NirN Protein from Pseudomonas aeruginosa is a Novel Electron-bifurcating Dehydrogenase Catalyzing the Last Step of Heme $d_1$ Biosynthesis*

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Julia Adamczack, Martin Hoffmann, Ulrich Papke, Kristin Haufschildt, Tristan Nicke, Martin Bröring, Murat Sezer, Rebecca Weimar, Uwe Kuhlmann, Peter Hildebrandt, and Gunhild Layer

From the Institute of Microbiology, Technische Universität Braunschweig, Spielmannstr. 7, 38106 Braunschweig, Germany; the Institute of Inorganic and Analytical Chemistry and Institute of Organic Chemistry, Technische Universität Braunschweig, Hagenring 30, 38106 Braunschweig, Germany; and the Institute of Chemistry, Technische Universität Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany

Background: Cytochrome $c_d$, nitrite reductase contains heme $d_1$ as an essential cofactor.

Results: P. aeruginosa lacking the NirN protein produces the heme $d_1$ precursor dihydro-heme $d_1$, which is converted by NirN to heme $d_1$.

Conclusion: NirN catalyzes the last step of heme $d_1$ biosynthesis.

Significance: The so far unknown function of NirN is revealed.

Heme $d_1$ plays an important role in denitrification as the essential cofactor of the cytochrome $c_d$, nitrite reductase NirS. At present, the biosynthesis of heme $d_1$ is only partially understood. The last step of heme $d_1$ biosynthesis requires a so far unknown enzyme that catalyzes the introduction of a double bond into one of the propionate side chains of the tetrapyrrole yielding the corresponding acrylate side chain. In this study, we show that a Pseudomonas aeruginosa PAO1 strain lacking the NirN protein does not produce heme $d_1$. Instead, the NirS purified from this strain contains the heme $d_1$ precursor dihydro-heme $d_1$, lacking the acrylic double bond, as indicated by UV-visible absorption spectroscopy and resonance Raman spectroscopy. Furthermore, the dihydro-heme $d_1$ was extracted from purified NirS and characterized by UV-visible absorption spectroscopy and finally identified by high-resolution electrospray ionization mass spectrometry. Moreover, we show that purified NirN from P. aeruginosa binds the dihydro-heme $d_1$ and catalyzes the introduction of the acrylic double bond in vitro. Strikingly, NirN uses an electron bifurcation mechanism for the two-electron oxidation reaction, during which one electron ends up on its heme $c$ cofactor and the second electron reduces the substrate/product from the ferric to the ferrous state. On the basis of our results, we propose novel roles for the proteins NirN and NirF during the biosynthesis of heme $d_1$.

The process of denitrification, i.e. the stepwise reduction of nitrate to molecular nitrogen, represents one important branch of the global biogeochemical nitrogen cycle. Moreover, for denitrifying organisms such as the human pathogen Pseudomonas aeruginosa, denitrification serves as a very efficient strategy for energy generation under anaerobic growth conditions (1). In the second step of the denitrification process, nitrite is reduced to nitric oxide. In P. aeruginosa, this reaction is catalyzed by the periplasmic cytochrome $c_d$, nitrite reductase NirS (2). This enzyme is a homodimer of two 60 kDa subunits, each carrying a heme $c$ and a heme $d_1$ cofactor (3). The non-covalently bound heme $d_1$ represents the catalytic site of the enzyme where the reduction of nitrite to nitric oxide takes place (4).

Heme $d_1$ belongs to the tetrapyrrole subfamily of isobacteriochlorins. It carries two unusual carbonyl functions on pyrrole rings A and B and an acrylate side chain on ring D that make heme $d_1$ unique among the naturally occurring tetrapyrroles (Fig. 1) (5, 6). The biosynthesis of heme $d_1$, which is converted by NirN from the common tetrapyrrole precursor uroporphyrinogen III requires six enzymatic steps (7). Most of the involved enzymes are encoded by the nir genes, which are located together in the nir operon in P. aeruginosa (nirSMCFDLGHJEN) and also in other denitrifying bacteria (Fig. 1A) (8–10). In the first step, the S-adenosylmethionine-dependent uroporphyrinogen III methyltransferase NirE catalyzes the methylation of uroporphyrinogen III to precorrin-2 (11, 12). Next, precorrin 2 is converted to siroheme in two enzymatic steps catalyzed by precorrin-2 dehydrogenase and a sirohydrochlorin ferrochelatase not encoded within the nir operon in P. aeruginosa. Siroheme is then decarboxylated to 12,18-didecarboxy-siroheme by the action of siroheme decarboxylase, which is composed of the four proteins NirD, NirL, NirG, and NirH (7). The penultimate step of heme $d_1$ biosynthesis consists of the removal of the two propionate side chains on pyrrole rings A and B, with the concomitant introduction of the carbonyl functions at these positions yielding the heme $d_1$ precursor dihydro-heme $d_1$. This transformation is believed to be catalyzed by the radical S-adenosylmethionine enzyme NirJ (7). However, this hypothesis still requires experimental proof. Similarly, the enzyme responsible for the two-electron oxidation reaction, during which one electron ends up on its heme $c$ cofactor and the second electron reduces the substrate/product from the ferric to the ferrous state. On the basis of our results, we propose novel roles for the proteins NirN and NirF during the biosynthesis of heme $d_1$.

