*Biochimica et Biophysica Acta*, 1144 (1993) 359–366



BBABIO 43885

# Nitric oxide reductase of *Achromobacter cycloclastes*

Alison M. Jones [1] and Thomas C. Hollocher

*Department of Biochemistry, Brandeis University, Waltham, MA (USA)*

(Received 4 January 1993)
(Revised manuscript received 28 April 1993)

Key words: Nitric oxide reductase; Cytochrome *bc*; Nitrite reductase; Denitrification; Nitric oxide; (*A. cycloclastes*)

Nitric oxide reductase from *Achromobacter cycloclastes* was solubilized with dodecyl maltoside from membranes and substantially purified by hydroxyapatite column chromatography. Preparations of enzyme had purities estimated to be 65–72% and specific activities of about 3 μmol NO/min per mg protein when measured at 30°C and pH 5.5 using ascorbate/phenazine methosulfate as the reducing system. Preparations lacked nitrite reductase activity. The enzyme consists of two peptides of approx. 38 and 17.5 kDa, and is identified as a cytochrome *bc* complex for which the mol ratio of cytochrome *c* to cytochrome *b* is about 1:1. A heme *c* containing band of approx. 55 kDa would appear to be a 1:1 complex or compound of the 38 and 17.5 kDa peptides. These and other observations suggest that *A. cycloclastes* possesses a nitric oxide reductase similar to that previously purified from *Pseudomonas stutzeri* and *Paracoccus denitrificans*. In contrast to the cytochrome $cd_1$-type nitrite reductase of the latter two bacteria, the nitrite reductase of *A. cycloclastes* is a copper protein. This is the first reported characterization of nitric oxide reductase from a denitrifier with a Cu-containing nitrite reductase, and provides further evidence that the denitrification pathway in such bacteria requires nitric oxide reductase and proceeds by way of NO as an intermediate.

## Introduction

The classical view of denitrification envisioned NO as an intermediate between nitrite and $N_2O$ [1–4,53]. This point has been a difficult one to prove experimentally and some controversy may yet remain. Support for the participation of NO as an intermediate comes from several lines of evidence, as outline below.

Denitrifying bacteria possess considerable nitric oxide reductase activity [5–7] and NO respiration is associated with proton translocation [8,9]. Nitric oxide reductase is associated with the cytoplasmic membrane in *Alcaligenes faecalis* [10], *Pseudomonas stutzeri* [11–13], *Paracoccus denitrificans* [14–16], *Pa. halodenitrificans* [17] and *Rhodobacter sphaeroides* [18,19], whereas the heme- or Cu-containing nitrite reductases in these organisms are soluble enzymes. Nitric oxide reductase has been purified from *P. stutzeri* [13] and *Pa. denitrificans* [15,16] and is composed of two polypeptides with a molecular mass of 37–38 and 17–18 kDa that are associated with a cytochrome *b* and *c*, respectively. The product of nitric oxide reductase is $N_2O$, perhaps via nitroxyl (NOH) [20], and the enzyme is highly active with $k_{cat}/K_m > 10^7\ M^{-1}\ s^{-1}$ [16]. NO has been judged to be a free intermediate in the denitrification pathway on the basis of its kinetic competence and isotopic criteria in vivo [7]. In addition, the steady-state concentrations of NO during denitrification (10–60 nM) could be explained in terms of the sequential action of nitrite and nitric oxide reductases [21,22], with the latter enzyme being the more active and having a very low $K_m$ [21–23]. A substantial fraction of the nitrogen entering the denitrification pathway in vivo can be trapped or recovered as extracellular NO [21,23]. Mutants of *P. stutzeri* defective in the respiratory heme-containing nitrite reductase (cytochrome $cd_1$) remained normal in nitric oxide respiration [24], but those defective in nitric oxide reductase were absolutely blocked at NO [25], which could accumulate to lethal levels. Mutants defective in the respiratory Cu-containing nitrite reductase of *Pseudomonas* sp. strain G-179 were similarly normal in nitric oxide reductase [26]. It is clear, however, that mutants defective in cytochrome $cd_1$ need not necessarily leave the levels of nitric oxide reductase unaffected. Ye et al. [27] demonstrated recently that Tn*5* mutants in the cytochrome $cd_1$ gene of *Pseudomonas fluorescens* were also diminished by about

Correspondence to: T.C. Hollocher, Department of Biochemistry, Brandeis University, Waltham, MA 02254, USA.
[1] Present address: Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada.
Abbreviation: PMS, phenazine methosulfate (the methylsulfate salt of *N*-methylphenazonium).

