Inhibitor Binding within the NarI Subunit (Cytochrome $b_{nr}$) of
Escherichia coli Nitrate Reductase A*

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We have used inhibitors and site-directed mutants to investigate quinol binding to the cytochrome $b_{nr}$ (NarI) of Escherichia coli nitrate reductase (NarGHI). Both stigmatellin and 2-<n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) inhibit menadionioxidoreductase activity with $I_{50}$ values of 0.25 and 6 $\mu$m, respectively, and prevent the generation of a NarGHI-dependent proton electrochemical potential across the cytoplasmic membrane. These inhibitors have little effect on the rate of reduction of the two hemes of NarI ($b_L$ and $b_H$), but have an inhibitory effect on the extent of nitrate-dependent heme reoxidation. No quinol-dependent heme $b_H$ reduction is detected in a mutant lacking heme $b_L$ (NarI-H56Y), whereas a slow but complete heme $b_L$ reduction is detected in a mutant lacking heme $b_H$ (NarI-H56R). This is consistent with physiological quinol binding and oxidation occurring at a site ($Q_p$) associated with heme $b_L$ which is located toward the periplasmic side of NarI. Optical and EPR spectroscopies performed in the presence of stigmatellin or HOQNO provide further evidence that these inhibitors bind at a heme $b_L$-associated $Q_p$ site. These results suggest a model for electron transfer through NarGHI that involves quinol binding and oxidation in the vicinity of heme $b_L$ and electron transfer through heme $b_H$ to the cytoplasmically localized membrane-extrinsic catalytic NarGH dimer.

Nitrate reductase A (NarGHI)$^1$ allows Escherichia coli to use nitrate as a terminal electron acceptor during anaerobic growth. This respiratory complex catalyzes quinol oxidation and proton release at the periplasmic side of the membrane and transfers electrons through various redox centers to the catalytic site where nitrate reduction and consumption of protons occurs (1). This leads to the generation of a proton electrochemical gradient across the cytoplasmic membrane (1). NarGHI comprises a catalytic subunit (NarG) containing a non-covalently bound molybdenum cofactor (2), an electron transfer subunit (NarH) containing multiple [Fe-S] clusters (3, 4), and a membrane anchor subunit (NarI) that is believed to be the location of quinol binding and oxidation (5). NarI (cytochrome $b_{nr}$) is a diheme $b$-type cytochrome (6, 7), the polypeptide of which is predicted to traverse the membrane bilayer five times (6). Such a transmembrane topology is supported by recent studies of site-directed mutants of NarI that identified the histidine axial ligands of the two $b$-type hemes on helices II (His$^{56}$ and His$^{66}$) and V (His$^{187}$ and His$^{205}$) (7). In NarI, the low potential heme (heme $b_L$, $E_{0,7} = +10$ mV) appears to be near the periplasmic surface of the membrane, whereas the high potential heme (heme $b_H$, $E_{0,7} = +120$ mV) appears to be near the cytoplasmic surface. These positions relative to the membrane surfaces were predicted from the disposition of the coordinating histidines within the transmembrane helices and corroborated by EPR and optical studies of wild-type and site-directed NarI mutants (7). Such topological characteristics enable the NarI protein to interact directly with the membrane-embedded quinols and facilitate the transfer of electrons from the periplasm to the cytoplasmic site of the membrane.

