Heme Axial Ligation by the Highly Conserved His Residues in Helix II of Cytochrome b (NarI) of Escherichia coli Nitrate Reductase A (NarGHI)*

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Optical spectroscopy and EPR studies confirm the existence of two b-type hemes in the NarI subunit (cytochrome b<sub>nar</sub>) of the membrane-bound nitrate reductase (NarGHI) of Escherichia coli. Replacement of His-56 by Arg and His-66 by Tyr results in the loss of the high-potential heme and of the low-potential heme, respectively. These data support the assignment of the axial ligands to the low-potential heme (His-66 and His-187) and to the high-potential heme (His-56 and His-205). This pairing is consistent with the model proposed for NarI of the nitrate reductase of Thiosphaera pantotropha (Berks, B. C., Page, M. D., Richardson, D. J., Reilly, A., Cavill, A., Outen, F., and Ferguson, S. J. (1995) Mol. Microbiol. 15, 319–331) in which the two bis-histidine ligated hemes are coordinated by conserved His residues of helix II and V. EPR and optical studies suggest that the low-potential heme (E<sub>m,7</sub> = +17 mV) and the high-potential heme (E<sub>m,7</sub> = +122 mV) are located near the periplasmic side and the cytoplasmic side of the membrane, respectively. Moreover, correct insertion of both hemes into NarI requires anchoring to NarGH.

Nitrates reductase A (NarGHI) is the predominant respiratory complex in the membrane of Escherichia coli when the cells are grown under anaerobic conditions in the presence of nitrate. This complex catalyzes electron transfer from quinol to nitrate coupled to proton release into the periplasm and the generation of a concomitant transmembrane proton electrochemical potential (1). NarGHI is complex of three non-identical subunits, NarG (150 kDa), NarH (60 kDa), and NarI (25 kDa), in the ratio of 1:1:1 as predicted by the operon organization. This complex is arranged in two catalytic domains; the NarGH subunits constitute the cytoplasmic domain and NarI the membrane anchor domain (2, 3). The NarGH domain contains four [Fe-S] centers (4, 5) and a molybdenum cofactor which is the site of nitrate reduction by the enzyme (6). The NarI subunit (cytochrome b<sub>nar</sub>) is a diheme b-type cytochrome required for attachment of the cytoplasmic domain to the inner side of the cytoplasmic membrane (3) and it mediates electron transfer from the membrane quinol pool (menaquinol or ubiquinol) to the molybdenum cofactor (2, 7). Optical redox titrations demonstrate the presence of two hemes in NarGHI with midpoint potentials (E<sub>m,7</sub> values) of +17 and +122 mV (8).

Of the well characterized membrane-intrinsic cytochromes b, the hemes are found to have bis-histidine ligation (9–11), and it is very likely that this is also the case with the hemes of NarGHI. The hydrophathy plot and sequence analysis of NarI using the "positive inside" rule of von Heijne (12) leads to a topological model in which five transmembrane α-helical segments (helices I-V) are arranged with a periplasmic N terminus and a cytoplasmic C terminus (13). Five His residues are present in the predicted transmembrane segments, at positions 56, 66, 74, 187, and 205. Sequence alignment of all known sequences of NarI from various bacterial membrane-bound nitrate reductases (4, 13, 14) reveals only four totally conserved histidines (His-56,His-66 in helix II and His-187,His-205 in helix V) as potential heme ligands, thereby excluding His-74 within helix II in heme ligation (13). Overall, this strongly suggests ligation of each heme by two histidine residues (13). Because of the different spacing of the conserved histidines on helix II (9 residues) and V (17 residues), the hemes probably lie in two planes with different angles relative to the plane of the membrane. Recently, an alternative model for heme ligation in NarI has been proposed by van der Oost et al. (15) on the basis of subunit stoichiometry and sequence analysis of various b-type cytochromes. This model predicts that the hemes are sandwiched in a NarI dimer and ligated only by His-187 and His-205 of helix V of each NarI subunit. Genetic procedures are, therefore, required for identifying the heme ligands and to distinguish the different models.

