

Modification of heme *c* binding motifs in the small subunit (NrfH) of the *Wolinella succinogenes* cytochrome *c* nitrite reductase complex

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Abstract The two multiheme *c*-type cytochromes NrfH and NrfA form a membrane-bound complex that catalyzes menaquinol oxidation by nitrite during respiratory nitrite ammonification of *Wolinella succinogenes*. Each cysteine residue of the four NrfH heme *c* binding motifs was individually replaced by serine. Of the resulting eight *W. succinogenes* mutants, only one is able to grow by nitrite respiration although its electron transport activity from formate to nitrite is decreased. NrfH from this mutant was shown by matrix-assisted laser desorption/ionization mass spectrometry to carry four covalently bound heme groups like wild-type NrfH indicating that the cytochrome *c* biogenesis system II organism *W. succinogenes* is able to attach heme to an SXXCH motif. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cytochrome *c* nitrite reductase; Respiratory nitrite ammonification; Heme *c* binding motif; NapC/NirT family; Cytochrome *c* biogenesis; *Wolinella succinogenes*

1. Introduction

Wolinella succinogenes can grow by respiratory nitrite ammonification using formate as electron donor [1,2]. The electron transport chain catalyzing this reaction consists of two membrane-bound enzyme complexes and menaquinone. The formate dehydrogenase complex catalyzes the reduction of menaquinone by formate, and menaquinol oxidation by nitrite is catalyzed by the cytochrome *c* nitrite reductase complex. The former reaction is known to be coupled to the generation of an electrochemical proton potential [3–5], whereas menaquinol oxidation by nitrite appears to be an electroneutral process [2,6,7]. The cytochrome *c* nitrite reductase complex consists of two *c*-type cytochromes, the pentaheme catalytic subunit NrfA and the tetraheme subunit NrfH [6–9]. NrfH anchors the complex in the bacterial membrane and mediates electron transport from menaquinol to NrfA [6,7].

NrfH and NrfA are encoded by the first two genes of the *nrfHAIJ* operon [6]. The *nrfI* gene product was shown to be required for the attachment of the active site heme group to NrfA [10] while the function of the *nrfJ* gene is unknown. A $\Delta nrfJ$ deletion mutant of *W. succinogenes* had wild-type properties with respect to nitrite respiration [6]. Recently, a genetic

system has been described that allows expression of mutated *nrfH* alleles in *W. succinogenes* [7]. The procedure is based on the integration of a *nrfH*-containing plasmid into the genome of the *W. succinogenes* $\Delta nrfH$ deletion mutant thus restoring the *nrfHAIJ* operon.

NrfH belongs to the NapC/NirT family of multiheme *c*-type cytochromes [2,7,11]. Members of this protein family are commonly found in bacteria and are generally thought to mediate electron transport from a respiratory quinone to a periplasmic oxidoreductase. To date, no high-resolution structure of any member of the NapC/NirT family is available although crystals of the *W. succinogenes* NrfHA complex have been obtained recently [12]. Furthermore, no data on site-directed modification of any NapC/NirT-like cytochrome have yet been reported. NrfH contains four heme *c* binding motifs (CXXCH) to which heme is covalently attached by forming two thioether bonds [7]. The detailed function of the four heme groups is unclear. It is not known how they are arranged in the protein or whether all four hemes are involved in electron transfer from menaquinol to NrfA. In this communication, the role of the NrfH heme groups was investigated by replacing the cysteine residues of the heme *c* binding motifs.

2. Materials and methods

2.1. Growth of *W. succinogenes*, cell fractionation and purification of the nitrite reductase complex

Strains of *W. succinogenes* were grown with formate and nitrate as described [13] but $(\text{NH}_4)_2\text{SO}_4$ (5 mM) was present in the medium instead of K_2SO_4 . The medium was supplemented with brain-heart-infusion broth (0.5%, w/v) which was only left out when the capability of growth by nitrite respiration was examined. The doubling time for growth with formate and nitrite was estimated from the growth curve of a batch culture containing 50 mM formate and 10 mM nitrate. Under these conditions, growth is solely dependent on nitrite respiration after nitrate has been completely reduced to nitrite. *W. succinogenes* cells harvested in the exponential growth phase were suspended (10 g cell protein/l) in an anoxic buffer (pH 8.0) containing 50 mM Tris-HCl and 1 mM dithiothreitol. 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 45 min at $100\,000\times g$ to yield the membrane fraction (sediment) and the soluble fraction. The NrfHA complex was purified from the membrane fraction as described [7].

