The nitric oxide reductase of *Paracoccus denitrificans*

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The nitric oxide (NO) reductase activity of the cytoplasmic membrane of *Paracoccus denitrificans* can be solubilized in dodecyl maltoside with good retention of activity. The solubilized enzyme lacks NADH-dependent activity, but can be assayed with isoascorbate plus 2,3,5,6-tetramethylphenylene-1,4-diamine as electron donor and with horse heart cytochrome *c* as mediator. Reduction of NO was measured with an amperometric electrode. The solubilized enzyme could be separated from other electron-transport components, including the cytochrome *bc*₁ complex and nitrite reductase, by several steps of chromatography. The purified enzyme had a specific activity of 11 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ and the $K_m^{\text{(NO)}}$ was estimated as less than 10 μM . The enzyme formed N₂O from NO with the expected stoichiometry. These observations support the view that NO reductase is a discrete enzyme that participates in the denitrification process. The enzyme contained both *b*- and *c*-type haems. The former was associated with a polypeptide of apparent molecular mass 37 kDa and the latter with a polypeptide of 18 kDa. Polypeptides of 29 and 45 kDa were also identified in the purified protein which showed variable behaviour on electrophoresis in polyacrylamide gels.

INTRODUCTION

Several recent investigations have shown that the reduction by denitrifying bacteria of nitrite to nitrous oxide (N₂O) involves nitric oxide (NO) as a free intermediate (Carr *et al.*, 1989; Goretski & Hollocher, 1988; Zafiriou & Hanley, 1989). In the case of *Paracoccus denitrificans*, it was shown that an inducible NO reductase was associated with the electron-transport system of the cytoplasmic membrane (Carr *et al.*, 1989). The activity was retained by cytoplasmic membrane vesicles. NADH could act as electron donor to NO in an antimycin- and myxothiazol-sensitive reaction that was coupled to ATP synthesis (Carr *et al.*, 1989). The presence in cytoplasmic membrane vesicles of good rates of NO reductase activity, in contrast with the absence of the periplasmic nitrite and N₂O reductases (Ferguson, 1987), suggested that an NO reductase must have the characteristics of an integral membrane protein. Reports of limited fractionations of NO reductase activities from other bacteria have given the same impression, but, with the exception of the enzyme from *Pseudomonas stutzeri*, for which some limited molecular characterization had been reported (Zumft *et al.* 1987), the enzyme has proved refractory to characterization and purification. In the present paper it is shown that use of an appropriate detergent and assay permits the substantial purification and characterization of NO reductase from *P. denitrificans*.

MATERIALS AND METHODS

P. denitrificans NCIB 8944 was grown anaerobically in 20-litre batch culture with succinate as carbon source and nitrate as electron acceptor as described previously (Burnell *et al.* 1975). The cells were harvested and cytoplasmic membrane vesicles prepared as detailed elsewhere (Burnell *et al.*, 1975).

Membrane vesicles (300–500 mg of protein) were solubilized by resuspension in 50 mM-Tris/HCl/1 mM-benzamidine/0.01 % 2-phenylethanol, pH 8.0 at 4 °C, followed by addition with stirring of dodecyl maltoside to 6 mg·ml⁻¹. Stirring was con-

tinued at 4 °C for 30 min. Insoluble material was removed from the solubilized protein by centrifugation at 200 000 *g* and 4 °C for 1 h in a Beckman L7 centrifuge using a 70.1 Ti rotor. The orange-brown supernatant was carefully removed with a syringe fitted with a long needle.

A stock solution of NO was prepared as described by Carr *et al.* (1989). The amperometric NO electrode (Carr *et al.*, 1989; Zimmer *et al.* 1985) was used to determine the NO concentrations in solution, whereas the silver cathode N₂O electrode (Alefounder & Ferguson, 1982; Carr *et al.*, 1989) was used to monitor the conversion of NO into N₂O. The response of the NO electrode tended to vary over the period required for monitoring fractions eluted from chromatography columns. Consequently the following two-step calibration procedure was adopted so that any such drift would not distort the profile of eluted NO reductase activity. First, the electrode response was calibrated by titration with NADH in the presence of membrane vesicles as described by Carr *et al.* (1989). Insufficient NADH was added to reduce all the NO present. Subsequent addition of isoascorbate/DAD (2,3,5,6-tetramethylphenylene-1,4-diamine)/cytochrome *c*, the reductant used for assay of solubilized NO reductase (see the Results section), permitted calculation of the specific rate of NO reduction by the vesicles with this substrate. Rates of NO reduction by solubilized membrane fractions could subsequently be internally calibrated by addition of a known amount of membrane vesicles before all the NO had been reduced by the soluble material. The addition of membrane vesicles thus caused an increase in the rate of NO reduction. This increase was known in terms of a specific rate from the first step of the calibration procedure. The use of this information then allowed calculation of the specific rate of NO reduction by the solubilized material. Typically between 100 and 400 μg (protein) of freshly thawed vesicles that had been stored in 25 % (v/v) glycerol were used in this calibration procedure. Vesicles stored in this manner maintained a constant rate of NO reduction when tested over at least 12 h after thawing. The buffer used in such assays was 2 ml of 20 mM-NaH₂PO₄/Na₂HPO₄ (pH 7.0)/16 mM-glucose/catalase

Abbreviation used: DAD, 2,3,5,6-tetramethylphenylene-1,4-diamine; LDS, lithium dodecyl sulphate.

