Periplasmic nitrate reduction in *Wolinella succinogenes*: cytoplasmic NapF facilitates NapA maturation and requires the menaquinol dehydrogenase NapH for membrane attachment

Melanie Kern^{1,2} and Jörg Simon^{1,2}

Correspondence

Jörg Simon

simon@bio.tu-darmstadt.de

¹Department of Microbiology and Genetics, Technische Universität Darmstadt, Schnittspahnstr. 10, 64287 Darmstadt, Germany

²Institute of Molecular Biosciences, Goethe University, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany

Various nitrate-reducing bacteria produce proteins of the periplasmic nitrate reductase (Nap) system to catalyse electron transport from the membraneous quinol pool to the periplasmic nitrate reductase NapA. The composition of the corresponding *nap* gene clusters varies but, in addition to *napA*, genes encoding at least one membrane-bound quinol dehydrogenase module (NapC and/or NapGH) are regularly present. Moreover, some *nap* loci predict accessory proteins such as the iron–sulfur protein NapF, whose function is poorly understood. Here, the role of NapF in nitrate respiration of the Epsilonproteobacterium *Wolinella succinogenes* was examined. Immunoblot analysis showed that NapF is located in the membrane fraction in nitrate-grown wild-type cells whereas it was found to be a soluble cytoplasmic protein in a *napH* deletion mutant. This finding indicates the formation of a membrane-bound NapGHF complex that is likely to catalyse NapH-dependent menaquinol oxidation and electron transport to the iron–sulfur adaptor proteins NapG and NapF, which are located on the periplasmic and cytoplasmic side of the membrane, respectively. The cysteine residues of a CX₃CP motif and of the C-terminal tetra-cysteine cluster of NapH were found to be required for interaction with NapF. A *napF* deletion mutant accumulated the catalytically inactive cytoplasmic NapA precursor, suggesting that electron flow or direct interaction between NapF and NapA facilitated NapA assembly and/or export. On the other hand, NapA maturation and activity was not impaired in the absence of NapH, demonstrating that soluble NapF is functional. Each of the four tetra-cysteine motifs of NapF was modified but only one motif was found to be essential for efficient NapA maturation. It is concluded that the NapGHF complex plays a multifunctional role in menaquinol oxidation, electron transfer to periplasmic NapA and maturation of the cytoplasmic NapA precursor.

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INTRODUCTION

Many different bacterial and archaeal species grow by anaerobic respiration in the absence of oxygen (Richardson, 2000). This type of energy metabolism usually requires a membrane-bound electron-transport chain that couples the catalysis of a redox reaction to the generation of a proton-motive force, and many variations of this common theme have been reported in the past (see Simon *et al.*, 2008 for a recent review). Especially interesting, though often poorly understood, are membrane-bound electron-transport modules that react with quinones or quinols. The most widespread of these modules belong to a

relatively small group of either *b*- or *c*-type cytochromes, iron–sulfur proteins or proteins that apparently lack any cofactors (Simon *et al.*, 2008; Simon & Kern, 2008). Prototypic examples are NapC, a tetrahaem *c*-type cytochrome, and the NapGH complex, which consists of two iron–sulfur proteins. Both modules are quinol dehydrogenases that are part of the periplasmic nitrate reduction (Nap) system, which catalyses nitrate reduction to nitrite using the iron–sulfur/molybdoprotein NapA as a terminal reductase (Potter *et al.*, 2001; Richardson *et al.*, 2001; González *et al.*, 2006). Some organisms contain either NapC or NapGH whereas others, such as *Escherichia coli*, use both of them. In *E. coli*, the NapGH complex has been postulated to function as a ubiquinol dehydrogenase that transfers electrons via the *c*-type cytochromes NapC and NapB to the periplasmic nitrate reductase NapA, whereas

Abbreviations: BV, benzyl viologen; Nap, periplasmic nitrate reductase; Nrf, cytochrome *c* nitrite reductase; Tat, twin-arginine translocation system.

menaquinol oxidation is most likely catalysed by NapC and occurs independently of NapGH (Brondijk *et al.*, 2002, 2004).

The composition of bacterial *nap* operons is diverse and many of them encode accessory proteins whose precise function is unclear (Marietou *et al.*, 2005; González *et al.*, 2006; Jepson *et al.*, 2006). The known *nap* operons of Epsilonproteobacteria do not include a *napC* gene but generally contain adjacent *napG* and *napH* genes directly downstream of *napA* (Kern & Simon, 2009). In line with this finding, nitrate respiration of the model bacterium *Wolinella succinogenes* (containing a *napAGHBFLD* gene cluster) has been shown to be independent of a NapC-type cytochrome *c* (Simon *et al.*, 2003). The absence of either NapG or NapH almost abolished growth by nitrate respiration of *W. succinogenes* cells without affecting the formation and nitrate-reducing activity of NapA, and NapG and NapH were shown to form a membrane-bound complex that very likely catalyses menaquinol oxidation and electron transfer to NapA (Kern *et al.*, 2007; Kern & Simon, 2008). NapG was detected in the periplasmic cell fraction of a *W. succinogenes* mutant that lacked the membrane-bound NapH, which is predicted to form four membrane-spanning domains. NapG and NapH contain four and two ferredoxin-type tetra-cysteine motifs, respectively. In addition, NapH carries two CX₃CP motifs of unknown function that, in principle, could serve in binding a redox-active metal centre or in catalysing thiol/disulfide chemistry. All cysteine-rich sequences of NapH are predicted to be located on the cytoplasmic side, implying that the NapGH complex harbours iron–sulfur cofactors on opposite sides of the membrane. Each of the four poly-cysteine motifs in *W. succinogenes* NapH was shown to be essential for nitrate respiration, as single cysteine to alanine modifications adversely affected either electron-transport activity from formate to nitrate or the assembly of the NapGH complex (Kern & Simon, 2008). NapG and -H proteins have not been purified from any organism and neither the number and location of quinol-binding site(s) nor the function of the predicted cytoplasmic iron–sulphur centres in NapH is known. It is assumed that the dihaem cytochrome *c* NapB forms a periplasmic complex with NapA that receives electrons from NapGH. NapB has been shown previously to be required for electron transport from the menaquinol pool to NapA in *W. succinogenes* (Kern *et al.*, 2007). In *E. coli*, NapD was shown to be a chaperone involved in directing cytoplasmic NapA to the twin-arginine translocation (Tat) system (Maillard *et al.*, 2007). This finding is supported by the fact that a *W. succinogenes* mutant deficient in NapD did not accumulate detectable amounts of NapA (Kern *et al.*, 2007). The function of periplasmic NapL is not known and inactivation of the *napL* gene in *W. succinogenes* only slightly affected cell growth in minimal nitrate medium despite the fact that NapA-dependent nitrate reductase activity was considerably reduced (Kern *et al.*, 2007; Kern & Simon, 2009).

