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Enzymology and bioenergetics of respiratory nitrite ammonification

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Abstract

Nitrite is widely used by bacteria as an electron acceptor under anaerobic conditions. In respiratory nitrite ammonification an electrochemical proton potential across the membrane is generated by electron transport from a non-fermentable substrate like formate or H_2 to nitrite. The corresponding electron transport chain minimally comprises formate dehydrogenase or hydrogenase, a respiratory quinone and cytochrome *c* nitrite reductase. The catalytic subunit of the latter enzyme (NrfA) catalyzes nitrite reduction to ammonia without liberating intermediate products. This review focuses on recent progress that has been made in understanding the enzymology and bioenergetics of respiratory nitrite ammonification. High-resolution structures of NrfA proteins from different bacteria have been determined, and many *nrf* operons sequenced, leading to the prediction of electron transfer pathways from the quinone pool to NrfA. Furthermore, the coupled electron transport chain from formate to nitrite of *Wolinella succinogenes* has been reconstituted by incorporating the purified enzymes into liposomes. The NrfH protein of *W. succinogenes*, a tetraheme *c*-type cytochrome of the NapC/NirT family, forms a stable complex with NrfA in the membrane and serves in passing electrons from menaquinol to NrfA. Proteins similar to NrfH are predicted by open reading frames of several bacterial *nrf* gene clusters. In γ-proteobacteria, however, NrfH is thought to be replaced by the *nrfBCD* gene products. The active site heme *c* group of NrfA proteins from different bacteria is covalently bound via the cysteine residues of a unique CXXCK motif. The lysine residue of this motif serves as an axial ligand to the heme iron thus replacing the conventional histidine residue. The attachment of the lysine-ligated heme group requires specialized proteins in *W. succinogenes* and *Escherichia coli* that are encoded by accessory *nrf* genes. The proteins predicted by these genes are unrelated in the two bacteria but similar to proteins of the respective conventional cytochrome *c* biogenesis systems. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Respiratory nitrite ammonification; Formate dehydrogenase; Hydrogenase; Cytochrome *c* nitrite reductase; NapC/NirT family; Cytochrome *c* biogenesis; *Wolinella succinogenes*; NrfA

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1. Introduction

Nitrite is a component of the biological nitrogen cycle which is provided by nitrate reduction or by ammonia oxidation in biological habitats (Fig. 1). Reduction of nitrite can be regarded as an assimilatory, respiratory or dissimilatory process [1]. Assimilatory nitrite reduction serves in the production of ammonia which is incorporated into cell material thus allowing growth with nitrate or nitrite as a nitrogen source. This process occurs in bacteria as well as in plants and fungi and is catalyzed by a cytoplasmic siroheme-containing nitrite reductase that uses either NAD(P)H or ferredoxin as electron donor [2–4]. Notably, this reaction can also be regarded as a dissimilatory process because it serves an NAD(P)-regenerating function [1]. In contrast to dissimilatory nitrite reduction, respiratory nitrite reduction is coupled to the generation of an electrochemical proton potential (Δp) across the membrane. The Δp is a prerequisite of ADP phosphorylation catalyzed by ATP synthase, according to the chemiosmotic mechanism. Growth by respiratory nitrite reduction is described only for bacteria where it results in the production of either dinitrogen (respiratory denitrification) or ammonia (respiratory nitrite ammonification) (Fig. 1). Both processes are carried out under anaerobic conditions but have not been reported to occur in the same bacterial species.

Enzymes involved in respiratory denitrification were the subject of previous reviews [5,6]. The nitrite reductase involved in this pathway is either a cytochrome cd_1 nitrite reductase or a copper-containing nitrite reductase. Both enzymes catalyze nitrite reduction to nitric oxide (NO). NO is subsequently reduced via nitrous oxide (N_2O) to dinitrogen by NO reductase and N_2O reductase, respectively. Dinitrogen is reduced to ammonia in prokaryotic nitrogen-fixing organisms. Nitrite ammonification can thus be regarded as a short circuit that bypasses denitrification and nitrogen fixation [7]. The nitrogen cycle is completed by respiratory nitrification with oxygen as electron acceptor. Bacteria of the genus *Nitrosomonas* catalyze ammonia oxidation to nitrite forming hydroxylamine as an intermediate. Species of *Nitrobacter* grow by nitrite oxidation to nitrate.

In respiratory nitrite ammonification, nitrite is reduced to ammonia without the release of intermediate products. The reaction is catalyzed by the cytochrome *c* nitrite reductase, the NrfA protein, which is clearly different from any NO-producing nitrite reductase. In the course of respiratory nitrite ammonification, a non-fermentable substrate (predominantly formate or H_2) is oxidized and electrons are transferred via the quinone pool to NrfA. Alternatively, many bacteria use nitrite as an electron sink, thereby replacing intermediates of fermentation which would be reduced in the absence of nitrite. This dissimilatory mode of energy conservation may be called fermentative nitrite ammonification (Fig. 1). In this case ATP is generated by substrate-level phosphorylation (mainly by acetate kinase) using organic substrates like glucose or lactate. Fermentative nitrite ammonification will not be further discussed in this review.

NrfA proteins with similar properties have been purified and characterized from many different organisms, e.g. *Escherichia coli* [8–10], *Desulfovibrio desulfuricans* [11], *Wolinella succinogenes* [12–14] and *Vibrio fischeri* [15,16]. For many years, the NrfA protein was considered to contain six heme *c* groups, mainly based on absorption and electron paramagnetic resonance (EPR) spectroscopy. In contrast, Schröder et al. [13] reported the presence of four heme *c* groups in NrfA isolated from the *W. succinogenes* membrane fraction. This view was supported at first by the *nrfA* gene sequence of *E. coli* that predicted four heme *c* binding motifs (CXXCH) [17]. The conflict was eventually solved in favor of five heme groups when the unprecedented heme attachment to a CXXCK motif was demonstrated for *E. coli* NrfA in 1998 [18]. Currently, high-resolution structures of NrfA proteins from three different bacteria have been determined and confirm the presence of five heme *c* groups [19–21].

It has long been known that the electron transport from formate (or H_2) to nitrite generates an electrochemical proton potential across the membrane in intact cells of different bacterial species [22–26]. A quinone seems to be an obligatory component for electron transport from the electron donor substrate to NrfA [13,27]. The complete electron transport chain from formate to nitrite was recently characterized for *W. succinogenes* where the coupled electron transport chain was reconstituted in liposomes from the purified components [26]. In this organism, the NrfA protein is anchored to the membrane by a tetraheme cytochrome *c* (NrfH) which oxidizes menaquinol [26,28]. However, different electron transfer routes from the quinone pool to NrfA seem to be present in phylogenetically distinct bacterial groups.

It is the aim of this article to review the structural and

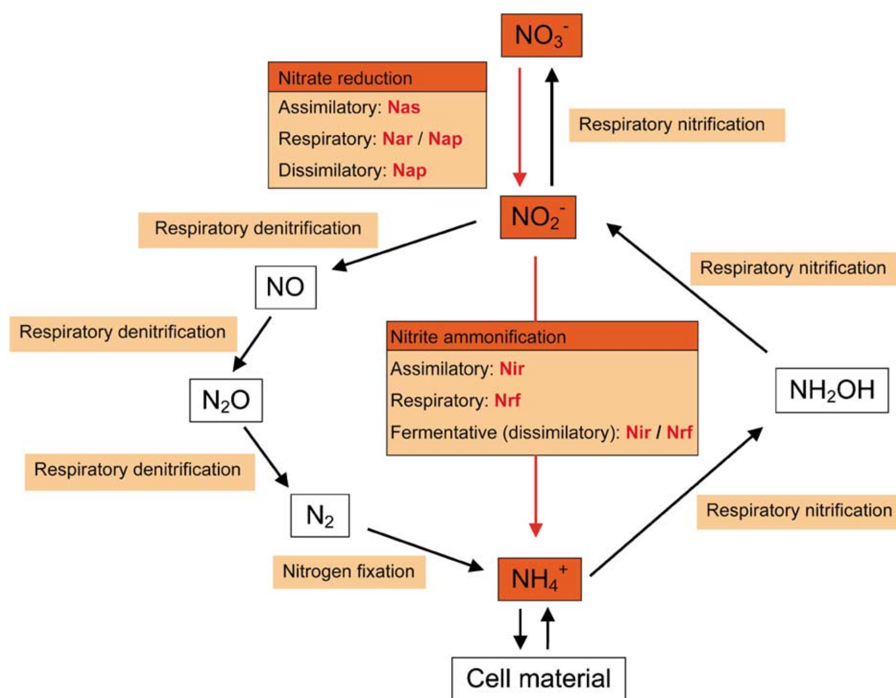


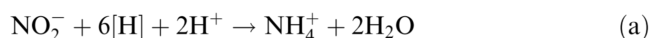
Fig. 1. The biological nitrogen cycle. Nitrate reduction and nitrite ammonification are categorized as assimilatory, respiratory or dissimilatory processes and the abbreviations of the corresponding enzymes are given. The reactions marked by the red arrows are the subject of this article. The designated assimilatory processes are carried out under both aerobic and anaerobic conditions, while the respiratory and dissimilatory processes of nitrate reduction, nitrite ammonification and denitrification are typical anaerobic processes. Nitrogen fixation and nitrification require the presence of oxygen. The 'anammox' process in which ammonia is oxidized anaerobically at the expense of nitrite to yield N_2 and 2 H_2O is left out for clarity. Nas, assimilatory nitrate reductase; Nar, respiratory nitrate reductase; Nap, periplasmic nitrate reductase; Nir, NADH-dependent nitrite reductase; Nrf, cytochrome *c* nitrite reductase.

functional aspects of enzymes involved in respiratory nitrite ammonification, with emphasis on the most recent developments. Respiratory nitrite ammonification and/or the biochemical and biophysical properties of cytochrome *c* nitrite reductase have been reviewed in [4,7,29–37], however, mostly in the broader context of anaerobic respiration or considering various reduced nitrogen compounds. In Section 2 of this article, the bioenergetic basis of respiratory nitrite ammonification is discussed and the relevant properties of bacteria which carry out respiratory nitrite ammonification are briefly summarized. Section 3 addresses the respiratory nitrite ammonification of the rumen bacterium *W. succinogenes* which, in that respect, is the most thoroughly studied organism. The results are compared to those obtained with other bacteria performing respiratory nitrite ammonification in Section 4, highlighting the composition of predicted electron transport chains. Genetic data derived from bacterial genome sequencing are compared in Section 5, laying emphasis on the arrangement of the open reading frames in the vicinity of *nrfA*. The corresponding gene products are predicted to be involved either in quinol oxidation and electron transfer to NrfA or in heme attachment to NrfA. The latter process contributes to the understanding of bacterial cytochrome *c* biogenesis and is discussed in Section 6. Finally, in Section 7, a short overview is given on respiratory nitrate reductases in *nrfA*-containing organisms.

2. Respiratory nitrite ammonification in bacteria

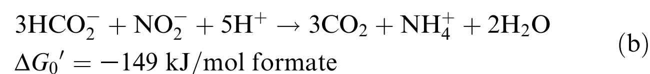
2.1. Bioenergetic considerations

The standard redox potential at pH 7.0 (E_0') of the redox pair NO_2^-/NH_4^+ is +0.34 V thus making nitrite a suitable electron acceptor for anaerobic respiration where it is reduced in a six-electron step to ammonia:

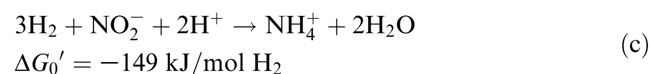


$$NO_2^- + 6[H] + 2H^+ \rightarrow NH_4^+ + 2H_2O \qquad (a)$$

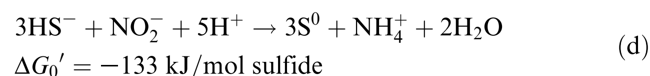
Formate and H_2 are common electron donors in respiratory nitrite ammonification. These substrates are oxidized according to reactions b and c. In addition, sulfide was shown to function as electron donor for respiratory nitrite ammonification in *Sulfurospirillum deleyianum* according to reaction d [38]. The E_0' values for the redox pairs formate/CO_2 (−0.42 V), H_2/H^+ (−0.42 V) and HS^-/S (−0.27 V) make reactions b–d strongly exergonic:



$$3HCO_2^- + NO_2^- + 5H^+ \rightarrow 3CO_2 + NH_4^+ + 2H_2O \quad \Delta G_0' = -149 \text{ kJ/mol formate} \qquad (b)$$



$$3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2H_2O \quad \Delta G_0' = -149 \text{ kJ/mol } H_2 \qquad (c)$$



$$3HS^- + NO_2^- + 5H^+ \rightarrow 3S^0 + NH_4^+ + 2H_2O \quad \Delta G_0' = -133 \text{ kJ/mol sulfide} \qquad (d)$$

The H^+/e ratio designates the number of protons apparently translocated across the membrane per mol electrons transported from the donor to the acceptor substrate. The theoretical maximum H^+/e ratio, $(n_{H^+}/n_e)_{max}$, can be calculated according to:

$$(n_{H^+}/n_e)_{max} = \Delta E_0'/\Delta p \quad (1)$$

Assuming a Δp of 0.17 V [26], $(n_{H^+}/n_e)_{max}$ for nitrite ammonification is nearly 4.5 with formate or H_2 as electron donor. This means that the actual H^+/e ratio could be up to 4, assuming that it has to be an integer number. The $(n_{H^+}/n_e)_{max}$ value with sulfide as electron donor is calculated to be 4.0.

