The effect of oxygen on chromatographic behavior and properties of nitrous oxide reductase

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Nitrous oxide reductase, a high-M_r copper protein, was purified under anaerobic conditions to yield a spectroscopically new species with 3–5-fold increased catalytic activity over the 'pink' form of the enzyme obtained thus far. The preparation was homogeneous by chromatographic and electrophoretic criteria and reduced N_2O to N_2. On aerobic gel filtration of a crude extract, the enzymatic activity was slightly shifted from the Cu-protein towards an apparent lower M_r. This effect was not observed under anaerobic conditions, nor was it observed with the purified enzyme on either aerobic or anaerobic chromatography. In crude extracts, oxygen appears to convert the Cu-protein to a lower activity form at the leading edge of a migrating chromatographic zone.

Nitrous oxide reductase *Copper protein* *Denitrification* *Nitrate respiration* Pseudomonas

1. INTRODUCTION

The terminal oxido-reductase of bacterial nitrous oxide respiration is labile and not readily detectable in vitro. The enzyme from *Pseudomonas perfectomarina* was recently identified as a novel type Cu-protein of M_r around 120000 [1]. In addition to the demonstration of catalytic turnover, the identification of N_2O reductase as the Cu-protein was based on several independent and consistent observations [2,3]. From a shift of enzymatic activity towards apparent lower M_r it was concluded, however, that the enzyme and the Cu-protein were different [4]. This shift, as shown here, was confined to aerobic chromatographic conditions and was only observed with crude extracts.

N_2O reductase isolated anaerobically and purified to homogeneity was spectroscopically and catalytically distinct from the enzyme species obtained aerobically. The anaerobically prepared enzyme was more active than aerobically purified material and clearly documented the association of N_2O reduction with the Cu-protein.

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2. MATERIALS AND METHODS

2.1. *Growth of cells*

P. perfectomarina (ATCC 14405) was grown with nitrate in a synthetic medium [2], with CaCl and $MgCl_2$ concentrations lowered by 50% to minimize formation of precipitates. Cultures were started from agar slants with 100 ml of this medium and were incubated for 24 h at 30°C in a gyratory shaker (120 rpm). For chromatographic studies, 1 l of medium in a 2-l Erlenmeyer flask was inoculated with the pre-culture and incubated under the same conditions. The low shaking frequency caused oxygen-limitation and led to expression of the N_2O-reducing system [1]. After 18 h of growth, cultures were harvested by centrifugation at 16270 × *g* for 10 min. The cell paste was washed once in 50 mM phosphate buffer (pH 7.1) and broken by two passages at 110 MPa in a French Press (Aminco). The homogenate was centrifuged at 111000 × *g* for 60 min. All steps, starting with the cell harvest, were done at 4°C under anaerobic

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conditions, and all solutions were thoroughly degassed on a vacuum line. The crude extract was kept under argon for immediate chromatography or stored in liquid nitrogen for further use.

2.2. *Enzyme purification*

N_2O reductase was purified to homogeneity from 400 g cells (wet wt) by modifying the procedure of [1]. The first two fractionation steps were inverted and the crude extract was applied directly to a 5 × 30 cm DE-52 (Whatman) column. After precipitation by $(NH_4)_2SO_4$, the enzyme solution was filtered through a 5 × 50 cm bed of Sephadex G-100. Final purification was obtained by preparative isoelectric focusing and gel filtration on Sephacryl S-200 [1]. The buffer used throughout the procedure was 50 mM Tris-HCl (pH 7.5). Enzyme purifications were done either entirely aerobic or under anaerobic conditions, except for isoelectric focusing which was run aerobically. Protein was determined by the Lowry method.

