Cytochrome \( c_b \)-Type Nitric Oxide Reductase with Cytochrome \( c \) Oxidase Activity from \textit{Paracoccus denitrificans} ATCC 35512

TAKE TOMO FUJIWARA AND YOSHIHIRO FUKUMORI

Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226, Japan

Received 9 October 1995/Accepted 22 January 1996

A highly active nitric oxide reductase was purified from \textit{Paracoccus denitrificans} ATCC 35512, formerly named \textit{Thiophaera pantotroph}a, which was anaerobically cultivated in the presence of nitrate. The enzyme was composed of two subunits with molecular masses of 34 and 15 kDa and contained two hemes \( b \) and one heme \( c \) per molecule. Copper was not found in the enzyme. The spectral properties suggested that one of the two hemes \( b \) and heme \( c \) were in six-coordinated low-spin states and another heme \( b \) was in a five-coordinated high-spin state and reacted with carbon monoxide. The enzyme showed high cytochrome \( c \)-nitric oxide oxido-reductase activity and formed nitrous oxide from nitric oxide with the expected stoichiometry when \textit{P. denitrificans} ATCC 35512 ferrocytochrome \( c-550 \) was used as the electron donor. The \( V_{\text{max}} \) and \( K_m \) values for nitric oxide were 84 \( \mu \)mol of nitric oxide per min/mg of protein and 0.25 \( \mu \)M, respectively. Furthermore, the enzyme showed ferrocytochrome \( c-550-\text{O}_2 \) oxidoreductase activity with a \( V_{\text{max}} \) of 8.4 \( \mu \)mol of \( \text{O}_2 \) per min/mg of protein and a \( K_m \) value of 0.9 mM. Both activities were 50\% inhibited by about 0.3 mM KCN.

\begin{center}
\textbf{MATERIALS AND METHODS}
\end{center}

Organisms and cultivation. \textit{P. denitrificans} ATCC 35512 was anaerobically cultivated as previously described by Robertson and Kuenen (21), with some modifications. The bacterium was grown at 37°C in a medium containing (per liter) 1.36 g of \( \text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}, 3.26 \text{g of KNO}_3, 0.8 \text{ g of K}_2\text{HPO}_4, 0.3 \text{ g of KH}_2\text{PO}_4, \) and 0.4 g of MgSO\(_4\) and a trace amount of heavy metals. During cultivation, pure nitrogen gas was gently bubbled into the medium. The cells were harvested in the stationary phase of growth by centrifugation at 10,000 \( \times \) g for 15 min. About 1 g (wet weight; centrifuged, packed state) of cells was obtained from 1 liter of medium. Harvested cells were stored at \(-80^\circ\text{C}\) until use.

Preparation of membrane and soluble fractions. All purifications were conducted at 4°C. Cells (12.4 g, wet weight) were suspended in 100 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.3 M KC1, 100 \( \mu \)M EDTA, and 10 \( \mu \)M phenylmethylsulfonyl fluoride and treated with a sonic oscillator (20 kHz, 250 W; model W-375; Heat Systems, Inc., Plainview, N.Y.) for 30 min with a 5-min interruption every 10 min. The resulting mixture was centrifuged at 5,000 \( \times \) g for 10 min to remove the unbroken cells. The supernatant was centrifuged at 190,000 \( \times \) g for 1 h. The pellet (the membrane fraction) and the supernatant (the soluble fraction) thus obtained were used as starting materials for purification of NO reductase and cytochrome \( c-550 \), respectively.

