J. Biochem. 125, 864-868 (1999)

# Anaerobic Purification and Characterization of Nitrous Oxide Reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106

**Kiichi Sato,[1] Akira Okubo, and Sunao Yamazaki**

*Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657*

Received December 9, 1998; accepted January 19, 1999

**The nitrous oxide reductase from the photodenitrifier, *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106, has been purified under anaerobic conditions. The specific activity of the enzyme was 78 μmol nitrous oxide reduced per min per mg protein, which was approximately 80% higher than that of the aerobic form. The enzyme purified anaerobically retained most of its activity after aerobic storage at 4°C for 2 months without any additives. Visible absorption spectra of the *Rhodobacter* nitrous oxide reductase resembled those of the enzymes from other origins. The enzyme retained its activity after reduction with sodium dithionite, and the enzyme activity could be determined using dithionite-reduced benzyl viologen. Turnover-dependent inactivation of the enzyme was suppressed by complete removal of oxygen from the reaction mixture, and promoted by zinc ions.**

**Key words: denitrification, multi-copper enzyme, $N_2O$ reductase, *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106.**

Denitrification is an energy-acquiring process of denitrifying bacteria, in which nitrogen oxyanions are sequentially reduced to molecular nitrogen under anaerobic conditions by a multiple enzyme system (*1, 2*). Nitrous oxide reductase ($N_2OR$, EC 1.7.99.6) catalyzes the final step of denitrification, *i.e.*, the reduction of nitrous oxide to nitrogen, in denitrifying bacteria. The enzyme has been purified from several bacteria under aerobic or anaerobic conditions so far (*1, 2*). Most of the purified 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 most enzymes to maintain high activity because of their instability in the presence of oxygen. Moreover, since the enzyme lost its activity on reduction with sodium dithionite, photoreduced viologen was used for the enzyme assay.

The first isolate of a photosynthetic denitrifying bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106, can grow depending on a variety of energy-generating systems, *i.e.*, not only denitrification but also $O_2$ respiration, dimethyl sulfoxide respiration and photosynthesis (*3*). $N_2OR$ was purified from *R. sphaeroides* f. sp. *denitrificans* IL106 aerobically (*4, 5*), and the aerobic form of *Rhodobacter* $N_2OR$ has molecular properties similar to those of the enzymes purified aerobically from other origins, except stability as to oxygen (*5*). While $N_2ORs$ from other origins lost most of the activity under aerobic conditions, *Rhodobacter* $N_2OR$ isolated aerobically exhibits relatively high activity and 80% of its activity was retained after aerobic storage at 4°C for 1 month (*5*). The anaerobic nature of this enzyme, however, has not been clear so far. In this paper we report the anaerobic purification of *R. sphaeroides* $N_2OR$ and its characteristics, *i.e.*, visible absorption spectra, the reaction with sodium dithionite and turnover-dependent inactivation of the enzyme.

## MATERIALS AND METHODS

*Organism and Growth Conditions*—A green mutant of *R. sphaeroides* f. sp. *denitrificans* IL106 was grown photoheterotrophically in a mineral salt medium comprising 0.3% sodium DL-malate, 20 mM potassium nitrate, and 1 μM $CuSO_4$ at 25°C for 30 h as reported previously (*5*). The cell yield from 20-liter medium was typically 80 g wet weight.

*Anaerobic Purification of Nitrous Oxide Reductase*—Nitrous oxide reductase from *R. sphaeroides* was purified anaerobically at 4°C, based essentially on the aerobic purification method described previously (*5*). Some modifications were made to prevent the loss of activity. Anaerobic purification was performed with degassed and $N_2$-saturated solutions in bags filled with $N_2$ gas.

Preparation of cell-free extracts and ammonium sulfate fractionation were performed as described previously (*5*). The precipitate obtained on ammonium sulfate fractionation was dissolved in 50 mM Tris-HCl buffer (pH 7.5), loaded onto a Q Sepharose FF column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and then eluted with a 0.15-0.4 M NaCl gradient in the same buffer (pH 7.5). Gel filtration chromatography on a Superdex 200 prep grade column (Pharmacia) and anion-exchange chromatography on a Resource Q column (6 ml, Pharmacia) were performed as described previously (*5*).

