Characterization by Electron Paramagnetic Resonance of the Role of the *Escherichia coli* Nitrate Reductase (NarGHI) Iron-Sulfur Clusters in Electron Transfer to Nitrate and Identification of a Semiquinone Radical Intermediate

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We have used *Escherichia coli* cytoplasmic membrane preparations enriched in wild-type and mutant (NarH-C16A and NarH-C263A) nitrate reductase (NarGHI) to study the role of the [Fe-S] clusters of this enzyme in electron transfer from quinol to nitrate. The spectrum of dithionite-reduced membrane bound NarGHI has major features comprising peaks at \( g = 2.04 \) and \( g = 1.98 \), a peak-trough at \( g = 1.95 \), and a trough at \( g = 1.87 \). The oxidized spectrum of NarGHI in membranes comprises an axial [3Fe-4S] cluster spectrum with a peak at \( g = 2.02 \) (g	ext{cyt}), and a peak-trough at \( g = 1.99 \) (g	ext{cyt}). We have shown that in two site-directed mutants of NarGHI which lack the highest potential [4Fe-4S] cluster (B. Guigliarelli, A. Magalon, P. Asso, P. Bertrand, C. Frixon, G. Giordano, and F. Blasco, Biochemistry 35:4828—4836, 1996), NarH-C16A and NarH-C263A, oxidation of the NarH [Fe-S] clusters is inhibited compared to the wild type. During enzyme turnover in the mutant enzymes, a distinct 2-n-heptyl-4-hydroxyquinoline-N-oxide-sensitive semiquinone radical species which may be located between the hemes of NarI and the [Fe-S] clusters of NarH is observed. Overall, these studies indicate (i) the importance of the highest-potential [4Fe-4S] cluster in electron transfer from NarH to the molybdenum cofactor of NarG and (ii) that a semiquinone radical species is an important intermediate in electron transfer from quinol to nitrate.

*Escherichia coli*, when grown anaerobically with nitrate as respiratory oxidant, develops a respiratory chain terminated by a membrane-bound quinol:nitrate oxidoreductase (NarGHI) (5, 7). The operon encoding nitrate reductase (narGHIJ) has been cloned (37), sequenced (7), and overexpressed (17). NarGHI is a heterotrimer comprising a molybdenum cofactor containing catalytic subunit (NarG; 139 kDa), an [Fe-S] containing electron transfer subunit (NarH; 58 kDa), and a heme-containing membrane anchor subunit (NarI; 26 kDa). The narG gene product is not part of the functional enzyme but is involved in its assembly and activation (8, 15). The catalytic and electron transfer subunits are also found as a soluble NarGHI dimer which accumulates in the cytoplasm of cells harboring the narG and narI genes on multicopy plasmids (2). NarGHI has been shown to oxidize both ubiquinol-1 and reduced benzyl viologen in vitro, whereas NarGHI oxidizes only reduced benzyl viologen (34). Thus, all three subunits appear to be necessary for the catalysis of physiological electron transfer from quinol to nitrate. The enzyme appears to be able to use both ubiquinol (UQH\(_2\)) and menaquinol (MQH\(_2\)) as physiological reductants (47), and at present it is unclear whether these quinols react at a single binding site or if there are two quinol-specific binding sites.

The electron paramagnetic resonance (EPR) properties of the NarGHI dimer have recently been reported (1, 2, 17, 24). Purified NarGHI contains one [3Fe-4S] cluster (24) and three [4Fe-4S] clusters with midpoint potentials (\( E_{m,n} \)) of +60 mV ([3Fe-4S] cluster) and +80, −200, and −400 mV ([4Fe-4S] clusters) (17). Ligands to the four [Fe-S] clusters are provided by 15 Cys residues in NarH which are arranged in four groups, I to IV. Groups I, II, and IV have sequences similar to those of the Cys groups ligating [4Fe-4S] clusters in bacterial ferredoxins (10) and respiratory chain enzymes (CAx2CBx2-11). Groups I, II, and IV each contain one cysteine residue instead of the consensus Val or Ile is found at the Cn position. Site-directed mutagenesis experiments have suggested that Cys groups II and III pair up to form a [4Fe-4S] cluster motif, and Cys groups I and IV pair up to form a [2Fe-4S] cluster motif (18). The assignment of the individual Cys groups to the four potentiometrically identified [Fe-S] clusters is as follows: Cys group I, \( E_{m,n} = +80 \text{ mV} \) [4Fe-4S] cluster; Cys group II, \( E_{m,n} = −200 \text{ mV} \) [4Fe-4S] cluster; Cys group III, \( E_{m,n} = +60 \text{ mV} \) [3Fe-4S] cluster; and Cys group IV, \( E_{m,n} = −400 \text{ mV} \) [4Fe-4S] cluster (1, 18). Mutations of either the first Cys of Cys group I (C16) or the last (fourth) Cys of Cys group IV (C263) result in the loss of the highest-potential \( E_{m,n} = +80 \text{ mV} \) [4Fe-4S] cluster (1, 18). It would be of interest to use these two mutants of NarH to study the role of the highest-potential [4Fe-4S] cluster in electron transfer from quinol to nitrate.

