Cytochrome c Nitrite Reductase from Wolinella succinogenes
STRUCTURE AT 1.6 Å RESOLUTION, INHIBITOR BINDING, AND HEME-PACKING MOTIFS*

Received for publication, July 13, 2000, and in revised form, September 11, 2000
Published, JBC Papers in Press, September 12, 2000, DOI 10.1074/jbc.M006188200

Oliver Einsle‡§, Petra Stach§, Albrecht Messerschmidt‡, Jörg Simon‡, Achim Kröger‡, Robert Huber‡, and Peter M. H. Kroneck**

From the ‡Max-Planck-Institut für Biochemie, Abteilung Strukturforschung, Am Klopferspitz 18a, 82152 Martinsried, the §Universität Konstanz, Mathematisch-Naturwissenschaftliche Sektion, Fachbereich Biologie, Fach M065, Universitätstr. 10, 78457 Konstanz, and the ¶Johann Wolfgang Goethe-Universität Frankfurt, Institut für Mikrobiologie, Marie-Curie-Str. 9, 60439 Frankfurt am Main, Germany

Cytochrome c nitrite reductase catalyzes the 6-electron reduction of nitrite to ammonia. This second part of the respiratory pathway of nitrate ammonification is a key step in the biological nitrogen cycle. The x-ray structure of the enzyme from the e-proteobacterium Wolinella succinogenes has been solved to a resolution of 1.6 Å. It is a pentaheme c-type cytochrome whose heme groups are packed in characteristic motifs that also occur in other multiheme cytochromes. Structures of W. succinogenes nitrite reductase have been obtained with water bound to the active site heme iron as well as with complexes with two inhibitors, sulfate and azide, whose binding modes and inhibitory functions differ significantly. Cytochrome c nitrite reductase is part of a highly optimized respiratory system found in a wide range of Gram-negative bacteria. It reduces both anionic and neutral substrates at the distal side of a lysine-coordinated high-spin heme group, which is accessible through two different channels, allowing for a guided flow of reaction educt and product. Based on sequence comparison and secondary structure prediction, we have demonstrated that cytochrome c nitrite reductases constitute a protein family of high structural similarity.

The biogeochemical nitrogen cycle represents a network of reactions catalyzed by enzymes with different metal cofactors, which allows for redox transitions of nitrogen between its oxidation state (+5), as in nitrate, and (−3), as in ammonia and its most abundant form dinitrogen. The one respiratory pathway that covers the whole range between nitrate and ammonia is the dissimilatory nitrate reduction to ammonia (1). Hereby, nitrate is first reduced to nitrite in a 2-electron step by a nitrate reductase, and subsequently the product nitrite is converted to ammonia in a 6-electron step catalyzed by a cytochrome c nitrite reductase (NiR), the subject of this study.

Cytochrome c nitrite reductases are pentaheme enzymes with a molecular mass of 55–65 kDa, encoded by a single gene termed nrfA (2). They have been found so far in proteobacteria belonging to the subdivisions γ (Escherichia coli (3) and Hae-mophilus influenzae Rd (4)) and ε (Sulfurospirillum deleyi-num (5) and Wolinella succinogenes (6)). The x-ray structure of NiR from S. deleyianum was solved recently to a resolution of 1.9 Å (5) and showed the enzyme to be a compact homodimer. The active site was localized at heme 1, and a Ca2+ ion was observed in close proximity. The heme group arrangement of nitrite reductase showed similarities to the one of hydroxylamine oxidoreductase (7), although sequence homologies were negligible, and structural homologies were limited to few regions of the protein (5). The conservation of the heme group arrangements in multiheme c cytochromes has been observed and discussed (8, 9), but the significance of such highly conserved motifs is not yet understood.

Here we report on the purification, crystallization, and structural analysis of cytochrome c nitrite reductase from the e-proteobacterium W. succinogenes. In addition to the water-ligated enzyme, we will present structures with two inhibitors of nitrite reductase activity, sulfate and azide, which act in different ways to impede substrate binding to the active site.

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection—NiR was isolated from the membrane fraction of W. succinogenes (10). Triton X-100 (0.05% w/v) used in the purification was removed by Extracti-Gel before crystallization.