Purification of NO reductase. The membrane fraction was suspended in 100 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.3 M KCl, 100 \( \mu \)M EDTA, and 10 \( \mu \)M phenylmethylsulfonyl fluoride to yield 2.7 mg of protein per ml. Solid sucrose mononanomol and 1% sodium cholate, 100 \( \mu \)M EDTA, and 10 \( \mu \)M phenylmethylsulfonyl fluoride for 12 h and subjected to anion-exchange chromatography on a DEAE-Toyopearl column (2.8 by 20 cm) which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% (wt/vol) each sucrose mononanomol and sodium cholate, 100 \( \mu \)M EDTA, and 10 \( \mu \)M phenylmethylsulfonyl fluoride (buffer A). After the column had been washed with 150 ml of buffer A containing 0.2 M NaCl, NO reductase was eluted by a linear salt gradient produced from 300 ml each of buffer A containing 0.2 and 0.45 M NaCl. The fractions with NO reductase activity were pooled and diluted with 10 vol of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% (wt/vol) each sucrose mononanomol and sodium cholate, 0.15 M NaCl, and 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% (wt/vol) each sucrose mononanomol and sodium cholate. The resulting fraction was subjected to anion-exchange chromatography on a DEAE-Toyopearl column (1 by 13 cm) which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% (wt/vol) each sucrose mononanomol and sodium cholate, and 0.15 M NaCl. After the column had been washed with 50 ml of buffer used for equilibration, NO reductase was eluted by a linear salt gradient produced from 200 ml each of buffer containing 0.15 and 0.25 M NaCl. The eluates with NO reductase activity were pooled and loaded onto a nonde-
TABLE 1. Purification of P. denitrificans ATCC 35512 NO reductase*

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Vol (ml)</th>
<th>Protein concn (mg/ml)</th>
<th>Total protein (mg)</th>
<th>NOR sp act (μmol/min/mg)</th>
<th>Total NOR activity (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane fraction</td>
<td>71.0</td>
<td>2.66</td>
<td>189</td>
<td>0.259</td>
<td>48.9</td>
</tr>
<tr>
<td>Solubilized fraction</td>
<td>72.0</td>
<td>2.10</td>
<td>151</td>
<td>0.690</td>
<td>104</td>
</tr>
<tr>
<td>DEAE-Toyopearl chromatography</td>
<td>63.0</td>
<td>0.087</td>
<td>5.47</td>
<td>13.7</td>
<td>75.2</td>
</tr>
<tr>
<td>DEAE-Biogel chromatography</td>
<td>1.26</td>
<td>0.192</td>
<td>0.243</td>
<td>84.0</td>
<td>20.4</td>
</tr>
<tr>
<td>Preparative PAGE</td>
<td>ND</td>
<td>0.084</td>
<td>ND</td>
<td>84.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* NO reductase (NOR) activity was measured as described in Materials and Methods. ND, not determined.

The copper atom contents of the purified enzyme preparations were measured by inductively coupled plasma atomic emission spectrometry with an SPS 1500VR Plasma Spectrometer (Seiko Instruments, Inc., Tokyo, Japan).

RESULTS

Purification of NO reductase. NO reductase was purified from P. denitrificans ATCC 35512 in the presence of sucrose mononcaprate SM-1080 and/or sodium cholate under aerobic conditions. As summarized in Table 1, solubilization of the enzyme with SM-1080 resulted in an about twofold increase in total activity. Activation of P. stutzeri NO reductase by a detergent has been reported (14). The enzyme preparation was homogeneous on non-denaturing PAGE (Fig. 1, lanes 2 to 4). The single protein band was stained with both heme-staining reagents and oxidase activity-staining (Nadi-staining) reagents. During purification of the enzyme, we found that the bacterium contained aa3-type cytochrome c oxidase, even though it was cultivated under highly anaerobic conditions with nitrate. As shown in Fig. 2, NO reductase was eluted at 0.18 M NaCl, while aa3-type cytochrome c oxidase was eluted at 0.4 M NaCl. Two enzymes were completely separated by ion-exchange col-

FIG. 1. Nondenaturing PAGE of NO reductase. The enzyme (4.4 μg of protein) obtained after the second ion-exchange chromatography step was loaded onto a 5% polyacrylamide gel in the presence of 0.5% Tween 20. Two bands were stained with Coomassie brilliant blue (lane 1). The red protein corresponding to the upper band was extracted from the gel and used as the final enzyme preparation. The purified NO reductase (1.8 μg of protein) was electrophoretically homogeneous (lanes 2 to 4). Lane 2, Coomassie brilliant blue staining of the purified enzyme; lane 3, heme staining of the purified enzyme; lane 4, cytochrome oxidase activity staining of the purified enzyme.