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![Diagram of heme $d_1$ biosynthesis](image)

**FIGURE 1. Heme $d_1$, biosynthesis.** A, the nir operon from *P. aeruginosa* comprises the genes coding for cytochrome $cd$, nitrite reductase (nirS), electron transferring $c$-type cytochromes (nirM and nirC), and proteins involved in heme $d_1$, biosynthesis and insertion into NirS (nirF, nirD, nirL, nirG, nirN, nirE, and nirN). The genes encoding proteins used in this study are shown as white arrows. B, the proposed last step of heme $d_1$, biosynthesis consists of the introduction of the double bond into the propionate side chain of pyrrole ring D of the precursor dihydro-heme $d_1$, yielding the acrylate side chain of heme $d_1$. Until now, the enzyme catalyzing this reaction was not known.

for the last step of heme $d_1$, biosynthesis has not yet been identified. In this step, the propionate side chain on pyrrole ring D is converted to the corresponding acrylate side chain (Fig. 1B). At some stage of its biosynthesis, heme $d_1$, or a precursor thereof, has to be transported across the cytoplasmic membrane to be incorporated into its target enzyme NirS in the periplasm. Currently it is thought that all heme $d_1$, biosynthesis steps, except the last one, take place in the cytoplasm and that the final step is catalyzed in the periplasm. This hypothesis is on the basis of the finding that NirF, which has been proposed to catalyze the acrylate group formation, is a periplasmic protein (13), whereas NirE, NirDLGH, and NirJ are located in the cytoplasm.

Depending on the organism, NirF is either a soluble periplasmic protein or attached to the outer face of the cytoplasmic membrane, most likely by a lipid anchor (14, 15). In addition to its periplasmic location, purified recombinant NirF from *Paracoccus pantotrophus* has been shown to bind heme $d_1$, in vitro (13). Moreover, early mutational studies with different denitrifying bacteria have shown that the deletion of the nirF gene results in bacterial strains unable to synthesize heme $d_1$ (8–10, 13). On the basis of all of these findings, NirF has been proposed to catalyze the formation of the double bond of heme $d_1$. However, so far there is no direct experimental evidence clearly attributing this enzymatic function to NirF.

Interestingly, another nir gene (nirN) is present in all denitrifying bacteria. The encoded NirN protein shares about 24% amino acid sequence identity with NirS. Moreover, NirN has been shown to be located in the periplasm and to contain a heme $c$ cofactor (16). The monomeric NirN was able to bind heme $d_1$, in vitro and transferred the cofactor to NirS when the two proteins were mixed (12). The deletion of the nirN gene in different denitrifiers led to a mild growth phenotype, and cell-free extracts prepared from the corresponding *Δ*nirN strains exhibited reduced NirS activity compared with the WT strains (9, 12, 16). From these observations it was deduced that NirN is not required for heme $d_1$, biosynthesis but, rather, might be involved in the insertion of the cofactor into NirS. In line with this proposal it has been shown that NirN from *P. aeruginosa* interacts with both NirS and NirF in *vivo*. However, in that study it was also observed by UV-visible absorption spectroscopy of periplasmic protein fractions that the cofactor content of NirS in the *P. aeruginosa* *Δ*nirN strain was different from that in the *P. aeruginosa* WT strain (15). Later, the same observation was reported for a *Δ*nirN strain of *Magnetospirillum gryphiswaldense* (17).

The apparently altered cofactor content of NirS in these *Δ*nirN strains can be rationalized by two different hypothetical scenarios: (i) NirN is involved in heme $d_1$, insertion into NirS, and the absence of NirN leads to an improper incorporation of the cofactor causing the observed spectral differences, or (ii) NirN is involved in the last step of heme $d_1$, biosynthesis (together with NirF?), and the absence of NirN leads to the accumulation of dihydro-heme $d_1$, which is nevertheless inserted into NirS, leading to a different form of holo NirS with different spectral properties and reduced enzyme activity. Therefore, the aim of this study was to investigate and clarify the still enigmatic function of NirN during either heme $d_1$, biosynthesis or insertion into NirS.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Chemicals and reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), or Thermo Fisher Scientific Inc. (Schwertze, Germany). Restriction endonucleases were purchased from New England Biolabs (Frankfurt am Main, Germany). QIAquick PCR purification and gel extraction kits were purchased from Qiagen GmbH (Hilden, Germany). Q-Sepharose Fast Flow and SP-Sepharose Fast Flow were obtained from GE Healthcare. Strep-Tactin-HC resin, Desthiobiotin, and Avidin were purchased from IBA GmbH (Göttingen, Germany). All oligonucleotide primers were obtained from Metabion International AG (Martinsried, Germany).