The ATP/e ratio designates the amount of ATP formed from ADP and inorganic phosphate per mol electrons transported from the donor to the acceptor substrate. The theoretical maximum ATP/e ratio, $(n_{ATP}/n_e)_{max}$, is determined according to:

$$(n_{ATP}/n_e)_{max} = \Delta E_0' \cdot F/\Delta G_P' \quad (2)$$

where F represents the Faraday constant. The term $\Delta G_P'$ designates the cellular phosphorylation potential at pH 7.0 which is in the range of 50 kJ/mol ATP [39]. The maximum ATP/e ratio is calculated to be 1.5 for nitrite ammonification with formate or H_2 and 1.3 with sulfide as electron donor. These values are in agreement with the obtained $(n_{H^+}/n_e)_{max}$ values when the number of protons translocated per ATP synthesized is assumed to be 3. It should be noted that the determination of the ATP/e and H^+/e ratios could be impaired by the transport of substrates across the energized membrane. Therefore, the theoretical values above were calculated based on the assumption that the catalytic sites for formate, H_2, sulfide and nitrite are located outside the cell. Alternatively, any transport process has to be ATP-independent or electroneutral.

2.2. Properties of bacteria performing respiratory nitrite ammonification

Bacterial species that were shown to carry out respiratory nitrite ammonification belong to either the γ-, δ- or ε-subclass of the proteobacteria (Table 1). All these bacteria contain menaquinone (or demethylmenaquinone) as the predominant quinone under anaerobic conditions, and use a variety of other electron acceptors for anaerobic respiration. It is likely that many more bacteria carry out respiratory nitrite ammonification. Candidates are bacteria which contain either a formate dehydrogenase or a hydrogenase, a respiratory quinone and a cytochrome *c* nitrite reductase.

The bacterium growing fastest at the expense of respiratory nitrite ammonification with formate as electron donor is the ε-proteobacterium *W. succinogenes* that was isolated by Wolin from bovine rumen fluid in the early 1960s [55]. Today, *W. succinogenes* is regarded as the only species of its genus [41]. The electron transport chains involved in *W. succinogenes* fumarate or polysulfide respi-

Table 1
Properties of organisms that perform respiratory nitrite ammonification

Organism[a]	Electron donor	Doubling time[b] (h)	Molar growth yield (Y_M)[b] (g dry cells (mol NO_2^-)$^{-1}$)	Alternative electron acceptors in anaerobic respiration	Major quinone under anaerobic conditions[e]	References
Wolinella succinogenes (ε)	Formate	2.0[c]	15.9[c], 25.5[d]	NO_3^-, N_2O, fumarate, polysulfide, DMSO	MK-6, MMK-6	[40–43]
Sulfurospirillum deleyianum (ε)	Formate	7.1[c], 9.0[f]	9.6[c] 6.0[f]	NO_3^-, fumarate, S^0, SO_3^{2-}, $S_2O_3^{2-}$, DMSO	MK-6, MMK-6	[38,44–46]
	Sulfide	11.0[f]	4.5[f]			
Campylobacter sputorum biovar *bubulus* (ε)[g]	Formate	5.8[h]	9.8	NO_3^-, fumarate, S^0, SO_3^{2-}, $S_2O_3^{2-}$, DMSO	n.r.	[47–49]
Desulfovibrio desulfuricans (δ)	H_2, formate	3.6[f]	8.8[f], 12.6[d]	NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, S^0, SO_3^{2-}, fumarate	MK-6, MK-5	[24,45,50–52]
Desulfovibrio gigas (δ)	H_2	n.r.	n.r.	SO_4^{2-}, $S_2O_3^{2-}$, S^0, SO_3^{2-}, fumarate	MK-6, MK-5	[25,45,53]
Escherichia coli (γ)	Formate[i]	n.r.	n.r.	NO_3^-, fumarate, DMSO, TMAO	MK-8, DMK-8	[22,23,32,54]

n.r.: not reported; MK: menaquinone; MMK: [5 or 8]-methylmenaquinone; DMK: demethylmenaquinone.
[a]The Greek letter in parentheses denotes the phylogenetic subclass of the proteobacteria.
[b]Doubling times and growth yields were usually determined in minimal medium with the indicated electron donor and nitrite as electron acceptor. Likewise, the parameters were determined in a batch culture with nitrate as electron acceptor when nitrate was completely reduced to nitrite.
[c]Growth with succinate as carbon source.
[d]Molar growth yield of a continuous culture extrapolated to infinite growth rate (Y_{max}).
[e]Numbers following the abbreviation of the quinone refer to the number of isoprene units in the side chain.
[f]Growth with acetate as carbon source.
[g]Growth was examined in a complex medium with nitrite as electron acceptor.
[h]Calculated from the dilution rate of a chemostat culture.
[i]Formate is the predominant electron donor in respiratory nitrite ammonification of *E. coli* (cf. Section 4.3.2).

ration were investigated in great detail (see [39,56] for recent reviews). Apart from formate, H_2 and sulfide were also shown to serve as electron donors for fumarate respiration [57,58]. It is likely that *W. succinogenes* can also grow with H_2 or sulfide and nitrite as sole energy substrates according to reactions c and d. Species of the genus *Campylobacter* are close relatives of *W. succinogenes*. In contrast to *W. succinogenes*, *Campylobacter* spp. grow only in enriched media because of their complex nutritional requirements. However, *Campylobacter sputorum* biovar *bubulus* was shown to grow by respiratory nitrite ammonification with formate [47]. Another ε-proteobacterium that grows by respiratory nitrite ammonification with formate or sulfide is the free-living *S. deleyianum* (formerly Spirillum 5175) which was originally isolated as a sulfur-reducing organism [38,49].

Many sulfate-reducing bacteria catalyze nitrite reduction to ammonia in a dissimilatory process using substrates that allow substrate-level phosphorylation [59,60]. Furthermore, nitrite ammonification at the expense of sulfide oxidation to sulfate was described for *D. desulfuricans* and *Desulfobulbus propionicus* [61]. Respiratory nitrite ammonification was reported only for *D. desulfuricans* and *Desulfovibrio gigas* (Table 1 and Section 4.2). *D. desulfuricans* was shown to prefer ammonification of nitrite (or nitrate) over sulfate reduction when both substrates were present [51]. Such behavior might reflect the fact that nitrate and nitrite are both energetically more favorable electron acceptors than sulfate [$E_0'(SO_4^{2-}/HS^-) = -0.22$ V] which has to be activated in an ATP-consuming process to adenosine 5′-phosphosulfate.

3. Electron transport chain and coupling mechanism of respiratory nitrite ammonification in *W. succinogenes*

Electron transport to nitrite in *W. succinogenes* was studied in great detail [13,26,28,40]. The reduction of nitrite by formate or H_2 (reactions b and c) is catalyzed by intact cells or by the bacterial membrane fraction. The electron transport activity from formate to nitrite in cells was measured as 3.6 μmol formate oxidized min^{-1} (mg dry cell wt)$^{-1}$ at 37°C [28]. This value is well above the theoretical electron transport activity (v_{min}) of 1.1 μmol formate oxidized min^{-1} (mg dry cell wt)$^{-1}$ calculated according to Eq. 3 from the growth rate (μ) and the molar growth yield (Y_M) (Table 1):

$$v_{min} = \mu / Y_M \quad (3)$$

The electron transport activities from formate or H_2 to nitrite were abolished upon extraction of menaquinone from the membrane, and were restored upon incorporation of vitamin K_1 into the extracted membrane [13,40]. The current view of the electron transport chain from formate or H_2 to nitrite is depicted in Fig. 2. The constituents of the electron transport chain (cytochrome *c* nitrite reductase, formate dehydrogenase and hydrogenase) are described in the following subsections. The three enzymes are multi-subunit complexes that react with quinones and are anchored in the membrane by either a cytochrome *c* or a cytochrome *b* subunit. The catalytic sites for nitrite, formate or H_2 are oriented towards the periplasmic side of the membrane. The electron transport chain from formate to nitrite was reconstituted from the isolated enzymes in proteoliposomes (cf. Section 3.4).

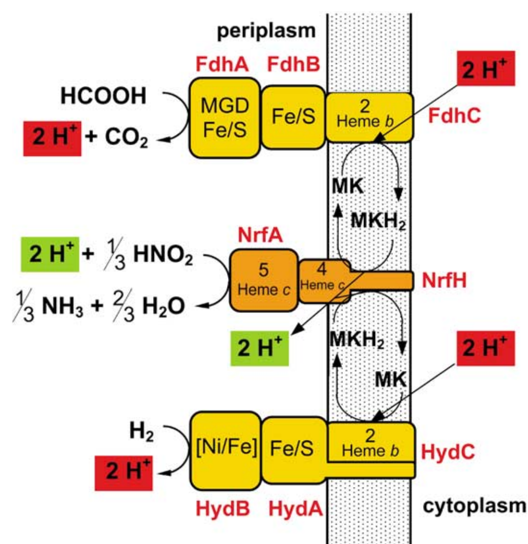
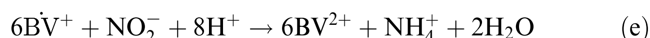


Fig. 2. Enzyme complexes involved in electron transport from formate or H_2 to nitrite in *W. succinogenes*. The names of the protein subunits making up formate dehydrogenase (Fdh), hydrogenase (Hyd) or cytochrome *c* nitrite reductase (Nrf) are shown in red. The hypothetical mechanism of Δ*p* generation is depicted by protons drawn with different color backgrounds. A red background denotes protons that are involved in the electrogenic oxidation of formate or H_2 by MK thus generating Δ*p* by a redox loop mechanism. Protons with a green background are involved in the electroneutral reduction of nitrite by MKH_2, and do not contribute to Δ*p* generation. Substrates and products of the redox reactions are drawn in their neutral forms for simplicity. MGD, molybdenum linked to molybdopterin guanine dinucleotide; Fe/S, iron–sulfur centers.

3.1. The cytochrome c nitrite reductase complex

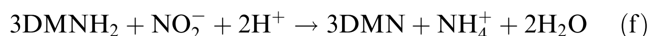
The cell homogenate of *W. succinogenes* grown with nitrate as electron acceptor catalyzes nitrite reduction to ammonia by benzyl viologen radical ($\dot{BV}^+$ in reaction e) with a specific activity of up to 50 μmol nitrite reduced min^{-1} (mg cell protein)$^{-1}$ at 37°C [62]. In contrast, the specific activity in homogenates of cells grown with nitrite or fumarate amounted to approximately 20% or 5% of that of nitrate-grown cells [62].



$$6\dot{BV}^+ + \underline{NO_2^-} + 8\underline{H}^+ \rightarrow 6\underline{BV}^{2+} + \underline{NH_4^+} + 2\underline{H_2O} \quad (e)$$

Reaction e is catalyzed by both the soluble cell fraction (∼30% of the total activity) and the membrane fraction (∼70%) of nitrate-grown cells [12,13]. The activity is absent in both fractions of a *nrfA* deletion mutant indicating

that the cytochrome *c* nitrite reductase (NrfA) is the only enzyme catalyzing reaction e [26,63]. NrfA is located in the periplasm or at the periplasmic membrane surface [26] which is typical for *c*-type cytochromes [64]. The soluble NrfA of *W. succinogenes* was first isolated by Liu et al. [12] and its crystal structure was solved recently at 1.6 Å resolution [20] (cf. Section 3.1.1). NrfA from the membrane fraction was isolated initially as a single polypeptide with similar properties to the soluble NrfA [13,14]. Later, it was also purified in a stable complex with a 22-kDa *c*-type cytochrome using two different isolation protocols [14,26]. The subunits of this complex could be separated by SDS–PAGE. The 22-kDa cytochrome *c* was identified as the *nrfH* gene product which mediates the electron transport between menaquinol and NrfA [26] (cf. Section 3.1.2). The *nrfH* and *nrfA* genes are part of the *nrfHAIJ* operon on the *W. succinogenes* genome [26] (cf. Section 5). It was found that only the NrfHA complex was capable of catalyzing the oxidation of the water-soluble menaquinol analogue 2,3-dimethyl-1,4-naphthoquinol ($DMNH_2$) by nitrite (reaction f) whereas the NrfA protein alone was not [26].



$$3DMNH_2 + NO_2^- + 2H^+ \rightarrow 3DMN + NH_4^+ + 2H_2O \quad \text{(f)}$$

The catalysis of reaction f was inhibited upon addition of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (R. Gross and J. Simon, unpublished). The results suggest that NrfH contains a menaquinone-binding site. When the *nrfH* gene was inactivated by the introduction of stop codons, the cells of the corresponding *W. succinogenes* mutant contained NrfA exclusively in the soluble cell fraction [28]. The NrfA protein of this mutant catalyzed nitrite reduction by benzyl viologen radical, but electron transport from formate to nitrite was absent. These results indicate that NrfH functions as the membrane anchor of the NrfHA complex and that it is an essential constituent of the electron transport chain catalyzing respiratory nitrite ammonification.

