NapF Is a Cytoplasmic Iron-Sulfur Protein Required for Fe-S Cluster Assembly in the Periplasmic Nitrate Reductase*

Received for publication, June 11, 2004, and in revised form, September 7, 2004
Published, JBC Papers in Press, September 15, 2004, DOI 10.1074/jbc.M406502200

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The periplasmic nitrate reductase (Nap) is widespread in proteobacteria. NapA, the nitrate reductase catalytic subunit, contains a Mo-bis-MGD cofactor and one [4Fe-4S] cluster. The nap gene clusters in many bacteria, including Rhodobacter sphaeroides DSM158, contain an napF gene, disruption of which drastically decreases both in vitro and in vivo nitrate reductase activities. In spite its importance in the Nap system, NapF has never been characterized biochemically, and its role remains unknown. The NapF protein has four polycysteine clusters that suggest that it is an iron-sulfur-containing protein. In the present study, a His₆-tagged NapF protein was overproduced in Escherichia coli and purified anaerobically. The purified NapF protein was used to obtain polyclonal antibodies raised in rabbit, and cellular fractionation of R. sphaeroides followed by immunoprecipitation with anti-NapF antibodies revealed that the native NapF protein is located in the cytoplasm. This contrast with the periplasmic location of the mature NapA. However, NapA could not be detected in an isogenic napF⁻ strain of R. sphaeroides. The His₆-tagged NapF protein displayed spectral properties indicative of Fe-S clusters, but these features were rapidly lost, suggesting cluster lability. However, reconstitution of the Fe-S centers into the apo-NapF protein was achieved in the presence of Azotobacter vinelandii cysteine desulfurase (NiFS), and this allowed the recovery of nitrate reductase activity in NapA protein that had previously been treated with 2,2'-dipyridyl to remove the [4Fe-4S] cluster. This activity was not recovered in the absence of NapF. Taking into account the cytoplasmic localization of NapF, the presence of labile Fe-S clusters in the protein, the napF⁻ strain phenotype, and the NapF-dependent reactivation of the 2,2'-dipyridyl-treated NapA, we propose a role for NapF in assembling the [4Fe-4S] center of the catalytic subunit NapA.

The periplasmic nitrate reduction (Nap) system has been found in many different bacteria, and several physiological

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* This work was funded in part by Ministerio de Ciencia y Tecnología (Grant BMC 2002-04126-C03-03) and Junta de Andalucía (Grant CVI-0117). The costs of publication of this article were defrayed in part by the payment of page charges. This must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequences reported in this paper have been submitted to the GenBank®/EBI Data Bank with accession number(s) Z46806.

† The abbreviations used are: Nap, periplasmic nitrate reductase; Amp⁶, ampicillin resistance; BSA, bovine serum albumin; DP, 2,2'-dipyridyl; DTT, dithiothreitol; IPTG, isopropyl β-d-thiogalactoside; LB, Luria-Bertani broth; MGD, molybdopterin guanine dinucleotide cofactor; MV, methyl viologen; NiFS, A. vinelandii cysteine desulfurase.

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severe growth defect in a NapG/H+ strain, but not in an napGH deletion mutant (13). It has been shown that electrons can flow from menaquinol to the NapC subunit in E. coli. A membrane-anchored NapGH complex could act as a proton translocating dehydrogenase transferring electrons from ubiquinol and catalyzing an effective electron transfer to the periplasmic NapAB complex. Nevertheless, no function has been assessed to NapF in the Nap system of E. coli (11–13). Fe-S clusters act as cofactors in many different proteins such as electron carriers, environmental sensors, substrate transporters, or regulatory proteins participating in control of gene expression (14, 15). Spontaneous in vitro Fe-S cluster assembly can occur, but in vivo Fe-S assembly requires accessory proteins (16). Studies of Fe-S cluster assembly in the Azotobacter vinelandii nitrogenase revealed the requirement of two proteins, NiFs and NiU (14). Homologues of these two proteins are found in almost all organisms, from bacteria to humans. NiF is a pyridoxal phosphate-dependent cysteine desulfurase that mobilizes sulfur from L-cysteine. NiU is the Fe-S cluster scaffold protein for assembly of iron-sulfur clusters and the Suf system is important for Fe-S biogenesis of different iron-sulfur proteins (17–20). The Isc system is the housekeeping Fe-S cluster assembly system, whereas the Suf system is important for Fe-S biogenesis under stressful conditions. In this system, SufA plays a role as scaffold protein for assembly of iron-sulfur clusters and delivery to target proteins. SufS is a cysteine desulfurase that mobilizes sulfur from cysteine and provides it to the cluster, and SufE binds to SufS and is responsible for a 50-fold stimulation of the cysteine desulfurase activity of SufS (19).