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(50 units)/glucose oxidase (4 units)/5 mM-sodium isoascorbate/0.3 mM-DAD/horse heart cytochrome *c*. (0.2 mg of protein). Once dissolved O_2 had been removed through the action of the glucose oxidase/catalase system, 100 μ l of a stock solution (held under positive pressure) of NO was added.

SDS and lithium dodecylsulphate (LDS)/polyacrylamide gels were run as described by Page & Ferguson (1989). Staining first with Coomassie Blue and then with silver was by the method of De Moreno *et al.* (1985). Haem staining was done as described by Thomas *et al.* (1976). Protein was determined by the method of Smith *et al.* (1985).

Spectra of NO reductase preparations were taken on a Aminco DW 2000 spectrophotometer.

DAD was purchased from Aldrich and recrystallized from ethanol. Dodecyl maltoside was from Boehringer Mannheim.

RESULTS

Selection of a suitable assay procedure

The amperometric NO electrode had already been established as very convenient for assay of NO reductase activity in whole cells or membrane vesicles (Zimmer *et al.*, 1985; Carr *et al.*, 1989). In those assays NADH, succinate or isoascorbate plus DAD was used as the reducing substrate, but in any purification procedure a reductant capable of donating electrons directly to the NO reductase was required. Possible alternatives to isoascorbate plus DAD that were tested included reduced Benzyl Viologens and isoascorbate plus phenazine methosulphate, but these donor systems gave unacceptably high rates of uncatalysed NO reduction. Consequently isoascorbate plus DAD was adopted as the reductant in assays of NO reduction, although it transpired (see below) that this system could not donate electrons directly to NO reductase, and thus horse heart cytochrome *c* had to be included as a mediator.

Selection of detergent for solubilization

Retention of high rates of NO reduction by cytoplasmic membrane vesicles suggested that the NO reductase would be a membrane-bound enzyme. The failure of NaCl concentrations as high as 800 mM to release NO reductase activity into a soluble fraction supported this view. Progress towards purifying the NO reductase from *P. denitrificans* depended upon identifying a suitable detergent for its solubilization. Some detergents were, as reported by Shapleigh *et al.* (1987) for Triton X-100, unsuitable because activity was almost completely lost. Dodecyl maltoside, as used by Berry & Trumpower (1985) for fractionation of the *P. denitrificans* plasma membrane, was found to be the most suitable detergent. The protein was effectively solubilized with good retention of activity (Table 1). However, the solubilized activity proved to be relatively unstable on storage at 4 °C with an approx. 50 % loss of activity over 2 days. The activity could be stabilized

by the inclusion of 0.01 % 2-phenylethanol, 1 mM-benzamidine and 0.1 μ M-pepstatin. EDTA did not provide any additional stabilization, whilst azide and phenylmethanesulphonyl fluoride had a deleterious effect on the activity.

Octyl thioglucoside was also effective at solubilizing the protein with good retention of activity. This detergent was evaluated for use in a purification of NO reductase because of its favourable critical micelle concentration and relatively low cost. However, it was found that this detergent could not be used in the presence of the salt concentrations that are needed for ion-exchange chromatography. Addition of salt caused considerable precipitation, especially at 4 °C, a feature not mentioned in publications advocating use of this detergent.

Ion-exchange chromatography of NO reductase

Preliminary trials established that activity that had been solubilized in dodecyl maltoside would bind to DEAE-Sephacel, but was not eluted until the NaCl concentration was raised to 400 mM, whereas a substantial amount of the remaining bound protein was eluted at 200 mM-NaCl. Consequently NO reductase was released from a DEAE-Sephacel column by elution with a gradient of NaCl (Fig. 1). Fractions with specific activities greater than 1 μ mol $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ were pooled and then concentrated on an Amicon PM 30 ultrafiltration membrane to a final volume of 4 ml. The specific activity of the pooled fractions increased 57-fold (other preparations were in the range 45–50-fold) over the activity of the solubilized membranes that were applied to the column. This increase is a little misleading, because the protein was applied to the column in dodecyl maltoside at 6 mg $\cdot$ ml⁻¹, but was eluted in the same detergent at 0.2 mg $\cdot$ ml⁻¹. The higher concentration of detergent allowed better extraction of protein from membrane vesicles, but inhibited NO reductase activity. Thus part of the increase in activity achieved by ion-exchange was a consequence of the dilution of the detergent. SDS/PAGE analysis showed that fractions con-

