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Variants of the tetrahaem cytochrome c quinol dehydrogenase NrfH characterize the menaquinol binding site, the haem c binding motifs and the transmembrane segment

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Abbreviations used: BV_{red}, reduced benzyl viologen; Nap, periplasmic nitrate reductase; Nrf, cytochrome c nitrite reductase.

SYNOPSIS

Members of the NapC/NrfH family are multihæm *c*-type cytochromes that exchange electrons with oxidoreductases situated at the outside of the cytoplasmic membrane or in the periplasmic space of many proteobacteria. They form a group of membrane-bound quinol dehydrogenases that are essential components of several electron transport chains, for example those of periplasmic nitrate respiration and respiratory nitrite ammonification. Knowledge on structure/function relationships of NapC/NrfH proteins is scarce and only one high-resolution structure (*Desulfovibrio vulgaris* NrfH) is available. In this study, several *Wolinella succinogenes* mutants that produce variants of NrfH, the membrane anchor of the cytochrome *c* nitrite reductase complex, were constructed and characterized in order to improve the understanding of the putative menaquinol binding site, the maturation and function of the four covalently bound hæm *c* groups and the importance of the N-terminal transmembrane segment. Based on amino acid sequence alignments, a homology model for *W. succinogenes* NrfH was constructed that underlines the overall conservation of tertiary structure in spite of a low sequence homology. The results support the proposed architecture of the menaquinol binding site in *D. vulgaris* NrfH, demonstrate that each histidine residue arranged in one of the four CX₂CH hæm *c* binding motifs is essential for NrfH maturation in *W. succinogenes* and indicate a limited flexibility toward the length and structure of the transmembrane region.

Key words: NapC/NrfH family; multihæm *c*-type cytochrome; quinol dehydrogenase; cytochrome *c* nitrite reductase NrfA, respiratory nitrite ammonification

INTRODUCTION

The membranous quinone pool, usually consisting of ubiquinone(s) and/or menaquinone(s), is a key component of electron transport chains involved in bacterial aerobic and anaerobic respiration [1]. Many different membrane-bound enzymes and enzyme complexes are capable of quinone/quinol turnover. Examples of such complexes operative in aerobic respiration are NADH dehydrogenase, succinate dehydrogenase and the cytochrome *bc*₁ complex. In anaerobic respiration, the oxidation of common electron-supplying substrates such as formate or H₂ is coupled to quinone reduction, respectively catalysed by membrane-bound formate dehydrogenase (Fdh) and hydrogenase (Hyd) complexes [1,2]. The generated quinol is often re-oxidised directly by a terminal reductase, e.g. during nitrate respiration by the membrane-bound nitrate reductase complex (Nar), completing a typically short bacterial electron transport chain. The mentioned Fdh, Hyd and Nar complexes all possess a membrane-integral di-haem cytochrome *b* subunit that harbours the quinone/quinol binding site and forms a stable complex with their corresponding redox partner proteins on either the in- or the outside of the bacterial cytoplasmic membrane [3,4]. Electron transport from quinols to various other terminal electron acceptors employed in anaerobic respiration, however, is independent of haem *b*-containing cytochromes and often relies on quinone-reactive proteins that belong to one of several protein families which form widespread and versatile electron transfer modules [5]. Examples of such quinol dehydrogenase groups are the NapC/NrfH, NapH and NrfD/PsrC families whose members are all envisaged to transfer electrons to redox partners located at the outside of the cytoplasmic membrane or in the periplasmic space of Gram-negative bacteria.

NapC/NrfH-type proteins are structurally related multihaem *c*-type cytochromes that contain either four or five haem *c* groups [6]. Each haem is bound covalently to the polypeptide chain via a CX₂CH haem *c* binding motif. The regio- and stereospecific attachment of haem *c* is catalysed by cytochrome *c* haem lyase, an enzyme that generates two thioether bridges from two haem vinyl side chains and the thiol groups provided by the two cysteine residues of the haem *c* binding motif [7,8]. The prototypic NapC and NrfH proteins contain four haem *c* groups each, catalyse quinol oxidation and donate electrons to either the periplasmic nitrate reductase system (Nap) or the ammonifying cytochrome *c* nitrite reductase (NrfA) [2,9]. Both proteins are likely to be anchored to the membrane via a hydrophobic N-

terminal transmembrane segment. When produced as a soluble periplasmic protein, i.e. when the N-terminal transmembrane domain was replaced by a cleavable signal peptide, the NapC protein from *Paracoccus pantotrophus* as well as a NapC homolog (CymA) from *Shewanella oneidensis* were shown to remain functional *in vivo* implying that the truncated variants can interact with the quinol pool in the membrane [10,11]. NapC proteins do not form stable complexes with their periplasmic redox partner which is usually another c-type cytochrome [12]. A soluble form of *Paracoccus denitrificans* NapC has been purified and was shown to contain four haem c groups [13]. Spectroscopic characterization supported the view that all four haem c groups exhibited axial *bis*-histidine haem iron coordination although the presence of His/Lys-ligated haem(s) could not be excluded. Similar results were obtained for native CymA from *Shewanella frigidimarina* [14]. The presence of four His/His-ligated haem c groups in *P. pantotrophus* NapC was also suggested by the finding that four histidine residues, which are conserved throughout the NapC primary structures, proved to be functionally essential [10].