Another auxiliary protein of many Nap systems is the iron–sulfur protein NapF, which, similar to NapG, contains four ferredoxin-type tetra-cysteine motifs. NapF proteins are encoded by *nap* operons from many Proteobacteria, including the majority of Epsilonproteobacteria (Kern & Simon, 2009). NapF from *E. coli* has been shown to be located in the membrane and the cytoplasmic fraction and to interact with NapA, suggesting a role in modification of NapA prior to its export into the periplasm by the Tat apparatus (Nilavongse *et al.*, 2006). In *Rhodobacter sphaeroides*, cytoplasmic NapF was proposed to play a role in the incorporation of the [4Fe–4S] cluster into NapA (Olmo-Mira *et al.*, 2004). These hypotheses are in line with the finding that a NapF-deficient mutant of *W. succinogenes* was hampered in growth by nitrate respiration, a phenotype that was apparently caused by a decreased amount of active NapA (Kern *et al.*, 2007). Two distinct forms of NapA were detected in cells of this mutant, suggesting retarded maturation of apo-NapA in the cytoplasm.

In this study, the localization and function of *W. succinogenes* NapF was investigated in more detail, providing evidence for the formation of a membrane-bound NapGHF complex. Furthermore, the four tetra-cysteine motifs of NapF were individually altered and the corresponding mutants were characterized with respect to growth properties, NapA maturation and interaction between NapF and NapH.

METHODS

Growth of *W. succinogenes*. *W. succinogenes* cells were grown by nitrate respiration in medium (pH 7.5) containing Tris (50 mM), sodium formate (80 mM), potassium nitrate (50 mM), K₂HPO₄ (1 mM), fumaric acid (5 mM), ammonium sulfate (5 mM), MgCl₂ (1 mM), CaCl₂ (0.2 mM), glutamic acid (0.68 mM), cysteine (0.57 mM) and the trace element solution (0.2 ml l⁻¹) of Pfennig & Trüper (1981). Brain-Heart-Infusion broth (0.25 % or 0.5 %, w/v) was added where appropriate. Growth by fumarate respiration was performed in medium described by Kröger *et al.* (1994). Potassium nitrate (20 mM) was added to the medium where appropriate.

Cell fractionation. To separate soluble and membrane fractions, *W. succinogenes* cells harvested in the exponential growth phase were suspended (10 g cell protein l⁻¹) in an anoxic buffer (pH 8.0) containing 50 mM Tris/HCl. The suspension was passed through a French press at 130 MPa and at 10 ml min⁻¹ flow rate. The resulting cell homogenate was centrifuged for 45 min at 100 000 g to yield the membrane fraction (sediment) and the soluble fraction. The periplasmic cell fraction was separated as described by Kern & Simon (2008). In brief, spheroplasts were produced by treatment of intact cells with EDTA and lysozyme in an isotonic sucrose-containing buffer. The periplasm was then obtained as supernatant after centrifugation of the spheroplasts. The separation success was examined by haem staining of SDS-polyacrylamide gels and determination of glutamate dehydrogenase activity (Kern & Simon, 2008). Soluble *c*-type cytochromes were found exclusively in the periplasmic fraction, which lacked glutamate dehydrogenase activity. After lysis of the spheroplasts, the cytoplasmic and membrane fractions were produced as described above. More than 97 % of the

total glutamate dehydrogenase activity was typically found in the cytoplasmic fraction.

Determination of specific enzyme activities and analytical procedures. Nitrate reductase activity was measured under anoxic conditions at 37 °C by photometrically recording the oxidation of benzyl viologen radical (BV) by nitrate at 546 nm. The test solution contained 50 mM potassium phosphate (pH 7.0), 1 mM BV and 10 mM sodium nitrate. BV was reduced by sodium dithionite and the reaction was started by addition of nitrate. Specific activities were calculated using an extinction coefficient, ϵ , of 19.5 mM⁻¹ cm⁻¹. Two micromoles of BV was oxidized per μ mol nitrate reduced to nitrite. The specific activity of electron transport from formate to nitrate was determined in *NrfHA*-deficient cells as described previously (Kern & Simon, 2008). Washed intact cells were incubated in a formate-containing Bicin buffer and, after addition of nitrate, the time-course of nitrite formation was followed. Protein was measured using the Biuret method with KCN (Bode *et al.*, 1968). Nitrate (Hartley & Asai, 1963) and nitrite (Rider & Mellon, 1946) were determined as described.