The ferrocyanochrome c-550-NO reductase activity of the enzyme was measured by the following method. A reaction mixture containing 20 mM phosphate buffer (pH 6.0), 1% sucrose mononcaprate SM-1080, and 0.1 M d-glucose in a total volume of 2.5 ml was placed in a cuvette. After liquid paraffin was floated on the surface of the solution, d-glucose (2 U) and catalase (50 U) were injected into the cuvette by a gas-tight syringe and the mixture was gently stirred for a few min at 20°C. Ferrocyanochrome c-550 and the enzyme were added to the anaerobic reaction mixture, and the reaction was then started by addition of NO-saturated ethanol. The reaction was monitored by measuring the decrease in $A_{550}$.

The ferrocyanochrome c-550-O2 oxidoreductase activity of the enzyme was measured by monitoring the oxidation of ferrocyanochrome c-550 with time in an air-saturated reaction mixture containing 20 mM phosphate buffer (pH 6.0), 0.1 M d-glucose, and 1% SM-1080. O2 consumption catalyzed by the enzyme was measured with a Clark-type O2 electrode (MP-1000; Iijima Products, Tokyo, Japan). All experiments were performed at 20°C.

Reagents. Cytochrome c-550 was purified from P. denitrificans ATCC 35512 by the method of Samy et al. (22), with a slight modification. Ferrocyanochrome c-550 was prepared by addition of a few grams of dithionite crystals. Pure NO, $N_2$, and CO gases were purchased from Nippon-Sanso Co. (Tochigi, Japan). SM-1080 was purchased from Mitsubishi-Kasei Foods Co. (Tokyo, Japan). DEAE-Toyopearl 650M and DEAE-Biogel were from Tosoh (Tokyo, Japan) and Bio-Rad Laboratories (Hercules, Calif., respectively). d-glucose oxidase was purchased from Oriental Yeast Co., Ltd. (Osaka, Japan), and catalase and Hb were from Sigma Chemical Co. (St. Louis, Mo.). All of the other reagents used in this study were of the highest grade commercially available.

Physical measurements. Absorption spectra were recorded with an MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) by using cuvettes with a 1-cm light path. Extraction of monocovalently bound hemes from the purified enzyme and analysis on high-performance liquid chromatography (HPLC) were performed by the method previously described by Sone and Fujisawa (25). Heme $b$ and $c$ contents were calculated on the basis of the peroxidase ferrohemochromes spectra obtained by using millimolar extinction coefficients of 34.4 mM$^{-1}$ cm$^{-1}$ at 557 nm for heme $b$ and of 29.1 mM$^{-1}$ cm$^{-1}$ at 550 nm for heme $c$ (28).

Nondenaturing polyacrylamide gel electrophoresis (PAGE) in the presence of 0.5% Tween 20 and sodium dodecyl sulfate (SDS)-PAGE in the presence of 0.1% SDS were performed as described by Davis (8) and Laemmli (19), respectively. Heme staining and oxidase activity staining (Nadi staining) were carried out as described by Connelly et al. (7) and Keilin (18), respectively. Protein concentrations were determined with a bichromonic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.
umn chromatography. Furthermore, the partially purified enzyme preparation obtained after a second anion-exchange chromatography showed a single heme-staining band on non-denaturing PAGE (Fig. 1, lane 1). These results indicate that the final enzyme preparation was not contaminated with \( \text{aa}_3 \)-type cytochrome \( c \) oxidase.

