Purification and initial kinetic and spectroscopic characterization of NO reductase from Paracoccus denitrificans

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Abstract

A new and relatively simple procedure to purify NO reductase from Paracoccus denitrificans by using the detergent lauryl maltoside has been developed. The purified enzyme consists of two subunits according to SDS polyacrylamide gel electrophoresis. Analysis of the content of prosthetic groups indicates the presence of non-haem iron in addition to the presence of cytochromes yielding a stoichiometry of haem b/haem c/non-haem iron = 2:1:1. The optical spectrum of reduced NO reductase shows bands of low-spin haem c and haem b with \( \alpha \)-band absorbance maxima at 551 nm and 558 nm, respectively. The optical spectrum of oxidized NO reductase shows a broad absorbance band around 590 nm which disappears upon reduction. This latter absorbance is ascribed to a high-spin haem b (charge-transfer) transition. The presence of high-spin haem b is also indicated by the shifts observed in the optical spectrum of oxidized NO reductase in the presence of NO or in the spectrum of reduced enzyme after addition of CO. The main features of the EPR spectrum of the oxidized enzyme are resonances from a highly anisotropic low-spin haem b \( g_z = 3.53 \) and from an anisotropic low-spin haem c with \( g_{z,y,x} = 2.99, 2.28, 1.46 \), the two haems being present in an approximate 1:1 stoichiometry. Minor signals representing about 1\% of the enzyme concentration due to high-spin haem b \( g = 5.8-6.2 \) and a novel type of signal with \( g = 2.09 \) ascribed to high-spin non-haem ferric iron were also observed. The analysis of steady-state kinetic measurements of the NO reductase activity shows a sigmoidal relation between rate of NO reduction and NO concentration, consistent with a model describing sequential binding of two molecules of NO to the reduced enzyme. At high NO concentrations substrate inhibition occurs \( K_{d,apparent} = 13.5 \ \mu M \) suggested to be due to binding of NO to oxidized enzyme. The absence from the EPR spectrum of signals originating from ferric non-haem iron and ferric high-spin haem b in stoichiometric amounts with respect to the enzyme concentration is suggested to be due to an antiferromagnetic coupling between these two centers. The steady-state kinetic behaviour and the optical and EPR spectroscopic properties of the NO reductase are incorporated into a tentative structural and mechanistic model.

Keywords: Denitrification; Nitric oxide reductase; Non-haem iron; Cytochrome oxidase; Nitric oxide; (Paracoccus denitrificans)

Abbreviations: EDTA, ethylenediaminetetraacetic acid; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; PMS, phenazine methosulfate.
1. Introduction

Denitrification is the process in which oxy anions of nitrogen are converted to dinitrogen. The properties of the enzymes involved in denitrification and the regulation of their expression are best characterized in the gram-negative bacteria Pseudomonas stutzeri and Paracoccus denitrificans (and the closely related Thiosphaera pantotropha) (see [1–5] for recent reviews). Denitrification does, however, also occur in gram-positive bacteria [6], in archaeabacteria [7] and in fungi [8]. In gram-negative bacteria, complete denitrification requires a nitrate/nitrite antiporter to allow for uptake of nitrate into the cytoplasm (cf. Ref. [1]). Nitrate is reduced to nitrite by the nitrate reductase at the cytoplasmic face of the cytoplasmic membrane. Thereafter nitrite is transported to the periplasm where the remaining reduction steps to dinitrogen occur. The redox reactions of denitrification are:

\[
\begin{align*}
\text{NO}_3^- + UQH_2 & \rightarrow \text{NO}_2^- + UQ + H_2O & \text{NAR, nitrate reductase} \\
\text{NO}_2^- + c^{2+}/\text{Cu}^+ + 2H^+ & \rightarrow \text{NO} + H_2O + c^{3+}/\text{Cu}^{2+} & \text{NIR, nitrite reductase} \\
2\text{NO} + 2c^{2+} + 2H^+ & \rightarrow N_2O + H_2O + 2c^{3+} & \text{NOR, NO reductase} \\
\text{N}_2O + 2c^{2+} + 2H^+ & \rightarrow N_2 + H_2O + 2c^{3+} & \text{NOS, N}_2\text{O} \text{ reductase}
\end{align*}
\]