2.3. *Chromatographic procedures*

Gel permeation chromatography was done on 2.5 × 50 cm columns of Sephacryl S-200 or S-300 (Pharmacia). The columns were equilibrated with 50 mM phosphate buffer (pH 7.1), and were run either aerobically or anaerobically. For the latter purpose, the buffer was degassed 10 times on a vacuum line and saturated each time with argon; the buffer reservoir that fed into the column was continuously flushed with argon. The columns were eluted with a buffer flow of 48 ml/h. About 3 ml of crude extract with approx. 60 mg protein/ml were usually applied to each column. Fractions of 2 ml were collected automatically in open test tubes from the aerobic column, and manually in argon-filled, capped vials from the anaerobic column. The purified enzyme was subjected to aerobic high performance gel chromatography on 7.5 × 300 mm TSK G-4000SW or G-3000SW columns at a flow rate of 0.5 ml/min. The solvent was 0.1 M KCl in 50 mM phosphate buffer (pH 7.0), or in 20 mM Tris-HCl (pH 7.3). The Tris buffer system was supplemented in several runs with 0.5% SDS. Detection was by dual wavelength spectrophotometry at 280 and 540 nm.

2.4. *Analytical methods*

Copper was determined by atomic absorption spectroscopy at 324.7 nm. The cytochrome content of chromatographic fractions was estimated spectrophotometrically at 410 nm. Reduction of N_2O to N_2 was assayed by monitoring the oxidation of photochemically reduced benzyl viologen at 600 nm [4]. 5-Deazaflavin at a final concentration of 16 μM substituted proflavin [5]. Samples from aerobic chromatographies were made anaerobic on a vacuum line prior to testing. Controls, consisting of the complete reaction mixture without N_2O, showed little or no bleaching of benzyl viologen after addition of the enzyme solution and mixing. SDS-polyacrylamide gel electrophoresis was done in 0.5 mm slab gels with a linear gradient of acrylamide from 9.5 to 22.6% [6]. The gels were fixed in ethanol-acetic acid overnight and stained with minor modifications according to [7].

3. RESULTS

Fractionation of a crude extract of *P. perfectomarina* on a 2.5 × 90 cm aerobic column (V_o = 172 ml) of Sephacryl S-300 (fig.1A) led to a difference of 4 ml (2 fractions) in the elution volume

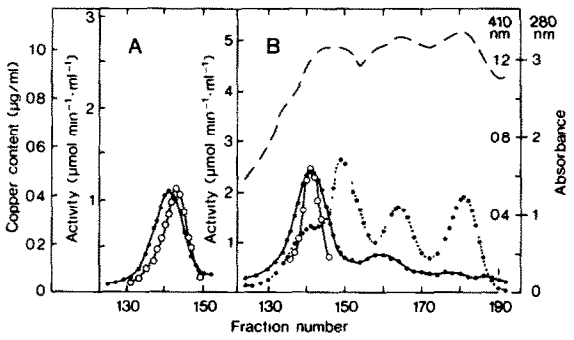

Fig.1. Gel permeation chromatographic profiles of a crude extract of *P. perfectomarina* on Sephacryl S-300. (A) Aerobic conditions, 3.6 ml crude extract with 150 mg protein, (B) anaerobic conditions, 3 ml crude extract containing 210 mg protein. Only the activity and copper profiles are shown in A. Representation of mainly particulate material eluting in the void volume of the column was omitted from the profiles. The specific activity of the peak fraction was 15.2 nkat/mg protein in A, and 32.3 nkat in B. (○) N_2O reductase activity, (●) copper content, (●---●) absorbance at 410 nm, (●——●) absorbance at 280 nm.

between the Cu-protein and N_2O-reducing activity, as had been observed similarly in [4]. Extract passed over the same column under anaerobic conditions did not show this effect. Rather, there was coincidence in the elution volumes of the Cu-protein and N_2O reductase (fig.1B). No further activity peaks were found in the chromatographic profile, either towards lower or higher M_r.

