates and to His-66. (ii) One electron is transferred to b2, the first redox cofactor in the Nar chain, and one proton is shuttled toward 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 Lys-86 could provide the pathway for the proton to the aqueous milieu. The fully oxidized ubiquinone must now dissociate to allow further enzyme turnover.

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