Spectroscopic properties of NO reductase. The enzyme showed an absorption spectrum in the visible region which is characteristic of so-called cytochrome \( bc \), as shown in Fig. 3A. The reduced form of the enzyme which was prepared by addition of small amounts of dithionite showed absorption peaks at 552, 523, and 420 nm. The absorption peak at 552 nm accompanied a shoulder around 559 nm, suggesting that the enzyme has six-coordinated low-spin heme \( b \) and heme \( c \) in the molecule. When pure CO gas was gently bubbled into the reduced enzyme for several seconds, the absorption peak at 420 nm in the reduced form was shifted to 421 nm and the absorbance of the \( g \) band increased by 30%. The \( a \) band was hardly changed. Furthermore, the difference spectrum, the CO-reduced complex minus the reduced form, of the enzyme showed a sharp peak at 422 nm, a broad absorption peak at 563 nm, and a trough at 615 nm, as shown in Fig. 3B. These spectral properties strongly suggest the presence of a five-coordinated high-spin heme \( b \) in the molecule.

Structural properties of NO reductase. The SDS-PAGE profile of the purified enzyme is shown in Fig. 4. Two bands corresponding to proteins with molecular masses of 34 (subunit I) and 15 (subunit II) kDa were observed in the final preparation. On the basis of the densitometric curve of the gel, the ratio of the area of subunit I to that of subunit II was estimated to be about 2.7:1. Therefore, the molar ratio of subunit I to subunit II in the enzyme seems to be about 1:1. When the gel was soaked with heme-staining reagents, only subunit II was stained, suggesting that heme \( c \) is covalently bound to subunit II of the enzyme. Uncovalently bound heme extracted from the enzyme by treatment with acidic acetone showed an absorption peak at 557 nm in its pyridine ferrohemochrome spectrum and had the same retention time as heme \( b \) which was prepared from Hb on HPLC (data not shown). Neither heme \( a \) nor heme \( o \) was detected in the extracted heme fraction on HPLC. The heme \( b \) and heme \( c \) contents were
estimated to be 24.9 and 14.2 nmol/mg of protein, respectively. The copper content was determined to be 0.7 nmol/mg of protein. The enzyme molecule seems to contain no copper atoms.

Enzymatic properties of NO reductase. In the present study, we monitored enzymatic NO consumption with the Hb-trapping method as described in Materials and Methods. As shown in Fig. 5, NO consumption was scarcely observed in the absence of the enzyme within 10 min after injection of NO into the reaction mixture, while NO was rapidly consumed by the purified NO reductase by using the electron-donating system involving the bacterial cytochrome c-550. Figure 6 shows the oxidation of ferrocytochrome c-550 coupled with reduction of NO catalyzed by the enzyme. When 6.0 nmol of NO was added to the anaerobic reaction mixture, 5.6 nmol of ferrocytochrome c-550 was oxidized by the enzyme. These results indicate that the enzyme catalyzes a one-electron reduction of NO to N₂O as cytochrome c-NO oxidoreductase. The maximum activity was about 84 μmol of NO per min per mg of protein. This value corresponds to a turnover for NO reduction of 67 mol of NO per mol of enzyme s⁻¹, assuming that each enzyme molecule is composed of 34- and 15-kDa subunits. The apparent affinity constants of the purified NO reductase for ferrocytochrome c-550 and NO were calculated to be 0.55 and 0.3 μM, respectively. The reaction catalyzed by NO reductase was 50% inhibited by 0.31 mM KCN.

The NO reductase purified from *P. denitrificans* ATCC 35512 showed cytochrome c oxidase activity. The maximum velocity was 10 mol of oxygen per mol of enzyme s⁻¹ in an air-saturated reaction mixture using ferrocytochrome c-550 as the electron donor. The cytochrome c oxidase activity was 50% inhibited by 0.32 mM KCN. Figure 7 shows the oxygen-consuming activity of the NO reductase in an assay system using ascorbate, N,N,N',N'-tetramethylphenylenediamine, and cytochrome c-550. Neither catalase nor superoxide dismutase plus catalase affected the O₂-consuming activity of the enzyme. These results suggest that the product of O₂ reduction catalyzed by the enzyme may be H₂O₂. The *Kₘ* value of the enzyme for O₂ was estimated to be about 0.9 mM. Nonlinearity observed in the rate of O₂ consumption may be due to very low affinity between the enzyme and O₂ molecules.