Globular proteins of M_r around 100000 are better resolved by Sephacryl S-200 than S-300, because the K_{av} term depends more strongly on the molecular mass for S-200 than for S-300. Fig.2 compares the elution profiles obtained with Sephacryl S-200 under aerobic and anaerobic conditions. N_2O-reducing activity was shifted on the aerobic column from the high-M_r Cu-protein by one or maximally two fractions towards lower M_r (fig.2A), but co-chromatographed on the anaerobic column (fig.2B). Sephacryl S-200 showed a substantially improved resolution for the soluble *c*-type cytochromes and a low-M_r Cu-protein [3], with near baseline separation for these components. Cytochrome cd_1, the first component in the 410 nm profile, which also has an M_r around 120000 (unpublished), was better resolved from the entailing split-α cytochrome. In contrast, no gain in resolution was achieved between the high-M_r Cu-protein and the putatively distinct activity of N_2O reductase (cf. fig.1,2).

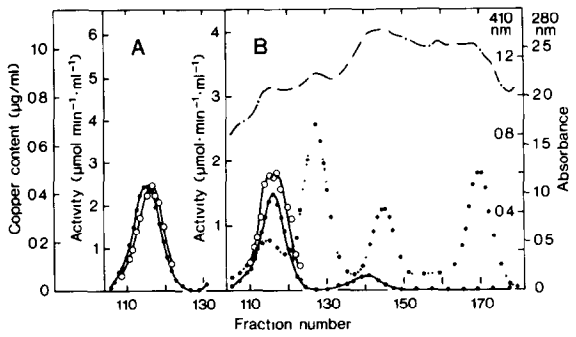

Fig.2. Gel permeation chromatographic profiles of crude extract of *P. perfectomarina* on Sephacryl S-200. (A) Aerobic conditions, 3 ml crude extract with 210 mg protein, (B) anaerobic conditions, 3 ml crude extract with 170 mg protein. The small, first peak in the 410 nm profile that overlaps with the activity peak and the high-M_r Cu-protein represents cytochrome cd_1. Other details as in fig.1.

N_2O reductase purified aerobically gave the already described 'pink' form of the Cu-protein [1] with a specific activity in the benzyl viologen assay of 15–35 nkat per mg protein. Chromatography on DEAE cellulose is the first step of this purification procedure to collect N_2O reductase in concentrated form from the crude extract. Although at times we noted an activity decrease, we have no evidence of irreversible binding of the enzyme to anion exchanger [4,8]. The activity that eluted from DEAE cellulose was associated with the major, Cu-containing component.

Cells broken anaerobically and fractionated under exclusion of O_2 from all chromatographic steps, yielding a spectroscopically new form of the Cu-protein (fig.3) whose catalytic activity was consistently 3–5-fold higher than that of the pink species. Its maximal specific activity was 115 nkat per mg protein. This form, as isolated, was purple and had absorption maxima at 540 and 780 nm. Maxima at 480 and 620 nm observed in the pink form were found only as shoulders. The dithionite-reduced form was blue with a single absorption maximum at 640 nm.

Chromatographic profiles of purified samples of the purple and pink forms of the enzyme on

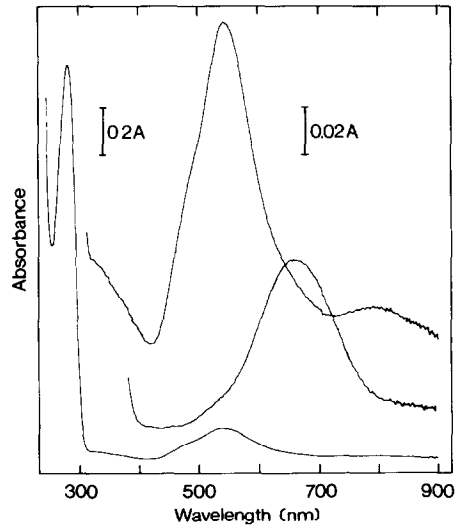

Fig.3. Electronic spectrum of the purple form of N_2O reductase from *P. perfectomarina* isolated under anaerobic conditions. The top expanded trace and the complete trace show the Cu-protein as isolated or after addition of ferricyanide; the middle trace was recorded immediately after adding dithionite.

