**Purification and Spectropotentiometric Characterization of *Escherichia coli* NrfB, a Decaheme Homodimer That Transfers Electrons to the Decaheme Periplasmic Nitrite Reductase Complex**

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*Escherichia coli* can reduce nitrite to ammonium via a 120-kDa decaheme homodimeric periplasmic nitrite reductase (NrfA) complex. Recent structure-based spectropotentiometric studies are shedding light on the catalytic mechanism of NrfA; however, electron input into the enzyme has not been addressed biochemically. This study reports the first purification of NrfB, a novel 20-kDa pentaheme c-type cytochrome encoded by the *nrfB* gene that follows the *nrfA* gene in many bacterial *nrf* operons. Analyses by gel filtration demonstrated that NrfB purifies as a decaheme homodimer. Analysis of NrfB by UV-visible and magnetic circular dichroism spectroscopy demonstrates that all five NrfB ferric heme iron ions are low spin and are most likely coordinated by two axial histidine ligands. Spectropotentiometry revealed that the midpoint redox potentials of five ferric hemes were in the low potential range of 0 to −400 mV. Analysis by low temperature EPR spectroscopy revealed signals that arise from two classes of *bis*-His ligated low spin hemes, namely a rhombic trio at \( g_{1,2,3} = 2.99, 2.27, \) and 1.5 that arises from two hemes in which the planes of histidine imidazole rings are near-parallel and a large \( g_{\text{max}} \) signal at \( g = 3.57 \) that arises from three hemes in which the planes of the histidine imidazole rings are near-perpendicular. NrfB was also overexpressed as a recombinant protein, which had similar spectropotentiometric properties as the native protein. Reconstitution experiments demonstrated that the reduced decaheme NrfB dimer could serve as a direct electron donor to the oxidized decaheme NrfA dimer, thus forming a transient 20-heme [NrfB]_4[NrfA]_2 electron transfer complex.

The reduction of nitrate to ammonium in the periplasm of *Escherichia coli* involves two enzymes, periplasmic nitrite reductase (*NarA*) and periplasmic cytochrome *c* nitrite reductase (*NrfA*). The process is found in many enteric bacteria and may be important for anaerobic nitrite and nitrate respiration at low nitrate concentrations (1, 2). The NrfA protein catalyzes the six-electron reduction of nitrite to ammonium but can also catalyze the five-electron reduction of NO and two-electron reduction of hydroxylamine, both of which may be bound intermediates in the catalytic cycle for nitrite reduction (3, 4). Indeed a possible physiological role of the enzyme in NO detoxification has recently been suggested (5).

The *E. coli* NrfA protein is a 52-kDa pentaheme cytochrome in which four hemes are covalently bound to the conventional motif CXXCH. The fifth heme is attached to the novel CXXCK motif that is essential for catalysis (6). The crystal structures of cytochrome *c* nitrite reductase from the sulfur-reducing bacterium *Sulfurospirillum deleyianum*, the closely related rumen bacterium *Wolinella succinogenes*, and the enteric bacterium *E. coli* have been determined recently (4, 7–9). In all three structures NrfA crystallized as a homodimer, with the hemes within each monomer closely packed to form arrangements of near-parallel and near-perpendicular heme pairs. In the absence of substrate, the NrfA active site heme displays a distal lysine ligand and proximal water or hydroxide ligand (8, 9).

Analysis of the organization of *nrfA* gene clusters from a range of bacteria reveals that they can be divided into two groups. In one group, which includes *W. succinogenes* and *S. deleyianum*, *nrfA* clusters with an adjacent gene, *nrfH*, that encodes a membrane-anchored tetraheme quinol dehydrogenase of the NapC family (10–13). In the second group, which includes *E. coli*, NrfA clusters with genes encoding a putative periplasmic pentaheme cytochrome (*nrfB*), a periplasmic (4 × [4Fe4S]) ferredoxin (*nrfC*) and an integral membrane putative quinol dehydrogenase (*nrfD*) (3, 5). Clearly, electron transfer from quinol to the NrfA in the different groups is distinct. The different protein-protein and cofactor-cofactor interactions implicit in this situation may be reflected by insertions and deletions in loop regions of the polypeptide chain in the two subgroups that can be identified in primary and tertiary structure analyses (9).