Detection of NapF by immunoblot analysis. Custom-made antibodies specifically directed against *W. succinogenes* NapF were raised in SPF rabbits by peptide immunization (Eurogentec). The primary structures of the two NapF peptides used were H₂N-

CLLRSKREEGGSNAPL-CONH₂ and H₂N-CTQAI SLTPLPLKEES-COOH, corresponding to residues 10–24 and 151–165, respectively. For Western blot analysis, protein was transferred from an SDS-polyacrylamide gel to PVDF membrane by electroblotting in a discontinuous buffer system. NapF was detected by indirect ELISA using anti-rabbit IgG coupled to horseradish peroxidase as second antibody (Cell Signaling Technology).

Construction of *W. succinogenes* mutants. Standard genetic procedures were used (Sambrook *et al.*, 1989). DNA was isolated from *W. succinogenes* using the DNeasy Tissue kit (Qiagen). PCR was carried out using BIOTAQ DNA polymerase (Bioline) or the Expand High Fidelity PCR System (Roche) with standard amplification protocols. A compilation of the *W. succinogenes* strains used in this study is shown in Table 1. *W. succinogenes* Δ napF was constructed using a deletion plasmid in order to replace 99 % of the genomic *napF* by an arrangement consisting of the kanamycin-resistance gene cartridge (*kan*) and a functional *nap* promoter (*P_{nap}*) to ensure transcription of the *napL* and *napD* genes situated downstream of *kan* in the Δ napF mutant strain (Fig. 1). For homologous recombination, the deletion plasmid p Δ napF contained the *kan/P_{nap}* region flanked by two DNA fragments obtained by PCR that were identical to appropriate regions in the *W. succinogenes* genome. The two PCR fragments were synthesized using the following primer pairs: 5'-GGGAGCTCAGCGCCAACTCTATAGCGAAGAGG-5031 and 5'-

Table 1. Strains of *W. succinogenes* used in this study

The genomes of strains 7–16 correspond to that of *W. succinogenes* N5 (complemented Δ napG-D mutant; see Fig. 1 in Kern *et al.*, 2007). See Methods for details of mutant construction.

Strain	Description and/or relevant properties*	Reference/source
Wild-type and deletion mutants serving as controls		
1. Wild-type	Type strain DSMZ 1740	DSMZ
2. Δ napG-D	Deletion mutant containing <i>napA</i> but lacking <i>napGHBFLD</i> ; Cm ^R	Kern <i>et al.</i> (2007)
3. Δ nrfHA	Deletion mutant lacking the cytochrome <i>c</i> nitrite reductase genes <i>nrfHA</i> ; Cm ^R	Simon <i>et al.</i> (2003)
Mutants that either lack NapF or produce NapF variants		
4. stopnapF	Wild-type <i>nap</i> locus containing full-length <i>napF</i> inactivated by four stop codons at positions 11, 12, 74 and 75; Cm ^R , Km ^R	Kern <i>et al.</i> (2007)
5. Δ napF	Deletion of <i>napF</i> ; the mutant genome contains a second <i>nap</i> promoter element upstream of <i>napL</i> ; Km ^R (Fig. 1)	This work
6. Δ napF Δ nrfHA	Deletion of <i>napF</i> (as in strain 5) in the genome of strain 3; Cm ^R Km ^R	This work
7. RRnapF	Wild-type <i>nap</i> locus encoding modified NapF (R3G/R4G)†; Cm ^R Km ^R	This work
8. NapF C42A/C46A	Wild-type <i>nap</i> locus encoding modified NapF (C42A/C46A)‡; Cm ^R Km ^R	This work
9. NapF C74A/C77A	Wild-type <i>nap</i> locus encoding modified NapF (C74A/C77A)‡; Cm ^R Km ^R	This work
10. NapF C113A/C116A	Wild-type <i>nap</i> locus encoding modified NapF (C113A/C116A)‡; Cm ^R Km ^R	This work
11. NapF C142A/C145A	Wild-type <i>nap</i> locus encoding modified NapF (C142A/C145A)‡; Cm ^R Km ^R	This work
Mutants containing His-tagged NapH		
12. NapH NHis ₆	Wild-type <i>nap</i> locus encoding NapH extended by an N-terminal hexahistidine tag; Cm ^R Km ^R	This work
13. NapH NHis ₆ C90A/C94A	Derivative of strain 12 encoding modified NapH (C90A/C94A)‡; Cm ^R Km ^R	This work
14. NapH NHis ₆ C185A/C189A	Derivative of strain 12 encoding modified NapH (C185A/C189A)‡; Cm ^R Km ^R	This work
15. NapH NHis ₆ C216A/C219A	Derivative of strain 12 encoding modified NapH (C216A/C219A)‡; Cm ^R Km ^R	This work
16. NapH NHis ₆ C247A/C250A	Derivative of strain 12 encoding modified NapH (C247A/C250A)‡; Cm ^R Km ^R	This work
Mutant containing a chimeric nap/nos operon		
17. napAG(StrepC)-nosH Δ nosG-H	Deletion mutant that contains a chimeric <i>nap</i> operon with <i>napH</i> replaced by <i>nosH</i> but lacks <i>nosG</i> , -C1, -C2 and <i>nosH</i> ; Cm ^R Km ^R	Kern & Simon (2008)

*Cm^R and Km^R denote resistance to chloramphenicol and kanamycin, respectively.

†Fig. 5(a) depicts the residues replaced in NapF.

‡See Fig. 5 in Kern & Simon (2008) for the position of residues replaced in NapH.

Fig. 1. Physical map of the *nap* gene locus in the genome of *W. succinogenes* $\Delta napF$. Black bars indicate regions used for homologous recombination during mutant construction (see Methods for details).

