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Characterization of a Multi-Copper Enzyme, Nitrous Oxide Reductase, from *Rhodobacter sphaeroides* f. sp. *denitrificans*

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The nitrous oxide reductase from the photodenitrifier, *Rhodobacter sphaeroides* f. sp. *denitrificans*, has been purified. The enzyme is composed of two identical subunits of 66 kDa, and contains four copper atoms per subunit. Copper supplementation of the medium resulted in a 3.5-fold increase in the enzyme yield with doubly enhanced specific activity. The activity of the purified nitrous oxide reductase was completely inhibited by 100 μM zinc ions.

Key words: denitrification, multi-copper enzyme, nitrous oxide reductase, *Rhodobacter sphaeroides* f. sp. *denitrificans*.

Denitrification is an energy-acquiring process of denitrifying bacteria in which nitrate or nitrite is sequentially reduced to dinitrogen gas under anaerobic conditions by a multiple enzyme system, *i.e.*, nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (*1-3*). This nitrate respiration or dissimilatory nitrate and nitrite reduction terminates with the reduction of nitrous oxide to dinitrogen by nitrous oxide reductase (N_2OR, EC 1.7.99.6). The enzyme has so far been purified from a number of denitrifying and non-denitrifying bacteria, including a photosynthetic bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* (*4*), which was reported as the first photodenitrifying bacterium. Most of the enzymes were obtained as soluble homodimeric proteins having subunit molecular masses of 66-68 kDa and four copper atoms per subunit. Anaerobic purification was necessary for these enzymes to maintain high activity because of their instability in the presence of oxygen (*1-3*).

R. sphaeroides f. sp. *denitrificans* is a facultative photosynthetic bacterium which can grow by means of a variety of energy-generating systems, *i.e.* not only denitrification but also O_2 respiration, dimethyl sulfoxide respiration and photosynthesis (*4, 5*). Michalski *et al.* reported that *Rhodobacter* N_2OR isolated aerobically was a monomer with a molecular mass ranging from 73 kDa (SDS-PAGE) to 95 kDa (gel filtration chromatography) (*6*). The enzyme was also reported to have four copper, two zinc, and 0.76 nickel g atoms per mol of enzyme and, different from the enzymes of other origins, the aerobically purified enzyme still exhibited high activity. From this unique nature of *Rhodobacter* N_2OR together with the ambiguity concerning the actual metal requirements for its activity and molecular weight including the subunit system, further characterization and the effects of metal ions on the enzyme were examined.

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Abbreviations: BV, benzyl viologen; $BV^{+}\cdot$, benzyl viologen cation radical (half-reduced form); N_2OR, nitrous oxide reductase.

MATERIALS AND METHODS

Organism and Growth Conditions—A green mutant of *R. sphaeroides* f. sp. *denitrificans* IL 106 was grown photoheterotrophically in a mineral salt medium containing 0.3% sodium DL-malate and 20 mM potassium nitrate at 25°C for 30 h as reported previously (*6*).

Purification of Nitrous Oxide Reductase—Nitrous oxide reductase from *R. sphaeroides* f. sp. *denitrificans* was purified aerobically at 4°C essentially by the purification method previously reported (*6*). Some modifications were made to prevent the loss of activity.

After being harvested by centrifugation, the cells were resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing lysozyme (0.5 mg/ml), DNaseI, RNase (2.5 μg/ml each), 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl (0.2 mg/ml), and leupeptin (5 μg/ml), in the buffer volume to cell wet weight ratio of 3:1. The cells were disrupted by use of a French press at 100 MPa, and the crude extract was centrifuged at 20,000 × *g* for 30 min. To the supernatant fraction was added solid $(NH_4)_2SO_4$, and the proteins precipitated between 40 and 60% saturation were recovered by centrifugation at 20,000 × *g* for 30 min. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and then dialyzed overnight against the same buffer. The dialyzed fraction was clarified by centrifugation, and the resulting supernatant was loaded onto a DEAE Sepharose FF column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and eluted with a 0.15-0.4 M NaCl gradient in the same buffer (pH 7.5).

The N_2OR-containing fractions were pooled and concentrated with a YM30 membrane filter (Amicon), and then loaded onto a Superdex 200 p.g. column (Pharmacia) equilibrated with 0.25 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with the same buffer and the fractions containing N_2OR activity were pooled.