Crystals were obtained by vapor diffusion using 12% polyethylene glycol (PEG 4000), 200 mM ammonium sulfate, and 15 mM YCl3 in 100 mM sodium acetate buffer, pH 5.7. The protein was stored at concentrations as high as 35 mg/ml and was diluted to 7.5 mg/ml immediately prior to crystallization experiments. Higher protein concentrations were beneficial for crystal growth, presumably by favoring dimerization of the enzyme. Without the addition of yttrium trichloride, only small plates were obtained whereas the addition of the compound yielded large, well diffracting single crystals. Yttrium ions took part in every single intermolecular contact in the crystal packing, including the contact between two monomers forming a dimer, where a Y3+ ion was coordinated by a propionate sidechain of heme 5 from each monomer. Another Y3+ ion bound close to the exit of the product channel, coordinated by propionate sidechains from both heme 3 and heme 4.

The crystals belonged to space group I 4 22, with unit cell constants of a = b = 119.5 Å and c = 186.0 Å. Despite a Matthews parameter of Vm = 2.83 Å3/Da that corresponds to a rather high solvent content of

* This work was supported in part by Deutsche Forschungsgemeinschaft (to P. K.), Volkswagenstiftung (to P. K.), EU-Biotech Project (to O. E., A. M., and R. H.), and Fonds der Chemischen Industrie (to P. K.).

** To whom correspondence may be addressed. Tel.: 49 7531 88 2966; E-mail: Peter.Kroneck@uni-konstanz.de.

1 The abbreviations used are: NiR, cytochrome c nitrite reductase; ICP, inductively coupled plasma emission spectroscopy; nrf, nitrite reductase with formate; r.m.s.d., root mean square deviation.
The relative orientation of the five heme groups corresponds exactly to the one observed in *S. deleyianum* nitrite reductase, including the fact that heme 1 is the five-coordinate active site heme group. In a superposition of the structures from *W. succinogenes* and *S. deleyianum*, the root mean square displacement of all Cα atoms is 1.2 Å with each of the three monomers in the asymmetric unit of the *S. deleyianum* crystals. This deviation is significant with respect to the one between the three monomers of *S. deleyianum* themselves (0.27 Å), but the structurally and functionally important features are conserved between both species.

Heme 1 of NiR is the active site, and it shows an unusual lysine- coordination, with an sp²-hybridized amine nitrogen (Lys-134) as the proximal ligand. The Lys-134 (N1-H1)-Heme 1 (Fe) bond distance was refined to 2.1 Å. The active site of cytochrome *c* nitrite reductases is the only known case where a lysine replaces the histidine within a regular Cys-X₁-X₂-Cys-His binding motif for *c*-type hemes. Substrates bind to the distal side of heme group 1 (see Fig. 4A), which is accessible from the protein surface through two narrow channels (see Fig. 3A). Hemes 2–5 are conventional *c*-type hemes, each being formed by a protoheme IX moiety whose vinyl groups are linked as thioethers to the S₃ sulfur atoms of two cysteine residues in a Cys-X₁-X₂-Cys-His motif in the peptide sequence. The histidine in this motif acts as the proximal ligand to iron, and the distal ligand is another histidine in all four cases.

Iron-to-iron distances between the heme groups (Fig. 1B) are in a range commonly found in redox proteins and would be short enough to allow for direct electron tunneling between the heme centers (16). Whereas heme 1 is clearly the site of nitrite reduction, it is more difficult to define the entry point for electrons delivered by the physiological redox partner, the *c*-type cytochrome NrfH (6). All heme groups cluster on one side of the dimer, and heme 2 as well as heme 5 have one edge of the porphyrin plane exposed to the solvent, although for heme 5, most of this area is in the dimer interface and is covered upon dimerization. Furthermore, the area where heme 2 reaches the protein surface is located within a patch of strong positive surface potential in the *W. succinogenes* structure. In *S. deleyianum*, the membranous nitrite reductase complex was described to be less stable than in *W. succinogenes* (10), and in accordance with this, the positive patch surrounding heme 2 is less pronounced. We therefore propose heme 2 to be the most likely entry point for electrons into the system.

The Calcium Site—The presence of a Ca²⁺ ion as an essential constituent in cytochrome *c* nitrite reductase was first discovered in the x-ray structure of the *S. deleyianum* enzyme and subsequently confirmed by ICP mass spectrometry (5). As all calcium-coordinating residues are strictly conserved between the *S. deleyianum* and *W. succinogenes* nrfA sequences, it was no surprise to find calcium at the same position in the enzyme from *W. succinogenes* (Fig. 2). The bond distances to the calcium ion are given in Table II.