To better understand the interaction of quinols with NarGHI, and more specifically with NarI, it is necessary to define functional domains and locate binding sites of inhibitors at key positions on the electron transfer pathway from quinol to nitrate. Specific inhibitors of cytochrome $b$-containing respiratory enzymes have been important tools for delineating the electron transfer mechanism of ubiquinol:cytochrome c oxidoreductase (cytochrome $b_1$, complex III) (8–10). It is noteworthy that the cytochrome $b$ mutations of the $bc_1$ complex that affect the inhibitor binding sites are all concentrated in four regions delineating the two quinol binding sites $Q_o$ and $Q_i$ located on the positive (outer) and negative (inner) sides, respectively, of the cytoplasmic membrane in prokaryotes or the inner mitochondrial membrane in eukaryotes (11). The recently determined crystal structure of the $bc_1$ complex from bovine heart mitochondria clearly shows that both antimycin A and myxothiazol-binding sites partly overlap the quinone-binding site at the $Q_o$ site and the $Q_i$ site, respectively (12). 2-<n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) is the most general inhibitor of the quinone-reacting cytochromes $b$, and its structure is reminiscent of physiological quinones. Such similarity suggests that its binding pocket may overlap quinone-interacting sites in the vicinity of the $b$-cytochromes. HOQNO has been shown to inhibit the quinol:quinone oxidoreductase activity from various organisms (13–15), as well as nitrate-de-
pendent proton extrusion into the periplasm (1). HQNO does not inhibit the benzyl violen:nitrate oxidoreductase activity (5), suggesting that its binding site is associated with NarI (this subunit is not essential for benzyl violen:nitrate oxidoreductase activity). Emerging structural data on NarI (7) combined with information obtained from the effects of specific inhibitors would be of importance in delineating the electron transfer and proton release mechanism of NarGHI.

Using optical and EPR spectroscopies of both wild-type and site-directed mutants of NarI, we report herein spectral shifts caused by several inhibitors of quinol oxidation. Our results suggest that physiological quinol oxidation occurs at a stigmatellin/HOQNO binding site (QP) which is located close to the low potential heme (heme b1), near the periplasmic side of the membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The E. coli strains and plasmids used in this study are listed in Table I. Plasmids pVA700 and pCD7 carry the narGHJI operon and the narI gene, respectively, under the control of the tac promoter (ptac).

**Growth of Bacteria and Preparation of E. coli Membranes Vesicles**—For studies of proton translocation, enzyme activity, and heme reduction/oxidation, cells were grown microaerobically at 37 °C as described previously (16). For EPR studies, cells were grown microaerobically overnight at 30 °C. Cultures were harvested when the A600 reached 1.0. Membrane vesicles were prepared by French pressure lysis in 100 mM MOPS buffer (pH 7.0) containing 5 mM EDTA and phenylmethanesulfonyl fluoride (0.2 mM) (16). Membranes were frozen in liquid N2 and stored at −70 °C until use.

**Enzyme Assays**—The benzyl violen:nitrate oxidoreductase activities were assayed by a method modified from Jones and Garland (17, 18). Menadion:nitrate and duroquinol:nitrate oxidoreductase activities were measured as described by Guigliarelli et al. (16). The Vmax values for inhibitors were defined as the concentration required to reduce the quinol:nitrate reductase activity by 50%. These values were deduced from the inhibitor titration curves of enzyme activity.

**Quinacrine Fluorescence Quenching**—Assays were performed as described (1) with a John-Yvon spectrophotometer model JY3D, using N2-saturated buffers. The reaction was initiated with 50 μM nitrate.

**Kinetics of Heme Reduction and Reoxidation**—Heme reduction and reoxidation were followed using a DW2A Chance AMINCO spectrophotometer. This instrument had a dual wavelength configuration, and heme reduction and reoxidation were followed using a wavelength of 560 nm with a reference wavelength of 575 nm. Reactions were observed at 25 °C in potassium phosphate buffer (K2HPO4/KH2PO4, 50 mM, pH 7.5) with quinol analogs (menadion or duroquinol) as electron donors (125 μM) and nitrate as electron acceptor (250 μM). Reaction conditions were as indicated in the individual figure legends.

**Inhibitor-induced Optical Shift Measurements**—For these experiments, the DW2A spectrophotometer was used in a dual-beam configuration. Difference spectra were recorded of membranes plus inhibitor minus membranes without inhibitor. Inhibitors were added from stock solutions made with ethanol as solvent, and equivalent volumes of this solvent were added to cuvettes that did not have inhibitor added. Membrane vesicles were first reduced by adding a few grains of sodium dithionite, and the base line was recorded. Inhibitors or ethanol were then added to the cuvettes. Experiments were carried out using a 50 mM MOPS buffer at pH 7.0.