In the present work, we have used site-directed mutagenesis, as well as optical and EPR spectroscopies, to test whether the two conserved histidine residues within helix II (His-56 and His-66) are heme ligands and, if so, to assign each histidine to either the low or the high-potential hemes. This work provides the first experimental evidence of the coordination of the hemes by His-56 and His-66 of the NarI subunit of E. coli NarGHI, and it is suggested that the two conserved histidine residues located in helix V (His-187 and His-205) are the other two heme ligands. It is also demonstrated by EPR studies that the low-potential heme is located close to the periplasmic side of the

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1 The abbreviations used are: NarGHI, nitrate reductase A holoenzyme; BV<sup>2-</sup>, reduced benzyl viologen; MOPS, 4-morpholinepropane sulfonic acid; NarGH, soluble catalytic dimer of nitrate reductase A; NarI, cytochrome subunit of NarGHI (cytochrome b<sub>nar</sub>)
2 R. Brasseur, unpublished results.
membrane and that the high-potential heme is near the cytoplasmic surface of the membrane. We have also studied the biogenesis of NarI by overexpressing this subunit in the absence of the membrane-extrinsic NarGH dimer.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli strains and plasmids used in this study are listed in Table I. Plasmid pVA700 and pCD7 carry the narGHJI operon and the narI gene, respectively, under the control of the tac promoter (ptac). These plasmids were used to transform E. coli LCB2048 (narGHJIΔ, narZYYVΔ) (16), and LCB79 (narGHJIΔ) (17).

Site-directed Mutagenesis—Oligonucleotide site-directed mutagenesis was performed by overlap extension (18) on plasmid pVA700 using the Expand Long Template PCR System (Boehringer). The polymerase chain reaction products were double-digested by SetII and SauI, and the restriction fragments were ligated with similarly cleaved expression plasmid pVA700. All mutated constructs were confirmed by restriction digestion and DNA sequencing. The resulting constructs were used to transform E. coli strains LCB2048 and LCB79. This permitted isopropyl-1-thio-β-D-galactopyranoside-induced overexpression of NarGH [H56R] and NarGH[H66Y].

Growth of Bacteria and Preparation of E. coli Membranes Vesicles— Cultures were grown microaerobically at 37 °C with isopropyl-1-thio-[H56R] and NarGHI[H66Y]. (77 K).

Enzyme Assays—The activity of nitrate reductase with benzyl viologen as substrate was assayed by a method modified from Jones and Garland (20, 21). Activities with menaquinol and ubiquinol were measured as described previously by Giugliarelli et al. (19).

Optical Spectroscopy—Spectra were recorded using a DW2A Chance AMINCO spectrophotometer. Samples of LCB2048 membranes enriched in wild-type or mutant NarGH or NarI were diluted with 100 mM MOPS buffer (pH 7.0) containing 5 mM EDTA and phenylmethylsulfolufonic acid (0.2 mM). Cells were subjected to French pressure lysis and MOPS buffer (pH 7.0) containing 5 mM EDTA and phenylmethylsulfonlfonic acid (0.2 mM). Cells were subjected to French pressure lysis and

EPR Spectroscopy and Redox Titrations—For studies of wild-type or mutant NarGH holoenzyme membranes were prepared from E. coli LCB79 transformed with the appropriate plasmids. For studies of NarI overexpressed from the plasmid pCD7, this plasmid was transformed into E. coli LCB2048. Redox titrations were performed as described previously (22). EPR spectra were recorded using a Bruker ESP300E spectrometer equipped with an Oxford Instrument ESR-900 flowing helium cryostat. Instrument conditions and temperatures were as described in the individual figure legends.