2.2. Determination of specific activities

All activities were determined at 37°C. Nitrite reduction by reduced benzyl viologen was measured photometrically [1]. One unit of enzyme activity (U) was equivalent to the oxidation of 2 μmol reduced benzyl viologen per minute. Nitrite reduction by 2,3-dimethyl-1,4-naphthoquinol (DMNH₂) was measured photometrically by recording

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Abbreviations: DMNH₂, 2,3-dimethyl-1,4-naphthoquinol

Table 1
Oligonucleotide primers used for site-directed mutagenesis of the *nrhH* gene in pBR-N2 [7]

Modification in NrhH	Forward primer sequence (5' → 3')
C42S	CGAGCGATCCAAAAGCG AG CATCAACTGTCATGTCATGAATCC-1253
C45S	GCGTGCATCAACT CT CATGTCATGAATCC-1253
C66S	GAGAGAGCCTCC CT GTGCGAATGTCACC-1315
C69S	CCTGTGTGGA CT CACCTCCCCACGGG-1325
C66S/C69S	CC CT GTGCGAAT CT CACCTCCCCACGGG-1325
C117S	GAGTTCAAGAGAA AG CATCTCCTGTCACG-1468
C120S	GAGAACTGCATCTCCT CT CACGCCTCTCTCTCC-1479
C149S	CGCATCCGAGAGGTG AG CTGGGAGTGCC-1561
C152S	GGTTGTGCTGGGAG AG CCACAAAAGTGTTC-1574

A pair of complementary primers was used for each modification of which only the forward primer is shown. The plasmid with modified codons 66 and 69 was constructed using the derivative of pBR-N2 as template that already contained the modification in codon 66. The altered nucleotides are printed in bold and the 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.

2,3-dimethyl-1,4-naphthoquinone formation [6]. One unit of activity is equivalent to the oxidation of 1 μ mol DMNH₂ per minute. The activity of electron transport from formate to nitrite was determined as described [7]. One unit of electron transport activity is equivalent to the consumption of 1 μ mol formate per minute. Protein was measured using the Biuret method with KCN [14].

2.3. Construction of *W. succinogenes* strains containing a mutated *nrhH* gene

All mutant strains were constructed as described previously for strain N2 [7]. Strain N2 was obtained by integration of plasmid pBR-N2 into the genome of *W. succinogenes* Δ *nrhH* thus restoring a wild-type copy of the *nrhH* locus. Site-directed mutagenesis of *nrhH* in pBR-N2 was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using pBR-N2 as template and specifically synthesized primer pairs (Table 1). Transformation of *W. succinogenes* Δ *nrhH* cells with plasmids was achieved by electroporation [15]. Transformants were selected in a 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 examined by PCR using suitable primer pairs. Each mutation was confirmed by sequencing the entire *nrhH* gene from a PCR fragment.

3. Results

NrhH contains eight cysteine residues that are arranged in four heme *c* binding motifs (CXXCH). Each cysteine was individually replaced by a serine residue and the corresponding *W. succinogenes* mutants were designated according to their NrhH modification (C42S, C45S, C66S, C69S, C117S, C120S, C149S and C152S). Furthermore, both cysteines of the second heme *c* binding motif were substituted by serine residues resulting in strain *W. succinogenes* C66S/C69S.

Of the nine mutants, only mutant C66S grew by nitrite respiration (Table 2), but the corresponding doubling time for growth with nitrite (10.2 h) was more than five times longer than that of the wild-type strain (1.8 h). The specific electron transport activity from formate to nitrite of mutant C66S amounted to 19% of the wild-type activity (Table 2). Nevertheless, a membrane potential of -150 mV (negative inside) was generated by the electron transport as estimated from the amount of tetraphenylphosphonium (TPP⁺) taken up by the mutant cells in the steady state of nitrite respiration (Fig. 1). The value of the potential is close to that obtained with wild-type cells (-160 mV) [6]. The other three mutants with SXXCH motifs (C42S, C117S and C149S) do not grow by nitrite respiration and their electron transport activity from formate to nitrite amounted to 6%, 2% and 3% of the wild-type activity, respectively (Table 2). All four mutants containing a CXXSH motif (C45S, C69S, C120S and C152S) as well

as mutant C66S/C69S did not catalyze electron transport from formate to nitrite, thus resembling mutant stopH (Table 2).