**Plasmids and Strains**—The *Escherichia coli* strain BL21(DE3) was used as a host for protein production of recombinant semi-native NirS (NirS<sub>sa</sub>), NirN, and NirF. For the production of NirS<sub>sa</sub> and NirN, *E. coli* BL21(DE3) was transformed with the plasmid pEC86 (provided by Dr. Linda Thöny-Meyer (18)) and either pET-22b(+)*nirF* (15) or pET-22b(+)*StrepII*nirN. For the production of recombinant NirN from *Dinoroeobacter shibae* NirN<sub>P. aeruginosa</sub>, *E. coli* BL21(DE3) was transformed with pET-22b(+)*nirH<sub>D.s.</sub>*PCSStrepII. The *P. aeruginosa* PA01 WT strain was used for protein production of native holo NirS (NirS<sub>WT</sub>). For protein production of native NirS containing dihydro-heme $d_1$, (NirS<sub>sa</sub>), the *P. aeruginosa* PA01 strain RM361 (nirN<sub>tet</sub>) was used (provided by Dr. Hiroyuki Arai (9)).

**Construction of Vectors**—For the construction of a NirN expression vector, the nirN gene from *P. aeruginosa* PA01 was PCR-amplified using the primers strepII*nirN<sub>fw</sub>*(TAT ACC ATG GCC TGG AGC AAC CCG CAG TTC GAA AAA GCT AGC GCC GAA GCC CCG AGG GAA G) and nirN<sub>rev</sub>* (CG GGA TCC TCA GTG CGA GTG TC), which contained NcoI and BamHI restriction sites (underlined), respectively. The di-
gested nirN insert was ligated into the correspondingly digested vector pET-22b(+) to generate pET-22b(+)StrepIinirN. For the construction of an expression vector for NirF from D. shibae, the plasmid pMK-RQpelBnirF/(D.s.)PCS was purchased from Invitrogen. Through restriction of the plasmid with Ndel and HindIII, the insert pelBnirF/(D.s.)PCSshs was obtained and ligated into the correspondingly digested plasmid pET-22b(+). A Strep tag coding insert was obtained by annealing the primers CTA GTA GCG CTT GGA GCC ACC GCC AGT TCG AAA AAG and GAT CCT TTT TCG AAC TGC GGG TGG CTC CAA GCG CTA. The His tag coding sequence was removed from pET-22b(+)pelBnirF/(D.s.)PCSshs using SpeI and BamHI restriction sites, and the Strep tag coding insert was ligated into pET-22b(+)pelBnirF/(D.s.)PCStrepI.

Growth Conditions—E. coli BL21(DE3) carrying the plasmids pEC86 and pET-22b(+)nirS or pET-22b(+)StrepIinirN for the production of NirSs.a. (containing the covalently bound heme c but no heme d1) and heme c containing NirN, respectively, were grown according to Studier (19) in self-inducing ZYM5052 medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for 4 h at 37 °C and, subsequently, for 26 h at 25 °C. E. coli BL21(DE3) carrying the plasmid pET-22b(+)nirF/(D.s.)PCStrepI was grown in terrific broth (20) containing 100 μg/ml ampicillin using BSA as a standard.

Extraction of Nitrite Reductase Cofactors—Nitrite reductase preparations were added to autoinducer bioassay minimal medium containing phase was removed and dried then centrifuged for 2 min at 13000 g. The organic cofactor-containing phase was removed and dried in vacuo. For HR-ESI-MS analysis,3 proteins were transferred to double distilled water prior to the extraction of the cofactors, and the dried cofactors were dissolved in methanol.

UV-visible Absorption Spectroscopy—UV-visible absorption spectra of purified proteins and extracted cofactors were recorded using a V-650 spectrophotometer (Jasco, Gross-Umstadt, Germany). The reduced forms of proteins and isolated cofactors were obtained by addition of a few grains of sodium dithionite.

Resonance Raman Spectroscopy—RR spectra with a spectral resolution of 2 cm⁻¹ were recorded using a confocal Raman spectrometer (Lab Ram HR-800, Jobin Yvon) equipped with a liquid nitrogen-cooled charge-coupled device camera. The 442-nm excitation line of a He-Cd laser (Soliton) was focused onto the sample (2 μm in diameter, 1 milliwatt at the sample). Using a Linkam Cryostage THMS600 cryostat, the temperature of the sample was set to 80 K throughout the measurement. Raman shifts were calibrated to an accuracy of 0.5 cm⁻¹ using toluene as an external standard. All spectra were corrected by polynomial background subtraction.

Heme d1 and Dihydro-Heme d1 Binding to NirN and NirF—For binding of heme d1 and dihydro-heme d1 to NirN and NirF, the in vacuo dried cofactors were directly dissolved in 150 μl of buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 20 μM of heme d1 to NirN at a concentration of 38 μM. Directly after mixing, UV-visible absorption spectra of the mixtures were recorded on a V-650 spectrophotometer (Jasco).