*Enzyme Assay*—By means of the previously described method (*5*), $N_2OR$ activity was routinely determined spectrophotometrically by monitoring the nitrous oxide-

[1] To whom correspondence should be addressed. Phone/Fax: +81-3-5802-3303

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

dependent rate of oxidation of the photochemically reduced benzyl viologen cation radical ($BV^{+}\cdot$) at 550 nm after anaerobic activation of the enzyme with $BV^{+}\cdot$ for 90 min, unless otherwise specified. Removal of oxygen from the reaction mixture was achieved by argon purging for 15 min.

In addition to the photoreduction assay described above, the enzyme activity was also estimated spectrophotometrically using dithionite-reduced $BV^{+}\cdot$. Reaction mixtures, comprising 3 ml of 0.4 mM benzyl viologen (BV) in 50 mM Tris-HCl buffer (pH 9.0), were prepared in 3.5 ml cuvettes fitted with a rubber stopper. The cuvettes were purged for 15 min with high-purity argon gas *via* syringe needles. After purging, BV was reduced to $BV^{+}\cdot$ by injection of 15 $\mu$l of 2% sodium dithionite in a 1% $NaHCO_3$ solution, and an aliquot of the enzyme solution (20 $\mu$l, typically) was then injected into each reaction mixture. For enzyme activation, the cuvettes were then allowed to stand at room temperature for 90 min. After the incubation, 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).

For complete removal of oxygen from the reaction mixture, an enzymatic deoxygenation system (*6*) was introduced to the photoreduction enzyme assay. The reaction mixture (*5*) containing 1% D-glucose was purged for 15 min with high-purity argon, and then 10 $\mu$l of a solution comprising glucose oxidase (10 mg/ml) and catalase (2 mg/ml) was injected to the reaction cuvette. After 1 min incubation, the $N_2OR$ solution was injected, followed by photoreduction, enzyme activation and the enzyme assay as described previously (*5*).

*Analytical Methods*—Protein concentrations were 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 was 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 an anaerobic solution of 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. Singly charged $(M+H)^{+}$ and doubly charged $(M+2H)^{2+}$ ions of BSA were used as standards to calibrate the mass spectrum ($m/z$ 66,431 and 33,216 respectively).

Visible spectra were obtained with a UV-3101 PC spectrometer (Shimadzu) using 1 cm path-length cuvettes at room temperature. The molar extinction coefficient of the purified enzyme was calculated for the dimeric enzyme.

## RESULTS AND DISCUSSION

*Anaerobic Purification of Nitrous Oxide Reductase from R. sphaeroides*—$N_2OR$ was purified to homogeneity from *R. sphaeroides* f. sp. *denitrificans* IL106 under anaerobic conditions. The dialysis of the ammonium sulfate fraction which is performed in the early stage of the aerobic purification procedure (*5*) was omitted, since enzyme inactivation and conversion to the aerobic form were particularly found in the crude extract. The fraction was dissolved in anaerobic Tris-HCl buffer and then directly applied to the anion-exchange column. The best results were obtained when the purification was performed as rapidly as possible (within 30 h from the French press step to the end of purification).

*Molecular Characteristics of $N_2OR$*—Table I summarizes several characteristics of the $N_2OR$ purified anaerobically from *R. sphaeroides* f. sp. *denitrificans* IL106. The enzyme was purified as a homodimer, and the estimated molecular mass of the subunit was 67 kDa on SDS-PAGE or 66,180 Da on MALDI-TOF, as was exactly the same as for the aerobic enzyme (*5*).