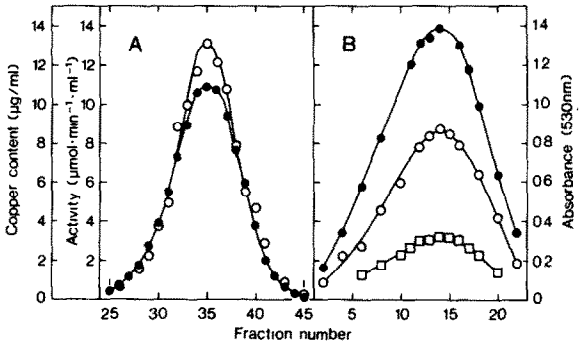

Fig.4. Cochromatography of N_2O-reducing activity with the Cu-protein on Sephacryl. (A) Chromatography of 3.5 ml with 60 mg purple Cu-protein on S-300 and anaerobic conditions, (B) chromatography of 2.6 ml with 83 mg pink Cu-proein on S-200 under anaerobic conditions. The fraction volume in B was only 1 ml. Specific activities of the peak fractions were 70 nkat/mg protein for A (purple form), and 25 nkat for B (pink form). (○) N_2O reductase activity, (●) copper content; (□) absorbance at 530 nm.

Sephacryl S-300 and S-200, respectively, are shown in fig.4. On both columns, N_2O-reducing activity migrated with the Cu-content of the fractions. In addition, the 530 nm absorbance of the pink Cu-chromophore also coincided (fig.4B). There was no difference in the elution profiles obtained under aerobic or anaerobic conditions.

The purity of the enzyme preparation was evaluated by gradient polyacrylamide gel electrophoresis and high performance liquid chromatography. In the latter technique, the Cu-protein eluted as a single symmetrical peak from TSK G-4000SW and G-3000SW columns in all solvents used including those with SDS. Retention times of the single component were identical, irrespective of whether detection was at 280 or 540 nm. The enzyme dissociates in detergent electrophoresis into two presumably identical subunits [1]. The pattern of an electrophoretically separated and silver-stained sample representing the protein monomer of the purple form of the enzyme is shown in fig.5.

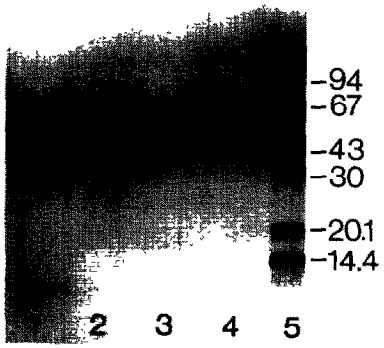

Fig.5. Gradient polyacrylamide gel electrophoresis of the purple form of the Cu-protein in the presence of SDS. Lanes 1–4 contained 25, 50, 75, and 100 ng protein, respectively. Lane 5 shows the pattern of 280 ng of a mixture of M_r marker proteins (Pharmacia): phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. Molecular masses are indicated in kDa.

4. DISCUSSION

The high-M_r Cu-protein of *P. perfectomarina* exists as two spectroscopically and catalytically distinct species. The pink form, obtained by aerobic purification, is less active than the purple form which is obtained when O_2 is excluded from the chromatographic steps. Both forms were purified to an electrophoretically homogenous state and each was associated with N_2O-reducing activity. The presently obtained maximal specific activity in the benzyl viologen-dependent assay was increased more than 200-fold over previous measurements without concomitant enrichment of a co-purifying component. The preparations were free of peptides of M_r 87000, 58000, or 25000, which have been suggested to represent N_2O reductase or its subunits [4,8].

The predominantly indirect approach for establishing the physical nature of the terminal oxido-reductase of N_2O respiration was necessitated by the difficulty to obtain consistently active enzyme in vitro. Oxygen has been identified as one inactivating agent [8]. Still, cell-free preparations often show no activity, or lose it rapidly, even though oxygen is excluded. This indicates a more complex process of enzyme inactivation. Differential inactivation at the leading edge of a migrating chromatographic zone, influenced by factors present in the crude extract, may account for the apparent, small separation observed on aerobic gel permeation chromatography. Purified samples of

the enzyme tend to maintain their activity level albeit in the presence of O_2. Ongoing characterization of the Cu-chromophore of N_2O reductase should provide keys for an understanding of the lability of this enzyme.

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