NapC is a member of a family of bacterial membrane-anchored tetra-heme c-type cytochromes that participate in a number of respiratory electron transport pathways. They are postulated to mediate electron transfer between membrane quinols/quinones and soluble periplasmic enzymes. The water-soluble heme domain of NapC has been expressed as a periplasmic protein. Mediated redox potentiometry and characterization by UV-visible, magnetic circular dichroism, and electron paramagnetic resonance spectroscopies demonstrates that soluble NapC contains four low spin hemes, each with bis-histidine axial ligation and with midpoint reduction potentials of −56, −181, −207, and −235 mV.

Many bacterial periplasmic oxidoreductases have been identified that couple respiratory electron acceptors or electron donors to energy conserving electron transport pathways (1). Often these enzymes need to interact with a redox protein that can mediate electron transport from, or to, the membrane QH2/Q3 pool (2). In some cases quinol oxidation can be facilitated by operation of the cytochrome bc1 complex, for example in electron transport to the periplasmic nitrite and nitrous oxide reductases of the denitrifying bacterium Paracoccus denitrificans (3, 4). However, the cytochrome bc1 complex is not ubiquitous in bacteria and when present is not always involved in electron transport to all the periplasmic enzymes. For example, electron transport to the periplasmic nitrate reductase of P. denitrificans proceeds independently of the cytochrome bc1 complex (4, 5). Over the last 5 years, sequencing studies have identified a family of periplasmic c-type cytochromes, the NapC/NirT family, which appear to provide the major, cytochrome bc1-independent, route for electron transport between the quinol pool and water-soluble periplasmic oxidoreductases. Such cytochromes are phylogenetically more widely distributed than the cytochrome bc1 complex among the Proteobacteria (6). However, no member of this important cytochrome family has been purified or characterized spectroscopically. Analysis of the primary structure derived from DNA sequences suggests that a common feature of the NapC/NirT-like cytochromes is the presence of a N-terminal transmembrane α-helical anchor that binds a tetra-heme globular domain to the periplasmic face of the plasma membrane (6). Some members of the family have an additional C-terminal extension that is predicted to bind a fifth heme (7).

The nirT gene product was the first member of the NapC/NirT family of membrane-anchored multiheme c-type cytochromes to be identified. nirT clusters with the nirS gene encoding the periplasmic cytochrome cd1 nitrite reductase of Pseudomonas stutzeri. Disruption of this gene results in the loss of the ability of the bacterium to reduce nitrite using endogenous electron donors (8). The napC gene encoding a homologue of NirT has since been identified in nap gene clusters that contain the napA gene for the catalytic subunit of the periplasmic nitrate reductase of Paracoccus denitrificans, Alcaligenes eutrophus, Rhodobacter sphaeroides, Hemophilus influenzae (6, 9–11), and Escherichia coli (37). Disruption of the napC gene in R. sphaeroides results in the loss of physiological electron transport to the nitrate reductase (10). In Shewanella putrefaciens disruption of the cymA gene, encoding another NapC/NirT homologue, results in the pleiotropic loss of nitrate, fumarate, and iron(III) reduction (12). The torC genes of E. coli and H. influenzae, the dmsC gene of R. sphaeroides, and the dorG gene of Rhodobacter capsulatus encode penta-heme members of the NapC/NirT family that are likely to be involved in electron transfer to the periplasmic trimethylamine N-oxide and dimethyl sulfoxide (MeSO) reductases (7, 11, 13, 14). Taken together, this combination of genetic evidence strongly implicates the members of the NapC/NirT family in electron transfer from quinols to periplasmic reductases. In addition to participating in quinol oxidation some members of the NapC/NirT family may also participate in quinone reduction. For example, the cycB gene of Nitrosomonas europaea encodes a NapC/NirT homologue and is clustered with genes encoding the hydroxylamine-cytochrome c oxidoreductase (15). Electron transport from hydroxylamine to the quinone pool is a prerequisite for lithoautotrophic growth using ammonia as electron donor.