2-fold in nitric oxide reductase activity, perhaps as an effect on the efficiency of down-stream transcription of the latter enzyme in the denitrification operon. The chelator, diethylthiocarbamate, can abolish the activity of the Cu-containing nitrite reductase in extracts of *Achromobacter cycloclastes* and *R. sphaeroides* without much inhibiting the nitric oxide reductase activity [28]. In addition, Shapleigh et al. [12] reported that Triton X-100 detergent can inhibit reduction of NO to $N_2O$ without effect on the reduction of nitrite to NO. In aggregate, the above results strongly suggest that nitrite and nitric oxide reductases of denitrifying bacteria are two physically distinctive enzymes.

Certain older isotope and kinetic results [5,6], which suggested that NO might be an enzyme-bound intermediate or was channeled from one enzyme to another, are now believed to have been due to artifacts related to gas transfer kinetics and the inhibition of NO uptake by NO itself [7].

An alternate pathway of denitrification, involving nitrosyl transfer to nitrite and entirely avoiding NO as an intermediate, was proposed by Averill and Tiedje [29]. It was proposed that nitrite reductase could activate nitrite to form an electrophilic nitrosyl ($NO^+$) donor species which would react with nitrite-N to form an enzyme-bound nitrite dimer, presumably $N_2O_3$. $N_2O_3$ would then be reduced at the active site to $N_2O$. It would appear, however, that the primary product in vitro of both the heme-and Cu-type nitrite reductases of denitrifying bacteria is NO [30–32,54], and this has been inferred to be the case also with nitrite reductases in vivo in cases where nitric oxide reductase was differentially inhibited or where its synthesis was not fully induced [7] and where it was abolished genetically [25]. On the other hand, there its much isotope data to show that nitrite reductases can catalyze anaerobic nitrosyl transfer reactions to N-nucleophiles (and water) and that the nitrosyl donor may lie on the denitrification pathway [30,33,34,54]. Additional support for the nitrite dimer mechanism comes from the observation [35,36,55] that denitrification and the nitrosation of azide are mutually competitive. Because the nitrosyl donor is presumed to be an enzyme-bound species derived from nitrite, competition implies involvement of a second molecule of nitrite in the denitrification process. But because of the high concentrations of nitrite and azide employed in these experiments and the accumulation of NO, the competitive action might arise from other causes, such as interactions at an effector site. It is significant in this regard that the Cu-containing nitrite reductase of *A. cycloclastes* (but not the heme-containing enzyme of other denitrifiers) can reduce nitrite to $N_2O$ in vitro but only in the presence of high concentrations of NO [32] and that removal of accumulated NO can inhibit nitrosyl transfer to azide in vivo [37]. These observations, and the recent observation that denitrifiers can catalyze $NO/H_2O$-$^{18}O$ exchange [38], suggest that NO may be able to stabilize the nitrosyl donor species on nitrite reductase, perhaps by mass action [32,37], and therefore permit the nitrite dimer pathway to occur under what might be considered to be physiologically extraordinary circumstances.

Although the weight of evidence presently favors the involvement of nitric oxide reductase and NO in the denitrification pathway, the situation with regard to denitrifiers that contain Cu-containing nitrite reductases remains somewhat perplexing. As mentioned above, the Cu-containing nitrite reductase of *A. cycloclastes* [39,40] can in fact reduce nitrite to $N_2O$ in presence of NO [32]; on the other hand, Goretski and Hollocher [7,23] reported evidence that the denitrifying pathway in this organism involves NO and nitric oxide reductase, which is present at high levels. Missing from that work however was a demonstration that *A. cycloclastes* contains an abundant and active nitric oxide reductase which is unambiguously different from nitrite reductase. Thus it was of interest to attempt to purify and characterize nitric oxide reductase from *A. cycloclastes*.

