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Nitric Oxide-Reductase Homologue That Contains a Copper Atom and Has Cytochrome *c*-Oxidase Activity from an Aerobic Phototrophic Bacterium *Roseobacter denitrificans*

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A cytochrome *cb*-type enzyme with cytochrome *c*-oxidase activity was purified from an aerobic phototrophic bacterium *Roseobacter denitrificans*. The enzyme was solubilized with sucrose monodecanoate from the membranes of *R. denitrificans* grown aerobically under light conditions, and purified to electrophoretic homogeneity. Absorption spectra of the purified enzyme showed peaks at 410 nm and 530 nm in the oxidized state, and peaks at 420, 522, and 551 nm and a shoulder at around 560 nm in the reduced state. The enzyme is composed of two subunits with apparent molecular weights on SDS-PAGE of 37,000 and 18,000, the latter positive to heme staining. The protein contains heme *c*, heme *b*, and copper in a 1:2:1 stoichiometry. The spectral properties indicated that the heme *c* and one heme *b* are in low-spin states, while the other heme *b* is in a high-spin state. The base sequences of the genes and the deduced amino acid sequences are similar to those of known NorB and NorC subunits of nitric oxide reductases from other bacterial species. The enzyme is similar to nitric oxide reductase, but differs in that it contains copper. Virtually no nitric oxide reductase activity was detected in the purified enzyme.

Key words: cytochrome *c* oxidase, denitrification, nitric oxide reductase, respiration, *Roseobacter denitrificans*.

Roseobacter denitrificans (*Erythrobacter* sp. Och114 (*1*)) is a marine aerobic phototrophic bacterium that contains bacteriochlorophyll *a*. It synthesizes a complete photosynthetic apparatus when grown under dark aerobic conditions, but the synthesis is suppressed when grown under light conditions (*2, 3*). This bacterium does not grow anaerobically unless the culture medium contains alternative electron acceptors, such as trimethylamine *N*-oxide or nitrate (*4, 5*).

Aerobic anoxygenic phototrophic bacteria do not form a phylogenetically distinct group. They are distributed rather widely within the class *Proteobacteria* together with facultative phototrophic bacteria and non-photosynthetic heterotrophic bacteria (*6, 7*). Aerobic anoxygenic phototrophs are thought to represent a transient state in the evolution from anaerobic phototrophs to aerobic heterotrophs (*6, 8*). *Roseobacter* is closely related to facultative phototrophs *Rhodobacter* and aerobic heterotrophs *Paracoccus* (*7, 9*). The respiratory electron transfer system of *R. denitrificans* has not been studied intensively. Recently, Candela *et al.* (*10*) reported the results of a kinetic and potentiometric study using membrane fragments of *R. denitrificans*. A characteristic feature of this bacterium is the absence of quinol oxidase, while the presence of aa_3-type cytochrome *c* oxidase was suggested (*10*).

Cytochrome *c* oxidase is a membrane-bound complex containing heme and copper that reduces oxygen to water as the terminal enzyme in aerobic respiration. An iron in a high-spin heme and an adjacent copper atom, called Cu_B (*11, 12*), form the binuclear center. These two metals are bound to a major catalytic subunit through conserved histidine residues. The redox reaction of the oxidase is coupled to the translocation of protons across the bacterial cell membrane or the mitochondrial inner membrane (*11, 13*). The most commonly distributed and well-studied member of the heme-copper oxidase family is aa_3-type cytochrome *c* oxidase, which contains two heme *a* and three copper atoms (one of them is Cu_B). The two heme *a* and Cu_B are bound to the catalytic subunit, Cox1. The remaining two copper atoms (Cu_A) are associated with a distinct subunit, Cox2, and receive electrons from cytochrome *c*. Depending on the organism, the enzyme contains additional subunits, the functions of which are not well understood.

Cytochrome cbb_3-type oxidase is considered to be the most distant member of the family (*14*). This enzyme shares some structural features with nitric oxide reductase. It was first identified in *Bradyrhizobium japonicum* as the protein complex derived from the *fixNOQP* gene cluster (*15–17*). This enzyme consists of three major subunits, FixN, FixO, and FixP (also called CcoN, CcoO, and CcoP depending on the bacterial species). FixN (CcoN) is a catalytic subunit and contains a low-spin heme *b*, a high-spin

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heme *b*, and one Cu_B. FixO (CcoO) and FixP (CcoP) are membrane bound *c*-type cytochromes, containing one and two heme *c*, respectively.

Nitric oxide reductase is a membrane-bound cytochrome *bc* complex, and is one of the terminal enzymes in denitrifying respiration. It has been purified from several bacterial species as a complex of NorB and NorC subunits (*18, 19*). NorB contains a low-spin heme *b*, a high-spin heme *b*, and one non-heme iron (*18, 19*). The latter two are components of a binuclear center in which nitric oxide (NO) is reduced to nitrous oxide (N_2O), using electrons derived from soluble cytochrome *c*. NorC is a membrane-bound cytochrome *c* subunit containing one heme *c*. The apparent molecular masses of NorB and NorC on SDS-PAGE are typically 38 and 18 kDa, respectively (*18*). Sequence data have shown that NorB is homologous to the catalytic subuint of heme-copper oxidases (*13, 20*). Nitric oxide reductases lacking the NorC subunit have been purified from two bacterial species. The one from *Ralstonia eutropha* is a single-component enzyme and accepts electrons from quinols (*21, 22*). In a gram-positive bacterium, *Bacillus azotoformans*, NorC is replaced by a Cu_A-containing subunit (*23*).

In the present paper, we report a NorBC homologue from *R. denitrificans*. It was found during the purification processes of cytochrome c oxidase as a copper-containing complex with cytochrome *c* oxidase activity. Its primary structure is quite similar to that of nitric oxide reductase, but the catalytic activity to reduce nitric oxide is too low to act as an enzyme. It is thought to be a novel member of the cytochrome *c* oxidase superfamily.

