Formation of a Dinitrosyl Iron Complex by NorA, a Nitric Oxide-binding Di-iron Protein from Ralstonia eutropha H16*§

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In Ralstonia eutropha H16, two genes, norA and norB, form a dicistronic operon that is controlled by the NO-responsive transcriptional regulator NorR. NorR has been identified as a membrane-bound NO reductase, but the physiological function of NorA is unknown. We found that, in a NorA deletion mutant, the promoter activity of the norAB operon was increased 3-fold, indicating that NorA attenuates activation of NorR. NorA shows limited sequence similarity to the oxygen carrier hemerythrin, which contains a di-iron center. Indeed, optical and EPR spectroscopy of purified NorA revealed the presence of a di-iron center, which binds oxygen in a similar way as hemerythrin. Diferrous NorA binds two molecules of NO maximally. Unexpectedly, binding of NO to the diferrous NorA required an external reductant. Two different NorA-NO species could be resolved. A minor species (up to 20%) showed an S = ½ EPR signal with g∥ = 2.041, and g⊥ = 2.018, typical of a paramagnetic dinitrosyl iron complex. The major species was EPR-silent, showing characteristic signals at 420 nm and 750 nm in the optical spectrum. This species is proposed to represent a novel dinitrosyl iron complex of the form Fe2⁺ –[NO]2⁻, i.e. NO is bound as NO⁻. The NO binding capacity of NorA in conjunction with its high cytoplasmic concentration (20 µM) suggests that NorA regulates transcription by lowering the free cytoplasmic concentration of NO.

Nitric oxide (NO)² is an obligate intermediate of denitrification (1, 2), formed by the reduction of nitrite by nitrite reductases and subsequently converted to nitrous oxide by NO reductases. In the denitrifier Ralstonia eutropha H16, formation of the NO reductase NorB is controlled by NorR, an NO-binding transcriptional activator (3, 4). The norB gene is cotranscribed with norA that encodes a protein of unknown function. Orthologs of NorA are present in many genomes of proteobacteria and firmicutes (5) and have been annotated as DnrN (Pseudomonas stutzeri), ScdA (Staphylococcus aureus), or YtfE (Escherichia coli and Salmonella enterica). The expression of DnrN, ScdA, and YtfE has been shown to be up-regulated by NO or nitrosating agents (6–9). The non-denitrifiers S. aureus, E. coli, and S. enterica, however, do not possess the norB gene, which demonstrates that norA-like genes do not necessarily co-occur with norB. The NO-dependent expression of norA and its coexpression with norB in R. eutropha suggest a function for NorA in NO metabolism. However, it was shown previously that a nonpolar in-frame deletion of the norA gene did not affect denitrification of R. eutropha cells in terms of growth, accumulation of nitrite or nitrous oxide, or formation of dinitrogen (4). In this study we report the purification and characterization of NorA from R. eutropha. We show that NorA is an NO-binding di-iron protein that modulates the NO-responsive expression of the norAB operon in denitrifying cells of R. eutropha.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strains and plasmids used in this study are listed in Table 1. E. coli JM109 was used for propagation of plasmids. E. coli S17-1 served as the donor in conjugative transfers. E. coli BL21(DE3) was used for overproduction of NorA-hexamhistidine fusion protein. Mutant strains of R. eutropha were constructed by a gene replacement strategy (15) using the suicide vector pLO1, and were verified by Southern blot analysis. The NorR, NorA, and NorB proteins referred to in this study are encoded by the norR1, norA1, and norB1 genes located on megaplasmid pHG1 of R. eutropha. A chromosomally encoded paralog nor gene cluster termed norR2A2B2 has not been extensively studied, but mutational analyses indicate that either of the two nor clusters is sufficient for denitrification (4).

All R. eutropha strains used in this study lack the norR2A2B2 paralogs. In mutant strains HF640, HF635, HF636, HF637, and HF658, the chromosomal norR2A2B2 region was replaced by a fragment containing a translational fusion of the norA1B1 promoter with the lacZ gene for β-galactosidase from E. coli. These strains were constructed by homologous recombination with pCH1018. HF637 contains an additional in-frame deletion of norA1 that was established using plasmid pCH698. HF698 contains an additional in-frame deletion of norR1 that was established using plasmid pCH699. Plasmids were constructed as follows. The upstream region of norA was amplified by PCR using pCH510 as the template. The oligonucleotide primers used were 5’-CGGGATCCGCCACGGCCGCAAGGTG-3’ and 5’-TAAAGCTTGTCAATGTTTCTCCGTCTG-3’, containing BamHI and HindIII sites (underlined), respectively. The amplified 320-bp fragment was cloned BamHI-HindIII into pPHU236 to give plasmid pGE750. pGE750 was digested with BamHI and Sall, and the resulting 3.5-kb fragment was

