Identification of two domains and distal histidine ligands to the four haems in the bacterial *c*-type cytochrome NapC; the prototype connector between quinol/quinone and periplasmic oxido-reductases

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NapC is a tetra-haem member of a family of bacterial membrane-anchored multi-haem *c*-type cytochromes implicated in electron transfer between membrane quinols and periplasmic enzymes. The water-soluble tetra-haem fragment of *Paracoccus pantotrophus* NapC has been expressed as a periplasmic protein (NapC^{sol}) in *Paracoccus denitrificans*, *P. pantotrophus* and *Escherichia coli*. Site-specific mutagenesis of NapC^{sol}, combined with spectroscopic studies, suggests that each haem iron centre has *bis*-histidinyl co-ordination. Four proximal ligands arise from each of four Cys-Xaa-Xaa-Cys-His haem-binding motifs; candidates for the four distal ligands are His⁸¹, His⁹⁹, His¹⁷⁴ and His¹⁹⁴. NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} mutants (with alanine substituted for each of the four candidate residues) have all been purified from *E. coli*. In each case, one of the haems has become high-spin, as judged by the presence of a broad absorption band between 620 nm and 650 nm for the oxidized cytochrome; this feature is absent for wild-type protein and presumably arises because of the absence of the distal histidine ligand from one of the haems. NapC^{H81A} and NapC^{H174A} are less

well expressed in *E. coli* than NapC^{H99A} and NapC^{H194A} and cannot be detected when expressed in *P. denitrificans* or *P. pantotrophus*. *In vitro* and *in vivo* complementation studies demonstrate that the soluble periplasmic NapC can mediate electron transfer from quinols to the periplasmic nitrate reductase. This capacity was retained *in vitro* with the NapC^{H99A} and NapC^{H194A} mutants but was lost *in vivo*. A model for the structural organization of NapC^{sol} into two domains, each containing a di-haem pair, is proposed. In this model, each haem pair obtains one distal haem ligand from its own domain and a second from the other domain. The suggestion of two domains is supported by observations that the 24 kDa NapC^{sol} cleaves to yield a 12 kDa haem-staining band. Determination of the cleavage site showed it was between two equally sized di-haem domains predicted from sequence analysis.

Key words: bacterial respiration, NapC/NirT family, periplasmic nitrate reductase, quinol oxidase, tetra-haem cytochrome.

INTRODUCTION

Bacterial nitrate reduction via a periplasmic respiratory nitrate reductase is widespread in proteobacteria [1]. In order to reduce nitrate in the periplasm, there has to be a means of transferring electrons from membrane-confined benzoquinols (or naphthoquinols) to the periplasmic nitrate reductase complex (NapAB). The candidate protein for fulfilling this is NapC, a member of a large family of bacterial tetra-haem and penta-haem cytochromes that have been proposed to participate in electron transfer between the quinol/quinone pool and periplasmic redox enzymes such as the trimethylamine N-oxide reductase, dimethylsulphoxide reductase, fumarate reductase, nitrite reductases and hydroxylamine oxidoreductase [1–6]. Topological analysis suggests that, in their native state, these cytochromes possess a single N-terminal transmembrane α -helix that serves to anchor one or more globular multi-haem domains on to the periplasmic surface of the cytoplasmic membrane. To facilitate biochemical and spectroscopic analyses, NapC has been expressed as a water-soluble periplasmic protein, NapC^{sol} [7]. Studies using magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) suggested that all four haems are low-spin and feature *bis*-

histidine axial ligation [7]. Redox potentiometric analysis indicated that the haems had low midpoint redox potentials ($E_{m,s.0}$) of –56 mV, –181 mV, –207 mV and –235 mV [7]. Primary structure analysis of NapC, and multiple sequence alignments of other members of the NapC family, led to the identification of four histidine residues as candidates for the distal haem ligands of the four covalently bound haems [7]. In the present study, each of these residues has been targeted for mutagenesis and the consequences for protein assembly, haem iron ligation and quinol oxidation assessed.

EXPERIMENTAL

Bacterial strains, media and growth conditions

Bacterial strains used in this work are listed in Table 1. *Escherichia coli* was routinely grown at 37 °C on Luria broth (LB). *Paracoccus denitrificans* CO10 and *Paracoccus pantotrophus* strains were cultured at 30 °C in minimal medium, as previously described in [7], or at 37 °C on LB. Antibiotics were added to plates and culture media at the following concentrations: ampicillin, 50 $\mu\text{g} \cdot \text{ml}^{-1}$; chloramphenicol, 34 $\mu\text{g} \cdot \text{ml}^{-1}$ (*E. coli* strains)

Abbreviations used: EPR, electron paramagnetic resonance; Fcc₃, soluble flavocytochrome *c*₃ fumarate reductase; HAO, hydroxylamine oxidoreductase; Ifc₃, iron-induced flavocytochrome *c*₃; LB, Luria broth; MCD, magnetic circular dichroism; Nap, periplasmic nitrate reductase; Nrf, formate-dependent nitrite reductase; SSC, Split-Soret cytochrome *c*.

