

A novel kind of multi-copper protein as terminal oxidoreductase of nitrous oxide respiration in *Pseudomonas perfectomarinus*

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The terminal oxidoreductase of nitrous oxide respiration in the marine, denitrifying bacterium, *Pseudomonas perfectomarinus*, was identified as multi-copper protein and purified to electrophoretic homogeneity. The enzyme reduced N_2O to N_2 with hydrogen, clostridial hydrogenase, and methyl viologen as electron-donating system. The copper content of the reductase corresponded to ~ 8 copper atoms/120 000 M_r . The subunit structure was dimeric with two peptides of equal size. Manganese, iron and zinc were absent, or were not found in stoichiometric amounts. The oxidized chromophore had absorption maxima at 350, 480, 530, 620 and 780 nm; addition of dithionite produced a blue protein form with maxima at 470, 635 and 740 nm. Both forms of the enzyme were paramagnetic. The same copper protein was also isolated from *Pseudomonas stutzeri*.

Nitrous oxide

Nitrous oxide reductase

Copper protein
Pseudomonas

Denitrification

Nitrogen cycle

1. INTRODUCTION

The marine, denitrifying bacterium *Pseudomonas perfectomarinus* reduces nitrate to N_2 under anaerobic, respiratory growth conditions. Like other denitrifying bacteria that form N_2 , it can respire at the expense of nitrous oxide (N_2O) only, which involves a single terminal reductase (no EC entry). High instability of this enzyme in cell-free extracts has thus far prevented its identification. However, in [1,2] N_2O respiration required Cu as trace element and subsequently a pink-colored, high- M_r copper protein was found in *P. perfectomarinus* as the likely oxidoreductase of N_2O respiration [3]. Here, we demonstrate catalytic activity for this copper protein and report some of its fundamental properties.

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2. MATERIALS AND METHODS

Pseudomonas perfectomarinus (ATCC 14405) was grown on a synthetic, nitrate-free medium [2], containing, besides iron, $1 \mu M$ $CuCl_2$ as the only trace element added. The activity of N_2O reduction was expressed under lowered oxygen tension, which was experimentally achieved by growing a 1 liter culture in a 2 liter Erlenmeyer flask at $30^\circ C$ in a gyratory shaker (120 rev./min), or sparging a 20 liter carboy with 200 ml air/h. Small-scale cultures were harvested by centrifugation at $12\,000 \times g$ for 15 min; cells from large batch cultures were separated from the medium by a continuous-flow centrifuge without precautions for anaerobicity. The cell paste was washed once with 0.85% NaCl solution. Cells were used at once, or otherwise stored at $-70^\circ C$. Denitrifying cells were also grown in the above medium on the basis of N_2O respiration only [2] or in a complex medium containing nitrate [4].

The molecular mass of N_2O reductase was determined by gel filtration on Sephadex G-200 [5], by sodium dodecyl sulfate (SDS)–polyacrylamide electrophoresis (PAGE) [6] and, under non-

denaturing conditions, by pore gradient PAGE as in [7].

Metal content was determined by atomic absorption spectroscopy. Protein was estimated by the Lowry method with bovine serum albumin as standard. For amino acid analysis, a protein sample was dialyzed against water and lyophilized. Weighed samples were hydrolyzed in 4 M methanesulfonic acid under N_2 for 24 and 72 h at 105°C. Hydrolyzates were adjusted to pH ~2 by adding calculated amounts of 3.5 N NaOH, and their amino acid composition was analyzed as in [7].

Clostridial hydrogenase (EC 1.12.7.1) was a by-product of nitrogenase preparations [8], and was collected from the first chromatographic step on Sephadex G-100. The enzyme was purified by anaerobic DEAE-cellulose and gel chromatography as in [9].

Gases were identified and quantitated by gas chromatography. Nitric oxide, N_2O and N_2 were separated on a dual column system of alumina and molecular sieve 13X across a single thermal conductivity detector (K. Frunzke, W.G.Z., unpublished).