NirN Activity Assay, Transfer of Cofactors to NirSs.a., and Reconstitution of NirSs.a.—The in vitro NirN activity assay was performed under aerobic or anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, MI). For the aerobic assay, dried dihydro-heme d1 was dissolved in buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) and transferred to a quartz cuvette (Hellma Analytics, Müllheim, Germany). NirN was subsequently added at a final concentration of 20 μM to give a final volume of 150 μl. For the anaerobic assay, the dried dihydro-heme d1 was dissolved in degassed buffer in the anaerobic chamber, followed by the addition of 20 μM NirN under anaerobic conditions. The NirN-catalyzed reaction was monitored using a V-650 spectrophotometer (Jasco). For the reconstitution of NirSs.a. with heme d1 or dihydro-heme d1, the transfer of the NirN reaction product to NirSs.a., the enzyme was added to the NirN assay or the cofactor solution at a final concentration of 20 μM. For the oxidation of heme d1, sodium persulfate was added.

HR-ESI-MS (Accurate Mass Measurements) of Nitrite Reductase Cofactors—The sample solutions obtained after cofactor extraction were used for accurate mass measurements on an LTQ-Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany). Electrospray measurements in positive mode were performed in direct infusion mode using a custom-made microspray device mounted on a Proxeon nanospray ion source. The sample solutions obtained after cofactor extraction were used for accurate mass measurements on an LTQ-Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany). Electrospray measurements in positive mode were performed in direct infusion mode using a custom-made microspray device mounted on a Proxeon nanospray ion source. The microspray device allows sample infusion without sheath gas through a stainless steel capillary (90-μm inner diameter) at a flow rate of ~1 μl/min. The spray voltage was set between 2.0 and 2.3 kV. The resolution of the Orbitrap mass analyzer was set to 100,000 full width at half-maximum (FWHM) at m/z 400. Accurate mass measurements were performed using the lock mass option of the instrument control software using the cation of tetradecyl-trimethylammonium bromide (256.29988 u) as an internal mass reference, which was added to the sample solution in appropriate amounts.

3 The abbreviations used are: HR-ESI-MS, high-resolution electrospray ionization MS; RR, resonance Raman.
RESULTS AND DISCUSSION

Purification of Native NirS from the P. aeruginosa PAO1 ΔnirN Strain RM361—To obtain more insights into the role of NirN, the first aim of this study was to purify NirS from the P. aeruginosa PAO1/H9004 strain RM361 and to characterize the cofactor content of this apparently different form of holo NirS. Previous attempts to purify native NirS from the P. aeruginosa PAO1 strain RM361 only resulted in the isolation of semi-apo NirS (NirSs.a.) completely lacking heme d1 (15). Therefore, the purification protocol was changed slightly, including the reduction of the column volume of the anion exchange chromatography column. Using the new purification procedure, NirS was purified to apparent homogeneity (Fig. 2A). The enzyme purified from strain RM361 (NirS/H9004) exhibited a less intense green color in contrast to the green color of NirS isolated from the WT strain (NirSWT) and the red colored semi-apo form of NirS lacking heme d1. This observation clearly supports the idea that a different form of holo NirS was produced in the P. aeruginosa H9004 strain.

UV-visible Absorption Spectroscopy of Purified NirS/H9004 and NirSWT—To characterize the cofactor content of NirS/H9004, UV-visible absorption spectra of the purified enzyme were recorded of the oxidized (as isolated) and dithionite-reduced forms and compared with the corresponding spectra of purified NirSWT (Fig. 2, B and C). The absorption spectra of NirS/H9004 are clearly different from those of NirSWT in both the oxidized and reduced forms. In fact, the spectra of the two enzymes differ, particularly in the region of the characteristic heme d1 absorption bands. Although the spectrum of oxidized NirSWT exhibits a typical heme d1 peak at 642 nm, the spectrum of NirS/H9004 shows a peak at 626 nm. In the reduced form, the heme d1 absorption band of NirSWT is shifted to 649 nm, whereas, for NirS/H9004, a peak shift to 631 nm is observed. These spectral differences between the purified NirS/H9004 and NirSWT confirmed the presence of two different forms of holo NirS depending on whether the enzyme was isolated from P. aeruginosa PAO1 strain RM361 or from the WT strain. There were two possible explanations for the observed spectral differences. Either heme d1 was not properly inserted into NirS/H9004 and the observed differences were due to a different ligation of the cofactor, or it was not heme d1 but the precursor dihydro-heme d1 that was present in NirS/H9004. On the basis of the observed UV-visible absorption spectra of NirS/H9004, we favored the second hypothesis. The conjugated system of dihydro-heme d1 lacks one double bond compared with heme d1. For this reason, the absorption maxima of dihydro-heme d1 can be expected to be located at shorter wavelengths than those of heme d1 (hypsochromic shift). Indeed, such a shift was observed for the absorption maximum of NirS/H9004 (626 nm, oxidized) compared with the heme d1-containing NirSWT (642 nm, oxidized).