in which c stands for cytochrome c and Cu for the blue-copper protein pseudoazurin. Although the presence of NO as a free intermediate has been questioned for a long time there is strong direct evidence that NO is a kinetically competent intermediate, its steady-state concentration varying between 1–65 nM, dependent on the species [9–11]. The reducing equivalents consumed in the reduction of nitrate to dinitrogen are derived from ubi- or menahydroquinone either directly as in the reduction of nitrate to nitrite, or indirectly in the other steps via the membrane-bound bc$_1$-complex which reduces c-cytochromes and/or possibly blue-copper proteins like pseudoazurin. Part of the free energy of the reduction of nitrate to dinitrogen is converted into a proton-motive force across the cytoplasmic membrane owing to the electrogenic reduction of nitrate by the membrane-bound nitrate reductase and the electrogenic action of the bc$_1$-complex. From a physiological point of view, denitrification is the anaerobic counterpart of the mitochondrial and bacterial aerobic respiratory chains — i.e., the removal of reducing equivalents generated in metabolism is coupled to the generation of a proton-motive force which can be used to drive ATP synthesis, the transport of metabolites or satisfies other bioenergetic demands of the cell.

The expression of enzymes specifically involved in denitrification are subject to control [1–3, 5, 12–14]. Except in T. pantotropha, a close relative of Pa. denitrificans, which displays both aerobic and anaerobic denitrification [12], the enzymes of denitrification are induced exclusively under anaerobic conditions in the presence of nitrate.

The primary sequences of the enzymes involved in denitrification from various sources have been determined from the DNA sequences of the corresponding structural genes (see Refs. [1–3, 5] and references therein). In total, approx. 40 genes are necessary to carry out denitrification. These include the structural and regulatory genes and genes for the biosynthesis of specific prosthetic groups. The properties of the two types of nitrate reductases, of the two different types of nitrite reductase and of the periplasmic N$_2$O reductases have been discussed recently in detail [5, 15].

NO reductase is the least characterized enzyme involved in denitrification. NO reductase is a membrane-bound enzyme and has been purified in the presence of detergent from P. stutzeri [16], Pa. denitrificans [17, 18], Achromobacter cycloclastes [19] and recently at a very small scale from Th. pantotropha [20]. The purified enzyme apparently consists of two subunits, one with molecular mass of 16 kDa carrying haem c, one carrying haem b with apparent molecular mass of about 35 kDa. The two haems have been reported to be present in a 1:1 ratio in Pa. denitrificans and P. stutzeri [16, 21] and in 2:1 (b/c) ratio in T. pantotropha. [20]. Direct information on the mechanism of action of the enzyme is lacking, but suggestions on the mechanism have been made [4].

Recently, the gene encoding the NO reductase from P. stutzeri has been sequenced [22] and also that of Pa. denitrificans [23]. The primary sequence of the cytochrome b subunit indicates a highly hy-
drophobic enzyme with 12 membrane-spanning helices. A structural similarity between this subunit of NO reductase and between subunit I of cytochrome-c oxidase or, more generally, between subunit I of the members of the so-called superfamily of haem-copper oxidases has been noted [24–26]. Both NO reductase and the oxidases are suggested to contain a similar pattern of membrane-spanning helices (and at least cytochrome-c oxidase does so [27–29]). Furthermore, six histidine residues which coordinate to the prosthetic groups in cytochrome-c oxidase are conserved in the primary sequence. Three histidine residues serve as ligands to the high-and low-spin heme centers and three are coordinated to Cu (27–29 and Ref. [30] for an overview) in the oxidases. Both high-spin (haem b) and low-spin haems (haem b and c) may actually be present in NO reductase, but copper is not [16,22]. Within the superfamily of haem-copper oxidases, NO reductase shows the highest degree of sequence similarity with the so-called cbb_3-type or cb-type oxidases [24,25,31,32] which are usually expressed under semi-anaerobic conditions. The cb-type oxidases lack Cu_A, contain Cu_B and also contain several c-type cytochromes and b-type cytochromes.