We report here that *A. cycloclastes* does indeed possess a nitric oxide reductase, and that this enzyme has characteristics typical of the nitric oxide reductases purified to date from two other denitrifiers which utilize the heme-containing nitrite reductase. These observations represent the first report on the physical characteristics of a nitric oxide reductase from a denitrifying bacterium with a Cu-type nitrite reductase.

## Materials and Methods

*Purification of nitric oxide reductase*

*Achromobacter cycloclastes* (ATCC 21921) was cultured semi-anaerobically in 6 liters of yeast extract-peptone medium [7] supplemented with 10 mM $KNO_3$ and 1 $\mu$M $CuSO_4 \cdot 7H_2O$ at 30°C for 18 h. Unless stated otherwise, subsequent steps were performed at 4°C using 50 mM potassium phosphate buffer (pH 7.0). Cells from cultures with $A_{660}$ values of about 0.85 were harvested, washed by centrifugation at $10\,000 \times g$ for 10 min, and resuspended in 20 ml of buffer containing 2 $\mu$g/ml of DNase. Phenylmethylsulfonyl fluoride (100 mM) dissolved in absolute alcohol was added to a final concentration of 1 mM immediately prior to cell breakage. Cells were disrupted by one passage through a French pressure cell (American Instrument, model 4–3398A) at 20.7 MPa (3000 psi). The suspension was centrifuged at $8000 \times g$ for 30 min to remove unbroken cells and heavy debris, and the resulting cell-free extract was centrifuged at $105\,000 \times g$ for 180 min. The supernatant fraction was discarded, and the pellet (membrane fraction) was suspended in buffer and cen-

trifuged again at 105 000 × *g* for 180 min. The supernatant fraction was again discarded and, using a tissue homogenizer, the pellet (washed membrane fraction) was suspended in buffer to a protein concentration of 1 mg/ml.

Dodecyl maltoside (Sigma) was slowly added with stirring to the washed membrane fraction to 0.01% (w/v), and the suspension was stirred for 30 min. This suspension was centrifuged at 105 000 × *g* for 60 min, the supernatant fraction was discarded, and the pellet was suspended in buffer to a protein concentration of approx. 1 mg/ml. More dodecyl maltoside was added with stirring to a final concentration of 0.05% (w/v) and the mixture was stirred for 30 min. The mixture was again centrifuged at 105 000 × *g* for 60 min, the supernatant fraction was discarded, and the pellet was again dispersed to a protein concentration of about 1 mg/ml. A second 0.05% dodecyl maltoside extraction was performed as described above and the resulting supernatant fraction was saved.

This supernatant fraction containing about 10 mg of protein was applied to an hydroxyapatite Bio-Gel HTP column (Bio-Rad, 1.5 × 3.5 cm) that had been equilibrated with 0.5 M potassium phosphate buffer (pH 7.0) containing 0.05% dodecyl maltoside. The column was washed with two bed volumes of this buffer at a flow rate of 0.5 ml/min, and then eluted at 0.075 ml/min with 20 ml of a linear gradient in potassium phosphate (0.5–1.2 M (pH 7.0)) in the presence of 0.05% dodecyl maltoside. The elution process was completed with passage of two column volumes of 1.2 M potassium phosphate/0.05% dodecyl maltoside (pH 7.0) at a flow rate of 0.075 ml/min. The fraction with greatest specific activity eluted at a phosphate concentration of about 1.05 M.

*Survey of detergents*

Various detergents were screened for their ability to solubilize and thus extract nitric oxide reductase activity from the membrane fraction. Aliquots (0.17 ml) of washed membrane fraction (1 mg protein/ml) were adjusted to the desired detergent concentrations, incubated at 4°C for 30 min, and then centrifuged at 122 000 × *g* for 10 min in the 18° rotor of a Beckman Airfuge. To monitor solubilization and stability of enzyme activity, assays for protein and nitric oxide reductase activity were performed on the membrane fraction before and after addition of detergent and on the supernatant fraction after centrifugation.