MATERIALS AND METHODS

Organism and Cultivation—*R. denitrificans* was cultured at 28°C under aerobic light conditions in the medium described previously (*4*). The bacteria were cultured under light conditions in order to suppress the synthesis of the photosynthetic apparatus and accompanying pigments (*1, 3*) because the pigments interfere with the purification procedure. The cells were harvested by centrifugation at 8,000 ×*g* for 20 min. About 2.9 g of cells was obtained from 1 liter of medium. For measurements of O_2 uptake by intact cells, the bacterium was cultured aerobically in the dark. The cells were harvested, washed once with and resuspended in 50 mM Tricine-NaOH (pH 8.0) containing 300 mM NaCl and 25 mM $MgCl_2$. The concentration of bacteriochlorophyll in the cell suspension was determined from the absorbance at 770 nm of an acetone–methanol (7:2, v/v) extract using a millimolar absorption coefficient of 75 mM^{-1} cm^{-1} (*24*).

Preparation of Membrane Fraction—Cells (about 55 g, wet weight) were suspended in 50 mM Tris-HCl buffer (pH 9.0) containing 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and disrupted through a French Pressure cell. The resulting extract was centrifuged at 12,000 ×*g* for 30 min to remove unbroken cells. The supernatant was centrifuged at 310,000 ×*g* for 2 h, and the precipitate was washed once with the same buffer and centrifuged again. The pellet (membrane fraction) obtained was used as the starting material for purification.

Purification Procedure—The membrane fraction was suspended in 50 mM Tris-HCl buffer containing 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and solid sucrose monodecanoate was added to a final concentration of 1% (w/v). The solution was stirred at 4°C for 1 h and then centrifuged at 370,000 ×*g* for 3 h. The precipitate was suspended in the same buffer containing 1% sucrose monodecanoate and centrifuged again. The supernatants obtained in the first and second centrifugations were combined and applied to anion-exchange chromatography on a DEAE-Sepharose CL-6B column (3.5 × 5.0 cm) that had been equilibrated with 50 mM Tris-HCl buffer (pH 9.0) containing 0.3% (w/v) sucrose monodecanoate. The column was washed with the same buffer containing 0.18 M NaCl, and proteins were eluted with 400 ml of a linear salt gradient from 0.18 to 0.35 M NaCl. The fractions containing cytochrome *c* oxidase activity were pooled and concentrated through DIAFLO YM-10 (43 mm diameter) and YM-30 (25 mm diameter) membranes (Amicon, Ireland) to about 1 ml. The preparation was applied to gel-filtration on a Sephacryl S-300 column (2.5 × 95 cm) that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 0.3% (w/v) sucrose monodecanoate. Cytochrome *c* oxidase activity was eluted with the same buffer and the eluates with activity were pooled. Finally, the preparation was applied to anion-exchange chromatography on a Mono Q HR5/5 column (1 ml, Pharmacia Biotech). The column, which was adapted to a FPLC system (Pharmacia Biotech, model GP-250 PLUS), had been equilibrated with 50 mM Tris-HCl (pH 7.0) containing 0.5% (w/v) sucrose monodecanoate, and the column was washed with the same buffer containing 0.18 M NaCl. Cytochrome *c* oxidase activity was eluted by a linear salt gradient from 0.18 to 0.7 M NaCl. The eluates at around 0.32 and 0.5 M NaCl with cytochrome *c* oxidase activity were pooled as Fractions A and B, respectively. Fraction A is the purified preparation used in this study. Samples thus obtained were used immediately for enzymatic assay, and the remainder was frozen in liquid nitrogen and stored at –80°C for other analyses.

Cytochrome c Oxidase Activity—Cytochrome *c* oxidase activity was measured with reduced horse heart cytochrome *c* as an electron donor, by monitoring the absorption change at 550 nm using an UV-240 or an UV-2400 spectrophotometer (Shimadzu, Kyoto). The reaction started when the sample was added to a reaction mixture containing 50 mM Tris-HCl (pH 7.0), 0.3% (w/v) sucrose monodecanoate, and horse heart cytochrome *c*. The reaction progress was measured at 25°C. One unit (U) of enzyme activity is defined as the oxidation of 1 μmol of cytochrome *c* per min.

O_2-Uptake Measurement—O_2 uptake by *R. denitrificans* whole cells was measured using a Clark-type electrode. The reaction vessel of the electrode was filled with 50 mM Tris-NaOH (pH 8.0) containing 100 mM KCl and 10 mM sodium succinate. The reaction was started by adding bacterial cells.

Nitric Oxide Reductase Activity—Nitric oxide reductase activity was measured using a Clark-type Pt electrode polarized at +0.85 V (*25*). The reaction vessel was filled with 2 ml of 50 mM Tris-HCl (pH 7.5) containing 0.2 M D-glucose, 10 mM sodium ascorbate, 500 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 5 μM horse heart cytochrome *c*, 200 U of D-glucose oxidase, and 100 U of catalase. To ensure oxygen–free conditions, the mixture was incubated for at least 5 min. Five microliters of NO-saturated ethanol was added to the reaction mixture to yield a final concentration of 30 μM [the concentration of NO in the NO-saturated ethanol is considered to be 11.9 mM (*26*)]. The re-

action was started by adding the sample. Nitric oxide reductase activities were calculated from the rate of the initial NO disappearance and corrected for nonenzymatic NO consumption measured with 50 mM Tris-HCl (pH 7.0) containing 0.3% sucrose monodecanoate instead of sample. The reaction was measured at 25°C. One unit of enzyme activity is defined as the consumption of 1 μmol of NO per min.

Spectroscopic Analysis—Absorption spectra of the purified enzyme were measured with an UV-2400 spectrophotometer (Shimadzu). The CO difference spectrum was obtained by the following procedure. Purified enzyme was diluted in 50 mM Tris-HCl buffer (pH 7.0) containing 0.3% (w/v) sucrose monodecanoate, reduced with sodium dithionite, and the absorption spectrum was recorded. CO gas was slowly bubbled through the sample for several minutes and a small amount of sodium dithionite was added again and the spectrum was recorded. The CO-reduced *minus* reduced difference spectrum was obtained by subtraction of the two spectra.