The pooled solution was concentrated by ultrafiltration and diluted with 50 mM Tris-HCl buffer (pH 7.5), and then loaded onto a Resource Q column (Pharmacia) equilibrated with the same buffer and eluted with a 0.15-0.35 M NaCl

gradient in 50 mM Tris-HCl buffer (pH 7.5). The fractions exhibiting maximal specific activity were pooled and concentrated by ultrafiltration.

Enzyme Assay—By means of Michalski's method (*6*), N_2OR activity was determined spectrophotometrically by monitoring the nitrous oxide-dependent rate of oxidation of the photochemically reduced benzyl viologen cation radical ($BV^{+}\cdot$) at 550 nm. The reaction mixture comprising 3 ml of 0.4 mM benzyl viologen (BV), 40 μM proflavin-HCl, and 10 mM triethanolamine in 40 mM Tris-HCl buffer (pH 9.0) was prepared in 3.5 ml cuvettes. The cuvettes, each fitted with a rubber stopper, were purged for 15 min with high-purity argon gas *via* syringe needles. The enzyme solution was then injected into the reaction mixture, and photoreduction of viologen was performed under irradiation with two 15 W fluorescent lamps for 7 min. For enzyme activation, the cuvettes were then allowed to stand at room temperature for 90 min. The viologen was then photoreduced again for 4 min and the low background autoxidation rate was measured for 15 s prior to initiating the enzyme reaction by injecting a saturated nitrous oxide solution (final concentration, 0.2 mM).

Analytical Methods—The protein concentration was estimated by the bicinchoninic acid method (*7*) using reagents from Pierce with bovine serum albumin as a standard. Metal analysis of the purified nitrous oxide reductase was performed by means of inductively coupled plasma atomic emission spectrometry with a SPS 1200 VR spectrometer (Seiko Instruments). SDS-PAGE were performed in 10.5% polyacrylamide slab gels by the method of Laemmli (*8*) with an electrophoresis calibration kit (Pharmacia) as markers, followed by staining for proteins with Coomassie Brilliant Blue R-250. HPLC gel filtration was carried out on a TSK gel G3000SW column (Tosoh), using 0.1 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) and a gel filtration calibration kit (Pharmacia) as molecular weight markers.

Determination of the molecular mass of the purified enzyme was performed with a Reflex-2 matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker). Data were acquired in the positive linear mode at 28.5 kV. A saturated solution of sinapinic acid in a mixture of 0.1% TFA and 30% acetonitrile was used as a matrix.

RESULTS AND DISCUSSION

Purification of Nitrous Oxide Reductase—Nitrous oxide reductase from *R. sphaeroides* f. sp. *denitrificans* was purified to homogeneity under aerobic conditions. Instead of the time-consuming purification in the previous study, which involved four chromatographic steps (*6*), more rapid purification steps were devised to prevent inactivation during the purification steps. For instance, a Resource Q column was used for the third chromatography instead of hydroxyapatite and the last gel filtration.

Metal Contents and N_2OR Activity—Since Michalski *et al.* reported that the enzyme contained 4 copper, 2 zinc, and 0.76 nickel atoms per enzyme, the actual requirements of these ions in relation to enzyme activity were studied. Four kinds of medium with different concentrations of these ions were used for cultivation, as shown in Table I. The metal ion concentrations in the normal medium were those of Michalski *et al.* (*6*). To determine the N_2OR activity of the cells from each culture, the yield of the enzyme per gram cell wet weight and the specific activity of the isolated enzyme from each medium were investigated, because the enzyme activity was very weak and variable in the crude extracts due to enzyme inhibitors or interference with the assay system.

Copper supplementation of the media resulted in a 2.6-fold increase in the copper content per enzyme and a 3.5-fold increase in the protein yield from the same amount of cells with 2-fold enhancement of the specific activity, indicating a 7-fold increase in unit activity (Table I). These effects were not observed with zinc and nickel supplementation. Low levels of zinc and nickel in the enzyme were only observed when the copper supplied was insufficient. These results suggest that a relatively high concentration (around 1 μM) of copper ions in the medium is needed for the production of mature nitrous oxide reductase. The growth rate and maximum density of the photodenitrifying cells were not affected by the copper concentration, from 0.05 to 1.0 μM, in the medium (data not shown).