Resonance Raman Spectroscopy of NirS/H9004, NirSWT and NirSs.a.—To further characterize the unusual cofactor content of NirS/H9004 and to obtain more insights into the question of whether dihydro-heme d1 is indeed bound in NirS/H9004, resonance Raman (RR) spectroscopy was performed. RR spectra of purified NirSWT, NirS/H9004, and semi-apo NirS were measured at low temperature to reduce the strong background scattering and to increase the signal-to-noise ratio. Although, upon 413-nm excitation, the heme c bands were selectively enhanced (data not shown), 442-nm excitation led to a substantial
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The absorption spectrum of the heme $d_1$ in NirS can readily be understood because the extension of the delocalized $\pi$-electron system affects both the absolute and relative RR intensities. The results of the experiments described so far strongly suggest that the heme $d_1$ precursor dihydro-heme $d_1$ might indeed be bound in NirS. To finally clarify this question, the non-covalently bound heme $d_1$ (or possibly dihydro-heme $d_1$) was extracted from the purified NirS and NirS as described under "Experimental Procedures." Then, UV-visible absorption spectra of the oxidized and dithionite-reduced forms were recorded (Fig. 4, A and B). The absorption spectrum of the heme $d_1$ cofactor extracted from NirS exhibits characteristic peaks at 408 and 683 nm and a shoulder at about 476 nm in the oxidized state, as reported previously (30, 31). In contrast, the spectrum of the oxidized form of the cofactor extracted from NirS shows absorption maxima at 397 and 671 nm and also a shoulder at about 476 nm. After dithionite reduction, the absorption bands of the heme $d_1$ extracted from NirS were shifted to 457 and 627 nm, whereas those of the cofactor extracted from NirS are shifted to 447 and 611 nm. Again, these observations strongly suggest that the cofactor isolated from NirS was indeed different from the heme $d_1$ extracted from NirS. As described above, we suspected that the heme $d_1$ precursor dihydro-heme $d_1$ lacking the acrylic double bond might be present in NirS. The UV-visible absorption spectra of the isolated cofactor are in line with this proposal because the characteristic heme $d_1$ absorption band at 683 nm in the oxidized state is shifted to a shorter wavelength by about 10 nm in the case of the cofactor from NirS, and the corresponding band of heme $d_1$ in the reduced state at 627 nm is shifted by about 15 nm in this case. Such a hypsochromic shift would be expected for dihydro-heme $d_1$ because of the lacking double bond (6).

FIGURE 3. RR spectra of NirS, NirS, and semi-apo NirS measured with 442-nm excitation at 80 K. A, oxidized heme $d_1$ of NirS. B, oxidized dihydro-heme $d_1$ of NirS. C, oxidized heme $c$ of semi-apo NirS. D, reduced heme $c$ of semi-apo NirS. The spectra in A and B were obtained by subtracting the heme $c$ contributions (spectra C and D) from the spectra of the (as-isolated) enzymes NirS and NirS, respectively. The spectra of the oxidized heme $c$ (C) and reduced heme $c$ (D) were generated by mutual spectra subtraction of the as-isolated (mainly oxidized) and dithionite-reduced semi-apo NirS because the spectrum of the pure oxidized semi-apo NirS cannot be measured directly because of photoreduction at low temperatures. Note that the ferrous hemes $c$ in semi-apo NirS undergoes an unprecedented reversible temperature-dependent transition from the low-spin to the high-spin configuration. The latter prevails at 80 K as indicated by the characteristic marker bands at 1353, 1470, and 1607 cm$^{-1}$ (24, 32).

increase of the spectral mass of heme $d_1$ and the putative dihydro-heme $d_1$ in NirS and NirS, respectively. Remaining contributions of heme $c$ could readily be removed by subtracting the spectra of NirS and NirS from those of NirS and NirS using the characteristic bands of heme $c$ as a reference (Fig. 3, C and D). Therefore, pure spectra of heme $d_1$ and the putative precursor were obtained in the oxidized (as-isolated) state (Fig. 3, A and B). The spectrum of ferric heme $d_1$ (Fig. 3A) differs substantially from that of heme $c$, which, because of its higher symmetry ($D_{4h}$), is dominated by the totally symmetric $A_{1g}$ modes (Fig. 3C) (23). In contrast, the low symmetry of heme $d_1$ leads to numerous RR-active modes that are not related to the heme $c$ bands. As a consequence, well-established empirical relationships for iron porphyrins between band frequencies above 1300 cm$^{-1}$ and the oxidation, spin, and coordination state of iron (24) cannot be extended to heme $d_1$, and, therefore, most of its bands must remain unassigned. Remarkable exceptions are the bands at 1687 and 1725 cm$^{-1}$, which originate from the C=O stretching modes of the keto groups and the carboxyl function of the acrylic side chain, respectively. Note that differences between the spectrum in Fig. 3A and a spectrum of oxidized $cd_1$ published previously (25) are likely to be due to different excitation lines and temperatures.