In fumarate-grown cells NrfA and the nitrite reductase activity with benzyl viologen radical are found in the membrane fraction but not in the soluble cell fraction. In this case, NrfA is apparently present only in the NrfHA complex. In nitrate-grown bacteria NrfA is possibly produced in excess of NrfH resulting in additional periplasmic NrfA.

3.1.1. *The catalytic subunit NrfA*

W. succinogenes NrfA is synthesized as a pre-protein carrying a typical Sec-dependent signal peptide of 22 residues [26]. The N-terminus of mature NrfA was determined and confirmed the predicted cleavage site [65]. The molecular mass of NrfA purified from either the soluble or the membrane fraction was determined to be 58 339 Da by MALDI mass spectrometry [28,63]. This value is in agreement with that calculated for mature NrfA (55 251 Da) carrying five covalently bound heme groups of mass 616 each. The size of NrfA was estimated from SDS-PAGE to be 63 kDa [12,13] or 55 kDa [14]. The presence of five covalently bound heme groups was unequivocally demonstrated by the crystal structure of *W. succinogenes*

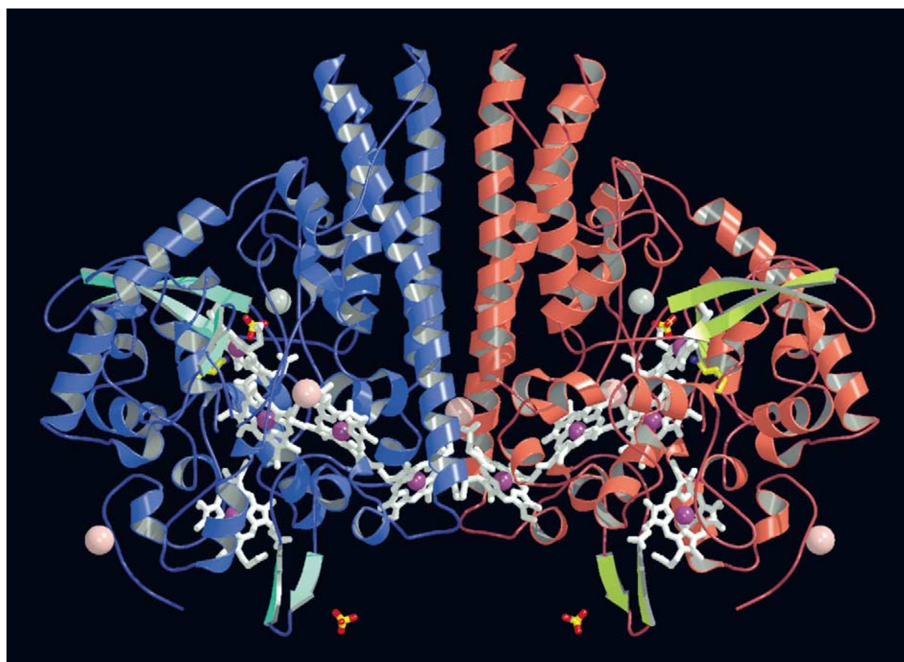


Fig. 3. Overall structure of the cytochrome *c* nitrite reductase (NrfA) homodimer of *W. succinogenes*. Monomers are shown in red and blue, respectively. Dimer formation is facilitated by the central helical segments. Heme groups are shown in white and their central iron atoms in purple. The active site is occupied by a sulfate ion, the nearby Ca^{2+} ion is shown in gray. The axial ligand to the active site heme group (lysine 134) is shown in yellow. Pink spheres designate yttrium ions (Y^{3+}) that derived from the crystallization buffer.

NrfA isolated from the soluble cell fraction (Fig. 3) [20]. Before the structure became available, the NrfA protein was considered to contain four or six heme groups [12,13,66].

The crystal structure of *W. succinogenes* NrfA was solved to a resolution of 1.6 Å and demonstrated the formation of a compact homodimer with an overall structure similar to those of the NrfA dimers from *S. deleyianum* [19] and *E. coli* [21] (cf. Section 4). The distances between the five heme groups of a monomer are suited for rapid electron transfer with the longest iron-to-iron distance being 12.5 Å. The iron-to-iron distance between the two nearest heme groups of two different NrfA monomers (11.7 Å) would allow electron transfer between the monomers. The iron atom of the active site heme group (heme 1) is co-ordinated by the lysine residue (K134) of a unique CWTCK motif. The cysteine residues of this motif serve in covalent heme attachment by forming two thioether bridges with the heme vinyl groups, similar to the attachment of hemes 2–5 to four conventional CXXCH motifs of NrfA (X meaning any residue). The iron atoms of heme groups 2–5 are axially ligated by two histidine residues each. Nitrite is thought to bind at the distal site of heme 1 which is occupied by a sulfate ion in the crystal structure. Sulfate is known as a weak inhibitor of nitrite reductase activity [67]. In addition, crystal structures with water or azide in the active center were obtained [20]. Near the active site, a Ca^{2+} ion was found which is required for catalysis although its detailed function is not clear [67]. All residues involved in Ca^{2+} binding are conserved in the available NrfA sequences [20]. Nitrite is reduced at the active site with bound NO and NH_2OH as probable intermediates [19,68]. It is notable that NrfA proteins are known to reduce added NO and NH_2OH, however at lower rates than nitrite [13,67].

Replacement by histidine of the active site lysine ligand (K134) in *W. succinogenes* resulted in a NrfA protein that still contained five heme groups as judged by MALDI mass spectrometry [63]. It is likely that the histidine residue of the generated CWTCH motif replaces the lysine residue as axial heme ligand. The activity of nitrite reduction by reduced benzyl viologen (reaction e) of the modified NrfA amounted to 40% of the wild-type activity [63]. The K_M value for nitrite (0.1 mM [13]) of the purified enzyme was not altered by the modification and ammonia was formed as the only product of nitrite reduction. The *W. succinogenes* mutant which produces the modified NrfA did not grow by respiratory nitrite ammonification and its rate of electron transport from formate to nitrite amounted to only 5% of that of the wild-type strain [63]. This drastic inhibition of electron transport may be explained by a decreased redox potential of heme 1 that lowers the velocity of electron transfer to the active site heme group from the other hemes which are involved in menaquinol oxidation. In contrast, reduced benzyl viologen is thought to react directly with heme 1. Site-directed mutagenesis was also carried out with *E. coli nrfA* which is discussed in Section 4.3.1.

Two different channels that lead from the enzyme surface to the active site were discovered in NrfA. A positively charged channel is thought to be the nitrite entry pathway, whereas ammonium possibly leaves the active site via the other, negatively charged channel. It is likely that the enzyme turnover benefits from two distinct pathways for the oppositely charged substrate and product molecules. The entry point for electrons delivered by NrfH is not clear. Heme 2 (the heme group nearest to the bottom in Fig. 3) is the most likely initial electron acceptor as it has contact with the enzyme surface within a patch of strong positive surface potential suitable for interaction with NrfH [20]. The stability of the NrfHA complex is thought to be largely dependent on such electrostatic interactions. Heme 5 (the heme group nearest the homodimer interface) is an alternative electron entry point although the corresponding surface area is largely covered by the second NrfA monomer within the dimer.

In recent years, certain structural arrangements of covalently bound heme groups (so-called heme packing motifs) were found to be conserved in multiheme *c*-type cytochromes even when the corresponding amino acid sequences are not similar [69]. In NrfA, hemes 2 and 3 as well as hemes 4 and 5 form a 'diheme elbow motif' with the heme planes situated almost perpendicular to each other [20]. This motif is found in a variety of other *c*-type cytochromes, e.g. in cytochrome c_3 of sulfate-reducing bacteria. Hemes 3 and 4 form a 'heme stacking motif' in which the porphyrin planes are nearly parallel to each other with an edge-to-edge distance below 4 Å. Interestingly, the structural arrangement of the five NrfA heme groups corresponds to that of five of the eight hemes of the hydroxylamine oxidoreductase of *Nitrosomonas europaea* that catalyzes the oxidation of hydroxylamine to nitrite [19,20,70].

A wealth of EPR spectroscopic data obtained with NrfA proteins, including *W. succinogenes* NrfA, has been published [14,21,31,34,66,71–76]. The spectra are broadly similar but surprisingly complex when compared to those of other *c*-type cytochromes. Typically, oxidized NrfA proteins exhibit perpendicular mode X-band EPR signals at $g \approx 2.9$, 2.3 and 1.5 that are likely components of a rhombic Fe(III) signal and are therefore assigned to low-spin bis-His-ligated ferric heme. Additional resonances at low field regions ($g \approx 10$ and 3.7) indicate the presence of high-spin ferric heme which was also confirmed by MCD spectroscopy [21,72,77]. Preparations containing NrfA purified from the membrane fraction contained a signal at $g = 4.8$ that probably arises by spin–spin interaction among heme groups and is found in samples from *W. succinogenes* [14,72], *S. deleyianum* [14], *D. desulfuricans* [71,73–75] and *Desulfovibrio vulgaris* [77]. The $g = 4.8$ signal is interpreted to be indicative of the complex between NrfA and its redox partner NrfH (cf. Sections 3.1.2 and

4.2). This signal does not appear in NrfA preparations from the soluble cell fraction of either *W. succinogenes* [14,66], *S. deleyianum* [14] or *E. coli* [21,66]. A detailed consideration of the spectroscopic data is beyond the scope of this review. Furthermore, most reports predated the determination of the NrfA crystal structures and were interpreted assuming the presence of six heme groups. The reader is referred to a recent re-examination of the spectroscopic data in the light of the *E. coli* NrfA crystal structure [21].

3.1.2. The membrane anchor subunit NrfH

The NrfH protein of *W. succinogenes* is a membrane-bound tetraheme cytochrome *c* that forms a quinone-reactive complex with NrfA in the membrane [26]. Its identity was confirmed by N-terminal sequencing of a peptide fragment obtained upon BrCN cleavage [26]. The molecular mass of NrfH was determined as 22 221 Da by MALDI mass spectrometry [28]. This value is consistent with that calculated from the *nrfH* gene (19 667 Da) assuming the covalent attachment of four heme *c* groups which is predicted by the presence of four CXXCH heme binding motifs in the NrfH sequence (Fig. 4). Crystals of the *W. succinogenes* NrfHA complex were reported recently [78] but structural information is not yet available.

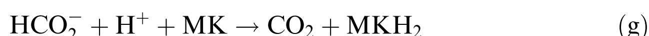
NrfH belongs to the NapC/NirT family of *c*-type cytochromes that are generally thought to be membrane-bound electron transfer mediators between the quinone pool and periplasmic oxidoreductases (see [28,79] for an overview). In that respect, NrfH seems to be an exceptional member of the family as it forms a stable complex with NrfA. Each member of the NapC/NirT family contains a hydrophobic stretch of approximately 20 amino acid residues near its N-terminus which is considered to function as a membrane anchor (Fig. 4). To date, about 30 members of the family are known, however, for most of them only the predicted primary sequence is available. Beside NrfH, at least two other members of the family are involved in reduction of nitrite or nitrate. The NirT protein is a possible electron donor for the periplasmic cytochrome cd_1 nitrite reductase involved in denitrification of *Pseudomonas stutzeri* [80]. However, this function is carried out by other redox proteins in cells of *Paracoccus pantotrophus* [81]. The NapC protein is thought to be the redox partner for the nitrate reductase complex (NapAB) found in the periplasm of various bacteria [82,83]. Other NapC-like proteins, namely DmsC, DorC or TorC, are likely electron donors for periplasmic dimethylsulfoxide (DMSO) reductase or trimethylamine *N*-oxide (TMAO) reductase. These proteins carry an additional C-terminal domain of about 185 residues that contains a fifth heme *c* binding motif (Fig. 4). The C-terminal domain is envisaged to function as a mediator of electron transport between the tetraheme domain and the TMAO or DMSO reductase [84].

So far, little biochemical knowledge about cytochromes of the NapC/NirT family is available and no structural information has been reported. After replacing the hydrophobic N-terminus by a cleavable signal peptide, the soluble tetraheme domain of *P. pantotrophus* NapC was purified from the bacterial periplasm [79]. Magneto-optical spectroscopic methods led to the identification of four bis-histidine-ligated low-spin heme groups. The spectrophotometric redox titration curve fitted to four Nernst curves centered at −56, −181, −207 and −235 mV, respectively. The CymA protein from *Shewanella frigidimarina* NCIMB400, a membrane-bound NapC homologue whose redox partner is not known, was purified in its native form [85]. All four heme groups were found to be bis-histidine-ligated with estimated midpoint potentials of +10, −108, −136, and −229 mV. The likely axial heme iron ligands of NapC and CymA are marked in Fig. 4. The DorC protein from *Rhodobacter capsulatus* which is involved in electron transfer to the periplasmic DMSO reductase was purified as a fusion protein [86]. DorC contains five heme *c* groups with estimated midpoint potentials of −34, −128, −184, −185, and −276 mV. All three purified members of the NapC/NirT family were reported to be at least partially reduced by water-soluble quinols [79,85,86].

