Overproduction, purification and novel redox properties of the dihaem cytochrome *c*, NapB, from *Haemophilus influenzae*

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The *napB* gene of the pathogenic bacterium *Haemophilus influenzae* encodes a dihaem cytochrome *c*, the small subunit of a heterodimeric periplasmic nitrate reductase similar to those found in other bacteria. In order to obtain sufficient protein for biophysical studies, we aimed to overproduce the recombinant dihaem protein in *Escherichia coli*. Initial expression experiments indicated that the NapB signal peptide was not cleaved by the leader peptidase of the host organism. Apocytochrome was formed under aerobic, semi-aerobic and anaerobic growth conditions in either Luria–Bertani or minimal salts medium. The highest amounts of apo-NapB were produced in the latter medium, and the bulk was inserted into the cytoplasmic membrane. The two haem groups were covalently attached to the pre-apocytochrome only under anaerobic growth conditions, and with 2.5 mM nitrite or at least 10 mM nitrate supplemented to the minimal salts growth medium. In order to obtain holocytochrome, the gene sequence encoding mature NapB was cloned in-frame with the *E. coli ompA* (outer membrane protein A) signal sequence. Under anaerobic conditions, NapB was secreted into the periplasmic space, with the OmpA signal peptide being correctly processed and with both haem *c* groups attached covalently. Unless expressed in the DegP-protease-deficient strain HM125, some of the recombinant NapB polypeptides were N-terminally truncated as a result of proteolytic activity. Under aerobic growth conditions, co-expression with the *E. coli ccm* (cytochrome *c* maturation) genes resulted in a higher yield of holocytochrome *c*. The pure recombinant NapB protein showed absorption maxima at 419, 522 and 550 nm in the reduced form. The midpoint reduction potentials of the two haem groups were determined to be −25 mV and −175 mV. These results support our hypothesis that the Nap system fulfils a nitrate-scavenging role in *H. influenzae*.

Key words: cytochrome *c* maturation, heterologous production, periplasmic nitrate reductase.

INTRODUCTION

In bacteria, three different types of nitrate-reducing system have been described (reviewed in [1]): cytoplasmic assimilatory nitrate reductases (Nas), cytoplasmic membrane-bound respiratory nitrate reductases (Nar) and periplasmic nitrate reductases (Nap). The last group contains heterodimeric enzymes consisting of the subunits NapA and NapB. NapA is the large (90 kDa) catalytic subunit that contains a molybdopterin guanine dinucleotide ('MGD') cofactor and one [4Fe–4S] centre. The small (13–19 kDa) subunit, NapB, is a dihaem cytochrome *c* that is involved in electron transfer to the NapA molybdoprotein. Both subunits are synthesized as precursors with N-terminal signal sequences, and are thus translocated across the cytoplasmic membrane into the periplasmic space. At least two other proteins, NapC and NapD, are part of the currently known Nap systems. NapC is a membrane-anchored tetrahaem cytochrome *c* that is a member of the NapC/NirT family of periplasmic multihaem *c*-type cytochromes, which appear to provide the major cytochrome bc_1-independent route for electron transfer between the quinol pool in the cytoplasmic membrane and water-soluble periplasmic oxidoreductases. The NapC/NirT homologue NrfH of *Wolinella succinogenes*, for instance, has been identified as the mediator between the quinol pool and cytochrome *c* nitrite reductase [2]. Also, disruption of the *napC* gene in *Rhodobacter sphaeroides* has been shown to result in the loss of physiological electron transport to the NapA–NapB complex [3]. The cytoplasmic NapD is essential for the maturation of NapA [4]. In the *nap* operons that have been characterized so far, five other *nap* genes can be found in different combinations with *napDABC*: *napF*, *napG* and *napH* encode iron–sulphur redox proteins, *napE* encodes an integral membrane protein with unknown function, and *napK* has only been found in *Rb*. *sphaeroides* [3,5].

Nap systems have been identified at the protein and/or the genetic level in an ever-growing number of bacterial species, including enterobacteria, aerobic denitrifiers and non-sulphur purple photosynthetic bacteria. The fact that these Nap systems are found throughout a phylogenetically diverse range of bacteria in which they are expressed under different conditions is an indication of their importance and physiological versatility. In particular, periplasmic nitrate reduction has been proposed to play a role in aerobic denitrification (*Paracoccus pantotrophus*, *Rb*. *sphaeroides* f. sp. *denitrificans*; [6–8]), in adaptation to anaerobic growth (*Ralstonia eutropha*; [9]), in redox balancing using nitrate as an electron sink to dissipate excess reductant (*Rb*.

Abbreviations used: *ccm*, cytochrome *c* maturation; ESMS, electrospray mass spectrometry; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani broth; Nap, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase; Nas, assimilatory nitrate reductase; OmpA, outer membrane protein A.

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capsulatus, *Rb. sphaeroides*, *Thiosphaera pantotropha*; [10–12]), or in scavenging nitrate in nitrate-limited environments (*E. coli*; [13]).