3. RESULTS

3.1. Expression of N_2O reductase

N_2O reductase was derepressed in the absence of nitrogenous oxides under low oxygen tension (table 1). Nitrite and NO reduction, other partial

Table 1

Activities and associated cytochrome steady-state oxidation levels of partial reactions of nitrite respiration in cells of *P. perfectomarinus* grown under reduced oxygen tension in the absence of nitrogenous oxides^a

Electron acceptor	Activity ^b	Cytochrome oxidation level ^c
Nitrite	0	4.6
NO	0.9–1.2	15.5
N_2O	14–20	59.6

^a Growth conditions as described in section 2

^b Activities are expressed as $\mu\text{mol electron acceptor reduced} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$

^c Determination of the steady-state oxidation level of cytochromes as in [2]

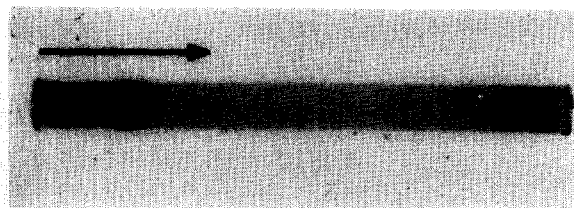


Fig. 1. SDS-Polyacrylamide gel electrophoresis of a purified sample of N_2O reductase from *P. perfectomarinus*.

reactions of denitrification, remained largely repressed, concomitant with a low steady-state oxidation level of the bulk cytochromes on addition of nitrite or NO to intact cells (table 1). To that extent, the low-aerobically grown cell resembled that grown with N_2O [2]. The advantage of cultivation under reduced tension of oxygen was found in ease of growth, low content of soluble cytochromes, and in particular the nearly complete absence of cytochrome *cd* (i.e., respiratory nitrite reductase, EC 1.9.3.2), an enzyme which tended to mask N_2O reductase.

3.2. Purification of N_2O reductase

This procedure refers to cells grown under low oxygen tension. The enzyme, with apparently the same properties, was also obtained from N_2O -grown cells [3], and from anaerobically, nitrate-grown cells which were rich in cytochrome *cd*. In the latter case the copper protein, because of equal mass and similar overall charge, co-purified with the intensely green cytochrome *cd*, and had already been isolated from it in 1979 by isoelectric focussing (using the procedure below) during the final purification step of cytochrome *cd* (W.G.Z., unpublished). When the necessity of Cu for N_2O respiration in *P. perfectomarinus* was recognized [2,3], this protein attracted our renewed attention, and is identified here as N_2O reductase.

For purification, cell paste was resuspended in 2 vol. 20 mM Tris-HCl (pH 7.5) and supplemented per gram cell mass with 1 mg each of DNase and RNase. Cells were broken by passing them twice at 110 MPa through a French press. The homogenate was spun for 90 min at $236\,000 \times g$, and the resulting supernatant was fractionated with solid $(\text{NH}_4)_2\text{SO}_4$ between 50–95% saturation. The pH was kept constant by addition of dilute NH_4OH . The precipitate was resuspended in a minimal

amount of water and dialyzed extensively in the cold against water and finally 10 mM Tris. A pH of 7.5 was used throughout the purification procedure. The dialysate was applied to a 2.5×35 cm column of DE-52 cellulose (Whatman), equilibrated with the dialysis buffer and washed with it, until the effluent was nearly protein-free. The column was developed with 600 ml of a linear NaCl gradient (0–0.4 M) in 10 mM Tris buffer. Two well-separated components contained copper. The later-eluting component was a low molecular mass protein (M_r 38 000) of unknown function [3]; fractions of the first-eluting one were concentrated by $(\text{NH}_4)_2\text{SO}_4$ or by ultrafiltration and chromatographed with 10 mM Tris on a 2.5×90 cm column of Sephacryl S-200 (Pharmacia). Fractions with absorbance at 780 nm were pooled and concentrated by ultrafiltration with an Amicon PM 10 membrane.