The similarity in primary structure suggested to exist between NO reductase and the superfamily of haem-copper oxidases is intriguing and the question is whether this similarity extends to their respective mechanisms of action [33–36]. For example what are the similarities and differences between oxygen reduction chemistry and NO reduction chemistry, is NO reductase like the haem-copper oxidases capable to couple the reduction of two molecules of NO to the formation of a membrane potential and/or to proton translocation or not [3–5,37,38].

To answer these questions procedures to routinely produce pure NO reductase are required. Current procedures to purify Pa. denitrificans NO reductase yield small amounts of relatively impure enzyme [17,18]. In this paper a new and relatively simple intermediate scale procedure to purify NO reductase is described. The purified enzyme is shown to contain stoichiometric amounts of non-haem iron in addition to the b and c cytochromes. Some aspects of the steady-state NO reduction kinetics are presented as well as the optical and EPR spectroscopic properties of the oxidized NO reductase. On basis of these findings a structural and mechanistic model for NO reductase will be presented.

2. Materials and methods

2.1. Growth of Paracoccus denitrificans

Paracoccus denitrificans (Pd 1222) was grown anaerobically with succinate (25 mM) as carbon source and nitrate (50 mM) as terminal electron acceptor in the medium described in [39] in 120 litre batch cultures at 30°C with pH control (pH 7.0). The 120 l batch was inoculated from an anaerobic preculture of 5 l which was allowed to grow for 24 h. Similarly, the 5 l preculture was obtained after inoculation from an aerobic 1 l culture and grown for 24 h. The 1 l culture was obtained by inoculation from an aerobic BHI agar plate. Normally, it took about 2–3 days before denitrification was induced as evidenced by the formation of nitrogen bubbles in the culture. At OD_600 nm = 1.0 the 1 l culture was used for inoculation. In all culture media except for the 120 l batch culture, rifampicin was present at 20 μg/ml. Cell yield was 7.1 g (wet weight) per litre culture.

2.2. Preparation of membranes

After harvesting and centrifugation for 15 min at 5000 × g, the cells were stored as a pellet at −40°C. For use the cells were thawed and washed once with excess Tris buffer (200 mM Tris-HCl, pH 8.0). Portions of 200 g of cells (wet weight) were diluted with 200 ml Tris buffer, 10 mM MgSO_4; solid DNAse was added and the cells were subsequently broken by passing them twice through a French Pressure Cell at 10 MPa. The solution was centrifuged at 5000 × g for 10 min. Subsequently, the supernatant was centrifuged at 175000 × g for 90 min. The red pellet, mainly membranes, was washed once with 50 mM Tris-HCl (pH 8.0) and centrifuged as before.

The membranes were further purified by sonication. Membranes, at 10 mg/ml of protein, were suspended in 50 mM Tris-HCl (pH 8.0), 150 mM KCl and sonicated four times for 15 min in portions of 50 ml, while the suspension was cooled in ice. Subsequently, the membranes were centrifuged as
described before and washed once with excess 50 mM Tris-HCl (pH 8.0) and finally dissolved in this buffer and stored at $-40^\circ$C until use.

2.3. Purification of NO reductase

The purification was carried out at 4°C except for chromatography on hydroxyapatite which was performed at room temperature (21 ± 2°C). NO reductase was purified from sonicated membranes. Sonicated membranes were suspended to 10 mg/ml of protein in 50 mM Tris-HCl (pH 8.0), 0.02% phenethyl alcohol and cooled in ice. While stirring, lauryl maltoside was added to a final concentration of 0.6% from a 10% (w/v) stock solution. After incubation for 15 min on ice the solution was centrifuged at 175,000 × g for 60 min. The clear supernatant was applied to a DEAE-Biogel A column (a column volume of 100 ml per gram of protein extracted was used) equilibrated with 50 mM Tris-HCl (pH 8.0), 0.02% lauryl maltoside, 0.01% phenethyl alcohol. The column was washed with 1.5 column volumes with this buffer before a linear NaCl gradient (0–0.5 M) was applied over 5 column volumes. Active fractions (i.e., those with activity > 40% of the peak fraction) eluting between approx. 0.3–0.4 M NaCl were pooled and concentrated in Amicon 30K concentrator centripreps to about 5 ml.