RESULTS

Physiological and Biochemical Characterization of the Wild-type and Mutant Enzymes—Each NarI mutant strain was assessed for its ability to support cell growth under conditions that require nitrate reductase activity. Only the strain carrying the wild-type plasmid pVA700 can grow under anaerobic conditions on a glycerol-nitrate minimal medium indicating that the NarI mutant holoenzymes encoded by plasmid pVA700-H56R and pVA700-H66Y fail to catalyze electron transfer from the quinol pool to the molybdenum cofactor of NarG (data not shown). E. coli SHSP18, a hemA mutant, is unable to grow under conditions requiring functional NarGH (data not shown), indicating that the presence of heme is necessary for electron transfer within NarGH.

To determine if the inability of the NarI mutants to grow anaerobically is due to an alteration of the assembly of the nitrate reductase complex to the membrane, the amount of enzyme present in each subcellular fraction was determined by rocket immunoelectrophoresis. The amount of overexpressed mutant enzymes is nearly 10 times that of the parental strain MC4100, and 60–70% of both mutant enzymes (as measured by NarGH content) are associated with the membrane (Table II), compared with 97% for the wild-type. In membranes from SHSP18/pVA700, only 40% of the enzyme is associated with the cytoplasmic membrane (Table II). Overall, these results indicate that the assembly or the stability of the NarGH holoenzyme has been modified as a consequence of the NarI-H56R and NarI-H66Y mutations and that both hemes are required for full assembly of the enzyme to the membrane.

In vivo, quinones are obligatory redox carriers for the nitrate reductase system of E. coli and both menaquinone and ubiquinone can act as electron donors in cells growing under anaerobic conditions (23). The quinol:nitrate oxidoreductase activities of wild-type and mutant NarGH-enriched membranes were determined using the quinol analogs menadion and duroquinol as electron donors. In both mutants, there is no activity (data not shown). The NarI mutant membranes were also assayed for the NO3⁻-dependent reoxidation of reduced benzyl viologen (BV⁺) for which NarI is not required (2, 24). Wild-type NarGH has a specific activity of 100 μmol of nitrate reduced min⁻¹ (mg of NarGH)⁻¹ (Table II). The specific activities are lowered in the H56R mutant (47 μmol of nitrate reduced min⁻¹ (mg of NarGH)⁻¹) and the SHSP18 mutant (43 μmol of nitrate reduced min⁻¹ (mg of NarGH)⁻¹) and to a lesser extent in the H66Y mutant (88 μmol of nitrate reduced min⁻¹ (mg of NarGH)⁻¹). Thus, each mutant NarGH is catalytically active with BV⁺ as electron donor, but inactive with quinol as electron donor. It appears that the absence of both h-type hemes in the SHSP18 mutant strain leads to a modification of the cytoplasmic domain (NarGH) of NarGH which results in a lower catalytic activity with BV⁺ as electron donor.

Liquid Nitrogen Temperature (77 K) Absorption Spectra of NarGH and NarI-enriched Membranes—Under the growth
conditions used herein, the cytochrome bd complex (25) is the major cytochrome component in the background strain LCB2048 (Fig. 1c). The amount of optically detectable NarI-associated heme present in the LCB2048/pVA700 membranes is substantially greater than the amount of cytochrome found in the background strain LCB2048 (cf. Fig. 1, a and c). This facilitates spectroscopic analysis of the hemes of NarGHI-enriched membranes without further purification of NarGH or the NarI cytochrome subunit. To characterize the hemes of wild-type NarI, we used the LCB2048/pVA700 and LCB2048/pCD7 strains which allow overexpression of NarGH and NarI alone, respectively. The low-temperature spectrum of membranes from LCB2048/pVA700 is characteristic of b-type hemes and exhibits a single a-band absorption peak with a maximum at 556 nm (Fig. 1a). Higher derivative analysis of the a-band absorption peak in the NarGH spectrum does not lead to the resolution of additional components (data not shown), suggesting that both hemes contribute equally to the spectrum. Instead of the single a-band absorption peak at 556 nm observed for the NarGH holoenzyme, the dithionite-reduced NarI spectrum of the LCB2048/pCD7 strain shows two a-band absorption peaks at 553 and 558 nm (Fig. 1d). Possible explanations for the presence of two a-bands in the NarI spectrum include: (i) the absence of the cytoplasmic dimer leads to a large modification of the optical properties of one heme, probably the heme located closest to the cytoplasmic side of the membrane (see below), leading to a blue shift of its a-band or (ii) one heme is missing and the remaining one exhibits a spectrum with a split a-band. Partial reduction of membranes enriched in NarI alone with ascorbate results in no detectable change in the line shape, suggesting that both absorption peaks arise from a single species.