Any insights gained into the electron transfer pathway through NarGHI would also be applicable to a range of closely related bacterial oxidoreductases. These include *E. coli* formate dehydrogenase (FdnGHI) (4), *Wolinella succinogenes*.
poly sulfide reductase (PsrABC) (27), and E. coli dimethyl sulfoxide reductase (DmsABC) (48). Each of these has an architecture comprising a molybdenum cofactor-containing catalytic subunit, an [Fe-S] cluster-containing electron transfer subunit, and a hydrophobic membrane anchor subunit. Electron transfer through the [Fe-S] clusters of the electron transfer subunit of E. coli DmsABC (DmsB) has been demonstrated via an EPR assay, the O- pool coupling assay (38, 39, 44), but a role in electron transfer for the [Fe-S] clusters in NarGHI has yet to be established. Based on redox potentiometric studies of the [Fe-S] clusters in the NarGHI dimer, it has been suggested that the two highest-potential clusters (a [4Fe-4S] cluster and the [3Fe-4S] cluster) mediate electron transport through NarH to the molybdenum cofactor of NarG (1, 17). It would therefore be interesting to study the physiological role of the [Fe-S] clusters of NarH in electron transfer from quinol to nitrate and to compare the results with those obtained for DmsABC.

In NarGHI, quinol oxidation appears to be mediated by NarI, a hydrophobic heme-containing membrane anchor subunit (6, 14, 34). The NarI subunits from both the E. coli and Paracoccus denitrificans nitrate reductases have been shown to contain two b6 domains with distinct redox potentials separated by approximately 110 mV for E. coli (Em 1 = +17 and +122 mV [19]; for P. denitrificans, Em = +95 and +210 mV [3]). Recently, Brito et al. (9) proposed the presence of a bound menaquinone within the NarGHI dimer. The initial site of quinol oxidation by NarGHI has been suggested to be located on the periplasmic side of the NarI cytochrome subunit (6, 25). Thus, it is likely that there are at least two distinct sites of quinol binding by NarGHI, one associated with NarI and another associated with the membrane-extrinsic NarGHI dimer. The role of the second quinol binding site is unknown.

In this study, we have used two site-directed mutants of the electron transfer subunit of NarGHI (NarH-C16A and NarH-C263A) to study the role of its [Fe-S] clusters in electron transfer from quinol to nitrate. By inducing enzyme turnover with nitrate, we have studied the potential role of a 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO)-sensitive semiquinone intermediate in the reaction mechanism of nitrate reduction by NarGHI. The potential role of this semiquinone in the reaction mechanism of nitrate reduction by NarGHI is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli LCB79 [ΔlacIΔ39 Δ(lacIPOZYA-argF) psl, thi d79(nar-lac)] was used throughout (35). LCB79 was transformed with plasmid pV700, which encodes the entire narGHI operon under the control of the tac promoter (18). pV700-C16A and pV700-C263A are mutants of pV700 encoding NarGHI(C16A) and NarGHI(C263A) (1, 18).

Growth of cells. Cells were grown anaerobically on a medium containing 10 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 2 g of glucose per liter, adjusted to pH 7.5 with NaOH (18). A 10% inoculum of a stationary-phase culture of LCB79 (pV700) encoding NarGHI(C16A) and NarGHI(C263A) (1, 18).

Preparation of membranes. Cells were harvested and washed, and membranes were prepared by French pressure cell lysis and differential centrifugation (38) in 100 mM morpholinepropanesulfonic acid (MOPS)-5 mM EDTA (pH 7.0). During the French pressing step, phenylmethylsulfonyl fluoride was added to a concentration of 0.2 mM. The membranes were assayed for protein content by the method of Lowry et al. (28), modified by inclusion of 1% (wt/vol) sodium dodecyl sulfate in the incubation mixture to solubilize membrane-bound proteins. Rocket immunoelectrophoresis was carried out as previously described (2).