The concentrated solution was applied to a Sephadex G 25 (Medium or Fine) column (50–100 ml) equilibrated with 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0), 0.02% lauryl maltoside, 0.01% phenethyl alcohol in order to remove salt and exchange the Tris buffer for phosphate. The red fractions were pooled, brought to 0.1% in lauryl maltoside and applied to a Bio-Scale Ceramic CHT20-I Hydroxyapatite column (Bio-Rad) equilibrated with 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0), 0.05% lauryl maltoside, 0.01% phenethyl alcohol. After washing with 2 column volumes a linear phosphate gradient (0.1–1.0 M) was applied. Although NO reductase tends to smear, most of the enzyme was recovered at high purity in the fractions eluting at 0.8–0.9 M of phosphate.

Pooled fractions were concentrated as described before and subsequently desalted in 0.5 ml portions on a PD10 column (Pharmacia) equilibrated with 20 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0), 0.05% lauryl maltoside. Before application to the PD 10 column, the concentrated protein was incubated for 15 min on ice with 5 mM CDTA, to remove adventitious iron, and 1 mM potassium ferricyanide, to oxidize the enzyme. Red fractions, omitting the tail fractions, were pooled and concentrated as described above. This concentrate constitutes the final preparation.

2.4. Enzyme activity assay

Continuous NO reductase activity measurements were made with a Clark electrode polarized at 0.85 V or with an IS0-NO electrode from World Precision Instruments. The Clark electrode was used for studies at high [NO] (10 µM–100 µM), the ISO-NO electrode for measurements below 20 µM NO. The Clark electrode also responds to the N$_2$O formed (in contrast to the ISO-NO electrode), but at 0.85 V polarization voltage the sensitivity for N$_2$O is about 25–times lower than that for NO, yielding a final signal amplitude (at zero [NO]) that was 2% or less than that of the initial response. NO reductase activity measurements were recorded with both electrodes simultaneously yielding very similar traces; differences between traces at the lower [NO] are due to some sensitivity of the Clark electrode for N$_2$O and also due to its longer response time ($\approx 3$ s). Note in Fig. 5 that the shape of the activity trace is sigmoidal and that the highest activity, which is 5–10-times higher than the initial activity, occurs at about 4–5 µM NO.

The reaction vessel was cylindrical in shape (3 cm long × 1.35 cm inner diameter). The Clark electrode was fitted from the bottom and stirring occurred from the side with a magnetic stirrer. The reaction vessel was closed with a movable cylindrical stopper — made gas tight with a rubber O-ring — in which the ISO-NO electrode was fitted and in which a small hole was bored that served as the entry port for the gasses as well for other additions made with a Hamilton syringe.

Measurements were made as follows. The reaction vessel was filled with about 3 ml of buffer (20 mM phosphate (pH 6.0), 10 mM ascorbate, 100 µM PMS and 20 µM horse heart cytochrome c), the stopper was placed on top of the vessel allowing a few millimeters of headspace to be present and nitrogen
was bubbled through the solution via a small needle through the entry port until anaerobiosis was obtained as registered by the Clark electrode. Subsequently gaseous NO was bubbled through the solution from a 95% He/5% NO containing cylinder until a stable reading (on both electrodes) was obtained indicating saturation of the solution with NO at this pressure. The needle was removed and the stopper was slowly moved downward while taking care that He/NO gas present in the headspace escaped via the entry port until the stopper reached the surface of the solution. The stopper was subsequently moved downward to the point where the reaction volume was equal to 2 ml. The reaction was now started by the addition of enzyme.