Despite the importance of periplasmic nitrite reductase to ammonium in enteric bacteria and the developing structure-informed biochemical understanding of the enzyme that catalyzes this process, the nature of electron delivery has never been addressed biochemically. In this paper we present the first purification and spectropotentiometric characterization of NrfB and demonstrate its competence as an electron donor to NrfA, with which it must transiently form a 20-heme [NrfB]_4[NrfA]_2 electron transfer complex.

**EXPERIMENTAL PROCEDURES**

*Growth of Bacteria*—The *E. coli* K-12 strain LB32048 is defective in the nitrate reductases NarA and NarZ and, because of a *narL* mutation, expresses the formate-dependent nitrite reductase NrfA at elevated concentrations (1, 2). The NrfA protein catalyzes the six-electron reduction of nitrite to ammonium but can also catalyze the five-electron reduction of NO and two-electron reduction of hydroxylamine, both of which may be bound intermediates in the catalytic cycle for nitrite reduction (3, 4). Indeed a possible physiological role of the enzyme in NO detoxification has recently been suggested (5).

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levels during anaerobic growth in the presence of nitrate (14). Cultures of strain LCB2048 were grown without aeration overnight at 37°C in minimal salts medium (14) supplemented with 0.4% glycerol, 20 mM nitrate, kanamycin (25 μg/ml), and spectinomycin (25 μg/ml). Initial cultures (250 ml) were inoculated with 0.5 ml of an overnight culture grown aerobically on Luria-Bertani broth medium. The 250-ml culture was successively transferred to 2, 6, and 100 liters of fresh medium.

**Purification of Native E. coli NrfB and NrfA—**Periplasmic proteins were extracted from the harvested cells as described previously (14). The enzyme was precipitated from the periplasm using 65% saturated ammonium sulfate and resuspended in 50 mM Tris- HCl, pH 7. The precipitate was dialyzed and applied to an anion exchange Q-Sepharose column (35 × 3 cm) equilibrated with 50 mM Tris-HCl, pH 7. The column was developed using a linear gradient of 0–200 mM NaCl. NrfB and NrfA fractions were applied separately to a Superdex G-75 HiLoad 16/60 fast protein liquid chromatography column equilibrated with 50 mM Tris- HCl, pH 7. NrfB and NrfA were then further purified on an anion exchange Dionex column (0.9 × 10 cm) equilibrated in the same buffer by using a linear gradient of 0–100 mM sodium sulfate and 80 mM NaCl. The NrfB and NrfA eluates were collected at 80 mM NaCl. Protein concentration was determined by the BCA method using bovine serum albumin as a protein standard. The NrfA and NrfB were judged pure on the basis of Coomassie Blue and heme-stained SDS-PAGE (Fig. 1).

**Expression of Recombinant nrfB—**The nrfB gene encoding NrfB was amplified from E. coli strain JM109 genomic DNA by PCR using gene-specific primers (5′-ATGGAGTTATCGGCTCCTG-3′ and 5′-TCTATG-GCTGTCCTCATTAGCA-3′) and the proofreading polymerase pico. The resulting DNA fragment was purified using a commercial gel extraction kit (Qiagen) and inserted by blunt end ligation into the EcoRV site of the plasmid pET21a (Novagen) to place the recombinant nrfB under the control of the IPTG-inducible T7 promoter. Recombinant plasmids were identified by blue-white screening, and the orientation of nrfB inserts was determined by restriction digests. Suitable recombinant plasmids were transformed into the expression host E. coli BL21(DE3)pETCS6. The accessory plasmid pETCS6 provided constitutive expression of the cytochrome c maturation (ccm) gene cluster, thus allowing expression of recombinant c-type cytochromes under aerobic conditions. E. coli BL21(DE3)pETCS6(pNrfB) was grown on Luria-Bertani medium plus ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml)1 at 37°C with aeration to an optical density of 0.4 at 600 nm. The culture was induced with 1 mM IPTG. Cells were harvested by centrifugation 16 h after induction.