EPR Spectroscopy of the Hemes of Wild-type NarGHI and NarI—The EPR spectrum of oxidized membrane-bound NarGHI has peaks at $g_z = 3.36$ and $g_{y} = 3.76$ corresponding to two low-spin heme iron atoms (Fig. 2A, ii and iii). This type of EPR signal (highly anisotropic low spin, HALS) with $g_z > 3.3$ is found for several membrane-intrinsic cytochromes $b$ for which His residues are proposed to serve as ligands (26–28). The EPR spectrum of the negative control sample (LCB79) (Fig. 2A, i) does not exhibit the $g_z = 3.76$ and 3.36 peaks, confirming that these can be assigned to the hemes of NarI. The line shapes of these two peaks and spectra recorded of site-directed mutants of the NarI subunit (see below) strongly suggest that they each arise from one of the hemes.

The EPR spectrum of NarI in the absence of the membrane-

extrinsic NarGHI dimer does not have a $g_z$ peak at 3.76 (Fig. 2B, ii), instead there are peaks at $g_z = 3.15$ and $g_{y} = 2.92$. The results of site-directed mutagenesis experiments (see below) suggest that the $g_z = 3.15$ peak arises from a slight modification of the environment of the heme responsible for the $g_z = 3.36$ peak of the NarGH holoenzyme (Fig. 2A, ii and iii). The $g_{y} = 2.92$ peak may be due to a dramatic modification of the environment of the heme responsible for the $g_z = 3.76$ peak of the NarGH holoenzyme, or it may be due to an altered conformation of the heme responsible for the $g_z = 3.15$ signal. EPR signals with a low-field component ($g_z$) at $g = 2.92$ have been observed for conformationally modified (or “relaxed”) membrane-bound b-type cytochromes (29, 30). Overall, the EPR spectra of membranes enriched in NarI demonstrate that the correct insertion of both b-type hemes requires the presence of the NarGH dimer.

![Fig. 1. Low-temperature light absorption spectra of wild-type and mutant NarGHI and NarI.](image-url)

The table below shows the levels of nitrate reductase in membrane and cytoplasmic fractions.

| Strains          | Immunoprecipitated nitrate reductase |
|------------------|-------------------------------------|
|                  | mg of NarGH | % NarGH | BV × g nitrate reductase activity |
| MC4100           | M 6         | 92      | M 598 |
|                  | S 0.5       | 8       | S 37  |
|                  |             |         |       |
|                  | M 63        | 97      | M 603 |
|                  | S 2         | 3       | S 128 |
|                  |             |         |       |
|                  | M 42        | 65      | M 1974 |
|                  | S 23        | 35      | S 1210 |
|                  |             |         |       |
|                  | M 41        | 63      | M 3608 |
|                  | S 24        | 37      | S 1943 |
|                  |             |         |       |
|                  | M 20        | 40      | M 860 |
|                  | S 30        | 60      | S 2100 |

Expressed as micromole of nitrate reduced min⁻¹.  
Expressed as percentage of the sum of the activities present in the soluble and membrane fractions.  
Expressed in micromole of nitrate reduced min⁻¹ (mg of NarGH)⁻¹.

