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NITROUS OXIDE REDUCTASE AND THE 120,000 MW COPPER PROTEIN OF N_2-PRODUCING DENITRIFYING BACTERIA ARE DIFFERENT ENTITIES

Stuart W. Snyder and Thomas C. Hollocher

Department of Biochemistry, Brandeis University, Waltham, MA 02254

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SUMMARY: Zumft and Matsubara ((1982) FEBS Lett. 148, 107-112) isolated a 120,000 MW copper protein from certain denitrifying bacteria which were capable of producing N_2. The presence of this protein correlated with a nutritional requirement of copper for growth on and reduction of N_2O by the bacteria. The copper protein was alleged by these workers to be nitrous oxide reductase. However, it is shown that the copper protein and nitrous oxide reductase have different molecular weights and exhibit different behavior upon anion exchange chromatography. The copper protein is therefore not nitrous oxide reductase.

Recently Zumft and Matsubara (1) concluded that a 120,000 MW copper protein was the nitrous oxide reductase of N_2-producing denitrifying bacteria. The basis for this assignment was threefold: a) a nutritional requirement of copper for growth of Alcaligenes spp. (2) and Pseudomonas perfectomarinus (3) on N_2O, b) the correlation of the copper protein with N_2O-reducing activity of intact cells for three bacteria (1,4), and c) the apparent catalysis of N_2O reduction by the pure copper protein from Ps. perfectomarinus (1). However, the specific activity of the pure copper protein was about 1,000 times lower (1) than that of the partially purified nitrous oxide reductase from Paracoccus denitrificans (5). Herein we determine whether nitrous oxide reductase activity and the 120,000 MW copper protein co-migrate together under column chromatography.

METHODS. Bacteria: Pseudomonas perfectomarinus (ATCC 14405) was grown at 30°C in the yeast extract-tryptone medium of Zumft and Vega (6). The starter culture (125 ml) was grown aerobically on a shaker overnight and was then used to inoculate a second culture (1350 ml) which was grown anaerobically on sparged N_2O for 24 h. Pseudomonas denitrificans (ATCC 13867) and Paracoccus denitrificans (ATCC 19367) were grown anaerobically on nitrate in yeast extract-peptone medium as described previously (7,8). Cells were harvested by centrifugation at 4°C after having reached a cell density of about 10^9 cells x ml^{-1} toward the end of exponential growth. The cells were washed twice with 50 mM potassium phosphate buffer, pH 7.1, at 4°C and resuspended in the same buffer to provide a cell density of 0.2 to 0.3 g wet weight of cells per ml.

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Cell-free extracts: The dense cell suspension was passed twice through a French press (American Institute Co.) at 13.6 to 20 MPa and centrifuged at 4°C for 1 h at 100,000xg. The resulting supernatant (cell-free extract) was then subjected to gel permeation or anion exchange chromatography.

Chromatography: Cell-free extract (5 ml) was combined with internal chromatographic markers (Blue Dextran, [^{3}H]acetate and [^{3}H]-labeled molecular weight standards), applied to the top of a Sephacryl S-300 (Pharmacia) column (2.6 x 95 cm) and eluted with use of 50 mM potassium phosphate buffer, pH 7.1, at 4°C and a flow rate of 40 ml x h^{-1}. Fractions of 5 ml were collected. The cell-free extract contained material which eventually degraded the performance of the column. Reproducible resolution was maintained by washing the Sephacryl with 0.2 M NaOH after every third experiment and repacking the column. The molecular weight standards were ferritin (440,000 MW), aldolase (158,000 MW), ovalbumin (43,000 MW) and RNase (13,700 MW), all from Sigma Chemical. These were labeled by acetylation with [^{3}H]acetic anhydride (New England Nuclear) according to the method of Wong and Marushige (9). Molecular weights of components eluted from the column were estimated by reference to K_{av} vs. log MW calibration curves (10).

Cell-free extract (15 ml) was also applied to a DE-52 (Whatman) anion exchange column (1.5 x 17.5 cm) which had been equilibrated with 10 mM Tris-HCl, pH 7.5. The column was washed with the equilibration buffer until the effluent was protein-free (60 ml) and then eluted with 100 mM Tris-HCl, pH 9.5. Fractions (6 ml) were collected and each was dialyzed overnight against 10 mM potassium phosphate, pH 7.1, which was sparged with nitrogen. The pH of the effluent had reached 9.5 by fraction number 50.