In contrast, sequence alignments and site-directed modification of NrfH from *Wolinella succinogenes* indicated an alternative haem c ligation pattern for members of the NrfH subgroup within the NapC/NrfH family [6]. *W. succinogenes* NrfH forms a stable, membrane-bound complex with cytochrome c nitrite reductase, NrfA, and was shown to be required for menaquinol oxidation and membrane attachment of the NrfHA complex, the terminal reductase of respiratory nitrite ammonification [2,15,16]. For *W. succinogenes*, a high-resolution crystal structure is available for the NrfA homodimer [17] and crystals have been obtained for the entire NrfHA complex [18]. However, the only crystal structure of a NapC/NrfH-type protein available to date is that of *Desulfovibrio vulgaris* NrfH, which was found in a (NrfHA₂)₂ complex arrangement with cytochrome c nitrite reductase [19]. Not surprisingly, two of the four haem c groups were found to deviate from standard *bis*-histidine haem c iron ligation. Haem 1, which is closest to the membrane and the likely entry point to a linear four-haem electron transfer wire, has an unusual proximal methionine ligand that derives from a CX₂CHXM sequence whereas an aspartate residue is located at the distal position, albeit without coordination to the haem iron. Haem 4 is His/Lys-coordinated with the distal lysine ligand being provided by a NrfA subunit. The presence of a menaquinol binding and oxidation site was proposed, forming a cavity lined by haem 1, a short α -helical region situated parallel to the membrane plane and the

periplasmic end of the N-terminal transmembrane α -helix (Fig. 1A, B) [19]. Several of the residues in close vicinity to the putative menaquinol binding pocket are conserved in NrfH proteins although the overall sequence identity between them is often fairly modest; for example, the NrfH proteins from *D. vulgaris* and *W. succinogenes* share only 23% identical residues.

Previously, several histidine, methionine and lysine residues of *W. succinogenes* NrfH have been modified and some of the corresponding mutants were found to be severely impaired in growth by nitrite respiration and specific activity of electron transport from formate to nitrite [6]. Here, we reassess our earlier data in the light of the *D. vulgaris* NrfH structure and present new NrfH variants that have been produced to further examine the structure and function of the assumed menaquinol binding site, the importance of the transmembrane α -helix and the significance of the histidine residues organised in haem *c* binding motifs. An alignment-based homology model for *W. succinogenes* NrfH was used to assess possible differences to NrfH from *D. vulgaris* regarding essential structural features such as haem ligands and active site geometry and to confirm the relevance of the selected modifications.

EXPERIMENTAL

Growth of *W. succinogenes* and cell fractionation

W. succinogenes DSMZ 1740 and derived mutant strains were grown in minimal medium with formate and nitrate as described [20], except that 5 mM $(\text{NH}_4)_2\text{SO}_4$ was present in the medium instead of K_2SO_4 . Cells were harvested in the exponential growth phase and suspended (10 g cell protein/l) 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 flow rate. The resulting cell homogenate was centrifuged for 1 h at 100,000 *g* to yield the sedimented membrane fraction and the soluble fraction. Typically, less than 10% of the cell protein were lost during the fractionation procedure and about one third of the total protein resided in the membrane fraction.

Determination of specific activities

The activity of electron transport from formate to nitrite was determined using whole cells as described [16]. This assay depends on the membrane-bound electron transport chain from formate dehydrogenase (FdhABC) to cytochrome *c* nitrite

reductase (NrfHA) [2]. The active sites of both enzymes are located in the periplasmic space. One unit of electron transport activity (U) is equivalent to the consumption of 1 μ mol formate (2 electrons) per min. Nitrite reduction by reduced benzyl viologen was measured photometrically [21]. This activity requires only the NrfA protein. Six molecules reduced benzyl viologen need to be oxidised in order to reduce one molecule of nitrite to ammonium. To normalise the two enzyme assays on the basis of a two-electron turnover, one unit of enzyme activity is defined as the oxidation of 2 μ mol benzyl viologen per min. All activities were determined at 37°C. Protein was measured using the Biuret method with KCN [22].

Detection of NrfH by immunoblot analysis

Custom-made antibodies specifically directed against *W. succinogenes* NrfH were raised in SPF rabbits by peptide immunization (Eurogentec). The primary structures of the two NrfH peptides used were H₂N-RNHQFNDPKGASERLC-CONH₂ and H₂N-CTATPDNLGVREVK-COOH corresponding to residues 134-149 and 165-177, respectively. The additional cysteine residue at the N-terminus of the second peptide was used for covalent coupling to a carrier protein in order to enhance antibody production. For Western blot analysis, protein was transferred from an SDS-gel to a PVDF membrane by electroblotting in a discontinuous buffer system. NrfH was detected by indirect ELISA using anti-rabbit IgG coupled to horseradish peroxidase as second antibody (Cell Signaling Technology).

Construction of *W. succinogenes* *nrfH* mutant strains

All *nrfH* mutants shown in Table 1 were constructed as described previously [16]. They were obtained upon integration of pBR-N2 derivatives carrying desired mutations into the genome of *W. succinogenes* $\Delta nrfH$, thereby restoring the wild-type *nrfHAIJ* locus. Site-directed mutagenesis of *nrfH* was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and pBR-N2 as template. For each modification, a pair of complementary primers was used, of which the forward primer sequence is as follows: D38N, 5'-CCTATCTATCGAGCAAATCCAAAAGCGTGC-1230; D38L, 5'-ACTCTCCTATCTATCGAGCCTTCCAAAAGCGTGCATCAAC-1236; G87A, 5'-CCAAGGCAAGGGATGCCTGGAATCACTCCGTC-1380; G87F, 5'-AAGTATATCTCCAAGGCAAGGGATTTTTGGAATCACTCCGTCGCC-1383, Q114L,

5'-GGGGCGAGAAGAGTTCTAGAGAACTGCATCTCC-1461; E115L, 5'-GGCGAGAAGAGTTCAATTGAACTGCATCTCC-1461; N116V, 5'-CGAGAAGAGTTCAAGAGGTCCTGCATCTCCTGTC-1465; H46L, 5'-AGCGTGCATCAACTGTCTTGTCATGAATCCGCAAT-1258; H70L, 5'-CTGTGTCGAATGTCTCCTCCCCACGGGTA-1327; H121L, 5'-CTGCATCTCCTGTCTCGCCTCTCTCTCCT-1480; H153L, 5'-TGTGCTGGGAGTGCCTCAAAGTGTTCCACAC-1578. Altered nucleotides are printed in bold and modified codons are underlined. The numbers drawn at the 3'-ends of the sequences correspond to the nucleotide positions given in the EMBL, GenBank and DDBJ data base entry with accession number AJ245540. The deletion of the codons for Phe-22, Val-23, Tyr-24 (Δ 22-24) and those for Val-26, Asn-27 (Δ 26-27) within the N-terminal transmembrane segment of NrfH was achieved by two consecutive rounds of site-directed mutagenesis as described above using the following forward primers: Δ 22-24, 5'-CCATCGCTTTGGGCTTGCTGGTGAATGCCTCC-1191 and Δ 26-27, 5'-CGCTTTGGGCTTGCTGGCCTCCAAGGCACTC-1200 (*cf.* Fig. 2).