**Purification of Recombinant E. coli NrfB—**Whole cell extracts of induced BL21(DE3)pETCS6(pNrfB) were generated by sonication of the harvested and washed cells in 10 mM Tris-HCl, pH 8. NrfB, as indicated by a red coloration, was precipitated by 10% saturated ammonium sulfate and then resuspended in and dialyzed against 10 mM Tris- HCl, pH 8. This dialyzed sample was then further separated on a DEAE-Sepharose column equilibrated in the same buffer by using a linear gradient of 0–1 mM NaCl over 5 column volumes. NrfB was found to elute at 0.30 mM NaCl.

**Activity Assays—**Nitrite reductase activity was measured spectrophoto metrically by substrate-dependent oxidation of reduced methyl viologen (ε10,000 = 13,700 M−1 cm−1). Assays (3.5-ml final volume) were performed by mixing at 25°C in anaerobic cuvettes containing 1 mM methyl viologen, 2 mM CaCl2, 50 mM Hepes, pH 7, and either nitrite or hydroxylamine. Methyl viologen was reduced by the addition of sodium dithionite, and turnover was initiated by the addition of NrfA (0.4 μM/mill).

**MCD and EPR Spectroscopy—**Perpendicular and parallel mode EPR measurements were performed with a Bruker EMX spectrometer equipped with an Oxford ESR-9 liquid helium cryostat and a dual mode cavity with microwave frequencies of 9.65 and 9.35 GHz for the perpendicular and parallel modes, respectively. MCD experiments were recorded on a JASCO spectrochroic Jasco J-810, for the wavelength range 280–1000 nm and a JASCO J-730 for the range 800–2000 nm. Samples were exchanged into deuterated buffer and placed in quartz cuvettes within an Oxford Instruments SM1 6T superconducting solenoid with an ambient temperature bore for room temperature measurements.

**Visible Absorption Spectra and Mediated Redox Potentiometry—**Ab

1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; EPR, electron paramagnetic resonance; LMCT, ligand-metal charge transfer; MCD, magnetic circular dichroism; NIR, near-infrared; STC small tetraheme cytochrome.
observed as evidenced by a decrease in absorbance of the reduced NrfB α peak at 552 nm (Fig. 2B). The initial rate of oxidation of NrfB under the experimental reaction conditions was 100 electrons s⁻¹. Spectra collected at the end of the experiment confirmed that full re-oxidation of NrfB had occurred. This finding indicates that, in the presence of the nitrite reductase NrfA, electrons can flow from NrfB via NrfA to nitrite. The full oxidation of NrfB via the 100-fold sub-stoichiometric NrfA shows that the oxidized NrfA dimer must interact transiently with a number of molecules of the reduced NrfB dimer during the time course of the experiment. In control experiments, oxidation of ferrous NrfB was not observed when nitrite was added to NrfB in the absence of NrfA, but NrfB oxidation was observed when NrfA was subsequently added (not shown). Not surprisingly for oxidation of a decaheme complex, the oxidation kinetics of NrfB were polyphasic, and, although not analyzed in detail, ionic strength appeared to have only marginal effect within the range 0–0.5 M NaCl. This observation suggested that the dominant forces stabilizing the interaction are not electrostatic in nature.

Spectropotentiometric Properties of NrfB—Spectra of NrfB were collected at a number of potentials in the range of 400 mV to ~400 mV. Plots of the increase in the absorbance change (measured at 552–700 nm) as a function of potential can be most simply fitted to five independently titrating (n = 1) low spin Nernstian components, each of which contribute approximately equally to the total reduced absorption peak (Fig 3A). The best fit to the data gave midpoint potentials at −63, −221, and −259 mV for three hemes and at −129 mV for two hemes with iso-midpoint potentials. Inspection of the spectra collected at each potential did not reveal any changes in the position of the 𝜆max (Fig 3B; note that at low potential, spectral features in the region of 600–650 nm become obscured by the absorption of reduced methyl viologen, which is present as a redox mediator).

