A Novel Copper A Containing Menaquinol NO Reductase from *Bacillus azotoformans*†

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ABSTRACT: The molecular biology and biochemistry of denitrification in gram-negative bacteria has been studied extensively. However, little is known about this process in gram-positive bacteria. We have purified the NO reductase from the cytoplasmic membrane of the gram-positive bacterium *Bacillus azotoformans*. The purified enzyme consists of two subunits with apparent molecular masses of 16 and 40 kDa based on SDS−PAGE. Analytical and spectroscopic determinations revealed the presence of one non-heme iron, two copper atoms and of two *b*-type hemes per enzyme complex. Heme *c* was absent. Using EPR and UV−visible spectroscopy, it was determined that one of the hemes is a low-spin heme *b*, in which the two axial histidine imidazole planes are positioned at an angle of 60−70°. The second heme *b* is high-spin binding CO in the reduced state. The high-spin heme center and the non-heme iron are EPR silent. They are proposed to form a binuclear center where reduction of NO occurs. There are two novel features of this enzyme that distinguish it from other NO reductases. First, the enzyme contains copper in form of copper A, an electron carrier up to now only detected in cytochrome oxidases and nitrous oxide reductases. Second, the enzyme uses menaquinol as electron donor, whereas cytochrome *c*, which is the substrate of other NO reductases, is not used. Copper A and both hemes are reducible by menaquinol. This new NO reductase is thus a menaquinol:NO oxidoreductase. With respect to its prosthetic groups the *B. azotoformans* NO reductase is a true hybrid between copper A containing cytochrome oxidases and NO reductases present in gram-negative bacteria. It may represent the most ancient "omnipotent" progenitor of the family of heme-copper oxidases.

Nitric oxide reductase (NOR)[1] in gram-negative bacteria is a membrane-bound enzyme that catalyzes the reduction of two molecules of nitric oxide (NO) to one molecule of nitrous oxide (N_2O) and one molecule of water. The purified NO reductases generally consist of two subunits: one has a molecular mass of 16 kDa containing heme *c* (NorC), and one 53 kDa, a heme *b*-containing subunit (NorB). Because of its hydrophobicity, the 53-kDa subunit migrates at an apparent molecular mass of about 37 kDa in SDS−PAGE (*1*−*7*). NO reductases from gram-negative bacteria use ferrocyotochrome *c*, located in the periplasm, as the physiological electron donor (*6*, *8*).

The primary sequence of NorC predicts that this subunit consists of a periplasmic heme-binding domain anchored to the cytoplasmic membrane with a single N-terminal transmembrane α-helix. NorB is predicted to have 12 transmembrane α-helices (*9*). On the basis of the NorB amino acid sequence, it belongs to the family of heme-copper terminal oxidases that include cbb_3 (or *bc*-type) oxidases, (*c*)aa_3 oxidases, and the quinol (bo_3, ba_3) oxidases (cf. ref *10*). The first oxidase was suggested to have evolved from NOR (*11*, *12*).

Cytochrome oxidases and NORs both contain a binuclear center consisting of a high-spin heme and a copper (Cu_B) or non-heme iron (Fe_B) center, respectively, where reduction of oxygen to water or of NO to N_2O is catalyzed (*4*, *13*−*15*). In NOR, the high-spin heme ferric iron and the Fe_B center are strongly antiferromagnetically coupled, which makes both metal centers EPR silent (*4*, *7*, *16*). Using resonance Raman spectroscopy, oxidized NOR was demonstrated to contain a pentacoordinated high-spin heme center forming an oxobridge with the non-heme iron center. Upon reduction, the bridging oxygen leaves as water, while the high-spin heme iron simultaneously coordinates to histidine. After this ligand change, the active site is ready to reduce NO (*14*, *15*).

The genes encoding two isofunctional NO reductases from the gram-negative bacterium *Ralstonia eutropha H16*, NorB and NorZ, have been sequenced. A His-tagged version of the NorB enzyme was purified as a single subunit enzyme that was fully functional in NO reduction (*17*, *18*). The NorB enzyme contains two *b*-type hemes and one non-heme iron atom. However, it lacks a *c*-type cytochrome, consistent with the absence of a linked gene homologous to *norC*. The *R. eutropha* NOR can use menaquinol as electron donor at a

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[1] Abbreviations: NOR, nitric oxide reductase.

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low rate, but it cannot use cytochrome *c* (*18*). A binding site for menaquinol was proposed to reside in the N-terminal extension of the NorB subunit, which comprises a periplasmic domain flanked by a transmembrane α-helix (*18*).