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‡ The abbreviations used are: NO, nitric oxide; PMS, phenazine methosulfate; Hr, hemerythrin; DNIC, dinitrosyl iron complex.
TABLE 1

Strains and plasmids used in this study

| Strains and plasmids | Relevant characteristics | Source or reference |
|----------------------|--------------------------|---------------------|
| E. coli strains      |                          |                     |
| JM109                | F\(^{-}\) traD36 lacI\(^{+}\), Δ(lacZ)M15 proA\(^{-}\) B\(^{+}\) recA4 Δ(lac-proA) thi gyrA96 endA1 recA1 relA1 hsdR17 | Novagen             |
| S17-1                | Tra\(^{+}\), recA pro thi hsdR chr::R4-2 |                     |
| BL21(DE3)            | F\(\sim\)ompT(gal[lac])::[F] (ø) hsdS\(\gamma\) r\(\gamma\); an E. coli B strain | This study           |

| R. eutropha strains |                          |                     |
|---------------------|--------------------------|---------------------|
| H16                 | Wild type                | DSM428, ATCC17699   |
| HF515               | ΔnorR2A2B2               | (4)                 |
| HF518               | ΔnorA ΔnorR2A2B2         | (4)                 |
| HF562               | NorRΔNTD                 | (4)                 |
| HF640               | ΔnorR2A2B2, PnorA1B1-lacZ| This study           |
| HF635               | ΔnorA ΔnorR2A2B2, PnorA1B1-lacZ| This study      |
| HF636               | NorRΔNTD ΔnorR2A2B2, PnorA1B1-lacZ| This study      |
| HF637               | NorRΔNTD ΔnorR2A2B2, PnorA1B1-lacZ| This study      |
| HF658               | ΔnorR1 ΔnorR2A2B2, PnorA1B1-lacZ| This study      |

| Plasmids            |                          |                     |
|---------------------|--------------------------|---------------------|
| pBluescript SK\(^{+}\)| Ap\(^{+}\), lac\(^{+}\), ColE1 ori, T7E10 promoter, fI ori | Stratagene          |
| pET22b (+)          | Ap\(^{+}\), lacI, T7 promoter, fI ori | Novagen             |
| pPHU236             | Te\(^{+}\), lac\(^{+}\) | (12)                |
| pBRR1MC5S-2         | Km\(^{+}\), lac POZ, Mob\(^{+}\) | (13)                |
| pEYD305             | Tc\(^{+}\) R2 ori, Mob\(^{+}\), promoterless lacZ gene | (14)                |
| pL15                 | Km\(^{+}\), sacB, Rps ori, ColE1 ori | (15)                |
| pCH515              | 7.0-kb Xhol fragment of pGE 26 in pBluescript SK\(^{+}\) | (16)                |
| pCH696              | 3.1-kb EcoRV fragment of norR2A2B2 region containing a 4.9-kb Stul deletion in pBluescript SK\(^{+}\) | (4)                 |
| pCH698              | 1.15-kb SpeI-SacI fragment with a 366-bp deletion (ΔnorA1) in pLO1 | (4)                 |
| pCH699              | 2.3-kb EcoRV-SnaBI fragment with a 1.1-kb deletion (ΔnorR1) in pLO1 | (4)                 |
| pCH1018             | 5.9-kb KpnI-SpeI fragment containing PnorA1B1-lacZ from pCH1128 in pLO1 | This study           |
| pCH1128             | 3.5-kb BamHI-Sall fragment from pGE570 treated with Klenow polymerase in pCH696 | This study           |
| pCH1095             | 0.716-kb Ndel-Sacl fragment containing promoterless norA1 in pET22b (+) | This study           |
| pGE26               | Cosmid from megaplasmid pHG1 containing norR1A1B1 | (17)                |
| pGE28               | Cosmid from megaplasmid pHG1 containing flp | (18)                |
| pGE570              | 0.320-kb BamHI-HindIII fragment containing norA1 upstream region in pPHU236 | This study           |
| pGE569              | 1.33-kb HindIII fragment containing Php::norA1 in pBRR1MC5S-2 | This study           |