Reaction temperature was 21°C ± 1°C. Taking a value of 2 mM for the solubility of NO under these conditions, equilibration with a 95% He/5% NO gas mixture corresponds to a maximum solubility of 100 μM NO in solution, a value which was routinely used to calibrate the extent measured on the recorder and used in all calculations described here. Independent measurements of the recorder sensitivity in which aliquots of saturated solutions of pure NO were titrated showed linear response of the Clark electrode and the ISO-NO electrode up to 150 μM NO and yielded values for [NO] within ± 15% of those determined with the 95% He/5% NO gas mixture.

The enzyme activities reported in the Table 1 were measured at the point of maximal slope in the trace (see, e.g., Fig. 5). This way of measuring the activity leads to errors of ± 20% between duplicate determinations.

2.5. Analytical determinations

Non-haem iron was determined using the ferene method [40] except that the heating step was omitted because it led to the formation of a precipitate. The amount of iron present in the buffer used for the PD10 column was less than 15% of the total amount of iron and was subtracted from the total amount of iron determined for the NO reductase preparation. Pyridine haemochrome was determined exactly as described in [41] calibrating the wavelength scale with the pyridine haemochrome spectra of horse heart cytochrome c and of beef hemoglobin. Protein was determined using the BCA Protein Assay Reagent of Pierce.

2.6. Materials

Column materials were from Pharmacia except the Bio-Scale Ceramic CHT20-I Hydroxyapatite column which was from Bio-Rad. N₂, NO and the He/NO mixture were from Air Products. Lauryl maltoside was from Anatrace.

2.7. Miscellaneous

Optical spectra were recorded with an SLM Am-inco DW-2000 spectrophotometer or an HP-8452 photodiode array spectrophotometer. EPR spectra were recorded with a Varian E-9 spectrometer equipped with a home-built He-flow system. Prior to freezing, samples were made anaerobic to remove oxygen which, in wide field scans, yields signals (for example at $g = 1.5$) that interfere with $g_z$-resonances of low-spin haem centers. Polyacrylamide SDS gel electrophoresis (8–25% gradient gel) was performed at 6°C to prevent aggregation of cytochrome $b$ with a Pharmacia Fast System.

3. Results

3.1. Purification

NO reductase could be purified from the membranes of anaerobically grown Pa. denitrificans using the detergent lauryl maltoside for extraction and solubilization followed by anion-exchange chromatography and chromatography on ceramic hydroxyapatite (see Section 2 and Table 1). Membranes were sonicated before detergent extraction because their purity increased by 20–30% as judged from the increase in specific activity (data not shown). The NO reductase activity was found to be quite stable in lauryl maltoside, even though after the first addition of the detergent the enzyme activity dropped by about 40%. This may be the result of (partial) delipidation. More than 90% of the enzyme activity could be reproducibly extracted with a concentration of 0.6% lauryl maltoside, provided that
care was taken to bring the protein to a concentration of 10 mg/ml. Anion-exchange chromatography was tested with several different DEAE-type columns such as DE-52, DEAE-Sephacel, DEAE-Sepharose and DEAE-Biogel A. The yield on the first three DEAE-types were generally low (<15%) mainly due to the tendency of the protein to smear. Elution on DEAE-Biogel A yielded in general 35–45% of the NO reductase activity in the pooled peak fractions (cf. Table 1).

For further purification several types columns were tried including Mono Q, hydroxyapatite (non-ceramic). A low recovery and/or poor purification were obtained mainly caused by smearing of the NO reductase over the column. As shown in Table 1 ceramic hydroxyapatite worked well giving both a good yield and a good purification. The problem of smearing could be quite well controlled on this type of column by eluting at lauryl maltoside concentrations between 0.05–0.1%.

Table 1 shows the results of a typical NO reductase purification. In general purification factors ranging between 30–60 were observed, overall yields varied between 5–15% in purifications starting with 1–5 g of sonicated membranes. Part of the spread in these values is due to uncertainties inherent in the activity measurements (see Section 2). In most cases the purification factor of the DEAE-Biogel A column was somewhat higher than that in Table 1 and that of the hydroxyapatite column correspondingly lower.