Enzymatic assays. Benzy lviologenand nitrate (Bv+ : NO3-) oxidoreductase activities were determined as previously described (25). Duroquinol (DOH2)- and menaquinol (MOH2)-dependent reduction of nitrate was determined as described previously (18).

Preparation of EPR samples. Membrane vesicles were suspended at a protein concentration of 30 mg ml-1 in 100 mM MOPS-5 mM EDTA (pH 7.0). Dithionite (5 mM)-reduced samples were incubated under argon at 23°C for 5 min. In some cases, nitrate (25 mM) was added and the samples were incubated for a further 2 min. Where indicated, redox dyes (quinhydrone [Em = +296 mV], 2,6-dichlorophenolindophenol [Em = +217 mV], 1,2-naphthoquinone [Em = +125 mV], tolylene blue [Em = +115 mV], phenazine methosulfate [Em = +80 mV], thionine [Em = +60 mV], duroquinone [Em = +7 mV], methylene blue [Em = −11 mV], resorufin [Em = −50 mV], indigotrisulfonate [Em = −80 mV], indigodisulfonate [Em = −125 mV], anthraquinone-2-sulfonic acid [Em = −225 mV], phenosafranine [Em = −255 mV], benzyl viologen [Em = −360 mV], and methyl viologen [Em = −440 mV]) were added to a final concentration of 50 μM. All samples were prepared in 3-mm-internal-diameter quartz EPR tubes and were rapidly frozen in liquid nitrogen-chilled ethanol prior to being stored under liquid nitrogen. EPR spectra were recorded by using a Bruker Spectrospin ESP-300 spectrometer equipped with an Oxford Instruments ESR-900 flowing helium cryostat. Instrument conditions and temperatures were as described in the figure legends.

RESULTS

EPR spectra of oxidized wild-type and mutant NarGHI in membranes. The EPR properties of E. coli NarGHI have been extensively studied in preparations comprising the soluble NarGHI dimer (1, 2, 17, 18, 24). While these studies have produced valuable insights into the ligation and redox chemistry of the [Fe-S] clusters, it is not known if the EPR properties of the soluble enzyme are equivalent to those of the physiologically relevant membrane-bound holoenzyme. We have investigated the EPR properties of membrane-bound NarGHI with the aims of (i) determining if there are any significant differences between the EPR properties of the iron-sulfur clusters of the membrane-bound holoenzyme and the soluble NarGHI dimer and (ii) determining the effect of two site-directed mutants, NarH-C16A and NarH-C263A, on electron transfer through the enzyme following nitrate addition. The enzymology and EPR characterization of the dimeric (NarGHI) forms of the two mutant enzymes have been described previously (1, 11, 18).

The specific activities of the membranes used in this work, determined by using reduced benzyl viologen and reduced quinol as reductants, as well as the content of wild-type and mutant NarGHI determined by rocket immunoelectrophoresis are shown in Table 1. The Bv+ : NO3- activities of the NarH-C16A and NarH-C263A mutants are much lower than in the wild type, indicating that the loss of the Em,2 = +80 mV [4Fe-4S] cluster has a significant effect on electron transfer.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme activitya</th>
<th>Dithionite-reduced [Fe-S] content (nmol mg of protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>BV+ : NO3-</td>
<td>DOH2 : NO3-</td>
</tr>
<tr>
<td>LCB79</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LCB79/pVA700</td>
<td>12.6</td>
<td>0.8</td>
</tr>
<tr>
<td>LCB79/C16A</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>LCB79/C263A</td>
<td>0.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Determined as described in Materials and Methods and expressed as micromoles of reductant oxidized per milligram of membrane protein per minute, the reductant being Bv+ , DOH2, or MOH2.

b Relative spin concentrations were determined by double integration of spectra of dithionite-reduced samples recorded under nonsaturating microwave power, using 1 mM CuEDTA as the standard (36).

c Estimated by rocket immunoelectrophoresis as previously (2). Values are percentages of total membrane protein.

d ND, not detected.
from BV\(^{+}\) to NO\(_{3}^{-}\). In agreement with our previous studies (18), loss of the highest-potential \([4Fe-4S]\) cluster results in the complete loss of the MQH\(_{2}\):NO\(_{3}^{-}\) activity but not the DQH\(_{2}\):NO\(_{3}^{-}\) activity. Also, in this study the amount of nitrate reductase accumulated in membranes is lower for the NarH-C263A mutant than for the NarH-C16A mutant and the wild type (Table 1).