**DISCUSSION**

We report here the purification and biochemical characterization of the highly active *cb*-type NO reductase of *P. denitrificans* ATCC 35512, which was formerly named *T. pantotropha*. NO reductases have been previously purified from the cytoplasmic membranes of two denitrifying bacteria, *P. stutzeri* (14, 17) and *P. denitrificans* ATCC 19367 (6, 9). The latter NO reductase has been solubilized with octylglucoside and purified by ion-exchange chromatography, hydroxyapatite column chromatography, and gel filtration in the presence of a detergent (6). Nonionic detergents such as Triton X-100 and Brij-35 have been reported to strongly inactivate the enzyme. On the other hand, Heiss et al. (14) have succeeded in the purification of NO reductase from *P. stutzeri* with Triton X-100 and reported that although the NO reductase activity of the purified enzyme is lower than that of the *P. denitrificans* ATCC 19367 enzyme purified with octylglucoside, the activity of *P. stutzeri* NO reductase is significantly enhanced by addition of soybean.
phospholipids and/or certain detergents (17). Therefore, it seems likely that the inactivation of the enzyme caused by Triton X-100 was due to complete depletion of the boundary phospholipid from the enzyme. Thus, the detergents used for purification of the membrane-bound NO reductase affect essentially stability and intactness. In the present study, we surveyed detergents for solubilization of NO reductase and found that sucrose monopalmitate SM-1080 is a suitable detergent for solubilization and stabilization of the enzyme. The solubilized extract retains full activity for over a week at 4°C under aerobic conditions, and furthermore, the enzyme purified with the detergent can be preserved at -80°C for several months without loss of activity.

In the present study, we obtained pure NO reductase by using nondenaturing PAGE. Although the enzyme was not denatured by the electrophoretic procedure, this was not applicable to large-scale purification. Therefore, a new method for large-scale purification should be developed.

The purified enzyme contains 24.9 nmol of heme b and 14.2 nmol of heme c per mg and is composed of two subunits with molecular masses of 34 and 15 kDa on SDS-PAGE. Recently, the structural genes of P. stutzeri NO reductase have been sequenced by Zumft et al. (31). The large subunit is a strongly hydrophobic membrane-bound protein involving 12 transmembrane helices. It has been known that some hydrophobic proteins show abnormal mobility on SDS-PAGE, resulting in underestimation of their molecular masses. In the case of the P. stutzeri enzyme, the molecular masses of the two subunits were calculated to be about 53 and 16.5 kDa on the basis of the primary structures deduced from the norBC genes (31), while the molecular masses of the same two subunits were determined to be 38 and 17 kDa on SDS-PAGE (14), respectively. These observations assume that the molecular mass of subunit I of the NO reductase purified from P. denitrificans ATCC 35512 is also higher than 34 kDa. Therefore, it may be concluded that the enzyme contains 2 mol of heme b and 1 mol of heme c per mol of the minimum structural unit, which is composed of one molecule each of the 34- and 15-kDa subunits.

NO reductases from P. stutzeri and P. denitrificans ATCC 19367 have been characterized as cytochrome bc on the basis of spectroscopic properties and heme composition (6, 14). Furthermore, the primary sequence of P. stutzeri NO reductase suggests that the large and small subunits contain hemes b and c, respectively (31). On the other hand, the P. denitrificans ATCC 35512 NO reductase molecule seems to contain two hemes b and one heme c. The absorption spectrum of the dithionite-reduced enzyme in the presence of CO suggests the presence of a five-coordinated high-spin heme b as the catalytic center of the enzyme for binding and reduction of the NO molecule. On the other hand, a sharp peak at 552 nm having a shoulder around 559 nm observed in the reduced enzyme is thought to result from the remaining heme b and heme c that may be in six-coordinated low-spin states. Although the presence of a high-spin heme center in the NO reductase should be confirmed by an electron paramagnetic resonance study as the same as in the P. stutzeri enzyme (17), P. denitrificans ATCC 35512 NO reductase can be referred to as a cytochrome ebb(CO)-type enzyme, where the b(CO) notation designates a CO-reactive heme b.