The purity as indicated by the SDS gel pattern in Fig. 1 was constant between preparations. The SDS gel shows two main bands with apparent molecular masses of 37 kDa and 17 kDa, representing the cytochrome b and the cytochrome c subunits, respectively [21,22]. The discrepancy between the molecular mass of cytochrome b, 52.5 kDa according to the sequence of the gene [22], and the apparent molecular mass has been noticed before and is due to its hydrophobic character [22,42]. Boiling the sample before electrophoresis or incubation at 37°C or 65°C led to some increase in the band migrating with an apparent molecular mass of 65 kDa and to aggregation of cytochrome b (cf. [42]). A minor band in addition to the two main bands with molecular mass of 22 kDa was also present in the preparations.

3.2. Analysis of the content of prosthetic groups

Pyridine haemochrome determination of the purified enzyme indicated the presence of haem b and haem c in an approximate 2 to 1 ratio. Furthermore, colorimetric determination of non-haem iron indicated an amount similar to the amount of haem c. Assuming one haem c per NO reductase, the following stoichiometry was determined: haem c/haem b/non-haem Fe = 1.0:2.01 ± 0.07:1.14 ± 0.10. No copper or haem a were detectable in the final enzyme preparation.

### Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific activity (µmol NO/mg·min)</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>2000</td>
<td>0.59</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>High-speed supernatant</td>
<td>664</td>
<td>0.83</td>
<td>47.1</td>
<td>1.4</td>
</tr>
<tr>
<td>DEAE-Biogel A</td>
<td>186</td>
<td>1.24</td>
<td>19.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Hydroxyapatite + PD10</td>
<td>4.2</td>
<td>25.8</td>
<td>9.2</td>
<td>43.5</td>
</tr>
</tbody>
</table>

Fig. 1. SDS gel electrophoresis pattern of purified NO reductase. Left lane: 1.5 µg purified NO reductase; right lane: molecular mass standards (94 kDa, 67 kDa, 43 kDa, 30 kDa, 20 kDa and 14.4 kDa).
The haem c content of the final preparation was 13.8 nmol/mg protein. The purity of the enzyme based on the haem c content is 96% assuming a total molecular mass of 69.5 kDa as calculated from the DNA sequence [23].

3.3. Optical spectroscopy

Fig. 2 shows the room temperature absolute optical spectra of purified NO reductase in the oxidized state and fully reduced using dithionite. The spectra are characteristic for a haemoprotein. High absorbance in the oxidized and fully reduced enzyme in the \( \gamma \)-band region with maxima at 411 nm and 422 nm, respectively. Contributions from c- and b-haems are not well separated in this region of the spectrum. Separate absorbance maxima are, however, seen in the \( \alpha \)-band region of the dithionite reduced enzyme showing a maximum at 551 nm due to haem c and a shoulder at about 558 nm due to haem b. The sharpening and increase in intensity of the bands at 551 nm and 558 nm upon reduction strongly suggests that these absorbance maxima belong to low-spin haems c and b, respectively.

In the oxidized spectrum a broad absorbance is observed with a maximum around 590 nm. The intensity of this absorbance decreases upon reduction with dithionite as is clearly seen in the reduced minus oxidized difference spectrum. Based on its position in the spectrum, its relative intensity and the effect of reduction on its intensity, the 590 nm absorbance is ascribed to a (charge-transfer) transition of a high-spin cytochrome \( b \) [43]. A similar transition at 628 nm has been observed in the cytochrome \( cb \)-type oxidases [31,32] or the cytochrome \( bo \) quinol oxidase [45] and similar to CO-difference spectra obtained with ferrous myoglobin or hemoglobin [46]. Addition
of NO to the oxidized NO reductase induced a small shift in the \(\gamma\)-band region and led to an increase of absorbance at 535 nm and 565 nm and to a decrease in the 590–600 nm region of the spectrum. The largest effect was, however, observed at 350 nm. In addition to a change in the shape of this broad absorbance, most notably, its intensity was increased by about 50%.