Only NO reductases from gram-negative bacteria have been characterized to date. *Bacillus azotoformans* is a gram-positive bacterium capable of complete denitrification yielding nitrogen gas from nitrate, nitrite, and nitrous oxide reduction (*19*). Therefore, *B. azotoformans* serves as a suitable model organism to study the properties of the enzymes involved in the denitrification pathway of gram-positive bacteria. In this paper, the purification and characterization of a novel highly active menaquinol-dependent NO reductase from *B. azotoformans* is reported. This enzyme distinguishes itself from other NORs by containing copper in the form of Cu_A in addition to heme *b* and non-heme iron. Heme *c* is absent. Thus, the *B. azotoformans* enzyme represents a new class of NO reductase that is proposed to be a hybrid between NOR and cytochrome oxidase enzymes.

EXPERIMENTAL PROCEDURES

Growth of B. azotoformans. B. azotoformans (NCCB 100003) was grown aerobically for 24 h at 30 °C in a medium consisting of 0.2% (w/v) yeast extract, 30 mM NH_4-Cl, 2 mM $MgSO_4$, 65 mM potassium phosphate buffer, pH 7.0, 1% (v/v) glycerol, and 0.001 (v/v) Lawford trace element solution (*20*). For anaerobic growth conditions, the medium was supplemented with 50 mM KNO_3 and sparged with nitrogen gas. Anaerobic medium (100 mL) was inoculated with 10% (v/v) of an aerobically grown culture. After 4−7 days, the cells began to grow anaerobically by denitrification, as judged from the formation of nitrogen gas. When the OD_{600} was approximately 1, the 100-mL anaerobic culture was transferred to a 1-L nitrate-containing medium and grown for 24 h. Finally, two anaerobic 1-L precultures were used to inoculate a 25-L fermentor, which was incubated for 24 h. The cells were harvested by centrifugation at 5000*g*. The yield was approximately 4 g of wet weight/liter culture. The cells were stored at −40 °C.

Preparation of Membranes. Approximately 50 g of cells (wet weight) were thawed and washed with 50 mM Tris-HCl buffer, pH 8.0. The cells were resuspended in 100 mL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM PMSF and a few grains of solid DNAse and immediately broken by passing the cell suspension twice through a French Pressure Cell operating at 10 MPa. The broken cells were centrifuged at 5000*g* for 10 min. To collect the membrane fraction, the supernatant was centrifuged at 150000*g* for 90 min. The red-colored membranes were washed twice with 50 mM Tris-HCl buffer, pH 8.0, and stored at −40 °C in 50 mM Bis-Tris, pH 6.5.

Purification of NO Reductase. The purification procedure was performed at 4 °C unless indicated otherwise. The membranes were diluted to 10 mg/mL of protein in 50 mM Bis-Tris buffer, pH 6.5, 1 mM EDTA. Dodecyl maltoside was added to a final concentration 0.6% (w/v) [from a 10% (w/v) stock solution], while stirring slowly. The suspension was then incubated on ice for 15 min and centrifuged at 150000*g* for 60 min. The clear supernatant was applied directly to a Q-sepharose column (2.5 × 20 cm) equilibrated with 20 mM Bis-Tris, pH 6.5, 1 mM EDTA, 0.03% dodecyl maltoside. The column was washed with two bed volumes of the equilibration buffer, before elution was performed with a linear NaCl gradient (0−0.5 M) at a flow rate of 1 mL/min. Active fractions eluted between 0.1 and 0.2 M NaCl. Those fractions with an activity greater than 40% of the maximum activity were pooled and concentrated using Amicon 30K concentrator centripreps to a final volume of 2.5 mL.

The solution was applied to a Sephadex G25 column equilibrated with 100 mM potassium phosphate buffer, pH 7.0, 0.03% dodecyl maltoside to remove salts and exchange the Bis-Tris buffer for phosphate buffer. The red-colored fractions from the Sephadex column were pooled and concentrated to a final volume of 2.4 mL. This solution was applied to a Bio-Scale Ceramic CHT20-I hydroxyapatite column equilibrated with 100 mM potassium phosphate buffer, pH 7.0, 0.1% dodecyl maltoside. The higher concentration of dodecyl maltoside in the eluent was used to avoid aggregation of NOR. Elution was performed with a linear phosphate gradient (0.1−1 M phosphate). Active fractions eluted at 0.6−0.7 M phosphate.

Analytical Determinations. (a) Activity Assays. Nitric oxide reductase activity was determined using a Clark electrode as described in ref *4*. Various combinations of electron donors including 10 mM ascorbic acid with or without 100 μM phenazine methosulfate (PMS) were added to the assay. In addition, 20 μM horse heart cytochrome *c* was added with or without the combination of other electron mediators. Menaquinol:NO reductase activity was determined using a Clark electrode. Menaquinol was prepared by reducing 100 μM menaquinone with 200 μM NADH using 0.2 U diaphorase (Sigma D 5540). To study the relation between activity and the concentration of menaquinol, the concentrations of NADH and diaphorase were chosen in such a way that the diaphorase reaction was not the rate-limiting factor. Alternatively, menaquinol was prepared by reducing menaquinone using a 10% molar excess of sodium borohydride under a nitrogen atmosphere. One unit of activity corresponds to 1 μmol of NO reduced/min.