treated with Klenow polymerase and cloned into the Stul site of pCH696 to give pCH1128. Following digestion of pCH1128 with KpnI and SpeI and treatment of the 5.9-kb fragment with Klenow polymerase, the fragment was cloned into the Pmel-digested vector pLO1, yielding plasmid pCH1018. For heterologous overproduction of a hexahistidine-tagged NorA, a 700-bp fragment was PCR-amplified from pCH510 as the template, using the primers 5’-TCAAGCTTTCAATGGTGATGGTGATGGTGTCGGTTT-3’ and 5’-GGAAGCTTCAAAGCTTACCTGCAGACGGAGAAGACATATGACCTGCAA-3’ and 5’-GGAGCTCCAGCTTTCAATGGTGATGGTGATGGTGTCGGTTT-GCTTGGTACGCGGCGGCAGCCGGCCGGTCGG-3’ containing Ndel and SalI sites (underlined), respectively. Insertion of the Ndel-Sacl fragment into the Ndel-Sacl-digested pET22b (+) yielded plasmid pCH1095. To construct a plasmid for homologous overproduction of NorA–His6, the promoter region of the flp gene from R. eutropha was PCR-amplified from pCH510 with primers 5’-GCTCCTAGACAGACCTTCTGCCAGCTCAGCTCAGTGTCGCGCGCGCAGAG3’ and 5’-GGGCTCAGATATGTCGGTCTCCATGCGCGCA-3’ containing XbaI, HindIII, and Ndel sites (underlined), respectively. The 632-bp PCR fragment was cloned Ndel-Xbal in pET22b (+) to gain an additional HindIII site, and subsequently inserted into the HindIII site of pBRR1MC5S-2. This construct was completed by a 700-bp Ndel-HindIII fragment from pCH1095 that contains a promoterless norA gene, yielding pGE569.

Media and Growth Conditions—E. coli strains were grown in Luria–Bertani (LB) broth at 37 °C. Aerobic starter cultures of R. eutropha strains were grown overnight at 37 °C in mineral salts medium with 0.4% (w/v) fructose as the carbon source (FN medium), as described previously (4). Denitrifying cultures of R. eutropha strains were grown without shaking at 30 °C in 150-ml septum-sealed flasks containing 100 ml of FN medium supplemented with 0.1% (w/v) potassium nitrate. The flasks were purged with helium prior to inoculation. Antibiotics were added as follows: for R. eutropha, tetracycline (15 µg/ml) and kanamycin (350 µg/ml); for E. coli, tetracycline (10 µg/ml) and ampicillin (100 µg/ml). Culture growth of E. coli and R. eutropha was monitored by following the optical density at 600 nm (A\(_{600}\)) and 436 nm (A\(_{436}\)), respectively.

Overproduction of NorA and NO Treatment on Cells—E. coli BL21(DE3) cells containing the NorA–His6 expression plasmid pCH1095 were grown from overnight cultures in 500 ml of LB medium at 37 °C with aeration (120 rpm). At A\(_{600}\) = 0.6, expression of norA was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The incubation was continued for another 4 h at 30 °C, and the cells were then harvested by centrifugation at 5000 × g for 15 min. For NO treatment on E. coli BL21(DE3) with plasmid pCH1095, cells were grown as described above. At A\(_{600}\) ~ 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside was added. After 1.5 h of incubation at 30 °C with shaking (120 rpm), the cells were spun down at 5000 × g and resuspended in 140 ml of prewarmed LB medium. The culture was transferred into a 150-ml sealed flask and incubated for 15 min at 30 °C without aeration to achieve anaerobic conditions. Nitric oxide-saturated solution was then added to the E. coli culture to a final concentration of ~5 µM. After further incubation of the sealed cultures for 5 min at 30 °C without aeration, cells were harvested at 5000 × g for 15 min. A saturated solution of NO (2 mM) was prepared by bubbling of NO gas through a sealed flask.
with water that was previously purged with helium. NO gas was purified by passage through a 5M NaOH solution and spectroscopically monitored for contaminating nitrite at 354 nm.

**Protein Purification** — *E. coli* cells were chilled and harvested by centrifugation at 5000 \( \times \) g for 15 min. Samples were kept at 4 °C. The cell pellet was washed once with 200 volumes of phosphate-buffered saline (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl). For purification under aerobic conditions, the cell pellet was resuspended in 5 volumes of buffer A (20 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole) and passed twice through a French pressure cell at 900 p.s.i. The resulting suspension was centrifuged at 13,000 \( \times \) g for 30 min, and the supernatant containing the His-tagged NorA was loaded onto a nickel-charged HiTrap column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 10 volumes of buffer B (20 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 100 mM imidazole) and eluted with 4 volumes of buffer C (20 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 150 mM imidazole). The presence of NorA in the eluate fractions was analyzed by SDS-PAGE followed by staining with Coomassie Blue. Fractions containing purified NorA-His6 were combined and concentrated using Centricon concentrators (cut-off, 10,000 Da).