As already expected from the weaker absorbance at 442 nm for NirS compared with NirS (Fig. 2), the RR spectrum of the putative heme $d_1$ precursor suffers from a substantially weaker resonance enhancement compared with heme $d_1$ (Fig. 3B). Despite the relatively poor signal-to-noise ratio, the comparison of both spectra demonstrates that most of the bands of heme $d_1$ also have counterparts within ±3 cm$^{-1}$ in the spectrum of the potential heme $d_1$ precursor, albeit with quite different relative intensities. Therefore, we conclude that the ground state structure of heme $d_1$ and its precursor are similar but not identical. This conclusion is consistent with the assignment of the heme $d_1$ precursor to a dihydro-heme $d_1$. Moreover, among the few bands of NirS for which conjugate bands in NirS are not immediately evident, the bands at 1611 and 1181 cm$^{-1}$ may be attributed to modes involving the C=C and C-(C=C) stretching coordinates, taking into account previous assignments of modes involving the vinyl substituents in protoporphyrin IX (26–29). Furthermore, assuming a dihydro-heme $d_1$ in NirS, the different intensity pattern compared with the spectrum of heme $d_1$ can readily be understood because the extension of the delocalized $\pi$-electron system affects both the absolute and relative RR intensities.

NirS Contains Dihydro-Heme $d_1$.—The results of the experiments described so far strongly suggest that the heme $d_1$ precursor dihydro-heme $d_1$ might indeed be bound in NirS. The RR spectra of NirS and NirS extracted from NirSWT exhibit characteristic peaks at 408 and 683 nm and a shoulder at about 476 nm in the oxidized state, as reported previously (30, 31). In contrast, the spectrum of the oxidized form of the cofactor extracted from NirS shows absorption maxima at 397 and 671 nm and also a shoulder at about 476 nm. After dithionite reduction, the absorption bands of the heme $d_1$ extracted from NirSWT are shifted to 457 and 627 nm, whereas those of the cofactor extracted from NirS are shifted to 447 and 611 nm. Again, these observations strongly suggest that the cofactor isolated from NirS was indeed different from the heme $d_1$ extracted from NirSWT. As described above, we suspected that the heme $d_1$ precursor dihydro-heme $d_1$ lacking the acrylic double bond might be present in NirS. The UV-visible absorption spectra of the isolated cofactor are in line with this proposal because the characteristic heme $d_1$ absorption band at 683 nm in the oxidized state is shifted to a shorter wavelength by about 10 nm in the case of the cofactor from NirS, and the corresponding band of heme $d_1$ in the reduced state at 627 nm is shifted by about 15 nm in this case. Such a hypsochromic shift would be expected for dihydro-heme $d_1$ because of the lacking double bond (6).
Finally, we determined the exact mass of the cofactor extracted from NirS/H9004 N by HR-ESI-MS. This method revealed a mass of 712.14675 for the \([M+H]^+\) ion, which is indeed in accordance with the calculated mass (712.14621), isotopic pattern and chemical formula of dihydro-heme \(d_1\) (Fig. 4C). For comparison, the mass of the heme \(d_1\) extracted from NirSWT was also determined by HR-ESI-MS. The measured value of 710.13075 for the \([M+H]^+\) ion is in excellent agreement with the theoretical mass (710.13065), isotopic pattern and chemical formula of heme \(d_1\) as expected (Fig. 4D). Moreover, the mass difference between the two cofactors isolated from either NirS\(_{AN}\) or NirSWT corresponds to the two hydrogen atoms that are present in dihydro-heme \(d_1\) (NirS\(_{AN}\)) but absent in heme \(d_1\) (NirSWT) because of the dehydrogenation of the propionate side chain of pyrrole ring D.

On the basis of all of the results described so far, we conclude that, in the absence of NirN in the bacterial cell, the formation of the acrylic double bond of heme \(d_1\) does not take place and that dihydro-heme \(d_1\) is inserted into NirS\(_{AN}\). This novel finding led to the question whether NirN itself catalyzes the dehydrogenation of the propionate side chain of pyrrole ring D, yielding the acrylate side chain of heme \(d_1\), or whether NirF catalyzes the double bond formation, as proposed previously, but with the help of NirN. These two possibilities were tested in vitro, and the results are described in the following section.