(b) Prosthetic Group Determination. The amount of heme was determined using the pyridine hemochrome method as described by Berry et al. (*21*). Non-heme iron was determined colorimetrically with the ferene method (*22*, *23*) as modified in ref *4*. Copper was determined colorimetrically as Cu^+ using 2,2′-bicinchoninic acid (BCA) as described in ref *24*. Heme *b* from myoglobin was used to correct for the background produced by the heme *b* in NOR. Copper was also determined colorimetrically as Cu^{2+} using biscyclohexanone oxalydihydrazone (cuprizone) as described in refs *25* and *26*.

(c) Other Methods. For N-terminal amino acid sequence analysis NO reductase (5 μg) was electroforesed as in ref *4* and transferred to a PVDF membrane as described in ref *27*. The N-terminal amino acid sequence analysis was performed by means of automatic Edman degradation at the protein sequencing facility of SILS at the University of Amsterdam.

Protein was determined using the BCA Protein Assay reagent of Pierce and BSA as the standard. The native molecular mass of the purified NO was determined using Superdex 200 HR 10/30 column chromatography (Pharmacia Biotech) in an eluent containing 50 mM potassium phosphate

Table 1: Summary of the Purification of the *B. azotoformans* NO Reductase

preparation	total prot (mg)	specific activity (μmol of NO mg^{-1} min^{-1})	yield (%)	purification
membranes	1739	0.67	100	1.00
extract[a]	350	1.92	57.6	2.9
Q-sepharose	32.9	6.15	17.4	9.2
hydroxyapatite	1.8	40.7	6.3	60.5

[a] High-speed supernatant of the dodecyl maltoside extraction of membranes.

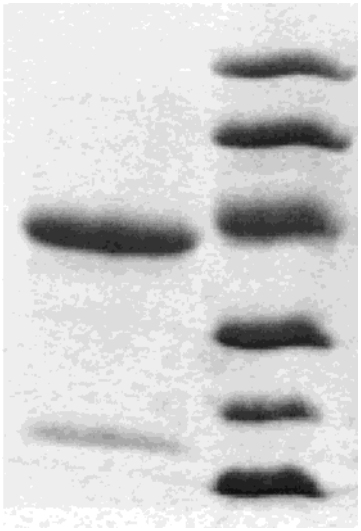

FIGURE 1: SDS−PAGE of NO reductase. Right lane, molecular mass standards (in kDa): 94; 67; 43; 30; 20.1; 14.4. Left lane, NO reductase (3 μg) from *B. azotoformans*. The calculated mobilities of the upper and lower bands correspond to molecular masses of 40 and 16 kDa, respectively.

buffer, pH 7.0, 0.15 M NaCl, and 0.1% dodecyl maltoside. The flow rate was 0.5 mL/min.

RESULTS

Purification of NO Reductase. NO reductase was extracted from the cytoplasmic membrane of *B. azotoformans* using dodecyl maltoside. To minimize proteolytic degradation during purification, the membranes were prepared in the presence of the protease inhibitors, PMSF and EDTA. Solubilization of NO reductase by dodecyl maltoside caused a drop in activity of about 40−50% presumably due to partial delipidation while more than 90% of the total activity was extracted (Table 1). The NO reductase was purified using Q-sepharose- and Ceramic hydroxyapatite-column chromatography resulting in a 60-fold increase in purity with respect to the membranes (Table 1). NOR purification was accomplished without intermediate freezing of the samples, and the activity was determined directly after each purification step because cycles of freezing and thawing caused a decrease of the activity.

On the basis of SDS−PAGE, the enzyme preparation after the last purification step consists of two polypeptides with apparent molecular masses of 40 ± 2 kDa and 16 ± 1 kDa (Figure 1). The elution profile of NOR on a Superdex 200 size exclusion column consisted of one single peak with an apparent molecular mass of 112 kDa (± 5 kDa) (data not shown).

Identity and Stoichiometry of Prosthetic Groups. The identity of the heme groups of purified NOR from *B. azotoformans* was determined with the pyridine hemochrome method. Spectral analysis indicated the presence of heme *b* but the absence of heme *c* in the purified NO reductase (data not shown). The ratio of the absorbance at 557 and 550 nm in the reduced minus oxidized pyridine hemochrome spectrum was 1.7, in good agreement with the ratio of the extinction coefficients for pure heme *b* at these same wavelengths (*21*). Colorimetric determinations were used to determine that NO reductase also contains non-heme iron and copper. The stoichiometric ratio between heme *b*, non-heme iron, and copper (with the BCA assay) was calculated to be 2.0:1.06:2.08 (or 2.26 for copper determined by the cuprizone assay). The heme *b* content of the purified NO reductase was 25.7 nmol/mg, the non-heme iron content was 13.7 nmol/mg, and the copper content was 26.7 nmol/mg of protein, consistent with a minimum molecular mass of 73.0 kDa.