For purification under anaerobic conditions, the cell pellet was resuspended in 2 ml of buffer A2 (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole). Cells were disrupted by sonication, transferred into an anaerobic chamber, and centrifuged at 8000 \( \times \) g for 10 min. NorA was purified from the supernatant by chelating chromatography on nickel-nitrilotriacetic acid spin columns (Qiagen) according to the manufacturer’s recommendation. The cell extract was applied to the columns, which were then washed with buffer B2 (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 50 mM imidazole). The NorA protein was eluted with buffer C2 (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole). For spectroscopic measurements, NorA samples were transferred to sealed cuvettes in the anaerobic chamber. The homogeneity of protein preparations was analyzed by SDS-PAGE and subsequent staining with Coomassie Brilliant Blue. To yield reduced NorA, 100 \( \mu \)M purified NorA protein was incubated with 10 mM ascorbate and 10 \( \mu \)M phenazine methosulfate (PMS) for 15 min in an anaerobic chamber. The reductant was removed by using a PD10 column (GE Healthcare), with buffer A2 as elution buffer. Eluted fractions with detectable 280 nm absorbance were combined, concentrated, and transferred into sealed cuvette. For the preparation of apoNorA, 0.5 ml of 0.3 mM aerobically purified NorA was transferred into an anaerobic chamber and 10 mM ascorbate and 10 \( \mu \)M PMS were added. Upon addition of 0.1 ml of 20 mM 2,2'-dipyridyl stock solution, the solution immediately developed a reddish pink color. This solution was incubated for 1 h at 6 °C and then loaded onto a PD10 desalting column (GE Healthcare) and eluted with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole. The eluting colorless fractions...
with detectable 280 nm absorbance were combined and constituted the apoNorA solution.

**Analytical Procedures**—Protein concentrations were determined by the method of Lowry (19) using bovine serum albumin as the standard. Titration with 5,5'-dithiobis(2-nitrobenzoic acid) was carried out in 100 mM sodium phosphate buffer, pH 8.0, 1 mM EDTA, and 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) using an absorption coefficient for 2-nitro-5-thiobenzoate of 14,150 M⁻¹ cm⁻¹ at 412 nm (20). The iron content of purified proteins was determined by the method of Fish (21). β-Galactosidase was assayed as described previously (22), and the activity was calculated according to Miller (23) except that the cell density was measured at 436 nm. NO consumption was assayed as described previously (16) using a Clark electrode. Nitrite was quantified using a colorimetric Griess assay (24). A modified Griess assay was used to determine NO bound to NorA and apoNorA. Proteins were incubated with three equivalents of NO and an excess of ascorbate/PMS in a sealed cuvette without headspace in an anaerobic chamber for 15 min. The solutions were transferred to a sealed Centricon concentrator (10-kDa cut-off) using a gas-tight syringe. The proteins were then separated from the reaction mixture by centrifugation for 1 min. Samples from both the concentrate and the flowthrough were injected with a gas-tight syringe into rubber-sealed reaction tubes containing Griess reagent and oxygen. After an incubation period of 5 h with shaking, NO was quantified by measuring the absorption at 550 nm. The concentration of protein-bound NO was calculated by subtracting NO in the flowthrough. Controls with nitrite and NO-saturated buffer (2 mM) showed that both compounds gave identical absorption values, thus proving the reliability of this method.

**Spectroscopy**—UV-visible spectra were recorded on a Cary 300 SCAN UV-visible spectrophotometer or a Hewlett Packard (HP8453) photodiode array spectrophotometer. EPR spectroscopy was performed as in a previous study (25).

**RESULTS**

**Genomic Comparison**—An alignment of selected NorA orthologs (Fig. 1) shows that these proteins have a bipartite structure. The N-terminal part harbors a domain of unknown function DUF542 (26) that is characterized by a conserved motif CCXG. The C-terminal part contains a histidine-rich region that resembles a hemerythrin domain. Hemerythrin (Hr), an oxygen carrier protein found in several marine invertebrates, contains a di-iron center (27). The two iron atoms in Hr are coordinated by five histidine residues and the carboxylates of a glutamate and an aspartate. Five histidine residues and two glutamate residues are strictly conserved within all NorA orthologs (His-86, His-107, His-131, Glu-135, His-162, His-206, and Glu-210 in *R. eutropha* NorA). Of these, His-86, His-131, His-162, and His-206 and both glutamates align with ligands coordinating the di-iron site in Hr. However, one histidine ligand that is conserved in all known Hr proteins (see Fig. 1) is not strictly conserved within NorA orthologs, whereas His-107 of NorA is not conserved in Hr. Hence the comparative analysis suggests that NorA, albeit not a typical hemerythrin, contains a di-iron center that is mainly coordinated by histidine ligands.