Figure 1 shows EPR spectra recorded at 12 K of oxidized LCB79 membranes containing wild-type and mutant NarGHI. Figure 1a shows the spectrum of membranes of LCB79 which contain no NarGHI. This spectrum is dominated by species having a spectrum with a peak at \(g = 2.02\) with a broad trough immediately upfield. This spectrum is similar to that reported for the \([3Fe-4S]\) cluster of FrdABCD (FR3) or succinate dehydrogenase (22).

Figure 1b shows the spectrum of oxidized wild-type NarGHI. Oxidized NarGHI exhibits a spectrum with a peak at \(g = 2.02\) and a peak-trough at \(g = 1.99\), and these features can be assigned to the \([3Fe-4S]\) cluster of NarGHI. We confirmed that the spectrum of nitrate oxidized NarGHI-enriched membranes arises from a single \([3Fe-4S]\) cluster and not from multiple species, for example, nitrosyl iron, by recording spectra under a range of microwave powers and temperatures (data not shown). The spectrum that we have assigned to the NarGHI \([3Fe-4S]\) cluster behaves as a single, axial species. This contrasts with the spectrum of oxidized purified NarGH dimer, which has a near-isotropic line shape (17) similar to spectra reported for the \([3Fe-4S]\) clusters of FrdABCD and succinate dehydrogenase (21, 29). The difference between the line shape of the \([3Fe-4S]\) cluster in the purified dimer and the line shape in membrane samples suggests that the environment of this cluster is altered in the dimer, possibly as a result of exposure of the protein close to the site of cluster ligation to the aqueous milieu (see Discussion).

Spectra of oxidized membranes containing NarGH[C16A]I and NarGH[C263]I are shown in Fig. 1c and d, respectively. These spectra are qualitatively very similar to the spectrum of the wild-type enzyme (Fig. 1b) and their relative intensities parallel the relative intensities of EPR spectra of the reduced membranes shown in Fig. 2.

**FIG. 1.** EPR spectra of oxidized membranes containing overexpressed NarGHI. (a) LCB79; (b) LCB79/pVA700; (c) LCB79/pVA700-C16A; (d) LCB79/pVA700-C263A. Membranes were suspended in 100 mM MOPS–5 mM EDTA buffer (pH 7.0). EPR spectrometer conditions: temperature, 12 K; microwave power, 20 mW at 9.45 GHz; modulation amplitude, 10 Gpp at 100 KHz. Spectra were corrected for differences in sample protein concentrations (to 30 mg ml\(^{-1}\)) and EPR tube calibrations.

**FIG. 2.** EPR spectra of dithionite-reduced membranes containing overexpressed NarGHI. (a) LCB79; (b) LCB79/pVA700; (c) LCB79/pVA700-C16A; (d) LCB79/pVA700-C263A. Samples were reduced with 5 mM dithionite for 5 min before being frozen in liquid nitrogen. EPR conditions were as described in the legend to Fig. 1.
[4Fe-4S] cluster corresponding to the $E_{m,3} = -400$ mV cluster of the purified NarGH dimer remains oxidized in dithionite-reduced membrane samples at pH 7. These results suggest that in membranes, only the three highest-potential clusters, viz., the [3Fe-4S] cluster and the $E_{m,8.3} = +80$ mV and $E_{m,8.3} = -200$ mV [4Fe-4S] clusters, become reduced in the presence of dithionite at pH 7.0. Further studies are in progress to determine the redox potentials of the NarH [Fe-S] clusters in membrane preparations (28a).

Figure 2c shows the EPR spectrum of dithionite-reduced membranes containing overexpressed NarGH[C16A]I. Mutation of NarH-C16 (the first Cys of group I) results in the loss of the $E_{m,8.3} = +80$ mV [4Fe-4S] cluster in studies of the soluble NarGH dimer (1). The spectrum of Fig. 2c is consistent with this; the features attributable to the high-potential [4Fe-4S] cluster (the peak at $g = 2.05$, the peak-trough at $g = 1.95$, and trough at $g = 1.87$) are modified or significantly reduced in intensity, indicating that this cluster is also missing in the membrane-bound form of NarGH[C16A]I.