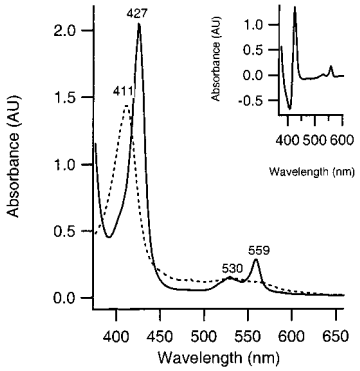

FIGURE 2: Absolute UV−Vis optical spectra of purified oxidized NO reductase (dotted line) and the dithionite reduced enzyme. The inset shows the dithionite reduced minus oxidized difference spectrum. [NOR] = 4.7 μM.

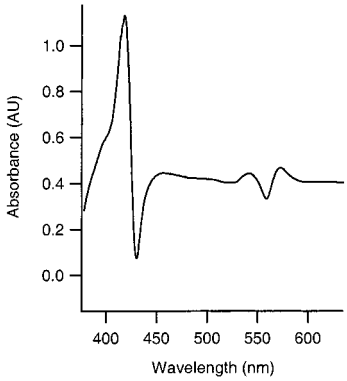

FIGURE 3: CO-difference UV−Vis optical spectrum of the purified NO reductase. Plotted is the difference [dithionite reduced NOR plus CO] minus [dithionite reduced NOR]. [NOR] = 2.7 μM.

Optical Spectroscopy. To examine the properties of the prosthetic groups further, the room-temperature UV−Vis spectrum of purified NO reductase in the oxidized state and after complete reduction with sodium dithionite was recorded (Figure 2). In the reduced enzyme, the absorbance maxima of the γ-, β-, and α-bands are at 427, 530, and 559 nm, respectively, consistent with the presence of heme *b* and absence of heme *c*.

The effect of CO on the dithionite-reduced NO reductase in the α- and γ-band regions is shown in Figure 3. The spectrum is very similar to the CO-difference spectra obtained for ferrous myoglobin (*28*) and for the NO reductase

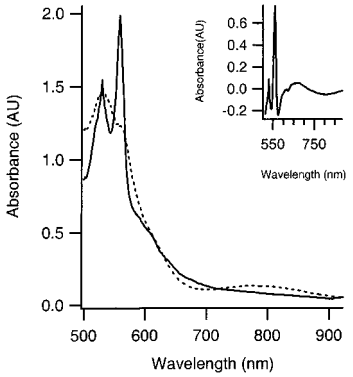

FIGURE 4: UV−Vis optical spectra in the near-infrared region of oxidized NOR (dotted line) and of the menaquinol-reduced enzyme. The inset shows the menaquinol reduced minus oxidized difference spectrum. [NOR] = 29.5 μM.

from *Paracoccus denitrificans* (*4*). This similarity indicating the presence of a high-spin heme *b* species changing to a low spin species upon binding of CO in the *B. azotoformans* NOR. The optical spectrum of the oxidized NO reductase also revealed a broad absorbance in the near-infrared region with an absorbance maximum at around 800 nm (Figure 4). Addition of menaquinol decreased the absorbance of the broad peak at 800 nm while the hemes *b* became fully reduced, i.e., to the same extent as obtained by sodium dithionite reduction. The disappearance of the 800 nm absorbance is indicative for the reduction of the Cu_A metal center (*29*).

EPR Spectroscopy. The wide scan EPR spectrum of the oxidized enzyme exhibits the resonances of an anisotropic low-spin heme center ($g_z = 3.16$, $g_y = 2.2$, $g_x = 1.27$) (Figure 5, panel A). The broad g_y resonance is in part obscured by the intense signal around $g = 2$. This latter signal is due to Cu_A. Minor signals representing no more than 1−2% of the enzyme concentration were present at $g = 6$ and $g = 4.3$ due to high-spin heme and adventitious non-heme iron, respectively.

The enlargement in Figure 5, panel B reveals the details of the Cu_A spectrum. The spin-Hamiltonian parameters $g_z = 2.172$, $g_{x,y} = 2.02$, $A_z = 3.54$ mT are characteristic for the dinuclear mixed-valence Cu_A centers of cytochrome oxidase and N_2O reductase (*30*−*34*). In the EPR spectrum of the *B. azotoformans* NOR, six of the seven hyperfine lines in the g_z peak of Cu_A center are well-resolved, and in this respect, the EPR spectrum is more similar to that of N_2O reductases than to that of cytochrome oxidases.