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Table 1 Bacterial strains and plasmids used in the present study

(a) Strain	Description	Reference
<i>P. denitrificans</i> C010	<i>cycA</i> ::Km ^r	[17]
<i>P. pantotrophus</i> GB17	Rif ^r , Sm ^r	[41]
<i>P. pantotrophus</i> NC1	<i>napC</i> :: Ω ; Rif ^r , Sm ^r , Spec ^r , Tc ^r	The present study
<i>P. pantotrophus</i> HJ52	Δ (<i>napEDABC</i>); Rif ^r , Sm ^r	H. J. Sears and D. J. Richardson, unpublished results
<i>P. pantotrophus</i> HJ510	<i>napA</i> :: Ω ; Rif ^r , Sm ^r , Spec ^r , Tc ^r	[42]
<i>E. coli</i> DH5 α	<i>recA</i> <i>endA</i> 1 Φ 80d <i>lacZ</i> dm15	Stratagene
<i>E. coli</i> pRK2013	PKR2013 carrying <i>tra</i> functions; Km ^r	[43]
<i>E. coli</i> JCB7123	Tc ^r	[10]
(b) Plasmid	Description	Reference
pBluescript KS ⁺	Expression vector; Amp ^r , LacZ ^r	Stratagene
pSUP202	Suicide vector; Amp ^r , Tc ^r , Cm ^r	[9]
pSUP202/ Ω nap	3.4 Kb <i>Pst</i> I DNA fragment containing Ω cassette (Sm ^r /Spec ^r) cloned into pSUP202	The present study
pLACsol-1	903 bp <i>Hind</i> III– <i>Bam</i> HI DNA fragment (wild type) cloned into pLA2917; Tc ^r	[7]
pLACsol-2	903 bp <i>Hind</i> III– <i>Bam</i> HI DNA fragment (His ⁹⁹ → Ala) cloned into pLA2917; Tc ^r	The present study
pLACsol-3	903 bp <i>Hind</i> III– <i>Bam</i> HI DNA fragment (His ¹⁹⁴ → Ala) cloned into pLA2917; Tc ^r	The present study
pLACsol-4	903 bp <i>Hind</i> III– <i>Bam</i> HI DNA fragment (His ⁸¹ → Ala) cloned into pLA2917; Tc ^r	The present study
pLACsol-5	903 bp <i>Hind</i> III– <i>Bam</i> HI DNA fragment (His ¹⁷⁴ → Ala) cloned into pLA2917; Tc ^r	The present study
pKKCsol-1	886 bp <i>Sal</i> I– <i>Pst</i> I DNA fragment (wild-type) cloned into pKK223-3; Amp ^r	The present study
pKKCsol-2	886 bp <i>Sal</i> I– <i>Pst</i> I DNA fragment (His ⁹⁹ → Ala) cloned into pKK223-3; Amp ^r	The present study
pKKCsol-3	886 bp <i>Sal</i> I– <i>Pst</i> I DNA fragment (His ¹⁹⁴ → Ala) cloned into pKK223-3; Amp ^r	The present study
pKKCsol-4	886 bp <i>Sal</i> I– <i>Pst</i> I DNA fragment (His ⁸¹ → Ala) cloned into pKK223-3; Amp ^r	The present study
pKKCsol-5	886 bp <i>Sal</i> I– <i>Pst</i> I DNA fragment (His ¹⁷⁴ → Ala) cloned into pKK223-3; Amp ^r	The present study
pLANap	4 Kb <i>Hind</i> III DNA fragment containing the <i>napEDABC</i> genes cloned into pLA2917; Km ^r Tc ^r	[7]

and rifampicin 100 $\mu\text{g} \cdot \text{ml}^{-1}$; kanamycin 50 $\mu\text{g} \cdot \text{ml}^{-1}$; tetracycline 0.5 $\mu\text{g} \cdot \text{ml}^{-1}$; streptomycin 25 $\mu\text{g} \cdot \text{ml}^{-1}$; spectinomycin 25 $\mu\text{g} \cdot \text{ml}^{-1}$ (*P. denitrificans* C010 and *P. pantotrophus* strains).