The *nap* genes have also been identified in *Pseudomonas putida* [14], *Ps. aeruginosa* [15], *Ps. stutzeri* [15], *Shewanella putrefaciens*, *H. influenzae* [16] and, more recently, in a number of other pathogenic bacteria, such as *Salmonella typhimurium*, *Vibrio cholerae*, *Yersinia pestis* and *Campylobacter jejuni*. Because more data will become available from genome-sequencing projects, we can expect that the number of bacterial species containing *nap* genes will continue to increase.

With the exception of a crystal structure of a NapA homologue that has been found in *Desulfovibrio desulfuricans* [17,18], no structural information on Nap proteins is available. Furthermore, initial attempts to overexpress Nap polypeptides in our laboratories were unsuccessful, possibly accounting for why biochemical characterization of Nap systems has so far been limited to those purified from only three genera. In the present study, we report the overproduction in *E. coli*, and the purification and the initial characterization, of the dihaem cytochrome *c* NapB from *H. influenzae*, a pathogenic bacterium that has only been found in human hosts. Although there are few reports of successfully overproduced multihaem cytochromes *c* in *E. coli* [19–22], we were able to produce a significant amount of recombinant NapB. Particular attention was paid to the redox properties of the recombinant NapB in an attempt to relate the midpoint redox potential of the two haem groups to the different physiological roles proposed for the Nap systems of aerobic denitrifiers and enteric bacteria.

EXPERIMENTAL

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1, and were routinely grown in Luria–Bertani (LB) medium or on LB agar plates at 37 °C. *E. coli* strain DH10B was used for the maintenance and propagation of plasmids pT10sompArPDI and pLPPsompArPDI. *E. coli* JM109 was used as a host for pQE-60 and its derivative, as well as for the production of recombinant cytochrome *c*. The *E. coli* strains M15(pREP4), MC1061, BL21(DE3), HM125 and JCB7123 were all used as hosts for cytochrome *c* production. pUC18 and pT10sompArPDI were used as cloning vectors. The pEC86 plasmid, which allows co-expression of the *ccm* genes, usually resulting in a higher yield of recombinant protein [19–22], was used in the present study. Plasmid GHICB54 (A.T.C.C. number 603052) was obtained from the TIGR/A.T.C.C. Microbial Genome Special Collection, Rockville, MD, U.S.A., and was used as the source of the *H. influenzae napB* gene.

Biochemical reagents

T4 DNA polymerase and all restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA, U.S.A.). *Taq* and *Pwo* polymerases were from Amersham Pharmacia Biotech (Uppsala, Sweden) and Roche Molecular Biochemicals (Mannheim, Germany) respectively. Oligonucleotides were synthesized at Amersham Pharmacia Biotech. T4 DNA ligase was obtained from Promega (Madison, WI, U.S.A.). Agarose was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, U.S.A.). Molecular-mass markers and reagents for protein electrophoresis were from Gibco BRL Life Technologies (Merelbeke, Belgium) and BioRad (Veenendaal, The Netherlands) respectively. Coomassie Brilliant Blue R-250, anti-foam and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma–Aldrich N. V. (Bornem, Belgium). Ni^{2+}-nitrilotriacetate Superflow matrix, DNA extraction and DNA purification kits were obtained from Qiagen Ltd (Crawley, West Sussex, U.K.). Vivaspin protein concentrators were from Vivascience Ltd (Lincoln, U.K.).

Table 1 Bacterial strains and plasmids used in this work

'+' indicates the presence of a hexahistidine tag coding sequence; '−' indicates its absence.

(a)

E. coli strain	Relevant characteristics	Reference or source
BL21(DE3)	Expression host, lack of Lon/OmpT proteases	Novagen
DH10B	Host for pT10sompArPDI and pLPPsompArPDI	[23]
HM125	Tc^r, Km^r, DegP protease-deficient strain	[24]
JCB7123	*narL*::Tn10 derivative of the cytochrome *c* overproducing strain JCB7120	[25]
JM109	Host for QE-60 and its derivative pQENapB$^+$	[26]
M15(pREP4)	Km^r, expression host for pQE-60 derivatives, constitutively expresses the *lac* repressor	Qiagen
MC1061	Expression host for the pLPPsompANapB plasmids	[27]

(b)

Plasmid	Relevant characteristics	Reference or source
GHICB54	Amp^r, pUC18 derivative carrying a 2.2 kb genomic fragment from *H. influenzae*	[16]
pUC18	Amp^r, cloning vector	[26]
pQE-60	Amp^r, *E. coli* expression vector, *T5* promoter, low-copy number	Qiagen
pEC86	Cm^r, pACYC184 derivative, constitutively expresses the cytochrome *c* maturation genes *ccmABCDEFGH* under control of the *tet* promoter	[19]
pT10sompArPDI	Amp^r, used as cloning vector	[28]
pLPPsompArPDI	Amp^r, used as expression vector, transcriptional control of both the lipoprotein and the *lac* promoter	[28]
pUCNapB	Amp^r, pUC18/640 bp *Rsa*I–*Spe*I DNA fragment containing the *napB* gene	This work
pQENapB$^+$	Amp^r, pQE-60 derivative containing the *napB* with own signal-peptide-coding sequence	This work
pT10sompANapB$^+$	Amp^r, pT10sompArPDI derivative, fusion *ompA–napB*	This work
pT10sompANapB$^-$	Amp^r, same cloning strategy as pT10sompANapB$^+$, without the His-tag coding sequence	This work
pLPPsompANapB$^+$	Amp^r, fusion *ompA–napB* under control of the lipoprotein and *lac* promoter	This work
pLPPsompANapB$^-$	Amp^r, same cloning strategy as pLPPsompANapB$^+$, without the His-tag coding sequence	This work