*Activity and purity*

At each step during purification, fractions were assayed for nitric oxide reductase activity. Absorption spectra were recorded and SDS-PAGE was carried out on selected column fractions. The gels were analyzed by densitometry in order to assess the relative abundance of peptide components.

*Assays.* Nitric oxide reductase activity was measured at 30°C with use of a Clark-type $O_2$/NO electrode (Pt/Ag, AgCl, Hansatech model DW-1) and ascorbate/PMS as the reducing system [16,41]. A mixed assay buffer was used that contained 150 mM each of Tris, potassium phosphate and sodium acetate [13,16]. This buffer was titrated with NaOH or glacial acetic acid to the desired pH, which was 5.5 for routine assays and diverse values for determination of the pH-rate profile. Because the nitric oxide reductase of *A. cycloclastes* exhibited substrate inhibition, just as did the enzyme from *Pa. denitrificans* (see Fig. 3A of Ref. 16), the kinetic progress curves observed during assays showed increasing rates with decreasing NO concentrations. Rates for enzymatic reduction of NO herein are average rates based simply on the time required to reduce 95% of the initial amount of NO [16], and not maximum rates. Maximum rates were about 3-times greater than average rates. The saturated (2 mM) solution of $NO_{aq}$ used to provide the NO for the assay was prepared by passing tank NO (Matheson) first through a column of NaOH pellets to remove traces of $NO_2$, and then bubbling through anaerobic buffer [16,41]. Nitrite reductase was assayed at pH 7.1 by following the rate of reoxidation of NADH-reduced phenazine methosulfate after the method of Robinson et al. [42], except that the initial excess of NADH over phenazine methosulfate was small.

Protein was determined by the bicinchoninic acid method (reagents from Pierce) using bovine serum albumin as the standard [43].

*Spectra*

Absorption spectra of column fractions were recorded at 25°C using a Perkin-Elmer model 559 UV/visible spectrophotometer. For wavelength calibration, horse heart cytochrome *c* (Sigma) and holmium oxide glass (Dow-Corning) were used. Chemical reduction and oxidation of cytochromes was achieved by use, respectively, of sodium dithionite and air or potassium ferricyanide.

*Electrophoresis*

SDS-PAGE was performed using the buffer system of Laemmli [44] and a mini-gel apparatus (Idea Scientific). Protein samples for gel electrophoresis were concentrated with use of Centricon-30 filter tubes (Amicon) and then dialyzed with stirring against deionized water in a Mega System Microdialyzer (Heath Products) which contained a Spectrapore membrane (Spectrum) having a cutoff at 6–7 kDa. Samples were electrophoresed with or without having been previously heated to 100°C in the sample buffer which contained SDS and mercaptoethanol. Electrophoresis was rou-

TABLE I

*Subcellular distribution of nitric oxide reductase activity in A. cycloclastes*

| Fraction | Specific activity (μmol NO/min per mg) | Total protein (mg) | Total activity (μmol NO/min) | (%) |
|---|---|---|---|---|
| Cell-free extract | 0.16 | 498 | 82 | |
| Soluble fraction | 0 | 329 | 0 | 0 |
| Membrane fraction | 0.38 | 146 | 55 | 100 |

tinely carried out at 200 V, except for gels subjected to heme staining, for which 100 V was used. In the latter case, the sample and running buffers contained 2 mM EDTA and the sample buffer lacked mercaptoethanol. Gels were stained for protein with Coomassie blue R-250 [44], and for heme with 3,3′,5,5′-tetramethylbenzidine/$H_2O_2$ [45]. An ISCO gel scanner (model 1312) was used for gel densitometry.

## Results

*Enzyme activities*

All of the nitric oxide reductase activity was associated with the membrane fraction of *A. cycloclastes* grown anaerobically on nitrate (Table I), and the enzyme was absent from cells grown aerobically. In comparison, 90% and 10% of the nitrite reductase activity was found, respectively, in the soluble and membrane fractions (specific activity was about 0.3 μmol nitrite/min per mg in the cell-free extract). Nitrite reductase activity was barely detectable in the washed membrane fraction and was not detected during the subsequent steps of the purification of nitric oxide reductase (Table II). These results are consistent with the fact that the Cu-containing nitrite reductase of *A. cycloclastes* is a soluble enzyme [39,40].