HPLC Heme Analysis—Non-covalently bound heme was extracted from the purified enzyme, horse muscle myoglobin (Nacalai, Kyoto), and the membrane fraction of *Escherichia coli* DH5α, according to a published procedure (*27*). The hemes were dissolved in a small volume of ethanol/acetic acid/water (70:17:13, v/v). The same solvent was used as the mobile phase for a C_{18} reverse phase column (Shinwa, Kyoto, 4.6 × 150 mm) and the hemes were eluted at a flow rate of 0.1 ml/min. The running temperature was 30°C. Absorbance was monitored at 402 nm.

Physical Measurement—Heme *b* and heme *c* contents of the purified enzyme were estimated by the formation of pyridine hemochromes, according to the procedure of Berry and Trumpower (*28*).

The contents of iron and copper in the enzyme preparations were measured using an inductively coupled plasma mass spectrometer (ICP-MS) (Yokogawa Electric, model PMS 2000, Tokyo) at the Center of Advanced Instrumental Analysis, Kyushu University. The sample was diluted in 0.1 N HNO_3 for ICP-MS.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*29*). The concentration of acrylamide was 12.0% and the proteins were treated with 2% SDS and 5% 2-mercaptoethanol at room temperature for 60 min before being loaded onto gels. Native polyacrylamide gel electrophoresis (PAGE) in the presence of 0.3% sucrose monodecanoate was carried out according to Davis (*30*). The concentration of acrylamide was 7.0%. The gels were stained with Coomassie Brilliant Blue for protein, and covalently bound heme was stained as described previously (*31*).

Protein concentrations were determined with a BCA Protein Assay Reagent (Pierce Chemical, Rockford, Ill.).

Amino Acid Sequence—Amino acid sequence analysis was performed on an Applied Biosystems model 477A sequencer using the manufacturer's program and chemicals. Polypeptides that had been separated by SDS-PAGE were transferred by electroblotting from the gel to a polyvinylidene difluoride (PVDF) membrane. The blotting buffer was 25 mM Tris/192 mM glycine/20% methanol. The transferred peptides on the membrane were visualized with Coomassie Brilliant Blue and bands of interest were excised and subjected directly to N-terminal sequence analysis. Internal amino acid sequences of the polypeptides of the purified enzyme were determined by a method involving the nonenzymatic fragmentation of proteins. After SDS-PAGE, the gel was stained with 0.3 M $ZnCl_2$ (adjusted to pH less than 6.0 with HCl) as described (*32, 33*), and negatively stained protein bands were excised. The gel slices were cut into small pieces and rinsed three times (10 min each) with 0.25 M EDTA/0.25 M Tris-HCl (pH 9.0). After destaining, the gel pieces were incubated for 10 min in the electrophoresis buffer used for SDS-PAGE. Polypeptides in the gel pieces were eluted electrophoretically into Centricon YM-10 (Amicon) using a Micro-electroelutor (Amicon) according to the manufacturer's procedure. The proteins obtained in the Centricon were concentrated to less than 100 μl by centrifugation and formic acid was added to a final concentration of 70% after removal to an eppendorf tube. Then, 1/10 volume of CNBr solution (0.18–0.6 g CNBr per ml of 70% formic acid) was added and N_2 gas was blown for 1 min. The mixture was incubated at 40°C for about 24 h in the dark, and diluted with about 10 volume of distilled water. Polypeptides thus cleaved at methionine residues (*34*) were separated by Tris-tricine SDS-PAGE (*35*) or on a reverse phase column (C_{18}; Waters, 2.0 × 150 mm, or C_8; Waters, 2.1 × 150 mm). Peptides separated by Tris-tricine SDS-PAGE were transferred from the gel to a PVDF membrane and applied to the amino acid sequencer as described above. The reverse phase column had been equilibrated with 0.06% trifluoroacetic acid, and 0.052% trifluoroacetic acid/80% methanol solution was used for elution. Peptides detected at 210 nm at a flow rate of 0.2 ml/min were applied directly to the amino acid sequencer.

DNA Sequence Determination—Total genomic DNA was prepared by the published method (*36, 37*). A set of primers was designed for PCR targeting the coding regions of the determined amino acid sequences. Primer A; 5′-AA(TC)AT(TCA)TT(TC)TA(TC)GG(TCAG)GG for NIFYGG (amino acid 12–17) in the smaller polypeptide as a mix of sense primers. Primer B; 5′-TAGAT(GA)AA(GC)AG(GATC)A(GG)(GATC)GC for ALLFXX (amino acid 430–435) in the larger polypeptide as a mix of antisense primers. The underlined residues were designed based on the corresponding region of the *norB* gene of *Paracoccus denitrificans* (accession number U28078) and *Rhodobacter sphaeroides* (AF000233), coding ALLFIY. PCR was performed using extracted genomic DNA as a template, and the product (1,731 bp) was cloned into pGEM-T Easy vector (Promega). T7 and SP6 primers (Promega) were used for sequencing, and the internal region of the DNA fragment was sequenced using another synthetic primer. DNA sequencing was performed on an automated DNA sequencer (Applied Biosystems model 3100) according to the manufacturer's protocol. The sequence data were analyzed with the Genetyx software package.