Quantitation of the low-spin heme and Cu_A signals yielded a ratio of 0.92 ± 0.03 consistent with the metal determination. This takes into account that Cu_A consists of two copper atoms and also that only one of the two *b* hemes could be detected in the EPR spectrum as a low-spin heme (*4*). The EPR silence of the non-heme iron center and the high-spin heme *b* can be explained by a strong antiferromagnetic coupling mediated by a bridging ligand as observed for the *bc*-type NOR from *Pa. denitrificans* (*4*, *10*, *16*). Direct spectroscopic evidence for a bridging oxo-ligand has been obtained very recently (*15*).

Steady-State Kinetic Properties. In general, NO reductases use cytochrome *c*, located in the periplasmic space of gram-negative bacteria, as the physiological electron donor. Alternatively, ascorbate can serve as artificial electron donor

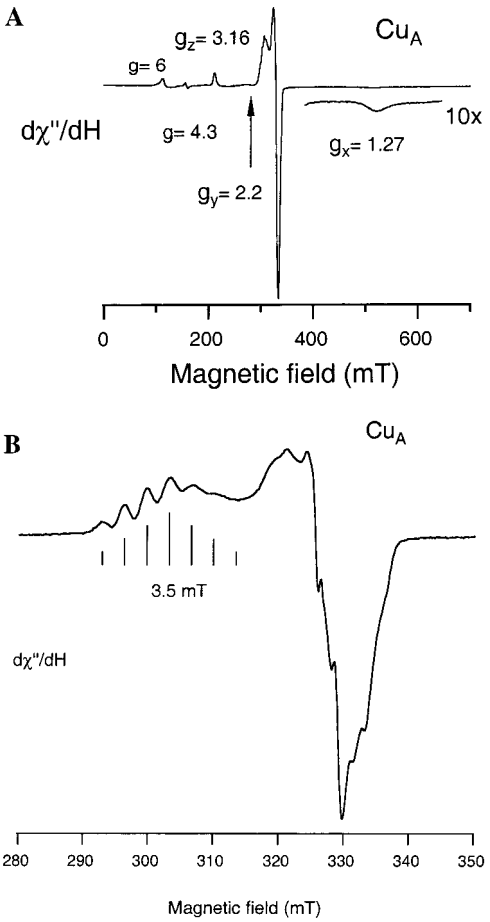

FIGURE 5: EPR spectra of purified oxidized NOR. (A) wide scan EPR spectrum; the arrow points to the positive part of the broad, derivative shaped, g_y resonance. The negative part is obscured by the Cu_A signal. The zero crossing of this resonance, i.e., the g_y value was estimated at $g = 2.2$ by simulation (not shown). The region of the heme g_x resonance was amplified 10 fold. (B) Enlargement of the $g = 2$ region showing the details of the Cu_A spectrum. [NOR] = 150 μM. EPR conditions: Frequency, 9.225 GHz; modulation amplitude, 1.0 mT (A) and 0.5 mT (B); microwave power, 2 mW in panel A and 0.2 mW in panel B; temperature: 19 K in panel A and 10 K in panel B.

directly or in combination with PMS or TMPD. To compare the *B. azotoformans* NOR with those from gram-negative bacteria, several electron donors were examined (Table 2). Like NO reductases from gram-negative bacteria, the *B. azotoformans* NOR can reduce NO with ascorbate. The addition of PMS resulted only in a moderate increase of activity (i.e., 1.3-fold). Significantly, reduced cytochrome *c* did not transfer electrons to this enzyme. However, NOR activity was stimulated by ferrocytochrome *c* when PMS and ascorbate were both present. The highest activity of the *B. azotoformans* NO reductase was measured with menaquinol as the electron donor. The activity using sodium borohydride-reduced menaquinone is about two times higher than obtained in the presence of ascorbic acid, PMS, and horse heart cytochrome *c*. With menaquinol regenerated from menaquinone by diaphorase in the presence of NADH, the menaquinol-dependent activity was about 2-fold lower. Increasing the diaphorase concentration caused turbidity in the assay system without further increasing the NO reductase activity.

N-Terminal Sequence Analysis. The N-terminal amino acid sequence of the 16-kDa polypeptide was determined to be MHKSEKIWLTLSFGM. Interestingly, this sequence is 65%

Table 2: Activity of NO Reductase with Various Electron Donors[a]

electron donor	specific activity (μmol mg^{-1} min^{-1})
ascorbic acid (10 mM)	12.2
ascorbic acid (10 mM) + PMS (100 μM)	16.2
horse heart ferrocytochrome *c* (20 μM)	no activity
ascorbic acid (10 mM) + horse heart cyt. *c* (20 μM)	11.9
ascorbic acid (10 mM) + horse heart cyt. *c* (20 μM) + PMS (100 μM)	26.4
menaquinol[b] (160 μM)	50.0
menaquinol[b] (160 μM) + ascorbic acid (10 mM) + horse heart cyt. *c* (20 μM) + PMS (100 μM)	48.6
menaquinone (1 mM) + NADH (200 μM) + diaphorase (0.2 U)	28.7

[a] The specific activities are lower by a factor of 2.5 (40.7 versus 16.2) as compared to the values in Table 1 due to cycles of freezing and thawing of the pure protein. [b] Menaquinol was generated from menaquinone by reduction with sodium borohydride.

identical to the N-terminal amino acid sequence of the bo_3-type cytochrome *c* oxidase subunit II, recently purified from the gram-positive bacterium, *Bacillus stearothermophilus*, (*35*, *36*). Homology to NO reductase subunits was not found. Determination of the N-terminal amino acid sequence of the 40-kDa polypeptide was unsuccessful since the N-terminus was blocked.