*Survey of detergents*

To obtain profiles for the release of nitric oxide reductase activity from the membrane fraction, three non-ionic detergents (Triton X-100, octyl glucoside and dodecyl maltoside) were tested from 0.01 to 1.0% (w/v). Triton X-100 was judged to be unsuitable for the enzyme from *A. cycloclastes* due to extensive losses of nitric oxide reductase activity that occurred at levels of detergent sufficient to solubilize the enzyme. Dodecyl maltoside and octyl glucoside were effective in solubilizing the enzyme and caused tolerable decreases in its activity. Dodecyl maltoside was the preferred detergent, however, because at concentrations of only 0.05% (w/v) it accomplished a rather sharp differential solubilization of nitric oxide reductase from other proteins and resulted in no more than a 33% loss of activity. With octyl glucoside, concentrations 10–20-times greater were required for enzyme solubilization and loss of activity was somewhat greater.

*Purification*

The purification of detergent solubilized nitric oxide reductase of *A. cycloclastes* involved column chromatography on hydroxyapatite and was based on the method which Dermastia et al. [16] used to purify the enzyme from *Pa. denitrificans*. The results for a representative enzyme purification are summarized in Table II. Dodecyl maltoside at 0.01% was effective in solubilizing 44% of the total protein of the washed membranes and leaving all of the nitric oxide reductase activity in the pellet. A second extraction with 0.05% detergent solubilized 74% of the remaining protein and 30% of the enzyme activity. The final extraction, again at 0.05% detergent, released about 85% of the remaining activity but only about half of the protein. This three-step process resulted in a 2-fold increase in specific activity relative to the washed membranes (Table II) in spite of the moderate but unavoidable decrease in activity caused by detergent.

Nitric oxide reductase activity eluted at 0.9–1.2 M phosphate as a single peak from the hydroxyapatite column (Fig. 1). A protein peak eluted first, followed in order by the peaks for heme protein ($A_{410\ nm}$), activity and specific activity which peaked at fraction 21. About 65% of the activity applied to the column could be recovered in the eluate. The $V_{max}$-pH profile showed a maximum at pH 5.5 rather than at pH 5.0 as was the

TABLE II

*Partial purification of nitric oxide reductase from A. cycloclastes*

The value in parentheses is the specific activity corrected for an apparent enzyme purity of about 65%. DM, dodecyl maltoside.

| Stage | Specific activity (μmol NO/min per mg) | Total protein (mg) | Total activity (μmol NO/min) | (%) |
|---|---|---|---|---|
| Washed membrane | 0.58 | 81 | 47 | 100 |
| 0.01% DM cut: | | | | |
| Supernatant fraction | 0 | 36 | 0 | 0 |
| Pellet | 0.89 | 45 | 40 | 85 |
| 1st 0.05% DM cut: | | | | |
| Supernatant fraction | 0.21 | 52 | 11 | 23 |
| Pellet | 1.4 | 18 | 25 | 53 |
| 2nd 0.05% DM cut: | | | | |
| Supernatant fraction | 1.2 | 11 | 13 | 28 |
| Pellet | 0.23 | 11 | 2.5 | 5.3 |
| Column fractions: | | | | |
| 19 | 1.8 | 0.82 | 1.5 | 3.2 |
| 20 | 2.7 | 0.56 | 1.5 | 3.2 |
| 21 | 3.0 (4.6) | 0.36 | 1.1 | 2.3 |
| 22 | 1.2 | 0.26 | 0.31 | 0.66 |