Magnetic Circular Dichroism of NrfB—The UV-visible MCD spectrum of NrfB exhibited an intense, derivative-shaped band centered at ~400 nm in the Soret region (Fig 4A). When normalized for the presence of five hemes, this derivative had a peak to trough intensity of ~150 m⁻¹ cm⁻¹ tesla⁻¹ heme⁻¹. This value is consistent with that observed for other low spin heme proteins (15, 16). Thus, the intensity of the MCD Soret band derivative is consistent with the presence of five low spin hemes in NrfB. This result is also supported by the α,β-MCD
bands at 500–600 nm, which are typical of low spin heme. No signals characteristic of high spin species in the region of 600–650 nm were apparent. The charge transfer band for low spin ferric heme occurs in the near-infrared (NIR) region of the electronic absorption spectrum in the region of 800–2500 nm. This LMCT band is rarely detected by absorption spectroscopy but can be readily detected by MCD spectroscopy, with the peak wavelength being an excellent indicator of the axial ligands to the heme iron (15, 22). In NrfB, a broad positive LMCT band at lower wavelength (1200–1300 nm), which reflects the fact that five hemes in side (g = 2.99, 2.27, and 1.5) are present, indicative of hemes with near-parallel and near-perpendicular imidazole ligands, and they integrate to yield a 3:2 ratio. Efforts to express cloned NrfB were unsuccessful. This result indicates that the products of nrfE/F/G genes (encoded on pEG86) that encode the proteins required for the attachment of hemes to CXXCH motifs. Expression under aerobic conditions, where the expression of the native nrfE/F/G genes is suppressed, resulted in synthesis of holo-NrfB in the soluble fraction, which was maximal following induction with IPTG in the presence of the ccm genes (pEG86) (Fig 1D, lanes 6–10). Fractionation of cells established that the recombinant NrfB was localized in the periplasmic fraction, and purification yielded 100 mg of pure protein from 10 liters of culture, a 200-fold higher yield than that for the native protein. Analysis of this recombinant protein, using the techniques applied for native NrfA described above, showed it to be competent as an electron donor to NrfA and to have similar spectroscopic and redox properties as the native protein. An illustration of this finding is shown in the EPR spectrum (Fig 5, C and D). As for native NrfB, both high g_max (g ~ 3.57) and rhombic signals (g ~ 2.99, 2.27, and 1.5) are present, indicative of hemes with near-parallel and near-perpendicular imidazole ligands, and they integrate to yield a 3:2 ratio. Efforts to express cloned nrfA under similar conditions were unsuccessful. This result indicates that the products of nrfE/F/G, which are required for correct folding and heme insertion into NrfA, are not required for NrfB. This observation is consistent with the spectroscopic evidence that, unlike NrfA, all five NrfB hemes are low spin bis-His coordinated species.

DISCUSSION

This work has provided the first purification and spectropotentiometric characterization of the NrfB cytochrome from any source. The genes for these novel pentaheme c-type cytochromes are only found in gene clusters that also encode the NrfA pentaheme nitrite reductase in enteric members of the γ-proteobacteria. The nrfA and nrfB genes are located contiguously, implicating a role for NrfB as an electron donor to NrfA, and the first biochemical evidence for this implication is now provided. The pentaheme NrfA nitrite reductase polypeptide is...
Characterization of the Pentaheme Cytochrome NrfB

NrfB concentration was 130 μM in 0.05 M Hepes, pH 7. Room temperature MCD spectrum of oxidized NrfB. A, visible region. NrfB concentration was 1.3 μM in 0.05 M Hepes, pH 7. B, near infrared region. NrfB concentration was 130 μM in 0.05 M Hepes, pH 7.