DISCUSSION

This communication describes the purification and characterization of the first NO reductase from a gram-positive bacterium, *B. azotoformans*. The enzyme was extracted from the cytoplasmic membrane with the detergent dodecyl maltoside and purified 60-fold. The maximal turnover number with menaquinol as the electron donor was calculated to be 153 NO/s. The pure enzyme consists of two subunits with apparent mobilities corresponding to molecular masses of 40 and 16 kDa, respectively. On the basis of its non-heme iron or copper content, the minimum protein molecular mass of NO reductase is calculated as 73.0 kDa. The discrepancy between the apparent molecular masses determined by SDS−PAGE and the estimation based on, for example, the non-heme iron contents, is probably caused by the abnormal, i.e., faster, migration of the hydrophobic 40-kDa subunit that only partially unfolds as reported previously for NO reductases from other denitrifiers (*3*−*5*, *7*, *9*, *18*). We conclude that the molecular mass of subunit I is approximately 57 kDa (i.e., 73 minus 16 kDa). Gel filtration chromatography of the native NOR gave a single molecular mass of 112 kDa. The difference between this value and 73.0 kDa is most likely due to the presence of both bound dodecyl maltoside to keep the enzyme in solution and perhaps to residual bound phospholipid.

UV−Vis absorbance spectroscopy and pyridine hemochrome analysis indicated that NOR contained heme *b* but lacked heme *c*. In this respect, the enzyme is similar to the single subunit, heme *b*-containing NOR from *R. eutropha* (*18*). The effect of CO in the UV−Vis spectrum of the dithionite-reduced *B. azotoformans* NO reductase is consistent with the presence of a high-spin heme *b* center. EPR spectroscopy indicated the additional presence of a low-spin heme *b* center. Non-heme iron is also present in stoichiometrical amounts. As in other NO reductases, this non-heme iron and the high-spin heme *b* are EPR-silent, most likely due to an antiferromagnetic coupling (*4*, *7*, *16*). Unlike NO reductases from gram-negative bacteria, the *B. azotoformans* NOR contains two additional copper atoms present as the mixed-valence dinuclear Cu_A, identified by its EPR spectrum and from the broad absorbance at around 800 nm (cf. refs *29*, *33*, and *34*). Cu_A has so far been identified as the redox center in subunit II of cytochrome oxidases or in nitrous oxide reductases (*29*, *32*−*34*, *37*−*40*). The NOR from *B. azotoformans* is the first NOR described to contain Cu_A.

The *g*-values of the low-spin heme *b* [$g_{z,y,x}$ = 3.16, 2.2, 1.27 (Figure 5)] are more anisotropic than those of the comparable low-spin heme *a*, *b*, or *o* centers in cytochrome oxidases and NO reductases [$g_{z,y,x}$ = 3.0, 2.2, 1.5 (± 0.05)] (*2*, *4*, *7*, *16*, *18*, *30*, *41*−*45*). Assuming a bis-histidine coordination for the low-spin heme *b* in the *B. azotoformans* NOR, as is the case for all other NORs and cytochrome oxidases, the *g*-anisotropy is dependent on the relative position of the two axial histidine ligands, i.e., whether these are mutually perpendicular or parallel and whether the two histidine planes are positioned over the pyrrole N-atoms or above the meso position of the porphyrin plane (*46*, *47*). The crystal structure of cytochrome oxidase from *Pa. denitrificans* (*38*) shows that the two histidine imidazole planes are nearly parallel and positioned over the meso position of the porphyrin, entirely consistent with the *g*-values determined by EPR and those calculated (*46*, *47*). In contrast, for the *B. azotoformans* NOR, the two histidine imidazole planes are calculated to make an angle of about 60−70°, pointing as much as possible into the direction of the meso position (*47*). With respect to the position of the histidines in cytochrome oxidase, both residues might be rotated away in opposite directions from their meso positions by 30−35°. Alternatively, one of the histidines has remained more or less in the same position while the other has rotated by 60−70°.