The mass spectra of the purified $N_2OR$ and the EDTA-treated enzyme are shown in Fig. 1. The purified $N_2OR$ exhibited a main peak at $m/z$ 66,180 with a shoulder at 66,010, while EDTA-treated $N_2OR$ exhibited a main peak at 66,010 with decreasing intensity at 66,180. The decrease in the heavier peak suggests removal of copper ions from the enzyme by EDTA, but the number of copper ions removed could not be determined because of the low resolution.

The native molecular mass was estimated to be 105 kDa by HPLC gel filtration, which agreed with that of the aerobic form.

The anaerobically purified enzyme contained 7.1 Cu

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

| | *R. sphaeroides* | *P. stutzeri* (*10*) | *P. denitrificans* (*9*) |
|---|---|---|---|
| Subunit molecular weight | 66,180[a] | 65,759[b] | 66,280[b] |
| Molecular weight determined by gel filtration | 105,000 | 118,000 | 143,000 |
| Atoms per enzyme | 7.1 Cu | 7.3 Cu | 7.5 Cu |
| Specific activity[c] | | | |
| Anaerobic form | 78 | 60 | 122 |
| Aerobic form | 44 | 1.8 | n.d. |
| Reduced form | 72 | 0 | 0 |
| Spectral bands | | | |
| Oxidized form | 549 nm | 540 nm | 550 nm |
| | 741 nm | 780 nm | 820 nm |
| Reduced form | 649 nm | 650 nm | 660 nm |

[a]Determined by MALDI-TOF; [b]estimated from the gene sequence; [c]$\mu$mol nitrous oxide reduced per min per mg protein; n.d., not determined.

atoms per dimeric enzyme as the prosthetic group, which was the same as for the aerobic form (*5*). No other elements were detected above background levels.

The specific activity of the anaerobic enzyme at 25°C was 78 $\mu$mol $N_2O$ reduced per min per mg protein, which was approximately 80% higher than the 44 $\mu$mol $min^{-1} \cdot mg^{-1}$ of the aerobically isolated enzyme (*5*). While this value was lower than that of *P. denitrificans* (*9*), it was similar to those of *Pseudomonas stutzeri* (*10*) and *Achromobacter cycloclastes* (*11*), and higher than those of *Pseudomonas aeruginosa* (*12*) and *Thiosphaera pantotropha* (*13*). The enzyme activity increased with increasing reaction temperature up to 60°C by a factor of 2.5, and most of the activity was lost at 70°C.

The anaerobically purified $N_2OR$ retained more than 80% of its activity after aerobic storage at 4°C for 2 months without any additives, while $N_2OR$ activity in the crude extract was completely lost in a week. The stability of the purified *Rhodobacter* $N_2OR$ was outstanding as compared with those of $N_2OR$s from other origins (*9, 10, 12*). The fact that the purified enzyme is more stable than the crude one under aerobic conditions implies the presence of either an inhibitor or something that reacts with the enzyme in the crude extract.

*Visible Absorption Spectra of Rhodobacter $N_2OR$*— Visible absorption spectra of the $N_2OR$ purified from *R. sphaeroides* are shown in Fig. 2. A purple form (Fig. 2a), from anaerobic preparations, exhibits principal absorption maxima at 549 nm ($\varepsilon$ 5,600) and 741 nm ($\varepsilon$ 3,000), with a slight shoulder at 630 nm. A pink form (Fig. 2c), from aerobic preparations, exhibits absorption maxima at 485 nm ($\varepsilon$ 3,000), 541 nm ($\varepsilon$ 3,100), 641 nm ($\varepsilon$ 2,800), and 758 nm ($\varepsilon$ 2,600). A faint blue form (Fig. 2e), which was obtained on the addition of an excess amount of sodium dithionite to the anaerobically purified enzyme, exhibits a principal absorption maximum at 649 nm ($\varepsilon$ 1,700) with a slight shoulder at 750 nm. In accordance with the terminology of Riester *et al.* (*14*), the anaerobic *Rhodobacter* $N_2OR$ is referred to as $N_2OR$ I, the aerobic form as $N_2OR$ II, and the dithionite-reduced form as $N_2OR$ III. These spectra resembled those of other $N_2OR$s, while *Rhodobacter* $N_2OR$ I exhibited stronger absorption around 740 nm than other $N_2OR$s. These bands are attributed to coordination to copper, although the detailed assignments are not clear at the moment.