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Plasmid constructions for the overexpression of the *napB* gene

Plasmid DNA isolations, transformations and other routine DNA manipulations were performed using standard procedures [29]. A 640 bp *Rsa*I–*Spe*I restriction fragment containing the *H. influenzae napB* gene was cut from plasmid GHICB54. The fragment was then inserted into the compatible *Hinc*II and *Xba*I cloning sites of pUC18, generating a recombinant plasmid, pUCNapB, carrying the complete *napB* gene. In order to construct a series of plasmids for the heterologous expression of *napB*, three sets of oligonucleotides were synthesized to amplify *napB* by PCR using pUCNapB as template DNA. First, *napB* was cloned as an *Nco*I–*Bgl*II fragment with its own signal sequence into the pQE-60 expression vector. The forward primer, QE2NCO (5′-TCAACCATGGCTAAACAGGTATCTAAAA-T-3′), contains the start codon. The codon ACT, encoding the second amino acid of the pre-apocytochrome NapB, was mutated into GCT to generate an *Nco*I site (underlined in the previous sequence). The reverse primer, QE2BGL (5′-GCTAGATCTGT-TTCCGTAACCTTTCAT-3′), was synthesized in order to mutate the stop codon, and thus to generate a *Bgl*II restriction site (underlined). Cloning of the amplified fragment in the *Nco*I–*Bgl*II sites of pQE-60 generated a hybrid gene consisting of the complete *napB* gene fused to the sequence coding for a C-terminal His_6-tag. Secondly, the gene sequence encoding the mature form of NapB was fused to the sequence encoding the signal peptide of the *E. coli* outer membrane protein A (OmpA), allowing export to the periplasmic space. The primers NAPNAE (5′-TCTGATGCACCAGCAGTGGGA-3′) and QECPCHIS (5′-TTAAGCTTA*GTGATGGTGATGGTGATG*GTTCCGTA-ACCTTTCATCGG-3′) were used to achieve the fusion with the OmpA signal peptide, and to add a hexahistidine tail to the C-terminal end of NapB by using PCR-based mutagenesis. NAPNAE starts with the first nucleotide of the mature protein. QECPCHIS contains a stop codon, a *Hin*dIII restriction site (underlined in the previous sequence), and allows the addition of the hexahistidine tag. The DNA sequence for the histidine codons (italicized in the previous sequence) consists of an alternating series of two codons used in *E. coli* for histidine. Primer QECPC (5′-TTAAGCTTAGTTTCCGTAACCTTTC-ATCGG-3′), containing a *Hin*dIII site and a stop codon, was used in a second PCR with NAPNAE to make the fusion of sompA–mature NapB without a histidine tag (where sompA corresponds to the OmpA signal sequence). Both amplified fragments were cloned into pT10sompArPDI, which had previously been digested with *Nae*I and *Hin*dIII. The two resulting plasmids were named pT10sompANapB$^+$ and pT10sompA-NapB$^-$, with and without the His_6-tag-coding sequence respectively. The two hybrid genes were transferred to the pLPP-sompArPDI vector as *Xba*I–*Hin*dIII fragments, bringing the expression under control of both the lipoprotein and the *lacUV5* promoter. The generated plasmids were called pLPPsompA-NapB$^+$ and pLPPsompANapB$^-$.

The DNA sequence of all hybrid genes was determined using Dye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA, U.S.A.).

Growth conditions in the overexpression experiments

Growth media were supplemented with the following antibiotics: carbenicillin, 100 μg/ml; kanamycin, 25 μg/ml; tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml. The recombinant plasmids were transformed into different *E. coli* strains. pQENapB$^+$ was introduced into strain M15(pREP4) according to the manufacturer's instructions (QIAexpressionist, Qiagen), and a 2 ml culture was used to inoculate 10 ml of LB medium and 10 ml of minimal salts medium [30] supplemented with 0.4 % (v/v) glycerol, 20 mM sodium fumarate, 5 % (v/v) LB medium, 10 μM selenate and 10 μM molybdate [31]. Either 2.5 mM sodium nitrite or increasing concentrations of potassium nitrate (1–50 mM) were also added. The cultures were grown aerobically at 300 rev./min, anaerobically in completely filled tubes, or 'semi-aerobically' in half-filled tubes shaken at 220 rev./min. When the D_{600} attained the value of 0.6, 1 mM isopropyl β-D-thiogalactoside (IPTG) was added, and the cultures were grown for a further 6 h. Both pLPPsompANapB$^+$ and pLPPsompANapB$^-$ were individually transformed into the *E. coli* strains JM109, HM125, MC1061, JCB7123 and BL21(DE3), which were subsequently grown anaerobically in the supplemented minimal salts medium [31]. Both expression plasmids were also co-transformed with pEC86 into the different strains, which were then grown aerobically in LB medium.