The participation of members of the NirT family in a wide range of periplasmic electron transfer systems in a phylogenetically diverse range of bacteria make the characterization of this protein of central importance to understanding a number of QH2/Q-linked periplasmic electron transport systems. In this study, we have developed an expression system for the heme-containing domain of NapC from the periplasmic nitrate reductase of P. denitrificans. This has provided a water-soluble form of NapC that can be easily purified and concentrated to the levels required for magneto-optical spectroscopies and has al-

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†To whom correspondence should be addressed. Tel.: 44-1603-593220; Fax: 44-1603-592250; E-mail: d.richardson@uea.ac.uk.

‡The abbreviations used are: QH2/Q, quinol/quinone; LB, Luria-Bertani; TAPS, N-tri(hydroxymethyl)methyl-3-aminopropanesulfonic acid; MCD, magnetic circular dichroism; CT, charge transfer; NIR, near-infrared; EPR, electron paramagnetic resonance; bp, base pair(s); T, tesla.
lowed the first spectroscopic characterization of a member of the NapC/NirT family.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions—**Bacterial strains and plasmids used and constructed in this work are listed in Table I. E. coli was routinely grown at 37 °C on LB medium (16). P. denitrificans strains were cultured at 30 °C in minimal media as described previously (17) or at 37 °C on LB medium. Antibiotics were added to plates and culture media at the following concentrations: 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 10 μg/ml tetracycline (E. coli strains); and 100 μg/ml rifampicin, 50 μg/ml kanamycin, 0.5 μg/ml tetracycline, 25 μg/ml streptomycin, and 25 μg/ml spectinomycin (P. denitrificans strains).

**Construction of a Soluble Form of NapC—**The vector pLA2917 carrying the napEDABC gene cluster (pLA2sp) was used as template in polymerase chain reactions performed with mutagenic primers that introduced EcoRI and PstI restriction sites into a DNA fragment encoding the putative periplasmic domain of NapC. The primers were: 5′-CGA TCC CAG ACG TCC CCG CGG-3′ (38) and 5′-ACG TCA GGC CAG GGC CAG GGC-3′ (39) (Fig. 1). The 575-bp polymerase chain reaction product was cloned into EcoRI-PstI fragment (pLA2917) and then into the broad host range vector pK2223–3, which contained the promoter and the DNA encoding the NapC/NirT family. A strategy for expressing a soluble form of NapC—NapC cytochrome could be separated from the 12-kDa cytochrome by phenyl-Sepharose chromatography (26 mm inside diameter and 40 cm in length). The fractions containing NapC were brought to 30% saturation with ammonium sulfate and loaded onto the phenyl-Sepharose column, which was developed using a 100 ml Tris-HCl, pH 8.0, dual gradient of ammonium sulfate (30–80%) and ethylene glycol (0–25%) v/v. Most of the bound proteins eluted in this first step, but the 12- and 24-kDa cytochromes remained bound. The 12-kDa cytochrome was subsequently eluted when a second gradient of ethylene glycol (25–60%) was applied. The 24-kDa NapC was eluted when the column was finally perfused with 70% ethylene glycol.

**Protein Assays and Heme Stains—**The protein was estimated using the bicinchoninic acid method (18) using bovine serum albumin Cohn fraction V as a standard. The proteins were stained with Coomassie Brilliant Blue R250. The stain for heme-linked peroxidase activity, which is specific for c-type cytochromes, was performed as described previously (19).

**Visible Absorption Spectra and Mediated Redox Potentiometry—**Absorption spectra were collected using an Amino SLM DW2000 spectrophotometer. The UV-visible spectra and redox titration of soluble NapC were carried out at 25 °C in 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl. Mediated redox potentiometry was performed as described (20). Dithionite and ferricyanide were used as reductant and oxidant, respectively. Redox mediators were phenazine methosulfate, phenazine ethosulfate, diaminodurene, 4-hydroxynapthoquinone, 5-anthraquinone 2-sulfonate, 6-anthraquinone 2,6-disulfonate, and benzylviologen (at a final concentration of 20 μM). Quinhydrone was used as a redox standard ($E_{m}^{o}$ = +295 mV). All potentials quoted are with respect to the normal hydrogen electrode.