Depending on the degree of purity, at this stage we either subjected the enzyme to preparative isoelectric focussing or chromatographed it once more on a DEAE–Sephacryl column (2.5×20 cm) under the above conditions for DEAE–cellulose. Isoelectric focussing was done with an LKB Multiphor apparatus. The preparation of the gel bed with 5 g Ultradex (LKB) followed the manufacturer's directions. Protein sample (3 ml) with <5 mM buffer were applied and separated under the following conditions: temp. 6°C; power, 7 W; duration, ~8 h; and pH gradient, 4–6. N_2O reductase focussed around pH 4.9 and was seen on completion of the focussing process as pink-to-violet band (often double band, but otherwise no discernible properties) in the gel trough. The colored material was scraped from the gel bed with a spatula, applied to the above Sephacryl S-200 column, and chromatographed with 50 mM Tris buffer. This procedure removed residual amounts of cytochromes and ampholytes, and gave, by electrophoretic criteria, a homogeneous preparation of N_2O reductase (fig.1).

3.3. Enzymatic activity

The homogeneous preparation of the copper protein reduced N_2O quantitatively to N_2 (fig.2). Methyl viologen was used as electron donor for the enzyme and was kept reduced by H_2 and clostridial hydrogenase. Dithionite-reduced methyl viologen was not a satisfactory system, as excess

$\text{Na}_2\text{S}_2\text{O}_4$ inhibited the enzyme. However, reductive activation of N_2O reductase was indicated by the observation that the blue form (see below) of the enzyme obtained after $\text{Na}_2\text{S}_2\text{O}_4$ addition and air oxidation of excess of reductant was required for the assay. The specific activity of the copper protein was ~0.5 nkat/mg protein. Comparing this activity to that of intact cells, and assuming a reasonable concentration of the enzyme in the N_2O -respiring cell, a potential for an activity increase by 10^2 – 10^3 appears possible. The low activity might be due to an inapt electron-donor system, or due to inactivation of the enzyme. At least the latter is indicated from the inability to demonstrate N_2O -reducing activity in cell-free extracts. Work is in progress to identify the physiological electron donor, and to clarify the mechanism of inactivation in order to improve assay conditions.

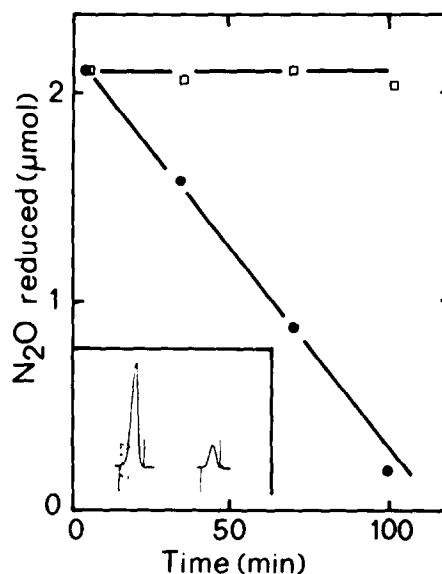


Fig.2. Reduction of N_2O by the *Pseudomonas* pink copper protein. The reaction mixture in 2 ml in total vol. was composed of 100 μmol Tris (pH 7.5), 0.5 μmol methyl viologen, 0.75 mg clostridial hydrogenase, and 0.6 mg *P. perfectomarinus* copper protein. The atmosphere was H_2 and contained 2.2 μmol N_2O : (●) complete reaction mixture; (□), copper protein omitted. The inset shows an example of original detector responses for N_2O of the gas chromatograph; left trace, at 3 min; right trace at 105 min.