**Table II**: Levels of nitrate reductase in membrane and cytoplasmic fractions

The amounts of total immunoprecipitated NarGH in membrane (M) and soluble (S) fractions were estimated by rocket immunoelectrophoresis as previously described by Augier et al. (21) and expressed in milligrams of immunoprecipitated NarGH or as a percent (%) of the sum of the activities present in the soluble and membrane fractions.
To assign a redox potential to each heme of the NarI subunit, redox titrations were performed on NarGHI-enriched membranes at pH 7. Redox titrations corroborated the presence of a high-potential and a low-potential heme component in the NarI subunit (Fig. 3). At a redox potential \( E_h \) of 281 mV, the two hemes of NarI are reduced (diamagnetic), and no \( g_z \) signals can be detected. At 18 mV, the heme with the \( g_z = 5.36 \) value is oxidized (paramagnetic), while the heme with the \( g_z = 5.76 \) is reduced. At 253 mV both hemes are oxidized and paramagnetic. The intensity of the \( g_z = 5.76 \) peak plotted versus \( E_h \) can be fitted to a single \( n = 1 \) component with an \( E_{m,7} \) of +110 mV (inset, Fig. 3). Due to the broadness of the \( g_z = 5.36 \) peak and baseline constraints, it was not possible to plot the intensity of this peak versus \( E_h \), but it is clear from the spectra of Fig. 3 that its \( E_{m,7} \) lies somewhere between -28 and +34 mV. Thus, the broad symmetrical \( g_z \) peak at 3.36 can be attributed to the low-potential heme of \( E_{m,7} = +17 \) mV, and the \( g_z \) peak at 3.76 can be attributed to the high-potential heme of \( E_{m,7} = +122 \) mV at pH 7 (\( E_{m,7} \) values from Hackett and Bragg (8)).

**Optical and EPR Spectroscopic Analysis of Site-directed Mutants of NarI**—To determine the location of the high- and low-potential hemes within the proposed transmembrane topology of NarI (13) and to assign His ligands to these hemes, we studied the effects of two mutations of conserved His residues, NarI-H56R and NarI-H66Y, on the optical and EPR properties of NarGHI. The NarI-H66Y mutant enzyme in membranes exhibits a symmetrical \( \alpha \)-band at 556.5 nm in reduced minus oxidized spectra (Fig. 1b), which has a lowered intensity compared with that exhibited by the wild-type enzyme (Fig. 1a). The optical spectrum of the NarI-H56R mutant exhibits a symmetrical \( \alpha \)-band at 556.5 nm in reduced minus oxidized spectra (Fig. 1b), which has a lowered intensity compared with that exhibited by the wild-type enzyme (Fig. 1a). The optical spectrum of the NarI-H56R mutant exhibits a symmetrical \( \alpha \)-band at 556.5 nm in reduced minus oxidized spectra (Fig. 1b), which has a lowered intensity compared with that exhibited by the wild-type enzyme (Fig. 1a).
3.15, similar to that observed in the spectrum of NarI expressed from pCD7 (Fig. 2B). This also correlates with optical spectra of membranes containing NarI and NarGHI[H56R] (Fig. 1, c and d), suggesting that the loss of the high-potential heme or the absence of the membrane-extrinsic NarGH dimer results in a modification of the environment of the low potential ($E_{m,T} = +17$ mV) heme. Fig. 4d shows the effect of the NarI-H66Y mutation. In this case the $g_z = 3.76$ peak is present, but the $g_z = 3.36$ peak is absent. These data are consistent with an assignment of the low potential heme to His-66 and the high potential heme to His-56, and with a proposed location of the high potential heme toward the cytoplasmic face of NarI in the model of Berks et al. (13).