Construction of site-specific mutants for NapC and interposon mutagenesis of the *napC* gene

The vector pBluescript KS⁺ carrying the *napC* gene was used as a template in PCR reactions performed with the mutagenic primers listed in Table 2. As described in [7], this *napC* gene has the DNA coding for the first 47 amino acids, which code for a predicted transmembrane helix, replaced by nucleotides coding for the periplasmic targeting sequence plus the first ten amino acids of the mature protein for *P. denitrificans* cytochrome *c*₅₅₀. Unfortunately, three of the first four amino acids of the resulting soluble form of NapC^{sol} were given incorrectly in the original publication [7]. They are QDGDAAKGEK, as confirmed by direct protein sequencing in the present work, rather than NQGQAAKGEK, as stated. The 903 bp PCR products were cloned into the high copy vector pKK223-3 to yield pKKCsol-1, pKKCsol-2, pKKCsol-3, pKKCsol-4 and pKKCsol-5 and thereby generate the restriction enzyme sites required for cloning into the broad host range vector pLA2917 [8] as described in [7]. These DNA constructs (pLACsol-1, NapC^{sol}; pLACsol-2, NapC^{H81A}; pLACsol-3, NapC^{H99A}; pLACsol-4, NapC^{H174A}; pLACsol-5, NapC^{H194A}) were transferred by conjugation from the donor strain DH5 α of *E. coli* to the recipient strains of *Paracoccus*. The transconjugants were selected on minimal medium plates containing rifampicin, streptomycin and tetracycline (*P. pantotrophus*) or rifampicin, streptomycin, tetracycline and kanamycin (*P. denitrificans* C010).

The *napC*:: Ω strain NC1 of *P. pantotrophus* was constructed by conjugating the suicide vector pSUP202 [9], carrying a

3.4 kb *Pst*I DNA fragment (pSUP/ Ω nap), into *P. pantotrophus*. The *Pst*I DNA fragment contained two different regions from the *napEDABC* operon of *P. denitrificans* which are located to the left and the right side of the Ω cassette (2000 bp) respectively: from *nap*4506 bp to *nap*4869 bp (363 bp, left side of Ω) and from *nap*4869 bp to *nap*5900 bp (1031 bp, right side of Ω). The transconjugants were selected on minimal medium plates containing rifampicin, streptomycin, and spectinomycin.

Expression in *E. coli*

The expression of NapC^{sol} and the mutant derivatives in *E. coli* was performed using the vector pKK223-3 as an expression system. The constructs pKKCsol-1, pKKCsol-2, pKKCsol-3, pKKCsol-4 and pKKCsol-5 were co-transformed with pEC86, which carried the *ccm* genes for cytochrome *c* maturation, into *E. coli* strain JCB7123 which was used for reasons described in [10]. Single colonies were picked straight into 500 ml of LB and grown for 18 h at 37 °C.

Isolation of periplasmic fractions and purification of NapC^{sol}, NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A}

Periplasmic fractions were prepared from 15 l batch cultures of *P. pantotrophus* (pLACsol-1), *P. pantotrophus* (pLACsol-3) and *P. pantotrophus* (pLACsol-5) grown anaerobically in minimal medium. The cells were harvested by centrifugation at 5000 *g* for 10 min (to *D*₆₁₀ of approx. 0.9) followed by a second centrifugation at 40000 *g* for 20 min at 4 °C. The periplasmic extract was prepared as described in [11]. In the case of expression in *E. coli*, the periplasmic extracts were prepared from cells grown aerobically in 10 l of LB for around 19 h. Cells were harvested by centrifugation at 5000 *g* for 10 min and incubated for 30 min at 37 °C in SET (0.5 M sucrose, 3 mM EDTA, 0.5 M

Table 2 Primers used in the present study

The alanine codon is marked in bold and italic. The remaining restriction enzyme (RE) sites (5 nucleotides) and the new restriction sites are in bold. *Represents nucleotide(s) that are not present in the wild-type sequence.

Residue Change	RE site inserted/removed	Oligonucleotide sequence
His ⁸¹ → Ala	<i>Aval</i>	5'-GACGGTGG GC TTCTCGAACCGA* TCGGG -3'
His ⁹⁹ → Ala	<i>BbrPI</i>	5'-GCC CACGTG *CCCG GC GCAATGGACCGAC-3'
His ¹⁷⁴ → Ala	<i>EcoRV</i>	5'-CCAGATT CCGC GAGCGA* TAT *CTGATCTCC-3'
His ¹⁹⁴ → Ala	<i>PvuII</i>	5'-GGGCATC GC GGCG CAGCTA *CCCCGAC-3'

Tris/HCl, pH 8) buffer containing 100 mg of lysozyme per litre of culture. The spheroplasts were then harvested by centrifugation at 40000 *g* for 1 h at 4 °C. The supernatant was then stored at -80 °C. The NapC proteins were purified using DEAE-Sepharose anion exchange and phenyl-Sepharose chromatography essentially as described in [7].

Protein gel electrophoresis and haem stains

SDS/PAGE was performed according to Schagger and von Jagow [12] using 15% gels. Samples that were to be stained for protein were boiled in the presence of 2-mercaptoethanol. The boiling step and 2-mercaptoethanol were omitted if haem staining was intended, as these treatments cause loss of the iron atom which is required for the staining procedure. Two types of molecular-mass markers were used if the gel was to be stained for protein, prestained broad range (7708S) and unstained broad range (7701S) both from New England BioLabs. If the gel was to be haem stained, only the prestained broad range markers were used. Horse heart cytochrome *c* was purchased from Sigma. The stain for haem-linked peroxidase activity, which is specific for *c*-type cytochromes, was performed as described in [7].