**RESULTS**

**Inhibitor Effects on the Quinol:Nitrate Reductase Activities**—Quinol:nitrate oxidoreductase activities, using menadion as electron donor, were measured in the presence of various potent inhibitors of electron transfer in the mitochondrial bc1 complex. It appears that all the compounds tested, the most effective are HQNO (I50 = 6 μM) and stigmatellin (I50 = 0.25 μM) whose structures are strongly reminiscent of quinones. DCMU and anticycin A are poor inhibitors and have measurable effects only at high concentrations (I50 > 40 μM). Finally, myxothiazol, atrazine, funiculosin, UHDBT and DBMBB appeared to have no effect on the quinol:nitrate oxidoreductase activity. Fig. 1 shows the concentration dependence of the HQNO and stigmatellin inhibition of menadion:nitrate reductase activity. The major part of the quinol activity is inhibited in a hyperbolic fashion, the remaining part of the activity constituting about 5 to 20% of the overall activity.

**Effects of Inhibitors on the Nitrate Reductase Mediated Proton Translocation**—Quinacrine hydrochloride distributes according to the transmembrane ΔpH and its accumulation within the membrane vesicles, in response to an inward translocation of protons, gives rise to a quenching of its fluorescence (19, 20). This phenomenon has been used herein to study the effects of the inhibitors on the nitrate reductase-dependent proton translocation in membrane preparations. These experiments were performed on membrane vesicles with overexpressed wild-type holoenzyme that possess an inside-out orientation with respect to the original cells.

**Addition of Nitrate to the Reaction Cuvette Containing the Membrane Vesicles and the Electron Donors (formate or quinol analogs) elicits a large quenching of fluorescence in rate and extent (Fig. 2). The energy-dependent quinacrine quenching in these particles can be prevented in three ways: (i) by uncouplers like carbonyl cyanide m-chlorophenylhydrazone (2 μM) suggesting that quinacrine distributes between the inner and outer compartments of the particles in response to a pH gradient rather than a membrane potential (21); (ii) by low concentrations of azide (25 μM) sufficient to inhibit specifically the nitrate reductase activity as previously reported (22) and (iii) by adding inhibitors of quinol binding and oxidation (1, 20). As expected, inhibitors such as myxothiazol and atrazine, which have no effect on the quinol-nitrate oxidoreductase activity, do not perturb the quenching of fluorescence (Fig. 2). Effective inhibitors of the nitrate reductase activity, HQNO, stigmatellin, DCMU and anticycin A, inhibit proton extrusion as observed by the absence of fluorescence quenching.

**Effect of Inhibitors on the Reduction and Reoxidation of the NarI Hemes**—The inhibitory effect of HQNO, stigmatellin
and, to a lesser extent DCMU, on the nitrate reductase activity was further assessed by observing NarI heme reduction by menadiol and subsequent reoxidation by nitrate. This was done to investigate which step is modified by the inhibitors in the overall steady-state electron transfer from quinol to nitrate. The results are shown in Fig. 3A. While quinol-dependent heme reduction is only slightly affected by the inhibitors, the extent of the nitrate-dependent heme reoxidation appears to be significantly decreased by HOQNO and stigmatellin (Fig. 3A), and to a lesser extent by DCMU (data not shown). Adding HOQNO and stigmatellin at the same time to the assays does not lead to further modification of the behavior of the hemes (data not shown), suggesting that both inhibitors act at the same level in the electron transfer pathway. This contrasts with what is observed in the mitochondrial cytochrome $b_{c_1}$ complex in which HOQNO and stigmatellin bind at separate sites on the opposite sides of the mitochondrial inner membrane (10).