To replace the N-terminal transmembrane segment of NrfH by a signal peptide similar to that of NrfA in strain NrfH (NrfA leader), a fragment of pBR-N2 was amplified by PCR using the following divergent primer pair; 5'-GAAGATCT**CAC**ATTCCCTCCTTCAGATGGTAAAACC (*Bgl*III restriction site underlined, complementary *nrfH* start codon shown in bold) and 5'-GCTCTAGAG**GCA**CTCTCCTATCTATCGAGCGATCC (*Xba*I site underlined, *nrfH* codon 31 in bold). The generated fragment (7.2 kb) represents the entire plasmid but lacks the partial *nrfH* sequence encoding residues 2-30. A double-stranded nucleotide stretch containing codons for residues 4 to 22 of NrfA as well as overhanging single-stranded regions (in italics) identical to those obtained after restriction with either *Bgl*III or *Xba*I was generated by annealing the following two primers; 5'-GATCTTTTAAACTTCTCTTGGCTGGCAGCTTGGTGGCTATCGTGAGCATGGGAC TTTTGGCGT (forward primer) and 5'-CTAGACGCCAAAAGTCCCATGCTCACGATAGCCACCAAGCTGCCAGCCAAGAG AAGTTTAAAA (reverse primer). Subsequently, the hybridisation product was ligated to the PCR product that had been restricted with *Bgl*III and *Xba*I. In the resulting

plasmid two codons are added at either end of the *nrfA* sequence due to the cloning procedure (the corresponding residues are boxed in Fig. 2).

Transformation of *W. succinogenes* $\Delta nrfH$ cells with the constructed plasmids was carried out by electroporation [23]. Transformants were selected in medium containing formate and nitrate, kanamycin (25 mg/l) and chloramphenicol (12.5 mg/l). The desired integration of the plasmid into the genome was confirmed by PCR using suitable primer pairs. Each mutation was verified by sequencing the entire *nrfH* gene from a PCR fragment.

Homology modelling

Based on an amino acid sequence alignment, the sequence of *W. succinogenes* NrfH was modelled onto the tertiary structure of *D. vulgaris* NrfH (PDB-ID 2J/A, chain C) using the SWISS-MODEL web server [24]. The alignment contained a total of four insertions within the *W. succinogenes* sequence. Three insertions were of a length of one residue (Lys-42, Arg-65 and Asn-77; *W. succinogenes* NrfH numbering), while the third, located around the distal side of haem IV, comprised 12 residues (Asn-133 through Lys-144). The distal ligand of haem I, His-90, was manually rebuilt to coordinate the haem iron with a typical bond distance of 2.1 Å. All figures showing protein structures were prepared using PyMOL (DeLano Scientific).

RESULTS AND DISCUSSION

Characterization of the menaquinol binding site and the transmembrane segment of *W. succinogenes* NrfH

Based on the *D. vulgaris* NrfH crystal structure, Rodrigues *et al.* [19] proposed the location of an active site for menaquinol oxidation by manually docking a menaquinol head group into a cavity near haem 1 which is likely to represent a menaquinol binding pocket (Fig. 1A, B). According to this model, the menaquinone hydroxyl groups are hydrogen-bonded to the side chains of residues Asp-89 and Lys-82, both located on a short α -helix predicted to be oriented nearly parallel to the membrane plane (Fig. 1C). These two residues correspond to His-90 and Lys-83 of *W. succinogenes* NrfH, both of which have been shown previously to be essential for growth by nitrite respiration and for the catalysis of electron transport from formate to nitrite [6]. In analogy to *D. vulgaris* NrfH, His-90 of *W. succinogenes* is likely to act as distal haem 1 iron ligand (note that an aspartate is found at this position in about one

fourth of all available NrfH sequences and that its side chain is apparently not suitable to act as a haem *c* iron ligand). The proximal haem 1 ligand of *D. vulgaris* NrfH is Met-49, organized in a CX₂CHXM motif, and the corresponding residue of *W. succinogenes* NrfH (Met-48) has also been demonstrated to be functionally essential [6]. From the *D. vulgaris* NrfH structure it can be inferred that efficient menaquinol binding is likely to depend on the small side chain of Gly-86 which leaves enough space for the menaquinol head group to enter the binding pocket (Fig. 1C). To investigate this prediction, the corresponding Gly-87 of *W. succinogenes* NrfH was replaced either by the slightly larger alanine or by bulky phenylalanine. Cells producing the G87A variant catalysed electron transport from formate to nitrite at a rate amounting to 62% of the wild-type activity while the G87F substitution led to a residual activity of only 9% (Table 1). This deficiency was also reflected by the growth properties of both mutants (Table 1). Cells of both mutants contained wild-type amounts of NrfH and NrfA, as shown by immunoblot analysis and NrfA-dependent nitrite reduction activity using reduced benzyl viologen as electron donor, and the formation of the membrane-bound NrfHA complex was apparently not impaired (Table 1; Fig. 3A). These properties were also found previously using cells producing other NrfH variants [6]. A homology model for NrfH from *W. succinogenes*, based on the *D. vulgaris* NrfH structure, indicates that the deep binding cavity for menaquinol in close proximity to haem I is structurally conserved (Fig. 1D). The entrance to the cavity is lined by Gly-87, Lys-83 and haem I, the putative immediate redox partner for menaquinol. The exchange of the distal aspartate ligand of haem I in *D. vulgaris* NrfH for His-90 in *W. succinogenes* is not expected to affect electron transfer from menaquinol, but it might play a role for its binding mode within the cavity. Taken together, the results are in line with the assumption that the *W. succinogenes* NrfH menaquinol oxidation site has a similar architecture as that proposed for *D. vulgaris* NrfH. If correct, the alanine and phenylalanine side chains introduced at position 87 are apparently flexible enough to not fully prevent menaquinol binding. Unfortunately, residues involved in the formation of quinone/quinol-binding sites in respiratory proteins cannot be accurately predicted due to the absence of conserved amino acid sequence motifs. Accordingly, structurally confirmed quinone/quinol-binding sites show a rather diverse architecture (discussed for example in [4, 19, 25]) and, ultimately, more structural evidence is needed to confirm our current model on NrfH-type quinol dehydrogenases.