3.4. Properties

Electrophoretically homogeneous samples of N_2O reductase contained 0.41% Cu. Mn was absent; the content of Zn was only 1/20 that of Cu, excluding Zn most likely as a stoichiometric component of the enzyme. Unlike Cu, Mn and Zn did not stimulate growth, and neither did Ni, Co nor Mo [2]. Fe was only found when the protein was not completely free of a contaminating heme component. The metal content corresponded to Cu ~ 8 atoms/enzyme molecule (M_r 120 000). As isolated, the protein was paramagnetic and showed at 20 K a copper EPR signal centered at $g = 2.023$.

The electronic spectrum of N_2O reductase as isolated in its oxidized form is shown in fig.3. It had a sequence of broad absorption maxima at 350, 480, 530, 620 and 780 nm, unlike any of the presently known copper enzymes. The protein maximum was at 279 nm. Addition of $\text{Na}_2\text{S}_2\text{O}_4$ produced a blue protein form, with an absorption maximum at 635 nm and two weak bands at 470 and 740 nm. This form was still paramagnetic ($g =$

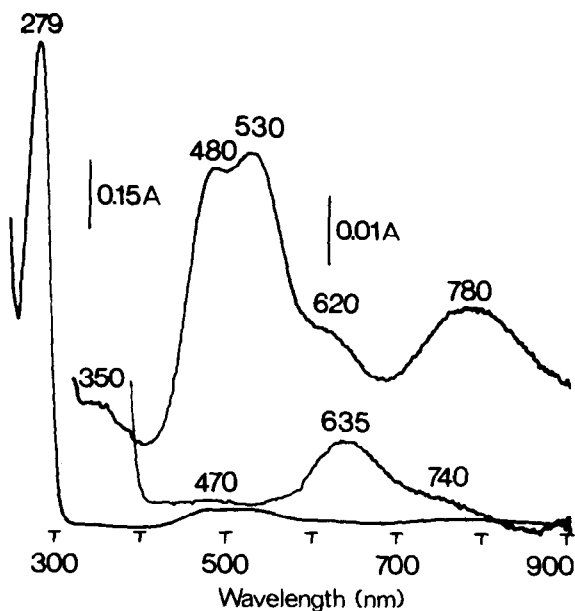


Fig.3. Electronic spectrum of *P. perfectomarinus* N_2O reductase. Solvent 50 mM Tris-HCl, (pH 7.5); band width 2 nm. The upper expanded, and the lower complete trace show the oxidized form; the middle, expanded trace shows the blue form obtained on dithionite addition.

2.052) and resembled a type 1 Cu (II) spectrum. However, assignment of the types of Cu in this enzyme is not being attempted at this stage and has to await ongoing physico-chemical characterization. The blue form was not autoxidizable; but addition of ferricyanide restored the original spectrum. Occasionally, the blue form was observed in the absence of added reductant on freezing of the pink form. The relative intensity of the absorption bands of both forms varied to certain extent (e.g., published spectrum in [3]) due to presently unidentified factors. This restricts the extinction co-efficient/Cu of $690 \text{ cm}^{-1} \cdot \text{M}^{-1}$ at 530 nm to tentative assignment. The purity index for N_2O reductase at the wavelength pair 280/530 nm was 14.

Pseudomonas stutzeri JM 300 (a gift from Professor J.L. Ingraham, Davis CA), like *P. perfectomarinus* a vigorous denitrifier, contained this copper protein as the single major copper component when grown in the *P. perfectomarinus* medium under low-aerobic conditions. The purified protein from *P. stutzeri* had an electronic spectrum that was, with maxima at 350, 480, 530, 620 (shoulder) and 780 nm, identical to that of *P. perfectomarinus*. The absorption bands showed some variation in intensity, thus the band at 350 nm was hardly visible and that at 620 nm was weak, as was the absorbance of the blue form on $\text{Na}_2\text{S}_2\text{O}_4$ addition. With respect to the blue form, it is important to note, that neither *P. stutzeri* nor *P. perfectomarinus* contained azurin, the 'Pseudomonas blue protein' [3].