GGGGATCCTCACTTCACCCCTTCATTGAGCGTG-5444 for amplifying the upstream fragment, and 5'-GGGTCGACATGAAAACAA-TTTTTCCTCTATC-5986 (*napL* start codon in bold) and 5'-GGCCATGGTCAAAGAGGGCGATTTCATTGCTGG-6392 for the downstream fragment (black bars in Fig. 1). Primers carried either a *SacI*, *BamHI* or *NcoI* restriction site (underlined) for cloning. The numbers at the 3'-ends of the primer sequences correspond to nucleotide positions in the EMBL, GenBank and DDBJ database entry with accession number AJ512686. Both fragments as well as the *nap* promoter region and the kanamycin-resistance gene cassette were consecutively inserted into the high-copy-number plasmid pPR-IBA1 (IBA BioTAGnology) as described previously for plasmids p $\Delta napG$ and p $\Delta napH$ (Kern & Simon, 2008). After sequencing of the inserted fragments, transformation of *W. succinogenes* wild-type cells was carried out by electroporation as described by Simon *et al.* (1998). Transformants were selected in the presence of kanamycin (25 mg l⁻¹) and the desired deletion in the transformant genome was confirmed by PCR. Furthermore, the identity of DNA stretches involved in recombination was confirmed by sequencing from suitable PCR products. Mutant $\Delta napF \Delta nrfHA$ was obtained after transformation of *W. succinogenes* $\Delta napF$ with p $\Delta nrfHA$ (Simon *et al.*, 2003).

Site-directed mutagenesis of *napF* was performed using either the QuikChange Site-Directed Mutagenesis Kit (Stratagene) or the Phusion Site-Directed Mutagenesis Kit (Finnzymes) with pnapG-Dkan as template and specifically synthesized primer pairs as described previously (Kern *et al.*, 2007). Derivatives of pnapG-Dkan were integrated into the genome of *W. succinogenes* $\Delta napG-D$ by a single recombination event via the 3'-end of *napA* present on both the plasmid and the mutant genome (see Fig. 1 in Kern *et al.*, 2007). Using this strategy, the complete genomic *nap* gene cluster was restored in the mutant, which lacked *napG*, *-H*, *-B*, *-F*, *-L* and part of *napD*. For modification of the double arginine motif, a pair of complementary primers was used, of which the forward primer sequence (5'→3') is as follows: RRnapF, 5'-GGTGAAGTGATG-GATGGAGGAGGGTTTTTCACC-5491. For the simultaneous mutagenesis of two *napF* cysteine codons, the following 5'-phosphorylated primer pairs were used (forward primer given): NapF_C42A, 5'-GCCTCAGCGGAGGGGCAAAGCTTGGAAGAAG-5567, and NapF_C46A, 5'-GCCCCGCCCAAGGCTTGCGAAGGAGGATC-5629; NapF_C74A, 5'-TTGGGCAAAGGTGCATCCCTCTCTTTG-AAAG-5661, and NapF_C77A, 5'-GCAAGCTGCGCTTGCCCTGCC-TAAGGG-5718; NapF_C113A, 5'-AGCGGCGAGGGTCTTTTGCC-ATGCAAGGCAC-5779, and NapF_C116A, 5'-TCCGCCAAGGA-GGTTTGTGATGAAGGGGCG-5839; NapF_C142A, 5'-CCCCGGCT-GCCGTGCAAGAGGGGGTG-5872, and NapF_C145A, 5'-CGAGCT-GTCGGAGTCTGCCCCACGCAAGCC-5926. Altered nucleotides are in bold and modified codons (see primer designations) are underlined. Transformation of *W. succinogenes* $\Delta napG-D$ cells with plasmid derivatives and genetic characterization of the transformants was performed as described by Simon *et al.* (2000). Each mutation was confirmed by sequencing an appropriate PCR fragment. The

preservation of desired stop codons was routinely confirmed after growth experiments.

Using the same strategy, a *W. succinogenes* mutant (strain NapH NHis₆) was constructed that encodes NapH carrying an N-terminal hexahistidine tag. The required plasmid (pNapH NHis₆), a derivative of pnapG-Dkan, contained *napH* extended by a nucleotide stretch encoding six histidine residues and an adjacent Gly-Ala-Ser linker. The plasmid was modified using the Phusion Site-Directed Mutagenesis Kit and the following 5'-phosphorylated primer pair: NapH_NHis-F, 5'-CATCACGGAGCCTCTAAATTTTAAAACACT-ATCGATTG-4111, and NapH_NHis-R, 5'-GTGATGGTGATGCAT-CTTACAACCTCCCGCTATTG-4065 (His-tag and linker in bold, *napH* start codon underlined). Mutants producing His-tagged NapH variants with modified poly-cysteine motifs (see Table 1) were constructed by transformation of *W. succinogenes* $\Delta napG-D$ with appropriate plasmid derivatives of pNapH NHis₆. Site-directed mutagenesis was performed using the primer pairs described by Kern & Simon (2008).