In this study we report the heterologous expression in E. coli of the R. sphaeroides NapF protein fused to an N-terminal Hisg motif. NapF purification allowed the study of its spectroscopic properties and the isolation of anti-NapF antibodies raised in rabbit to assess the subcellular localization of the native protein in R. sphaeroides. Demonstration of the implication of the R. sphaeroides NapF protein in the assembly of the iron-sulfur center of the catalytic subunit (NapA) is also presented.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The bacterial strains and plasmids used in this work are listed in Table I. The E. coli strains were cultured in Luria-Bertani (LB) medium or on LB agar plates at 37 °C (22). pBluescript and pSVB25 were used routinely in gene manipulation, and the pKD60 vector was used for the construction of alkaline phosphatase gene fusions. E. coli cells harboring any of the plasmids used in this work were cultured in the presence of ampicillin at a final concentration of 100 μg ml⁻¹.

**TABLE I**

| Strains and plasmids | Phenotype or characteristics | Source or reference |
|----------------------|-----------------------------|--------------------|
| **Bacterial strains** |                             |                    |
| R. sphaeroides DSM158 | Wild type, Nap⁺          | 21                 |
| R. sphaeroides DSM158 (pF88 Km1/2) | NapF⁻, defective strains | 7                  |
| E. coli JM109        | Host strain for plasmids carrying the pQE derivatives | 22                 |
| E. coli DH5a         | Lac⁺, host strain for plasmids carrying the lacZ or phoA genes | 22                 |
| E. coli XLI-blue     | Host strain for plasmids carrying the phoA gene | 22                 |
| **Plasmids**         |                             |                    |
| pBluescript SK(+/−)  | Cloning vector, AmpR⁺, lacZ, flori, T7 promoter⁺ | Stratagene         |
| pSVB25               | Cloning vector, AmpR⁺, lac⁺ | 23                 |
| pGEM-T               | Poly-T cloning vector for PCR fragments, AmpR⁺ | Promega            |
| pKD60                | From pKK223–3 with a cycA81'-phoA fusion, AmpR⁺ | Qiagen             |
| pQE22                | Cloning vector carrying the Hisg motif, AmpR⁺ | This work          |
| pFR24               | 2.17-kb PstI-BamHI fragment with the napKEFD genes cloned into pUC18 SaI, AmpR⁺ | This work          |
| pALTER               | Site-directed mutagenesis vector, AmpR⁺ | This work          |
| pALTER-PB            | 2.17-kb PstI-BamHI fragment with the napKEFD genes cloned into pALTER, AmpR⁺ | This work          |
| pKD60X               | From pKD60 with a napF⁺-phoA fusion | This work          |
| pKD60Δ SX            | From pKD60 with a deletion of the cycA sequence | This work          |
| pKD60B⁺·X⁻          | From pKD60 with a napA⁺·phoA fusion | This work          |
| pFR24               | 2.17-kb PstI-BamHI fragment cloned into pUC18 SaI, AmpR⁺ | This work          |
| pQE32/napF           | 0.8-kb BamHI fragment with the napFD genes cloned into pQE32 | This work          |

⁺ AmpR⁺, ampicillin-resistance marker.