Figure 2d shows the spectrum of dithionite-reduced membranes containing overexpressed NarGH[C263A]I. Mutation of NarH-C263 (the fourth Cys of group IV) also results in the loss of the $E_{m,8.3} = 80$ mV [4Fe-4S] cluster in studies of the soluble NarGH dimer (18), indicating that this cluster is ligated by the first three Cys residues of group I and the fourth Cys of group IV. The spectrum of Fig. 2d is similar to that of Fig. 2a, indicating that the level of expression and assembly of NarGH[C263A]I into the cytoplasmic membrane is significantly lower than that observed for wild-type NarGH.

The relative spin concentrations of the reduced membrane samples used to obtain the spectra in Fig. 2 were determined by double integration of spectra recorded at nonsaturating microwave power at 12 K, using 1 mM CuEDTA as the standard (36) (Table 1). In the case of NarGH[C263A]I, it is clear both from the spin quantitation, the rocket immunoelectrophoresis, and the intensity of the reduced EPR spectrum that this mutant does not assemble well into the cytoplasmic membrane compared to the wild-type and NarGH[C16A]I enzymes.

To confirm the presence of the lowest-potential ($E_{m,8.3} = -400$ mV) [4Fe-4S] cluster in membranes containing overexpressed wild-type NarGH, we recorded EPR spectra of membrane samples containing a range of redox mediators such as those used in previously reported redox titrations of the NarGH dimer (17, 18) in which this cluster was observed (Fig. 3). Figure 3a shows the spectrum of membranes containing wild-type NarGH in the absence of redox mediators. The spectrum in Fig. 3b (dithionite reduced in the presence of redox mediators) corresponds to that reported by Guigliarelli et al. (17) for the fully reduced soluble NarGH dimer.

Nitrate-induced enzyme turnover. To study the effect of the loss of the highest-potential [4Fe-4S] cluster of NarH on electron transfer through NarGH, we studied the effect of nitrate addition to dithionite-reduced membranes. Addition of nitrate to membranes devoid of NarGH (LCB79 membranes) elicits no significant oxidation of the reduced [Fe-S] clusters (data not shown). Figure 4 shows the effect of nitrate on the EPR spectrum of membranes containing wild-type NarGH. Figure 4a is a spectrum of dithionite-reduced NarGH in membranes. Following addition of nitrate (25 mM) to a dithionite-reduced sample, the features of the reduced spectrum are essentially eliminated and are replaced with a spectrum corresponding to that of an incompletely oxidized NarH [3Fe-4S] cluster (Fig. 4b).

Figure 4c shows the spectrum of membranes treated with 25 mM nitrate. This spectrum is equivalent to that shown in Fig. 1b.

![FIG. 3. Effects of redox mediators on dithionite-reduction of membranes containing wild-type NarGH. Membranes were reduced with 5 mM dithionite for 5 min in 100 mM CHES [2-(cyclohexylamino)ethanesulfonic acid]-5 mM EDTA (pH 9.0). (a) Spectrum recorded in the absence of redox mediators; (b) spectrum recorded in the presence of redox mediators (see Materials and Methods). EPR conditions were as reported for Fig. 1.](https://jb.asm.org/)

![FIG. 4. Nitrate-induced turnover of wild-type NarGH. (a) Membranes were reduced with 5 mM dithionite for 5 min at 23°C. (b) Following dithionite reduction, membranes were incubated with 25 mM nitrate for 2 min. (c) Membranes were oxidized with 25 mM nitrate for 2 min. EPR instrument conditions were as described for Fig. 1.](https://jb.asm.org/)
Figure 5 shows the effect of nitrate oxidation on the NarH-C16A mutant in which the highest-potential [4Fe-4S] cluster is missing. Figure 5a shows the dithionite-reduced spectrum of NarGH[C16A]I in LCB79 membranes, which is equivalent to that shown in Fig. 2c. Following addition of 25 mM nitrate to dithionite-reduced membranes, there is incomplete oxidation of the remaining NarH [Fe-S] clusters, manifested by an incomplete disappearance of the spectrum of the reduced enzyme (Fig. 5b). In addition, there is a concomitant appearance of an intense peak-trough centered at \( g = 2.00 \), corresponding to a radical species that appears during enzyme turnover of the NarGH[C16A]I mutant enzyme. The NarH-C263A mutant also lacks the highest-potential [4Fe-4S] cluster. Figure 6 shows that the effect of nitrate oxidation in this mutant is equivalent to that observed in the NarH-C16A mutant (Fig. 5b), suggesting that the appearance of the radical species is related to the loss of the [4Fe-4S] cluster and the consequently lower rates of electron transfer observed in the two NarH mutants (1, 18). This correlates with the lower quinol:nitrate oxidoreductase activities observed in membranes enriched in the two mutant enzymes compared to the wild-type (Table 1).