The sequences of seven NrfH proteins are currently known (Fig. 4). The corresponding genes were identified upstream of *nrfA* homologs and a function similar to that demonstrated for *W. succinogenes* NrfH is expected. Interestingly, only two of the likely axial heme ligands in NapC and CymA are conserved in the NrfH sequences. Therefore, NrfH proteins possibly possess a heme ligation pattern different from that of NapC proteins. Beside histidine, conserved methionine or lysine residues which may serve as axial heme ligands are highlighted in Fig. 4. To clarify this point more information is required which may derive from future site-directed mutagenesis experiments, spectroscopic studies or redox potentiometry. It is notable that NrfH of *Carboxydothermus hydrogenoformans* is predicted to contain only three histidine residues in addition to those arranged in CXXCH motifs. Therefore, at least one heme group is assumed not to be bis-histidine-ligated. This may also hold true for DorC from *R. capsulatus* which contains only three histidine residues in the N-terminal tetraheme domain, apart from those within the CXXCH motifs. In this case, however, four other histidines are present at the C-terminal side of the fifth CXXCH motif.

3.2. Formate dehydrogenase

The *W. succinogenes* formate dehydrogenase catalyzes the reduction of menaquinone by formate:



$$HCO_2^- + H^+ + MK \rightarrow CO_2 + MKH_2 \quad \text{(g)}$$

The enzyme consists of two hydrophilic (FdhA and FdhB)

Fig. 4. Multiple sequence alignment of representative members of the NapC/NirT family. The upper seven sequences are those of NrfH proteins from different bacteria. The next five sequences belong to the 'NapC subgroup' which is characterized by conserved histidine residues that possibly serve as axial heme iron ligands. Encircled histidine residues belong to proteins for which bis-histidine ligation of all four heme groups was shown [79,85]. The last two sequences belong to the 'pentaheme subgroup' of the family containing an additional C-terminal monoheme domain. Sequences of the latter two subgroups were only considered when the corresponding protein was characterized biochemically. The putative N-terminal transmembrane domain and the heme binding motifs are highlighted in yellow. Conserved histidine (pink), methionine (green) and lysine (blue) residues are possible axial heme ligands. *Ws*: *Wolinella succinogenes*; *Sd*: *Sulfurospirillum deleyianum*; *Cj*: *Campylobacter jejuni*; *Dv*: *Desulfovibrio vulgaris*; *Gs*: *Geobacter sulfurreducens*; *Pg*: *Porphyromonas gingivalis*; *Ch*: *Carboxydothermus hydrogenoformans*; *Pp*: *Paracoccus pantotrophus*; *Ec*: *Escherichia coli*; *Rs*: *Rhodobacter sphaeroides*; *Ps*: *Pseudomonas stutzeri*; *So*: *Shewanella oneidensis*; *Rc*: *Rhodobacter capsulatus*.

and one hydrophobic subunit (FdhC) (Fig. 2). The three subunits are encoded by the corresponding genes within the *fdhEABCD* operon that is found twice on the *W. succinogenes* genome [87,88]. The catalytic subunit FdhA contains molybdenum coordinated by molybdopterin guanine dinucleotide and is predicted to carry a [4Fe–4S] iron–sulfur cluster [87,89]. FdhB most likely carries four [4Fe–4S] or, alternatively, one [3Fe–4S] and three [4Fe–4S] iron–sulfur centers. FdhC is a diheme cytochrome *b* that reacts with menaquinone and anchors the enzyme in the membrane [90,91]. The catalytic subunit of the formate dehydrogenase is oriented towards the periplasmic side of the membrane [92]. This observation is in line with the fact that the pre-protein of FdhA is predicted to contain an N-terminal double-arginine export sequence [93]. The functions of the putative *fdhD* and *fdhE* gene products are not known. It is likely that these proteins play a role in the biogenesis of the formate dehydrogenase complex.

The structure of *W. succinogenes* formate dehydrogenase is not known but is expected to be similar to that of the *E. coli* FdnGHI complex (formate dehydrogenase-N) [94]. The catalytic subunit (FdnG) contains molybdenum coordinated by two molybdopterin dinucleotide molecules. The catalytic site is accessible through a large cleft allowing substrate and products to enter and leave the enzyme. Similar three-dimensional structures were determined for other catalytic subunits of molybdoenzymes like DMSO reductase from *Rhodobacter* species [95], periplasmic nitrate reductase from *D. desulfuricans* [96] and *E. coli* formate dehydrogenase-H [97]. The high-resolution structure of the FdnGHI complex implies that the electrons obtained from formate oxidation are transported via five [4Fe–4S] clusters (one in FdnG and four in FdnH) to the diheme cytochrome *b* subunit FdnI. The five iron–sulfur centers are arranged in a straight line that is extended by the two heme *b* groups which are situated nearly on top of each other when viewed along the membrane normal. The four FdnI histidine ligands of the heme iron are provided by three of the four transmembrane helices (one histidine each by helix I and II and two histidines by helix IV). The axial heme ligands are conserved in the cytochrome *b* subunits of various formate dehydrogenases and [NiFe]-hydrogenases including the *W. succinogenes* enzymes (see [98] for an alignment). A

diheme cytochrome *b* membrane anchor with the two heme groups oriented to different sides of the membrane was also reported for the *W. succinogenes* quinol:fumarate reductase complex [99] and for the cytochrome *b* subunit of cytochrome bc_1 complexes. The same heme arrangement is also likely to apply for the membrane anchor subunit of respiratory nitrate reductase (NarI). The structure of formate dehydrogenase-N revealed a likely quinone binding site which was occupied by a HQNO molecule [94]. This site is located near the cytoplasmic surface of the membrane near the distal heme *b* group. It is therefore suggested that both heme groups are involved in electron transport and that the protons required for menaquinone reduction are taken up from the cytoplasm. Several residues that are in contact with the HQNO molecule in FdnI are conserved in the cytochrome *b* subunits of formate dehydrogenases and [NiFe]-hydrogenases [98].

The *E. coli* FdnGHI complex is anchored in the membrane by the four transmembrane domains of FdnI and by the C-terminal helix of the iron–sulfur subunit FdnH. In contrast, *W. succinogenes* FdhB is not predicted to contain the C-terminal hydrophobic segment. Instead, FdhC is predicted to span the membrane five times with segments II–V corresponding to the four segments of *E. coli* FdnI [98]. Despite the alternative ways of membrane anchoring, the catalytic sites for formate and MK as well as the connecting electron transfer pathways are expected to be essentially identical.

3.3. Hydrogenase

The membrane-bound [NiFe]-hydrogenase complex of *W. succinogenes* catalyzes menaquinone reduction by H_2 according to:

$$H_2 + MK \rightarrow MKH_2 \quad \text{(h)}$$

The enzyme consists of two hydrophilic (HydA and HydB) and one hydrophobic subunit (HydC) (Fig. 2) [100]. HydB carries the catalytic site of H_2 oxidation and HydA is predicted to contain three iron–sulfur clusters that are thought to mediate the electron transport from HydB to HydC. HydC is a membrane-bound diheme cytochrome *b* that carries the site of MK reduction [39]. The isolated trimeric enzyme catalyzed the reduction of the water-soluble MK analogue DMN by H_2 whereas a preparation lacking HydC did not [100]. Both forms catalyzed the reduction of viologen dyes by H_2.

The subunits of the *W. succinogenes* [NiFe]-hydrogenase are encoded by the first three genes of the *hydABCDE* operon [100,101]. A *W. succinogenes* mutant that lacks the *hydABC* genes did not grow by anaerobic respiration with H_2 as electron donor and either fumarate or nitrate as electron acceptor [101]. Furthermore, the mutant did not contain any hydrogenase activity demonstrating that the HydABC complex is the only hydrogenase in *W. succinogenes*. The *hydD* gene product is similar to proteins encoded by other hydrogenase operons. HydD is most likely the protease responsible for processing the C-terminus of HydB after cofactor incorporation [102]. Proteins similar to the predicted HydE protein are encoded on the genomes of *Campylobacter jejuni* [103] and *Helicobacter pylori* [104] which are close relatives of *W. succinogenes*. The function of the *hydE* gene product is unclear in any of these organisms.

W. succinogenes HydC is predicted to form four transmembrane domains. The sequence of HydC is similar to *W. succinogenes* FdhC and *E. coli* FdnI including the four conserved histidine residues in helices I, II and IV that axially ligate the two heme groups in FdnI [94,98]. Mutants of *W. succinogenes* lacking one of these histidine residues did not catalyze H_2 oxidation by DMN [105]. The mutant membranes still contained HydC and catalyzed H_2 oxidation by benzyl viologen.

The *W. succinogenes* HydABC complex is anchored in the membrane by both HydC and the hydrophobic C-terminus of HydA which is predicted to traverse the membrane similar to the C-terminus of *E. coli* FdnH (Fig. 2) [101]. Mutants that could synthesize only one anchor contained a catalytically active HydB that was still bound to the membrane. In the absence of both anchors, however, HydB and the activity of viologen reduction by H_2 were located in the periplasm. This observation strongly indicates the periplasmic orientation of HydB which is also supported by the presence of a double-arginine export sequence at the N-terminus of the HydA pre-protein. The two arginine residues were shown to be essential for the translocation of both HydA and HydB across the membrane [106]. It was proposed that a functional HydAB complex is exported by the twin-arginine-translocation (Tat) apparatus [107–109]. After its translocation, the HydAB complex might be retained in the membrane by the C-terminus of HydA before the trimeric complex with HydC is formed as the last step of hydrogenase biosynthesis. The incorporation of heme in HydC as well as the localization of HydC in the membrane were not impaired after modifying the double-arginine motif of pre-HydA [106].

A conserved histidine residue (H305) in the HydA membrane anchor was replaced by methionine [105]. The corresponding *W. succinogenes* mutant could reduce neither cytochrome *b* nor quinol by H_2 although the hydrogenase was found in the membrane fraction. The *W. succinogenes* mutant that lacks the membrane anchor of HydA had similar properties [101]. The simplest explanation for these results is the involvement of the HydA C-terminus in the formation of a functional complex between HydAB and HydC. H305 of HydA is conserved in *E. coli* FdnH where it is hydrogen-bonded to a conserved tyrosine residue of the cytochrome *b* subunit [94].

The crystal structures of the periplasmic hydrogenases from several *Desulfovibrio* species are known [110–112]. These enzymes consist of two hydrophilic subunits that

are similar to *W. succinogenes* HydA and HydB. The residues involved in Ni and Fe binding as well as in ligation of the three iron–sulfur clusters are conserved. Therefore, a common structure and catalytic mechanism for these hydrogenases can be assumed. The periplasmic localization or the orientation to the periplasmic side of the membrane implies that the protons derived from H_2 oxidation are released into the periplasm. The electrons are probably guided by the three consecutive iron–sulfur clusters to the respective electron acceptor which is a cytochrome *c* in the *Desulfovibrio* species (cf. Section 4.2.2), in contrast to the cytochrome *b* of *W. succinogenes*.

3.4. The coupling mechanism of respiratory nitrite ammonification

Intact cells of *W. succinogenes* grown with formate and nitrate catalyze electron transport from formate to nitrite at high rates [28]. When electron transport was started by the addition of formate and nitrite, cells were observed to take up tetraphenylphosphonium (TPP^+). During the experiment, the external TPP^+ concentration was recorded with an ion-selective TPP^+ electrode. Addition of a protonophore abolished the ability to take up TPP^+ whereas the electron transport activity was not affected. The TPP^+ uptake indicated that electron transport from formate to nitrite is coupled to the generation of a membrane potential ($\Delta\psi$, negative inside) across the membrane. The $\Delta\psi$ generated by the electron transport from formate to nitrite was determined as −160 mV [26]. This value should be close to Δp because the ΔpH across the membrane was found to be very small [113,114]. An identical $\Delta\psi$ was obtained in an experiment with formate replaced by H_2 (S. Biel and J. Simon, unpublished).

The formate dehydrogenase and the NrfHA complex from *W. succinogenes* were incorporated into liposomes that also contained menaquinone isolated from the *W. succinogenes* membrane [26]. The resulting proteoliposomes catalyzed electron transport from formate to nitrite at about 0.3 mmol nitrite reduced min^{-1} mg^{-1} nitrite reductase. The turnover number of the nitrite reductase in the electron transport (780 electrons s^{-1}) amounted to about 30% of that of the enzyme in growing *W. succinogenes* cells. Using the TPP^+ electrode, the $\Delta\psi$ across the liposomal membrane was determined as −120 mV (negative inside) [26]. Interestingly, the NrfA protein could be incorporated into liposomes even in the absence of NrfH [13]. The resulting proteoliposomes, however, did not catalyze electron transport from H_2 to nitrite, supporting the view that the NrfH protein is required for menaquinol oxidation by nitrite.