Protein assays and UV/visible spectroscopy

The protein was estimated using the bicinchoninic acid ('BCA') method with bovine serum albumin Cohn fraction V as a standard. Absorption spectra were taken using a Lambda 2 spectrophotometer (Perkin-Elmer) using buffer in the reference cuvette.

In vivo and *in vitro* analysis of NapC quinol dehydrogenase activities

The DNA constructs pLACsol-1, pLACsol-2, pLACsol-3, pLACsol-4 and pLACsol-5 were introduced into the *napC::Ω* strain of *P. pantotrophus* to perform studies of complementation *in vitro*. The strains were cultured aerobically in 100 ml of LB and their periplasmic fractions were isolated (1 ml final volume). A diaphorase-dependent assay for regeneration of duroquinol through the reduction of duroquinone by NADH was used essentially as described in [13]. Anaerobic assays were set up in 1 ml cuvettes with the following contents: 900 µl of Tris/HCl, pH 8, 100 µl of periplasm containing 8 µM NapC, 4 µM NapAB, 1 mM NADH, 1 unit diaphorase and 0.5 mM duroquinone. Samples were degassed for 15 min under a nitrogen atmosphere and reactions were initiated by the addition of KNO₃ (15 mM). After 15 min, nitrite production, which changed linearly with the alteration of the concentration of added wild-type NapC^{sol} from 2 to 10 µM, was determined colorimetrically by determining the absorbance at 540 nm [14]. No nitrite accumulated if NapC was

omitted. The soluble NapAB complex was purified from the periplasmic fraction of *P. pantotrophus* as described in [11]. Diaphorase from *Clostridium kluyveri* was obtained from Sigma.

Studies of complementation *in vivo* were carried out by transferring the pLACsol DNA constructs and pLAnapEDABC (carrying the *napEDABC* operon) into the *P. pantotrophus* *napC::Ω* strain NC1. The wild-type strain, the NC1 (pLACsol1-5) and NC1 (pLAnapEDABC) strains were cultured aerobically in 100 ml of minimal medium with caproate as a carbon source and nitrate present as an electron acceptor [15]. The nitrite released into the medium was determined colorimetrically [14].

N-terminal amino acid sequences were determined by stepwise Edman degradation using a gas-phase sequencer (Applied Biosystems model 470A) after SDS/PAGE and transfer of proteins to PVDF.

RESULTS

Expression of NapC^{sol}, NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} in *Paracoccus denitrificans*

The water-soluble periplasmic form of NapC (NapC^{sol}) and the engineered His → Ala derivatives were expressed from the pLACsol series of expression vectors (Table 1). These plasmids contain the *Paracoccus denitrificans* *cycA* promoter, which is highly active under anaerobic denitrifying growth conditions [7,16]. Expression of NapC^{sol}, NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} was initially assessed in the *P. denitrificans* C010 cytochrome *c*₅₅₀ mutant [17], as the absence of the 17 kDa cytochrome *c*₅₅₀ allows for unambiguous resolution of the 24 kDa NapC^{sol} on haem-stained SDS/PAGE gels of periplasmic fractions. These four mutations were chosen because they correspond to the four, other than those from the Cys-Xaa-Xaa-Cys-His motifs, conserved histidine residues in NapC (Figure 1). A 24 kDa haem-staining band could be clearly resolved in periplasmic fractions prepared from *P. denitrificans* C010 carrying pLACsol-1 (NapC^{sol}), pLACsol-3 (NapC^{H99A}) and pLACsol-5 (NapC^{H194A}) (Figure 2). This was absent in the plasmid-free parental strain, which expresses only the membrane-anchored native NapC (NapC^{mem}), and the strains carrying pLACsol-2 (NapC^{H81A}) and pLACsol-4 (NapC^{H174A}) (Figure 2). These results suggest that His⁸¹ and His¹⁷⁴ are more important for the assembly of a stable holo periplasmic NapC complex than are His⁹⁹ and His¹⁹⁴. In a similar series of experiments the same pattern of results was observed when the five pLACsol plasmids were introduced into wild-type *P. pantotrophus*.

Expression of NapC^{sol}, NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} in *E. coli*

Since NapC^{H81A} and NapC^{H174A} did not assemble in *P. denitrificans* or *P. pantotrophus*, expression in *E. coli* was attempted. In this case 24 kDa haem-staining bands were detected in all five cases for expression of NapC^{sol}, NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} (results not shown). This showed that the *P. denitrificans* cytochrome *c*₅₅₀ signal peptide could direct export of the multi-haem cytochromes into the periplasm of *E. coli* and that, for an unresolved reason, NapC^{H81A} and NapC^{H174A} were more stable when expressed in *E. coli* than in the *Paracoccus* strains. All five cytochromes were purified using DEAE-Sepharose anion exchange (elution maximum, 300 mM NaCl) chromatography followed by phenyl-Sepharose hydrophobic interaction chromatography (elution maximum, 70% ethylene glycol). In all cases, the elution profiles were similar. The UV/visible absorption spectra of all five oxidized cytochromes revealed the presence of spectral features characteristic of low-spin *c*-type haems, λ_{max}