To obtain further information on the location(s) of the inhibitor-binding sites, we studied heme reduction and reoxidation in a NarI-H66Y mutant devoid of heme $b_L$, and in a NarI-H56R mutant devoid of heme $b_H$ (7). The reduction by menadiol of the heme $b_L$ in the NarI-H56R mutant cannot be reoxidized by nitrate (Fig. 3B). The slow but total reduction of the heme $b_L$ in this mutant is not significantly impaired by HOQNO or stigmatellin (data not shown), in agreement with the results presented for the wild-type enzyme (see above). The kinetic behavior of each NarI mutant is fully in agreement with their inability to sustain anaerobic
growth on nitrate and underlines the importance of the presence of both hemes for electron/proton transfer within NarGHI.

**Effect of Inhibitors on the Optical Spectrum of the NarI Hemes**—It is generally accepted that in the presence of effective inhibitors, the most affected heme is that closest to the inhibitor binding site. We therefore examined the effect of inhibitors on the optical spectrum of the NarI hemes. As expected, no shifts are observed in the heme α-bands of reduced NarGHI in the presence of saturating amounts of myxothiazol, funiculosin, DBMIB, atrazin or UHDBT (data not shown). With the wild-type strain LCB2048/pVA700, the difference spectra observed after adding saturating amounts of HOQNO or stigmatellin to the reduced membrane vesicles are similar in shape (Fig. 4, traces 1 and 4). These S-shaped curves have maxima and minima at 555.5 nm and 550.5 nm, respectively. The difference spectrum obtained after adding antimycin A or DCMU are similar in shape but much lower in amplitude in comparison to those observed with HOQNO and stigmatellin (data not shown).

The spectral shifts obtained with the NarI-H66Y and NarI-H56R site-directed mutants devoid of heme b₁ and heme b₃L, respectively, are also illustrated in Fig. 4. Similar HOQNO and stigmatellin-induced spectral shifts are obtained with both wild-type and heme b₁L-deficient mutants, suggesting that the heme located near the periplasmic side of the membrane (b₁L) is affected by the inhibitors (Fig. 4, traces 2 and 5). In the absence of heme b₁L, no spectral shifts are observed in the presence of HOQNO and stigmatellin (Fig. 4, traces 3 and 6). In the absence of heme b₁L, spectra observed in the presence of HOQNO and stigmatellin correspond to a slight oxidation of hemes due to the addition of inhibitors (Fig. 4, traces 3 and 6), suggesting that there is no interaction of the inhibitors with the remaining heme (heme b₃L).

**EPR Spectral Shifts Caused by the Inhibitors in the Wild-type and NarI Mutant Enzymes**—Fig. 5 shows the effects of some of the inhibitors used herein on the EPR lineshapes of oxidized hemes b₁ and b₁L in the presence of HOQNO (B), stigmatellin (C), DCMU (D), and antimycin A (E). HOQNO elicits a change in the Gₓ of heme b₁ from approximately 3.36 to 3.50, whereas stigmatellin appears to have the opposite effect on this heme, moving its Gₓ from 3.36 to approximately 3.31. DCMU and antimycin A appear to have no detectable effect on the EPR lineshape of heme b₁L. None of the inhibitors tested appeared to have any effect on the Gₓ of heme b₃L at approximately 3.76.

In order to further characterize the position of the HOQNO/stigmatellin binding site(s) within NarI, we also studied the effects of these inhibitors on EPR spectra of membranes containing overexpressed NarI in the absence of the membrane-extrinsic catalytic dimer (7). Fig. 6 shows the effect of the two most potent inhibitors, HOQNO and stigmatellin, on the lineshape of heme b₁L, which has a Gₓ of 3.15 in the absence of NarGHI. Neither inhibitor appears to have any effect on the Gₓ = 2.92 feature that we have previously attributed to conformationally relaxed or modified heme b. HOQNO elicits a change in the Gₓ of the remaining signal from 3.15 to approximately 3.45. In contrast to what was observed in the holoenzyme in Fig. 5, stigmatellin elicits a similar, but smaller shift in Gₓ to that observed with HOQNO, moving it to approximately 3.27.