**MCD and EPR Analyses—**The MCD and EPR spectra were carried out in 20 mM TAPS, pH 8.0, and 100 mM NaCl. MCD spectra were measured using a split-coil superconducting solenoid, type SM-4 (Oxford Instruments), capable of generating a maximum magnetic field of 5 T and spectropolorimeters JASCO J-500D and J-730 for the wavelength ranges 240–100 and 800–2000 nm, respectively. EPR spectra were recorded using an ER-2000 X-band spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid-helium flow cryostat (ESR-9; Oxford Instruments).

**RESULTS**

**Expression and Purification of a Water-soluble Form of NapC—**A strategy for expressing a soluble form of NapC was designed (Fig. 1). P. denitrificans was used to express NapC instead of E. coli, because E. coli has a very limited capacity for synthesis of c-type cytochromes and efforts to express NapC in this bacterium failed. The protein produced must be posttranslationally modified by covalent attachment of four hemes, and this occurs after export of the polypeptide into the periplasmic

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**Table I**

| Strain/plasmid | Description | Reference/source |
|----------------|-------------|------------------|
| P. denitrificans 1222 | Rif" enhanced conjugation frequencies; cyca "Km" | (38) |
| C010 | plACsol; Rif"Tc" | This work |
| 1222/pLACsol | plACsol; cyca "Km"Tc" | This work |
| E. coli DH5a | recA1 endA1 4800 lacZ dm15 | This work |
| pKR2013 | pKR2013 carrying tra functions; Km" | (40) |
| Plasmid | Broad host range mobilizable cloning vector; Km"Tc" | (41) |
| pLA2917 | 4-kb HindIII DNA fragment containing the napEDABC genes cloned into pLA2917; Km"Tc" | This work |
| pLITMUS 38 | Cloning vector; Amp" | New England Biolabs |
| pLTIC | 575-bp EcoRI-PstI PCR fragment cloned into pLITMUS 38; Amp" | This work |
| pKK223–3 | Expression vector; Amp" | Amersham Pharmacia Biotech |
| pKPD1 | 1028-bp DNA fragment containing cyca gene cloned into a pKK223–3 derivative vector; Amp" | (21) |
| pKKCsol | 903-bp SalI-PstI DNA fragment cloned into PKK223–3; Amp" | This work |
| pLACsol | 903-bp HindIII-BamHI DNA fragment cloned into PLAC2917; Tc" | This work |
compartment. Thus, having removed the DNA encoding the N-terminal membrane anchor region to construct a napC gene encoding a soluble form of NapC (pLITC), it was necessary to introduce a DNA fragment coding for a signal peptide to direct export to the periplasm. The DNA region encoding the CycA (cytochrome c\textsubscript{550}) P. dentrificans signal peptide and the first 12 amino acids of this cytochrome were used in the gene fusion that was then expressed under the control of the cycA promoter (pLACsol; Fig. 1). The cycA promoter and signal peptide were used because cytochrome c\textsubscript{550} is expressed at high levels under a variety of growth conditions and has been previously shown to direct efficient export of passenger mono-heme c\textsubscript{550}-type cytochromes (21).

Periplasmic extracts were prepared from P. denitrificans 1222 carrying pLACsol. The cells were grown under anaerobic denitrifying conditions, with succinate as carbon substrate and nitrate as electron acceptor, and periplasmic fractions examined by SDS-polyacrylamide gel electrophoresis (Fig. 2A). Comparison of heme-stained gels revealed the presence of a 24-kDa c-type cytochrome that was absent from the parent strain (Fig. 2A, lanes 1 and 2). The calculated molecular mass of the mature NapC-soluble protein with the leader sequence cleaved is 22,035 Da, rising to 24,493 Da if the expected four heme groups are covalently bound. Thus, the presence of a 24-kDa heme-staining protein in periplasmic fractions of P. denitrificans 1222/pLACsol is strong evidence for successful expression of a soluble form of NapC. The 24-kDa heme staining band corresponding to NapC could also be resolved in periplasmic extracts of P. denitrificans C010/pLACsol (Fig. 2A, lane 4), which carries a cycA::km insertion resulting in the loss of the cytochrome c\textsubscript{550} heme-staining band at 17 kDa on the SDS-polyacrylamide gels (Fig. 2A, lanes 3 and 4). Indeed, the NapC-soluble heme staining band was more intense in periplasmic extracts from P. denitrificans C010/pLACsol compared with P. denitrificans 1222/pLACsol. Moreover, the absence of cytochrome c\textsubscript{550} aided purification of the NapC, so the strain C010/pLACsol of P. denitrificans was selected for further analysis. The highest
levels of expression of NapC were observed in periplasmic extracts prepared from cells grown aerobically in LB media (Fig. 2B, lane 2). Much lower levels of expression of this protein were observed in cells grown aerobically in minimal media with succinate as carbon source (Fig. 2B, lane 1) or grown anaerobically in LB media (Fig. 2B, lane 3). Growth in LB media offered the best yield combined with an easy preparation of the batch cultures and purification, so this medium was selected for purification of the enzyme, which was performed using DEAE-Sepharose anion exchange and phenyl-Sepharose hydrophobic chromatographies. The purified NapC (Fig. 2C) was stable at 4 °C at pH 8 (Tris-HCl or TAPS) provided that the sodium chloride concentration was maintained at 100 mM or that the ethylene glycol was maintained at 70% (v/v). The yield was 5 mg of NapC/100 liters of culture.