The specific BV$^+$:NO$_3^-$ oxidoreductase activity of the NarGHI[H56R] and NarGHI[H66Y] holoenzymes are 47 and 88% of that of the wild-type, respectively (Table II). We have previously shown that the BV$^+$:NO$_3^-$ oxidoreductase activity appears to be independent of the presence of NarI (2, 24). This prompted us to investigate whether the NarI-H56R mutation has any effect on the assembly or conformation of the NarH [Fe-S] clusters. Fig. 5 shows EPR spectra of these [Fe-S] clusters. Fig. 5, a-c, shows dithionite-reduced spectra of the wild-type, NarI-H56R, and NarI-H66Y mutants, respectively. These spectra are essentially identical to that reported for dithionite-reduced wild-type NarGHI in membranes (31). Although there are minor differences in their line shape and intensities, the reduced spectra correlate with the concentrations of NarGHI estimated by rocket immunoelectrophoresis (Table II). In the ferricyanide oxidized spectra (Fig. 5, d-f), it is clear that the NarI-H56R mutation results in a significant modification of the line shape of the [3Fe-4S] cluster, indicating that its oxidation is incomplete at the concentration of ferricyanide used here. This suggests that the loss of the high potential heme in the NarI-H56R mutant modifies the environment of the [3Fe-4S] cluster, which is consistent with a location of this heme close to the NarI-NarGH interface region in the NarGHI holoenzyme.

**DISCUSSION**

Two different models for the ligation of the hemes of NarI have been generated on the basis of sequence analyses and theoretical rules on the ligation of $b$-type hemes (13, 15). In such models, the functional role of the well conserved His residues of helix II (His-56 and His-66) is contentious. In the model of van der Oost et al. (15), these residues are not involved in heme ligation. In the model of Berks et al. (13), they act as ligands in conjunction with the His-187 and His-205 of helix V. The data presented herein favor the latter model.

The functional importance of the His residues of helix II in heme ligation was established here by replacing His-56 and His-66 with Arg or Tyr, respectively. As shown by low-temperature optical and EPR spectroscopy, the NarI-H56R mutant is devoid of the high-potential heme, whereas the NarI-H66Y mutant is devoid of the low-potential heme. These results are consistent with the proposed location of these hemes in the NarI-NarGH interface region in the NarGHI holoenzyme.
His-187 and His-205 mutants supports this model for heme ligation by NarI.\(^3\)

Comparison of the potentiometric titration data from the wild-type enzyme (Fig. 3) with the effect of the H56R mutation on the heme EPR line shape (Fig. 4) indicates that the high-potential heme is located toward the cytoplasmic side of NarI in close juxtaposition with the [3Fe-4S] cluster of NarH (Fig. 5). That the heme optical and EPR spectra in the pCD7-overexpressed NarI are similar to those of the NarGH[H56R] holoenzyme also suggests that the conformation of the NarI subunit is dependent on the presence of the NarGH dimer. These results support a model for the NarI-NarGH interface region in which electron transfer occurs between the high-potential heme and the [3Fe-4S] cluster of NarH.

EPR analyses of both hemes of NarGHI show \(g\) values > 3.0. Attempts to rationalize such large \(g\) values within a number of membrane-bound b-type cytochromes have relied upon the assumption of distortion at the heme center controlled by the orientation of the imidazole rings (29, 32). Palmer (27) has proposed that if the two imidazole-ring planes of the ligating histidines are gradually rotated from a parallel orientation, which gives a \(g\) value of about 3.0, toward a perpendicular orientation, the \(g\) increases, and the \(g_x\) and \(g_y\) components of the EPR signal become progressively smaller. Hence, the highly anisotropic heme of NarGHI with a \(g\) value of 3.76 reflects an asymmetrical ligand field at the heme due to a near perpendicular orientation of the two axial ligands. This is in disagreement with the model of Berks et al. (13) in which both hemes are ligated with the two imidazole rings approximately coplanar. Recently, Banci and co-workers (33) have proposed a correlation between ligand-field parameters and the reduction potential of the iron atom. It seems that in our case the EPR \(g\) values of both hemes follow the same trend as predicted by Banci et al., as the heme with the highest \(g_z\) (\(g_z = 3.76\)) has the higher \(E_m,\gamma\) value (+120 mV).