### RESULTS

**Overall Structure**—Cytochrome *c* nitrite reductase from *W. succinogenes* was crystallized in space group I 4; 22 with one monomer per asymmetric unit of the crystal unit cell. Using a twofold crystallographic symmetry axis, this monomer forms a similar dimer as the homologous enzyme from *S. deleyianum* (5). As it has been found in all crystal forms observed so far with heme-heme interactions across the interface, it is presumed to be the physiologically active form of the enzyme (Fig. 1). The structure is dominated by three long a-helical elements in each monomer, two of which (Fig. 1A, helices ø22 and ø25) are key elements in dimer formation through a mainly hydrophobic contact surface. As in the *S. deleyianum* structure, helix ø27 runs along the protein surface with a kink at Lys-470. Whereas this helix does not participate in the formation of any functional features or in covering the heme groups, its conservation indicates a possible stabilizing effect on the protein as a whole.
The Ca\textsuperscript{2+} binding site appears to be an essential structural feature in the overall architecture of the enzyme, and the region surrounding the calcium binding site is one of the most highly conserved parts of the whole sequence. This is easily understood for Tyr-218, which is an active site residue that can directly interact with substrate. It can be rationalized that the calcium ligands Lys-274 and Gln-276 immediately precede another active site residue, His-277, such that the calcium bridges two stretches of protein that hold key residues for catalysis. Furthermore, both Lys-274 and Gln-276 take part in forming the active site cavity, whose electrostatic surface potential is presumably essential for guiding substrate influx and product efflux. Another remarkable feature close to the active site is a set of tyrosine residues, which are conserved in all cytochrome \textit{c} nitrite reductase sequences known to date: Tyr-219 follows directly on the active site residue Tyr-218, whose backbone carbonyl oxygen is a calcium ligand. The O\textsubscript{h} atom of Tyr-219 is hydrogen-bonded to O\textsubscript{e} of Tyr-254 at 2.5 Å, and Tyr-255 is again hydrogen bonded to O\textsubscript{e} of Glu-217, which is another ligand of the calcium ion. Asp-262 binds to the two water ligands of the calcium at 2.7 Å and 2.8 Å, and one of them, H\textsubscript{2}O-1, is also close to O\textsubscript{e} of Tyr-255 (3.0 Å) (Fig. 2). Note that every single one of the residues mentioned above is conserved throughout all NrrA sequences reported so far (see Fig. 6).

The tyrosine residues close to the active site might play a role in dealing with possible radical intermediates of the stepwise...
reduction of nitrite to ammonia by forming tyrosyl radicals during the catalytic cycle. This hypothesis is supported by the observation that in all structures of nitrite reductase from *W. succinogenes* partial ortho-hydroxylation of Tyr-219 seems to occur (data not shown).

Substrate and Product Channels—A charged substrate/product channel that traverses the protein and passes the active site has been discovered in the structure of *S. deleyianum* nitrite reductase (5). A similar channel is present in the *W. succinogenes* enzyme (Fig. 3A). Oxyanions, such as nitrite or sulfite, enter the active site cavity via a funnel with significantly positive electrostatic surface potential. It would be rather unfavorable for the physiological reaction product, the ammonium cation, to leave the enzyme on the same way, not only because of electrostatic repulsion, but also because a shared, narrow path would lower the enzyme’s efficiency by product inhibition. Consequently, a second channel leads to the protein surface on the opposite site of the substrate entrance.

In contrast to the latter and the active site cavity itself, the exit channel shows a predominantly negative electrostatic surface potential, thus assisting efflux of the cationic product.

Heme 1 forms the bottom of the positively charged active site cavity whose sides are made up by the primary active site residues Arg-114, Tyr-218, and His-277, and by the propionate sidechains of heme 1. The roof of the cavity is formed by Gln-352 and Phe-92, and the cavity as a whole shows a strong positive surface potential. As the active site cavity leads into the exit channel, a ring of conserved residues (Lys-274, the backbone carbonyl oxygen of Ala-275, Ala-398, Tyr-96, Trp-87, and Asp-394) induces a change toward a mainly negative surface potential. Subsequently, a negatively charged channel leads to the protein surface, lined by Met-76, Lys-80, Leu-83, Lys-348, Gln-352, Ile-397, and His-400. Residue Gln-352 corresponds to Asn-358 in the NrfA sequence of *S. deleyianum*, and there the shorter sidechain leads to a branching of the exit channel which is absent in the *W. succinogenes* structure.
Because of the conservation of the essential residues involved in channel formation (see Fig. 6, yellow arrows) it is assumed that the channels themselves as well as their electrostatic surface potential properties represent a conserved feature in all cytochrome c nitrite reductases.