The reduction of $N_2OR$ I to $N_2OR$ III on the addition of dithionite was reported to be a biphasic reaction consisting of a first rapid reduction phase (within 1 min) and a second slow phase (duration more than 20 min) (*12, 14*), or a slow reaction (*9*) with an isosbestic point around 635 nm. Similar biphasic spectral changes for *Rhodobacter* $N_2OR$ I to $N_2OR$ III were observed with a major change to the semi-reduced form (Fig. 2d) within the initial few seconds and a small change in the second phase from spectrum d to e in Fig. 2. In the second phase, the absorbance at 548 nm decreased slowly, but the absorbance at 623 nm hardly increased. An isosbestic point, therefore, did not clearly appear for *Rhodobacter* $N_2OR$.

On aerobic incubation at 4°C for 3 days, the spectrum of *Rhodobacter* $N_2OR$ I was intermediate between those of $N_2OR$ I and II (Fig. 2b). $N_2OR$ III was autoxidized on overnight incubation at 4°C under aerobic conditions, giving a spectrum intermediate between those of $N_2OR$ I and II, which resembled that in Fig. 2b.

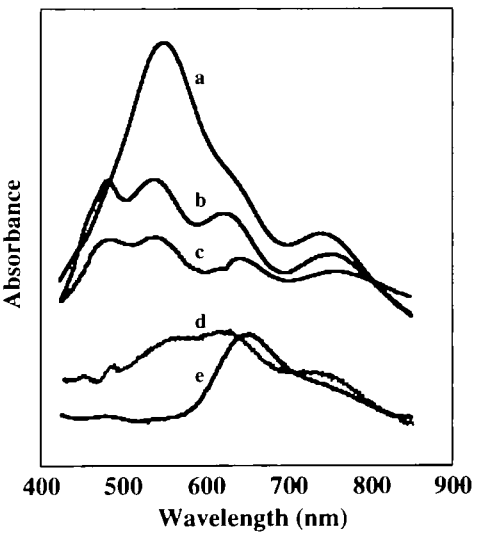


Fig. 2. **Visible absorption spectra of the purified nitrous oxide reductase.** a, the enzyme purified anaerobically (native form); b, the anaerobic enzyme after aerobic incubation for 3 days; c, the enzyme purified aerobically; d, the anaerobic enzyme immediately after the addition of sodium dithionite (semi-reduced form); e, the enzyme fully reduced by dithionite (reduced form).

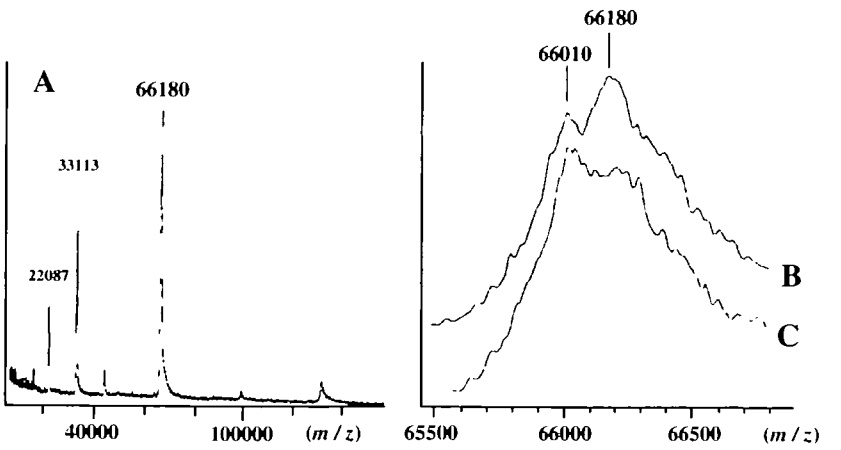


Fig. 1. **MALDI-TOF mass spectra of the purified nitrous oxide reductase.** A and B, native; C, EDTA-treated.