When the strain was cultured in the normal medium (Michalski's medium), three metal ion species were detected in the enzyme, although their contents were fairly low compared to those reported by Michalski *et al.* (*6*). However, in both cases, *i.e.* supplementation with copper alone and combined with the two other metal species, no zinc or nickel was found in the protein. The finding that only copper was incorporated into the enzyme in the medium supplemented with the three metal species indicates that copper is the most favored for the active site of N_2OR. The enhancement of the activity in the absence of zinc and nickel indicates that copper is the sole metal species responsible for the activity.

All well studied N_2ORs, *i.e.* the enzymes from *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrificans*, contain 8 copper atoms per enzyme (*3*). In *Rhodobacter* N_2OR, the copper content of the enzyme from

TABLE I. **Effects of metal ions in the medium on nitrous oxide reductase of *R. sphaeroides* f. sp. *denitrificans*.** Enzyme activity is expressed in μmol nitrous oxide reduced per min.

Medium	Ion concentration added to the medium (μM)			Enzyme yield per gram cell wet weight		Specific activity (U/mg)	Metal content (atoms/enzyme)		
	Cu^{2+}	Zn^{2+}	Ni^{2+}	Total activity (U)	Protein (μg)		Cu^{2+}	Zn^{2+}	Ni^{2+}
Normal medium	0.17	0.83	0.00	1.0	45	22	2.7	0.5	0.1
Cu^{2+}-supplemented medium	0.83	0.83	0.00	7.1	161	44	7.0	0.0	0.0
Cu^{2+}, Zn^{2+}, Ni^{2+}-supplemented medium	0.83	4.20	0.83	6.8	155	44	6.9	0.0	0.0
Zn^{2+}, Ni^{2+}-supplemented medium	0.17	4.20	0.83	1.0	49	20	2.6	0.4	0.1
Michalski *et al.* (*6*)	0.17	0.83	0.00	1.4	56	25	4.0	2.0	0.8

cells grown in copper-supplemented medium was 7.0 g atoms per mol of enzyme, indicating *Rhodobacter* N_2OR in the mature form contains 8 copper g atoms per mol of enzyme, *i.e.*, 4 atoms of copper per subunit as the prosthetic group. The shortage of copper may be due to loss during the purification.

Molecular Mass of Nitrous Oxide Reductase—The molecular characteristics of *Rhodobacter* N_2OR are compared with those of Michalski's and the *P. stutzeri* enzymes in Table II. The enzyme used was that from the copper-supplemented medium. The molecular mass of the enzyme was estimated to be 105 kDa by HPLC gel filtration and 67 kDa by SDS-PAGE (Fig. 1). MALDI-TOF mass spectrometry of *Rhodobacter* N_2OR gave a molecular ion at m/z 66,180, which is close to the electrophoretic value. Different from Michalski's assumption that the enzyme was a monomer (*6*), these results together with the fact that there were no other bands except for the 67 kDa one on SDS-PAGE (Fig. 1) indicated that *Rhodobacter* N_2OR is composed of two identical subunits, just like the N_2ORs of other origins. The little smaller value obtained on gel filtration in either case in Table II might be due to the enzyme structure or another characteristic of N_2OR.

As to specific activity, the enzyme purified under aerobic conditions in the present work still exhibited a level of 44 μmol N_2O reduced per min per mg protein, which is higher than that of Michalski's enzyme. Moreover, the purified enzyme retained 80% of its activity after aerobic storage at 4°C for 1 month. In contrast, *Pseudomonas* or *Paracoccus* N_2OR loses its activity almost completely under aerobic conditions (*1, 2*). The difference in stability as to oxygen between the two enzymes is of utmost importance for understanding the higher order structure of this enzyme.

Effects of Metal Ions on the Enzyme Activity—Table III summarizes the effects of various metal ions on the activity of the purified N_2OR, which contains 7 copper atoms per enzyme. Each metal ion or combination was added to the reaction mixture at the initial stage of the assay, and then the enzyme activity was estimated in the same way as described under "MATERIALS AND METHODS." The metal concentrations in this experiment were more than 10 to 100 times higher than those in the bacterial culture medium summarized in Table I.

The metal ions, copper, zinc, and nickel, caused decreases in activity at concentrations between 10 and 100 μM. Among them, zinc showed the highest inhibition (100% at 100 μM, and 60% at 10 μM), compared to that by 100 μM copper and nickel ions, which caused 60 and 50% reductions in activity, respectively.