In *W. succinogenes*, the mechanism of formate-dependent nitrite respiration was elucidated experimentally by reconstituting the electron transport chain consisting of formate dehydrogenase (FdhABC), menaquinone-6 and cytochrome *c* nitrite reductase (NrfHA) in proteoliposomes [15]. It was demonstrated that the proton motive force generated during nitrite respiration is generated exclusively by formate oxidation coupled to menaquinone reduction (accomplished by a redox loop mechanism) whereas menaquinol oxidation by nitrite is electroneutral [2, 15]. The latter finding indicates that the two protons generated during menaquinol oxidation are released towards the periplasmic side of the membrane where they compensate the amount of protons taken up by NrfA in order to reduce nitrite. Electroneutral nitrite reduction by menaquinol is supported by the crystal structure of the *D. vulgaris* NrfHA complex that effectively rules out known mechanisms of proton motive force generation such as proton pumping or a quinone cycle. Concerning the pathway the protons may take to leave the menaquinone binding pocket, Rodrigues *et al.* [19] hypothesised that Asp-38, located at the C-terminal end of the transmembrane α -helix (Fig. 1C), might be involved in this process as its side chain is in hydrogen-bonding distance to Lys-82. Asp-38 of *W. succinogenes* NrfH, however, is not essential for nitrite respiration as two variant enzymes (D38N and D38L) were not adversely affected in either growth, electron transport catalysis or NrfH content (Table 1; Fig. 3A). Therefore, protons deriving from menaquinol oxidation in *W. succinogenes* may possibly enter the periplasmic space directly via Lys-83 and His-90.

Prior to the publication of the *D. vulgaris* NrfHA structure, the functional participation of the N-terminal transmembrane segment in quinone/quinol turnover of NapC/NrfH-type proteins was often assumed despite the fact that variants of NapC and CymA were shown to be capable of quinol oxidation even in the absence of the membrane anchor (see Introduction). Regarding the *D. vulgaris* NrfH structure and the above mentioned modification of Asp-38 in *W. succinogenes* NrfH, it seemed conceivable that the transmembrane segment is only structurally but not functionally significant. This hypothesis was tested by successively truncating the transmembrane domain to produce NrfH variants lacking 3 or 5 residues (Fig. 2). Deletion of residues 22 to 24 did not influence the membrane attachment of the NrfHA complex although the electron transport activity from formate to nitrite in the corresponding mutant was only about 60% of that of wild-type cells (Table 1). In

contrast, the additional deletion of residues 26 and 27 apparently prevented complex formation between NrfH and NrfA as active NrfA resided in the soluble cell fraction while NrfH was still detectable in the membrane fraction by immunoblot analysis (Table 1; Fig. 3B). These results suggest that the N-terminal transmembrane domain is essential for complex formation between NrfH and NrfA. According to this model, NrfH is first adjusted in the membrane, followed by haem *c* attachment and subsequent NrfHA complex formation. The crystal structure of *D. vulgaris* NrfH shows that the transmembrane domain is tilted relative to the membrane plane implying that more than twenty residues contribute to α -helix formation (note that residues 1-13 are not resolved in the structural model). In case of *W. succinogenes* NrfH, the loss of five residues from that stretch might therefore straighten up the orientation of the α -helix in a way that it becomes more perpendicular to the membrane which could negatively affect the binding of the NrfA monomer (or dimer) as well as the dimerization of the NrfHA (or NrfHA₂) assembly.

Although the N-terminal 23 residues of NrfH from mutant NrfH Δ 22-24 Δ 26-27 resemble those of the Sec-dependent signal peptide of NrfA (Fig. 2), the electrophoretic mobility of the variant NrfH suggests that the protein is not processed (Fig. 3A). It remains unclear how the Sec system discriminates in this case between a genuine signal peptide and a similar structure that eventually forms an N-terminal transmembrane segment. Using a different approach, we aimed at the production of a soluble NrfHA complex by replacing the transmembrane region of NrfH by the entire NrfA signal peptide (Fig. 2). In the resulting mutant, NrfA was found in the soluble cell fraction but NrfH was not detectable by immunoblot analysis (Table 1). The reason for the absence of the modified NrfH is not known. Possibly, it was not correctly dealt with by either the Sec system or the cytochrome maturation apparatus (see below) or, if soluble holo-NrfH was formed, it might have been degraded due to misfolding. Taken together, these results imply that the transmembrane domain is required for the production of active NrfH and that its length is crucial for enzyme function and/or NrfHA complex formation.

Role of the canonical histidine residue in NrfH haem *c* binding motifs

Bacterial cytochrome *c* maturation is accomplished by proteins that belong to complex enzyme systems [7,8,26]. The most widespread systems are known as system I (or Ccm system; e.g. in *E. coli*) and system II (or Ccs system, e.g. in *W.*

succinogenes, *Campylobacter* and *Bordetella* species). These systems employ unrelated cytochrome *c* haem lyases and it is unclear how such enzymes recognise haem *c* binding motifs in apo-cytochromes. In case of *E. coli*, it was shown that both cysteines and the histidine residue of the canonical CX₂CH motif are strictly required for haem *c* attachment by the system I haem lyase [27,28]. In contrast, the system II organism *W. succinogenes* was shown to be capable to attach haem covalently to a modified SX₂CH motif in NrfH [29]. *W. succinogenes* is rather exceptional because its genome encodes three different cytochrome *c* haem lyases designated CcsA1, CcsA2 and NrfI [30]. NrfI has been shown to be involved specifically in haem attachment to the unusual CX₂CK motif that binds the haem *c* group in the active site of NrfA [17,31] while CcsA1 was suggested to attach haem *c* to both cysteine residues of a CX₁₅CH sequence in the octahaem cytochrome *c* MccA [32]. This would leave the CcsA2 enzyme to handle the standard CX₂CH motif.