*Effect of Dithionite on the Enzyme Activity*—Although reduction of *Rhodobacter* $N_2OR$ by sodium dithionite resulted in a change in the spectrum, as shown in Fig. 2, the reduced enzyme retained its activity. The enzyme reduced with an excess amount of dithionite, or even the enzyme dissolved in a 1% sodium dithionite solution, retained more than 90% of the original activity. The enzyme activity of the reduced $N_2OR$ did not vary after anaerobic incubation at 4°C with dithionite for more than 3 h, or after autoxidation by agitation with a vortex tube mixer. The same results were obtained on reduction with dithionite in the presence of BV.

$N_2OR$ activity was also assayed spectrophotometrically using dithionite-reduced $BV^{+}\cdot$ (for details see "MATERIALS AND METHODS"). The estimated activity with dithionite was approximately 80% of that with photoreduced $BV^{+}\cdot$ as an electron donor. This might be the first case of detection of high activity of $N_2OR$ by means of a spectroscopic assay with dithionite-reduced viologen. When excess dithionite remained in the reaction mixture at the time of injection of $N_2O$, the enzyme activity could not be measured correctly.

These results indicate that *Rhodobacter* $N_2OR$ III, which was obtained by reduction of the purified enzyme with dithionite, has almost the same activity as $N_2OR$ I, that is to say, dithionite is not an inhibitor of *Rhodobacter* $N_2OR$.

The behavior and stability of *Rhodobacter* $N_2OR$ as to dithionite are distinguished from those of the enzymes from other origins, which were reported to be inactivated by dithionite (*9-12*). *Thiosphaera* $N_2OR$, however, resembled *Rhodobacter* $N_2OR$ and retained 70% of its activity after reduction with dithionite, and dithionite-reduced viologen was usable for the enzyme assay with an electrode if viologen was present in excess over dithionite (*13*).

*Enzyme Activation and Turnover-Dependent Inactivation*—Nitrous oxide reductases are generally activated by anaerobic incubation under weak basic conditions (*4, 5, 10, 12, 13*). *Rhodobacter* $N_2OR$ II, *i.e.*, the aerobically-purified form, required 90 min to show maximum activity on anaerobic incubation with $BV^{+}\cdot$ at pH 9.0 (*4, 5*), whereas the anaerobically isolated enzyme ($N_2OR$ I) and reduced enzyme ($N_2OR$ III) were activated within 30 min (Fig. 3). The enzyme was also activated by anaerobic incubation at pH 9.0 without $BV^{+}\cdot$, but the activity increased only about 50% from the original level on 90 min incubation (data not shown). Almost the same results were obtained with the spectroscopic assay involving dithionite-reduced $BV^{+}\cdot$, except that the obtained activities were lower than those with the photoreduction assay.

Turnover-dependent inactivation of the enzyme was observed (Fig. 4), as has been reported previously (*4, 9, 10, 12, 13*). $t_{1/2}$ for turnover-dependent inactivation of *Rhodobacter* $N_2OR$ in the presence of $N_2O$ and $BV^{+}\cdot$ is about 5 min, which is longer than the 1 min for *Paracoccus* $N_2OR$ (*9*). The aerobic enzyme tended to show stronger turnover-dependent inactivation.

When oxygen was removed from the enzyme reaction mixture completely by means of a glucose oxidase-catalase system (see "MATERIALS AND METHODS"), turnover-dependent inactivation was not observed (Fig. 4). On the other hand, stronger turnover-dependent inactivation was observed if deoxygenation of the $N_2O$ solution was incomplete (data not shown). The addition of catalase solely or superoxide dismutase to the reaction mixture had no effect on the enzyme activity. These results suggest that *Rhodobacter* $N_2OR$ retains its activity during the enzyme reaction without oxygen, and that oxygen causes turnover-dependent inactivation of the enzyme.