Analytical methods

Samples were subjected to SDS/PAGE using 15 % or 18 % (w/v) resolving gels, and 6 % (w/v) stacking gels in a BioRad Mini-PROTEAN II electrophoresis cell (BioRad). For whole-cell samples, the bacterial pellet was resuspended in SDS-loading buffer containing 12 % (v/v) 2-mercaptoethanol, and was then incubated for 5 min at 95 °C before loading. Protein bands were stained with either Coomassie Brilliant Blue or silver. Holocytochrome *c* was detected by staining the SDS gels for assessment of haem-linked peroxidase activity, as described in [32].

Reduced, oxidized and 'reduced-minus-oxidized' spectra were recorded at room temperature using a double-beam Uvikon 943 spectrophotometer (Kontron Instruments, München, Germany). Dithionite was used as a reductant.

For N-terminal sequencing, recombinant proteins were transferred on to PVDF membranes after SDS/PAGE. The blotting buffer consisted of 25 mM Tris, 192 mM glycine and 10 % (v/v) methanol. Analysis was performed on a 476A pulsed liquid sequenator (Applied Biosystems, Foster City, CA, U.S.A.).

Molecular masses of recombinant proteins were determined by electrospray mass spectrometry (ESMS) on a BIO-Q triple quadruple instrument (Micromass, Altrincham, Cheshire, U.K.).

Direct electrochemistry was performed in the three-electrode microcell with an inverted working electrode, as described previously [33]. The working electrode was activated glassy carbon (Le Carbon Loraine, Paris, France), the counter electrode was platinum wire (P-1312; Radiometer, Copenhagen, Denmark) and the reference electrode was Ag/AgCl (R-201; Radiometer). The glassy carbon disc was oxidatively activated by immersion in concentrated nitric acid at 60 °C to obtain a highly activated hydrophobic surface. The electrochemical cell was controlled with a digital potentiostat (Autolab-PGSTAT 10) running under GPES 4.8 (Eco Chemie, Utrecht, The Netherlands). Potentials have been recalculated here with reference to the normal hydrogen electrode, NHE, taking $E = -197$ mV as the potential of the Ag/AgCl electrode in saturated KCl at 22 °C. No promoter was used.

Cell fractionation

In order to check the subcellular localization of the produced apocytochrome, 40 ml of a full-grown pQENapB$^+$ culture was inoculated in 4 litres of minimal salts medium [31], supplemented with either 2.5 mM nitrite or 50 mM nitrate. Both cultures were grown anaerobically in completely filled 1-litre flasks, induced at

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$D_{600} \approx 0.6$ with 1 mM IPTG, and then grown for a further 6 h. The cell pellet was obtained after centrifugation of the cultures for 5 min at 1600 ***g***, resuspended in 4 ml of sucrose buffer [0.5 M sucrose/5 mM EDTA/50 mM Tris/HCl (pH 7)] containing 200 μg/ml lysozyme, and then incubated for 30 min at 30 °C. After centrifugation for 5 min at 19000 ***g***, the spheroplasts were resuspended in 10 mM Tris/HCl, pH 7, and disrupted by sonication. The crude extracts were centrifuged at 19000 ***g*** for 5 min to remove cell debris and unbroken cells, and subsequently at 100000 ***g*** for 1 h to pellet the membranes. The periplasmic, cytosolic and membrane fractions were analysed on an SDS/15 % polyacrylamide gel.

Production of NapB

A 2-ml 50 % (v/v) glycerol slant of HM125/pEC86/pLPPsompANapB^{-} was used to inoculate 100 ml of LB medium, and was grown at 37 °C with vigorous shaking until the D_{600} reached the value 1. The inoculum was used as a starter culture for growth in a 7-litre Bioreactor from Applikon Dependable Instruments (Schiedam, The Netherlands) filled with 6 litres of LB medium, the appropriate antibiotics and 10 μl of anti-foam. The culture was aerated and stirred at 300 rev./min. The pH was monitored, but not adjusted.