Proteoliposomes containing *W. succinogenes* hydrogenase, menaquinone and the NrfHA complex developed a $\Delta\psi$ of −143 mV in the steady state of electron transport from H_2 to nitrite (S. Biel and J. Simon, unpublished). In the absence of nitrite, these proteoliposomes catalyzed the oxidation of H_2 by DMN which led to a similar $\Delta\psi$. $DMNH_2$ was oxidized upon the addition of nitrite but this reaction did not generate a $\Delta\psi$ under the experimental conditions. It was shown previously that H_2 oxidation with DMN by *W. succinogenes* hydrogenase is coupled to $\Delta\psi$ generation in intact cells, inverted cell vesicles or proteoliposomes [114,115]. The corresponding H^+/e ratio in proteoliposomes was experimentally determined to be close to 1.0 [115]. In similar experiments formate oxidation with DMN by *W. succinogenes* formate dehydrogenase was shown to generate a $\Delta\psi$ in cells and proteoliposomes [92,114]. Again, the corresponding H^+/e ratio measured with proteoliposomes was close to 1.0 [115].

In conclusion, the results strongly suggest that the oxidation of formate or H_2 by menaquinone is coupled to Δp generation whereas menaquinol oxidation by nitrite is not (Fig. 2). This view is also supported by the localization of the quinone binding sites. MK is probably reduced near the inner side of the membrane as deduced from the similarity of both FdhC and HydC to *E. coli* FdnI (cf. Sections 3.2 and 3.3). Therefore, the protons consumed by MK reduction are most likely taken up from the cytoplasmic side. This process is coupled to the release of two protons into the periplasm upon oxidation of formate or H_2 thereby generating Δp by a so-called redox loop mechanism (Fig. 2). The quinone binding site of the NrfHA complex is expected to be near the periplasmic surface of the membrane close to the heme *c* groups as it appears unlikely that the single transmembrane domain of NrfH could accommodate a quinone binding site near the cytoplasmic membrane surface. The protons produced by MKH_2 oxidation are therefore thought to be liberated into the periplasm where they balance the protons consumed by nitrite reduction. The theoretical H^+/e ratio for respiratory nitrite ammonification derived from this mechanism is 1.0. This is only 25% of the theoretical ratio calculated in Section 2.1. According to the hypothesis of Δp generation, the low efficiency is largely due to the fact that the free energy of menaquinol oxidation by nitrite is not conserved.

In fumarate respiration of *W. succinogenes*, the electron transport from formate or H_2 to fumarate is also coupled to Δp generation [39,57]. In this case, the standard redox potential at pH 7 of the electron acceptor fumarate is about 300 mV more negative than the acceptor in respiratory nitrite ammonification [E_0'(fumarate/succinate) = +0.03 V]. Nevertheless, it appears that the coupling mechanism of fumarate respiration and respiratory nitrite ammonification is principally identical. The formate dehydrogenase and hydrogenase involved are the same in both electron transport chains, but in fumarate respiration MKH_2 oxidation is catalyzed by fumarate reductase. The latter enzyme is a membrane-bound complex that consists of the catalytic flavoprotein FrdA, the iron–sulfur subunit FrdB and of FrdC, a diheme cytochrome *b* membrane anchor [99,116,117]. The subunits FrdA and FrdB

are oriented to the cytoplasmic side of the membrane. Using intact cells, inverted cell vesicles or proteoliposomes, fumarate reduction by menaquinol was shown to be electroneutral [114,115]. Furthermore, the H^+/e ratio for H_2 oxidation by fumarate was determined as 1.0 in proteoliposomes containing hydrogenase, menaquinone and fumarate reductase [115].

4. Respiratory nitrite ammonification in other proteobacteria

The components of the electron transport chain of respiratory nitrite ammonification are well known in case of the ε-proteobacterium *W. succinogenes*. In contrast, the information on the corresponding electron transfer pathways in other bacteria is much more limited. As a common feature, different proteobacteria contain or encode similar NrfA proteins (Table 2, see [20] for an alignment of NrfA sequences). In this section, these bacteria are categorized according to their proteobacterial subclass and the available data is compared to that described for *W. succinogenes* in Section 3. The following topics are discussed: (I) properties and localization of NrfA, (II) enzymology and role of electron donating enzymes such as formate dehydrogenase or hydrogenase in nitrite ammonification, (III) requirement for quinones in electron transfer from a donor substrate to nitrite and (IV) characterization of proteins involved in electron transfer from the donor substrate to the quinone pool and from the quinone pool to NrfA.

In the absence of a comprehensive characterization of these enzymes and their interaction, data obtained from genome sequencing proved to be very useful for establishing hypothetical models of the electron transport chain of respiratory nitrite ammonification in different bacteria.

Table 2
Structural Nrf proteins predicted from corresponding genes

Name	Organism	Residues[a]	Identity in %	Characteristic features	Database accession number[b]
NrfA	*Wolinella succinogenes* (ε)	507	100	CWTCK and 4 CXXCH motifs	CAB53160
	Sulfurospirillum deleyianum (ε)	503	75	CWTCK and 4 CXXCH motifs	CAB37320
	Campylobacter jejuni NCTC11168 (ε)	610	31	5 CXXCH motifs	NP_282503
	Desulfovibrio vulgaris Hildenborough (δ)	524	29	CWNCK and 4 CXXCH motifs	–
	Geobacter sulfurreducens (δ)	490	30	CLTCK and 4 CXXCH motifs	–
	Carboxydothermus hydrogenoformans (Gram+)	400	32	CMTCK and 4 CXXCH motifs	–
	Carboxydothermus hydrogenoformans (Gram+)	416[c]	33	CMTCK and 4 CXXCH motifs	–
	Porphyromonas gingivalis W83 (Bacteroides)	498	45	CWVCK and 4 CXXCH motifs	–
	Escherichia coli (γ)	478	46	CWSCK and 4 CXXCH motifs	CAA51048
	Salmonella typhi CT18 (γ)	478	46	CWSCK and 4 CXXCH motifs	NP_458575
	Salmonella typhimurium LT2 (γ)	478	47	CWSCK and 4 CXXCH motifs	NP_463142
	Shewanella oneidensis MR-1 (γ)	467	47	CWSCK and 4 CXXCH motifs	–
	Haemophilus influenzae Rd KW20 (γ)	538	43	CWTCK and 4 CXXCH motifs	NP_439227
	Pasteurella multocida PM70 (γ)	510	43	CWSCK and 4 CXXCH motifs	NP_244960
NrfB	*Escherichia coli* (γ)	188	100	5 CXXCH motifs	CAA51042
	Salmonella typhi CT18 (γ)	188	88	5 CXXCH motifs	NP_458576
	Salmonella typhimurium LT2 (γ)	188	89	5 CXXCH motifs	NP_463143
	Haemophilus influenzae Rd KW20 (γ)	226	42	5 CXXCH motifs	NP_439226
	Pasteurella multocida PM70 (γ)	231	43	5 CXXCH motifs	NP_244961
NrfC	*Escherichia coli* (γ)	223	100	16 conserved cysteine residues	CAA51043
	Salmonella typhi CT18 (γ)	223	91	16 conserved cysteine residues	NP_458577
	Salmonella typhimurium LT2 (γ)	223	91	16 conserved cysteine residues	NP_463144
	Haemophilus influenzae Rd KW20 (γ)	225	63	16 conserved cysteine residues	NP_439225
	Pasteurella multocida PM70 (γ)	226	66	16 conserved cysteine residues	NP_244962
NrfD	*Escherichia coli* (γ)	318	100	8 conserved hydrophobic segments	CAA51044
	Salmonella typhi CT18 (γ)	318	86	8 conserved hydrophobic segments	NP_458578
	Salmonella typhimurium LT2 (γ)	318	86	8 conserved hydrophobic segments	NP_463145
	Haemophilus influenzae Rd KW20 (γ)	321	49	8 conserved hydrophobic segments	NP_439224
	Pasteurella multocida PM70 (γ)	319	52	8 conserved hydrophobic segments	NP_244963
NrfH	*Wolinella succinogenes* (ε)	177	100	4 CXXCH motifs; NapC homologue	CAB53159
	Sulfurospirillum deleyianum (ε)	177	68	4 CXXCH motifs; NapC homologue	CAD19316
	Campylobacter jejuni NCTC11168 (ε)	171	36	4 CXXCH motifs; NapC homologue	NP_282504
	Desulfovibrio vulgaris (δ)	159	23	4 CXXCH motifs; NapC homologue	–
	Geobacter sulfurreducens (δ)	154	31	4 CXXCH motifs; NapC homologue	–
	Carboxydothermus hydrogenoformans (Gram$^+$)	137	36	4 CXXCH motifs; NapC homologue	–
	Porphyromonas gingivalis W83 (Bacteroides)	203	43	4 CXXCH motifs; NapC homologue	–

[a]The size of the NrfA pre-proteins is given. In case of alternative start codons the most likely translational start site is considered.
[b]Primary protein sequences derived from preliminary genome sequences are not referenced.
[c]Denotes the protein predicted by the *C. hydrogenoformans nrfA2* gene (Table 3).

The organization of bacterial *nrf* loci is presented in Table 3 (see also Section 5). As the most notable result, the *nrfHA* arrangement is found in proteobacteria belonging to the ε- and δ-subclasses (Sections 4.1 and 4.2) as well as in two non-proteobacteria, the bacteroid *Porphyromonas gingivalis* and the thermophilic Gram-positive *C. hydrogenoformans*. Thus, a NrfHA complex similar to that of *W. succinogenes* is predicted to be present in many different bacteria. Members of the γ-proteobacteria appear to be exceptional since it was proposed that the pathway of electron transfer from the quinone pool to NrfA is independent of a NrfH-like protein (Section 4.3).

4.1. ε-Proteobacteria

The free-living bacterium *S. deleyianum* is similar to *W. succinogenes* in many physiological aspects (Table 1) [49]. NrfA of *S. deleyianum* was the first cytochrome *c* nitrite reductase whose crystal structure was determined [19]. The NrfA sequences from *W. succinogenes* and *S. deleyianum* are very similar (75% identity) including the five heme binding motifs and conserved residues near the active site that are thought to be involved in catalysis (Table 2). The three-dimensional structures of the two enzymes are nearly identical with respect to the location of the five heme groups, the substrate and product channels and the architecture of the catalytic site [20]. Furthermore, the catalytic properties and EPR spectra of both NrfA proteins were found to be nearly identical [14,29,67]. *S. deleyianum* NrfA is found in the periplasm as well as in the membrane fraction where it forms a NrfHA complex similar to that of *W. succinogenes* [14]. The NrfH proteins of the two bacteria share 68% identical residues (Fig. 4 and Table 2). Both organisms contain a *nrfHAIJ* locus on the genome (Table 3) [20]. It is likely that the coupling mechanism of *S. deleyianum* respiratory nitrite ammonification is the same as in *W. succinogenes* although little is known about *S. deleyianum* formate dehydrogenase or [NiFe]-hydrogenase [46]. It is expected that the latter two enzymes are also highly similar to those of *W. succinogenes*.

Among members of the genus *Campylobacter*, only *C. sputorum* biovar *bubulus* was shown to grow by respiratory nitrite ammonification (Table 1) [47]. It was proposed that the electron transport chain from formate to nitrite is similar to that depicted for *W. succinogenes* in Fig. 2. [48]. Bacterial electron transport was shown to be coupled to proton translocation and the corresponding H^+/e ratio was estimated to be 1.0 [48]. When grown with lactate instead of formate as electron donor, the growth yield of *C. sputorum* biovar *bubulus* per mol nitrite was increased by a factor of 2.6 indicating additional ATP synthesis by substrate level phosphorylation [47]. The NrfA protein from *C. sputorum* biovar *bubulus* was not isolated and the corresponding gene was not sequenced.