Here, the histidine residue in each of the four CX₂CH motifs of *W. succinogenes* NrfH was individually replaced by leucine, an experiment that has not yet been described for a system II organism. Each of the resulting mutants did not grow by nitrite respiration and the variant NrfH was not detectable by immunoblot analysis (Table 1; Fig. 3A). Wild-type amounts of active NrfA were located almost exclusively in the soluble cell fraction (Table 1). The results suggest that the presence of the histidine residue is required by the *W. succinogenes* cytochrome *c* maturation system. Replacement of the second cysteine in any of the NrfH CX₂CH motifs was shown previously to result in mutants with similar properties while substitution of the first cysteine did not completely abolish electron transport activity and NrfHA complex formation [29]. The results further suggest that the haem lyase recognizes primarily the CH signature whereas the presence of (or the distance to) the second cysteine seems to be less important.

His-46 of *W. succinogenes* NrfH, located in the haem 1-binding CX₂CHXM motif, is apparently essential for haem *c* attachment despite the fact that the corresponding His-47 in *D. vulgaris* NrfH is displaced as haem *c* iron ligand by Met-49 in the crystal structure. A similar scenario has been also observed in an octahaem cytochrome *c* from *Shewanella oneidensis* in which a distant lysine residue replaced the histidine residue of a CX₂CH motif as haem *c* iron ligand [33]. Therefore, it appears that even in the event of subsequent haem *c* ligand switching, the histidine of the haem *c* binding motif is obligatorily required for haem attachment. Notably,

system I of *E. coli* has been shown not to attach haem to a CX₂CM motif implying that there is no cytochrome *c* haem lyase that would recognize this motif [28].

We also replaced three residues located directly upstream of the third haem *c* binding motif in *W. succinogenes* NrfH, resulting in mutants Q114L, E115L and N116V. These residues are conserved to a relatively high degree and situated in a polypeptide stretch that connects the two dihaem halves of NrfH (Fig. 1C). Mutants E115L and N116V were impaired in nitrite respiration (and in case of E115L also in the amount of produced NrfA) but not in NrfH content or NrfHA complex formation while the Q114L grew by nitrite respiration with the same doubling time but to an even higher cell density as compared to the wild-type (Table 1; Fig. 3A). The most likely explanation for the decreased electron transport activity in mutants E115L and N116V is perturbation of the electron transferring multahaem *c* arrangement.

The NrfH and NapC subgroups of the NapC/NrfH family – a reassessment

As of January 2008, 44 different NrfH sequences have been identified in protein data bases which mostly derive from ϵ - and δ -proteobacteria (like *W. succinogenes* and *D. vulgaris*) but corresponding genes have been also found in the genomes of Gram-positive bacteria, members of the Bacteroidetes/Chlorobi and Chlamydiae/Verrucomicrobia groups as well as in Planctomycetes (note that the *nrfH* gene was only classified as such when accompanied by an adjacent *nrfA* homolog). Therefore, it seems that the NrfH-type quinol dehydrogenase is more widespread than NapC as *nap* gene clusters are apparently restricted almost exclusively to Proteobacteria. The differences in the primary structures of NapC and NrfH proteins discussed by Gross *et al.* [6] were further underpinned by the wealth of new sequences as well as by the *D. vulgaris* NrfH structure. Nevertheless, *W. succinogenes* NrfH is still the only such protein for which data from site-directed modification are available and we have presented here new experimental evidence on the major structural and functional features of *W. succinogenes* NrfH. Hopefully, the elucidation of a NapC structure in the future will clarify the assumed differences within the NapC/NrfH family with respect to axial haem *c* iron ligation, especially for haems 1 and 4, and the structure of the menaquinone binding site which does not necessarily have to be similar to that of NrfH. Furthermore, a crystal structure of *W. succinogenes* NrfHA and its comparison with that of *D. vulgaris* would help to define the significance of the various primary structure differences found between them.