Purification of recombinant NapB

When the 6-litre batch cultures reached a D_{600} of 2.0, the cells were harvested by centrifugation at 1600 ***g*** for 5 min. The periplasmic protein fraction was prepared by the cold osmotic-shock procedure [28], except that 5 mM instead of 10 mM EDTA was used. The periplasmic protein fraction was then gradually saturated with ammonium sulphate. The red-coloured NapB protein precipitated at 35–70 % saturation, and was re-suspended in a small volume of PBS buffer. This fraction was brought to a saturation of 20 % (w/v) ammonium sulphate, and loaded on to a 20 ml butyl-Sepharose FF column connected to a Äkta chromatographic system (Amersham Pharmacia Biotech). NapB was recovered by gradient elution from 20 % to 0 % ammonium sulphate in 50 mM sodium phosphate buffer, pH 7. After concentration, the sample was dialysed against 10 mM Mes, pH 5.5, and loaded on to a 4 ml Source S30 cation exchanger (Amersham Pharmacia Biotech) equilibrated with 50 mM Mes, pH 5.5. NapB was clearly visible as a red band at the top of the column, and was eluted at approx. 0.6 M NaCl. After gel filtration on an HL-Superdex75 column (1.5 cm × 60 cm; Amersham Pharmacia Biotech) in 50 mM NaCl and 50 mM Tris/HCl, pH 7, three contaminating proteins were still present. At neutral pH, two of these proteins were retained on a 1 ml Q Sepharose and a 1 ml CM Sepharose column (Amersham Pharmacia Biotech) respectively, while NapB was found in the flow-through fraction. The third protein was only partially retained and co-eluted with NapB.

RESULTS

Initial overexpression experiments

The gene encoding the NapB protein of *H. influenzae* was initially cloned with its own signal-peptide-coding sequence into the low-copy-number plasmid pQE-60. In the resulting plasmid pQENapB^{+}, the expression of NapB is based on the *T5* promoter transcription–translation system, in which the production of recombinant protein is tightly regulated by the high levels of Lac repressor protein constitutively expressed on the pREP4 plasmid present in the *E. coli* M15(pREP4) host strain. The expression of recombinant NapB was studied under aerobic, 'semi-aerobic' or anaerobic conditions, in either LB medium or minimal salts medium supplemented with nitrite or increasing concentrations of nitrate [31]. Under all growth conditions, apocytochrome was visible as a distinct band on the Coomassie-Blue-stained SDS/polyacrylamide gels of whole-cell samples of the different cultures. Apocytochrome was produced in the largest amounts in minimal medium. A small fraction of the apocytochrome was found in the cytoplasm and the periplasm, whereas the bulk was found in the membrane fraction (results not shown).

Haem staining, indicative of the formation of holocytochrome *c*, was only present when the cultures were grown anaerobically in the presence of either 2.5 mM nitrite or at least 10 mM nitrate. Under anaerobic conditions, the Fnr-dependent promoter (where Fnr corresponds to the transcriptional regulator of *E. coli*) of the *aeg-46.5* operon [34] (renamed *nap–ccm* operon) is induced, resulting in the expression of the *E. coli* endogenous *ccm* genes, which are necessary for the maturation of holocytochromes *c*. In addition, this promoter is induced more strongly by nitrite than by nitrate. However, N-terminal amino acid sequence analysis of the holocytochrome indicated that the signal sequence was not cleaved off, signifying that the *E. coli* leader peptidase was not able to recognize the cleavage site of the *H. influenzae* pre-apocytochrome NapB protein. This also provides the explanation for why the protein migrated on the gels with a higher molecular mass than the calculated value of the mature His_6-tagged NapB protein (15571 Da). It is therefore likely that the uncleaved signal peptide serves as an N-terminal anchor that is inserted into the cytoplasmic membrane. This would explain why apocytochrome was also found in the membrane fraction. The fact that haem groups were covalently bound to pre-apocytochrome confirms that haem binding is not a prerequisite for the cleavage of the signal peptide, but that both these processes occur independently of each other [35].

Overexpression experiments with altered signal peptide

In order to obtain mature holo-NapB, we replaced the NapB signal sequence by the OmpA signal sequence of *E. coli*. According to the method of von Heijne [36], *H. influenzae* NapB was predicted to have a signal peptide of 23 amino acids. The gene fragment encoding mature NapB was cloned in-frame with the OmpA signal-peptide-coding sequence in the pLPPsompA expression vector. Two different clones were made: one with, and one without, a C-terminal sequence encoding a His_6 affinity tag. Both clones were transformed into different *E. coli* strains, either with or without the plasmid pEC86, which constitutively expresses the *E. coli ccm* genes. The strains without pEC86 were grown anaerobically in supplemented minimal salts medium; the ones with pEC86 were grown aerobically in LB medium. Expression levels of recombinant NapB were compared under these different conditions, and the following conclusions could be drawn.

In all cases, the *E. coli* signal peptidase properly removed the signal peptide from the precursor sompA–NapB molecule: the N-terminal amino acid sequence of recombinant NapB was determined, and revealed the expected sequence of Ser-Asp-Ala-Pro-Ala. Haem staining after SDS/PAGE yielded positive results, and MS of the partially purified untagged and His_6-tagged proteins indicated that both haem groups were attached covalently. In the periplasmic protein fraction that was prepared from the different cultures, a peak at 550 nm was observed in the UV–visible absorption spectrum upon reduction with dithionite.