The membrane fraction of *C. jejuni* NCTC 11168 catalyzes nitrite reduction by benzyl viologen radical with a

Table 3
Organization of *nrf* genes and corresponding system of cytochrome *c* biogenesis

Organism[a]	*nrf* locus	Cytochrome *c* biogenesis	Dissimilatory nitrate reductase
Wolinella succinogenes (ε)	*nrfHAIJ*	system II[f]	Nap[g]
Sulfurospirillum deleyianum (ε)	*nrfHAIJ*	system II[f]	n.r.
Campylobacter jejuni NCTC 11168 (ε)	*nrfHA*[b]	system II	Nap
Desulfovibrio vulgaris Hildenborough (δ)	*nrfHA*	system II	Nap
Geobacter sulfurreducens (δ)	*nrfHA*	system II	?[h]
Porphyromonas gingivalis W83 (Bacteroidaceae)	*nrfHAKLM*	system II	n.p.
Carboxydothermus hydrogenoformans (Gram+)	*nrfHA* and *nrfA2*[c]	system II	?[h]
Escherichia coli (γ)	*nrfABCDEFG*	system I	Nap/Nar
Salmonella typhi CT18 (γ)	*nrfABCDEFG*	system I	Nap/Nar
Salmonella typhimurium LT2 (γ)	*nrfABCDEFG*[d]	system I	Nap/Nar
Shewanella oneidensis MR-1 (γ)	*nrfA*	system I	Nap
Haemophilus influenzae Rd KW20 (γ)	*nrfABCD* and *nrfE-ccmG2-nrfFG*[e]	system I	Nap
Pasteurella multocida PM70 (γ)	*nrfABCDE-ccmG2-nrfFG*	system I	Nap

The data are compiled from published genome sequences [103,118–121] or from searchable preliminary genome sequences presented on the website of The Institute for Genomic Research (TIGR). Genomes were searched using the BLAST algorithm and the following queries: NrfA from *W. succinogenes* and *E. coli*, CcmE and CcmH from *E. coli* [64], CcsA from *Mycobacterium leprae* [122], CcdA from *Bacillus subtilis* [123], NarG and NapA from *E. coli* [82].

n.r.: not reported; n.p.: not present on the genome.

[a]The phylogenetic classification of the bacteria is denoted in parentheses; for proteobacteria the corresponding subclass is given.

[b]The predicted NrfA protein contains five CXXCH motifs.

[c]The genome sequence of *C. hydrogenoformans* reveals a second copy of *nrfA*, named *nrfA2* here, which is not accompanied by a copy of *nrfH*. The predicted NrfA2 protein is 50% identical to *C. hydrogenoformans* NrfA including the conserved CMTCK motif.

[d]The *S. typhimurium nrfE* and *nrfF* genes are fused in one open reading frame.

[e]The *H. influenzae nrfF* and *nrfG* genes are fused in one open reading frame.

[f]The classification is based on the similarity of NrfI to various CcsA proteins.

[g]R. Gross and J. Simon, unpublished.

[h]Presence not known since the genome sequence was in a too preliminary state at the time of writing this article.

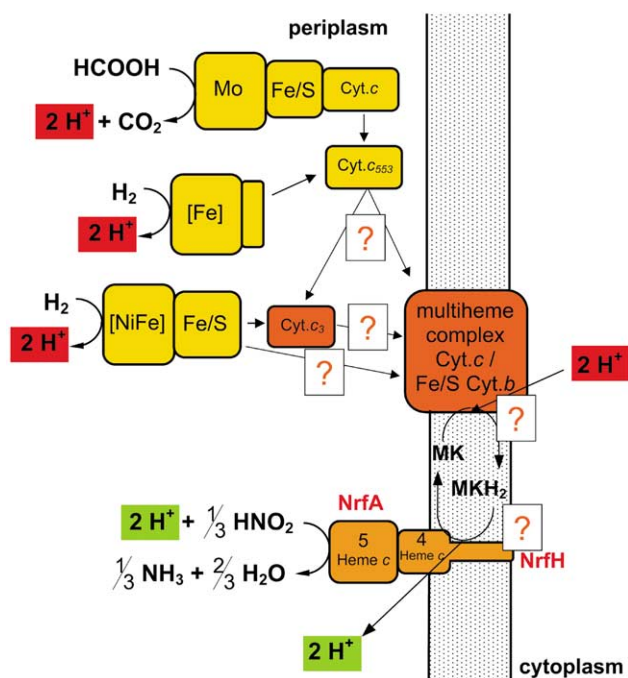


Fig. 5. Hypothetical electron transport chains from formate or H_2 to nitrite in *D. desulfuricans*. Note that the figure is based on results obtained with different strains of *D. desulfuricans* (see text). The membrane-bound cytochrome *c* complex is designated 'Hmc' in *D. desulfuricans* Essex and '9Hc' in *D. desulfuricans* ATCC 27774. Question marks denote speculative reactions or protein interactions. Further explanations are given in the legend of Fig. 2.

higher specific activity than that of *W. succinogenes* (R. Gross and J. Simon, unpublished). The *nrfA* gene of *C. jejuni* NCTC 11168 is located immediately downstream of a *nrfH* homologue (Table 3 and Fig. 4) as seen from the genome sequence [103]. Surprisingly, the predicted NrfA sequence of *C. jejuni* contains a CXXCH motif instead of the CXXCK motif although the overall sequence is similar to those of *W. succinogenes* and *S. deleyianum* (Table 2). The *C. jejuni* genome also contains two gene clusters that are highly similar to the *W. succinogenes hyd* and *fdh* operons suggesting the existence of electron transport chains similar to those shown in Fig. 2 [103].

4.2. δ-Proteobacteria

Within the δ-proteobacteria, respiratory nitrite ammonification was shown only for *D. desulfuricans* and *D. gigas* (Table 1). Proton translocation was shown to be coupled to electron transport from formate or H_2 to nitrite in *D. desulfuricans* (Table 1) [24]. Furthermore, electron transport from H_2 to nitrite in *D. gigas* was reported to develop a Δp and to drive ATP synthesis [25]. The H^+/e ratio for nitrite reduction by H_2 was experimentally determined as approximately 1.0 for both *D. desulfuricans* and *D. gigas* [24,25]. A model electron transport chain from formate or H_2 to nitrite in *D. desulfuricans* is depicted in Fig. 5. The presence of a membrane-bound NrfHA complex is suggested in different *Desulfovibrio* species (Section 4.2.1). The hypothetical chain of electron transport from formate dehydrogenase or hydrogenase to the nitrite reductase is the subject of Section 4.2.2.

4.2.1. Cytochrome *c* nitrite reductase

The activity of nitrite reduction by reduced viologen dyes was found exclusively in the membrane fraction of *Desulfovibrio* spp. [29,76]. In contrast, at least part of the activity is located in the periplasm in proteobacteria of the ε- or γ-subclass. The nitrite ammonifying enzyme (NrfA, 66 kDa) was purified from the membrane fraction of *D. desulfuricans* ATCC 27774 and proved to be a cytochrome *c* nitrite reductase [11,50,71]. The enzyme was reported to be membrane-bound and was proposed to carry six heme *c* groups as concluded from EPR and Mössbauer spectroscopy (see [31] for review). However, the enzyme used for Mössbauer spectroscopy [73,74] showed the low-field EPR signal at $g = 4.8$ indicative of the NrfHA complex of both *W. succinogenes* and *S. deleyianum*. Therefore, the presence of a multiheme NrfHA complex in the *D. desulfuricans* membrane could not be excluded [74]. Indeed, *D. desulfuricans* NrfA was purified in a complex with another *c*-type cytochrome of 19 kDa [75] which is probably a homologue of the NrfH proteins found in the ε-proteobacteria (Fig. 5). This situation is reminiscent of the initial purification attempts of the membrane-bound nitrite reductase from *W. succinogenes* where NrfH was lost during enrichment of NrfA [13]. Unfortunately, the genes encoding the *D. desulfuricans* nitrite reductase complex have not yet been reported but it is likely that they will encode typical NrfA and NrfH proteins. As crystals of the *D. desulfuricans* cytochrome *c* nitrite reductase have been reported [124], hopefully, this point will be clarified upon structure determination.

Recently, a two-subunit nitrite reductase complex was purified from the membrane fraction of *D. vulgaris* Hildenborough [76], an organism that contains a *nrfHA* gene cluster on the genome (Table 3). The small subunit of this complex was identified as the NrfH protein by comparison of the reported N-terminus [76] to that predicted by the *nrfH* gene (Fig. 4). The *D. vulgaris nrfH* and *nrfA* genes predict a tetraheme and a pentaheme cytochrome *c*, respectively (Table 2). The deduced sequence of NrfA contains the conserved CXXCK motif. A similar *nrfHA* locus was also revealed by sequencing the genome of the δ-proteobacterium *Geobacter sulfurreducens* (Tables 2 and 3).

It is not known why cells of different *Desulfovibrio* species synthesize NrfA during sulfate respiration in the absence of nitrite [51,60,76]. This is even more surprising in the light of the fact that *D. vulgaris* Hildenborough did not grow upon nitrite reduction with either lactate or formate as electron donor [76]. A possible explanation, beside a function in nitrite detoxification, would be that NrfA serves in periplasmic sulfite reduction. Cytochrome *c* ni-

trite reductase catalyzes the reduction of sulfite to sulfide (reaction i), which is a six-electron reaction isoelectronic to nitrite ammonification (Eq. a):

$$HSO_3^- + 6[H] \rightarrow HS^- + 3H_2O \quad \text{(i)}$$

In fact, the NrfHA complex of *D. desulfuricans* was isolated by enrichment of the sulfite reductase activity from the membrane fraction [75]. The activity was determined with methylviologen as electron donor which was reduced by H_2 in the presence of hydrogenase. The specific activity of the purified NrfHA complex (2 μmol H_2 oxidized min^{-1} mg $protein^{-1}$) was even higher than that of the cytoplasmic siroheme sulfite reductase [125]. Sulfite reductase activity was also reported for the NrfHA complex from *D. vulgaris* [76] and for *S. deleyianum* NrfA [67]. In all cases, the specific activity with sulfite was less than 0.5% of that with nitrite [67,75,76]. The physiological function of periplasmic sulfite reduction is not clear since it is not considered to be involved in Δp generation or ATP synthesis.

4.2.2. The electron transport chain from formate or H_2 to nitrite

The presence of a NrfHA complex in *Desulfovibrio* spp. makes it plausible that a respiratory quinone like menaquinone-6 (Table 1) is involved in electron transfer to nitrite, although this has not been demonstrated experimentally (Fig. 5). As in *W. succinogenes*, the NrfH protein is expected to function as the menaquinol oxidizing subunit in the electroneutral oxidation of menaquinol by nitrite. If true, Δp has to be generated upon menaquinone reduction by either H_2 or formate (Fig. 5). Hydrogenase and formate dehydrogenase were described as periplasmic enzymes in *D. desulfuricans* and *D. gigas* [24,53]. Unfortunately, the electron transport from hydrogenase or formate dehydrogenase to the quinone pool is essentially unclear in *Desulfovibrio* spp., largely due to the fact that menaquinone is assumed not to be a constituent of the electron transport chain from H_2 or formate to sulfate.

Within the genus *Desulfovibrio* various [NiFe]-, [Fe]- or [NiFeSe]-hydrogenases were described which are located either in the periplasm, in the membrane or in the cytoplasm [126,127]. The crystal structure of the periplasmic [NiFe]-hydrogenase from *D. desulfuricans* ATCC 27774 was determined recently [112]. The enzyme is a heterodimer of two subunits (62 kDa and 27 kDa) that are similar to those of other [NiFe]-hydrogenases (cf. Section 3.3). The low-potential tetraheme cytochrome c_3 is thought to interact with the *D. desulfuricans* [NiFe]-hydrogenase and to receive electrons from the distal [4Fe-4S] cluster of the small hydrogenase subunit [112,128–130]. A further known structure is that of the periplasmic [Fe]-hydrogenase from *D. desulfuricans* ATCC 7757 [131]. This enzyme also consists of two subunits but the active site is unrelated to that of the [NiFe]-hydrogenase. It was suggested that the [Fe]-hydrogenase transfers electrons to the periplasmic monoheme cytochrome c_{553} [132].

Intact cells of several *Desulfovibrio* species catalyze nitrite reduction by formate at a rather low rate of less than 20 nmol nitrite reduced min^{-1} (mg dry cell $wt)^{-1}$ [60]. Periplasmic formate dehydrogenases were isolated from *D. desulfuricans* ATCC 27774 [133] and *D. vulgaris* Hildenborough [134,135]. The enzymes from both organisms consist of a molybdenum-containing catalytic subunit, an iron–sulfur protein and a *c*-type cytochrome (Fig. 5). The first two proteins are similar to *W. succinogenes* FdhA and FdhB (Section 3.2). The diheme cytochrome *b* (FdhC) of *W. succinogenes* seems to be replaced by a cytochrome *c* subunit as electron acceptor. The *fdh* gene cluster from *D. vulgaris* Hildenborough is known from genome sequencing by The Institute for Genomic Research. It encodes the three subunits of formate dehydrogenase, as revealed by comparison of the predicted primary sequences to the reported N-termini [134]. The cytochrome *c* subunit contains four heme *c* binding motifs, in contrast to the biochemical characterization of this protein where only one heme *c* per peptide chain was determined [134]. The cytochrome *c* subunit of the *D. desulfuricans* formate dehydrogenase was reported to be a tetraheme cytochrome *c* [133]. The trimeric formate dehydrogenase of *D. vulgaris* is thought to interact with the periplasmic cytochrome c_{553} [135, 136].