~50 kDa and, thus, has a heme/protein ratio of ~1 heme per 10 kDa. By comparison, the NrfB cytochrome is very small, having a polypeptide mass of ~17 kDa and, hence, a heme/protein ratio of only ~1 heme per 3.4 kDa. This will lead to a very close packing of the five hemes that will most likely allow for rapid electron transfer through the protein to the redox partner in a manner also suggested for small tetraheme cytochromes (STCs) such as the Shewanella oneidensis STC (18). The MCD analysis of NrfB is consistent with all five hemes being low spin hexacoordinate ferric species. The NIR MCD peak at 1500 nm suggests that these hemes all have bis-histidinyl ferric iron axial ligation, which would require 10 histidine residues to be present in the NrfB polypeptide chain. Sequence alignments of NrfB members reveal 11 conserved His residues, five in the CXXCH heme binding motifs that provide the proximal ferric iron ligands and six elsewhere that are candidates for the five distal ferric iron ligands (Fig. 6). A conserved methionine residue identified in the alignment can be excluded as a distal heme ferric iron ligand by the MCD, because a His-Met ligand pair would give rise to a NIR MCD peak of ~1800 nm, and no such peak was apparent. However, it should be noted that a His-Lys ligand pair can also give rise to a peak in the 1500 nm region of the NIR MCD spectrum (17), and there are conserved Lys residues in NrfB (Fig. 6), one of which is close to the first heme binding motif. Given that the active site heme iron in the NrfA nitrite reductase has a Lys proximal ligand, the possibility of a Lys ligand to a ferric heme iron in NrfB cannot be excluded. However, if a low spin ferric heme iron possesses a His-Lys axial ligand pair, it characteristically has a sharp signal at g ~ 3.5 in the EPR spectrum (17). Such a signal was not apparent in the NrfB EPR spectrum. If, as in NrfA, there is a Lys-coordinated heme that does not have a second protein-derived ligand, it is usually a high spin species. Again, no evidence for such a species was apparent in freshly prepared samples. Finally, the requirement for only the standard cytochrome c maturation genes to facilitate the synthesis of recombinant holo-NrfB during anaerobic growth also argues against an unusual Lys coordination to the heme iron. Thus, taking the MCD, EPR, sequence alignment, and expression experiments together, we favor a model for NrfB in which all five ferric hemes are bis-histidinyl-coordinated low spin species attached to CXXCH heme binding motifs. This model distinguishes the pentaheme core of NrfB from that of its enzymatic redox partner, NrfA, in which only four or five hemes are low spin, with the fifth heme iron being the high spin Lys-coordinated species to which nitrite binds and at which catalysis takes place. The absence of such a site in NrfB was consistent with the failure to observe any direct nitrite reduction by the reduced protein in the absence of NrfA.

The electron-donating properties of the imidazole ring nitrogen ligands stabilize the ferric state of heme iron with bis-His axial coordination (17). Consequently, bis-His-ligated hemes usually have low Fe(III)/Fe(II) midpoint redox potentials. The reduction of all five NrfB hemes in the region of 0 to ~400 mV is thus consistent with bis-His heme iron coordination. The low equilibrium midpoint potentials of these hemes overlap with those of the hemes of NrfA (one at ~37 mV, two at ~107 mV, and two at ~323 mV), such that a simple exergonic movement of electrons in an energetically favorable manner from one electron carrier to the next cannot be envisaged. Rather, the electron transfer to the active site of NrfA through the NrfAB hemes must involve both exergonic and endergonic electron transfer.

Fig. 4.