**Purification and Spectroscopic Characterization of NorA**—NorA was overproduced in *E. coli* as a His-tagged protein and purified under aerobic conditions from the cytoplasmic fraction. SDS-PAGE of isolated NorA under non-reducing conditions showed a band with an apparent molecular mass of 28 kDa (Fig. 2) and an additional band with an apparent molecular mass of 60 kDa. Both bands were identified as NorA by mass spectrometry (matrix-assisted laser desorption ionization-time of flight; not shown); the 60-kDa band disappeared upon incubation with excess dithiothreitol, indicating that NorA homodimers are formed due to oxidation of cysteine residues and subsequent disulfide bridging between monomers. Chemical iron determination of various NorA preparations yielded 1.5–2.2 mol of iron per mol of NorA.

The optical spectrum of as-isolated NorA shows a maximum at 353 nm (ε ~ 4500 M⁻¹ cm⁻¹) and a shoulder at 430 nm (Fig. 3, *trace* 1). Similar spectral features have been observed in other di-iron proteins and were attributed to absorbance of the (µ-oxo)-bridged diferric center (28–30). However, the EPR spectrum of as-isolated NorA displayed a single signal with *g*₁ = 1.9665, *g*₂ = 1.9230, and *g*₃ = 1.8732 (Fig. 4A), typical of the S = ½ state of a mixed-valent antiferromagnetically coupled dinuclear iron site (31, 32). Quantification of the signal revealed that the fraction of mixed-valence NorA was low (<7%) and varied from preparation to preparation thus confirming that the majority of aerobic isolated NorA contained a diferric rather than a diferrous or a mixed-valence di-iron center.

To assess the *in vivo* oxidation state of NorA, the protein was purified under anaerobic conditions. SDS-PAGE under non-reducing conditions yielded only monomeric NorA, indicating that a disulfide bridge is not formed in the cytoplasm of *E. coli* cells. Titration of NorA with 5,5'-dithiobis(2-nitrobenzoic acid) under non-denaturing conditions yielded a ratio of 3.9 free thiols per NorA monomer, showing that all four cysteines of NorA are solvent-accessible. Iron determination of anaerobically

![Dinitrosyl Iron Complex of a Di-iron Protein](image-url)
Dinitrosyl Iron Complex of a Di-iron Protein

purified NorA yielded 1.6–2.0 mol of iron per mol of NorA. The EPR spectrum of anaerobically purified NorA did not show the mixed-valence signal, indicating that this form of NorA is not present in the intracellular environment. In the optical spectrum, weak absorbances at 316 nm and ~370 nm were observed (Fig. 3, trace 2). The low intensity of these absorbances is consistent with the presence of mainly diferrous di-iron centers, lacking absorption between 300 and 1000 nm (28, 33, 34). Complete reduction of NorA with ascorbate and PMS led to bleaching of the optical spectrum (Fig. 4C, see also Fig. 5, trace 2).

Admission of air to the anaerobic sample resulted in an increase of absorbance at 316 and 370 nm, and the appearance of a new signal at 520 nm \((\lambda = 1153 \text{ nm}^{-1} \text{ cm}^{-1})\) (Fig. 3, trace 3) within 10 min. This pattern of spectral features is reminiscent of that of the oxygenated form of Hr. Upon oxygenation, the diferrous center in the deoxygenated form of Hr is oxidized to the diferric form in oxygenated Hr, and the bound dioxygen is reduced to the peroxide oxidation state, giving rise to absorbance bands at 330, 360, and 500 nm (28). Although the bands between 300 and 400 nm are attributed to absorption of the \((\mu\text{-oxo})\text{Fe(III)}\) di-iron center (35), the band at 500 nm is due to the hydroperoxo-Fe(III) ligand-metal charge transfer transition (36). The presence of the 520 nm band in the optical spectrum of NorA thus indicates formation of a stable \(\text{O}_2\) adduct. The low wavelength of this band suggests the presence of a hydroperoxo ligand, rather than a peroxo ligand, because generally \((\mu\text{-1,2-peroxo})\text{di-iron units give rise to bands at or above 600 nm}\) (37).

Upon prolonged (16 h) aerobic incubation of the protein on ice, the resulting spectrum was identical to that of aerobically purified NorA, indicating that a \((\mu\text{-oxo})\text{Fe(III)}\) di-iron center was formed by a process analogous to the auto-oxidative conversion of oxygenated Hr to diferric Hr by loss of the peroxide ligand (28, 38). Reduction of the diferric center of NorA to the diferrous state was accomplished by addition of ascorbate and PMS under anaerobic conditions, as indicated by the complete bleaching of the optical spectrum and the absence of mixed-valence signal in the EPR spectrum of ascorbate/PMS-treated NorA (Fig. 4B, trace 1). Reduced NorA shows a tiny signal (3400 G) of unknown origin.
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Upon readmission of air, formation of the absorbance bands of oxidized NorA at 316, 370, and 520 nm was observed in the optical spectrum. Taken together, our data show that the anaerobically purified NorA contains a diferrous di-iron center, which binds oxygen upon exposure to air and is finally converted to the d ferric state by autooxidation.