Figure 1 Sequence alignment for NapC proteins

The four haem-binding motifs are boxed and the four putative additional histidine ligands to the haem groups are shaded. The NapC sequences originate as follow: ppNapC, *Paracoccus pantotrophus* [28]; atNapC, *Agrobacterium tumefaciens* [29]; smNapC, *Sinorhizobium meliloti* [30]; paNapC, *Pseudomonas aeruginosa* [31]; abNapC, *Azospirillum brasilense* [32]; rsNapC, *Rhodobacter sphaeroides* [33]; mmNapC, *Magnetospirillum magnetotacticum* (GenBank® accession no. AB055444); seNapC, *Salmonella enterica* [34]; stNapC, *Salmonella typhimurium* [35]; ecNapC, *Escherichia coli* [36]; ypNapC, *Yersinia pestis* [37]; hiNapC, *Haemophilus influenzae* [38]; pmNapC, *Pasteurella multocida* [39]. *, identity; :, strongly similar; ., weakly similar.

Figure 2 Haem-linked peroxidase stain of periplasmic fractions resolved by SDS/PAGE (15% gels) of *P. denitrificans* C010 carrying plasmids for expression of different forms of NapC^{sol}

Lane 1, *P. denitrificans* C010; lane 2, *P. denitrificans* C010/NapC^{sol} (wild-type); lane 3, *P. denitrificans* C010/NapC^{sol} (His⁸¹ → Ala); lane 4, *P. denitrificans* C010/NapC^{sol} (His¹⁷⁴ → Ala); lane 5, *P. denitrificans* C010/NapC^{sol} (His⁹⁹ → Ala); lane 6, *P. denitrificans* C010/NapC^{sol} (His¹⁹⁴ → Ala). Protein (20 µg) was loaded on to each lane.

oxidized, 410 nm, 530 nm (Figure 3A) with $\lambda_{\max}$ reduced, 420 nm, 525 nm, 551 nm (results not shown). However, an additional absorption feature between 620 and 660 nm was apparent in the spectra of ferric NapC^{H81A}, NapC^{H99A}, NapC^{H174A} and NapC^{H194A} (Figure 3B). This is characteristic of charge transfer bands of high-spin ferric haem iron and would arise from a haem co-ordinated by a proximal histidine and either no distal ligand or water or hydroxide in the distal position. In all four cases, a clear peak at around 650 nm can be resolved; this $\lambda_{\max}$ is characteristic of penta-co-ordinate haems with a histidine proximal ligand, for example cytochrome *c'* [18–20]. For NapC^{H81A}, the ratio of absorbance at 650 nm to that at 620 nm is smaller than for the other three variant proteins (Figure 3B). The greater relative absorbance at 620 nm in this case may arise from a greater population of His–H₂O/HO[−] co-ordinated haem [18–20].

Quinol oxidation assays and complementation studies

The functionality of NapC^{sol}, NapC^{H99A} and NapC^{H194A} was assessed by measuring duroquinol-dependent nitrite production

in periplasmic fractions of *P. pantotrophus* (Table 3). This could not be done with NapC^{H81A} and NapC^{H174A}, as these did not assemble in *P. pantotrophus*. The reaction depends on the presence of a water-soluble NapC^{sol} in the periplasm to mediate electron transfer from duroquinol to the added periplasmic NapAB complex, which can then reduce nitrate to nitrite. Near maximal accumulation of nitrite is observed in periplasmic fractions prepared from *P. pantotrophus* (pLACSol-1), which expresses the NapC^{sol} and NapAB (Table 3). In periplasmic fractions prepared from the parent strain and the *napC::Ω* mutant NC1, the accumulation of nitrite at the end point of the assay was only 15% of that of *P. pantotrophus* (pLACSol-1) (Table 3). These strains do not express a periplasmic NapC^{sol}, and so the nitrite produced represents the background level of NapC-independent nitrite accumulation in this assay; the molecular basis for this accumulation is not understood. The nitrite production was identical in strains carrying pLACSol-2 (NapC^{H81A}) and pLACSol-4 (NapC^{H174A}), which also do not assemble holo periplasmic NapC (Table 3). However, nitrite accumulation was observed at similar levels to *P. pantotrophus* (pLACSol-1) in the periplasmic fractions prepared from the strains carrying pLACSol-3 (NapC^{H99A}) and pLACSol-5 (NapC^{H194A}) (Table 1). A control without the added periplasmic NapAB complex was performed; nitrite was not detectable. Taken together, these results demonstrate that NapC^{sol} can mediate electron transfer from the duroquinol to the NapAB complex and this ability is retained by NapC^{H99A} and NapC^{H194A}.