The absence of the cytoplasmic dimer in samples containing NarI overexpressed from pCD7 results in the appearance of new EPR features at \(g_z = 3.15\) and \(g_z = 2.92\) and in the absence of the \(g_z = 3.76\) signal corresponding to the high-potential heme located closest to the cytoplasmic side of the membrane. Two possible interpretations of these results can be proposed.

(i) First, both hemes are present in the pCD7-overexpressed NarI, but the \(g_z = 3.76\) heme is in a relaxed conformation (\(g_z = 2.92\)), and the \(g_z = 3.36\) peak moves to \(g_z = 3.15\). This could be explained by a perturbation (or “relaxation”) of the orientation of the imidazole rings of the heme bis-histidine ligands located at the NarI-NarGH interface, yielding a \(g_z = 2.92\) peak in the EPR spectrum. This correlates with the NarI-H56R mutant in which the \(g_z = 3.76\) peak is absent, but \(g_z = 3.15\) peak corresponding to the other heme is present. However, this interpretation is not in accordance with the optical data obtained with membranes containing NarGHI[H56R] and NarI alone under the same conditions.

(ii) Alternatively, the absence of the NarGH dimer leads to the high-potential heme not being inserted as suggested by the absence of the \(g_z = 3.76\) signal. The resulting modified structure of NarI leads to a modification of the \(g\) values of the remaining heme (i.e. \(g_z = 3.36\) toward \(g_z = 3.15\)), and the partial formation of conformationally modified (or relaxed) heme as evidenced by the \(g_z = 2.92\) peak. Thus, both EPR features in the NarI spectrum (from LCB2048/pCD7 membranes) may arise from the same heme species, resulting in essentially identical optical spectra observed for NarI alone and the NarGHI[H56R] holoenzyme. Unfortunately, due to the broadness of the heme EPR spectra, interfering baseline signals, and baseline constraints, it was not possible to further test this hypothesis by carrying out spin quantitations on the various membrane samples used here. However, we favor the second hypothesis as it is in accordance with both EPR and optical data.

Anchoring of NarGH to NarI probably leads to conformational changes within the heme binding pocket, facilitating heme insertion and stabilization of the whole structure of the NarGHI holoenzyme. When the NarI-H56R and NarI-H66Y mutants are overexpressed from a plasmid construct encoding only the mutated narI gene (pCD7), both hemes are absent (data not shown). It seems that the structure of the mutant NarI is not competent for stabilization or insertion of the hemes in absence of the cytoplasmic dimer. Therefore, heme insertion must depend on some aspects of the quaternary interaction within NarGHI. Such a phenomenon has already been described for the assembly of the Bacillus subtilis and E. coli succinate:quinone oxidoreductases (34, 35) and for the cytochrome \(d\) terminal oxidase complex of E. coli (36). In the latter case, heme insertion in the different membrane-bound subunits can only occur after the complete formation of the cytochrome \(bd\) oxidase complex. In the case of the cytochrome \(b_{160}\) of bovine heart succinate-ubiquinone reductase, the pronounced changes in EPR properties that accompany isolation of the cytochrome \(b_{160}\) are attributed to perturbation of the orientation of the imidazole rings of the heme bis-histidine ligands (29). In the case of the B. subtilis cytochrome \(b_{556}\), the hemes in the whole complex II and in the isolated cytochrome subunit are not in the same environment as judged by differences in the optical absorption and EPR spectra (32).

The location of the low-potential heme toward the periplasmic side and the high-potential heme toward the cytoplasmic side of NarI suggests that the flow of electrons through this subunit is from the periplasmic side inverts, as proposed by Berks et al. (13). The data presented here indicates the importance of both hemes of NarI in mediating electron transfer from quinol to nitrate within NarGHI. The effect of the NarI-H56R mutation on the [3Fe-4S] cluster of NarH strongly implies that this center accepts electrons from the high potential heme of NarI. The ultimate goal of our structural studies on this protein is to model the electron transfer within NarGHI and locate the reaction sites on the protein of external ligands such as quinones and inhibitors. These studies are currently underway in our laboratories.

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