**Enzyme Assays, Analytical Methods, and Spectroscopic Analyses**—Nitrate reductase was assayed with reduced methyl viologen as artificial electron donor and the nitrite produced was determined colorimetrically (25). Alkaline phosphatase activity was assayed in subcellular fractions of E. coli with p-nitrophenyl phosphate (24, 26). Malate dehydrogenase activity was assayed in subcellular fractions of R. sphaeroides by following NADH oxidation in a spectrophotometer at 340 nm (27). Succinate dehydrogenase was assayed in subcellular fractions of R. sphaeroides by the phenazine methosulfate-dependent reduction of dichlorophenolindophenol (28). Labile sulfur determination was performed by a reaction with N-N-dimethyl-p-phenylenediamine and FeCl₃ following described methods (29, 30) and using a calibration curve with Na₂S₉H₂O in NaOH (under nitrogen atmosphere) as standard. Labile iron was assayed in samples heated at 80 °C for 10 min, by reaction with sodium methasulfite and bathophenanthroline, as previously described (31), and using a calibration curve with FeSO₄·7H₂O as standard. To determine iron and sulfur labile in the apo-NapF recombinant protein, the apo-NapF was treated with 20 μl of 0.55 M Tris-HCl (pH 8.0). The oxidized and reduced UV-visible spectra of NapF were obtained by oxidizing the protein with potassium ferricyanide or using dithionite as reductant, respectively. The spectra were recorded in a DU7500 (Beckman) spectrophotometer. The form B molybdopterin derivative was extracted from the native NapA protein and the dipyriddyli-treated NapA samples by a modification of the procedure of Johnson and Rajagopalan (33). 1 ml of sample (0.5 mg ml⁻¹ of enzyme) in 10 mM Tris-HCl (pH 7.0) was acidified to pH 2.5 with concentrated HCl. The samples were incubated in a boiling water bath for 20 min and centrifuged at 19,000 × g for 10 min. Fluorescence excitation and emission spectra of the supernatants were recorded using a PerkinElmer Life Sciences LS-5 luminescence spectrometer. Surface-enhanced laser desorption ionization mass spectrometry was used to determine, through time of flight, the Hisg-tagged NapF molecular mass by using an immobilized metal affinity capture surface.

**Subcellular Fractionation**—Subcellular fractions of E. coli and R. sphaeroides were carried out as previously described (34). Cells were harvested by centrifugation and washed in 100 ml of 0.05 M Tris-HCl buffer (pH 8.0). A cell pellet was obtained by centrifugation and resuspended in 50 ml of sucrose buffer (75 mM Tris-HCl, pH 8.0; 20 mM EDTA; 100 mM NaCl; 0.5 mM succrose) and 100 μg ml⁻¹ lysozyme and then incubated for 30 min at 30 °C. After centrifugation for 30 min at 19,000 × g, the supernatant (periplasmic fraction) was separated from the pellet (spheroplasts), which was resuspended in 75 mM Tris-HCl, pH 8.0, buffer. The spheroplasts were then broken by cavitation (3)
pulses of 5 at 90 watts), and the cell extract was centrifuged at 200,000 × g for 45 min. Then, the supernatant (cytoplasmic fraction) was separated from the pellet (membrane fraction). Several enzymatic activities were measured as markers of purity of each subcellular fraction of \( \textit{R. sphaeroides} \). Thus, periplasmic nitrate reductase (25) was found in the periplasmic fraction, malate dehydrogenase activity (27) was detected in the cytoplasmic fraction, and succinate dehydrogenase (28) was found in the membrane fraction.

Western Blots and Heme or Protein Staining—For electrophoretic separation, samples were loaded onto polyacrylamide gels, with 14% (w/v) resolving gels and 5% stacking gels. These gels were used in Western blots, heme analysis, or protein staining with Coomassie Brilliant Blue or silver. Immunoprobing analyses to detect the His6-tagged NapF protein in \( \textit{E. coli} \) were performed by using monoclonal anti-polyhistidine clone his-1 from mouse ascites fluid as primary antibody and anti-mouse IgG alkaline phosphatase conjugate from goat as second antibody. Immunoprecipitation was achieved by using agarose-agarose to detect the tail-extended NapF protein in \( \textit{R. sphaeroides} \) were carried out with polyclonal anti-NapF antibodies raised in rabbit and anti-rabbit IgG alkaline phosphatase conjugate from goat. Immunoprecipitation analyses to detect the NapA protein in \( \textit{R. sphaeroides} \) were carried out with polyclonal anti-NapA antibodies and anti-rabbit IgG alkaline phosphatase conjugate from goat. Heme-staining gels were loaded with dimethoxybenzidine dihydrochloride (35). The reaction was developed with 0.5 mM sodium citrate (pH 4.4) and 300 mM of 30% \( \text{H}_2\text{O}_2 \). Silver-staining gels were developed in the presence of sodium acetate, sodium thiosulphate, 25% glutaraldehyde, 2.5% silver nitrate, and 37% formaldehyde (36).