To assess the physiological competence of NapC^{sol} and the His → Ala derivatives, nitrite accumulation during aerobic growth of *P. pantotrophus* on the highly reduced carbon substrate caproate was assessed (Table 3). Expression of the periplasmic nitrate reductase is maximal during aerobic growth on reduced carbon substrates, and nitrite accumulation can be used as a measure of nitrate reduction via the periplasmic nitrate reductase system [15]. In comparison with wild-type cells, nitrite accumulation by the *napC::Ω* strain NC1 of *P. pantotrophus* is severely compromised (Table 3). The low levels of nitrite found in this strain are likely to reflect the activity of an alternative electron transport route by which electrons can proceed from quinols to the periplasmic nitrate reductase. No nitrite was detected in the growth medium of *P. pantotrophus* HJS1 ($\Delta napA$) or HJS2

Figure 3 Absorption spectra for *P. pantotrophus* NapC cytochromes

(A) Visible absorption spectra of oxidized wild-type (Wt) *P. pantotrophus* NapC and four histidine mutants. Spectra were recorded at 25 °C, in 20 mM Hepes/NaOH, pH 7.5; in all cases, the protein concentration was approximately 12 µg/ml. (B) Difference of absorption between the oxidized spectra of the NapC^{sol} mutants and the wild-type protein at equal protein concentration.

($\Delta napEDABC$) (Table 3), which excludes the possibility that this low rate arises from the membrane-bound nitrate reductase that is normally expressed under anaerobic growth conditions in *Paracoccus* species. Nap activity, and therefore nitrite

accumulation, was substantially recovered by reintroducing the membrane-anchored NapC present on pLAnapEDABC (Table 3). A partial complementation of nitrite accumulation was also observed when pLACsol-1, expressing NapC^{sol}, was introduced into the *napC::Ω* mutant *P. pantotrophus* NC1 (Table 3). The results are not directly comparable, because the native *nap* promoter drives transcription in wild-type and NC1 (pLAnapEDABC) strains of *P. pantotrophus*, whereas the *cycA* promoter drives transcription in the NC1 strains containing the pLACsol vectors. Nevertheless, the reproducibly obtained approx. 45% recovery of nitrate reduction represents a significant complementation activity and suggests that even in the absence of the membrane anchor the globular domain of NapC can interact with the membrane and catalyses quinol dehydrogenation. This complementation activity was not apparent in either *P. pantotrophus* NC1 (pLACsol-3; NapC^{H99A}) or *P. pantotrophus* NC1 (pLACsol-5; NapC^{H194A}; Table 3). Thus, despite retaining quinol dehydrogenase activity with the water-soluble duroquinol, the His⁹⁹ → Ala and His¹⁹⁴ → Ala mutants cannot catalyse oxidation of membrane-entrapped quinols. Direct assay of NapAB, using viologen as an electron donor, showed that the amount of enzyme present was constant, except where it was expected to be absent in the HJS1 and HJS2 strains (Table 3).

Proteolytic cleavage of NapC^{sol}

When handling NapC^{sol} and the engineered derivatives purified from *E. coli*, it was apparent that the proteins were susceptible to degradation. NapC^{H81A} and NapC^{H174A} were the most unstable, which underlies the failure to detect these proteins at all when expressed in *P. denitrificans* or *P. pantotrophus*. It was notable, however, that the most common degradation pattern was for the 24 kDa haem-staining band on SDS/PAGE gels to break down into a 12 kDa haem-staining band (Figure 4). Note that in Figure 4(B), prestained marker proteins had to be used in order to calibrate the haem stain. In the 15% gel system used, these markers appear to give an inaccurate estimate for the lower-molecular-mass material. Consequently, a sample of horse heart cytochrome *c* was also run. It is clear that this protein electrophoreses very similarly to the NapC^{sol} fragment in both the gels that were haem stained (Figure 4B) or protein stained (Figure 4A). This additional marker therefore confirms the molecular mass of the fragment of NapC^{sol} as approx. 12 kDa. It is evident that the intact NapC^{sol} shows a different mobility on the haem-

Table 3 The effect for *P. pantotrophus* of variants of NapC on a (i) a quinol-dependent nitrate reductase activity in periplasmic extracts and (ii) *in vivo* production of nitrite by the Nap enzyme

ND, not determined; n.d., not detected; †, when nitrate reductase was present it had similar activities, measured in a viologen-dependent but NapC-independent assay, in all cases. *A relative value of 1 corresponds to accumulation of 2 µM nitrite.