The NO reductase from *B. azotoformans* could be assayed with artificial electron donors but also with menaquinol, the natural quinone in *Bacillus* species (*48*). Reduced horse heart cytochrome *c* could not donate electrons to the enzyme. However, horse heart cytochrome *c* stimulated the activity in the presence of PMS. The nature of this stimulation is unknown, but polylysine, which might mimic the positive charge of cytochrome *c*, did not elicit any effect on the rate (data not shown). The menaquinol:NO reductase activity is dependent on how the menaquinol is prepared. The activity with menaquinol regenerated by diaphorase in the presence of NADH is about 2-fold lower than the activity with menaquinol reduced by sodium borohydride. Both activities are 20-fold higher than reported for the NOR from *R. eutropha* (*18*). In addition, the *B. azotoformans* NOR does not react directly with the diaphorase in contrast to the NOR from *R. eutropha* pointing to important differences between

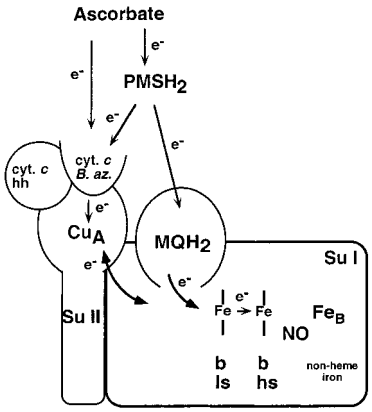

FIGURE 6: Subunit topology of and possible electron-transfer pathways in the MQH_2:NO reductase from *B. azotoformans*. Ascorbate is proposed to reduce the enzyme directly. Either via a site in subunit II where normally the natural cytochrome *c* donates or via reduction of PMS which in turn can reduce the enzyme via two sites, the putative cytochrome *c* site and the menaquinol binding domain in subunit I. Binding of horse heart (hh) cytochrome *c* to subunit II is at or close to the proposed *B. azotoformans* cytochrome *c* (B. az.) electron accepting domain, stimulating the activity of reduction in the presence of ascorbate and PMS. Possible sequential electron transfer (see also text) with menaquinol as substrate involving Cu_A as an intermediate acceptor of the electron en route to the low-spin (ls) heme *b* is not indicated in the drawing. The double-headed arrow connecting Cu_A and the low-spin heme *b* indicates reversible electron transfer, allowing thermodynamic redox equilibration (cf. Figure 4). The binuclear active site consisting of the high-spin (hs) heme and the non-heme iron is proposed to react with NO. Electron transfer from the high-spin heme to the non-heme iron center Fe_B has been omitted for reasons of clarity of the drawing. Arrows, both straight and curved, indicate electron transfer.

the quinol accepting substrate sites of the two enzymes.

N-terminal sequence analysis of subunit II (16 kDa) revealed high homology to the *B. stearothermophilus* bo_3-type cytochrome *c* oxidase subunit II (*35*, *36*). Although *B. azotoformans* and *B. stearothermophilus* exhibit 88% similarity in their 16S rRNA sequences, the 65% identity in the N-terminal region of an oxidase and a NO reductase is remarkably high. Subunit II of the bo_3 cytochrome *c* oxidase is a small Cu_A-binding subunit containing only one instead of two transmembrane α-helices that are found in mitochondrial oxidases or the quinol binding subunit of cytochrome *bo* oxidases (*39*, *49*, *50*). On the basis of the high N-terminal sequence identity and the similar molecular masses of the *B. stearothermophilus* bo_3 cytochrome *c* oxidase subunit II (i.e., 17 kDa), we propose that the Cu_A metal center is located in the 16-kDa subunit of the *B. azotoformans* NOR (Figure 6). Subunit I of the *B. stearothermophilus* bo_3 cytochrome *c* oxidase migrates in SDS−PAGE with an apparent molecular mass of 43.0 kDa, but its molecular mass derived from the DNA sequence was calculated to be 60.6 kDa (*35*). Similarly, the apparent molecular mass of subunit I of the *B. azotoformans* NOR is approximately 40 kDa, but its calculated molecular mass is approximately 57 kDa (see above). The reduced minus oxidized spectrum as well as the CO difference spectrum of the bo_3 cytochrome *c* oxidase are almost identical to that measured for the NOR of *B. azotoformans* (compare Figures 2−4 with Figure 3 of ref *36*) again indicating a very close structural similarity between NO reductase and cytochrome oxidases in general and in particular between these two enzymes in the two *Bacillus* species.