DNA Manipulations—DNA manipulations were performed by using standard procedures (22). A fusion between the 5'-end of the \( \text{napF} \) gene and the alkaline phosphatase gene (\( \text{phoA} \)) from the plasmid pKP60 (24) has been performed. For this purpose, the 2.17-kb PstI/BamHI fragment, which contains the \( \text{napF} \) gene, was ligated into the vector pALTER to generate pALTER-\( \text{NB} \). To create the 26th triplet of the \( \text{napF} \) gene, an amplification by PCR with the primers 5'-TGTCGGCGCTCGACCTACGG-3' (Xhol site underlined) and 5'-CCACGCAGCTTGCTCCGGATC-3' was performed. The restriction enzyme XhoI was used to digest the isolated PCR product, generating a 1.2-kb fragment that contains the \( \text{napKE} \) genes and the 5'-end of the \( \text{napF} \) gene. This fragment was then ligated into the pKP60 vector previously digested with Xhol and Sall to generate the pKP60X construct. In the alkaline phosphatase measurements, the pKP60 vector was used as a positive control, because this plasmid contains the signal peptide of the periplasmic cytochrome \( \text{c550} \) of \( \text{Paracoccus denitrificans} \) used to the \( \text{phoA} \) gene. A negative control was performed by digestion of the pKP60 vector with Xhol and Sall and further re-igation to generate the pKP60X plasmid, thus removing a 0.5-kb fragment, which contains the cytochrome \( \text{c550} \) signal peptide. A \( \text{napA-phoA} \) gene fusion was also constructed by inserting the 2.17-kb PstI/BamHI fragment, which contains the \( \text{napF} \) and the \( \text{napA} \) genes, into the unique XhoI site in the plasmid pSVB25X60. This construct was digested with BamHI and, the linear fragment was partially filled-in with the Klone polymerase in the presence of dGTP and dATP. Thus, compatible ends were generated to be cloned into the vector pKP60, previously digested with Xhol and partially filled-in with the Klone polymerase in the presence of dCTP and dTTP, to produce the last construct pKP60B'X'-X' with the desired in-frame \( \text{nap-phoA} \) gene fusion. The plasmids pKP60, pKP60X, pKP60AX, and pKP60B'X'-X' were sequenced to check that all the constructions were made in the correct reading frame, and were introduced into \( \textit{E. coli} \) strains DH5α and XL1-blue. To generate the His6-tagged NapF recombinant protein, the PCR mutagenesis technique was performed by using as template the plasmid pFR24 carrying the \( \text{napF} \) gene and the 5'-end of the \( \text{napA} \) gene, which presents a terminal end, which could act as a possible signal for its translocation to the periplasmic protein in the Tat pathway (Fig. 1A). However, this twin arginine motif is not followed by the conserved region XFLK and the hydrophobic sequence required for the exportation to the periplasm (40, 41). Thus, a cytosolic localization of NapF is more likely. The hydropathy profile reveals that most of NapF is very hydrophobic, with the cytosolic regions of iron-sulfur centers located in these
hydrophobic regions (Fig. 1B), although NapF does not contain transmembrane helices.

_Overproduction and Purification of the NapF Protein—_ A His$_6$-tagged NapF protein was overproduced in _E. coli_ and purified, as described under “Experimental Procedures.” The _R. sphaeroides_ NapF protein was synthesized as a fusion protein with a His$_6$ motif at the N terminus of the protein. This fusion was constructed in the pQE32 vector (Qiagen) under the lac promoter, for IPTG induction when expressed in _E. coli_ JM109. Subcellular fractions of the strains JM109 (pQE32) and JM109 (pQE32/napF) of _E. coli_ were isolated from cells cultured anaerobically with IPTG. Western blots of the subcellular fractions using polyclonal anti-His tag antibodies revealed that His$_6$-tagged NapF protein was expressed and localized in the cytoplasm of the strain JM109 (pQE32/napF) but was absent in the cytoplasmic fraction of the control strain JM109 (pQE32) of _E. coli_ (Fig. 2A). The His$_6$-NapF protein was not detected in the periplasmic fraction of the strain JM109 (pQE32) or in the periplasmic fraction of the strain JM109 (pQE32/napF) (Fig. 2A). This result indicates that in _E. coli_ the recombinant NapF protein is only localized in the cytoplasmic fraction. Purification of this soluble His$_6$-tagged NapF protein was undertaken from the cytoplasmic fraction, which was isolated and loaded onto a nickel-nitrilotriacetic acid-agarose column. The recombinant NapF protein was eluted using a gradient between 5 and 250 mM imidazole. The different chromatographic fractions were analyzed in SDS-polyacrylamide gels by either Coomassie Blue stain (not shown) or silver stain (Fig. 2B). These gels