RESULTS

The absence of NapF impairs NapA maturation

A non-polar *napF* deletion mutant of *W. succinogenes* (strain $\Delta napF$) was constructed (Table 1, Fig. 1) using the genetic strategy described by Kern & Simon (2008). The doubling time and final optical density of the *W. succinogenes* $\Delta napF$ cells grown in either minimal formate/nitrate medium or in the same medium supplemented with Brain-Heart-Infusion broth were found to be equivalent to the corresponding parameters of the wild-type (Table 2). Notably, these properties are in contrast to those obtained earlier using strain *W. succinogenes* stopnapF, which contained four stop codons in a *napF* gene located in an otherwise unaltered *nap* gene cluster (Kern *et al.*, 2007). In the previous study, the growth rate of *W. succinogenes* stopnapF cells in minimal medium was about half that of the wild-type, with the final optical density being only 36% relative to the wild-type (Kern *et al.*, 2007). Reassessment of the *W. succinogenes* stopnapF mutant largely confirmed these values (Table 2). The decreased final optical density of *W. succinogenes* stopnapF is now explained by the finding that the cells, for unknown reasons, do not produce NrfA, the catalytic subunit of the cytochrome *c* nitrite reductase complex, and therefore do not reduce any nitrite formed by nitrate respiration to ammonium (Table 2; Fig. 2). The *nrfA* mRNA was not detectable by quantitative (real-time) reverse transcription

Table 2. Growth parameters of *W. succinogenes* *nap* mutants

Representative data from at least two independent cultures are shown. NA, Not applicable; ND, not detectable.

Strain	Growth in minimal medium*		Growth in supplemented medium†		Nitrate reduction rate (mM h ⁻¹)‡	Specific activity of electron transport from formate to nitrate [U min ⁻¹ (mg protein) ⁻¹]§	NrfA present
	Doubling time (h)	Final OD ₅₇₈ (%)	Doubling time (h)	Final OD ₅₇₈ (%)			
Wild-type	1.9	≡100	1.7	≡100	17.0	NA	+
<i>ΔnapG-D</i>	∞	≡0	∞	≡0	ND	NA	—
<i>ΔnrfHA</i>	3.2	55	3.0	59	16.0	0.14	—
<i>stopnapF</i>	5.4	56	4.9	58	5.3	0.04	—
<i>ΔnapF</i>	2.0	108	1.7	119	5.4	NA	+
<i>ΔnapF ΔnrfHA</i>	5.3	54	5.0	58	5.7	0.04	—
<i>RRnapF</i>	3.8	55	3.0	59	17.0	0.15	—
<i>NapF C42A/C46A</i>	3.6	54	2.9	59	16.4	0.14	—
<i>NapF C74A/C77A</i>	3.7	55	2.8	59	16.8	0.15	—
<i>NapF C113A/C116A</i>	5.0	53	4.8	56	4.4	0.04	—
<i>NapF C142A/C145A</i>	3.6	55	2.9	59	16.1	0.15	—

*Cells were incubated at 37 °C in anoxic minimal medium containing formate (80 mM) and nitrate (50 mM) as energy substrates in the presence of 10 mM ammonium. Fumarate (5 mM), present as carbon source, allowed limited growth by fumarate respiration during the initial stage (2–3 h) of growth. Cultures were inoculated using cells grown in the same medium, but supplemented with 0.25 % (w/v) Brain-Heart-Infusion broth. Doubling times were calculated from the exponential growth phase. The OD₅₇₈ of wild-type cells entering the stationary growth phase was typically about 0.55 (corresponds to 100 %), while that of mutant cells unable to grow by nitrate respiration was 0.08 (set as 0 %). All growing cultures entered the stationary growth phase after 7–9 h. ∞ denotes no growth.

†Cells were grown as described in footnote * but the medium was supplemented with 0.25 % (w/v) Brain-Heart-Infusion broth. Cultures were inoculated with cells grown in formate/fumarate medium supplemented with 50 mM potassium nitrate and 0.5 % (w/v) Brain-Heart-Infusion broth. The OD₅₇₈ of wild-type cells entering the stationary growth phase was typically about 0.6 (corresponds to 100 %), while that of mutant cells unable to grow by nitrate respiration was 0.09 (set as 0 %).

‡Calculated from the time-course of the nitrate concentration in the medium (see Fig. 2).

§Determined with NrfA-deficient cells grown by nitrate respiration in medium with 0.25 % Brain-Heart-Infusion broth. Harvested and washed cells were incubated in an isotonic buffer in the presence of formate, the reaction was started by addition of nitrate and the formation of nitrite was followed as described previously (Kern & Simon, 2008). One unit of electron transport activity (U) is equivalent to the consumption of 1 μmol formate min⁻¹.

||Judged from SDS gels stained for covalently bound haem (see Fig. 2).

PCR (qRT-PCR) experiments in cells of *W. succinogenes* *stopnapF*, whereas the *ΔnapF* mutant was found not to be impaired in *nrfA* transcription (data not shown). Although nitrite respiration contributes only slightly to wild-type growth under the tested conditions (Fig. 2a), it becomes increasingly important in cells with a lowered nitrate reduction rate. Such cells instantly reduce any formed nitrite to ammonium instead of accumulating it in the medium. Cells of both the *stopnapF* and *ΔnapF* mutants exhibited only about one-third of the wild-type nitrate consumption rate determined in cultures grown in supplemented formate/nitrate medium (Table 2; Fig. 2a) and this rate is also reflected in the decreased electron-transport activity from formate to nitrate measured with washed suspensions of intact cells as compared to cells of *W. succinogenes* *ΔnrfHA* (Table 2). In both the *stopnapF* and *ΔnapF* mutants, the nitrate reductase activity determined with BV as artificial electron donor was found to be

less than 50 % of that of the wild-type (Table 3) despite the fact that the cells formed wild-type amounts of *napA* mRNA (data not shown).