Purified, Recombinant NirN from P. aeruginosa Catalyzes the Conversion of Dihydro-heme \(d_1\) to Heme \(d_1\) in Vitro—To test whether NirN is able to catalyze the dehydrogenation of the propionate side chain of pyrrole ring D, yielding the acrylate side chain of heme \(d_1\), the extracted dihydro-heme \(d_1\) was mixed with purified NirN, and UV-visible absorption spectra were recorded at different time points after mixing. As shown in Fig. 5A, the absorption band of the free dihydro-heme \(d_1\) at 671 nm disappeared upon addition of NirN, and a new absorption band at 609 nm developed into a broad band at about 635 nm that did not change further during prolonged incubation. On the basis of this observation, we speculated that NirN might have catalyzed the formation of the acrylic double bond after the initial binding of the dihydro-heme \(d_1\) to NirN. Therefore, we then oxidized the NirN/dihydro-heme \(d_1\) reaction mixture and compared the final absorption spectrum with the absorption spectra of a NirN/heme \(d_1\) mixture in the oxidized and reduced states (Fig. 5B). Strikingly, the final spectrum of the NirN/dihydro-heme \(d_1\) reaction mixture was identical to the spectrum of the NirN/heme \(d_1\) mixture in the reduced state, both showing an absorption band at 635 nm. Furthermore, we then oxidized the NirN
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FIGURE 5. Binding of dihydro-heme $d_1$ to purified NirN and transfer to semi- apo NirS. A, UV-visible absorption spectra of the free dihydro-heme $d_1$, (dd$_{d_1}$), of purified NirN, and of the NirN/dihydro-heme $d_1$ mixture measured immediately after mixing the two components (NirN addition) and measured after 15 s of incubation. Characteristic absorption bands are labeled. B, UV-visible absorption spectra of NirN/heme $d_1$, (dd$_{d_1}$) mixtures in the oxidized and reduced forms and of the NirN/dihydro-heme $d_1$ mixture after incubation for several minutes and subsequent oxidation with sodium persulfate. The spectra in A indicate that dihydro-heme $d_1$ first bound to NirN, resulting in a new absorption band at 609 nm, and was then transformed into a new species exhibiting an absorption band at 635 nm. Upon oxidation with sodium persulfate, this species showed an absorption band at 626 nm. The comparison of these spectra with those of NirN/dihydro-heme $d_1$ after incubation of NirN with dihydro-heme $d_1$ (Fig. 5B) suggested that NirN catalyzed the transformation of dihydro-heme $d_1$ into heme $d_1$. C and D, UV-visible absorption spectra of the NirN/dihydro-heme $d_1$, mixture after several minutes of incubation and subsequent addition of Nir$_{S,a}$ (NirN + dd$_{d_1}$ + Nir$_{S,a}$) and of Nir$_{S,a}$/heme $d_1$ and Nir$_{S,a}$/dihydro-heme $d_1$ mixtures in the oxidized (C) and reduced (D) forms. The comparison of the obtained spectra suggested that the cofactor that was transferred from NirN to Nir$_{S,a}$ after incubation of NirN with dihydro-heme $d_1$ was indeed the newly formed heme $d_1$. AU, absorption units.

reaction mixture initially containing dihydro-heme $d_1$ by the addition of sodium persulfate, which resulted in a shift of the 635 nm absorption band to 626 nm. The obtained absorption spectrum was now identical to the spectrum of the NirN/heme $d_1$ mixture in the oxidized state (Fig. 5B). These results suggest that NirN is indeed able to catalyze the conversion of dihydro-heme $d_1$ to heme $d_1$.

To further substantiate this finding, we added Nir$_{S,a}$ to the NirN/dihydro-heme $d_1$ reaction mixture after the potential conversion of the precursor to heme $d_1$ was completed. Because it has been reported previously that heme $d_1$-loaded NirN is able to transfer the cofactor to Nir$_{S,a}$ (12), we assumed that this transfer should also take place in our reaction mixture. After several minutes of incubation with Nir$_{S,a}$, UV-visible absorption spectra were recorded. As shown in Fig. 5C, the absorption band at 626 nm potentially representing heme $d_1$-loaded NirN (oxidized) was shifted to 641 nm upon addition of Nir$_{S,a}$. Indeed, the newly obtained spectrum was identical to the spectrum of heme $d_1$-containing NirS. Moreover, the same was true after the reduction of the cofactor with sodium dithionite (Fig. 5D). These observations clearly supported the idea that NirN catalyzed the conversion of dihydro-heme $d_1$ to heme $d_1$. The subsequent transfer of the formed heme $d_1$ to Nir$_{S,a}$ yielding holo-NirS took place as described previously (12).

Finally, to unambiguously demonstrate that heme $d_1$ was formed out of dihydro-heme $d_1$ by the action of NirN, we extracted the cofactor from the NirN/dihydro-heme $d_1$ reaction mixture after the reaction was completed and analyzed the formed reaction product by UV-visible absorption spectroscopy and HR-ESI-MS (Fig. 6). The extracted reaction product exhibited an absorption band at 683 nm identical to heme $d_1$. Moreover, a mass of 710.13043 was determined for the reaction product ([M + H$^+$]), which is in agreement with the theoretical mass (710.13065) of heme $d_1$. Therefore, we concluded that NirN indeed catalyzed the formation of the acrylic double bond, therefore converting dihydro-heme $d_1$ to heme $d_1$. Purified, Recombinant NirF from D. shibae Binds Dihydro-Heme $d_1$ without Conversion to Heme $d_1$—The finding that NirN catalyzes the conversion of dihydro-heme $d_1$ to heme $d_1$ was completely unexpected because NirF was originally proposed to be responsible for this reaction. This proposal was made on the basis of the observation that the periplasmic NirF from P. pantotrophus was able to bind heme $d_1$ in vitro, and the obtained NirF-heme $d_1$ complex was interpreted to represent an enzyme-product com-
plex (13). Therefore, we incubated purified, recombinant NirF from *D. shibae* with dihydro-heme d1 and heme d1, and the binding of the cofactors to the protein was followed by UV-visible absorption spectroscopy (Fig. 7). The spectrum of the NirF/heme d1 mixture exhibits characteristic absorption bands at 429 and 629 nm in the oxidized state and at 448 and 627 nm in the reduced form, which is in agreement with the absorption features of heme d1-containing NirF from *P. pantotrophus* reported previously (13). The spectrum of the NirF/dihydro-heme d1 mixture displays absorption bands at 421 and 593 nm in the oxidized form and at 438 and 608 nm in the reduced form, showing that NirF is also able to bind the dihydro-heme d1 precursor because the observed absorption features of the NirF/dihydro-heme d1 mixture clearly differ from those of the free dihydro-heme d1. More importantly, the absorption spectrum of the NirF/dihydro-heme d1 mixture recorded directly after mixing the protein with the cofactor did not change further upon prolonged incubation, in contrast to what was observed for the NirN/dihydro-heme d1 mixture (see above). Therefore, we concluded that NirF was able to bind dihydro-heme d1 but did not convert it to heme d1.