**DISCUSSION**

The sequence of NarI is remarkably conserved among the membrane-bound nitrate reductases from various organisms,
intermediate arising from the succinate-de-
ning site(s) within NarGHI in much the same way as has
turnover.
quinol site-heme motif in proton release during enzyme
mic surface and heme
b
L is located near the periplasmic sur-
H is located near the cyto-
L deficient NarI-H56R mutant in comparison with the
E
m,7
s of the heme
b
L (28). Experiments
for the mechanism of quinol oxidation at the Q
b
site of NarI. In the presence of HOQNO, the
first oxidation step of the quinol (QH2 to Q) might occur
allowing the reduction of the hemes, whereas the second step
(Q2 to Q) would be suppressed. This model for HOQNO inhibi-
tion would explain the residual enzyme activity observed at
high concentrations of HOQNO (Fig. 1). The similarity between
the inhibition profiles of HOQNO and stigmatellin suggests
that their mechanisms of inhibition are identical.

A proposed mechanism of HOQNO-inhibition of B. subtilis
SQR has been suggested by Smirnova et al. (28) in which
HOQNO acts as a semiquinone anion analog that displaces the
physiological Q2
a
intermediate arising from the succinate-de-
pendent quinone reduction reaction at the quinone binding
site. Such a proposal can be applied to the mechanism of quinol
oxidation at the Q
b
site of NarI. In the presence of HOQNO, the
observed midpoint potentials of the two hemes with
b
L (Em,7
+17mV) located toward the periplasmic side and
b
H (Em,7
+122mV) located toward the cytoplasmic side of NarI, respec-
tively, suggests that physiological quinol oxidation occurs at
the Q
b
site, as previously suggested (6). This would account for
the proton release into the periplasm reported herein and else-
where (1). Heme
b
H deficient NarI-H56R mutant in comparison with the
wild-type enzyme, and no reoxidation of heme
b
H by nitrate is
observed. On the other hand, no quinol-dependent reduction of
heme
b
H is observed in the heme
b
H deficient NarI-H66Y mu-
tant enzyme, in agreement with what has been observed in B.
subtilis SQR (30). These observations support a model for qui-
Inhibitor-binding Sites of E. coli Nitrate Reductase A

nol binding and oxidation in which dissociable binding occurs only at the heme $b_1$-associated $Q_p$ site. In this model the electron flow through NarI occurs successively via heme $b_L$ and heme $b_H$ from the periplasmic side to the cytoplasmic side of the membrane, allowing subsequent reduction of the [Fe-S] clusters of the NarH subunit.

It has been suggested that there is a second non-dissociable site of quinol binding associated with the NarGH catalytic dimer (31). This $Q_{nr}$ site may be located between the hemes of NarI and the [Fe-S] clusters of NarH. The quinone normally localized at this site may be functioning as an electron conduit in much the same way as the $Q_A$ site of the bacterial photoreduction system. The possible presence of a $Q_{nr}$ site located between heme $b_H$ of NarI and the [Fe-S] clusters of NarH bears interesting comparison with results reported for a similar anaerobic reductase of *E. coli*, Me$_2$SO reductase (DmsABC) (32).

This enzyme has a similar subunit and prosthetic group composition to that of NarGH, except that its membrane anchor subunit (DmsC) does not contain heme. In DmsABC, the EPR lineshape of a [3Fe-4S] cluster introduced into DmsB by site-directed mutagenesis is significantly altered by HQQNO binding. It is possible that the NarGHI $Q_{nr}$ site might correspond to the site observed in the [3Fe-4S] mutant of DmsABC, although in the latter case the site appears to be dissociable.

Overall, we have clearly demonstrated by kinetic, optical, and EPR measurements, the presence of a quinol binding site (Q$_D$) within NarGHI that is associated with heme $b_L$ of NarI, and is located toward the cytoplasmic side of NarI. Our results suggest that this Q$_D$ site is responsible for physiological quinol oxidation and proton release into the periplasm (29 and this work). These results represent an important step in delineating the mechanisms of quinol oxidation, electron transfer, and proton release by NarGHI.

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