Active Site Structure and Inhibitor Binding—Crystals of the W. succinogenes enzyme were grown in a buffer containing ammonium sulfate. Sulfate acted as a weak inhibitor of nitrite reductase activity (17) and was found to occupy the substrate binding site at the distal position of heme 1. The binding mode of sulfate differs slightly in S. deleyianum (5) and W. succinogenes, most obviously by a torsion around the $\chi_1$ angle of Tyr-218 of 5° away from the bound substrate in W. succinogenes.

For the sulfate ion, this difference causes a rotation such that its $O_1$ atom is bound to both His-282 (at 2.6 Å) and Tyr-217 (at 2.8 Å) in the S. deleyianum enzyme, but only to Tyr-218 (2.6 Å) in the W. succinogenes enzyme. Hereby, the distance to $N_{e2}$ of His-277 has increased to 3.3 Å (Fig. 4B). The distance between the fourth oxygen of sulfate and iron is 2.05 Å in both structures, and the Fe(III) retains the high-spin state according to EPR spectroscopy (17). A possible explanation for this difference can be found in the pH of the crystals: whereas the S. deleyianum enzyme was crystallized at pH 7.5, the crystals of the enzyme from W. succinogenes were grown at pH 5.7. As the $pK_a$ of histidine is around 6.5, the two crystal forms might differ in the protonation state of the imidazole nitrogen of His-277.

At low concentrations, sulfate does not bind to the enzyme and is replaced by a water molecule. The structure of the water-bound form at 1.6 Å clearly shows the complete removal of sulfate and the oxygen atom of a water bound to the iron at the same distance, 2.05 Å (Fig. 4A). Without the bulky sulfate ion, the imidazole moiety of His-277 moves closer to the heme iron, and a hydrogen bond with a length of 2.88 Å is formed between its $N_{e2}$ and the water molecule at the active site. The positions of both Tyr-218 and Arg-114 remain unchanged.

Azide, a known inhibitor of nitrite reductase (10), was expected to bind to the active site heme iron as a competitive inhibitor just like sulfate. Instead, the structure at 2.0 Å showed a water bound to iron at 2.05 Å, hydrogen bonded to His-277 as described above. However, a characteristic, elongated difference electron density was observed in close proximity, which could be assigned to an azide anion (Fig. 4C). This ion binds to residues lining the active site entrance, with hydrogen bonds to Gln-276 (3.0 Å) and Tyr-218 (2.8 Å) and to $N_{e1}$ (3.0 Å) and $N_{e2}$ (2.9 Å) of Arg-114 (Fig. 4B). With azide bound in this fashion, the active site cavity can no longer accommodate sulfate. In a parallel soaking experiment with 200 mM sulfate and 100 mM azide, the stronger inhibitor azide quanti-
tatively replaced sulfate. The substrate channel is effectively blocked, and although azide and nitrite do not compete for the same site, their binding is mutually exclusive for steric reasons.

**Structural Modules in Multiheme c Cytochromes**—With the increasing number of multiheme c cytochrome structures, common motifs of heme-heme interaction begin to emerge from a protein family, which is highly diverse in amino acid sequence and protein fold. Indeed, characteristic structural modules are observed in the heme arrangement of cytochrome c nitrite reductase, at least one of which has been detected in every other multiheme c protein structure described so far. The single exception hereof is the cytochrome subunit of the photosynthetic reaction center of *Rhodopseudomonas viridis* (18).

In cytochrome c nitrite reductase, hemes 2 and 3 as well as hemes 4 and 5 are almost perpendicular to each other. Interestingly, both of these heme pairs are arranged very similarly, and this design of heme-heme interaction is observed in the heme arrangement of cytochrome c nitrite reductase, at least one of which has been detected in every other multiheme c protein structure described so far. The single exception hereof is the cytochrome subunit of the photosynthetic reaction center of *Rhodopseudomonas viridis* (18).