**NirN Catalyzes the Double Bond Formation using Electron Bifurcation**—As outlined above, our studies revealed a new and unexpected function of NirN during heme d1 biosynthesis, namely that of the terminal dehydrogenase introducing the double bond into the propionate side chain of pyrrole ring D of dihydro-heme d1 to yield the acrylate side chain of heme d1. However, NirN must be a quite unusual dehydrogenase because it did not require the addition of any cofactor such as NADH or FAD/FMN typically employed by common dehydrogenases catalyzing two-electron oxidation reactions. Interestingly, in our initial NirN/dihydro-heme d1 reaction described above, we observed that the produced heme d1 was in the reduced Fe(II) state, although the substrate dihydro-heme d1 entered the reaction mixture in the oxidized Fe(III) form. Therefore, it seemed that one electron of the two-electron oxidation remained on the reaction product itself. Nevertheless, the fate of the second electron was still unclear. In this context, it is important to note that NirN contains a covalently attached heme c cofactor (16), and, therefore, we speculated that the second electron might be transferred to the heme c. In the initial reaction mixture, no reduction of the heme c in NirN was observed. However, this reaction was performed under aerobic conditions, which might have led to a rapid reoxidation of any reduced heme c. Therefore, we repeated the NirN activity assay under anaerobic conditions in an anaerobic chamber. NirN in its oxidized form (i.e. containing Fe(III)-heme c) was mixed with oxidized Fe(III)-dihydro-heme d1, and the reaction pro-

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**FIGURE 6.** Characterization of the cofactor extracted from the NirN/dihydro-heme d1 mixture. A, UV-visible absorption spectra of the cofactor extracted from the NirN/dihydro-heme d1 reaction mixture after several minutes of incubation (NirN assay) and spectra of extracted heme d1 (d1) and dihydro-heme d1 (dd1) for comparison. Characteristic absorption bands are labeled. B, HR-ESI-MS analysis of the cofactor extracted from the NirN/dihydro-heme d1 reaction mixture. The top panel shows the experimental mass and isotopic pattern of the [M+H]+ ion, and the bottom panel shows the calculated mass spectrum of heme d1. Note that the measured masses 711.57397 and 712.57680 originate from an uncharacterized impurity present in the sample. AU, absorption units.

**FIGURE 7.** Binding of heme d1 and dihydro-heme d1 to purified NirF. A and B, UV-visible absorption spectra of purified NirF, NirF incubated with heme d1 (d1), and NirF incubated with dihydro-heme d1 (dd1). Spectra were measured in the oxidized (A) and dithionite-reduced (B) forms. Characteristic absorption bands are labeled. AU, absorption units.
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Considering the finding that NirN catalyzes the last step of heme \(d_1\) biosynthesis, the question arises what the function of NirF might be that was assumed previously to play the role now assigned to NirN. In this study, we observed that NirF is able to bind dihydro-heme \(d_1\). Together with the previous observation that NirF from \(P.\ aeruginosa\) is a periplasmic, membrane-anchored lipoprotein that interacts with both NirN and NirS (14, 15), one might speculate that NirF could be involved in the uptake of dihydro-heme \(d_1\) into the periplasm from a still unknown membrane transporter and then further transfer the precursor to NirN. NirN then catalyzes the formation of heme \(d_1\), which is finally transferred to NirS, yielding the active holo form of the enzyme. Such a role of NirF in the uptake of the heme \(d_1\) precursor into the periplasm is, of course, speculative at this stage and will require further research.

FIGURE 8. NirN activity assay under anaerobic conditions. UV-visible absorption spectra of free dihydro-heme \(d_1\) (dd1), of purified NirN, and of the NirN/dihydro-heme \(d_1\) reaction mixture incubated under anaerobic conditions in an anaerobic chamber. For the NirN/dihydro-heme \(d_1\) reaction mixture, spectra were measured immediately after mixing the two components (light gray) and then every 15 s (from light to dark gray). Characteristic absorption bands are labeled. The increase or decrease of absorption bands during the reaction progress is indicated by arrows. AU, absorption units.

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