Fig. 1. Elution profile for NO reductase from DEAE-Sephacel

A DEAE-Sephacel column (2.6 cm $\times$ 12.5 cm) was equilibrated with 50 mM-Tris/HCl/1 mM-benzamidine/0.01 % 2-phenylethanol/dodecyl maltoside (0.2 g $\cdot$ l⁻¹), pH 8.0 (buffer A) at 4 °C, containing 190 mM-NaCl. Solubilized protein (300 mg) in buffer A supplemented with 190 mM-NaCl was applied to the column at a flow rate of 50 ml $\cdot$ h⁻¹. The column was subsequently washed with 200 ml of 190 mM-NaCl in buffer A to remove weakly bound protein. A 190–300 mM linear gradient of NaCl (158 ml) in buffer A was then applied. Development of the column was continued with 300 mM-NaCl in buffer A. Fractions were collected for 3 min. The absorbance at 280 nm due to protein was monitored continuously with a u.v. monitor.

Table 1. Summary of purification of NO reductase

Step	Total protein (mg)	Specific activity (nmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	Overall purification (fold)
Vesicles	420.0	272	—
Solubilized vesicles	300.4	89	—
DEAE-Sephacel	18.6	5130	19
Hydroxyapatite	14.5	6100	22.4
Gel filtration	3.5	12700	46.7

Fig. 2. Elution profile for NO reductase from hydroxyapatite

An Ultragel hydroxyapatite column (1.0 cm $\times$ 17.4 cm) was equilibrated with 50 mM-NaCl/1 mM-benzamidine/0.01 % 2-phenylethanol/100 mM-pepstatin A/dodecyl maltoside (0.2 g $\cdot$ l $^{-1}$) (solution A) in 150 mM-NaH₂PO₄, pH 7.0 at 4 °C. After exchange into the same buffer, 28 mg of protein collected from DEAE-Sephacel chromatography and containing NO reductase activity was loaded at 12.5 ml $\cdot$ h $^{-1}$. This was followed by washing the column with solution A supplemented with 150 mM-NaH₂PO₄, pH 7.0, to remove weakly bound protein. A 150–400 mM linear gradient of NaH₂PO₄, pH 7.0 (136 ml), in solution A was then applied. Development of the column was completed with 400 mM-NaH₂PO₄, pH 7.0, in solution A. Fractions were collected for 5 min. The absorbance at 280 nm due to protein was monitored continuously with a u.v. monitor.

Fig. 3. Elution profile for NO reductase from Sephacryl S300

A Sephacryl S300 HR column (2.6 cm $\times$ 98 cm) was equilibrated with 50 mM-Tris/HCl/100 mM-NaCl/1 mM-benzamidine/0.01 % 2-phenylethanol/dodecyl maltoside (0.2 g $\cdot$ l $^{-1}$), pH 8.0 at 4 °C. 4 ml (13.6 mg) of NO reductase, purified by sequential chromatography on DEAE-Sephacel and hydroxyapatite, was applied. The column was eluted at 11 ml $\cdot$ h $^{-1}$ in the same buffer. Fractions were collected for 15 min. Absorbance at 280 nm due to protein was monitored continuously with a u.v. monitor.

taining NO reductase activity also contained a complex mixture of polypeptides.

Hydroxyapatite chromatography

To allow protein to bind to hydroxyapatite, the NO reductase from the DEAE-Sephacel step had to be transferred to phosphate buffer. This was achieved by loading the concentrated enzyme on to a Sephadex G-25 (medium grade) desalting column (1.6 cm $\times$ 20 cm) containing 150 mM-NaH₂PO₄/Na₂HPO₄,

50 mM-NaCl, dodecyl maltoside (0.2 mg $\cdot$ ml $^{-1}$), pH 7.0 at 4 °C plus proteinase inhibitors as specified previously. Trial experiments had established that higher concentrations of phosphate were required for elution of the enzyme from hydroxyapatite. It was during such trial experiments that it was noted that, whereas the NO reductase activity of material applied to hydroxyapatite could be detected with isoascorbate plus DAD as reductant, the activity of the eluted material could be measured only if the assay was supplemented with horse heart cytochrome *c*. This comparison, therefore, indicates that the hydroxyapatite chromatography must have separated the NO reductase from one or more electron-transport components that could mediate electron transfer between reduced DAD and the enzyme.