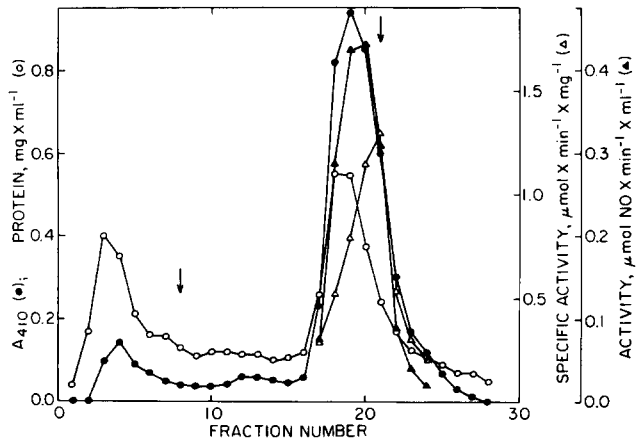


Fig. 1. Elution profile for nitric oxide reductase from the hydroxyapatite column. The column was developed with a linear gradient in potassium phosphate ((pH 7.0) 0.5–1.2 M) with 0.05% dodecyl maltoside. The arrows at left and right indicate, respectively, the start and end of the gradient elution.

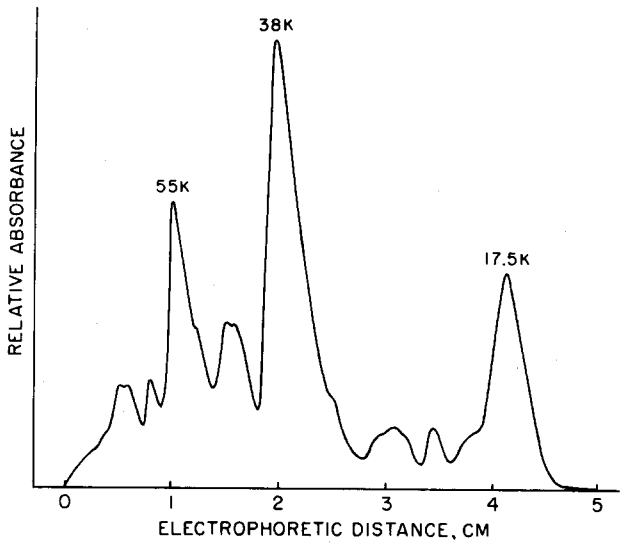


Fig. 2. Densitometric analysis of SDS-PAGE of nitric oxide reductase, fraction 21 of Fig. 1 and Table II, after staining with Coomassie blue. Zero distance corresponds to the beginning of the running gel. The densitometer was set at a sensitivity of 0.5 on the absorbance scale, rise time was 2.5 s and scan speed was 2.5 cm/min. The area integral provided by the components of 17.5, 38 and 55 kDa was estimated to be 65% of the total integral from 0–5 cm. The protein sample was not heated in the electrophoretic buffer prior to electrophoresis. The peptides at 17.5 and 55 kDa stained strongly for heme, whereas that at 38 kDa stained only weakly for heme.

case with the enzyme from *Pa. denitrificans* [16], but otherwise the profiles were very similar (data not shown). Column fractions 19–22 lost not more than 10% of their activity over 24 h at 0°C, and were stable for at least 3 weeks in liquid nitrogen. Evidence presented below suggested that enzyme in the fraction with greatest specific activity was between 65 and 72% pure.

*Electrophoresis*

SDS-PAGE of fractions 20–22 showed after staining two major peptides of 38 and 17.5 kDa (Fig. 2). These are also the molecular mass values, respectively, of the cytochrome *b* and *c* subunits of nitric oxide reductase from *P. stutzeri* [13] and *Pa. denitrificans* [15,16]. As with the enzyme from *Pa. denitrificans*, the 38 kDa peptide was not detected in samples previously heated to 100°C in the electrophoretic sample buffer and the 17.5 kDa peptide stained strongly for heme. The third most intense electrophoretic band had a molecular mass of 55 kDa, stained for heme and is presumed, by analogy with the enzyme from *P. stutzeri* [13], to be a 1:1 complex or compound between the 17.5 and 38 kDa peptide. The column fractions clearly contained proteins with heme groups that dissociate in SDS, because heme stain revealed the presence of free heme migrating near the electrophoretic front [16]. The mol ratio for the 17.5 to 38 kDa peptides in the fraction with maximum specific activity was 1:1 by densitom-