A second type of heme interaction is found in nitrite reductase (Fig. 5), but the motif also appears in hemes 2/3, 5/6 and 7/8 of hydroxylamine oxidoreductase (7), hemes 3/4 of cytochrome c<sub>554</sub> (23), and in hemes 3/4 of flavocytochrome c fumarate reductase from *Shewanella* (24). The repeated occurrence of this heme arrangement has been noted before ("di-heme elbow motif"); however, the properties and function remain to be understood.

A second type of heme interaction is found in nitrite reductase, which is also present in several other proteins. Hemes 3/4 of nitrite reductase show a parallel stacking of their porphyrin planes with an edge-to-edge distance below 4 Å, thus close enough for direct resonance electron transfer (Fig. 5). The same arrangement is found in hemes 1/2, 3/5, and 7/8 of hydroxylamine oxidoreductase (7), in hemes 2/4 of cytochrome c<sub>554</sub>, in hemes 2/4 of flavocytochrome c fumarate reductase (24), and in the two hemes of di-heme split-Soret cytochrome c from *Desulfovibrio desulfuricans* ATCC 27774 (25). In the structures of both nitrite reductase and hydroxylamine oxidoreductase, the active site heme group is the only one that is not part of one of these motifs. All other hemes are arranged in di-heme elbow motifs, and all of these are connected among each other in a parallel stacking similar to the one observed in the split-Soret cytochrome c (9).

**Cytochrome c Nitrite Reductase from Different Bacterial Families**—The NrfA protein of *W. succinogenes* has been purified as a stable complex with the tetraheme c cytochrome NrfH, a member of the NapC/NirF family (6, 10). As this complex was able to catalyze the oxidation of menaquinol with nitrite in proteoliposomes, NrfH was proposed to be the physiological electron donor of NrfA (6). However, the nrf operon of *E. coli* does not contain a nrfH homolog, and here a hydrophilic pentaheme c cytochrome, NrfB, was proposed to take over this function (3). Electron transfer from menaquinol to NrfA is an essential determinant of the efficiency of the nitrite reductase system, and thus the different operon contexts of NrfA need to be compared and analyzed.

In addition to the four sequences of cytochrome c nitrite reductase available in the GenBank<sup>TM</sup> / EBI sequence database (*E. coli, H. influenzae, S. deleyianum, and W. succinogenes*), a search of preliminary genome sequences yielded another five related operon sequences, which can be assumed to encode for proteins involved in dissimilatory nitrite respiration (Figs. 6 and 7). Whereas all of these operons contain an nrfA gene, they differ strongly in the other genes. Most common are the operon types nrfABCDEFG, as found in γ-proteobacteria (3) and nrfHALJ, as found in *S. deleyianum* and *W. succinogenes* (6), but further variations occur: The bacteroid *Porphyromonas gingivalis* has an nrfHAKLM operon, and the γ-proteobacterium *Shewanella putrefaciens* has an nrfA gene which does not seem to be organized in a polycistronic operon at all. The e-proteobacterium *Campylobacter jejuni* (26) only shows an nrfHÂ® operon, and here the asterisk is used to indicate that the putative nrfA<sup>®</sup> does not contain a Cys-X<sub>1</sub>-X<sub>2</sub>-Cys-Lys motif but five conventional Cys-X<sub>1</sub>-X<sub>2</sub>-Cys-His motifs (Fig. 6).

Also, a preliminary genome sequence for the δ-proteobacterium *D. vulgaris* Hildenborough has become available from the TIGR homepage. This species possesses an nrf operon which starts with an nrfH gene followed by nrfA. At the time this paper was completed; however, the sequence was in a very preliminary state and the nrfA gene contained several obvious frameshifts. Thus a detailed comparison should not be attempted at this point. Nevertheless, the homology to other nrfA genes is strong, and the gene can be included in the nrfA family tree (Fig. 7).

**DISCUSSION**

*Architecture of the Cytochrome c Nitrite Reductase Complex in Vivo*—In the nitrite reductase dimer, the active sites of both monomers are presumed to be functional and are not supposed to act cooperatively. This raises the question of whether dimer formation is essential for the function of the enzyme. *S. deleyianum* nitrite reductase has been shown to be soluble and active as a monomer although in those activity assays small molecules were used as electron donors, usually methyl or benzyl viologen (27). Hemes 2 and 5 and also the propionate sidechains of hemes 3 and 4 reach the protein surface, and so there is a multitude of possible entry points for electrons. Whereas this is desirable when nitrite reductase activity is measured, and the reaction at the active site is supposed to be the limiting step, it does not allow us to draw conclusions about physiological electron transfer pathways in an environment with a positive redox potential such as the bacterial periplasm.