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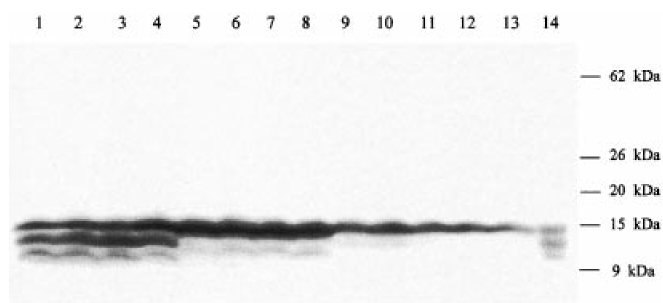

Figure 1 Haem-stained SDS/15% (w/v) polyacrylamide gel of samples taken from different aerobically and anaerobically grown NapB over-expression cultures

The lanes were loaded with different samples as follows: lanes 1–4, MC1061/pEC86/pLPPsompANap$^+$, without or with 1 mM IPTG for 2, 4 and 6 h respectively; lanes 5–8, HM125/pEC86/pLPPsompANapB$^-$, without or with 1 mM IPTG for 2, 4 and 6 h respectively; lanes 9–12, HM125/pLPPsompANapB$^+$, without or with 1 mM IPTG for 2, 4 and 6 h respectively; lanes 13 and 14, MC1061/pLPPsompANapB$^+$, D_{600} 0.7 and 2.0 respectively. All strains containing the pEC86 plasmid were grown aerobically in LB medium; the other strains were grown anaerobically in minimal salts medium supplemented with 0.4% (v/v) glycerol, 20 mM sodium fumarate, 5% (v/v) LB medium, 10 μM selenate, 10 μM molybdate and 2.5 mM sodium nitrite.

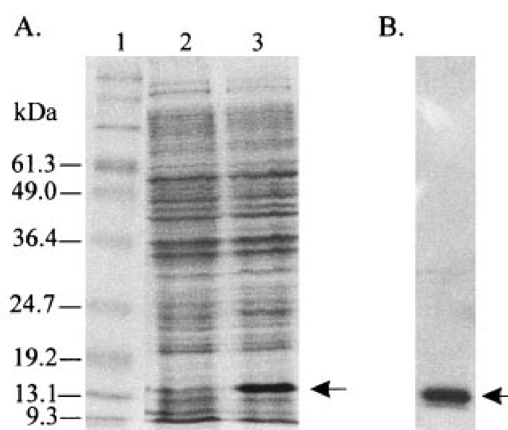

Figure 2 SDS/PAGE analysis of NapB before and after purification

(**A**) Coomassie-Brilliant-Blue-stained 15% gel of: lane 1, molecular-mass markers (masses in kDa are shown to the left of the gel); lane 2, total cell lysate of HM125/pEC86; lane 3, total cell lysate of HM125/pEC86/pLPPsompANapB$^-$. (**B**) Silver-stained 18% gel of purified untagged recombinant NapB.

From the moment that the cultures of the different transformed strains reached a D_{600} value of 0.7, two haem-staining polypeptides with molecular masses lower than holo-NapB appeared on the gels (Figure 1, lanes 13 and 14). To identify these polypeptides, a periplasmic protein extract of MC1061/pLPPsompANapB$^+$ was loaded on to a Ni^{2+}-nitrilotriacetic acid metal-affinity matrix. Because the two polypeptides were retained on the column, they were considered to be proteolytic degradation products of NapB. The degradation must have occurred at the N-terminus, since the C-terminal His_6-tag was apparently retained. Using ESMS, their molecular masses were determined to be 14296 Da and 11770 Da respectively. In strain HM125, the degradation was reduced to a minimum (Figure 1, lanes 5–12). HM125 is a strain deficient in the *degP* gene encoding a periplasmic protease. In this respect, it was remarkable that NapB was degraded in BL21(DE3) as well, considering that this strain lacks the Lon and OmpT proteases.

As expected, expression levels were approx. 5-fold higher when the cultures were grown aerobically in the presence of pEC86 expressing the *ccm* gene products (Figure 1, lanes 1–8 as compared with lanes 9–14).

As is also evident from Figure 1, the expression levels were independent of the addition of IPTG. The same observation has been made by other groups [28,37,38], using pLPPsompArPDI as an expression vector. De Sutter et al. [28] concluded that the plasmid-encoded LacI level was not sufficient to ensure a noticeable repression of the lipoprotein promoter P_{lpp} under non-inducing conditions.

Remarkably, the His_6-tagged protein was produced in lower concentrations than the untagged protein. The same results were obtained by David et al. [39]: the overall yield of the untagged form of cytochrome *c*-552 of *Bacillus subtilis* per volume of culture was also higher than the tagged form, although purification of the latter was easier. Despite the different expression levels, the reduced, oxidized and 'reduced-minus-oxidized' spectra of the tagged and untagged forms of NapB were identical, indicating that the tagged protein retained its redox activity. Therefore we can conclude that the C-terminal histidine tag did not cause any major changes in either the folding or the conformation of the protein.

It can be concluded that the recombinant production of mature NapB was highest in *E. coli* strain HM125/pEC86/pLPPsompANapB$^-$, aerobically grown in LB medium.