The enzyme was 120 000 M_r by gel filtration and 113 000 M_r by pore-gradient PAGE. The minimal mass, 15 600, from Cu analysis extrapolated to 124 800, assuming 8 Cu atoms/enzyme molecule. Under the denaturing conditions of SDS-PAGE, a uniformly migrating, single protein band was observed with M_r 62 000. This suggested a dimeric structure for N_2O reductase with an α_2 subunit assembly.

Table 2 shows the amino acid composition of N_2O reductase. The low pI of the enzyme was reflected in the prevalence of acidic residues. The enzyme monomer contained 4 residues of cysteine which might participate in binding of the Cu chromophore. Comparison of the amino acid composition with that of the bacterial Cu enzymes nitrite reductase [10] and L-tryptophan-2,3-dioxygenase [11] did not reveal a resemblance. A pink copper

Table 2

Amino acid composition of *P. perfectomarinus* N₂O reductase^a

Amino acid	Residue/subunit ^b	Nearest interger
Asp	65.1 ± 2.1	65
Thr ^c	28.8 ± 0.9	29
Ser ^c	28.0 ± 1.0	28
Glu	48.2 ± 1.5	48
Pro	30.5 ± 1.6	30
Gly	47.1 ± 1.7	47
Ala	44.4 ± 1.9	44
Cys ^d	3.7 ± 0.5	4
Val ^e	44.4 ± 0.7	44
Met	18.1 ± 1.2	18
Ile	28.9 ± 0.6	29
Leu ^e	33.0 ± 0.9	33
Tyr	15.5 ± 0.8	16
Phe	23.9 ± 0.9	24
Lys	38.7 ± 0.6	39
His	21.6 ± 1.1	22
Arg	21.4 ± 0.7	21
Trp ^f	4.1 ± 0.4	4
Total	545.2	545

^a Unless otherwise indicated, residue values were based on 4 samples hydrolyzed for 24 h and 3 hydrolyzed for 70 h

^b Moles amino acid/60 000 *M_r* subunit ± SD of all determinations

^c Corrected for time-dependent losses by extrapolating to zero time of hydrolysis

^d Includes oxidized forms

^e Determined as the 70 h value

^f Determined as the 24 h value

protein from human erythrocytes had absorption maxima at 503 and 760 nm, but otherwise substantially different properties [12].

4. DISCUSSION

This communication fills a void in the enzymology of the nitrogen cycle with the molecular identification and beginning characterization of N₂O reductase. The expression and activity attenuation of this enzyme is an important factor in determining the microbial emission of N₂O into the atmosphere, where it is a major contender for a possible ozone reduction by several percent [13].

Within this context, it was of high interest to find expression of N₂O reductase in response to diminished oxygen tension even in the absence of nitrogenous oxides and prior to the synthesis of nitrite and NO reductase.

The assay of the purified enzyme indicated a necessity for activation. This might explain the absence of activity in cell-free extracts, which has long delayed advancement in this area. The previously presented physiological evidence for a copper requirement of N₂O respiration in *P. perfectomarinus* [2], the consistent association of the pink protein with nitrite-respiring bacteria and denitrifying growth conditions [3], and the present finding of enzymatic activity are the basis for ascribing to this copper protein the function of terminal oxidoreductase in N₂O respiration.

In 1972, a copper protein of unknown function was isolated from *Alcaligenes faecalis*, which was at that time not related to N₂O reduction and has not been reported from any other bacterium since. Its molecular mass and copper content were different from the protein found in *P. perfectomarinus*; however, its similar electronic spectrum makes it likely that it will be found to have an identical chromophore and, possibly, have the same catalytic activity. Molecular mass, subunit structure, and lack of a metal chromophore of a 60-fold purified N₂O reductase from *Paracoccus denitrificans* [15] are at variance with the corresponding properties of the *P. perfectomarinus* enzyme. Further work will have to show, whether properties of an insufficiently purified sample were assigned to the *Paracoccus* enzyme, or, more interestingly, whether a second type of respiratory N₂O reductase exists.

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