Characterization of the turnover-induced radical signals in NarGH[C16A]I and NarGH[C263A]I. The appearance of a radical species in EPR spectra of NarGH[C16A]I- and NarGH[C263A]I-enriched membranes during enzyme turnover under the conditions of temperature and microwave power used to record the spectra of Fig. 5 and 6 (12 K and 20 mW) is somewhat unexpected (see Discussion). As such radicals are usually more readily observable at higher temperatures or at very low microwave powers (32). Figure 7 shows the temperature dependence of this radical signal observed in NarGH[C16A]I-enriched membranes. The signal reaches optimal intensity at 0.2-mW microwave power at approximately 8 K and tails off with increasing temperature until, at about 80 K, only approximately 30% of the signal intensity remains. The line width of the signal is 10.0 G below 10 K and decreases to 8.9 G above 30 K. The radical signal in NarGH[C263A]I-enriched membranes behaves in an essentially identical manner (data not shown). The line width values of the radical species are similar to those reported for semiquinol radicals in the cytochrome bc₁ complex (31, 33, 45, 50), NADH dehydrogenase (complex I) (12, 46), cytochrome bo (23, 41), and cytochrome bd (20). Flavin semiquinones typically have EPR line widths of approximately 19 to 20 G (16).
The turnover-induced radical signal observed in the NarGH[C16A]I- and NarGH[C263A]I-enriched membranes reaches maximum intensity between 30 s and 2 min after addition of 25 mM nitrate to 5 mM dithionite-reduced membranes (Fig. 8) (there is some variation from preparation to preparation). After this incubation time, the radical signal intensity diminishes, and this diminution is followed by the appearance of EPR spectrum of the [3Fe-4S] cluster (Fig. 8), suggesting that the radical species is located before the [3Fe-4S] cluster in the electron transfer pathway to nitrate.

HOQNO is a potent quinone analog inhibitor that acts on a number of respiratory chain enzymes. To further study the origin of the radical signal, we studied the effect of HOQNO on its appearance. Figure 9 shows the effect of HOQNO on the appearance of the NarGH[C16A]I turnover-induced radical species, showing that the radical signal is diminished in the presence of a quinol analog inhibitor. NarGH[C263A]I-enriched membranes behave in these experiments in the same way as the NarGH[C16A]I-enriched membranes (data not shown). HOQNO also appears to elicit an increase in the intensity of the Mo(V) signal observed during nitrate-induced enzyme turnover, suggesting that the electron of the semiquinol may be displaced to the Mo cofactor, resulting in the observation of an increase in the intensity of its EPR spectrum.

**DISCUSSION**

The EPR properties of NarGHI in membrane vesicles bear interesting comparison with the EPR properties of the soluble NarGH dimer. Guigliarelli et al. (17) reported that NarGH contains four [Fe-S] clusters, viz., three [4Fe-4S] clusters ($E_{m,8,3} = +80, -200, and -400$ mV) and one [3Fe-4S] cluster ($E_{m,8,3} = +60$ mV). Our results with the NarGHI holoenzyme in situ indicate that dithionite reduction is not sufficient to elicit full reduction of all the NarH [Fe-S] clusters at pH 7.0 in the absence of redox mediators. Comparison of the dithionite-reduced spectrum of Fig. 2b with Fig. 1 in reference 17 suggests that it is the [4Fe-4S] cluster equivalent to the $E_{m,8,3} = -400$ mV cluster of the NarGH dimer which remains oxidized in membranes reduced with dithionite. This observation suggests either that this cluster has a very low midpoint potential (less than approximately −500 mV at pH 7 [30]) or that it is in redox isolation from the other redox-active prosthetic groups of the enzyme. That the lowest-potential cluster appears to be reducible by dithionite in the presence of redox mediators (Fig. 3) suggests that this cluster may be redox isolated from the other [Fe-S] clusters, hemes, and molybdenum cofactor of the enzyme. Given these results, it is unlikely that this cluster is able to cycle between the [4Fe-4S]$^{2+}$ and [4Fe-4S]$^{3+}$ states during enzyme turnover. The low potential cluster of *E. coli* fumarate reductase, FR2 ($E_{m,8,3} = -320$ mV) (26, 29), has been proposed to have a structural role in the assembly of the electron transfer subunit of this enzyme. It is possible that the $E_{m,8,3} = -400$ mV [4Fe-4S] cluster of NarGHI plays a similar structural role, as has been suggested by Guigliarelli et al. (18).