Analyses: Fractions collected from gel permeation chromatography were assayed for radioactivity, copper, nitrous oxide reductase activity and protein. Those from anion exchange chromatography were assayed for copper, nitrous oxide reductase activity and protein. Radioactivity was determined by use of a Beckman model LS-7500 scintillation counter and a xylene-based scintillation fluid (Amersham ACS). Typically 100 to 1000 background-corrected counts were accumulated for each sample to give a counting error of about 10% at the lowest radioactivities to 3% at the highest. Copper was determined by means of atomic absorption spectroscopy (Perkin-Elmer Model 305). Nitrous oxide reductase activity was assayed by a modification of the procedure of Kristjansson and Hollocher (11). Instead of reducing benzyl viologen (Sigma Chemical) with sub-stoichiometric amounts of dithionite, the new procedure used photochemical reduction as follows. To each 3 ml optical cuvette was added 2.5 ml of 10 mM potassium phosphate buffer, pH 7.1; 0.1 ml of 1 M triethanolamine; 25 µl of 0.4 mM proflavin (Sigma Chemical); and 25 µl of 10 mM benzyl viologen (ICN). The cuvette was then sealed with a rubber septum and sparged with N_2O for 10 min, after which benzyl viologen was photochemically reduced to the blue cation radical (12) by exposure to a 15 watt Sylvania Blue fluorescent bulb (F15T8/B) at a distance of 10 to 15 cm. The assay was started by the injection of 50 or 100 µl of a chromatographic fraction, which had been subjected to the reactivation procedure (see below), and followed by the decrease in absorbence at 600 nm. This modification avoids the use of dithionite which in excess can inactivate or inhibit nitrous oxide reductase (5). Protein was estimated by the method of Lowry et al. (13).

Reactivation of nitrous oxide reductase: Inactivation of nitrous oxide reductase by O_2 is largely, but perhaps not entirely, reversible by exposure to benzyl viologen cation radical at room temperature for several hours (11). The following reactivation procedure was used. To 1 ml of a chromatographic fraction in a 9 ml vial was added 0.1 ml of 1 M triethanolamine, 25 µl of 0.4 mM proflavin and 25 µl of 10 mM benzyl viologen. The vial was sealed with a rubber septum and made anaerobic by evacuation and back filling with argon. The benzyl viologen was then photochemically reduced at room temperature by exposure to the aforementioned fluorescent bulb, after which the vial was allowed to stand at least 3 h before its contents were assayed for nitrous oxide reductase activity.

RESULTS AND DISCUSSION. Gel permeation Chromatography: Fig. 1 is representative of experiments performed using cell-free extract from Ps. perfectomarinus and shows the elution profiles of protein-bound copper and nitrous oxide reductase activity relative to the [^{3}H]acetylated protein standards and Blue Dextran. In every one of numerous experiments using cell-free extract from this and other denitrifying bacteria, the copper peak eluted before the enzyme activity peak. The specific activity of the fraction having maximum enzyme activity was typically between 0.2 and 4.0 µmol N_2O x min^{-1} x mg $protein^{-1}$. These values are similar to those obtained by Kristjansson and Hollocher (5) but are 10 to 200 times greater than that reported by Zumft and Matsubara (1) for the 120,000 MW copper protein. In addition, there was no evidence that the copper peak was associated with a secondary activity peak or that the activity peak was associated with a secondary copper component. The second copper peak represents the 38,000 MW copper protein of Ps. perfectomarinus (4). That protein does not correlate with N_2O-reducing activity of cells and is absent from some other denitrifiers (4).

When the elution profile of the unlabeled molecular weight standards (primary standards) was compared carefully with that of their [^{3}H]acetylated