Finally, the spectrum of dithionite reduced NO reductase sometimes showed a broad absorbance with low intensity around 615 nm (data not shown, but cf. Ref. [20]). This band is absent in completely reduced enzyme as obtained after two or three additions of excess solid dithionite and after prolonged incubation (Fig. 2) but was clearly visible in spectra obtained after short incubation periods or after addition of relatively small amounts of dithionite (data not shown).

### 3.4. EPR spectra of purified NO reductase

Fig. 4 shows the low-temperature EPR spectrum of NO reductase as isolated. The spectrum shows two signals due to anisotropic low-spin haem centers, one with \(g_z = 3.53\) (the corresponding \(g_y, g_x\) being too broad to be observed) and one with \(g_z = 2.99, g_y = 2.28, g_x = 1.46\). The signal with \(g_z = 3.53\) is highly anisotropic and has an asymmetric line shape very similar to the EPR signal of cytochrome \(b_1\) of the \(bc_1\) complex [47,48]; the signal is proposed to originate from a low-spin haem \(b\) with bis-histidine coordination. The signal with \(g_z, g_y, g_x = 2.99, 2.28, 1.46\) is ascribed to low-spin haem \(c\) with histidine-methionine coordination. The amount of haem \(c\) determined from the EPR spectrum employing single-peak \((g_z)\) integration is similar to that determined by the pyridine

![EPR spectra of purified NO reductase](image)

**Table 2**

<table>
<thead>
<tr>
<th>Species</th>
<th>(g)-value</th>
<th>Concentration (^a)</th>
<th>Stoichiometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-spin haem (c)</td>
<td>2.99, 2.28, 1.46</td>
<td>230.8 (\mu)M</td>
<td>1.00</td>
</tr>
<tr>
<td>Low-spin haem (b)</td>
<td>3.53</td>
<td>163.8 (\mu)M</td>
<td>0.71</td>
</tr>
<tr>
<td>High-spin haem</td>
<td>6.2–5.8</td>
<td>&lt; 2.4 (\mu)M</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-haem (Fe^{3+})</td>
<td>2.009</td>
<td>2.7 (\mu)M</td>
<td>0.01</td>
</tr>
<tr>
<td>Adventitious (Fe^{3+})</td>
<td>4.3</td>
<td>&lt; 0.8 (\mu)M</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The concentration haem \(c\) determined by the pyridine haemochrome method of the preparation used for EPR studies was 222.0 \(\mu\)M, that of haem \(b\) 430.5 \(\mu\)M (\(b/c = 1.94\)).
haemochrome method (see Table 2), the amount of cytochrome \( b \) calculated by integrating the area under the asymmetric \( g = 3.53 \) resonance and using the procedure outlined in [47] accounts for 0.71 haem per haem c.

The \( g = 4.3 \) signal is due to high-spin adventitious iron. The signal around \( g = 6.0 \) is due to a small amount of high-spin haem iron.

The signal with an apparent \( g \)-value at \( g = 2.009 \) is a new type of signal (Fig. 4, lower trace). Some structure or ‘asymmetry’ is visible in the signal. Its apparent \( g \)-value is outside the range normally observed for organic radicals (2.000–2.005). Further, its rate of relaxation is very high, unlike that of an organic radical; i.e., the signal was not saturated at full microwave power (200 mW) at 15 K. This latter property and the apparent \( g \)-value suggest that the signal is due to high-spin, non-haem iron. The \( M_S + 1/2 \Rightarrow M_S + 3/2 \) transition of such a 3d\(^5\) iron center in a tetrahedral or octahedral environment is known to yield an almost isotropic signal close to \( g = 2 \) in case the Zeeman interaction is the leading term in the spin Hamiltonian [49]. The positions of the other possible transitions, \( M_S + 1/2 \Rightarrow M_S + 3/2 \) and \( M_S + 3/2 \Rightarrow M_S + 5/2 \), are dependent on the value of the cubic splitting parameter \( a \) [49,50]. These transitions are not likely to be observed in a powder spectrum at this low concentration. Small deviations from perfect cubic symmetry would introduce more terms in the spin Hamiltonian such as zero-field terms described by the parameters D and E. As long as these parameters are small compared to the Zeeman interaction, the EPR spectrum might actually show some structure or asymmetry as observed for the \( g = 2.009 \) species.