Fig. 2 shows a typical elution profile for NO reductase activity from hydroxyapatite. The sharp peak of NO reductase activity eluted at 400 mM-phosphate was red in colour. Fractions with activities greater than 4 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$ were pooled and then concentrated to a final volume of 4 ml on an Amicon PM 30 ultrafiltration membrane. There was a small increase in specific activity (Table 1), but both non-denaturing and SDS/PAGE showed that a variety of polypeptides was present and that a further purification step was required. The loss of an electron-transport protein at this step may explain why the specific activity of NO reductase increased by only 1.2-fold, despite the removal, in this chromatography step, of half of the contaminating protein.

Gel filtration

The third stage of chromatography was gel filtration on Sephacryl S-300 (Fig. 3). Typically, 10–15 mg of protein was applied in 4 ml and eluted as four or five peaks. The second and largest peak contained the NO reductase activity. However, the highest specific activities were usually found in the trailing edge of this peak, suggesting contamination of the enzyme by the preceding protein. The two fractions with the highest specific activities were pooled and concentrated by ultrafiltration. This gave a further 2-fold purification (Table 1). Other preparations had similar activities at this stage, with the average being 11 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$. The active fraction from this gel-filtration column appeared homogeneous, as judged by both non-denaturing PAGE and isoelectric focusing. In the latter procedure the single band (pI 4.3) that stained for protein could also be identified by its red colour. SDS/PAGE indicated that as many as ten polypeptides were in the NO reductase fraction, which suggested that the protein may not be homogeneous. Consequently the enzyme was applied to an h.p.l.c. gel-filtration column.

Three peaks eluted from a Protein-Pak 300SW gel-filtration column (Fig. 4), with the largest, in terms of absorbance at 278 nm, containing the NO reductase activity. Only small samples could be applied to this column, but, by chromatographing several samples, sufficient pooled NO oxide reductase was obtained for further study. After a concentration step this material was orange-red in colour and ran as one large peak with only two very small other peaks upon reapplication to the h.p.l.c. column. This strategy permitted the acquisition of several hundred micrograms of NO reductase protein, which was used for characterization of the enzyme that is described here. The h.p.l.c. step led to a significant simplification of the pattern of polypeptides observed upon analysis by SDS/PAGE, as is shown below.

Purified NO reductase catalyses reduction of NO to N₂O

It has been shown previously that, at an appropriate polarizing voltage, a silver-cathode electrode responds to both NO and N₂O (Carr *et al.*, 1989). This electrode was used to study whether the

Fig. 4. H.p.l.c. gel-filtration profile for NO reductase

A portion (20 μ l; 40 μ g of protein) of NO reductase collected from the Sephacryl S300 HR column was applied, at 0.5 ml $\cdot$ min $^{-1}$, to a Protein-Pak 300 SW h.p.l.c. gel-filtration column equilibrated with 100 mM- NaH_2PO_4 / Na_2HPO_4 /dodecyl maltoside (0.2 g $\cdot$ l $^{-1}$), pH 7.0 at 25 $^{\circ}\text{C}$. The eluent was monitored continuously at 278 nm by means of a u.v. monitor. The central fractions were collected for each peak of protein eluted.

Fig. 5. Reduction of NO to N_2O by purified NO reductase

The reaction chamber of the NO electrode contained, in 2 ml: 20 mM- NaH_2PO_4 / Na_2HPO_4 , 16 mM-glucose, 50 units of catalase, 4 units of glucose oxidase, 0.2 mg of horse heart cytochrome *c*, 5 mM-sodium isoascorbate and 0.3 mM-DAD. At 'a', 100 μ l of NO solution, held at 20 $^{\circ}\text{C}$ and 101 kPa (1 atm), were added, followed by, at 'b', 20 μ l (2 μ g of protein) of NO reductase that had been equilibrated in an atmosphere of argon; 50 μ l (0.3 mg) of deoxygenated cytoplasmic membrane vesicles from *P. denitrificans* were added at 'c', followed by, at 'd', 2 μ l of N_2O -saturated solution at 20 $^{\circ}\text{C}$ and 101 kPa. The drift of the electrode record before addition of the reductase was ascribed to loss by diffusion of NO from the reaction chamber of the electrode. This loss of NO by diffusion was taken into account and thus the NO remaining at 'b' was taken for the calculation of the stoichiometry of NO reduction to N_2O . The rate of loss of NO declines as the NO concentration decreases and as NO is reduced by the reductase activity, and therefore no correction is made for loss of NO after addition of the enzyme. The baseline established before addition of NO applies to calculation of both NO and N_2O concentrations.

purified enzyme catalysed the stoichiometric reduction of 2NO to give 1 N_2O with isoascorbate/DAD/cytochrome *c* as electron donor. Fig. 5 shows that a residual signal was observed after reduction of NO catalysed by the purified enzyme. The residual signal is attributed to N_2O , because it was not diminished by