When P. denitrificans ATCC 35512 has been cultivated under anaerobic conditions in the presence of nitrate, two kinds of soluble c-type cytochromes, cd, and c-550, are predominately synthesized in the cells (20). Recently, Samyn et al. have determined the primary structure of cytochrome c-550 and indicated that the cytochrome c is categorized as being in the cytochrome c2 group (22). Although the involvement of cytochrome c2 in the electron transfer pathway for NO reduction has been proposed in some photosynthetic denitrifying bacteria (3, 16), no one has reported that the purified NO reductase shows cytochrome c-NO reductase activity.

In the present study, we monitored the purification of NO reductase with an assay system using ascorbate–N,N,N’,N’-tetramethylphenylenediamine–ferrocytochrome c-550. It is important to use cytochrome c-550 purified from the bacterium as an electron donor for the enzyme assay. The maximum velocity of the cytochrome c-NO reductase activity of the enzyme is about 84 μmol of NO per min/mg of protein in the absence of phospholipids, which is about 1.4-fold and 8.4-fold relative to that of the P. stutzeri enzyme and the P. denitrificans ATCC 19367 enzyme (9, 17), respectively. The Km value of the purified enzyme for NO is about 0.25 μM, while that of the P. stutzeri enzyme for NO has been reported to be about 60 μM (14). The high affinity of the P. denitrificans ATCC 35512 enzyme for NO is meaningful in terms of not only smooth linkage through successive reactions in the denitrification process but also protection against the cytotoxicity of NO.

P. stutzeri NO reductase has been reported to have no O2-reducing activity (14). However, surprisingly, P. denitrificans ATCC 35512 NO reductase can utilize oxygen as an alternative electron acceptor when bacterial ferrocytochrome c-550 is the electron donor. The Vmax of cytochrome c oxidase activity is 8.4 μmol of O2 per min/mg of protein under air-saturated conditions. The calculated turnover is about 27 mol of ferrocytochrome c-550 per mol of enzyme s⁻¹. The absence of heme a in the enzyme preparation and the high Km and Kc of the
enzyme for O₂ and for KCN show that the aa₃-type cytochrome c oxidase eluted at a higher concentration of NaCl on the first ion-exchange chromatography is not contaminated in the preparation of NO reductase. Therefore, it is concluded that the cytochrome c oxidase activity of the NO reductase is an innate enzymatic feature, although the activity may have no physiological functions even under aerobic conditions because of the high Kₘ for O₂.

Recently, cytochrome c oxidases with heme b, heme c, and copper have been purified from Rhodobacter sphaeroides (10), R. capsulatus (13), and Magnetospirillum magnetotacticum (26). These enzymes are induced under microaerobic conditions and function as respiratory terminal oxidases. Furthermore, the recent progress in determining the structure of P. stutzeri NO reductase shows significant homology between NO reductase and the bc₂-type cytochrome c oxidase described above (23, 27). In the present study, we found that although P. denitrificans ATCC 25512 NO reductase contains high-spin heme b, low-spin heme b, and heme c and shows relatively high cytochrome c oxidase activity, as well as NO reductase activity, the enzyme molecule has no copper. Therefore, it seems likely that the bc₂-type cytochrome c oxidase in the aerobic respiratory chain evolved from an ancestral terminal respiratory enzyme as NO reductase in the anaerobic respiratory chain, accompanying incorporation of copper.

ACKNOWLEDGMENT

This work was supported by a grant-in-aid for scientific research C (06680580) to Y. F. from the Ministry of Education, Science and Culture of Japan.

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