It was reported previously that cells of *W. succinogenes* *stopnapF* produced two different immunodetectable forms of NapA and it was argued that the larger form (which was not detectable in wild-type cells) represented the cytoplasmic NapA precursor protein containing an N-terminal Tat signal peptide with a predicted mass of about 3 kDa (Kern *et al.*, 2007). Both forms of NapA were also clearly visible in the cell homogenate of *W. succinogenes* *ΔnapF* cells, with the larger form dominating (Table 3; Fig. 3a). The sizes of the two bands were estimated from Western blot analysis as 95 and 98 kDa, which is in good agreement with calculated masses of 102 and 105 kDa derived from the primary structures of pre-NapA and N-terminally processed NapA, respectively. Minor amounts of the larger NapA form were

Fig. 2. Growth of *W. succinogenes* wild-type and NapF-deficient mutants by nitrate respiration in formate/nitrate medium (supplemented with 0.25 % Brain-Heart-Infusion broth) and presence of the cytochrome c nitrite reductase NrfA. (a) Time-course of nitrate (■) and nitrite (▲) concentrations in growing cultures. (b) Detection of NrfA by peroxidase activity staining in cell homogenates separated by SDS-PAGE; 100 μ g protein was applied to each lane.

also detected in wild-type cells during the exponential growth phase (Fig. 3a). However, the corresponding protein band disappeared when the cells reached the stationary phase, indicating efficient NapA maturation. After cell fractionation the smaller form was found to be located in the periplasm, where it was catalytically active

towards nitrate reduction (Fig. 3b). A minor amount of this form was also detected in the membrane fraction. In contrast, the larger NapA band was found exclusively in the cytoplasmic fraction (Fig. 3b). This form of NapA did not reduce nitrate as it probably lacked at least one of its cofactors, which are known to be incorporated in the

Table 3. Specific nitrate reductase activity of *W. succinogenes* wild-type cells and *nap* mutants

Representative values from at least two independent cultures are shown. Cells were grown in medium containing formate (100 mM), fumarate (90 mM) and nitrate (20 mM) as energy substrates and supplemented with 0.5 % (w/v) Brain-Heart-Infusion broth. Cells and membrane fractions were suspended in an anoxic buffer (pH 7.5) containing 50 mM Tris/HCl. NA, Not applicable.

Strain	Specific nitrate reductase activity $BV_{red \rightarrow nitrate}$ [μ mol nitrate reduced min^{-1} (mg cell protein) $^{-1}$]			NapA precursor detectable in stationary-phase cells*
	Cell homogenate	Membrane fraction	Soluble fraction	
Wild-type	1.8	0.5	1.2	No
$\Delta napG-D$	0.3†	0.3†	0.06	NA
$\Delta nrfHA$	1.9	0.4	1.3	No
<i>stopnapF</i>	0.8	0.2	0.5	Yes
$\Delta napF$	0.8	0.2	0.6	Yes
$\Delta napF \Delta nrfHA$	0.7	0.2	0.5	Yes
<i>RRnapF</i>	1.7	0.4	1.3	No
NapF C42A/C46A	1.7	0.4	1.2	No
NapF C74A/C77A	1.9	0.6	1.1	No
NapF C113A/C116A	0.7	0.2	0.5	Yes
NapF C142A/C145A	1.7	0.5	1.2	No

*See Figs 3 and 5(b).

†NapA-independent nitrate reduction activity. Such an activity has been reported previously for *W. succinogenes napA::kan* cells (Simon *et al.*, 2003).

Fig. 3. Immunoblot detection of *W. succinogenes* NapA using an antiserum raised against NapA from *Paracoccus denitrificans* (Berks *et al.*, 1994). Cells of the indicated strains were grown in formate/fumarate medium supplemented with 0.25 % Brain-Heart-Infusion broth. After 3 h growth ($OD_{578} \sim 0.3$), 20 mM nitrate was added; the cultures reached the stationary growth phase after 7 h incubation at 37 °C. For Western blot analysis, proteins were transferred onto PVDF membrane by electroblotting in a discontinuous buffer system. NapA was detected by indirect ELISA using goat anti-rabbit IgG coupled to peroxidase (Cell Signaling Technology) as second antibody (Simon *et al.*, 2003). (a) After inoculation of the cultures, samples of cell suspensions were taken at the indicated time points and SDS-PAGE was performed using intact cells (150 µg protein per lane). (b) Detection and specific nitrate reductase activity of NapA in subcellular fractions; 100 µg protein samples of the periplasmic (PF), membrane (MF) and cytoplasmic (CF) fractions were applied. Representative specific nitrate reductase activities are shown ($n=3$).

cytoplasm prior to Tat-dependent export and signal peptide cleavage. Notably, mutants lacking either NapH or NapG (strains *W. succinogenes* $\Delta napH$, *stopnapH*, $\Delta napG$ and *stopnapG*; see Table 1), possess only mature NapA in cells of the early stationary growth phase, suggesting that NapF is functional in the absence of either NapG or NapH (data not shown).

Cytoplasmic NapF requires functional NapH for membrane attachment

Using an antiserum directed against two NapF polypeptides, the NapF protein was detected in nitrate-grown wild-type cells at the expected size of 19 kDa (Fig. 4a). The vast majority of NapF was found to be located in the membrane fraction. In cell fractions of either *W. succinogenes* $\Delta napH$ or *W. succinogenes* *stopnapH*, however, NapF was present almost exclusively in the cytoplasmic fraction, suggesting that NapF is associated to the membrane as a subunit of a postulated NapGHF complex (Fig. 4a). Absence of NapG in *W. succinogenes* *stopnapG* or *W. succinogenes* $\Delta napG$ had

no effect on NapF localization, as NapF was detected in the membrane fraction of these mutants (data not shown). NapF could be washed off the membrane using increasing concentrations of sodium chloride dissolved in 50 mM Tris/HCl buffer, pH 8.0 (not shown). Treatment with 1 M NaCl was sufficient to achieve complete solubilization of NapF, indicating that the interaction between NapF and NapH is more sensitive to increasing salt concentrations than the previously reported binding of NapG to NapH, which required 2.5 M NaCl for complete dissociation (Kern & Simon, 2008). Previously, cells of *W. succinogenes* *napAG*(StrepC)-*nosH* $\Delta nosG-H$ were shown to form a membrane-bound complex between NosH and NapG in the absence of NapH (Kern & Simon, 2008). NosH is a homologue of NapH whose gene is part of the *nos* gene cluster encoding the respiratory cytochrome *c* nitrous oxide reductase system (Simon *et al.*, 2004; Kern & Simon, 2009). Cells of this mutant contained NapF in the membrane fraction, indicating that NapF binds specifically to NapH but not to NosH (not shown).