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REFERENCES

- 1 Richardson, D.J. (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiology* **146**, 551-571
- 2 Simon, J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol. Rev.* **26**, 285-309
- 3 Richardson, D. and Sawers, G (2002) PMF through the redox loop. *Science* **295**, 1842-1843
- 4 Gross, R., Pisa, R., Sanger, M., Lancaster, C.R.D. and Simon, J. (2004) Characterization of the menaquinone reduction site in the diheme cytochrome *b* membrane anchor of *Wolinella succinogenes* NiFe-hydrogenase. *J. Biol. Chem.* **279**, 274-281
- 5 Simon, J. and Kern, M. (2008) Quinone-reactive proteins devoid of haem *b* form widespread membrane-bound electron transport modules in bacterial respiration. *Biochem. Soc. Trans.* **36**, in press
- 6 Gross, R., Eichler, R. and Simon, J. (2005) Site-directed modifications indicate differences in axial haem *c* iron ligation between the related NrfH and NapC families of multiheme *c*-type cytochromes. *Biochem. J.* **390**, 689-693
- 7 Ferguson, S.J. (2001) Keilin's cytochromes: how bacteria use them, vary them and make them. *Biochem. Soc. Trans.* **29**, 629-640
- 8 Stevens, J.M., Daltrop, O., Allen, J.W.A. and Ferguson, S.J. (2004) C-type cytochrome formation: chemical and biological enigmas. *Acc. Chem. Res.* **37**, 999-1007
- 9 Richardson, D.J., Berks, B.C., Russell, D.A., Spiro, S. and Taylor, C.J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell. Mol. Life Sci.* **58**, 165-178
- 10 Cartron, M.L., Roldan, M.D., Ferguson, S.J., Berks, B.C. and Richardson, D.J. (2002) 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. *Biochem. J.* **368**, 425-432
- 11 Schwalb, C., Chapman, S.K. and Reid, G.A. (2003) The tetrahaem cytochrome CymA is required for anaerobic respiration with dimethyl sulfoxide and nitrite in *Shewanella oneidensis*. *Biochemistry* **42**, 9491-9497
- 12 Jepson, B.J.N., Marietou, A., Mohan, S., Cole, J.A., Butler, C.S. and Richardson, D.J. (2006) Evolution of the soluble nitrate reductase: defining the monomeric periplasmic nitrate reductase group. *Biochem. Soc. Trans.* **34**, 122-126.
- 13 Roldán, M.D., Sears, H.J., Cheesman, M.R., Ferguson, S.J., Thomson, A.J., Berks, B.C. and Richardson, D.J. (1998) Spectroscopic characterization of a novel multihem c-type cytochrome widely implicated in bacterial electron transport. *J. Biol. Chem.* **273**, 28785-28790
- 14 Field, S.J., Dobbin, P.S., Cheesman, M.R., Watmough, N.J., Thomson, A.J. and Richardson, D.J. (2000) Purification and magneto-optical spectroscopic characterization of cytoplasmic membrane and outer membrane multihem c-type cytochromes from *Shewanella frigidimarina* NCIMB400. *J. Biol. Chem.* **275**, 8815-8522
- 15 Simon, J., Gross, R., Einsle, O., Kroneck, P.M.H., Kröger, A. and Klimmek, O. (2000) A NapC/NirT-type cytochrome c (NrfH) is the mediator between the quinone pool and the cytochrome c nitrite reductase of *Wolinella succinogenes*. *Mol. Microbiol.* **35**, 686-696
- 16 Simon, J., Pisa, R., Stein, T., Eichler, R., Klimmek, O. and Gross, R. (2001) The tetraheme cytochrome c NrfH is required to anchor the cytochrome c nitrite reductase in the membrane of *Wolinella succinogenes*. *Eur. J. Biochem.* **268**, 5776-5782
- 17 Einsle, O., Stach, P., Messerschmidt, A., Simon, J., Kröger, A., Huber, R. and Kroneck, P.M.H. (2000) Cytochrome c nitrite reductase from *Wolinella succinogenes*: Structure at 1.6 Å resolution, inhibitor binding and heme-packing motifs. *J. Biol. Chem.* **275**, 39608-39616
- 18 Einsle, O., Stach, P., Messerschmidt, A., Klimmek, O., Simon, J., Kröger, A. and Kroneck, P.M.H. (2002) Crystallization and preliminary X-ray analysis of the membrane-bound cytochrome c nitrite reductase complex (NrfHA) from *Wolinella succinogenes*. *Acta Cryst. D* **58**, 341-342

- 19 Rodrigues, M.L., Oliveira, T.F., Pereira, I.A.C. and Archer, M. (2006) X-ray structure of the membrane-bound cytochrome *c* quinol dehydrogenase NrfH reveals novel haem coordination. *EMBO J.* **25**, 5961-5960
- 20 Lorenzen, J.P., Kröger, A. and Unden, G. (1993) Regulation of anaerobic respiratory pathways in *Wolinella succinogenes* by the presence of electron acceptors. *Arch. Microbiol.* **159**, 477-483
- 21 Bokranz, M., Katz, J., Schröder, I., Robertson, A.M. and Kröger, A. (1983) Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor. *Arch. Microbiol.* **135**, 36-41
- 22 Bode, C., Goebell, H. and Stähler, E. (1968) Zur Eliminierung von Trübungsfehlern bei der Eiweißbestimmung mit der Biuretmethod. *Z. klin. Chem. u. klin. Biochem.* **6**, 418-422
- 23 Simon, J., Gross, R., Ringel, M., Schmidt, E. and Kröger, A. (1998) Deletion and site-directed mutagenesis of the *Wolinella succinogenes* fumarate reductase operon. *Eur. J. Biochem.* **251**, 418-426
- 24 Schwede, T., Kopp, J., Guex, N. and Peitsch, M.C. (2003) SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.* **31**, 3381-3385
- 25 Lancaster, C.R.D., Gross, R., Haas, A., Ritter, M., Mäntele, W., Simon, J. and Kröger, A. (2000) Essential role of Glu-C66 for menaquinol oxidation indicates transmembrane electrochemical potential generation by *Wolinella succinogenes* fumarate reductase. *Proc. Natl. Acad. Sci. USA* **97**, 13051-13056
- 26 Kranz, R., Lill, R., Goldman, B., Bonnard, G. and Merchant, S. (1998) Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. *Mol. Microbiol.* **29**, 383-396
- 27 Allen, J.W.A., Tomlinson, E.J., Hong, L. and Ferguson, S.J. (2002) The *Escherichia coli* cytochrome *c* maturation (Ccm) system does not detectably attach heme to single cysteine variants of an apocytochrome *c*. *J. Biol. Chem.* **277**, 33559-33563
- 28 Allen, J.W.A., Leach, N. and Ferguson, S.J. (2005) The histidine of the *c*-type cytochrome CXXCH haem-binding motif is essential for haem attachment by the *Escherichia coli* cytochrome *c* maturation (Ccm) apparatus. *Biochem. J.* **389**, 587-592