Production and purification

E. coli strain HM125/pEC86/pLPPsompANapB$^-$ expressing the untagged protein was grown in 6-litre batches using a bioreactor. Production of recombinant NapB was maximal when the culture was aerated and stirred at 300 rev./min (Figure 2A). Although the stationary phase was attained much more quickly, almost no recombinant NapB was detected at a higher speed. Under such conditions, the pEC86-encoded Ccm proteins are produced in insufficient amounts to ensure the maturation of the high numbers of apocytochrome polypeptides that are produced constitutively as a consequence of the 'leaky' promoter of the pLPP vector.

During the preparation of the periplasmic protein fraction, about 5% of the recombinant NapB was located in the sucrose fraction obtained after treatment of the bacterial cell pellet with sucrose buffer. The bulk of the protein was found in the periplasmic fraction. Both fractions were pooled and gradually saturated with ammonium sulphate in 5% increments. The red-coloured NapB precipitated in a wide range of 35–70% saturation, but only the fractions between 40% and 65% were pooled after resuspension in a small volume of PBS buffer. After hydrophobic-interaction chromatography, concentration and dialysis, NapB-containing fractions were purified further by cation-exchange chromatography at pH 5.5. Gel filtration was used to remove the high-molecular-mass-contaminating proteins. After this step, three contaminants were still present, of which two were removed completely after ion-exchange chromatography on a 1 ml Q Sepharose column followed by a 1 ml CM Sepharose column at neutral pH. Despite numerous attempts, we were unable to completely remove the third contaminant (Figure 2B).

Characterization

The UV–visible absorption spectrum of both the tagged and untagged forms of dithionite-reduced recombinant NapB revealed a spectral pattern typical of a *c*-type cytochrome, with

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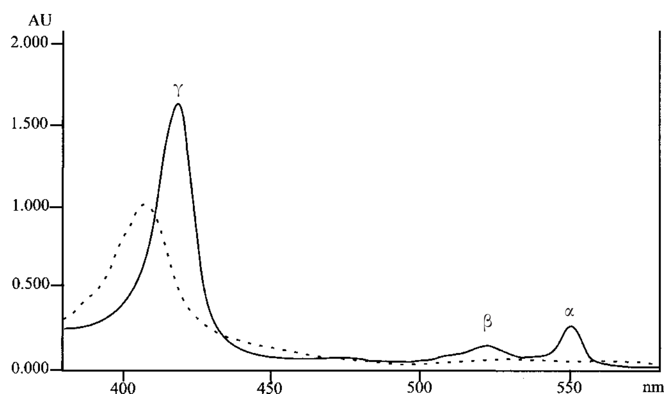

Figure 3 UV–visible absorption spectra of the untagged form of NapB

Spectra were recorded at room temperature using partially purified NapB in 10 mM Tris/HCl, pH 7. The air-oxidized spectrum is indicated by a dashed line, whereas the dithionite-reduced spectrum is shown as a continuous line. AU, absorbance units.

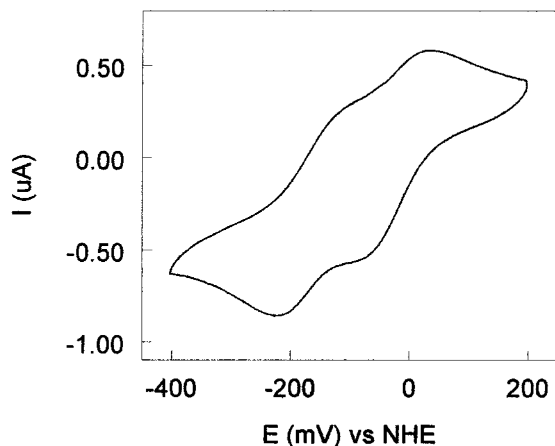

Figure 4 Cyclic voltammogram of the untagged form of NapB at 'super-activated' glassy carbon

The recombinant NapB was used at a concentration of 0.5 mg of protein ml^{-1} in 25 mM Hepes, pH 7.0, in the absence of any promoter. The glassy carbon was pre-activated by immersion in concentrated nitric acid at 60 °C for 30 min. The potential scan rate was $v = 2$ mV/s and the temperature was 22 °C. NHE, normal hydrogen electrode.

absorption maxima at 419 nm (γ, Soret peak), 522 nm (β) and 550 nm (α) (Figure 3).

The surface of monohaem mitochondrial *c*-type cytochromes is predominantly positively charged, contributing to a pI of the proteins in the range of 9–10. They typically exhibit rapidly developing, fast and reversible electron transfer on activated glassy carbon in the absence of promoters [33]. NapB is a dihaem cytochrome *c* with bis-histidine axial co-ordination for each haem. However, except for the two conserved Cys-X-Y-Cys-His haem-binding and iron co-ordination patterns (in which X and Y represent any amino acid), no part of the primary structure shows similarity to any other *c*-type cytochrome. Also, the calculated pI of the mature NapB, with a value of 8.4, is lower than that of the mitochondrial cytochromes.