Dimer formation brings heme groups 5 from each monomer...
into very close contact. This includes almost direct interaction of their propionate sidechains, mediated only through a single coordinated water molecule.

The functional nitrite reductase system of the e-proteobacteria \textit{W. succinogenes} and \textit{S. deleyianum} (in view of similarity) is a complex of the enzyme, NrfA, and a tetraheme c-type cytochrome, NrfH, which acts as a quinol oxidase to receive electrons from the membranous quinone pool \cite{6}. Our working model thus implies that in \textit{W. succinogenes}, the NrfA dimer is associated with the peripheral membrane protein NrfH, with the positively charged surrounding of heme 2 as an interaction area (Fig. 8).

Electron Transfer and Heme Packing—Most likely, heme 2 is the entry point for electrons, which then are transferred to heme 1 through a set of closely packed heme centers. As shown in Fig. 5, hemes 2/3 and 4/5 each constitute a di-heme elbow motif, both of which are connected by the parallel stacking motif. In the dimer, these four motifs seem to form an efficient electron transfer chain, which involves both monomers, and possibly extends to the electron donor of the complex, NrfH. Both active sites of the dimer then can obtain electrons from this system.

Looking for through-bond or through-space electron transfer pathways, the enzymes from both organisms offer multiple...
possibilities. Cytochrome c nitrite reductase has a rather high number of coordinated water molecules within the protein, and several putative electron-transfer pathways can be found between each of the hemes. On the other hand, according to Page et al. (16), effective electron transfer to the active site may not depend on a gradient of tuned redox sites as long as the distance between redox centers does not exceed approximately 14 Å. This condition is fulfilled for all the iron centers of nitrite reductase.

Nevertheless, the extent of conservation of these heme-packing motifs is striking. The list of multiheme cytochrome c structures that contain the perpendicular di-heme elbow motif and/or the parallel heme stacking motif is rapidly growing (9, 28), and whereas the relative orientations of heme groups are nearly invariant, the surrounding protein shows hardly any similarity at all. Obviously the feature conserved through evolution is indeed the heme packing itself, and although there are many possible arrangements in which not only iron-iron distances but also the relative orientation of heme planes can be conserved, the exact motifs as described above are found with only minor deviations (Fig. 5). To date, theoretical approaches cannot describe the properties of large heme group arrangements in a satisfying way, but it can be expected that the significance of the special packing arrangements found here go beyond simple distance considerations.

Diversity in Organization of the nrf Operon—The two available crystal structures of cytochrome c nitrite reductase together with the highly consistent secondary structure predictions for all sequences show that the NrfA protein is a specialized and highly conserved entity (Fig. 6), and we can expect a very similar structure for all members of this enzyme class. It is thus remarkable in which variable operon contexts the nrfA gene was found in different organisms. Whereas NrfA and NrfH have been shown to form a stable membrane-associated complex in W. succinogenes (6), NrfA was found to be soluble in enteric bacteria containing an nrfABCDEFG operon, connected to the quinol oxidase NrfCD through the soluble cytochrome Nrb (3). In terms of electron transfer, this second system is presumably less effective, as it involves diffusion of reaction partners within the periplasm (Fig. 8). In E. coli, the nrfEFG gene products were shown to be specifically involved in the covalent attachment of the active site heme group of NrfA (29). Each of these proteins resembles components of the regular cytochrome c maturation system (30, 31), and the same holds true for NrfI and NrfJ in W. succinogenes and S. delayoutanum and at least for NrfM in the nrfHALKL operon of P. gingivalis.

With this, all cases can presumably be brought down to three basic elements, which form the nitrite reductase system. These are (i) the enzyme (NrfA), (ii) a system to oxidize membranous menaquinol and transport the electrons to NrfA (NrfH, Ref. 6, or NrfBCD, Ref. 3), and (iii) a modified heme lyase needed for the covalent attachment of the active site heme group (NrfI, NrfEFG, or NrfKLM). Although a direct involvement of these genes in attachment of the active site heme group of NrfA has only been shown for E. coli (29), a nrfIJ deletion mutant of W. succinogenes produced an enzymatically inactive NrfA protein with a reduced heme content (6). Finally, both NrfI and NrfM contain the conserved tryptophan-rich motif (WGXXWXXW) which is typical for type II heme c maturation systems (31).