Fig. 4. Immunoblot detection of *W. succinogenes* NapF using a peptide antiserum. (a) NapF detection in cell homogenates and cell fractions of different strains grown in formate/nitrate medium. (b) Immunoblot detection of NapF and NapH in mutants that produce NapH variants. Left: immunoblot detection of NapF. Right: detection of His-tagged NapH using a HisTag antibody HRP conjugate (Novagen) and the SuperSignal West Pico Chemoluminescent substrate system (Pierce). A 100 μ g protein sample of the indicated cell fractions was applied to each lane. Wt, wild-type; CH, cell homogenate; PF, periplasmic fraction; CF, cytoplasmic fraction; MF, membrane fraction; SF, soluble fraction.

In a previous report, mutants were presented that encoded NapH variants, each of which contained one modified poly-cysteine motif out of the two CX₃CP motifs and the two tetra-cysteine motifs situated near the C terminus (Kern & Simon, 2008). Only one such variant (NapH C247A/C250A) was capable of forming the membrane-bound NapGH complex. In contrast, the other three variants (NapH C90A/C94A, C185A/C189A and C216A/C219A) contained not membrane-bound, but soluble NapG, and it remained unclear whether or not the modified NapH was present in the membrane. To directly detect NapH, a hexahistidine tag was attached to the N terminus of wild-type NapH and each of the four cysteine variants (see Table 1 for designation of the corresponding mutants). As judged from immunoblot analysis, tagged NapH was present in the membrane fractions of the wild-type as well as in mutants NapH C90A/C94A and NapH C247A/C250A but was absent in strains C185A/C189A and C216A/C219A (Fig. 4b). All four NapH mutants contained the majority of NapF in the soluble cell fraction (Fig. 4b),

indicating that at least the first CX₃CP motif and the second tetra-cysteine motif of NapH are required for NapF/NapH interaction and for electron transport during nitrate respiration.

Characterization of strains producing NapF variants

W. succinogenes NapF contains two adjacent arginine residues near the N terminus (resembling those in Tat signal peptides) and four tetra-cysteine motifs that presumably serve in binding four iron-sulfur centres (Fig. 5a). The genetic system presented by Kern *et al.* (2007) was used to generate mutants producing NapF variants in the context of the complete *nap* operon (strains RRnapF, NapF C42A/C46A, NapF C74A/C77A, NapF C113A/C116A and NapF C142A/C145A; Table 1). Similar to the stopnapF strain described above, each of these mutants lacked NrfA, thus causing a reduced final optical density after growth in formate/nitrate medium (Table 2). With the exception of *W. succinogenes* NapF C113A/C116A, the mutants producing NapF variants had properties that were similar to the *nrfHA* deletion strain in terms of doubling time, nitrate consumption, specific electron-transport activity from formate to nitrate and specific nitrate reductase activity (Tables 2 and 3). Furthermore, the majority of NapF was found in the membrane fraction and only one NapA band was present in the stationary growth phase of these cells (Table 3; Fig. 5b). The NapF protein of mutant NapF C113A/C116A was also found to be attached to the membrane but the cells grew with a doubling time similar to that of the $\Delta napF \Delta nrfHA$ mutant (Table 2; Fig. 5b). Moreover, the NapA precursor was clearly visible in cell fractions of the NapF C113A/C116A mutant, along with a reduced nitrate consumption rate and lowered specific activities of electron transport from formate to nitrate and of NapA-dependent nitrate reduction (Tables 2 and 3; Fig. 5b). Taken together, these results demonstrate that only the third poly-cysteine motif is essential for NapF function and that the N-terminal two arginine residues are functionally dispensable, which is in line with the fact that NapF is not exported by the Tat system.

DISCUSSION

Like many other bacteria, the Epsilonproteobacterium *W. succinogenes* grows by anaerobic respiratory nitrate ammonification using formate or H₂ as electron donor substrates (Bokranz *et al.*, 1983; Simon, 2002). This mode of respiration depends on two terminal periplasmic reductases, nitrate reductase (NapA) and cytochrome *c* nitrite reductase (NrfA), and mutants deficient in either *napA* or *nrfA* did not grow by nitrate or nitrite respiration, respectively (Simon *et al.*, 2000, 2003). While the NapC-type tetrahaem cytochrome *c* NrfH protein was shown to form a membrane-bound complex with NrfA that catalyses

Fig. 5. Arrangement of tetra-cysteine clusters in NapF and detection of NapF and NapA in *W. succinogenes* mutants producing NapF variants. (a) Features of *W. succinogenes* NapF. Cysteine residues are arranged in tetra-cysteine motifs (Fe/S) and residues replaced by alanine are underlined (see Table 1 for mutant designation). Peptide stretches that were synthesized for antiserum production are indicated by grey boxes. (b) Immunoblot detection of *W. succinogenes* NapF (top row) and NapA (bottom row) as described in Figs 3 and 4; 100 µg protein samples of the indicated cell fractions were applied. CH, cell homogenate; SF, soluble fraction; MF, membrane fraction.