**UV-visible, MCD, EPR, and Mediated Redox Potentiometry of NapC**—The UV-visible absorption spectrum of air-oxidized NapC (Fig. 3A) and ferricyanide-oxidized (Fig. 4, inset, top) NapC showed a Soret peak at 406.5 nm with an extinction coefficient of 434 mM⁻¹ cm⁻¹. This wavelength, the bandshape of the α, β region (500–600 nm), and the absence of bands in the 630–650 nm region suggest that the hemes are in the low spin state. The Soret intensity is consistent with the presence of four such species. The NapC hemes could be reduced by dithionite but not by ascorbate (Em,1.0 = +80 mV), even in the presence of the mediators tetramethyl-p-phenylenediamine or phenazine methosulfate. Absorption maxima at 416, 525, and 551 nm were observed in the dithionite reduced form (Fig. 4, inset, bottom).

The room temperature MCD spectrum of air-oxidized NapC (Fig. 3B) could be assigned unambiguously to ferric heme; none of the characteristic signatures of ferrous heme were observed. Low spin ferric MCD bands dominate those of high spin throughout the 300–600 nm region (22). This is especially so for the Soret region (~400 nm), where a single low spin heme gives rise to a derivative shaped band with a peak-to-trough intensity of approximately 150 mM⁻¹ cm⁻¹ T⁻¹. The MCD Soret feature of NapC (Fig. 3B) had an intensity of 573 mM⁻¹ cm⁻¹ T⁻¹, implying the presence of four low spin ferric hemes. The MCD for the α, β bands were also consistent with this interpretation. All the bands between 300 and 600 nm, arise from π–π* transitions of the porphyrin but are sensitive to the spin-state of the iron (22). At wavelengths longer than 600 nm, CT bands (largely porphyrin → ferric) are observed, one for low spin ferric heme and two for the high spin state (23, 24). The first of the high spin CT bands often appears in the absorption spectrum as a shoulder at ~630 nm, and its observation flags the presence of this spin-state. However, its apparent absence can be due to overlap from adjacent and more intense α, β bands, and the transition is more reliably located in the MCD spectrum, where it gives rise to a derivative shaped feature. The MCD spectrum of NapC showed no trace of this band, implying that there were no significant levels of high spin heme.

The CT band for low spin ferric heme, which occurs in the near-infrared region (NIR) (800–2000 nm), is rarely detectable by absorption spectroscopy but is also readily located by MCD and the peak wavelength is diagnostic of the heme ligands (22, 25). The NIR-MCD of NapC revealed a positive band at 1490 nm with side structure to higher energy (Fig. 3C). This band is characteristic of the low spin CT band and the wavelength is typical for bis-histidine ligation (25). However, examples of both histidine-amine (26, 27) and methionine-histidine (28) co-ordination are known, and these give CT bands at similar wavelengths. Met-His⁺ can be ruled out in this case since the co-ordination of sulfur to low spin ferric heme iron, either as methionine or cysteine, always gives rise to additional ligand → Fe(III) CT MCD bands in the 650–750 nm region (29–31). No such bands were observed in the NapC MCD spectrum. Furthermore, EPR analysis revealed a typical low spin ferric heme spectrum with features at gzz = 2.96, gxx = 2.28, gxz = 1.5 (data not shown). These g values are characteristic of heme with two histidine ligands orientated with parallel planes (32) and rule out the presence of histidine-amine co-ordination, where the g values are substantially different, i.e. gzz = 3.3–3.55, gxz = 1.8–2.05 (26, 27).