Acknowledgments—The authors thank G. P. Bourenkov and H. D. Bartunik (Deutsches Elektronen-Synchrotron Hamburg) for help with synchrotron data collection and K. Sulger (Universität Konstanz) for assistance in protein purification.

REFERENCES
1. Cole, J. A., and Brown, C. M. (1980) FEMS Microbiol. Lett. 7, 65–72
2. Darwin, A., Hussain, H., Griffiths, L., Grove, J., Sambongi, Y., Busby, S., and Cole, J. (1993) Mol. Microbiol. 9, 1253–1265
3. Hussain, H., Grove, J., Griffiths, L., Busby, S., and Cole, J. (1994) Mol. Microbiol. 12, 153–163
4. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J.-F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L. I., Godek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, K., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrman, J. L., Geoghagen, N. S. M., Ghem, C. L., McDonalid, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) Science 269, 496–512
5. Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Kroneck, P. M. H. (1999) Nature 400, 476–480
6. Simon, J., Gross, R., Einsle, O., Kroneck, P. M. H., Kruger, A., and Klumke,
Wolinella succinogenes Nitrite Reductase

O. (2000) Mol. Microbiol. 35, 686–696
7. Igarashi, N., Moriyama, H., Fujitake, T., Fukumori, Y., and Tanaka, N. (1997) Nat. Struct. Biol. 4, 276–284
8. Narager, S., Legrand, P., Pielke, L., Hatchikian, C., and Roth, M. (1999) J. Mol. Biol. 290, 881–902
9. Barker, P. D., and Ferguson, S. J. (1999) Structure 7, 281–290
10. Schumacher, W., Hole, U., and Kroneck, P. M. H. (1994) Biochem. Biophys. Res. Comm. 205, 911–916
11. Otwinski, Z., and Minor, W. (1996) Methods Enzymol. 276, 307–326
12. Navaza, J. (1994) Acta Crystallogr. A 50, 157–163
13. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 281–290
14. Schumacher, W., and Kroneck, P. M. H. (1991) Arch. Microbiol. 156, 70–74
15. Einsle, O. (2000) in Handbook of Metalloproteins (Messerschmidt, A., Huber, R., Poulos, T., and Wieghardt, K., eds), Wiley & Sons, NY
16. Eaves, D. J., Grove, J., Staudenmann, W., James, P., Poole, R. K., White, S. A., Griffiths, I., and Cole, J. A. (1998) Mol. Microbiol. 28, 205–216
17. Thony-Meyer, L. (1997) Microbiol. Mol. Biol. Rev. 61, 337–376
18. Kraulis, P. (1991) J. Appl. Crystallogr. 24, 946–950
19. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524
20. Guex, N., and Peitsch, M. C. (1996) PDB Quart. Newslett. 77, 7–10
21. Rost, B. (1996) Methods Enzymol. 266, 525–539
22. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584–599
23. Iverson, T. M., Arciero, D. M., Hsu, B. T., Logan, M. S. P., Hooper, A. B., and Rees, D. C. (1998) Nat. Struct. Biol. 5, 1005–1012
24. Schumacher, W., and Kroneck, P. M. H. (1994) Arch. Microbiol. 162, 255–260
25. Matias, P. M., Morais, J., Coelho, A. V., Meijers, R., Gonzalez, A., Thompson, A. W., Sieker, L., Legall, J., and Carrondo, M. A. (1997) J. Biol. Chem. 272, 507–514
26. Nørager, S., Legrand, P., Pieulle, L., Hatchikian, C., and Roth, M. (1999) J. Mol. Biol. 290, 881–902
27. Matias, P. M., Morais, J., Coelho, A. V., Meijers, R., Gonzalez, A., Thompson, A. W., Sieker, L., Legall, J., and Carrondo, M. A. (1997) J. Biol. Inorg. Chem. 2, 507–514
28. Parkhill, J., Wren, B. W., Mungall, K., Kettle, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshen, A. V., Moule, S., Fallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S., and Barrett, B. G. (2000) Nature 403, 665–668
29. Schumacher, W., and Kroneck, P. M. H. (1994) Arch. Microbiol. 162, 255–260
30. Schumacher, W., and Kroneck, P. M. H. (1994) Arch. Microbiol. 162, 255–260
31. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291