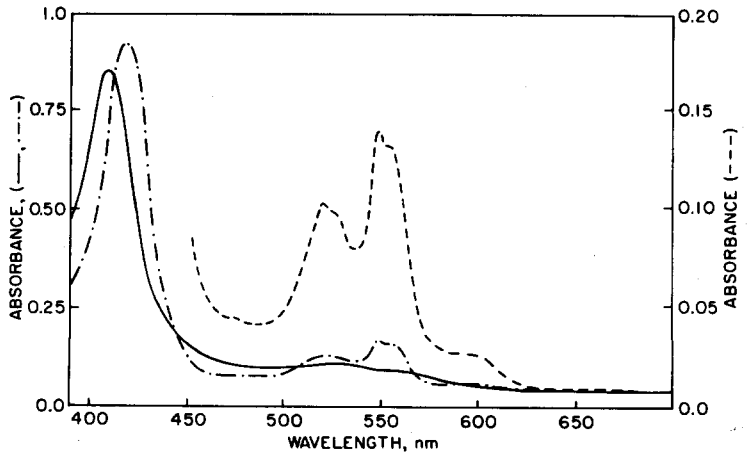


Fig. 3. Absorption spectra of nitric oxide reductase in a mixture of fractions 18 and 19. Solid curve, protein as prepared; dashed and dot-dashed curves, reduced with a few crystals of sodium dithionite. Protein concentration was about 0.55 mg/ml.

etry with an error of about 10% (mass ratio was about 1:2 (Fig. 2)). The densitometric integrals under the 17.5, 38 and 55 kDa bands (Fig. 2) represented together about 65% of the total integral for fraction 21 of Table II and Fig. 1. This apparent level of purity was typical over several enzyme preparations.

The 17.5 and 38 kDa peptides could be clearly identified in gels following SDS-PAGE of the washed membrane fraction, in spite of the large number of other peptides present. Densitometry suggested that these two peptides could comprise together 5% very roughly of the total membrane proteins. On this basis, a 20-fold increase in specific activity might be realized for the complete purification of nitric oxide reductase, assuming that it is in fact a cytochrome *bc* complex [13,15,16]. The largest increase in specific activity that our data admit is about 8-fold (Table II; $3.0/0.58 \times 0.65 = 8$ where 0.65 represents the apparent electrophoretic purity of the enzyme). A substantial part of the difference between 8- and 20-fold increases in specific activity can be accounted for by the depression of activity caused by the detergent.

*Spectra*

The column fraction with highest specific activity exhibited a reddish color and spectra characteristic of cytochromes *b* and *c* (Fig. 3). The enzyme as prepared (oxidized) showed a strong Soret band at 412 nm and broad, weak bands at about 525 and 551 nm. Upon reduction with dithionite, strong bands were observed at 420, 523 and 551 nm, a prominent shoulder with inflection of slope ($dA^2/d^2\lambda = 0$) at 559.5 nm * and a lesser band at about 600 nm. The 551 and 559.5 nm bands are ascribed to the reduced $\alpha$-bands of a cytochrome *c* and *b*, respectively. The reduced Soret band at 420 nm was unusually broad and had a truncated appearance, as one would expect if the Soret bands of a cytochrome *b* and *c* overlapped but were separated by 8–12 nm. Based on published extinction coefficients for the reduced $\alpha$-band of well-characterized cytochromes *b* and *c* [46–51], the mol ratio of cytochrome *c* to *b* was estimated to be about 1:1 in the fractions having maximum activities. The heme proteins of the nitric oxide reductase preparation underwent autoxidation only slowly in the presence of air. The preparation also exhibited little or no oxidase activity when air replaced NO in the amperometric assay system. In these regards, the enzyme of *A. cycloclastes* and *Pa. denitrificans* are similar.

Using a cytochrome *c/b* mol ratio of 1, a weighted value of 112000 $M^{-1}cm^{-1}$ for the extinction coefficient of the composite oxidized Soret band [16], molecular masses of 38 and 17.5 kDa for, respectively, the cytochrome *b* and *c* subunits, and the measured values for $A_{412}$ and protein concentration, fraction 21 was calculated to have a purity of about 72% with respect to the sum of cytochromes *b* and *c*.

*Comparative specific activities*

For the column fraction with maximum specific activity, values of 4–5 $\mu$mol NO/min per mg were commonly obtained (Table II) after having corrected for protein impurities. These values are 4–5-times lower than those reported for nitric oxide reductase for *Pa. denitrificans* [16] for which enzyme activities were measured in the same apparatus and under essentially the same conditions.