Fig. 6. Visible absorption spectra of NO reductase

Cuvettes of 1.5 ml capacity and fitted with a Suba-Seal contained 100 μ g of NO reductase, obtained after two steps of h.p.l.c. chromatography, in 1 ml of 50 mM-Tris/HCl/dodecyl maltoside (0.2 g $\cdot$ l $^{-1}$), pH 8.0 at 25 $^{\circ}\text{C}$. The contents of the cuvette were stirred and a train of argon gas was continuously passed over the surface of the enzyme solution. a, Enzyme as prepared; b, after addition of 8 μ l of sodium ascorbate (100 mg $\cdot$ ml $^{-1}$); c, after addition of 8 μ l of $\text{K}_3\text{Fe}(\text{CN})_6$ (1 mg $\cdot$ ml $^{-1}$); d, after addition of 8 μ l of sodium dithionite (40 mg $\cdot$ ml $^{-1}$) in 10 mM-NaOH.

addition of membrane vesicles, which can reduce NO but not N_2O . Calibration of the electrode response by addition of a known volume of N_2O -saturated buffer showed that NO had been reduced to N_2O with close to the expected stoichiometry of 2:1. Fig. 5, together with related experiments using the gold-cathode electrode, which responds solely to NO, also shows that the rate of NO reduction was linear essentially until the limit of reliable detection by the electrode was reached. From analysis of such linear rates of NO reduction and consideration of the sensitivity of the electrode, it can be estimated that the K_m for NO must be 10 μM or less in this assay system.

Spectroscopic properties of NO reductase

Fig. 6 shows the spectra of enzyme purified with h.p.l.c. as the final step. Four sets of conditions were used: (a) as prepared; (b) with sodium isoascorbate; (c) with potassium ferricyanide; (d) with sodium dithionite. The enzyme was purified in a near-fully-oxidized form, as judged from the similarity of its spectrum to that obtained in the presence of ferricyanide (cf. Fig. 6, a, with Fig. 6, c). Addition of either sodium dithionite or sodium isoascorbate reduced the oxidized enzyme, with the former being more effective (Fig. 6), whereas addition of potassium ferrocyanide had no effect (result not shown). The ability of ascorbate, but not ferrocyanide, to reduce the enzyme suggests that the midpoint potentials of the detectable redox groups lie closer to that of ascorbate (+58 mV) than that of ferrocyanide (+420 mV).

Spectra of the reduced enzyme indicated the presence of b- and c-type haems, as judged by the absorption maxima at 558.8 nm and 552.2 nm respectively (Fig. 6). This was partially substantiated by acetone extraction of the b-type haem and production of a pyridine haemochrome. The reduced-oxidized

Fig. 7. Effect of NO on the spectra of oxidized NO reductase

The conditions were as described in Fig. 6. Spectra are: a, enzyme as prepared; b, after addition of 100 μ l of NO-saturated buffer.

spectra of this haemochrome had maxima at 420.5 nm, 524 nm and 556 nm, as expected for a *b*-type haem (Berry & Trumpower, 1987). It was not possible to detect the *c*-type haem by the pyridine-haemochrome method because, after extraction of the *b*-type haem, it proved impossible to identify conditions for solubilization of the protein to which the *c*-type haem would be attached. The spectra in oxidized and reduced forms shown in Fig. 6 were identical with those of the material that was applied to the h.p.l.c. column, thus indicating that the contaminants removed by the h.p.l.c. step did not contain haem or other chromophores that absorbed in the visible region.

Addition of nitric oxide to the enzyme as prepared (i.e. in the substantially oxidized form) resulted in a shift in the absorption maxima at 526.5 nm to 528.5 nm, and the shoulder at 559 nm became a peak at 560 nm (Fig. 7). The addition of NO also caused a decrease in absorbance of approx. 30% in the wavelength range 500–580 nm.

Apparent molecular mass of the NO reductase–dodecyl maltoside micelle

A Protein-Pak 300 SW column was calibrated with anionic protein standards. Subsequent passage through the column, after equilibration with buffer containing dodecyl maltoside, of a sample of NO reductase indicated that its apparent molecular mass was between 160 and 170 kDa.