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**Fig. 1.** Sequence alignment of the NapF proteins from several bacteria and hydropathy plot of the _R. sphaeroides_ DSM158 NapF. A, amino acid sequence comparison of the NapF proteins from _R. sphaeroides_ (7), _Sinorhizobium meliloti_ (accession number NP435922), _Pseudomonas aeruginosa_ (accession number NP249867), and _E. coli_ (11). Identical amino acid residues in at least three of the four sequences are marked in bold. The putative twin arginine motif for periplasmic targeting (40) is underlined, and the four-cysteine motifs for binding of the iron-sulfur clusters are indicated with double bars. B, the hydropathy plot of the _R. sphaeroides_ NapF protein is shown according to Kyte and Doolittle (44). The amino acid sequence is also shown, and the four-cysteine motifs are marked with double bars.
reduced NapF. Protein concentration was 0.4 mg ml$^{-1}$ and eluted by using an imidazole gradient were loaded onto a polyacryl-triacetic acid-agarose column, and several chromatographic fractions were collected. These fractions were then silver-stained.

**UV-visible Spectra of the Iron-Sulfur Protein NapF**—The UV-visible spectra of the recombinant NapF purified under anaerobic conditions showed a peak at 303 nm when oxidized with potassium ferricyanide (not shown) and two peaks at 310 nm and 420 nm when reduced with dithionite (Fig. 2C), indicating that NapF is an iron-sulfur protein. However, the 420-nm peak, which showed an absorbance value of 0.18, was rapidly lost suggesting that iron-sulfur centers of NapF are very labile. An approach for in vitro attachment of iron-sulfur clusters to the apo-protein was undertaken (38), in which apo-NapF protein was incubated anaerobically in the presence of L-cysteine, ferrous ammonium sulfate, and DTT. The spectra were recorded at several times from 350 to 700 nm during 90 min. An assay control replacing the protein NapF for BSA is also shown. Lines c and d correspond to spectra of 0 and 90 min of this assay control, respectively.

**Subcellular Localization of NapF in E. coli**—To investigate the NapF localization, a fusion between the napF gene and the periplasmic cytochrome _c_550 ([ _A. vinelandii_ ](http://www.ncbi.nlm.nih.gov/nuccore/522798530?report=genbank)) gene was expressed in E. coli cells. It has been shown that the NapA protein is translocated to the periplasm by the Tat pathway (41, 42). However, cells carrying the napF-phoA...
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The top dots

ing the primary antibodies, and the napF cytoplasmic fraction isolated from the DSM158.

A (0.5 mg ml
described under "Experimental Procedures." Dilutions of purified NapF antibodies. Anti-NapF polyclonal antibodies were raised in rabbit as described under "Experimental Procedures.

and alkaline phosphatase assays were performed as described under "Experimental Procedures." From the wild-type, napF-, and napB- strains, loaded onto a polyacrylamide gel, and stained to detect the heme groups. B, immunoprobing with anti-NapA antibodies of soluble (periplasmic and cytoplasmic) fractions from the wild-type and napF- strains. A control with the purified NapA protein was also included. These analyses were performed four times with a total protein concentration in the different subcellular fractions in a range of 0.1–0.4 mg ml⁻¹.

Fig. 5. Heme staining gel of periplasmic fractions and immunoprobing of soluble fractions isolated from R. sphaeroides strains. A, the periplasmic fractions were isolated as described under “Experimental Procedures” from the wild-type, napF-, and napB- strains, loaded onto a polyacrylamide gel, and stained to detect the heme groups. B, immunoprobing with anti-NapA antibodies of soluble (periplasmic and cytoplasmic) fractions from the wild-type and napF- strains. A control with the purified NapA protein was also included. These analyses were performed four times with a total protein concentration in the different subcellular fractions in a range of 0.1–0.4 mg ml⁻¹.