RESULTS

Purification of the Cytochrome cb-Type Enzyme—The enzyme with cytochrome *c* oxidase activity was purified from aerobically cultivated *R. denitrificans* by DEAE-Sepharose CL-6B anion-exchange chromatography, Sephacryl S-300 gel filtration, and Mono Q anion-exchange chromatography. A typical elution profile of the Mono Q anion-exchange chromatography is shown in Fig. 1. A small peak of cyto-

chrome *c* oxidase activity was found at around 0.32 M NaCl while the bulk of the activity was eluted at around 0.5 M NaCl. The samples containing the activity were pooled separately as Fractions A and B, respectively, and examined by electrophoresis. The Fraction A preparation showed a single band on native PAGE with Coomassie Brilliant Blue and heme staining (Fig. 2). SDS-PAGE of the same preparation revealed two bands with apparent molecular masses of 37 and 18 kDa (Fig. 3, lane 1). The latter was also visualized by heme staining (lane 2), indicating that the smaller polypeptide has a covalently-bound heme *c*. Since the 37-kDa band disappeared when the loading sample was heated, the samples were incubated for 60 min at room temperature in the presence of SDS and 2-mercaptoethanol. The Fraction B preparation revealed several bands on native PAGE by protein staining (data not shown), indicating that this preparation was not homogeneous. After SDS-PAGE, protein staining of the gel revealed at least six bands (Fig. 3, lane 3) and heme staining showed two clear bands and one weak band (lane 4). The bands shown in lanes 1 and 2 were also visualized in lanes 3 and 4, respectively. This implies that the same protein complex was included in both Fractions A and B. In addition, Fraction B probably contained other species of cytochrome *c* oxidase. The reduced *minus* oxidized absorption spectrum showed peaks at 422, 444, 522, 550, and 606 nm and a shoulder at around 560 nm (data not shown). The peak at 606 nm suggests that the fractions contained heme-*a*–associating oxidase. On the other hand, N-terminal sequence analysis suggested that the fractions also contained a cbb_3-type oxidase, as described later. These oxidases were not separated during the whole purification procedure. In gel filtration, the cytochrome *c* oxidase activity eluted as a broad single band with an apparent molecular mass ranging from 60 to 180 kDa.

A typical result of purification is summarized on Table I. The specific activities of the two fractions were 1.2 and 9.2

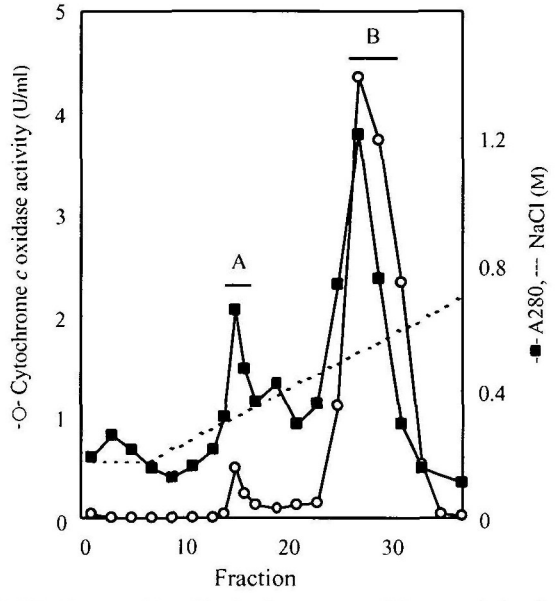

Fig. 1. **Elution profile of cytochrome *c* oxidase activity from a Mono Q column.** The column was equilibrated with 50 mM Tris-HCl (pH 7.0) containing 0.5% (w/v) sucrose monodecanoate. Horizontal lines labelled A and B show the fractions collected as Fractions A and B, respectively. Fraction A is considered to be the purified preparation in this study. Open circles, cytochrome *c* oxidase activity; filled squares, A_{280}; dashed line, NaCl concentration (M).

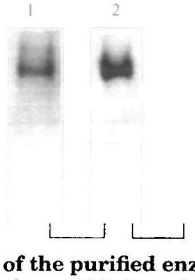

Fig. 2. **Native PAGE of the purified enzyme.** The polyacrylamide concentration in the gel was 7.0%. Lane 1, Coomassie Brilliant Blue staining (1.5 μg of protein); lane 2, heme staining (3.8 μg of protein).

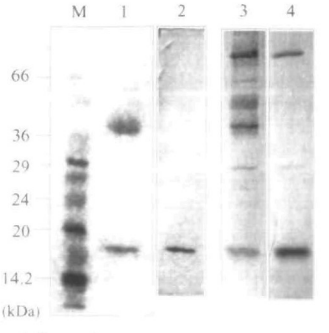

Fig. 3. **SDS-PAGE of protein(s) from Fractions A and B as shown in Fig. 1.** The polyacrylamide concentration in the gel was 12.0%. Purified enzyme (2.6 μg) from Fraction A was loaded into lanes 1 and 2; proteins (3.4 μg) from Fraction B were loaded into lanes 3 and 4. Standard proteins were loaded into lane M. Lanes M, 1, and 3: Coomassie Brilliant Blue staining; lanes 2 and 4: heme staining. Proteins were treated previously with 2% SDS and 5% 2-mercaptoethanol at room temperature for 1 h.

TABLE I. **Purification of the cytochrome *cb*-type enzyme.**

Step	Total protein (mg)	Cytochrome *c* oxidase		Nitric oxide reductase	
		Total activity (U)[a]	Specific activity (U/mg)	Total activity ($\times 10^{-3}$ U)[b]	Specific activity ($\times 10^{-3}$ U/mg)
Membrane fraction	1,902	351.6	0.18	7,188	3.8
Solubilized fraction	550	593.2	1.08	5,455	9.9
DEAE-Sepharose CL-6B	58.2	138.9	2.38	200	3.4
Sephacryl S-300	13.9	65.5	4.72	127	9.1
Mono Q					
Fraction A	1.29	1.53	1.2	10	7.6
Fraction B	3.24	29.9	9.2	24	7.3

[a,b]Enzymatic activities were measured as described in "MATERIALS AND METHODS."

(U/mg), respectively. Fraction B revealed 3.5–7.5 times higher specific activity than Fraction A, depending on the preparation. The specific activity of Fraction A was even lower than that after gel filtration. One probable reason of this is that the complex purified in Fraction A has a lower specific activity than other type(s) of cytochrome *c* oxidase contained in Fraction B.