SDS/PAGE of NO reductase

The polypeptide profile found after SDS/PAGE and staining with Coomassie Blue of the NO reductase purified with h.p.l.c. as the final step is shown in lane 1 of Fig. 8(a). The two most strongly stained bands were found at mobilities corresponding to molecular masses of 37 and 45 kDa. Neither of these polypeptides electrophoresed as a tightly focused band. This can be seen by comparison with lane 2 (Fig. 8a), which was taken from the same gel and shows many well-focused bands in the complex polypeptide profile found for the detergent-solubilized membranes. Several other fainter bands could be observed in lane 1, two of which, running with apparent molecular masses of 29 and 18 kDa, were relatively more strongly stained by silver (Fig. 8a, lane 3) than by Coomassie Blue. The results of the electrophoresis were strongly dependent on the manner in which the sample was prepared. If it was treated with dithiothreitol, with or without

Fig. 8. Electrophoresis of NO reductase in polyacrylamide gels in the presence of dodecyl sulphate

(a) Protein staining. Samples were prepared in 10 mM-Tris/HCl/12.5% (v/v) glycerol/1% (w/v) SDS/0.001% Bromophenol Blue and incubated at 25 °C for 30 min. Lanes 1 and 3 contained 50 μ g of protein of purified (after h.p.l.c.) NO reductase; lane 2 contained 395 μ g of protein of detergent-solubilized cytoplasmic membranes of *P. denitrificans*. Lanes 1 and 2 were stained for protein with Coomassie Blue. Lane 3 was stained sequentially with Coomassie Blue and silver. (b) Haem staining. Samples were prepared as follows: lane 1, 50 μ g of NO reductase in 10 mM-Tris/HCl/12.5% glycerol/1% LDS/0.001% Bromophenol Blue, pH 6.8, was incubated at 100 °C for 2 min before being applied to the gel; lane 2, as for lane 1, except that 150 μ g of protein was used and the sample was incubated for 30 min at 25 °C; lane 3, as for lane 1, except that the sample was incubated for 30 min at 25 °C.

boiling, then the bands at 37 and 45 kDa became very diffuse and almost merged. A number of higher-molecular-mass bands also became apparent, indicating that the reductase has a tendency to aggregate, even in the presence of SDS. The cytochrome *bc*₁ complex was also identified in the same later fractions obtained from the DEAE chromatography step (Fig. 1) as described by Berry & Trumpower (1985) or Xiaohang & Trumpower (1986). SDS/PAGE analysis of this material showed that it had the composition reported by Xiaohang & Trumpower (1986) and was distinct from the NO reductase in this respect as well as chromatographically.

Samples of NO reductase were also subjected to LDS/PAGE at 4 °C. The advantage of this procedure is that non-covalently-bound haem can be retained by a polypeptide and detected by a staining procedure. Fig. 8(b), lanes 2 and 3, shows that, when the reductase was electrophoresed without preheating, several bands stained positively for haem. When the sample was heated before the electrophoresis, only one band stained for haem (Fig. 8b,

lane 1). After such heating, non-covalently-bound *b*-type haem is lost, and consequently haem stains under such conditions indicate the presence of *c*-type haem. Therefore, the haem-staining band of higher mobility (excluding that which ran with the dye front in lanes 2 and 3, which is attributed to free haem) indicated a polypeptide carrying a *c*-type haem group, and the less-mobile bands must have carried a *b*-type haem. Subsequent staining with Coomassie Blue showed that the polypeptide carrying the *c*-type haem corresponded to the polypeptide of apparent molecular mass 18 kDa identified in Fig. 8(a), whereas the most mobile *b*-type haem band (Fig. 8b, lane 3) could be identified with the polypeptide of apparent molecular mass 37 kDa. The nature of the other polypeptides that stained for *b*-type haem (Fig. 8, lanes 2 and 3) is not clear, but they were relatively less prominent when the loading of protein was reduced (cf. lanes 2 and 3 in Fig. 8b). The polypeptide of apparent molecular mass 45 kDa did not clearly match with a band that stained for haem. In view of the tendency of NO reductase to aggregate, it is possible that these bands represented material that contained the 37 kDa polypeptide, but other explanations, including adventitious association of haem with polypeptides on the gel, or the presence of contaminating proteins that carry haem, cannot be eliminated. It should be noted that relative intensities of haem strains cannot be used as a reliable guide to the relative amounts of polypeptides present.

DISCUSSION

The preparation of NO reductase reported here was apparently homogeneous by two criteria even before the h.p.l.c. step that fractionated the material further. After this step the enzyme clearly contained *b*- and *c*-type haems and, if usual absorption coefficients apply, in approximately equimolar amounts. Four polypeptides are considered to be components of the enzyme. There are the polypeptides of apparent molecular masses 45, 37, 29 and 18 kDa. This assignment is made on the following basis. The polypeptides of 45 and 37 kDa are those which stain most strongly with Coomassie Blue on SDS/PAGE. The 37 kDa polypeptide also carries *b*-type haem, whereas only the 18 kDa polypeptide, which is more strongly stained by silver, carries the *c*-type haem. The fourth relatively prominent polypeptide seen by silver staining is that which would have a molecular mass of 29 kDa. There is no evidence that the 29 and 45 kDa polypeptides contain haem, but the possibility that *b*-type haem is not retained under the conditions of electrophoresis (Fig. 8b) cannot be definitely excluded. The sensitivity of the SDS/PAGE profile to the treatment of the sample before the electrophoresis indicates that the electrophoresis of the polypeptides of NO reductase is sensitive to the exact conditions used, and thus the apparent molecular masses obtained may not correspond to the true values, as is often found for membrane proteins. Some hydrophobic membrane proteins also stain weakly with Coomassie Blue and, as the NO reductase is clearly an integral membrane protein, this consideration may also apply to the current work. It is thus unreliable to assign polypeptides as contaminants on the basis of weak staining on SDS/PAGE.