nitrite reduction using menaquinone as electron donor (Simon *et al.*, 2000, 2001; Gross *et al.*, 2005), the electron-transfer route from menaquinol to NapA was less obvious until the essential role of the putative menaquinol dehydrogenase complex NapGH in nitrate respiration was demonstrated (Kern *et al.*, 2007; Kern & Simon, 2008). Fig. 6 depicts the current model of the electron-transport chain from menaquinol to NapA and the postulated function of the NapF protein. According to this model, NapF is a cytoplasmic redox partner of NapH, thus forming the counterpart of NapG on the periplasmic side of the membrane. Consequently, the NapGHF complex can be envisaged as an electron-transport bifurcation device that delivers electrons from the quinone/quinol pool to both the cytoplasmic and periplasmic cell compartments. Such a complex might be an evolutionarily ancient electron-transfer module, assuming that iron-sulfur proteins predated the emergence of cytochromes. The interaction between NapH and NapF in *W. succinogenes* is dependent on the presence of the first CX₃CP and the second tetra-cysteine motif of NapH, and it is notable that the first CX₃CP motif was also found to be required for the interaction of NapH with NapG (Kern & Simon, 2008). On the other hand, modification of each NapF tetra-cysteine cluster did not abolish membrane attachment, suggesting that the general folding of the NapF variants is not disturbed. The NapGHF complex is likely to be present in other organisms that contain the corresponding genes. One example would be *E. coli* where the majority of NapF was detected in the membrane fraction (Nilavongse *et al.*, 2006). However, the NapH/NapF interaction could not be shown experimentally using a bacterial two-hybrid assay (Nilavongse *et al.*, 2006).

What is the function of NapF in nitrate-respiring *W. succinogenes* cells? The data presented here suggest that NapF plays a crucial role in NapA maturation rather than in bioenergetics of nitrate respiration, since the growth parameters of the $\Delta napF$ mutant are comparable to those of the wild-type strain. NapF is possibly involved in the generation of a NapA precursor competent for cofactor insertion or in delivering NapA to the Tat system (Fig. 6). It cannot be excluded that some sort of electron transfer is involved in such a process. The data suggest that only the third tetra-cysteine motif of NapF is required for efficient NapA maturation, while any of the other three tetra-cysteine clusters are dispensable. Therefore, it is also possible that a putative [4Fe-4S] centre ligated by the third motif is transferred from NapF to NapA as part of the NapA maturation process. Such a cofactor transfer was suggested by *in vitro* experiments with purified NapF and NapA from *R. sphaeroides* (Olmo-Mira *et al.*, 2004). Interestingly, *W. succinogenes* NapF was still functional as a soluble protein in the absence of NapH, indicating that there is at least one functional alternative to NapH (Fig. 6). This would also be the case in NapF-encoding organisms, such as *R. sphaeroides*, that lack the *napH* and *napG* genes.

There are many examples of organisms that lack *napF* (but contain *napG* and *-H*), suggesting that electron transport from the quinol pool via NapGH to NapA is catalysed independently of NapF (Marietou *et al.*, 2005; Pittman *et al.*, 2007; Kern & Simon, 2009). In *W. succinogenes*, the absence of NapF did not completely abolish nitrate reduction or NapA maturation, and therefore at least one protein (possibly another cytoplasmic iron-sulfur protein) is expected to be present in *W. succinogenes* that is functionally equivalent to NapF, albeit less efficient in

Fig. 6. Working model illustrating the proposed role of the *W. succinogenes* NapGHF complex in periplasmic nitrate reduction and NapA maturation. Menaquinol is oxidized by NapH and electrons are transferred via the iron–sulfur protein NapG and the *c*-type cytochrome NapB to NapA (Kern & Simon, 2008). The number of menaquinol binding sites in NapH and the side to which the generated protons are released are not known. The first CX₃CP signature and the second tetra-cysteine motif (Fe/S-2) of NapH are essential for NapF binding. The predicted iron–sulfur protein NapF is hypothesized to be involved in electron transfer from NapH and in maturation of the cytoplasmic NapA precursor (with or without cofactors attached). NapA is exported by the Tat system after cofactor incorporation. Only the third tetra-cysteine motif of NapF (Fe/S-3, underlined) is required for NapA maturation. The hypothetical protein Alt1 is predicted to replace absent NapF in NapA maturation. Protein Alt2 is suggested to interact with soluble NapF when NapH is absent. Black and blue arrows indicate electron transfer and protein–protein interaction respectively. Fe/S, tetra-cysteine motif that likely binds a [4Fe–4S] centre; the number denotes the order of the motif in the primary protein structure.

NapA maturation (Fig. 6). A similar scenario is conceivable for *Campylobacter* species that are devoid of *napF*. Here, the *napF* gene might have been lost during evolution if its gene product proved to be functionally redundant. Although an *E. coli* $\Delta napF$ mutant that produced only ubiquinone but no menaquinone (which made NapA-dependent nitrate respiration rely solely on NapGH) showed a growth defect in glycerol/nitrate medium, its NapA-dependent nitrate reduction rate was not significantly affected (Brondijk *et al.*, 2002; Nilavongse *et al.*, 2006). This would suggest that NapA maturation in *E. coli* is not dependent on NapF. In contrast, NapA was not detected in a NapF-deficient strain of *R. sphaeroides* (Reyes *et al.*, 1998; Olmo-Mira *et al.*, 2004).

The differing phenotypes of NapF-deficient bacterial species make it difficult to unequivocally deduce the function of NapF from physiological experiments. Future studies in our lab will therefore focus on the purification of

NapF, NapGH and NapGHF in order to reveal structure–function relationships and to characterize the various proposed protein–protein interactions.

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