Spectroscopic Analysis—Absorption spectra of the purified enzyme in the air-oxidized and dithionite-reduced states are shown in Fig. 4A. In Fig. 4B, the dithionite-reduced *minus* air-oxidized difference spectrum is shown. The enzyme shows absorption peaks at 410 and 530 nm in the oxidized form, and peaks at 420, 522, and 551 nm, and a shoulder at around 560 nm in the reduced form. The spectral features in the α-band region suggest that the enzyme contains both heme *c* and heme *b*. The dithionite-reduced + CO *minus* dithionite-reduced difference spectrum of the enzyme is shown in Fig. 4C. The spectrum shows peaks at 417, 537, and 570 nm and troughs at 433, 522, and 550 nm. The relatively high wavelength of the γ-trough (433 nm) and the profiles in the 500–600 nm region are characteristic of a CO-binding high-spin heme *b* (*38, 39*).

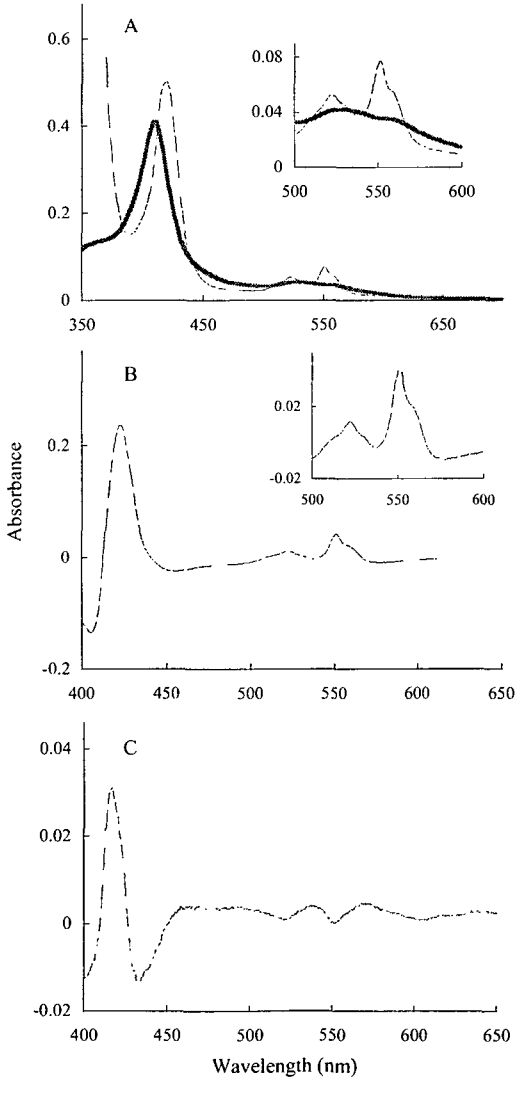

Fig. 4. **Absorption spectra of the purified enzyme.** Measurements were performed using 0.14 mg protein per ml. (A) Air oxidized (bold line) and dithionite reduced (normal line) forms. (B) Reduced *minus* oxidized difference spectrum. (C) CO-reduced *minus* reduced difference spectrum. The insets in A and B show enlarged spectra in the range from 500 to 600 nm.

Structural Properties—The non-covalently bound heme was extracted from the purified enzyme with HCl-acetone and applied to a C_{18} reverse-phase column. As references, heme *b* extracted from myoglobin and heme *b* and heme *o* extracted from *E. coli* membranes were also applied. The purified enzyme showed a single peak at a position corresponding to heme *b*. Heme *o* was not detected. The composition of heme in the enzyme was further analyzed by forming pyridine hemochrome. Determinations with four different preparations gave a mean value for heme *c*:heme *b* of 1:1.63 ± 0.20 (SD). Heme *a* was not detected.

The concentrations of iron and copper in the purified preparation were measured by ICP-MS. Combined with the results of pyridine hemochrome analysis, the ratio of heme *c*:heme *b*:Fe:Cu was calculated to be 1:1.63:3.00 ± 0.76 (SD):1.01 ± 0.40 (SD). The protein measured using the BCA protein assay reagent was 89.2 ± 17.9 (SD) kg/mol of heme *c*. The results indicate that the enzyme contains heme *c*, heme *b*, and Cu, in a stoichiometry of 1:2:1. The measured ratio of heme *b* to heme *c* was somewhat lower than 2, possibly indicating that part of the non-covalently bound heme *b* may have been lost during the purification steps. On the contrary, the Fe content was somewhat higher than the value expected from the heme content. The values for Fe content varied to some extent depending on the preparation, while the values for Cu per heme *c* were reproducible. One possible reason for this is that the determination of Fe with ICP-MS is less accurate due to the high background caused by ArOH, which is inevitably included in water.

DNA and Amino Acid Sequences—N-terminal and internal amino acid sequences of two subunits of the purified enzyme were determined directly by an amino acid sequencer. Some of the sequences obtained showed homology to known sequences of nitric oxide reductases. Therefore, the alignments of the *norC* and *norB* genes of nitric oxide reductase were referred to design the set of primers for PCR to obtain a DNA fragment coding the purified enzyme. The obtained sequence coding the two subunits (1,731 bp) is available with the derived amino acid sequences (accession number AB078896). The amino acid sequences contain regions that agree completely with the directly determined sequences (Fig. 5). The smaller subunit was composed of 150 amino acids with a molecular mass, including heme *c*, calculated to be 17.6 kDa. A sequence specific to heme-*c* attachment (CXXCH) was found in the translated sequence (Fig. 5). For the larger subunit, 434 amino acids were deduced from the nucleotide sequence and the molecular mass was calculated to be 49.0 kDa. The C-terminal sequence of this protein has not yet been determined. The obtained amino acid sequences are shown aligned in Fig. 5 with those of the NorC, NorB, CcoO, and CcoN proteins from *P. denitrificans* and *R. sphaeroides*. The sequences of the smaller subunit and the larger subunit are 68.7 and 78.6% identical to those of NorC and NorB, respectively, from *P. denitrificans*. The purified enzyme is thus closely related to the NorBC complex of nitric oxide reductase. On the other hand, the smaller subunit and the larger subunit are only 20.4 and 21.5% identical to CcoO and CcoN, re-