- 29 Simon, J., Eichler, R., Pisa, R., Biel, S. and Gross, R. (2002) Modification of haem c binding motifs in the small subunit (NrfH) of the *Wolinella succinogenes* cytochrome c nitrite reductase complex. FEBS Lett. **522**, 83-87
- 30 Hartshorne, S., Richardson, D.J. and Simon, J. (2006) Multiple haem lyase genes indicate substrate specificity in cytochrome c biogenesis. Biochem. Soc. Trans. **34**, 146-149
- 31 Pisa, R., Stein, T., Eichler, R., Gross, R. and Simon, J. (2002) The *nrfI* gene is essential for the attachment of the active site haem group of *Wolinella succinogenes* cytochrome c nitrite reductase. Mol. Microbiol. **43**, 763-770
- 32 Hartshorne, R.S., Kern, M., Meyer, B., Clarke, T.A., Karas, M., Richardson, D.J. and Simon, J. (2007) A dedicated haem lyase is required for the maturation of a novel bacterial cytochrome c with unconventional haem binding. Mol. Microbiol. **64**, 1049-1060
- 33 Mowat, C.G., Rothery, E., Miles, C.S., McIver, L., Doherty, M.K., Drewette, K., Taylor, P., Walkinshaw, M.D., Chapman, S.K. and Reid, G.A. (2004) Octaheme tetrathionate reductase is a respiratory enzyme with novel heme ligation. Nat. Struct. Mol. Biol. **11**, 1023-1024

Table 1 Properties of *W. succinogenes* wild-type and *nrfH* mutants

The cells were grown in minimal medium containing formate (50 mM) and nitrate (10 mM) as energy substrates in the presence of 5 mM fumarate as carbon source and 10 mM ammonium to ensure growth even in the absence of nitrite reduction to ammonium. To grow cells for subsequent NrfH immuno-detection or determination of BV-dependent enzyme activity, the medium was supplemented with Brain-Heart-Infusion broth (0.5%, w/v). After nitrate has been completely reduced to nitrite, a growth of a batch culture is solely dependent on nitrite respiration, thus allowing to estimate the capability of growth by nitrite respiration from the growth curve. The optical density at 578 nm (OD₅₇₈) of wild-type cells entering the stationary growth phase was typically about 0.45 (corresponding to 100%), while that of the stopH mutant was 0.22, resulting from nitrate and fumarate respiration (set as 0%). The *nrfH* gene in the control strain *W. succinogenes* stopH is inactivated by the introduction of two consecutive stop codons [16]. Representative values are presented that derived from at least two independent cultures of each strain.

| Strain | Growth by nitrite respiration (%) | Electron transport activity formate → nitrite [U/mg protein] | Specific nitrite reductase activity BV _{red} → nitrite [U/mg protein] | | |
|-----------|-----------------------------------|--|--|-------------------|------------------|
| | | | Cell homogenate | Membrane fraction | Soluble fraction |
| Wild-type | = 100 | 3.5 | 41 | 27 | 34 |
| stopH | = 0 | < 0.01 | 31 | 0.5 | 48 |
| D38N | 95 | 3.2 | 33 | 31 | 34 |
| D38L | 100 | 3.3 | 39 | 37 | 45 |
| G87A | 59 | 2.2 | 40 | 32 | 47 |
| G87F | 14 | 0.3 | 41 | 39 | 44 |
| Q114L | 150 | 3.5 | 38 | 25 | 31 |
| E115L | 26 | 0.6 | 18 | 14 | 14 |
| N116V | 25 | 0.3 | 39 | 30 | 22 |

| | | | | | |
|------------------------------------|----|--------|----|---|----|
| H46L | 0 | < 0.01 | 35 | 2 | 60 |
| H70L | 0 | < 0.01 | 36 | 3 | 52 |
| H121L | 0 | < 0.01 | 37 | 3 | 48 |
| H153L | 0 | < 0.01 | 37 | 4 | 52 |
| NrfH Δ 22-24 | 78 | 2.2 | 35 | 1 | 32 |
| NrfH Δ 22-24 Δ 26-27 | 0 | 0.07 | 24 | 2 | 28 |
| NrfH (NrfA leader) | 0 | 0.06 | 28 | 4 | 39 |

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FIGURE LEGENDS

Figure 1 Structural features of *D. vulgaris* NrfH and homology model of *W. succinogenes* NrfH