TABLE II

Alkaline phosphatase activities of periplasmic and cytoplasmic fractions of E. coli strains carrying different phoA gene fusions

| Bacterial strains | Periplasm | Cytoplasm |
|------------------|-----------|-----------|
| E. coli DH5α     | 18.1      | 3.1       |
| E. coli DH5α/pKPD60 | 159.2    | 9.0       |
| E. coli DH5α/pKPD60ΔX | 16.2    | 3.2       |
| E. coli DH5α/pKPD60X | 14.3    | 3.4       |
| E. coli DH5α/pKPD60B*X* | 17.6    | 3.3       |
| E. coli XLI-Blue | 20.1      | 2.8       |
| E. coli XLI-Blue/pKPD60 | 92.3    | 4.4       |
| E. coli XLI-Blue/pKPD60ΔX | 16.3    | 1.7       |
| E. coli XLI-Blue/pKPD60X | 15.4    | 1.3       |
| E. coli XLI-Blue/pKPD60B*X* | 22.5    | 3.2       |

Fig. 4. Subcellular localization of NapF in R. sphaeroides DSM158. A, titration of serum extraction in the presence of primary antibodies. Anti-NapF polyclonal antibodies were raised in rabbit as described under “Experimental Procedures.” Dilutions of purified NapF (0.5 mg ml⁻¹) were used: a, 1:50 dilution; b, 1:10 dilution; c, 1:5 dilution. The top dots represent the serum extraction at a 1:100 dilution containing the primary antibodies, and the bottom dots represent the pre-immune serum at a 1:100 dilution. B, localization of NapF in R. sphaeroides DSM158 by immunoprobing with the anti-NapF polyclonal antibodies: a, cytoplasmic fraction isolated from the wild-type strain; b, cytoplasmic fraction isolated from the napF- strain; c, 5 μg of purified NapF; d, periplasmic fraction isolated from the wild-type strain; e, periplasmic fraction isolated from the napF- strain. The immunodetection analysis was carried out four times with a total protein concentration in the different subcellular fractions in a range of 0.1–0.4 mg ml⁻¹.

fusion (pKPD60X) or the napA-phoA fusion (pKPD60B*X*) were devoid of alkaline phosphatase activity in the periplasm (Table II). This result indicates that the twin arginine motif does not allow the translocation of alkaline phosphatase, thus making inadequate this approach to investigate the subcellular location of NapF. Therefore, to determine the subcellular localization of the R. sphaeroides NapF protein, the purified NapF was used to obtain polyclonal antibodies, which were titrated by dot blotting (Fig. 4A). After a partial purification, the polyclonal anti-NapF antibodies raised in rabbit were used in Western blots with the subcellular fractions isolated from R. spha-
eroides. This immunological analysis showed that NapF is only present in the cytoplasm of the wild-type cells and is absent in the cytoplasm of the isogenic napF- mutant strain (Fig. 4B). The NapF protein was not detected in the periplasmic fractions of the wild-type or the napF- strains (Fig. 4B). When the membrane fractions of the wild-type or the napF- strains were used, NapF was also undetectable (not shown). As a positive control, 5 μg of purified NapF was used (Fig 4B). To check the purity of each subcellular fraction of R. sphaeroides, several enzymatic activities were measured. The periplasmic nitrate reductase was only found in the periplasm, malate dehydrogenase was only detected in the cytoplasm, and succinate dehydrogenase was only found in the membrane fraction (not shown). This immunological analysis was carried out four times with different amounts of total protein in a range of 0.1–0.4 mg ml⁻¹, indicating that the native NapF protein is localized in the cytoplasm of R. sphaeroides DSM158.