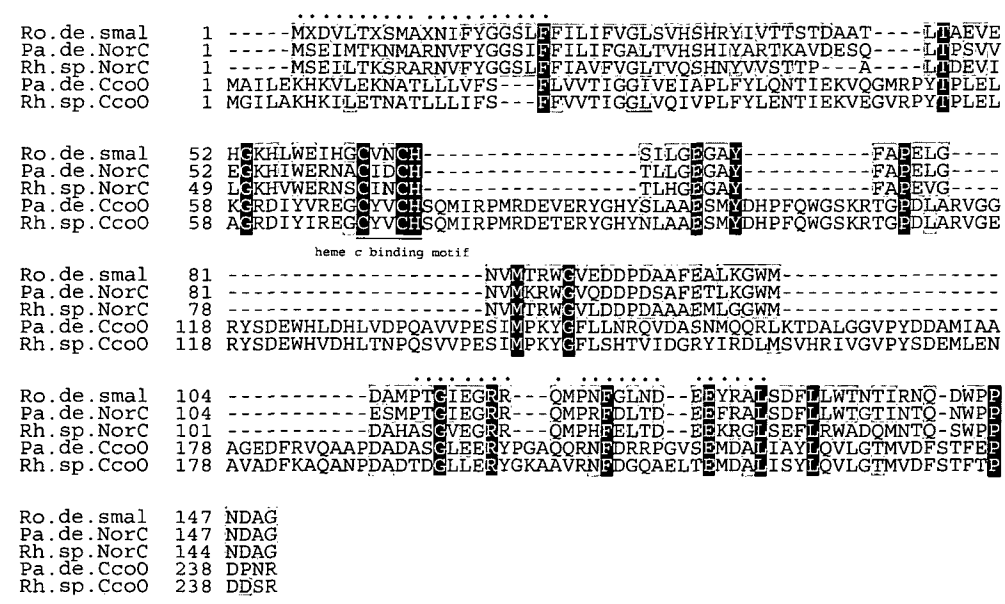

Fig. 5. **Amino acid sequence alignment of the purified enzyme with nitric oxide reductase and cbb_3-type oxidase.** (Ro. de. smal) and (Ro. de. larg) represent the smaller and larger subunits, respectively, of the purified enzyme from *R. denitrificans*. (Pa. de. NorC) and (Pa. de. NorB) indicate the NorC and NorB proteins, respectively, from *P. denitrificans* (accession number U28078). (Rh. sp. NorC) and (Rh. sp. NorB) indicate the NorC and NorB proteins, respectively, from *R. sphaeroides* (AF000233). (Pa. de. CcoO) and (Pa. de. CcoN) indicate the CcoO and CcoN proteins, respectively, from *P. denitrificans* (U34353). (Rh. sp. CcoO) and (Rh. sp. CcoN) indicate the CcoO and CcoN proteins, respectively, from *R. sphaeroides* (U58092). Conserved residues are boxed in black and residues identical to those of the smaller or larger subunits are boxed in gray. 'X' represents uncertain amino acids. Amino acids considered to ligate the hemes or a non-heme metal are indicated. Directly determined amino acids from the purified enzyme are indicated by dots.

spectively, from *P. denitrificans*.

The proteins contained in Fraction B from the Mono Q step were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue and the bands were excised and applied to an amino acid sequencer. We were able to determine the N-terminal sequences of two polypeptides appearing in the 18 and 28 kDa region on SDS-PAGE (Fig. 3, lane 3). The sequence of the 18-kDa polypeptide was XVLTXXMAXN, indicating that this polypeptide is identical to the smaller subunit of the purified enzyme. The 28-kDa polypeptide, which was also visualized by heme staining, had an N-terminal sequence of XILAKXXFIXXNATL. This is similar to that of the mono-heme subunit of cbb_3-type cytochrome *c* oxidase (MAILEKHKVLEKNATL in *P. denitrificans*), indicating that *R. denitrificans* has a cbb_3-type cytochrome *c* oxidase. It is presumed that the N-terminal sequences of the two polypeptides were originally "MXXVL" and "MXILA," respectively, and that the underlined residues were cut by an aminopeptidase present in the bacterium itself.

Nitric Oxide Reductase Activity—The purified enzyme with cytochrome *c* oxidase activity resembled the NorBC complex in subunit composition, in heme composition, and in primary structure. Since nitric oxide reductase from *P. denitrificans* has been reported to have cytochrome *c* oxidase activity (*40*), we measured nitric oxide reductase activities in the preparations at each step of purification (Table I). Most of the nitric oxide reductase activity in the membrane fraction was lost during purification. The residual activity in the purified enzyme preparation was very low, 7.6×10^{-3} U/mg, corresponding to about 0.5 mol NO consumed per mol of enzyme per min. We also investigated whether the enzyme catalyzed the oxidation by NO of reduced horse heart cytochrome *c* or cytochrome c_2 from *R. denitrificans* under anaerobic conditions, by monitoring the absorption change at 550 nm. The oxidation of neither horse heart cytochrome *c* nor *R. denitrificans* cytochrome c_2 was detected (data not shown). Girsch and de Vries (*25*) reported that high concentrations of NO inhibit the activity of the nitric oxide reductase from *P. denitrificans*. We did not observe similar phenomenon with the membrane fraction in the range of NO concentration up to 30 μM. Therefore, the low activity of the purified enzyme is not likely to be caused by the inhibition by NO.