Reaction of NorA with NO—Addition of NO to NorA as isolated aerobically or anaerobically did not result in major changes of the UV-visible spectrum above 400 nm (cf. Fig. 5, trace 3). Only weak absorbance around 420 nm was observed in the optical spectrum upon addition of a 2.5-fold excess of NO to diferrous NorA, prepared by reduction with ascorbate/PMS and subsequent anaerobic removal of reductant (Fig. 4C, trace 2). The EPR spectrum shows a broad signal around 3500 G due to free NO in solution (Fig. 4B, trace 2). In addition, the spectrum contains an $S = \frac{1}{2}$ signal with $g_1 = 2.041$, and $g_0 = 2.018$, characteristic for dinitrosyl iron complexes (DNICs) of the type Fe(NO)$_2$ (39, 40). Similar signals have been detected with other iron proteins (41–44). Quantification of the EPR signal revealed that only 1.0–1.5% of NorA protein contributed to the DNIC.

Upon addition of ascorbate/PMS to diferrous NorA incubated with a 2.5-fold excess of NO, an approximate 3-fold increase of the signal at $g_1 = 2.041$, and $g_0 = 2.018$ was observed in the EPR spectrum (Fig. 4B, trace 3). As determined by integration from various NorA preparations, the $S = \frac{1}{2}$ species varied between 5 and 10% of the total amount of NorA. The EPR line shape of protein-bound DNICs is independent of temperature, whereas the EPR signals of low molecular weight DNICs, formed upon reaction of thiol- or nitrogen-based ligands and ferrous iron with NO, exhibit multiline patterns at room temperature (40). The EPR spectrum of NorA-NO obtained in frozen solution between 13 and 100 K was identical to that recorded at room temperature (not shown). The absence of resolved hyperfine structure indicates slow tumbling, proving that the DNIC is associated to NorA. Control experiments showed that the DNIC was not formed in the absence of NorA. Note that the signal of free NO in solution decreased greatly, indicating that almost all free NO (250 μM) had been complexed by NorA (100 μM) with a stoichiometry of 2 NO/NorA. In agreement with EPR, the corresponding optical spectra (Fig. 4C) show that full formation of the NorA-NO adduct required additional electrons (Fig. 4C, trace 3).

The optical spectrum of diferrous NorA in the presence of excess ascorbate/PMS and NO is shown in Fig. 5, trace 4. The difference spectrum (inset in Fig. 5) revealed two prominent features: a shoulder at 420 nm ($\epsilon_{420} = 1360$ M$^{-1}$ cm$^{-1}$) and a broad absorption centered around 750 nm ($\epsilon_{750} = 126$ M$^{-1}$ cm$^{-1}$). This spectrum did not change significantly within 1 h, indicating that the NorA-NO complex is stable. In fact, NorA did not show catalytic turnover of NO with ascorbate and PMS as electron donor system in an assay using an NO-responsive Clark-type electrode (not shown). The number of NO molecules bound to NorA was determined by a modified nitrite determination assay using Griess reagent. ApoNorA was used as a control to assess whether NO is bound exclusively to the di-iron center. Upon incubation with a 3-fold excess of NO, NorA bound 1.8 molecules per protein, whereas 0.8 equivalent of free NO was detected. ApoNorA did not bind NO. In conclusion, up to 10% of NorA-NO can be characterized as an EPR-visible DNIC, whereas the majority of NorA-NO is an EPR-silent iron-dinitrosyl.

To determine if similar NorA-NO adducts are formed in vivo, the protein was purified from E. coli cultures treated with NO. Anaerobic purification of NorA from these cells yielded a monomeric yellow-green protein. The optical spectrum revealed an absorbance at 420 nm and a broad signal at 750 nm (supplemental Fig. S1), demonstrating NO binding to the di-iron center in the cell upon addition of exogenous NO. Using the extinction coefficients given above, this in vivo prepared NorA-NO complex contained ~70% NorA-NO. The EPR spectrum of this sample was similar to that of in vitro prepared NorA-NO, but now ~20% of NorA protein contributed to the paramagnetic DNIC.
**Dinitrosyl Iron Complex of a Di-iron Protein**

| TABLE 2 Impact of NorA on norAB promoter activities |
|-----------------------------------------------|
| Strain            | Relevant properties | β-Galactosidase activities* |
|-------------------|---------------------|-----------------------------|
| HF640             | NorA+               | 497 ± 62                    |
| HF635             | NorA−               | 1438 ± 159                  |
| HF640(pGE569)     | NorA overexpression | 129 ± 17                    |
| HF640(pBBR1MCS2)  | Empty vector        | 407 ± 41                    |
| HF635(pGE569)     | NorA overexpression | 308 ± 41                    |
| HF635(pBBR1MCS2)  | Empty vector        | 1288 ± 132                  |
| HF36              | NorA+ NorΔNTD       | 254 ± 28                    |
| HF37              | NorA− NorΔNTD       | 242 ± 31                    |
| HF658             | NorA+ NorΔNTD       | 14 ± 3                      |