It was reported that several metal ions, such as $Zn^{2+}$, $Cu^{2+}$, and $Ni^{2+}$, showed inhibitory effects on the activity of *Rhodobacter* $N_2OR$ (*5*). These ions obviously change the turnover-dependent inactivation rate of the enzyme. In particular zinc ions promoted turnover-dependent inactivation strongly at the concentration of 10 $\mu$M (Fig. 4), whereas the ions had no effect on $N_2OR$ I or a weaker inhibitory effect on the enzyme with photoreduced $BV^{+}\cdot$ without $N_2O$ ($t_{1/2}$ for inactivation was about 75 min at 10 $\mu$M) (*5*).

The enzyme lost its activity with inhibitors (oxygen and metal ions) during the enzyme reaction, while both the

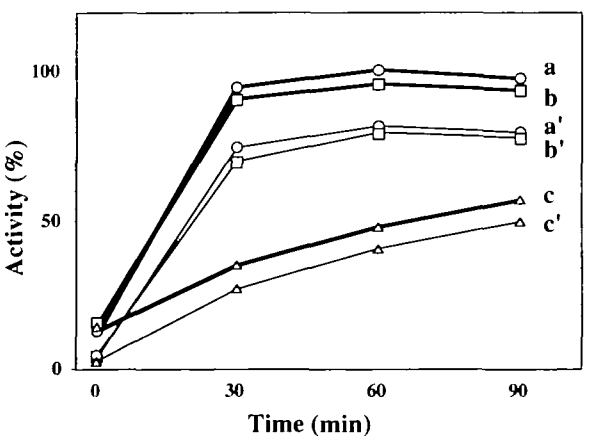


Fig. 3. **Activation of nitrous oxide reductase in different states, as compared with two assay systems.** a and a', the anaerobic enzyme; b and b', the reduced enzyme; c and c', the aerobic enzyme; a, b, and c, photoreduction assay; a', b', and c', assay involving dithionite. The enzymes were activated by basic incubation with $BV^{+}\cdot$. Activity is expressed as a percentage of the activity of the anaerobic enzyme with 90-min activation.

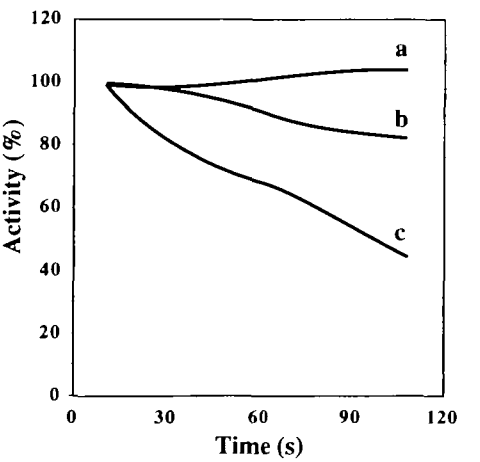


Fig. 4. **Turnover-dependent inactivation of nitrous oxide reductase.** a, assay with glucose oxidase and catalase; b, standard assay; c, assay with 10 $\mu$M $Zn^{2+}$. Activity is expressed as a percentage of the activity at 10 s.

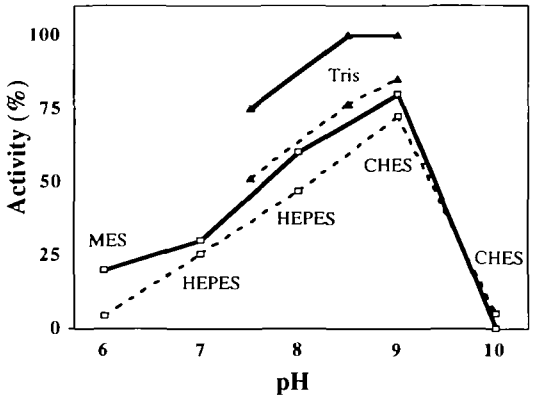


Fig. 5. **Variation of the activity of nitrous oxide reductase with pH.** Solid lines, photoreduction assay; broken lines, assay involving dithionite. Good's buffer was used as sodium salts. The anaerobically purified enzyme was used. Activity is expressed as a percentage of the activity observed with the photoreduction assay in Tris-HCl, pH 9.0.

oxidized and reduced enzymes were hardly affected. It seems that the enzyme may have a structure that is easily affected by inhibitors in the enzyme reaction turnover.