The large subunit of the nitrite reductase complex (NrhA) catalyzes nitrite reduction by reduced benzyl viologen even in the absence of NrhH [6,7]. The corresponding specific activity in the cell homogenate of all nine cysteine mutants was in the same range as those of the wild-type strain (Table 2). The presence of wild-type amounts of NrhA in each mutant was also demonstrated by immunoblot analysis and by heme staining (not shown). In wild-type cells and cells of strain N2, the majority of nitrite reductase activity is located in the membrane fraction. The residual activity found in the soluble cell fraction was shown previously to be catalyzed by periplasmic NrhA [6,7]. The stopH mutant contained almost all of the activity in the soluble cell fraction indicating

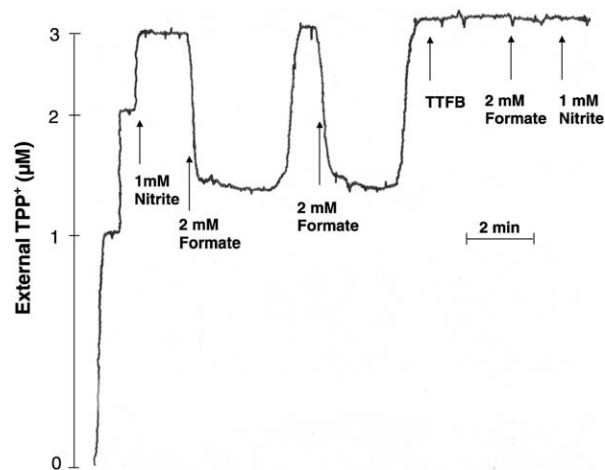


Fig. 1. TPP⁺ uptake by cells of *W. succinogenes* C66S. The cells were grown with formate and nitrate, harvested in the exponential growth phase and washed twice with an N₂-saturated buffer (pH 7.5) containing 50 mM HEPES and 0.5 M mannitol. Subsequently, the cells were suspended (0.7 g cell protein/l) in the same buffer and were incubated for 10 min at 37°C. The TPP⁺ electrode [16,17] was calibrated upon addition of TPP⁺ (3×10^{-6} M). Cells were observed to take up TPP⁺ when the electron transport was started upon addition of nitrite and formate. After consumption of one of the substrates, TPP⁺ was liberated into the medium. The cycle of TPP⁺ uptake and release could be repeated by adding the missing substrate and the duration of the cycle was consistent with the electron transport activity. The membrane potential was calculated as described previously [5,6]. The cycle was abolished in the presence of the protonophore 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB; 30 μ mol/g protein).

Table 2
Properties of *W. succinogenes* strains

Strain	Growth by nitrite respiration	Electron transport activity formate → nitrite (U/mg dry cell weight)	Specific nitrite reductase activity reduced benzyl viologen → nitrite (U/mg cell protein)			NrfH present ^a
			Cell homogenate	Membrane fraction	Soluble fraction	
Wild-type	+	3.9	30	24	9.0	+
stopH	—	< 0.01	34	0.3	36	n.d.
N2	+	3.8	36	23	7.0	+
C42S	—	0.25	28	4.3	22	+
C66S	+	0.74	32	26	11	+
C117S	—	0.09	26	21	6.0	+
C149S	—	0.11	28	21	6.0	+
C45S	—	< 0.01	31	3.2	29	n.d.
C69S	—	< 0.01	36	2.6	28	n.d.
C66S/C69S	—	< 0.01	29	4.0	34	n.d.
C120S	—	< 0.01	23	2.1	23	n.d.
C152S	—	< 0.01	32	3.1	26	n.d.

The cells were grown in a medium containing formate and nitrate as energy substrates in the presence of ammonium. The growth of cells incapable of nitrite respiration ceased after complete reduction of nitrate to nitrite. The membrane fraction was washed with an anoxic buffer (pH 8.0) containing 50 mM Tris-HCl and 0.5 M mannitol. Strain N2 contains the restored wild-type *nrfHAIJ* operon whereas the *nrfH* gene is inactivated in strain stopH by the introduction of consecutive stop codons [7].