*β-Galactosidase activities were determined when nitrite excreted to the medium reached a maximum. Activities are given in Miller units from the mean ± S.D. of at least three independent experiments.

**Physiological Characterization**—To determine if the presence of NorA alters the level of cytoplasmic NO in denitrifying R. eutropha, an NO-sensitive reporter system was constructed by using the transcriptional activator NorR. Because NorR responds to NO (3, 4), the availability of free NO in the cytoplasm of denitrifying R. eutropha cells is reflected by the activity of the NorR-dependent promoter of the norAB operon, norA. A single copy of a NorA-lacZ fusion was established in both the wild-type and the NorA mutant, and promoter activation by NorR was recorded as β-galactosidase activity expressed in Miller units. The results are assembled in Table 2. Compared with the wild-type strain (HF640), the absence of NorA (HF635) resulted in an ~3-fold increased promoter activity. This increase was nullified by overexpression of NorA-His6 in trans (HF635 pGE569). The presence of plasmid pGE569 in the wild-type HF640 led to an approximate 4-fold decrease in promoter activity and thus shows the opposite effect of a NorA mutation. A NorR derivative that lacks the N-terminal signaling domain (NorRΔNTD) activates transcription from the norAB promoter constitutively and hence does not rely on a signal molecule (4). In a NorRΔNTD background, promoter activation was not significantly affected in the absence (HF636) or presence (HF637) of NorA. These results indicate that, in wild-type cells, NorA attenuates the NO-dependent activation of the transcriptional regulator NorR on the level of signal perception.

A ratio of 0.33% NorA per total soluble protein was determined by immunoblot analysis of denitrifying R. eutropha wild-type cells. Taking average values for the cytoplasmic volume and protein concentration of an E. coli cell (45), the cytoplasmic concentration of NorA was estimated to be around 20 μM. This concentration is well above the free steady-state concentration of NO, which is proposed to be in the nanomolar to low micromolar range in denitrifying bacteria (2, 46). Thus binding of two NO per NorA may result in a significant decrease of cytoplasmic NO concentration.

**DISCUSSION**

In this study the NorA protein from R. eutropha was characterized as a di-iron protein on the basis of UV-visible and EPR spectroscopy. The EPR spectrum of the mixed-valence form of NorA with g value components <2.0 shows that NorA contains a di-iron center (47–49). Regarding the low amount of the mixed-valence signal, being highest in aerobically purified NorA, we consider the mixed-valence state as a non-physiological intermediate.

In the presence of ascorbate/PMS, NorA reacts with NO to form a major and a minor species. Although the minor species (<10%) can be safely attributed to a paramagnetic DNIC on the basis of its EPR spectrum, the majority of NorA-NO is an EPR-silent complex, which contains two NO molecules per di-iron center and shows characteristic absorption bands at 420 and 750 nm in the visible spectrum. Although Mössbauer parameters for two strongly non-equivalent iron sites in NorA-NO2 would unambiguously rule out an [Fe(NO)]2 configuration in this complex, the UV-visible and EPR data presented here strongly favor an EPR-silent [Fe-Fe(NO)₂] DNIC configuration (50, 51). For example, the EPR-active low molecular weight DNIC synthesized from S-nitrosothiolamine, cysteamine hydrochloride, and Fe(II) displayed absorption maxima at 392, 603, and 772 nm in the UV-visible spectrum (52). In the presence of air or hydrogen peroxide, the paramagnetic complex converted into an EPR-silent complex showing a shoulder at 440 nm and bands at 305, 362, and 755 nm.