Strain	Details of NapC encoded	24 kDa haem-staining band in periplasm	Relative DQH ₂ -dependent nitrite accumulation in periplasmic fractions*	Nitrite accumulation during aerobic growth on caproate (µM)	Periplasmic nitrate reductase activity in caproate-grown cells†
Wild-type	Membrane-anchored	No	1	80	+
NC1	None	No	1	18	+
NC1 (pLAnapEDABC)	Membrane-anchored	No	1	70	+
HJS1	Membrane-anchored	No	ND	n.d.	—
HJS2	None	No	ND	n.d.	—
NC1 (pLACsol-1)	Periplasmic	Yes	6.1	35	+
NC1 (pLACsol-2)	Periplasmic His ⁸¹ → Ala	No	1	18	+
NC1 (pLACsol-3)	Periplasmic His ⁹⁹ → Ala	Yes	4.6	18	+
NC1 (pLACsol-4)	Periplasmic His ¹⁷⁴ → Ala	No	1	18	+
NC1 (pLACsol-5)	Periplasmic His ¹⁹⁴ → Ala	Yes	6.4	18	+

Figure 4 SDS/PAGE analysis (15% gels) of cleavage of NapC^{sol} into two fragments

(A) Gel stained for proteins; lane 1, prestained marker proteins with nominal molecular masses 32.5 kDa, 25 kDa and 16.5 kDa; lane 2, aged sample of NapC^{sol}; lane 3, marker proteins with molecular masses of 26.6 kDa, 20 kDa and 14.3 kDa; lane 4, horse heart mitochondrial cytochrome *c*. (B) Gel stained for haem; lane 1, prestained marker proteins as in lane 1 of (A); lane 2, aged sample of NapC^{sol}; lane 3, horse heart mitochondrial cytochrome *c*. In all cases approx. 10 µg of protein was loaded. As explained in the text, NapC^{sol} material that ran with a mobility corresponding to a molecular mass of 12 kDa was subjected to N-terminal sequencing. Note that a fraction of the horse heart cytochrome *c* ran as an apparent dimer in this gel system. This is always seen in this gel system (M. L. Cartron and S. J. Ferguson, unpublished work), and a similar phenomenon has been remarked upon by others [40].

and protein-stained gels. This can be explained by the protein not being fully unfolded under the conditions, omission of boiling and 2-mercaptoethanol, that are required to retain the iron needed for the haem-staining procedure. Other multi-haem *c*-type cytochromes have been observed to run anomalously on SDS/PAGE [21]. When the 12 kDa NapC^{sol} band was analysed for the N-terminal sequence, two sequences were recovered, QDGDAAKGEKEF and LELAKHEWARLKA. These correspond respectively to the N-terminal of NapC^{sol}, which has 12 amino acids from cytochrome *c*₅₅₀ at the N-terminus (note that this is the N-terminus of *P. denitrificans* cytochrome *c*₅₅₀, as described in [7]), and a sequence that commences in between the two di-haem domains, exactly as predicted by Roldán et al. [7]. The ageing of NapC^{sol} that resulted in the cleavage of the two domains was accompanied by an increase in absorbance at 650 nm, indicating the concomitant formation of high-spin haem iron. Separate characterization of the N- and C-terminal fragments has not been possible, as conditions for purifying the two identically sized molecules have not been identified.

DISCUSSION

This work has presented the first mutagenesis programme of a member of the NapC family of membrane-anchored multi-haem *c*-type cytochromes. The mutagenesis studies have taken advantage of previous work in which we developed a protocol for expressing a soluble periplasmic form of NapC with a cleavable signal peptide substituting for the membrane anchor [7]. Mediated potentiometric titrations and MCD spectroscopy were used to demonstrate that the NapC^{sol} covalently bound four haems, each with *bis*-histidine axial ligation [7]. Our previous primary structure analysis of the NapC family led us to propose that the globular region comprised two structurally similar di-haem binding domains. Approx. 50 amino acids of the N-terminal and C-terminal segments, each containing two Cys-Xaa-Xaa-Cys-His motifs, can be aligned as motif I with motif III and motif II with motif IV. The two Cys-Xaa-Xaa-Cys-His

motifs in each segment are spaced approx. 28 amino acids apart. Two conserved histidine residues (His⁸¹ and His¹⁷⁴; *P. pantotrophus* numbering) lie between the two motifs in each segment. These are candidates for the distal ligand of two of the *bis*-histidine co-ordinated haem irons predicted from EPR/MCD. A second pair of conserved histidine residues (His⁹⁹ and His¹⁹⁴) lie three or five amino acids respectively after the second Cys-Xaa-Xaa-Cys-His motif in each segment and are candidates for the ligand of the second *bis*-histidine-ligated haem in each domain. The present study has supported this latter assignment, since alanine substitutions, NapC^{H99A} and NapC^{H194A}, both assemble with a high-spin haem present that presumably arises as a consequence of the loss of a distal haem ligand. Attempts to analyse NapC^{sol} for haem content by a variety of mass-spectrometric procedures were unsuccessful. The failure of NapC^{H81A} and NapC^{H174A} to assemble in *P. denitrificans*, and their lower stability after expression in *E. coli*, suggests that these two histidine residues play important structural roles in addition to providing haem iron ligands. The separation of His⁹⁹ from the Cys-Xaa-Xaa-Cys-His motif II by only two intervening residues means that this histidine cannot be an axial ligand to the haem bound by this particular motif. In hydroxylamine oxidoreductase (HAO), one histidine is similarly separated from a Cys-Xaa-Xaa-Cys-His motif by two residues. This histidine is a ligand to a nearby, but not the closest, other haem [22].