Inhibition by Cyanide—*R. denitrificans* may have several types of enzymes with cytochrome *c* oxidase activity. In order to assess the contribution of oxidases to aerobic respiration, the sensitivities of Fraction A, Fraction B, and the membrane fraction to cyanide were compared (Fig. 6). The activities of the latter two were inhibited similarly by relatively low concentrations of KCN. On the other hand, the activity of the Fraction A preparation was less sensitive to cyanide. This suggests that the contribution of the NorBC-type enzyme to aerobic respiration is small. We also examined the effect of cyanide on oxygen uptake by intact cells. Candela *et al.* (*10*) reported that the oxidation of NADH by *R. denitrificans* membranes was inhibited almost completely by antimycin A or myxothiazole, indicating that the bacterium does not have a quinol oxidase. Thus the oxygen uptake depends predominantly on the activity of cytochrome *c* oxidase. The inhibition pattern by cyanide of oxygen uptake by intact cells in the presence of succinate differed from those of the oxidation of cytochrome *c* by the membrane fraction or by Fraction B. With 50 to 100 μM KCN, the cytochrome *c* oxidation was almost completely inhibited, while 100 μM KCN inhibited only 60% of the oxygen uptake by intact cells. This suggests that electrons may pass through the NorBC-type enzyme when other terminal oxidases are inhibited.

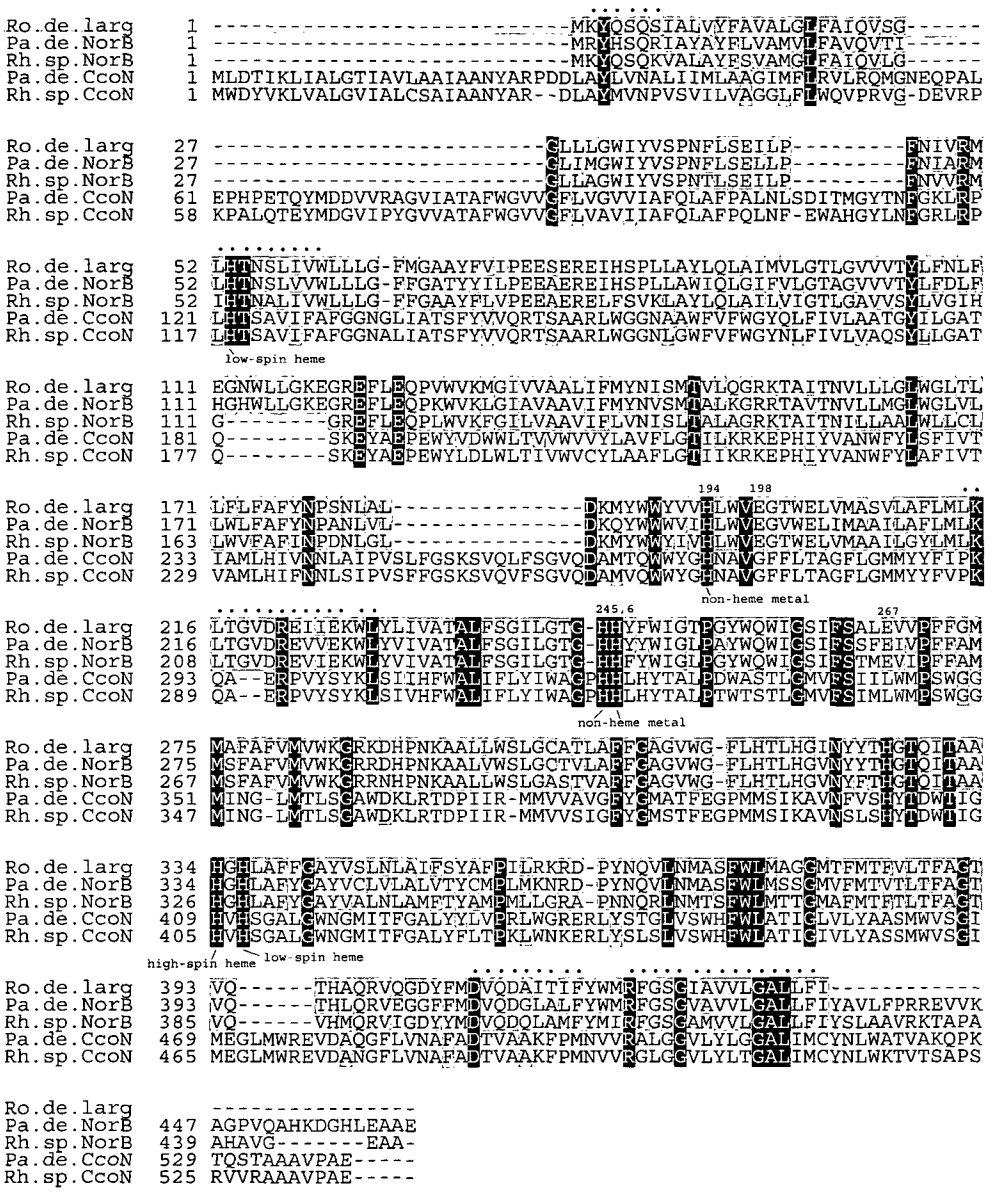

Fig. 5-2

DISCUSSION

We purified a cytochrome *cb*-type enzyme containing a copper atom to an electrophoretically homogeneous state. This enzyme was characterized as to subunit composition, heme and metal composition, primary structure, and enzymatic activity. Some details of our experiments and considerations of the results are described here.

The smaller subunit of the purified enzyme consists of 150 amino acids and the larger subunit comprises at least 434 amino acids. We assume that 20 to 30 amino acids remain to be determined at the C-terminus of the larger polypeptide based on comparison with known sequences of NorB. Therefore, this enzyme is believed to be composed of 600 to 610 amino acids with a molecular weight greater than 70,000. This value is comparable to the molecular weight of 89,000 calculated from the measured protein and heme concentrations assuming that one complex possesses one heme *c*. On the other hand, the sum of the apparent molecular weights of the two subunits on SDS-PAGE is 55,000. This discrepancy is probably due to an underestimation of the molecular mass of the larger subunit on SDS-PAGE because of its high hydrophobicity. A similar case was observed for NorB from *P. denitrificans* with a molecular mass of 52.5 kDa (*41*). This polypeptide has many hydrophobic regions and is detected in the 37–38 kDa region on SDS-PAGE (*42, 43*).