Although inorganic DNICS have been fairly well characterized, this is not the case for protein-bound DNICS. The best characterized to date are the protein-bound paramagnetic and EPR-silent DNICS formed upon reaction of NO with the E. coli iron uptake regulator Fur (42), which, in contrast to NorA, contains a mononuclear iron center. The optical spectrum of the paramagnetic DNIC of Fur, the major component, in contrast to NorA is characterized by a shoulder at 540 nm, and three bands at 410, 650, and 830 nm. ~5% of Fur-NO represented an EPR-silent Fe(NO)₂ complex, showing a shoulder at 590 nm and three bands at 310, 360, and 790 nm in the optical spectrum. The paramagnetic DNIC of Fur-NO has been assigned a [Fe(NO)₂]₈ electronic structure, according to the formalism of the Enemark-Feltham notation (53) and was suggested to arise from the EPR silent [Fe(NO)₂]₈ intermediate by reduction. Because three NO molecules per Fur monomer were required to produce the paramagnetic Fur-NO, it was proposed that two NO molecules are ligands of the iron center and a third one acts as reductant, i.e. formally reducing Fe(II) to Fe(I). In contrast to Fur, for the reaction of NorA with NO, the presence of a reductant is not only required for the formation of the paramagnetic, but also of the EPR-silent DNIC. In general, NO can serve as a ligand to ferrous iron in three ways: NO⁺, NO⁻, or NO⁻/NO₂⁻ (54). We propose that one of the two iron atoms in reduced NorA combines with NO in the presence of an external reductant according to Reaction 1.

\[
\text{Fe}^{2+} + 2\text{NO} + 2e^- \rightarrow \text{Fe}^{2+} - \left[\text{NO}_2^+\right]^{-1}
\]

**REACTION 1**

The Fe²⁺(NO)₂⁻ complex, which is EPR-silent, corresponds to a [Fe(NO)₂]₁⁰ complex. NO is thus proposed to bind as NO⁻ mainly, while the iron remains ferrous (Fe(II), d⁸). The NorA EPR-silent complex is thus different from that in Fur ([Fe(NO)₂]₈). In the paramagnetic form of the DNIC from NorA the iron becomes Fe(I), d⁶ ([Fe(NO)₂]₈), i.e. is one electron more reduced, and the complex is proposed to be similar to that in Fur. Although the NorA contains a di-iron center, the EPR spectrum of the DNIC (Fig. 4) is a hallmark for the binding
of two NO (NO\(^{-}\)) to a single iron atom. It is possible that one of the NO ligands would be bridging the two iron atoms.

There is little we can say at present about the ferrous ion not involved in binding NO. Although the Fe\(^{2+}\) is most likely \(S = 2\), parallel mode EPR spectroscopy of reduced NorA with or without bound NO did not reveal any signal. Although this might be due to, e.g., a large value of the zero-field parameter \(\mathcal{E}\), the spin of the total system might be zero, something that could be investigated by magnetic susceptibility measurements. The finding that the EPR signal of the DNIC of NorA is so similar to that in mono-iron Fe(NO)\(_2\) systems might be taken as an indication that the magnetic coupling between the two iron atoms in NorA is weak.

The need for additional reductant opens the possibility that formation of NorA-NO is modulated by the redox state of the cell. In this context it is interesting to note that the NorA ortholog of *Pelobacter propionicus* is fused with a ferredoxin domain (Fig. 1). It is tempting to speculate that a cytoplasmic ferredoxin is the physiological electron donor of NorA. At this stage, the physiological role of DNIC formation by NorA remains unclear. DNICs have been suggested to act as storage and transport forms of NO and its redox derivatives in mammalian cells (40, 55). In addition, the conversion of NO into its iron center, which is coordinated by the N-terminal domain of NorA (57), and putative ligands of the iron have been identified by oxidase and nitrosative stress (59, 60). DNIC formation by NorA may facilitate interconversion between relatively stable DNICs and more reactive nitrogen species that are instrumental in NO signaling.

The presence of high amounts of NorA (~20 μM) in the cytoplasm opens the possibility that the protein may significantly lower the intracellular level of free NO. Evidence for NO-scavenging by NorA came from two observations: (i) activity of the norA promoter was elevated in a NorA-negative mutant, and (ii) this effect was not seen in the presence of an NO-insensitive derivative of NorR. The orthologous NorR from *E. coli* has been recently shown to bind NO by a monooiron center, which is coordinated by the N-terminal domain of the protein (57), and putative ligands of the iron have been identified for *R. eutropha* NorR by site-directed mutagenesis (3). These data suggest that, in *R. eutropha*, NorR and NorA compete for available NO. In *E. coli* a NorA ortholog YtfE (58) is required for repair of iron-sulfur clusters that have been damaged by oxidative and nitrosative stress (59, 60). DNIC formation with YtfE has not yet been reported. Although the repair of nitrosylated iron-sulfur clusters might involve NO binding by YtfE, repair of oxygen-damaged clusters is unlikely to depend on NO. Thus it has to be considered that the physiological function of YtfE (and perhaps of other NorA-like proteins) differ from that of NorA in *R. eutropha*.

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