The subsequent electron transport from the *c*-type cytochromes to the quinone pool is not known. In the case of *D. desulfuricans* it was suggested that the electrons are eventually transferred to a putative membrane-bound multiheme cytochrome complex which was postulated to consist of four subunits (Fig. 5) [137]. The initial electron acceptor is thought to be a nonaheme cytochrome *c* that is encoded by the *9hcA* gene in *D. desulfuricans* ATCC 27774 and whose crystal structure is known [138–140]. Further subunits of the complex might be encoded by the adjacent *9hcB–D* genes that predict an iron–sulfur protein, a possible diheme cytochrome *b* and a hydrophobic protein of unknown function, respectively [137]. One can speculate that the latter two proteins may function in menaquinone reduction thus making electrons available for nitrite reduction (Fig. 5). In *D. desulfuricans* Essex, the nonaheme cytochrome *c* was reported to interact directly with the [NiFe]-hydrogenase without the mediation of cytochrome c_3 [141,142]. This observation is supported by the fact that a cytochrome c_3 mutant of *D. desulfuricans* G20 was not affected in growth with H_2 and sulfate [143].

The function of the *D. desulfuricans* 9hc complex may be carried out by the putative Hmc complex in *D. vulgaris* Hildenborough and *D. gigas* [144,145]. HmcA is a high-molecular-mass cytochrome with 16 heme *c* moieties, HmcB and HmcF are predicted to be iron–sulfur proteins while the function of the hydrophobic membrane proteins HmcC, HmcD and HmcE is not known. As suggested by the phenotype of a corresponding deletion mutant, the Hmc complex is involved in electron transport from H_2

to sulfate [146]. However, cells of the *hmc* mutant were not examined for electron transport to nitrite.

4.3. γ-Proteobacteria

Nitrite ammonification is widespread in γ-proteobacteria [147]. However, the corresponding enzymes or the capability of respiratory nitrite ammonification were characterized in only a few cases. Cytochrome *c* nitrite reductases (NrfA) with similar properties were isolated from the soluble cell fractions of *E. coli* (Section 4.3.1), *Vibrio fischeri* [15,16] and *Vibrio alginolyticus* [148]. Putative *nrfA* genes were identified in several γ-proteobacterial genomes (Table 3, see also Section 5).

4.3.1. The cytochrome c nitrite reductase of E. coli

E. coli NrfA (cytochrome c_{552}) was isolated from the soluble cell fraction [8–10] but minor amounts were also identified in the membrane fraction [21,149]. The periplasmic cytochrome c_{552} was described in the early 1960s [150] and was suggested to function as a nitrite reductase as the reduced heme *c* in the protein was oxidized by nitrite [151–153]. It is now clear that NrfA is a pentaheme *c*-type cytochrome which is synthesized as a pre-protein with a signal peptide of 26 or 33 amino acid residues [17,18,21]. The covalent attachment of heme to the conserved CXXCK motif was first demonstrated for *E. coli* NrfA [18]. The crystal structure of *E. coli* NrfA was determined recently [21] and was found to be essentially identical to those of the NrfA proteins from *W. succinogenes* and *S. deleyianum*. *E. coli* NrfA contains all characteristic features discussed in Section 3.1.1 for *W. succinogenes* NrfA, although the primary sequences of these two NrfA proteins share only 46% identical residues (Table 2). It is notable, however, that *W. succinogenes* NrfA more closely resembles any γ-proteobacterial NrfA than NrfA of the fellow ε-proteobacterium *C. jejuni* (Table 2).

Replacement of the lysine ligand of the active site heme group in *E. coli* NrfA resulted in a mutant protein with lowered nitrite reductase activity measured with methyl viologen radical as electron donor [18]. Similar results were obtained for *W. succinogenes* NrfA (cf. Section 3.1.1). As in the ε-proteobacterial NrfA proteins, heme 2 of *E. coli* NrfA was favored as the entry point for electrons donated by its partner cytochrome *c* (cf. Section 4.3.2) although its solvent accessibility is lower and the electropositive patch in the vicinity of heme 2 is less pronounced in the *E. coli* protein [21]. Optical and EPR spectra of *E. coli* NrfA are similar to those of the NrfA protein from the soluble cell fraction of *W. succinogenes* or *S. deleyianum* [21,66].

During anaerobic growth in the presence of nitrite, *E. coli* produces a second nitrite ammonifying enzyme (NirBD) that is regulated differently from NrfA [4,154,155]. NirBD is a cytoplasmic NADH-dependent nitrite reductase that is responsible for more than 80% of the

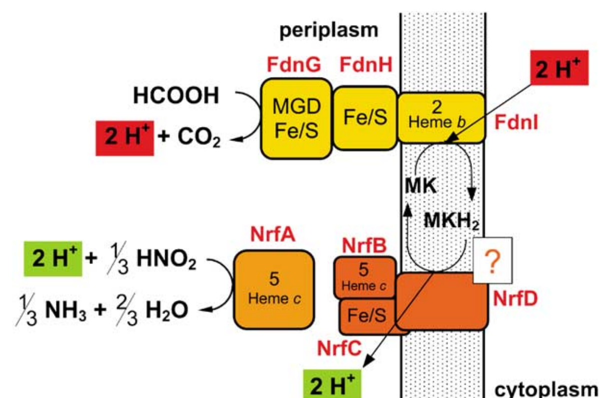


Fig. 6. Enzyme complexes involved in electron transport from formate to nitrite in *E. coli*. The question mark indicates that the quinol-oxidizing NrfBCD complex is hypothetical. Note that demethylmenaquinone can functionally replace MK. Further explanations are given in the legend of Fig. 2.

total nitrite reductase activity while the residual activity is catalyzed by NrfA [23,83,147]. NirB is an iron–sulfur protein that also carries FAD and a siroheme cofactor [156]. The function of NirBD was assigned primarily to detoxification of nitrite which is produced by the cytoplasmically oriented membrane-bound nitrate reductase complex (NarGHI) [4]. The NirBD enzyme is not regarded as a respiratory enzyme as energy is apparently not conserved during nitrite reduction by NADH [7,23].

4.3.2. Electron transport to NrfA in E. coli

NrfA was initially thought to be involved in nitrite reduction by formate thus establishing the abbreviation 'nrf' [147,157,158]. At minor rates, pyruvate, ethanol, glucose and lactate were also reported to serve as electron donors for NrfA [23,147,158,159]. Under anaerobic conditions, formate is readily formed from pyruvate by pyruvate formate lyase. Growth of *E. coli* cells with formate and nitrite as sole energy substrates has not been reported and the activity of nitrite reduction by formate was measured to be below 0.1 μmol nitrite reduced min^{-1} (mg dry cell $wt)^{-1}$ [147,157,159]. Nevertheless, 'formate-dependent nitrite ammonification' was shown to be coupled to the generation of a membrane potential in intact *E. coli* cells [22,23]. Nitrite reduction by formate is observed only with intact cells. The loss of activity after cell disruption can be explained by the fact that NrfA is primarily located in the periplasmic space. The requirement for naphthoquinones (demethylmenaquinone and/or menaquinone) for electron transport from formate to nitrite has been demonstrated [27]. The involvement of any of the at least three *E. coli* hydrogenases in the Nrf pathway has not yet been shown.

The hypothetical electron transport chain from formate to nitrite of *E. coli* is shown in Fig. 6. The main difference from *W. succinogenes* (Fig. 2) is the electron transfer pathway from quinol to NrfA which was proposed not to involve a cytochrome of the NapC/NirT family [160]. *E. coli* NrfA is encoded by the first gene of the *nrfA-G* gene

cluster [17,160] which does not contain a *nrfH* homologue. Furthermore, all members of the NapC/NirT family encoded on the *E. coli* genome (NapC, TorC and TorY) are probably not involved in electron transport to NrfA as their genes are organized in clusters that encode catalytic subunits of other electron transport enzymes involved in anaerobic respiration, i.e. periplasmic nitrate reductase or TMAO reductase [161–163]. Instead, the products of the *nrfB*, *C* and *D* genes might carry out the function of NrfH in *E. coli* but neither this function nor any interaction of the NrfA, B, C and D proteins has been experimentally demonstrated so far. The NrfB sequence contains five CXXCH motifs and the corresponding protein was therefore predicted to be a pentaheme cytochrome *c* (23.8 kDa). The sequence of NrfB is unrelated to any NrfH sequence. NrfB was detected by heme staining of *E. coli* proteins after SDS–PAGE [164] and was found to be membrane-associated [149]. The hydrophobic N-terminus of NrfB might function either as a membrane anchor or as a signal peptide [160]. NrfC (24.5 kDa) is likely to be a Fe/S protein as it contains 16 cysteine residues that are conserved in various Fe/S proteins involved in electron transport of anaerobic respiration [160]. NrfC was thought to be a membrane-bound protein [160] although the presence of a double-arginine motif might suggest a periplasmic localization [83]. NrfD (37 kDa) is predicted to be a membrane-bound protein with eight transmembrane traversions. It is similar to PsrC, the membrane anchor of the *W. succinogenes* polysulfide reductase complex (PsrABC) that is thought to bind MMK-6 [165]. Assuming a membrane-bound NrfBCD complex (Fig. 6), NrfB might be the direct electron donor to NrfA with NrfC as an electron mediator and NrfD as the naphthoquinol oxidase. Protons obtained upon quinol oxidation by nitrite are thought to be liberated into the periplasmic space where they balance the protons taken up in the course of nitrite reduction. It cannot be excluded, however, that NrfD is a proton pump, in contrast to *W. succinogenes* NrfH. The *nrfE*, *F* and *G* gene products of *E. coli* are not considered to be involved in electron transfer to cytochrome *c* nitrite reductase. The corresponding gene products are discussed in Section 6. Differences in the enzyme surface in the vicinity of heme 2 of NrfA might indicate whether a NrfA protein interacts with a NrfB or a NrfH protein. In fact, *E. coli* NrfA contains a striking seven residue insertion (residues 169–175 in the pre-protein) located near the heme 2 binding motif (residues 160–164) and the putative docking region with NrfB [21]. This insertion is conserved in NrfA sequences of *nrfB*-containing organisms whereas it appears to be absent in those with a *nrfH* gene [20,21].

E. coli contains three different formate dehydrogenases (see [166] for review). All three enzymes were reported to donate electrons into the Nrf pathway as suggested from the rates of nitrite reduction by formate in intact cells of appropriate formate dehydrogenase mutants [167]. Formate dehydrogenase-N (FdnGHI) is the predominant formate dehydrogenase under anaerobic conditions in the presence of nitrate in the mM range (Fig. 6) [167]. The enzyme is oriented to the periplasm and its high-resolution structure is known (cf. Section 3.2) [94,168]. Formate dehydrogenase-N catalyzes the reduction of respiratory naphthoquinones which is coupled to apparent proton translocation across the membrane by a redox-loop mechanism (Fig. 6) [98,169]. Notably, formate dehydrogenase-N might also reduce ubiquinone which, to a minor extent, is present in anaerobically grown *E. coli* cells. However,

Table 4
Accessory Nrf proteins predicted from the corresponding genes

Name	Organism	Residues	Identity in %	Characteristic feature	Database accession number[a]
NrfI	*Wolinella succinogenes* (ε)	902	100	W-rich motif: WGRYWAWD	CAB53161
NrfJ	*Wolinella succinogenes* (ε)	217	100		CAB53162
NrfE	*Escherichia coli* (γ)	552	100	W-rich motif: WGGWWFWD	NP_418498
	Salmonella typhi CT18 (γ)	578	78	W-rich motif: WGGWWFWD	NP_458579
	Salmonella typhimurium LT2 (γ)	740	78	W-rich motif: WGGWWFWD	NP_463146
	Haemophilus influenzae Rd KW20 (γ)	635	41	W-rich motif: WGGWWFWD	NP_439096
	Pasteurella multocida PM70 (γ)	641	44	W-rich motif: WGGWWFWD	NP_244964
NrfF	*Escherichia coli* (γ)	127	100		NP_418499
	Salmonella typhi CT18 (γ)	170	74		NP_458580
	Salmonella typhimurium LT2 (γ)	740	74	NrfF is fused to NrfE	NP_463146
	Haemophilus influenzae Rd KW20 (γ)	384	49		NP_439094
	Pasteurella multocida (γ)	161	47		NP_244966
NrfG	*Escherichia coli* (γ)	198	100		CAA51047
	Salmonella typhi CT18 (γ)	206	69		NP_458581
	Salmonella typhimurium LT2 (γ)	206	70		NP_463147
	Haemophilus influenzae Rd KW20 (γ)	384	30	NrfG is fused to NrfF	NP_439094
	Pasteurella multocida (γ)	278	36		NP_244967
NrfK	*Porphyromonas gingivalis* W83 (Bacteroides)	208	–		–
NrfL	*Porphyromonas gingivalis* W83 (Bacteroides)	238	–		–
NrfM	*Porphyromonas gingivalis* W83 (Bacteroides)	271	–	W-rich motif: WGTYWNWD	–

[a]Primary protein sequences derived from preliminary genome sequences are not referenced.

ubiquinol apparently does not serve as electron donor to NrfA [27].