## Discussion

This study demonstrates that *A. cycloclastes*, a denitrifier with a Cu-containing, soluble, nitrite reductase, also contains a separate, membrane-bound, nitric oxide reductase at high levels of activity. This nitric oxide reductase was substantially purified from the membrane fraction by a simple and rapid method involving a single column and has characteristics similar to those of the enzyme from *P. stutzeri* [13] and *Pa. denitrificans* [15,16]. The enzyme from *A. cycloclastes* would appear to be a hydrophobic cytochrome *bc* complex composed of two different peptides. In its spectrophotometric characteristics, strong retention on hydroxyapatite, peptide molecular mass values and lack of oxidase activity, it is clearly distinguishable from the respiratory cytochrome $bc_1$ complex and *co*-type cytochrome oxidases [13,15] which are also examples of membrane-associated cytochrome *bc* complexes of bacteria.

The specific activity of the membrane fraction from *A. cycloclastes* was observed to be very similar to that of the membrane fraction from *Pa. denitrificans* [16], thus suggesting that the cellular levels of nitric oxide reductase activity can be essentially the same between denitrifiers that contain the heme- or Cu-containing nitrite reductase. Nitric oxide reductase from *A. cycloclastes*, although similar to the enzyme previously purified in most ways, differed in at least four particulars: (a) Both spectra and SDS-PAGE showed that the *A. cycloclastes* enzyme was relatively enriched in cytochrome *b* (38 kDa peptide). The cytochrome *c/b* (17.5/38 kDa peptide) mol ratios were about 1, 1.7 and 2 for the purified enzyme from *A. cycloclastes, Pa. denitrificans* and *P. stutzeri*, respectively [13,16]. (b) The dithionite reduced enzyme from *A. cycloclastes* exhibited a spectral band at 600 nm which was not

* The inflection of slope was reported to be located at 562 nm with the nitric oxide reductase from *Pa. denitrificans* [16], but the correct value is 559–559.5 nm. The maximum negative value of $dA^2/d^2\lambda$ is located at 562 nm.

observed with the enzymes from *P. stutzeri* and *Pa. denitrificans*. (c) The specific activity of the purified *A. cycloclastes* enzyme, when corrected for apparent degree of purity, was typically 4–5-times smaller than for the *Pa. denitrificans* enzyme in direct comparisons. (d) The pH optimum for activity was about 0.5 unit higher than for the enzyme from *P. stutzeri* and *Pa. denitrificans*.

It is not known at present whether the decrease of about 30% in specific activity of nitric oxide reductase brought about by dodecyl maltoside was due to inhibition or inactivation. If the specific activity were to increase upon removal of detergent or the replacement of detergent with phospholipid, the effect may be interpreted as inhibition [13]. Such experiments may be difficult, however, because of the precipitation of enzyme that attends the removal of detergent.

It should be noted that a nitrite reductase exists in *Wolinella succinogenes*, *Escherichia coli* and certain other bacteria that can in fact reduce nitrite to $N_2O$ [52] The hexaheme cytochrome *c* nitrite reductase has the primary role in *W. succinogenes* of catalyzing the dissimilatory reduction of nitrite to ammonia, but it has been demonstrated in vitro also to reduce nitrite to NO and NO to $N_2O$ at appreciable rates [52]. NO production seems to depend on the particular reducing system used and especially on its redox potential. In general, low potential reductants favor $NH_3$ production, whereas higher potential ones favor NO production. As far as we know, the hexaheme enzyme does not play a dissimilatory role in denitrifying bacteria, but it does play an essential dissimilatory role in *W. succinogenes*.

The results reported here are consistent with the idea that nitric oxide reductase is an enzyme of major importance in the denitrification pathway of all denitrifiers, including those which exploit the Cu-containing nitrite reductase, and is not limited to bacteria which use the heme-type nitrite reductase. This finding suggests that the major and perhaps only pathway of denitrification is one in which NO is an intermediate.

## Acknowledgement

This work was supported by Grant DCB 88–16273 from the National Science Foundation.

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