The EPR line shape of the oxidized [3Fe-4S] cluster in the NarGHI holoenzyme is altered compared to its line shape in the NarGH dimer. This result bears interesting comparison with studies of site-directed mutants of the DmsABC electron transport chain of *E. coli*. The turnover of the holoenzyme reduces the EPR spectrum of the [4Fe-4S] cluster, suggesting that the [3Fe-4S] cluster is located before the [4Fe-4S] cluster in the electron transfer pathway. This result is consistent with the observation that the [3Fe-4S] cluster is more readily reduced by dithionite than the [4Fe-4S] cluster. The EPR properties of the oxidized [3Fe-4S] cluster in the NarGHI holoenzyme are altered compared to its line shape in the NarGH dimer. This result bears interesting comparison with studies of site-directed mutants of the DmsABC electron transport chain of *E. coli*.

![Figure 8](http://jb.asm.org/.../FIG. 8. Time course of the appearance of the radical signal following addition of nitrate to dithionite-reduced membranes enriched in NarGH[C16A]I. (a) Dithionite-reduced membranes; (b to h) membranes after incubation with nitrate for 30 s (b), 1 min (c), 1.5 min (d), 2 min (e), 4 min (f), 6 min (g), and 10 min (h). Spectra were recorded at 12 K at 0.2-mW microwave power. Other EPR conditions were as described for Fig. 1.)

![Figure 9](http://jb.asm.org/.../FIG. 9. Effect of HOQNO on the appearance of the turnover-induced radical EPR spectrum. Samples of NarGH[C16A]I-enriched membranes were reduced with 5 mM dithionite for 10 min, then 25 mM nitrate was added, and the oxidation reaction was allowed to proceed for 15 s prior to freezing in liquid nitrogen. Samples were prepared in the absence (a) and presence (b) of 0.5 mM HOQNO. EPR conditions were as described for Fig. 1 except that the microwave power was 0.2 mW.)
transfer subunit (DmsB) containing a [3Fe-4S] cluster ligated primarily by Cys group III rather than the [4Fe-4S] cluster of the wild-type enzyme. In one of these mutants, DmsB-C102W, the Cg of Cys group III is changed to a Trp, making the sequence in this region of the protein very similar to that found in NarH (38), resulting in the assembly of a [3Fe-4S] cluster into DmsB in place of one of the [4Fe-4S] clusters of the wild-type enzyme. The line shape of the [3Fe-4S] cluster of NarGH in membranes is much more axial than that found in the NarGH dimer and is qualitatively similar to that found for the [3Fe-4S] cluster in the DmsB-C102W and DmsB-C102S mutants. The DmsB-C102S mutant does not assemble a [3Fe-4S] cluster when it is part of a DmsAB[C102S] dimer but does assemble one when it is part of the DmsAB[C102S]C holoenzyme (39), indicating that the soluble DmsAB dimer also has altered EPR properties compared to the DmsABC holoenzyme. Overall, these results strongly suggest that the environment of the NarH [3Fe-4S] cluster is altered in the NarGH dimer compared to the NarGH holoenzyme, possibly as a result of its exposure to the aqueous milieu or of a local structural modification. Further potentiometric studies are in progress to determine if the $E_{m_F}$ of the [3Fe-4S] cluster (and the [4Fe-4S] clusters) is altered in the holoenzyme compared to the NarGH dimer (28c).

We have shown that the dithionite-reducible [Fe-S] clusters of NarGH respond to the addition of the physiological oxidant nitrate (Fig. 3), suggesting that these clusters are in redox equilibrium with the site of nitrate reduction, the molybdenum cofactor of NarG. The rapid oxidation of the [Fe-S] clusters in the wild-type enzyme and the inhibition of this oxidation in the two site-directed mutants that lack the highest-potential [4Fe-4S] cluster (Fig. 4 and 5) suggest that this cluster is on the pathway of electron transfer from quinol to the cofactor of NarG. The residual quinol/nitrate oxidoreductase activity of the NarH-C16A and NarH-C263A mutants (Table 1) may be catalyzed by the remaining high-potential [Fe-S] cluster, viz., the [3Fe-4S] cluster.