The purified enzyme has two heme *b* and one copper atom. The shoulder at around 560 nm in the absorption spectrum in the reduced state (Fig. 4A) suggests that the enzyme has a low-spin heme *b*. The second heme *b* should be high-spin type and forms a catalytic center together with the copper atom. The heme *b* and the copper atom should be associated with the larger subunit.

The larger polypeptide of the purified enzyme possesses the conserved-histidine residues, H53, H194, H245, H246,

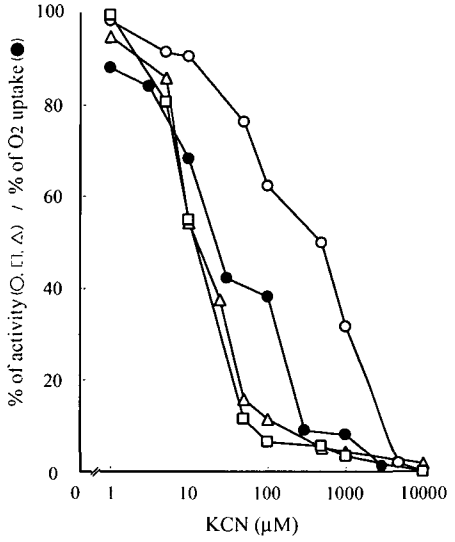

Fig. 6. **Inhibition by cyanide of cytochrome *c* oxidase activity and O_2 uptake by intact cells.** Open circles, cytochrome *c* oxidase activity of Fraction A; open squares, cytochrome *c* oxidase activity of Fraction B; open triangles, cytochrome *c* oxidase activity of the membrane fraction; closed circles, O_2 uptake by *R. denitrificans*; the concentration of bacteriochlorophyll in the reaction vessel was 5.9 μM.

H334, and H336 (Fig. 5), that are known to be required for binding two hemes and a non-heme metal. H194, H245, and H246 are believed to be the ligands for binding the non-heme metal, and the metal must be Cu for this polypeptide. However, the NorB protein, a homologue of the larger subunit, contains Fe at the catalytic center as a non-heme metal. Hendriks *et al.* (*18*) proposed that some additional ligands might create the specificity for Fe binding. As candidates they suggested E198 and E267, which are peculiar to NorB proteins and absent from all heme-copper cytochrome *c* oxidases. However, these glutamates are present in the larger subunit of the purified enzyme. A14, V72, G273, A397, and A411 in the larger subunit are also found in CcoN but are absent from NorB; however, these amino acids are probably not close to the Cu-binding site.

The physiological role of this enzyme is not clear. N-terminal sequence analysis of the 28-kDa polypeptide on SDS-PAGE (Fig. 3, lane 3) suggested that *R. denitrificans* has a cbb_3-type oxidase. The absorption spectrum of the Fraction B preparation suggests that the bacterium has a heme-*a* associating oxidase. Recently, Candela *et al.* (*10*) reported that the membranes of *R. denitrificans* contain two *a*-type cytochromes with midpoint redox potentials of +335 mV and +218 mV, indicating that the bacterium has an aa_3-type cytochrome *c* oxidase. The specific activity of the purified NorBC-type enzyme as cytochrome *c* oxidase is low. Although it is possible that some degree of inactivation occurred during the purification procedure, the enzyme by nature probably has low activity. Its cellular content appears to be high in the growth conditions of this study, because the 18-kDa band on SDS-PAGE was always stained deeply during purification. On the other hand, the difference in the sensitivity to cyanide between the purified enzyme and the membrane fraction indicates that its contribution to aerobic respiration is small.

R. denitrificans grows anaerobically in the presence of nitrate producing N_2O instead of N_2 (*5*). The bacterium appears not to have a nitrous oxide reductase. The activities of nitrate reductase and nitrite reductase have been demonstrated in cell-free extracts (*5*). In addition, two types of cytochrome cd_1 with nitrite reductase activity have been purified (*44*). Here we have demonstrated nitric oxide reductase activity in the membrane fraction, while the purified NorBC-type enzyme has virtually no nitric oxide reductase activity. It is possible that the bacterium has a distinct nitric oxide reductase with a non-heme iron in the catalytic center. The *bona fide* nitric oxide reductase and the copper-containing homologue would have similar primary structures. However, preliminary Southern blot analyses with a gene probe for the larger polypeptide of the purified enzyme always revealed only a single band. An alternative possibility is that the copper-containing NorBC-homologue functions as a nitric oxide reductase *in vivo*, and that the nitric oxide reductase activity is specifically inactivated during purification. Fujiwara and Fukumori (*40*) reported that the nitric oxide reductase from *P. denitrificans* ATCC 35512 has cytochrome *c* oxidase activity. Giuffrè *et al.* (*45*) showed that the heme-copper oxidase from *Thermus thermophilus* has nitric oxide reductase activity. Recently, the cbb_3-type oxidase from *Pseudomonas stutzeri* was also reported to have nitric oxide reductase activity (*46*). From these examples, it will be expected that the non-heme metal in the catalytic center does not necessarily determine exclusively whether the center reacts with O_2 or NO. However, the non-heme metal may determine with which substrates the enzyme reacts more efficiently (*45*). The enzyme we purified has the primary structure of a nitric oxide reductase and the catalytic center of a cytochrome *c* oxidase. It may not be a good catalyst of either reaction.

It is generally accepted that the heme-copper oxidase family evolved from a non-heme iron–containing nitric oxide reductase, although there is no conclusive evidence that the common ancestor had iron instead of copper in the catalytic center. Replacement of iron by copper would have been a critical event that made the complex adapt more to O_2 reduction than to NO reduction. The purified enzyme contains a copper atom, while the primary structure indicates that it is more closely related to nitric oxide reductases than to cytochrome oxidases. This suggests that the replacement of the non-heme metal in the catalytic center of nitric oxide reductase probably occurred a second time and made this peculiar enzyme in *R. denitrificans*. Its characterization will provide new information for understanding the structure-function relationship and evolution of terminal oxidases.

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