Fig. 5. Perpendicular mode X-band EPR spectra of air-oxidized native and recombinant NrfB. Spectra were collected at a temperature of 10 K and microwave power of 2 milliwatts (A and C) and at a temperature of 10 K and microwave power of 40 milliwatts (B and D). For both spectra, the modulation amplitude was 1 millitesla (mT), the microwave frequency was 9.67 GHz, and the NrfB concentration was 40 μM in 0.05 M Hepes, pH 7. A and B, native NrfB. C and D, recombinant NrfB. The features at g = 3.57, 2.99, 2.27, and 1.5 are discussed under “Results.” The feature at g = 4.3 arises from adventitious iron. The gap in the data at ~325–350 milliteslas is due to removal of a cavity signal.
transfer, with rapid flux ensured by close interaction of the hemes within the protein milieu. NrfB has been shown, through the use of analytical gel filtration, to occur as a decaheme homodimer in solution. As NrfA is also a decaheme cytochrome, this finding is consistent with the formation of a decaheme NrfB dimer in solution. NrfB has been shown, through the use of analytical gel filtration, to occur as a decaheme cytochrome in the current data bases. However some decaheme cytochromes have only been characterized spectrophotometrically (21). They are ~40 kDa proteins in which the 10 hemes are bound to the histidine imidazole rings of the protein. These decaheme cytochromes have only been characterized spectrophotometrically (21). They are ~40 kDa proteins in which the 10 hemes are bound to the histidine imidazole rings of the protein. These decaheme cytochromes have only been characterized spectrophotometrically (21). They are ~40 kDa proteins in which the 10 hemes are bound to the histidine imidazole rings of the protein.

In conclusion then, this paper has presented the first purification and characterization of NrfB from any organism and has provided biochemical evidence for the role of this protein as a direct electron donor to the NrfA nitrite reductase with which it must form a transient 20-heme [NrfB]2[NrfA]2 complex. The high heme:protein ratio of NrfB strongly suggests that the NrfB dimer would dock in a large pocket at the base of the protein with heme 2 and/or heme 5 being potential electron input sites.

In the NrfA structure, the hemes are organized as parallel or perpendicular heme pairs in which the tetrapyrrole rings can be as close as 4 Å. The high heme:protein ratio of NrfB strongly suggests that such closely interacting hemes will be a feature of this cytochrome group. The NrfB primary structure does not have significant homology with any structurally defined multiheme cytochrome in the current data bases. However some features are suggestive of parallel heme pair organization. In the structurally defined STC (18) and the tetra-heme domains of flavocytochrome c furmarate reductases (19, 20) a broadly conserved primary structure feature can be identified that binds a parallel heme pair, CXXCHX₃₋₅HXXHX₃₋₅CXXCHXXH (Fig. 5), where the second His between the two CXXCH motifs provides a distal ligand to the heme covalently attached to the second CXXCH motif. The His 9–17 amino acids after the first CXXCH motif and the His two amino acids after the second CXXCH motif provide distal ligands for hemes that are not part of the heme pair. This feature, with similar amino acid spacing, can be identified in NrfB with the sequence CXXCHX₉₋₁₂HXXHₓ₃₋₅CXXCHXXH (Fig. 6). In STC and flavocytochrome c, two of the four hemes have near-perpendicular His ligands, the other two have near-parallel His ligands, and all of the hemes titrate in the low potential domains, features that are in common with that observed for NrfB in this study. It is notable that one STC also has a CXXCHH motif at the first heme binding site similar to that of NrfB, and from best fit alignments it appears that NrfB and STC may have diverged in evolution through a deletion/insertion of the second heme binding region of the polypeptide (Fig. 6).

Another group of multiheme cytochromes that merit consideration in the context of NrfB are the periplasmic decaheme cytochromes (e.g. MtrA) of the Fe(III)-respiring bacteria of the Shewanella genus. These decaheme cytochromes have only recently been characterized spectrophotometrically (21). They are ~40 kDa proteins in which the 10 hemes are bound to the polypeptide in two pentaheme segments. Given that the decaheme NrfB dimer is also ~40 kDa, the possibility that the decaheme cytochromes arose from gene duplication and fusion of an nrfB-like gene is apparent. Indeed, the arrangement of the CXXCH motifs in the first pentaheme segment of MtrA is rather similar to that of NrfB, including a CXXCHH motif at heme binding site 1. Spectrophotometric analysis of S. oneidensis decaheme MtrA reveals that, like the decaheme NrfB dimer, all ten hemes exhibit bis-His axial coordination with both near-parallel and near-perpendicular imidazole ring geometries and titrate in the range 0 to ~400 mV (21).

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Purification and Spectropotentiometric Characterization of *Escherichia coli* NrfB, a Decaheme Homodimer That Transfers Electrons to the Decaheme Periplasmic Nitrite Reductase Complex

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