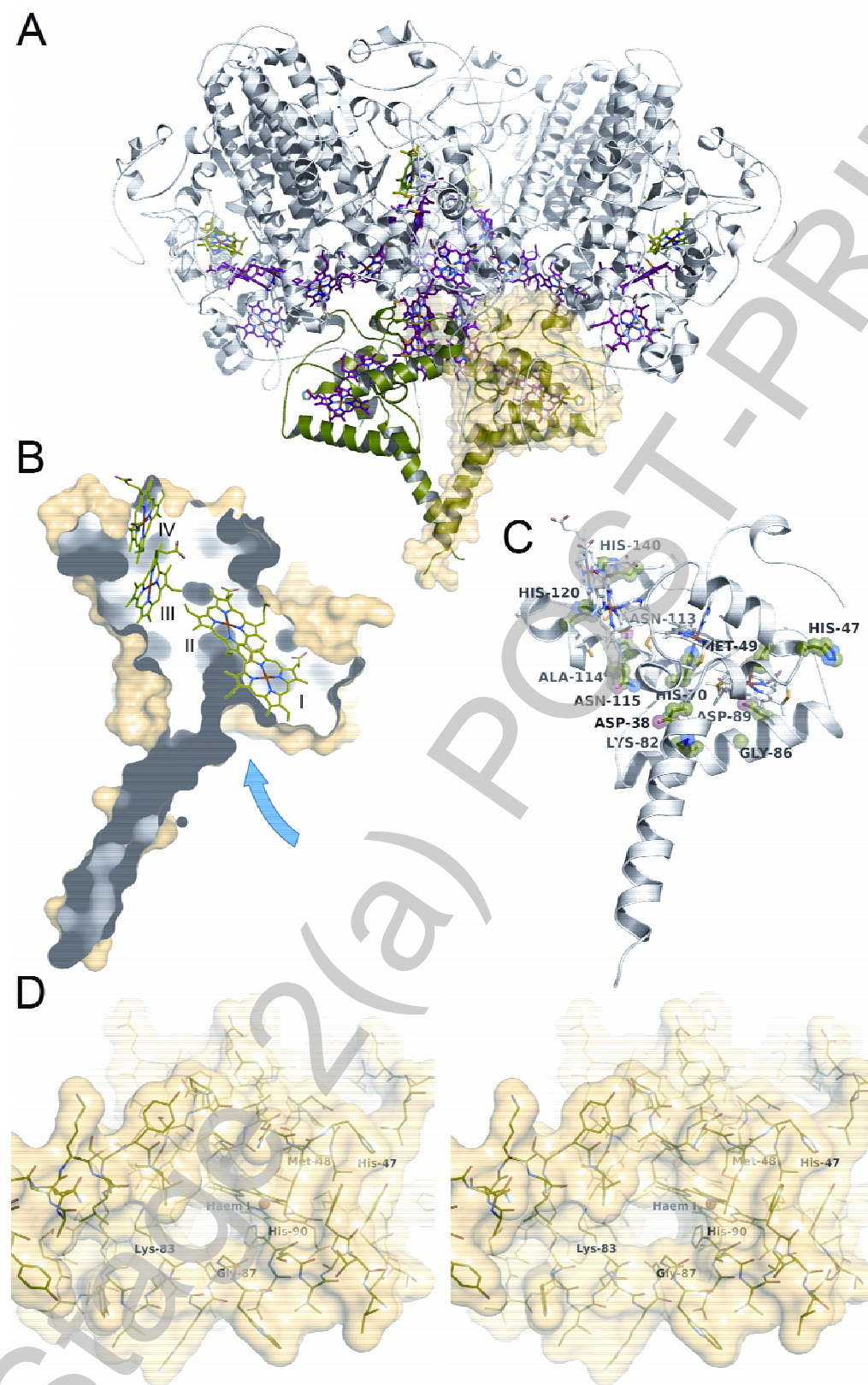
(A) The (NrfHA₂)₂ complex of *D. vulgaris* (PDB ID 2J7A) in cartoon representation. NrfH is shown in green, NrfA in gray, with the haem groups in stick representation in red (electron transfer haems) and green (active site haems). A molecular surface is shown for one NrfH monomer (yellow). (B) Surface representation of *D. vulgaris* NrfH displaying the location of the four haem groups and the deep binding cavity for menaquinol (arrow) that is only accessible from within the membrane. Haem groups are numbered I-IV according to their sequence of attachment to the protein chain. (C) Structure of *D. vulgaris* NrfH showing residues at positions that were subject to site-directed modification in *W. succinogenes* NrfH, as discussed in the text. (D) Homology model of *W. succinogenes* NrfH. The stereo representation shows a view from within the membrane into the binding cavity for menaquinol, rotated 90° with respect to the orientation shown in (B). The entrance of the cavity is lined by Gly-87, Lys-83 and haem I.

Figure 2 N-terminal primary structures of *W. succinogenes* NrfH (wild-type and variants) and NrfA

The doubly underlined residues of NrfH were successively deleted resulting in variants NrfH Δ 22-24 and NrfH Δ 22-24 Δ 26-27. The third NrfH variant structure carries residues 4-22 of the NrfA signal peptide (residues identical to NrfA are underlined). The boxed residues were introduced due to the cloning procedure. The aspartate printed in bold indicates the terminus of the transmembrane segment as adopted from the *D. vulgaris* NrfH structure. Positively charged residues typically found in Sec-dependent signal peptides are shaded. The experimentally determined leader peptide cleavage site of NrfA is indicated by a vertical arrow [15].

Figure 3 Detection of NrfH by immunoblot analysis using an anti-NrfH peptide antiserum

(A) NrfH detection in cell homogenates (100 μ g protein) of *W. succinogenes* wild-type and mutants producing NrfH variants. (B) Localisation of NrfH in the membrane fraction (MF) of mutant *W. succinogenes* Δ 22-24 Δ 26-27. 100 μ g protein were applied to each lane. CH, cell homogenate; SF, soluble cell fraction.

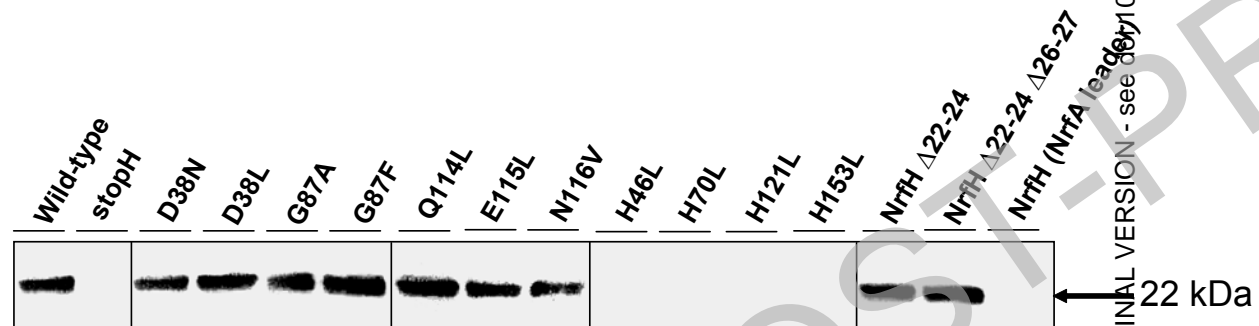
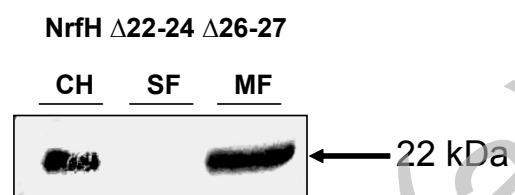
Kern *et al.*, Fig. 1

Kern *et al.*, Fig. 2

| | |
|--------------------|--|
| NrfH | VNKSFLVYSSLVVFAIALGLFVYLVNASKALSYLSSD-38 |
| NrfH Δ22-24 | VNKSFLVYSSLVVFAIALGLLVNASKALSYLSSD-35 |
| NrfH Δ22-24 Δ26-27 | VNKSFLVYSSLVVFAIALGLLASKALSYLSSD-33 |
| NrfH (NrfA leader) | VRSEKLLLAGSLVAIVSMGLLASRALSYLSSD-32 |
| <hr/> | |
| NrfA | MTKFKLLLAGSLVAIVSMGLLASNINEREKER-32 |

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