While the present paper was in preparation, further details of the NO reductase from *Pseudomonas stutzeri* became available (Heiss *et al.*, 1989). The enzyme from this organism is reported to possess two polypeptide chains with relative molecular masses of 38 and 17 kDa, as judged by SDS/PAGE, with the former carrying a *b*-type haem and the latter a *c*-type haem. However, if the enzyme was not heated before electrophoresis, then a polypeptide of apparent molecular mass 45 kDa was observed, whereas in heated samples the 38 kDa polypeptide appeared as a diffuse band. Boiling before electrophoresis apparently also

caused aggregation, which was indicated by the appearance of a band that electrophoresed with an apparent molecular mass of 67 kDa. Thus, in common with the enzyme from *P. denitrificans*, it appears that the behaviour of polypeptides of NO reductase from *P. stutzeri* on SDS/PAGE is very dependent on the experimental conditions.

The results of staining LDS/ or SDS/PAGE gels for haem and protein suggest that the NO reductases of *P. denitrificans* and *P. stutzeri* are very similar to one another. The estimated molecular masses (about 170 kDa in each case) and the almost identical visible absorption spectra of the two enzymes reinforce the conclusion that the two enzymes are closely related. However, the stronger inhibition of the *P. denitrificans* enzyme by Triton X-100 (Shapleigh *et al.*, 1987) could be an indication of some structural difference between the reductases from the two organisms, and thus the enzyme from *P. denitrificans* could have four subunits, whereas that from *P. stutzeri* may have only two. The difference could also be related to the fact that the enzyme from *P. denitrificans* is active with a *c*-type cytochrome as electron donor, whereas this had not been demonstrated for the enzyme from *P. stutzeri*. Nevertheless, it may be premature to draw firm conclusions about polypeptide structure. There have been instances of membrane proteins being purified without their full complement of polypeptides. For example, the cytochrome *aa₃* oxidase of *P. denitrificans* was first thought to comprise only two polypeptides, but subsequent modified preparative procedures have provided evidence for a third subunit (Halita *et al.*, 1988). The combination of results obtained for NO oxide reductases of *P. denitrificans* and *P. stutzeri* clearly establishes *b*- and *c*-type haem respectively, borne by polypeptides with molecular masses 37–38 kDa and 17–18 kDa, as components of NO reductase. Although the results with *P. stutzeri* indicate that the 45 kDa band might correspond to a different electrophoretic behaviour of the 37 kDa band (Heiss *et al.*, 1989), we have not made any observations to support this view with *P. denitrificans* NO reductase. Very substantial further work is likely to be necessary to finalize the characterization of the polypeptide composition of the NO reductases, which will involve reconstitution of a fully functionally enzyme in a phospholipid bilayer and able to accept electrons from a physiological electron donor. It should also be noted that seven or eight polypeptides were reported recently, but without details of their apparent molecular masses, for a preparation of NO reductase from *Rhodobacter sphaeroides* f.sp. (forma species) *denitrificans* (Itoh *et al.*, 1989).

It is difficult to explain why Hoglen & Hollocher (1989) reported, as the present paper was being written, a preparation of NO reductase from *P. denitrificans* that differs so strikingly from that reported here. The latter authors found that NO reductase lacked haem centres and purified as large aggregates or vesicles (molecular mass approx. 1000 kDa). Further work will be necessary to resolve this discrepancy and to establish the relationship between the reported candidate peptide (34 kDa) for the catalytic subunit of the latter preparation with the polypeptides identified in the present work.

In view of the past arguments that a discrete NO reductase may not exist, it was important in the present work that the reductase was shown to be distinct from not only the nitrite and N₂O reductases but also the cytochrome *bc₁* complex. The latter complex was suspected as having a role in NO reductase in *R. sphaeroides* f.sp. *denitrificans*, but more recent work has indicated that the activity can be separated from this complex (Itoh *et al.*, 1989).