In addition to the observed proteolytic cleavage of NapC in approximately the middle of the molecule, further support for a two domain model, in which each domain binds a di-haem pair, can be obtained from analysis of the primary structure of NapC in the context of the recent X-ray crystal structures of the octa-haem HAO [22], the penta-haem formate-dependent nitrite reductase (Nrf) [23], the tetra-haem flavocytochrome *c* fumarate reductases [Ifc₃ (iron-induced flavocytochrome *c*₃) and Fcc₃ (soluble flavocytochrome *c*₃ fumarate reductase)] [24] and the di-haem Split-Soret cytochrome *c* (SSc) [25]. All of these cytochromes bind at least one di-haem pair, in which the closest haem-haem distance is around 4 Å and the Fe-Fe distance is around 9 Å. The HAO binds two such haem pairs and the SSc is a homo-dimer in which each monomer binds a di-haem pair. The segment of the polypeptide of each protein that binds the di-haem pair through two adjacent Cys-Xaa-Xaa-Cys-His motifs is variable in length, with the spacing between the two binding motifs being 10–90 amino acids. Thus there is little similarity between these di-haem polypeptide segments for the different cytochromes. Despite this poor sequence conservation, comparison of the di-haem binding segments of the flavocytochrome *c* fumarate reductases with the C- and N-terminal segments of NapC suggests that each segment may be of the type that bind a di-haem pair. In Ifc₃/Fcc₃, the conserved histidine residue that lies between the two Cys-Xaa-Xaa-Cys-His motifs serves as a distal haem ligand to the second haem of the haem pair [24]. We suggest that this histidine residue does likewise in NapC. The similar effect of the substitution in each segment also supports the view that His⁸¹ and His¹⁷⁴ are functionally equivalent in each domain. The histidine that lies after the second Cys-Xaa-Xaa-Cys-His motif of the di-haem polypeptide segment of Ifc₃/Fcc₃ provides an iron ligand to a haem that is not part of the di-haem pair. We suggest that this histidine does likewise in NapC, except that, in this case, the co-ordinated haem is one of the di-haem pairs of the adjacent domain. The domain-domain interactions must be strong enough to tolerate the loss of these haem ligands. As with His⁸¹/His¹⁷⁴, the similar effects of the His⁹⁹ and His¹⁹⁴ mutations point towards equivalent roles in each domain of the protein. If the tetra-haem NapC^{sol} does comprise two cross-linked haem domains, it would resemble the tetra-haem Sec

dimer. In this latter case the two di-haem monomers are 'cross-linked' by a C-terminal histidine from one monomer providing a distal haem-Fe ligand to one of the haems of the second monomer.

Previous studies on the DorC (a penta-haem *c*-type cytochrome electron donor protein to dimethyl sulphoxide reductase) and NrfH members of the NapC family have demonstrated quinol oxidation activity [2,3]. The complementation analysis presented here suggests that the site of quinol oxidation by NapC lies in the water-soluble globular tetra-haem segment of the protein. The complementation of a NapC^{mem} mutant by NapC^{sol} suggests that the periplasmic form of the protein can still interact with the membrane in the absence of the membrane anchor. In this respect, it is notable that NapC^{sol} binds very tightly to a phenyl-Sepharose column, requiring 70% ethylene glycol to release it. This suggests that it may have a very hydrophobic region on the surface that allows it to embed into the membrane. The failure of NapC^{H99A} and NapC^{H194A} to complement intact cells for electron transport to NapAB, despite retaining quinol oxidation activity in *in vitro* assays, suggests a perturbation in the surface properties of the protein that serves to decrease its avidity for the cytoplasmic membrane. Studies into the nature of the quinol binding site and the mechanism by which NapC interacts with quinols in the membrane will be the subject of future studies, aided by the protein expression methodologies outlined in this paper.

Inspection of 13 currently available NapC sequences (Figure 1) reveals some notable features. First, the sequences of the second, third and fourth haem binding motifs, Cys-Xaa-Xaa-Cys-His, are completely conserved. This is very unusual among families of *c*-type cytochromes and implies that some particular structural features or tuning of redox potential are required here. Although there is no conserved histidine that might form part of a 'triad' sequence for quinol binding [26], there are a number of other conserved residues of interest in particular, the EW (Glu-Trp) sequence that lies in the region between haems II and III. A motif including EW has been discussed as a key structural element in quinol oxidase sites in cytochrome *bf* and *bc*₁ complexes [26]; this provisionally implicates that this inter-domain region of NapC is important for interactions with quinols.

During revision of the present paper, another report of mutagenesis of a NapC-like protein appeared by Simon et al. [27], and, surprisingly, showed that one cysteine in the second haem binding motif is dispensable so that the haem was attached to the protein by only one thioether bound.

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