Lowering the rate of electron transfer within NarGH results in the observation of a radical species during enzyme turnover (Fig. 8). The observation of this species suggests that it is an important intermediate in electron transfer from quinol to nitrate. The line width and $g$ value of this species are consistent with it arising from a semiquinol radical, as is its sensitivity to HOQNO. The observation of a radical species with an EPR spectrum that reaches maximum intensity at around 8 K is unexpected, as such species usually have much higher temperature optima ($\approx$100 K) (32). In NADH dehydrogenase (complex I), such a radical has also been observed at relatively low temperature and high microwave power (12, 43, 46). In complex I, these unusual properties have been explained by the existence of a strong interaction between the radical species and the reduced N-2 [4Fe-4S] cluster (12, 43, 46). It is unlikely, given the method of sample preparation in the presence of dithionite and the reported redox potentials of the hemes (19) ($E_{m_h} = +122$ and $+17$ mV), that the hemes become oxidized (and paramagnetic) during sample preparation. Optical studies of the redox of the NarI hemes suggest that under the conditions used herein, there is little or no redox of the NarI hemes following addition of 25 mM nitrate to 5 mM dithionite-reduced membranes containing NarGH[C16A] and NarGH[C263A] (data not shown), suggesting that the interacting species could be one or more of the NarH [Fe-S] clusters. In membranes containing wild-type NarGH, the hemes become oxidized almost immediately following nitrate addition (data not shown). In NarGH, it is possible that the radical observed in the NarH-C16A and NarH-C263A mutants interacts with the reduced [4Fe-4S] clusters or the reduced S = 2 [3Fe-4S] cluster. However, given the existing data, it is not possible to speculate how close the putative interacting species would have to be to the radical to induce a temperature optimum for the semiquinone EPR signal of around 8 K.

The observation of the turnover-induced radical species represents an important step in delineating the electron transfer intermediates in nitrate reduction by NarGH. Given our results, it is likely that the radical is located before the [Fe-S] clusters in the electron transfer pathway from quinol to nitrate. This suggests that there may be a quinol binding site (at which a semiquinol radical can be stabilized) between the hemes of NarI and the [Fe-S] clusters of NarH. It has recently been shown that in E. coli fumarate reductase, the [3Fe-4S] cluster is closely or functionally associated with a menaquinol binding site (13). In E. coli dimethylsulfoxide reductase (DmsABC), mutants in which one of the four [4Fe-4S] clusters of the wild-type enzyme is converted to a [3Fe-4S] cluster have been generated (38). This [3Fe-4S] cluster has been shown to be conformationally linked to an MQH, binding site of DmsC (39). It has been proposed that in NarGH, there is a tightly bound menaquinone associated with the NarGH dimer (9), and it is possible that this is the location of the radical observed in the turnover experiments reported herein. The presence of a tightly bound quinol species that occupies a high-affinity site that is able to stabilize a ubisemiquinol radical species (23, 41) has also been demonstrated in cytochrome bo (42). This site interacts with the low-spin heme of cytochrome bo. If it is assumed that the semiquinol radical species of NarGH is located in the NarGH dimer, it may function as an electron transfer intermediate between the hemes of NarI and the [Fe-S] clusters of NarH.

The effect of the quinol inhibitor HOQNO on the appearance of the turnover-induced radical in the two mutants is consistent with the proposal that the radical arises from a bound semiquinol intermediate. However, the effects do not distinguish between two possibilities: (i) that the semiquinol is directly displaced by the inhibitor resulting in the loss of the radical signal and (ii) that the inhibitor affects electron transfer from the hemes, resulting in a lower effective availability of electrons to generate the observed semiquinol. Studies are in progress to assess the involvement of the hemes of NarI in electron transfer to the [Fe-S] clusters of NarH by using optical spectroscopy and to determine if the turnover-induced radical and the HOQNO have the same binding site within NarGH (28b).

Overall, the results reported herein show that there are significant differences between the EPR properties of the NarGH holoenzyme compared to the NarGH dimer. The low-potential [4Fe-4S] cluster of NarH reported to have a midpoint potential ($E_{m_{BO}}$) of $-400$ mV by Guigliarelli et al. (17) appears to be in redox isolation in the holoenzyme. In addition, there is a significant difference between the EPR line shape of the oxidized [3Fe-4S] cluster in the holoenzyme compared to the dimer. We have shown that under turnover conditions of two mutants lacking the highest-potential [4Fe-4S] cluster, a mechanistically significant semiquinol radical appears during inhibited enzyme turnover.

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