^aThe presence of the NrfH protein in the membrane fraction was judged from heme stain experiments, +: NrfH present in wild-type amounts, n.d.: NrfH not detected.

that NrfH is the membrane anchor of the NrfHA complex [7]. Wild-type distribution of nitrite reductase activity was found in strains C66S, C117S and C149S. Although most of the activity was detected in the soluble fraction of the other *nrfH* mutants, the activity in the membrane fraction was 7–14-fold higher than that of the stopH mutant and was retained after washing the membrane fraction (Table 2). This result suggests that a certain amount of the NrfHA complex is present in the membrane of every mutant with a modified CXXCH motif. However, some of the modifications seem to severely affect the stability of the NrfHA complex. The NrfH protein was detected in the cell homogenate of strains C42S, C66S, C117S and C149S by staining an SDS–polyacrylamide gel for covalently bound heme (Table 2). Using the same procedure NrfH was not detected in mutants containing a CXXSH motif or in mutant C66S/C69S. This might be due to a decreased amount of NrfH and/or to a decreased heme content.

It was possible to isolate the NrfHA complex from the membrane fraction of mutant C66S, but not from mutants C117S and C149S, probably owing to insufficient stability of the complex. The specific activity of reduced benzyl viologen oxidation by nitrite of the preparation from mutant C66S (360 U/mg protein) was about three times lower than that of the wild-type enzyme complex. The NrfHA complex from mutant C66S catalyzed the oxidation of DMNH₂ by nitrite at 0.14 U/mg protein while the activity of the wild-type NrfHA complex was 1.9 U/mg protein. DMNH₂ is a water soluble menaquinol analogue that reacts with the NrfHA complex but not with NrfA [6]. The decreased activity of nitrite reduction by DMNH₂ is in line with the decrease in electron transport activity from formate to nitrite of mutant C66S (Table 2). The activity of DMNH₂ oxidation by nitrite was determined only for purified NrfHA complexes as it is too low to be accurately measured with intact cells or membrane fractions. The nitrite reductase preparation from mutant C66S consisted mainly of two *c*-type cytochromes that corresponded in size to NrfH and NrfA as judged by SDS–PAGE followed by Coomassie or heme staining. The preparation was analyzed by

delayed-extraction matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described previously for the wild-type NrfHA complex [7]. The mass spectrum was essentially identical to that of the wild-type NrfHA complex (see figure 2 in [7]). The two most prominent peaks in the spectrum corresponded to NrfH and NrfA containing, respectively, four and five heme groups (not shown). The precision of mass determination (± 10 Da for a protein of the size of NrfH), however, is not sufficient to verify the expected mass difference of 16 Da resulting from the cysteine to serine substitution in NrfH. To check for the possible presence of non-covalently bound heme, the enzyme preparation was treated with 2% (v/v) HCl in acetone, followed by diethyl ether extraction. No heme was spectrophotometrically detected in the ether phase (not shown). Furthermore, the masses of NrfH and NrfA were not altered after such treatment indicating that all four heme groups of the variant NrfH are covalently attached. The mass spectrum did not contain any signals that would correspond to NrfH species carrying less than four heme groups.

4. Discussion

4.1. Modification of NrfH

NrfH from mutant C66S was shown to form the complex with NrfA and to contain all four covalently bound heme groups. Most likely one heme is bound to the cysteine residue of the SXXCH motif via a single thioether bond. Although not experimentally proven, this might also hold true for the NrfH proteins from mutants C42S, C117S and C149S since the modification of any of the N-terminal cysteine residues within a CXXCH motif did not completely abolish electron transport from formate to nitrite. In contrast, the presence of a CXXSH motif in NrfH of mutants C45S, C69S, C120S and C152S prevented electron transport despite the fact that a portion of NrfA remains membrane-bound, probably by the modified NrfH protein. It is possible that heme is not attached to any CXXSH motif thus resulting in NrfH proteins with three heme *c* groups at the most. If true, electron transport

from menaquinol to NrfA would require the presence of all four NrfH heme *c* groups.