Phenotype of the napF- Strain from R. sphaeroides—Nitrate reduction is severely impaired in an isogenic napF- mutant strain of R. sphaeroides both in vivo and in vitro (7). It is worth noting that MV donates electrons directly to the active site of the nitrate reductase (9, 10), and, for this reason, a mutant in the napF gene shows the same MV-dependent Nap activity as the wild-type strain (7). On the contrary, the napF- mutant shows only a very low activity with MV as artificial electron donor (7). This low Nap activity of the napF- mutant strain is not due to a decrease on napF gene expression, because this mutant showed even a slightly higher level of the c-type cytochrome NapB than the wild-type in a heme-stained gel (Fig. 5A). In addition, an immunological analysis revealed that levels of the catalytic subunit NapA in the soluble (periplasmic and cytoplasmic) fractions of the napF- strain are undetectable (Fig. 5B). NapA was also undetectable in membrane fractions.
of the napF strain (not shown). The absence of the catalytic subunit NapA in the napF strain is in agreement with an essential role of NapF in the NapA maturation, probably in the assembly of its [4Fe-4S] cluster.

Reconstitution of the Nitrate Reductase Activity of the DP-treated NapA Protein by NapF—The generation of an inactive NapA protein devoid of its [4Fe-4S] cluster was undertaken as previously described for the nitrogenase (39). The purified NapA protein was incubated anaerobically in the presence of 2,2'-dipyridyl (DP), as indicated under “Experimental Procedures.” About 88% of the MV-dependent nitrate reductase activity was lost after 20 min of incubation with this iron chelator. The molybdenum cofactor was still present in the DP-treated sample. The reconstitution of the nitrate reductase activity was carried out anaerobically with the DP-treated NapA, NapF and NifS (circles). Two negative assays without NapF, either with only DP-treated NapA and NifS (triangles) or with DP-treated NapA, NifS, and BSA instead of NapF (squares) are also shown. The NapF concentration used was 40 µg.

The periplasmic nitrate reduction system has been found in a phylogenetically wide range of bacteria. Although most of these Nap systems include the napF gene, no function has been assessed for this component (7, 11–13). The NapF sequence analysis reveals that polycysteine clusters are indicative of the presence of iron-sulfur clusters (Fig. 1). The conserved twin arginine motif in the N terminus of NapF suggests that this protein could be translocated to the periplasm through the export Tat pathway. This system is specific for folded metalloproteins, which presents a hydrophobic signal peptide (28–58 amino acids) with the conserved RXRXFLK motif (40–42). However, the NapF sequence lacks the conserved residues and the hydrophobic region following this twin arginine motif (Fig. 1A), and thus, a cytoplasmic localization can be considered. In addition, the NapF hydropathy plot reveals that NapF does not contain transmembrane helices but is a very hydrophobic protein (Fig. 1B).

To investigate this further, an anaerobic overproduction of a His6-tagged NapF protein was carried out in E. coli cells. The recombinant protein was localized in the cytoplasm with anti-His antibodies (Fig. 2A) and purified with an imidazole gradient (Fig. 2B). The dithionite-reduced spectrum of the His-tagged NapF showed a peak at 420 nm (Fig. 2C) that suggests the presence of Fe-S centers in the molecule (43). Because the spectrum was performed with a total protein concentration of 22.7 µM, and the absorbance at 420 nm was 0.18, it can be deduced an absorption coefficient (ε) at 420 nm of about 8 mM⁻¹ cm⁻¹. It has been described previously that the ε420 nm is 4 mM⁻¹ cm⁻¹ for proteins with one [4Fe-4S] center (43), and therefore, only 50% of the expected [4Fe-4S] centers are present in the purified NapF. Further attempts to determine labile iron and sulfur by chemical procedures were unsuccessful, because the sample lost the 420-nm peak very rapidly, thus making it difficult to assign a specific type of Fe-S centers to the NapF protein. In light of this, the in vitro attachment of iron-sulfur clusters to NapF has been successfully undertaken (38), increasing the peak at 420 nm during reconstitution (Fig. 3). This correlates with the fact that the sample was turned brown-colored mostly within 90 min. In addition, iron and sulfur labile determinations in the reconstituted NapF protein reveals that 50% of the expected [4Fe-4S] are present in the protein. The presence of the 420-nm peak in the optical spectrum and the development of the yellow-brown color are typical for [4Fe-4S] clusters formation (38, 43). However, the presence of other types of Fe-S clusters, such as [2Fe-2S], or the formation of dimers/multimers of NapF that may act as a functional unit can not be excluded.