The membrane-bound formate dehydrogenase-O is expressed constitutively but at a low level [4]. It is under debate whether the catalytic site of this enzyme is oriented to the cytoplasm or to the periplasm [168,170]. Formate dehydrogenase-H is part of the cytoplasmic formate hydrogen lyase which is repressed by the presence of nitrate. The electron transfer pathway from formate dehydrogenase-H to NrfA is essentially unclear but is assumed not to contribute to Δp generation [167].

5. Organization of *nrf* genes

Table 3 summarizes the organization of bacterial *nrf* loci. The deduced primary sequences are compared in Tables 2 and 4. Many of the Nrf proteins are only predicted from genome sequences. All *nrfH* genes are located upstream of *nrfA* homologs (cf. Section 4). The capability of nitrite ammonification by the *nrfH*-containing non-proteobacteria *P. gingivalis* and *C. hydrogenoformans* has not yet been examined. In Gram-positive bacteria, *c*-type cytochromes are always anchored to the membrane with their hydrophilic parts facing the outside of the cell. It is therefore not surprising to find the *nrfHA* genes in the Gram-positive bacterium *C. hydrogenoformans* rather than the *nrfABCD* arrangement. The *nrfABCD* genes form a conserved entity that appears to be restricted to γ-proteobacteria (Table 3). An exception is the *nrfA* gene of *Shewanella oneidensis* MR-1 (formerly *S. putrefaciens*) that seems not to be organized in a polycistronic operon. The electron transfer pathway to NrfA in this organism remains to be elucidated. The corresponding genome contains separated *nrfC* and *nrfD* homologs and also encodes a variety of *c*-type cytochromes including a member of the NapC/NirT family, the *cymA* gene product (Fig. 4) [85,171] which was shown to be essential for various modes of anaerobic respiration including nitrate respiration [171,172]. The *cymA* gene is also not part of a gene cluster. The open reading frames downstream of the *nrfHA* or *nrfABCD* modules as shown in Table 3 are discussed in the following section as they are considered to function in NrfA biogenesis.

6. Heme attachment to NrfA

All NrfA proteins are expected to be similar cytochromes and most of them are considered to contain the lysine-ligated active site heme *c* group. A complex cytochrome *c* biogenesis apparatus is required for the maturation of bacterial *c*-type cytochromes for which, depending on the organism, two completely different systems (named system I and II) emerged in recent years [173]. Evidence was provided that both systems can serve in heme attachment to CXXCH motifs of NrfA but neither system serves in the attachment of the CXXCK-bound active site heme group. This holds true for the system I organism *E. coli* [18] as well as for the system II organism *W. succinogenes* [63]. Instead, at least some of the open reading frames that follow the structural *nrf* genes in ε- or γ-proteobacteria (*nrfIJ* or *nrfEFG*; Table 3) were shown to be specifically required for heme attachment to CXXCK. Some properties of these accessory *nrf* gene products are compared in Table 4.

The biogenesis of *c*-type cytochromes minimally comprises the following steps: (1) transport of apo-cytochrome and of heme across the membrane, as *c*-type cytochromes are typically located either in the periplasm or at the outer aspect of the membrane [64], (2) maintaining the cysteine residues of the heme binding motif in a reduced state, (3) the covalent attachment of heme catalyzed by a cytochrome *c* heme lyase. Several gene loci were identified that are involved in the synthesis of *c*-type cytochromes in different bacteria and their classification led to the establishment of system I and system II of cytochrome *c* biogenesis [173,174]. According to the nomenclature of Kranz and co-workers [173], *E. coli* is prototypic for system I in containing the *ccmA–H* locus (Table 3). System I is found in α- and γ-proteobacteria, in plant and protozoal mitochondria and in various archaea. Components of system II are the *ccsA*, *ccsB*, *ccsX* and *ccdA* gene products [175] which share no overall similarity to any of the *ccm* gene products. System II is present in members of the β-, δ- and ε-proteobacteria, in several Gram-positive bacteria, in the cyanobacterium *Synechocystis* sp. PCC 6803 as well as in chloroplasts. Every gene product of both systems is predicted to be membrane-bound and proved to be essential for the synthesis of each *c*-type cytochrome of the respective organism. The only similarity between proteins that belong to different systems is a short tryptophan-rich stretch (consensus motif WGXϕWXWD with ϕ representing an aromatic residue; Table 4) together with a couple of conserved histidine residues. These features are present in CcmC, CcmF and NrfE (system I) as well as in CcsA and NrfI (system II) [18,63,173,176]. The tryptophan-rich motif was suggested to play a role in either heme export and/or in heme delivery to the heme lyase [122,176].

An *E. coli* mutant unable to produce NrfE, F and G was found to synthesize an inactive NrfA protein that lacked the active site heme group while at least three of the other hemes were covalently bound [18]. Using *E. coli* strains containing only a single inactivated *nrf* gene, NrfE as well as NrfG (but not NrfF) were demonstrated to be required for formate-dependent nitrite reduction [149]. In contrast, none of the *nrfE*, *F* and *G* genes appeared to be involved in the production of *c*-type cytochromes with CXXCH motifs [149]. The NrfE protein is similar to CcmF whereas NrfF and NrfG are similar to the N-terminal and C-terminal region of CcmH, respectively. CcmF and CcmH most likely belong to a heme lyase complex

that is required for heme attachment to CXXCH motifs [64,177]. It is not known how a heme lyase recognizes a heme binding motif or how the discrimination between histidine and lysine in such a motif is achieved. Recent evidence suggested that CcmH is the best candidate for the recognition of the CXXCH motif [177]. Possibly, this role is taken over by NrfG (or by a NrfFG complex) in case of the CXXCK motif. CcmF and NrfE might be involved in heme delivery to the site of heme ligation rather than in the attachment process itself [177].

The arrangement of the *nrfEFG* genes is not strictly conserved in other γ-proteobacteria (Table 3). In *S. typhimurium* LT2, *nrfE* appears to be fused to *nrfF* while the *H. influenzae* Rd KW20 genome revealed a likely gene fusion of the *nrfF* and *nrfG* genes. The closely related bacteria *H. influenzae* and *P. multocida* PM70 each contain an additional copy of *ccmG* between *nrfE* and *nrfF* (Table 4). CcmG of *E. coli* is a periplasmic thiol:disulfide oxidoreductase that probably serves in keeping the heme binding cysteines of apo-cytochrome *c* in a reduced state [178,179]. In *H. influenzae*, the *nrfE-ccmG2-nrfFG* cluster is not located downstream of *nrfABCD* but is found elsewhere on the genome.

How do system II organisms handle the specific task of heme attachment to the NrfA CXXCK motif? In *W. succinogenes*, a mutant containing an inactivated *nrfI* gene had properties similar to those of the *E. coli nrfEFG* deletion mutant [63]. The enzymatically inactive NrfA was isolated from the *nrfI* mutant and was found to contain the four CXXCH-ligated heme groups while the active site heme group was missing. When the lysine residue of the CXXCK motif was altered to a histidine residue, NrfA was produced with five covalently bound heme groups. Heme attachment to the five CXXCH motifs in the variant NrfA was found to occur also in the absence of the intact *nrfI* gene [63]. It was concluded that distinct heme lyases exist that recognize either the CXXCH or the CXXCK heme binding motif and that the *nrfI* gene product might be the CXXCK-specific heme lyase. The C-terminal third of NrfI is similar to various CcsA proteins which were proposed to function as CXXCH-specific heme lyases in system II. The N-terminal part of NrfI resembles CcsB (Ccs1) proteins, another system II component. The *W. succinogenes* NrfJ protein has some similarity to thioredoxin-like CcsX proteins of system II organisms but it lacks the common CXXC signature. As a *nrfJ* deletion mutant of *W. succinogenes* had wild-type properties with respect to nitrite respiration and nitrite reductase activity, *nrfJ* might be a pseudogene or its function is taken over by another system II protein in the mutant cells [26].

Like *W. succinogenes*, *S. deleyianum* also possesses a *nrfHAIJ* locus (Table 3). The *nrfHA* genes of *P. gingivalis* are followed by three genes (*nrfKLM*) whose function might be similar to that of *W. succinogenes nrfI* [20]. The predicted NrfM protein resembles the C-terminal region of *W. succinogenes* NrfI including the tryptophan-rich stretch (Table 4). In contrast, accessory *nrf* genes were not found downstream of *nrfA* on the genomes of *C. jejuni*, *G. sulfurreducens*, *D. vulgaris* and *C. hydrogenoformans* (Table 3). In case of *C. jejuni* this observation is not unexpected as the corresponding NrfA protein lacks the CXXCK motif. However, it is unclear how the δ-proteobacteria and the Gram-positive organism manage heme attachment to the CXXCK motif of NrfA. Possibly, accessory *nrf* genes are situated elsewhere on the genome. Alternatively, it cannot be excluded that in these organisms a single heme lyase is able to attach heme to both the CXXCH and CXXCK motifs.

Taken together, the results indicate that, at least in *E. coli* and *W. succinogenes*, heme ligation to the CXXCK heme binding motif requires specialized heme lyases which cannot be replaced by those serving in heme ligation to the CXXCH motif. The specific heme lyases probably evolved from the existing cytochrome *c* biogenesis system resulting in *nrfEFG* genes in system I and *nrfIJ* genes in system II. The function of some of these genes, namely *nrfF* and *nrfJ*, might also be carried out by proteins of the conventional cytochrome *c* biogenesis system. Surprisingly, the occurrence of the *nrfHA* genes correlates strictly with the presence of system II as do the *nrfBCD* genes with system I (Table 3). It is not known whether this finding has any biological significance.

7. Respiratory nitrate reductases in *nrfA*-containing organisms

Organisms capable of nitrite ammonification usually also catalyze nitrate reduction to nitrite in dissimilatory metabolism (Table 1). A notable exception are δ-proteobacteria where the ability of nitrate reduction is restricted to only a few species like *D. desulfuricans* and *D. propionicus* [51]. Thus, the majority of sulfate-reducing *Desulfovibrio* species use nitrite, but not nitrate [60]. Like nitrite ammonification, nitrate reduction may be categorized as an assimilatory, dissimilatory or respiratory process (Fig. 1) [1]. Assimilatory nitrate reduction is catalyzed by the cytoplasmic Nas enzyme using NAD(P)H as electron donor [82]. Respiratory nitrate reduction is dependent either on a membrane-bound nitrate reductase, the NarGHI complex [180], or on a periplasmic nitrate reductase, the NapAB complex [82,83]. The NarGHI complex is oriented to the cytoplasmic side of the membrane, and a Δp is generated by a redox loop mechanism during reduction of nitrate by a respiratory quinol [98]. NarG is the molybdopterin-containing catalytic subunit that receives electrons from the iron–sulfur subunit NrfH. The enzyme is anchored in the membrane by NarI, a diheme cytochrome *b* quinol oxidase. The periplasmic NapA is also a molybdoprotein that usually forms a complex with the diheme cytochrome *c* NapB. The crystal structure of NapA from

D. desulfuricans is known [96]. It is assumed that the tetraheme cytochrome *c* NapC is the electron mediator between the quinone pool and the NapAB complex (cf. Section 3.1.2). The *napA*, *B* and *C* genes are commonly found in gene clusters that consist of up to seven open reading frames [82,83]. The composition of the *nap* loci varies considerably in different bacteria and the function of genes additional to *napA*, *B* and *C* remains to be elucidated. The electron transfer from quinol to nitrate catalyzed by the Nap enzyme is not considered to contribute to Δp generation. However, Nap might be involved in respiratory nitrate reduction when a membrane-bound dehydrogenase complex couples substrate oxidation by quinone to Δp generation. Such a function would be similar to that of the NrfHA cytochrome *c* nitrite reductase in respiratory nitrite ammonification.

Bacteria containing the *nrfHA* genes often possess a *nap* gene cluster whereas *nar* genes were not reported so far (Table 3). All γ-proteobacteria with a *nrfA* gene also contain *nap* genes but only some of them have a *nar* gene cluster. The presence of the Nap and Nrf pathways should enable periplasmic nitrate ammonification without transport of nitrate and nitrite across the membrane. The presence of both Nar and Nap enzymes was reported for α-, β- and γ-proteobacteria but was not yet shown to occur within the δ- or the ε-subclass [82]. Different functions were assigned to Nap and Nar when present in the same cell. In *E. coli*, for example, the Nar enzyme is the dominant nitrate reductase in the presence of nitrate in the mM range while Nap appears to play a role in nitrate scavenging at low nitrate concentrations [4,82,83,181].

8. Concluding remarks

The NrfA protein is a key enzyme in the presented pathways of respiratory nitrite ammonification. The electron transport chain catalyzing nitrite reduction by formate (or H_2) is well understood only in the case of *W. succinogenes* where it was shown to consist of only two enzyme complexes with menaquinone as the redox mediator. The composition of the electron transport chains leading to NrfA in other bacteria is less clear. Possibly, additional electron transfer proteins are involved or the mechanism of Δp generation differs from that of *W. succinogenes*. Furthermore, it is uncertain whether the quinone pool is generally required for electron transport to NrfA. Future work will hopefully elucidate the structure and interaction of those proteins involved in the different electron transfer pathways to cytochrome *c* nitrite reductase.

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