There is no simple explanation for the decreased electron transport activity from formate to nitrite in mutants with an SXXCH motif, especially when the presence of all four hemes and a three-dimensional structure similar to wild-type NrfH is assumed. Possibly, the position of one or more heme groups in the variant NrfH proteins is slightly altered or the midpoint potential of the atypically bound heme group is shifted. It is also possible that the modification of a heme *c* binding motif might affect the quinone binding site of NrfH. It was pointed out before that efficient electron transfer between heme groups might depend on certain three-dimensional heme arrangements, so-called heme packing motifs [9,18,19]. Such motifs were found to be conserved in multiheme *c*-type cytochromes even when the corresponding primary sequences were not related. Conserved heme packing motifs might be a general feature of cytochromes of the NapC/NirT family.

4.2. Cytochrome *c* biogenesis in *W. succinogenes*

Covalent attachment of heme to an apo-cytochrome usually requires a complex system of cytochrome *c* biogenesis (see below). However, spontaneous heme attachment to either an AXXCH or a CXXAH motif was observed for monoheme cytochrome *c*₅₅₂ from *Hydrogenobacter thermophilus* after the production of the corresponding modified proteins in the cytoplasm of *Escherichia coli* [20]. Both variant proteins resembled wild-type cytochrome *c*₅₅₂ with respect to thermal stability and to heme redox potential but the modified proteins were not tested in an electron transport assay. Spontaneous heme attachment has not yet been reported to occur in the bacterial periplasm or to a cytochrome from a mesophilic bacterium. Therefore, it seems to be more likely that heme is enzymatically attached by the cytochrome *c* biogenesis system to the SXXCH motif in NrfH from *W. succinogenes* C66S although spontaneous heme attachment cannot be excluded.

Two bacterial systems of cytochrome *c* biogenesis were described that differ in their enzymic components (for review see [21–23]). While α - and γ -proteobacteria, e.g. *Paracoccus denitrificans* or *E. coli*, make use of system I, system II is present in several Gram-positive bacteria, in the cyanobacterium *Synechocystis* sp. PCC 6803, in the β -proteobacterium *Bordetella pertussis*, and in the ϵ -proteobacteria *Helicobacter pylori*, *Campylobacter jejuni* and *W. succinogenes* [2,6,22,24,25]. System II comprises at least four gene products (CcsA, CcsB, CcsX and CcdA) that are essential for the formation of *c*-type cytochromes [22,24,26]. Unfortunately, the detailed function of any of the proteins making up system II is not yet clear. This report describes the so far unprecedented modification of a CXXCH motif in a cytochrome *c* biogenesis system II organism. The results suggest that the *W. succinogenes* heme lyase is able to attach heme to an SXXCH motif whereas there is no evidence for heme binding to a CXXSH motif. Enzymic attachment of heme to an SXXCH motif indicates that the formation of a disulfide bond from the thiol groups of the two cysteines of a CXXCH motif is not obligatory in the process of cytochrome *c* maturation. Such a disulfide bond was suggested to be a prerequisite for heme attachment in the system I organism *E. coli* [27,28]. Both cysteine residues of the CXXCH motif were found to be required for heme binding when corresponding variants of the monoheme cytochrome *c*₅₅₀ from *P. denitrificans* were pro-

duced in *E. coli* [27]. In contrast, there are various reports of covalent heme attachment to naturally occurring single-cysteine motifs in eukaryotes. Heme attachment in eukaryotes is thought to be catalyzed by a heme lyase which is located in the mitochondrial intermembrane space. This enzyme is not related to any protein of the two prokaryotic systems [22]. It has been demonstrated that heme is attached to the AAQCH motif of *Euglena gracilis* cytochrome *c*₅₅₇ [29], to the AAQCH motif of *Crithidia oncopelti* cytochrome *c*₅₅₈ [29] and to the cytochrome *c*₁ FAPCH motif of both *E. gracilis* [30,31] and *Crithidia fasciculata* [32]. Furthermore, the production of human cytochrome *c* with the heme *c* binding motif modified to ASQCH led to a functional holo-cytochrome in yeast [33]. It is notable that in all cases the C-terminal cysteine residue of the conventional CXXCH motif was retained. Thus cumulative evidence suggests that an (A/F/S)XXCH motif is sufficient for heme attachment in eukaryotes as well as in prokaryotic organisms containing the cytochrome *c* biogenesis system II, but not in organisms with system I.

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