To determine the relationship between the decrease in activity and the active site copper ions in the enzyme, a replacement experiment was conducted. The purified enzyme was dissolved in an oxygen-free reaction mixture containing 100 μM $CuSO_4$ or $ZnSO_4$, and then the cuvettes were allowed to stand at room temperature for 90 min. The resulting solutions were ultrafiltrated with a Centricon 30 (Amicon) four times to remove free metal ions completely.

TABLE II. **General properties of nitrous oxide reductases.**

	R. sphaeroides		*P. stutzeri*
	This work	Michalski *et al.* (*6*)	(*9, 10*)
Molecular weight on			
SDS-PAGE	67,000	73,000	74,000
DNA sequence	n.d.	n.d.	65,759
Mass spectrometry	66,180[a]	n.d.	65,800[b]
Gel filtration	105,000	95,000	118,000
Atoms per enzyme	7.0 Cu	4.0 Cu 2.0 Zn 0.76 Ni	7.3 Cu
Specific activity			
Anaerobic preparation	n.d.	n.d.	60
Aerobic preparation	44	25	1.8

[a]MALDI-TOF, [b]electrospray, n.d., not determined.

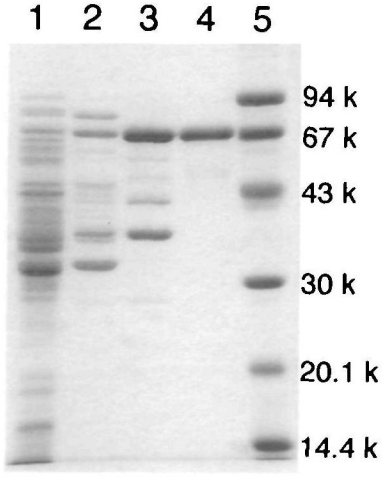

Fig. 1. SDS-PAGE of nitrous oxide reductase fractions obtained during purification. Lane 1, ammonium sulfate fraction; lane 2, DEAE Sepharose FF eluate; lane 3, Superdex 200 p.g. eluate; lane 4, purified enzyme; lane 5, standard molecular weight markers.

TABLE III. **Effects of metal ions on nitrous oxide reductase activity.** The enzyme concentration in the reaction mixture was 0.1 μM. Relative activity is expressed as a percentage of the control activity (without the addition of metal ions).

Metal ion[a]	Concentration (μM)	Relative activity (%)
Cu^{2+}	100	40
Cu^{2+}	10	80
Zn^{2+}	100	0
Zn^{2+}	10	40
Ni^{2+}	100	50
Ni^{2+}	10	90
Fe^{2+}	100	95
Mg^{2+}	100	100
Ca^{2+}	100	85
Zn^{2+} and Cu^{2+}	100 (each)	40
Zn^{2+} and Fe^{2+}	100 (each)	0
EDTA	100	0
Control		100

[a]*Metal ions were added as the sulfate form except for* $NiCl_2$ *and* $CaCl_2$.

TABLE IV. **Nitrous oxide reductase incubated with metal ions.** Relative activity is expressed as a percentage of the control activity.

Metal ion	*Relative activity* (%)	*Metal content (atoms/enzyme)* Cu	Zn
Cu^{2+}	100	7.1	0.0
Zn^{2+}	100	6.4	0.3
Cu^{2+} and Zn^{2+}	100	6.5	0.2
Control	100	6.5	0.0

The enzyme activity and metal contents of the obtained enzyme were assayed. As shown in Table IV the relative activity and copper content were not affected by the addition of copper and zinc. These results indicate that metal replacement does not occur in the oxidized form of the enzyme.

Another possibility for the decrease in activity could be in the process of the enzyme assay. In the present system, photo-reduced proflavin transfers electrons to the enzyme *via* the mediator, BV. On the addition of excess copper ions to the reaction solution, rapid decolorization of $BV^{+}\cdot$ occurred, indicating oxidation of $BV^{+}\cdot$. The inhibition of the enzyme activity by external copper ions could be ascribed to electron transfer from $BV^{+}\cdot$ to copper ions, not to the active site copper of the enzyme.

On the other hand, the addition of zinc did not result in decolorization of $BV^{+}\cdot$, indicating that the effect of zinc on the enzyme activity was not the same as that of copper ions. Although the detailed mechanism is unknown, the finding that $BV^{+}\cdot$ was maintained in a half-reduced state in the presence of excess zinc ions indicates that zinc ions could affect the later part of the electron transfer system, *i.e.*, $BV^{+}\cdot$ to N_2OR, or N_2OR to the substrate, N_2O.

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