The assay used in the present work with isoascorbate plus DAD as electron donor depended upon the presence of horse heart cytochrome *c*. The latter is therefore assumed to have acted as electron donor to the NO reductase. As there is some similarity

between mitochondrial cytochrome *c* and cytochrome c_{550} of *P. denitrificans*, it is possible that the latter is the physiological electron donor to the reductase. This concept is supported by the evidence that the related cytochrome c_2 of *R. sphaeroides* f.s. *denitrificans* is active in transferring electrons to the NO reductase of that organism (Itoh *et al.*, 1989). The nature of the electron donor to the NO reductase of *P. denitrificans* needs to be investigated further. The low K_m observed for NO is indicative of use of an assay that mimics physiological conditions. Cells also reduce NO with a very low apparent K_m , which accounts for the failure of NO to accumulate during reduction of nitrate to N_2 (Carr *et al.*, 1989). The higher $K_m^{(NO)}$ (60 μ M) reported by Heiss *et al.* (1989) in their recent paper on *P. stutzeri* may reflect the use of an assay (ascorbate plus phenazine methosulphate as electron donor) that less closely corresponds to the physiological conditions.

The preparations of NO reductase reported here and by Heiss *et al.* (1989) contain both *b*- and *c*-type haem. NO has strong affinity for haem centres, and thus it is not unexpected that NO reductase is a haem protein. However, neither in the present work nor in that of Heiss *et al.* (1989) is there evidence that these haem centres are directly involved in the reduction of NO. Conceivably they could be present in order to provide a pathway for electron flow to the centre at which NO is reduced. The midpoint potential for reduction of NO to N_2O is +1175 mV. Thus the suggestion that the haem groups of the NO reductase have midpoint potentials lower than +420 mV is compatible with their possible role not only as direct electron donors in the reduction of NO but also as electron carriers between other components of the electron-transport system and the active centre of the reductase. The thermodynamics of NO reduction are compatible with electron donation to the reductase from the cytochrome *c* region of the electron-transport chain, as suggested previously (Carr *et al.*, 1989). A recent investigation with membrane vesicles strongly suggests that the NO reductase enzyme is active towards NO in the presence of O_2 (Carr & Ferguson, 1990), whereas some other enzymes of denitrification are inactive in the presence of O_2 (Ferguson, 1987). Activity in the presence of O_2 is reasonable, because NO can usually bind to haem centres with greater affinity than O_2 .

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REFERENCES

- Alefunder, P. R. & Ferguson, S. J. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1149–1155.
- Berry, E. A. & Trumpower, B. L. (1985) *J. Biol. Chem.* **260**, 2458–2467.
- Berry, E. A. & Trumpower, B. L. (1987) *Anal. Biochem.* **161**, 1–15.
- Burnell, J. N., John, P. & Whatley, F. R. (1975) *Biochem. J.* **150**, 527–536.
- Carr, G. J. & Ferguson, S. J. (1990) *Biochim. Biophys. Acta*, in the press.
- Carr, G. J., Page, M. D. & Ferguson, S. J. (1989) *Eur. J. Biochem.* **179**, 683–692.
- De Moreno, M. R., Smith, J. F. & Smith, R. V. (1985) *Anal. Biochem.* **151**, 466–470.
- Ferguson, S. J. (1987) *Trends Biochem. Sci.* **12**, 354–357.
- Goretski, J. & Hollocher, T. C. (1988) *J. Biol. Chem.* **263**, 2316–2323.
- Halita, T., Puustinen, A. & Finel, M. C. (1988) *Eur. J. Biochem.* **172**, 543–546.
- Heiss, B., Frunzke, K. & Zumft, W. G. (1989) *J. Bacteriol.* **171**, 3288–3297.
- Hoglen, J. & Hollocher, T. C. (1989) *J. Biol. Chem.* **264**, 7556–7563.
- Itoh, M., Mizukami, S., Matsuura, K. & Satoh, T. (1989) *FEBS Lett.* **244**, 81–84.
- Page, M. D. & Ferguson, S. J. (1989) *Mol. Microbiol.* **3**, 653–661.
- Shapleigh, J. P., Davies, K. J. P. & Payne, W. J. (1987) *Biochim. Biophys. Acta* **911**, 334–340.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, F. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
- Thomas, P. E., Ryan, D. & Levin, W. (1976) *Anal. Biochem.* **75**, 168–176.
- Xiaohang, Y. & Trumpower, B. L. (1986) *J. Biol. Chem.* **261**, 12282–12289.
- Zafriou, O. C. & Hanley, Q. S. (1989) *J. Biol. Chem.* **264**, 5694–5699.
- Zimmer, W., Danneberg, G. & Bothe, H. (1985) *Curr. Microbiol.* **12**, 341–346.
- Zumft, W. G., Gotzmann, D. J., Frunzke, K. & Viebock, A. (1987) in *Inorganic Nitrogen Metabolism* (Ullrich, W. R., Aparicio, P. J., Syrett, P. J. & Castillo, F., eds.), pp. 61–67, Springer-Verlag KG, Berlin.

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