To determine the subcellular localization of NapF in R. sphaeroides DSM158, the purified NapF protein was used to obtain

**DISCUSSION**

The periplasmic nitrate reductase activity was not found when NapF was omitted or replaced by BSA (Fig. 6). In addition, in the absence of NifS, DTT, Fe²⁺, or cysteine, reconstitution of the reductase activity was not observed. The reactivation of NapA was also sensitive to pretreatment of NapF with air, and this is in accordance with the absence of the 420-nm peak in the NapF reconstitution assay under aerobic conditions. These results indicated that iron-sulfur centers in NapF are required for the recovery of the nitrate reductase in the DP-treated NapA protein.

**FIG. 6.** Reactivation of the DP-treated NapA form by the NapF protein. The whole assay was carried out as indicated under “Experimental Procedures” and included the DP-treated NapA, NapF and NifS (circles). Two negative assays without NapF, either with only DP-treated NapA and NifS (triangles) or with DP-treated NapA, NifS, and BSA instead of NapF (squares) are also shown. The NapF concentration used was 40 µg.

**FIG. 7.** A model for the NapF-dependent assembly of the [4Fe-4S] cluster in the NapA protein. A similar model has been described for the Suf system of E. coli (19).
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specific antibodies raised in rabbit against the recombinant NapF protein. Immunoprobings with the polyclonal antibodies anti-NapF of the subcellular fractions of R. sphaeroides revealed that the native NapF protein is localized in the cytoplasm of the R. sphaeroides cells (Fig. 4B) and is absent in the periplasmic fraction (Fig. 4B) or in the membrane fraction of this strain (not shown). As a negative control, subcellular fractions of an isogenic napF\(^{-}\) mutant strain were used, and NapF was never detected. As a positive control, 5 \(\mu\)g of purified NapF was used (Fig. 4B). This result confirms that NapF is localized in the cytoplasm of R. sphaeroides DSM158. A different approach to demonstrate the NapF localization by alkaline phosphatase fusion was unsuccessful due to the inability of the Tat pathway to export a functional alkaline phosphatase, as revealed by the napA-phoA fusion (Table II). The PhoA protein is translocated by the Sec route in an unfolded state and acquires its native conformation in the periplasm, whereas the Tat pathway seems to translocate folded proteins that acquire their cofactors in the cytoplasm (40), as described for the NapA protein (41, 42). Therefore, it can be deduced that the twin arginine signal peptides do not allow the periplasmic translocation of a functional alkaline phosphatase. However, the immunoprobings with polyclonal antibodies anti-NapF demonstrated that NapF has a cytoplasmic localization in R. sphaeroides DSM158. This cytoplasmic location of NapF contrasts with the periplasmic localization of the mature NapA but is compatible with a role of this protein in processing or assembling the iron-sulfur center of NapA, rather than as an electron donor to the periplasmic NapAB complex.

On the other hand, nitrate reduction is severely impaired in a nap\(^{-}\) mutant strain of R. sphaeroides, and only a very low nitrate reductase activity is observed both in vivo and in vitro (7). Interestingly, the NapA protein was undetectable in the nap\(^{-}\) strain (Fig. 5B), probably because, in this mutant, the NapA protein lacks its [4Fe-4S] cluster and this inactive protein is degraded in the cytoplasm. This result agrees with a possible role of NapF in the assembling of the [4Fe-4S] center of NapA prior to its translocation to the periplasm. To confirm this function, an inactive DP-treated NapA protein was obtained by incubation of the native NapA protein with 2,2\'-dipyridyl. Fluorescence spectra of the extracted form B molybdenum cofactor and one [4Fe-4S] cluster, and both cofactors are attached to the protein in the cytoplasm prior to export by the Tat translocase. This raises the possibility of a role for NapF in the biogenesis of the [4Fe-4S] center of NapA. This is also in agreement with the presence of highly labile iron-sulfur clusters in NapF and was clearly demonstrated by the NapF-dependent reconstitution of the DP-treated NapA form. We propose that NapF interacts with NapA in the cytoplasm for the assembly of the [4Fe-4S] center and avoids its translocation via the Tat machinery, probably by the interaction of the NapF N-terminal twin arginine motif with the Tat components, until the biogenesis of the [4Fe-4S] cluster of NapA is completed.

Acknowledgments—We thank Pablo Porras and Dr. M. Emilia Martínez for assistance in the isolation of the NapF-polyclonal antibodies.

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