*The Effect of pH on $N_2OR$ Activity*—Variation of the $N_2OR$ activity with the pH of the reaction mixture is shown in Fig. 5. The activity showed an optimum pH of around pH 8.5-9, when photoreduced $BV^{+\bullet}$ was used as the electron donor. The observed activity was higher in Tris-HCl buffer than in Good's buffer at the same pH value. Moreover, with dithionite as a reductant, the activity variation with pH was similar to that observed with the photoreduction assay, while the activity decreased around 20% at every pH.

The turnover-dependent inactivation rate of the enzyme was relatively low and scarcely changed from pH 6 to 10, whereas turnover-dependent inactivation at neutral or lower pH has been reported for $N_2ORs$ from other origins (*9, 10, 12, 13*).

We are grateful to Dr. S. Nagata (The University of Tokyo) for the support in the MALDI-TOF measurements.

REFERENCES

1. Berks, B.C., Ferguson, S.J., Moir, J.W.B., and Richardson, D.J. (1995) Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta* **1232**, 97-173
2. Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**, 533-616
3. Satoh, T., Hoshino, Y., and Kitamura, H. (1976) *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. *Arch. Microbiol.* **108**, 265-269
4. Michalski, W.P., Hein, D.H., and Nicholas, D.J.D. (1986) Purification and characterization of nitrous oxide reductase from *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*. *Biochim. Biophys. Acta* **872**, 50-60
5. Sato, K., Okubo, A., and Yamazaki, S. (1998) Characterization of a multi-copper enzyme, nitrous oxide reductase, from *Rhodobacter sphaeroides* f. sp. *denitrificans*. *J. Biochem.* **124**, 51-54
6. Englander, S.W., Calhoun, D.B., and Englander, J.J. (1987) Biochemistry without oxygen. *Anal. Biochem.* **161**, 300-306
7. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85
8. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
9. Snyder, S.W. and Hollocher, T.C. (1987) Purification and some characteristics of nitrous oxide reductase from *Paracoccus denitrificans*. *J. Biol. Chem.* **262**, 6515-6525
10. Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Koerner, H., and Jakob, W. (1985) Nitrous oxide reductase from denitrifying *Pseudomonas perfectomarina*: Purification and properties of a novel multicopper enzyme. *Eur. J. Biochem.* **153**, 459-468
11. Hulse, C.L. and Averill, B.A. (1990) Isolation of a high specific activity pink, monomeric nitrous oxide reductase from *Achromobacter cycloclastes*. *Biochem. Biophys. Res. Commun.* **166**, 729-735
12. SooHoo, C.K. and Hollocher, T.C. (1991) Purification and characterization of nitrous oxide reductase from *Pseudomonas aeruginosa* strain P2. *J. Biol. Chem.* **266**, 2203-2209
13. Berks, B.C., Baratta, D., Richardson, D.J., and Ferguson, S.J. (1993) Purification and characterization of a nitrous oxide reductase from *Thiosphaera pantotropha*: Implications for the mechanism of aerobic nitrous oxide reduction. *Eur. J. Biochem.* **212**, 467-476
14. Riester, J., Zumft, W.G., and Kroneck, P.M.H. (1989) Nitrous oxide reductase from *Pseudomonas stutzeri*: Redox properties and spectroscopic characterization of different forms of the multicopper enzyme. *Eur. J. Biochem.* **178**, 751-762