Mediated potentiometric titrations of *P. denitrificans* NapC...
monitored spectroscopically at the α-band maximum at 551 nm could only be satisfactorily fitted with four n = 1 Nersat components (Fig. 4, main panel). The titer was fully reversible in both oxidative and reductive directions. The best fit was obtained when it was assumed that all the hemes contributed equally to the absorption amplitude of the fully reduced sample. The estimated midpoint potentials (pH 7.0) of the four hemes were −56, −181, −207, and −235 mV. These potentials are consistent with the spectral evidence for bis-His-ligated hemes, which tend to have mid-point potentials in the range of −400 to 0 mV, whereas the redox potentials of His-Met ligated hemes tend to lie in the range of 0 to +400 mV (33). Spectra were collected at each redox potential in the titer and analysis of difference spectra indicated that each component had identical reduced absorption maxima.

**DISCUSSION**

This paper has presented the first spectroscopic characterization of a member of the NapC/NirT family of membrane-anchored multi-heme cytochromes. The technique of substituting a signal peptide for the membrane-anchor to yield a stable soluble periplasmic multi-heme cytochrome should prove useful for the characterization of other membrane-anchored c-type cytochromes. The soluble NapC is capable of mediating electron transfer between water-soluble quinols and the periplasmic nitrate reductase, demonstrating that it retains functionality in this form. The combined approach of electron transfer between periplasmic sulfide dehydrogenase and the QH2/Q pool, has also suggested a duplication of two di-heme domains (34). However, this analysis argued for a bis-His ligated heme in each segment and not the ligand of the second bis-His ligated heme in each domain. Therefore, we suggest that in the N-terminal domain His-81 provides the distal ligand to the heme bound to CXX CH motif I and that in the C-terminal domain His-194 provides the distal ligand to the heme bound to CXX CH motif III (Fig. 5).

A previous analysis of the primary structure of a NapC/NirT homologue from *Chromatium vinosum*, which may be involved in electron transfer between periplasmic sulfide dehydrogenase and the QH2/Q pool, has also suggested a duplication of two di-heme domains (34). However, this analysis argued for a His-Met and bis-His-ligated heme in each domain with Met-68, His-81, Met-161, and His-194 (P. denitrificans numbering) providing the axial ligands. Although the residues Met-68 and Met-161 are highly conserved in all the members of the NapC/NirT family (Fig. 5), our spectroscopic data exclude these amino acids as candidates for the ligands of any of the hemes of NapC. In our analysis, the heme ligands suggested are all amino acids as candidates for the ligands of any of the hemes of NapC. Our analysis of the globular region of the protein, which includes the four CXXCH heme binding motifs, supports the possibility of a gene duplication leading to two structurally similar di-heme binding domains (Fig. 5). 60–70 amino acids of the N-terminal and C-terminal segments, each containing two CXXCH motifs, were aligned at CXXCH motif I with motif III and CXXCH motif II with motif IV (Fig. 5). The two CXXCH motifs in each segment are spaced around 28 amino acids apart. A conserved His residue (His-81 and His-174; *P. denitrificans* numbering) lies between the two motifs in each segment. These are candidates for the distal ligand of two of the bis-His hemes predicted from the EPR/MCD. A less well conserved His residue (His-99 and His-194) lies close to the CXXCH motif in each segment and is a candidate for the ligand of the second bis-His ligated heme in each domain. Therefore, we suggest that in the N-terminal domain His-81 provides the distal ligand to the heme bound by CXXCH motif II and His-99 provides the distal ligand to the heme bound by CXXCH motif I and that in the C-terminal domain His-194 provides the distal ligand to the heme bound to CXXCH motif IV and His-194 provides the distal ligand to the heme bound to CXXCH motif IV.

2 M. D. Roldán and D. J. Richardson, unpublished data.
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