What is the evolutionary relation between the Cu_A-containing NOR from *B. azotoformans*, the *bc*-type NOR from gram-negative bacteria such as *Pa. denitrificans*, the single subunit menaquinol-dependent NO reductase from *R. eutropha*, and cytochrome *c* oxidases and quinol oxidases? Assuming that denitrification predated aerobic respiration, NO reductases are the oldest members of the heme-copper oxidase family (*10*−*12*). The *B. azotoformans* NOR contains all the prosthetic groups present in the various oxidases and NO reductases, except that a *c*-type cytochrome is absent. In addition, the enzyme exhibits (mena)quinol-dependent activity. In these respects, the *B. azotoformans* NOR can be considered as the "omnipotent" precursor of the family of heme-copper oxidases. In time, two important structural and functional changes have occurred. One at the electron-donor site, the other at the electron-acceptor site. As to the latter, the scalar NO reductase has evolved into the electrogenic proton-pumping cytochrome oxidase in which the non-heme iron has been replaced by a Cu_B center, and several other structural changes in subunit I took place allowing the transmembrane movement of protons (cf. refs *13* and *38*−*40*). Regarding the electron-donor site, the Cu_A-containing subunit II with the single transmembrane α-helix subunit may be the evolutionary precursor version of subunit II with two transmembrane α-helices which in some organisms has lost its copper-binding residues and became a quinol accepting site as for the *Escherichia coli* bo_3 quinol oxidase (*39*, *49*, *50*). In other organisms, subunit II may altogether have been replaced by a membrane-anchored *c*-type cytochrome, yielding the *bc*-type NO reductases and the cbb_3-type oxidases. The high structural similarity between the bo_3 cytochrome *c* oxidase from *B. stearothermophilus* and the MQH_2:NO reductase from *B. azotoformans* thus suggests that these two enzymes are closer to the root of the family of heme-copper oxidases than the *c*-type NORs and the cbb_3-type oxidases in contrast to what has been proposed previously (*11*, *12*, *51*, *52*). As a further consequence, the Cu_A center from the NO reductase could have been acquired by a N_2O reductase via exchange of genetic material, an evolutionary scenario opposite to that proposed previously (*11*).

UV−Vis spectra of the *B. azotoformans* NO reductase reduced by menaquinol demonstrate clearly that both hemes *b* and Cu_A become completely reduced. In which subunit the menaquinone binding site is located, how electron transfer occurs from the substrate to the various redox centers, and what the pathway is of internal electron transfer are currently unknown. In view of its small size, we consider it unlikely that subunit II contains the menaquinone binding motif in addition to the Cu_A center. Thus the menaquinone binding site may be located in subunit I (Figure 6). Its calculated molecular mass of 57 kDa indicates that it is much smaller than the single subunit NO reductase from *R. eutropha* that migrates as 75 kDa in SDS−PAGE and has a molecular mass of 84.5 kDa (*18*). In the NOR from *R. eutropha*, the quinol binding domain was suggested to be located in the N-terminal extension. This N-terminal extension is approximately 25 kDa in size and is predicted to consist of a periplasmic loop flanked by two transmembrane α-helices. In view of the relatively small apparent molecular mass of subunit I of the *B. azotoformans* NOR, considerable differences between the menaquinone binding domains of this NOR and that of *R.*

eutropha are expected, consistent with our conclusion drawn above.

For the electron transfer within the *B. azotoformans* NOR, several routes may exist (Figure 6). One possibility would be a direct pathway from Cu_A to the low-spin heme *b* as present in oxidases. The electrons would subsequently be transferred via the high-spin heme to the non-heme iron center. When both these redox centers are reduced, reduction from NO to N_2O occurs. Alternatively, a direct route from menaquinol to the low-spin heme *b* might exist. Both of these routes may be present as "parallel" reduction pathways or as sequential electron-transfer routes from menaquinol via Cu_A to the low-spin heme *b* center (not shown in Figure 6). As long as internal redox equilibration between Cu_A and the low-spin heme b can occur, all of these pathways are consistent with the spectroscopic results presented in Figure 4 and the NO reductase activity measurements (Table 2). The activities obtained with borohydride reduced menaquinol were the highest, and the further addition of ascorbate, PMS and horse heart cytochrome *c* did not stimulate the rate. This suggests that either an internal electron-transfer step is limiting the rate, or that a single substrate site exists for menaquinol and the artificial electron donors, ascorbate or reduced PMS (Figure 6). Given, however, that ascorbate itself can act as a substrate, that horse heart cytochrome *c* stimulates the rate in the presence of ascorbate plus PMS, and that many Cu_A-containing oxidases can be assayed in this way, it seems likely that two separate substrate binding sites exist, one for menaquinol and one for cytochrome *c*. The latter is in analogy to the site in Cu_A-containing cytochrome *c* oxidases. It is possible that only an endogenous cytochrome *c* of *B. azotoformans* can donate electrons efficiently to this site. Horse heart cytochrome *c* may not serve as electron donor directly because of its high redox midpoint potential. However, the structure of horse heart cytochrome *c* might be sufficiently close to that of the natural donor to elicit some type of PMS induced-fit by which NOR activity is stimulated. The existence of alternative or additional electron-transfer pathways in the *B. azotoformans* NOR may be supported by the, so far unique, relative position of the two histidine imidazole planes of the low-spin heme *b* center facilitating electron transfer from several distinct directions. Future pre-steady-state kinetic analysis is planned to provide more insight into the various electron-transfer pathways in the MQH_2:NO reductase from *B. azotoformans*.

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