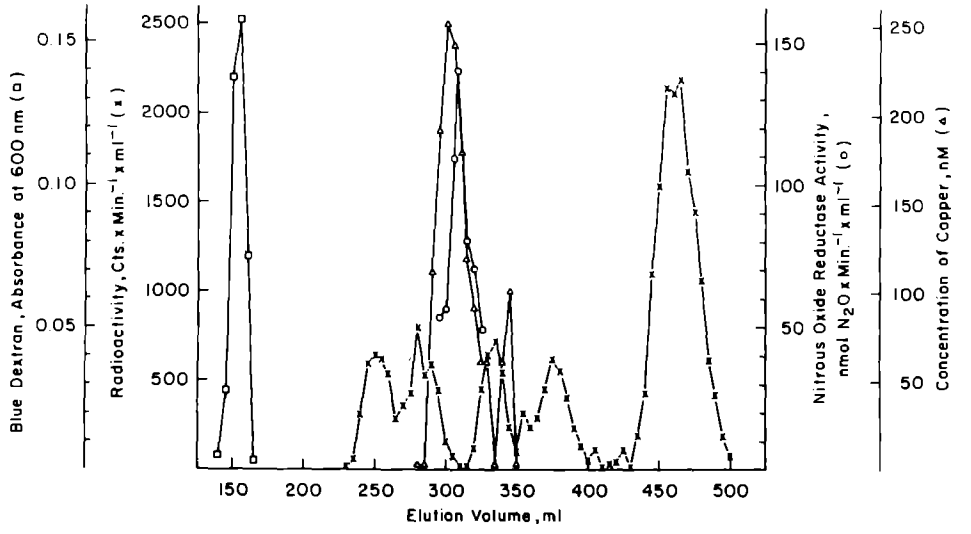

Figure 1: Elution profile from the Sephacryl S-300 column of a cell-free preparation derived from denitrifying Ps. perfectomarinus. See text for technical details. The five radioactivity peaks (X) correspond, left to right, to tritiated ferritin, aldolase, ovalbumin, RNase, and acetate.

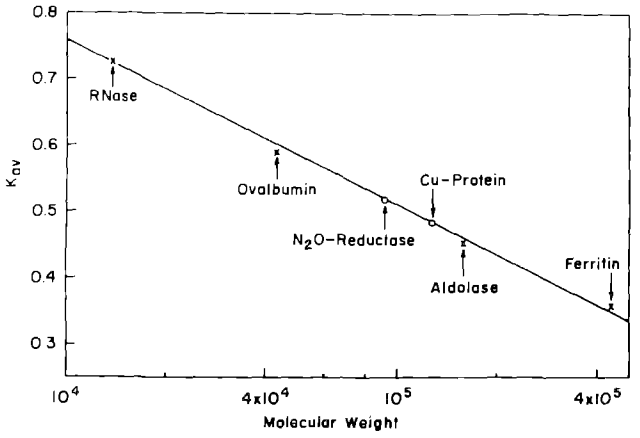

Figure 2: Molecular weight estimates. Over the molecular weight range of 10^4 to 5×10^5, it was assumed that K_{av} is linear in log MW when using Sephacryl S-300 ($K_{av} = (V_e - V_o)/(V_t - V_o)$). The elution volumes of Blue Dextran and [^{3}H]acetate were taken to be the void volume (V_o) and the total volume (V_t), respectively. The eluted volumes (V_e) of the [^{3}H]acetylated protein standards were adjusted by factors of 1.023, 1.036, 1.007, and 1.006 for ferritin, aldolase, ovalbumin, and RNase, respectively, in order to reference them to the unacetylated parent proteins (see text). Thus, K_{av} refers to the unacetylated standards. The data are those of Fig. 1.

derivatives (internal secondary standards), small differences in elution volume (V_e) of 1 to 10 ml were found. In each case the acetylated protein eluted before the parent protein and thus exhibited a slightly greater apparent molecular weight. The elution volumes were then corrected so that K_{av} *vs.* log MW curves (Fig. 2) referenced the primary standards. The data of Fig. 1, when reduced (Fig. 2), gave molecular weights of 92,000 and 126,000 for the nitrous oxide reductase activity and the first copper protein, respectively, based on least-squares linear regression analysis. Molecular weights of 83,000 and 115,000 were obtained based on a smooth curve connecting the four calibration points. The compendium of molecular weights (Table 1) is based on least-squares linear regression analyses. The mean molecular weight of 89,000 for nitrous oxide reductase from *Pa. denitrificans* agrees with earlier estimates of 83,000 to 87,000 (5), and the copper-protein molecular weights (overall average, 123,000 MW) agree with those reported by Zumft and Matsubara (1,4) for *Ps. perfectomarinus*, *Ps. stutzeri* and *Ps. fluorescens*.

Anion exchange chromatography: Although the 120,000 MW copper protein eluted in fractions 14 to 17 (data not shown), there was no evidence of nitrous oxide reductase activity in fractions 1 to 50 at which point flow was

Table 1

GEL PERMEATION MOLECULAR WEIGHT ESTIMATES FOR NITROUS OXIDE REDUCTASE AND HIGH MOLECULAR WEIGHT COPPER PROTEIN FROM DENITRIFYING BACTERIA

Organism	Molecular Weight ($\times 10^{-3}$) with the number of separate determinations in parentheses	
	Nitrous Oxide Reductase	Copper Protein
Pa. denitrificans	89 ± 3 (4)	132 ± 10 (2)
Ps. denitrificans	87 ± 5 (4)	127 ± 6 (2)
Ps. perfectomarinus	87 ± 4 (7)	111 ± 8 (4)

stopped. This result is in agreement with Kristjansson and Hollocher (5), who found that nitrous oxide reductase is strongly retained by commercial tertiary amine anion exchange materials.

It is clear that nitrous oxide reductase and the 120,000 MW copper protein are different and separable components in the three denitrifiers studied. The role of the copper protein is unknown, but it may be a unique electron transport component on the pathway to nitrous oxide reductase.

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