On the activated glassy carbon electrode, NapB was found to give sluggish and poorly defined responses. Following 'super-activation' of the glassy carbon by heating the immersing oxidizer nitric acid to 60 °C for 15–30 min, the NapB response became reproducible and quasi-reversible. A fully reversible response, i.e. fast electron transfer between protein and electrode, is typically characterized by a cathodic-to-anodic peak potential difference $\Delta E = 57$ mV at ambient temperatures for potential scan rates of the order of $v \approx 10$ mV/s [40]. The cyclic voltammogram of NapB in Hepes buffer, pH 7.0, exhibits two well-separated redox waves (Figure 4). However, the peak separations are $\Delta E \approx 75$ mV for a slow scan rate of $v = 1$ mV/s, and they increase to $\Delta E \approx 82$ mV for $v = 2$ mV/s (Figure 4) and to $\Delta E \approx 86$ mV for $v = 5$ mV/s. This corresponds to a heterogeneous electron transfer rate constant at midpoint potential $k^0 \approx 6 \times 10^{-4}$ cm^2/s [40], assuming a diffusion coefficient $D \approx 1 \times 10^{-6}$ cm^2/s for NapB.

The midpoint reduction potentials of the untagged protein were determined to be -172 and -25 mV (Figure 4), with an uncertainty of ± 10 mV. These potentials are within the normal range for bis-histidine-ligated *c*-type cytochromes, unlike the relatively high potentials ($E_{m,7} = -15$ mV and $+80$ mV) reported for the NapB protein from *T. pantotropha* [41].

DISCUSSION

Although Nap enzymes have been identified in a wide range of bacterial species, the exact role of several of the Nap proteins in periplasmic nitrate reduction is still unclear. Moreover, the different Nap proteins are not well characterized, with the NapA homologue from *D. desulfuricans* being an exception. The crystal structure of this protein was solved by Dias et al. [18], and contributed to a better understanding of the molecular basis of nitrate reduction by the periplasmic reductase. The soluble domain of the NapC protein from *P. pantotrophus* has also been characterized, though only spectroscopically and electrochemically [42]. Mediated redox potentiometry and characterization by UV–visible, magnetic CD and EPR spectroscopies have demonstrated that NapC contains four low-spin haems, each with bis-histidine axial ligation and with midpoint reduction potentials of -56, -181, -207 and -235 mV.

NapA and NapC are the redox partners of the NapB protein, and they constitute an electron-transport chain that functions in the transfer of electrons from the quinone/quinol pool in the cytoplasmic membrane to the soluble NapA, and ultimately to a nitrate molecule. In the present paper, we demonstrate the heterologous production of the NapB protein from *H. influenzae* in *E. coli*. NapB cytochromes are involved in the direct electron transfer to NapA, and are essential for Nap activity, since it has been shown that NapB deletion mutants of *Rb. sphaeroides* DSM158 and *E. coli* have very low nitrate reductase activity [3,4]. The amino acid sequences of NapB cytochromes share no significant similarity with any other *c*-type cytochrome. Moreover, the two haem-binding sites are only 35 residues apart, and their positioning is unprecedented: although the mature proteins never consist of more than 140 amino acids, the first cysteine of the N-terminal haem-binding site is generally positioned between residues 56–64 of the mature protein [4,9,16,43,44]. These characteristics suggest that NapB proteins belong to a novel class of cytochrome *c*.

The two haem *c* groups of the Nap enzyme from *T. pantotropha* have midpoint reduction potentials of -15 mV and $+80$ mV [41]. These potentials are relatively high for bis-histidine-ligated *c*-type cytochromes, but are in agreement with the calculated and experimentally determined potentials of bis-histidine-ligated *b*-type cytochromes. The low-potential haem, presumably the immediate electron acceptor [45], has a midpoint potential ($E_{m,7.0} = -15$ mV) that is lower than that of the ubiquinol/ubiquinone couple ($E_{m,7.0} = +80$ mV). This suggests that the Nap enzyme will only be fully active when the quinone pool becomes very

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reduced. Two of the possible explanations of this observation are not mutually exclusive. First, the lower midpoint redox potential of both of the haems of the *Haemophilus* NapB, compared with the corresponding haems of the *Thiosphaera* NapB, strongly supports the previous proposal that these enzymes fulfil different physiological roles in the two organisms. We may ask whether *Haemophilus* NapB evolved to function at a redox potential lower than that of the *Thiosphaera* counterpart, for example in environments like the gastrointestinal tract of warm-blooded animals, in which oxygen and nitrate are scarce, but reduced carbon compounds are abundant. A second relevant observation is that naphthoquinones are more abundant than benzoquinones in anaerobic cultures of enteric bacteria, but the predominant quinones in *Thiosphaera* are benzoquinones. The midpoint redox potentials of naphthoquinones are lower than those of benzoquinones, and so it is possible that Nap in enteric bacteria preferentially accepts electrons from the menaquinone pool rather than from ubiquinone. Whatever the explanation, it is clear that, as we proposed previously [13], the redox properties of NapB make it suitable for fulfilling the nitrate-scavenging role, rather than serving in a redox-balancing role.

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