Quantitation of the \( g = 6.0 \) and the \( g = 2.009 \) signals indicates that they are minor components (see Table 2). After reduction by excess dithionite the EPR signals present in Fig. 4 disappeared (cf. Ref. [16]).

3.5. Steady-state kinetics of NO reductase

An example of an NO consumption trace at high concentration of NO (100 \( \mu \)M) recorded with the Clark electrode is shown in Fig. 5A. Such a sigmoidal shape of the curve is displayed by NO reductase present in membranes as well as in purified preparations containing the detergent lauryl maltoside. The characteristic sigmoidal shape of the curve is independent of pH. The curvature of the activity trace indicates that the enzyme is inhibited at high concentrations of the substrate NO. As the concentration of NO decreases, the rate increases up to a maximum and finally decreases due to depletion of NO. The latter decrease is more clearly seen in Fig. 5B, recorded with the ISO-NO electrode at low (\( < 5 \mu \)M) NO concentrations.

The steady-state activity of NO reductase was not affected by \( \text{N}_2\text{O} \) at 2 mM nor by azide or cyanide (0.5 mM) or by EDTA or CDTA (5 mM). Addition of lauryl maltoside (0.6–1.0%) to membranes prior to measurement of the activity resulted in an inhibition by about 40%.

The steady-state kinetic properties of NO reductase as present in membranes were analysed from kinetic traces as shown in Fig. 5 by measuring the slope at different times and plotting these as rate versus [NO] for low concentrations of NO or as a Dixon plot; i.e.,

Fig. 5. Experimental NO consumption traces at high concentration of NO recorded with the Clark electrode (A) and at low concentrations of NO recorded with the ISO-NO electrode (B). In trace A the arrow marks the addition of an aerobic aliquot of NO reductase; the upward deflection is due to some oxygen present in the aerobic NO reductase preparation which is subsequently rapidly reduced by the NO leading to an initial rapid downward deflection. The rest of the trace shows the enzymic reduction of NO. In trace B only the last part of the NO consumption trace is shown. Closed circles in trace B indicate points where slopes were measured in order to calculate the rate. See also Section 2 for experimental details.
Fig. 6. Dixon plot (upper) and rate versus [NO] plot (lower) calculated from traces like those shown in Fig. 5. The upper curve contains data obtained from both the Clark electrode and the ISO-NO electrode traces, the points in the lower curve were measured from traces recorded with the ISO-NO electrode only. The lines through the points are simulations using Eq. 4 with the parameters listed in the text; (a.u.), arbitrary units.

1/(rate) versus [NO] (Fig. 6). The latter plot indicates that the substrate inhibition observed at the higher NO concentrations is fairly well approximated as competitive inhibition [51]. The plot of rate versus [NO] at low [NO] (Fig. 6) is sigmoidal as may be expected for the reaction in which two molecules of NO are required to form one molecule of N₂O. These considerations have been incorporated in the following minimal scheme to describe the steady-state kinetics of NO reductase and the phenomenon of substrate inhibition at higher [NO]:

\[
\begin{align*}
E_{\text{red}} + \text{NO} & \rightleftharpoons E_{\text{red}}(\text{NO}) + \text{NO} \rightleftharpoons E_{\text{red}}(\text{NO})_2 \\
& \xrightarrow{k_{\text{cat}}} E_{\text{ox}} + \text{N}_2\text{O} \\
E_{\text{ox}} & \rightarrow E_{\text{red}} \\
E_{\text{ox}} + \text{NO} & \rightleftharpoons E_{\text{ox}}(\text{NO})
\end{align*}
\]

(1)

(2)

(3)

Eq. (1) describes the overall reaction catalysed by the enzyme; the binding of NO is described by the two (equilibrium) dissociation constants \( K_1 \) and \( K_2 \), conversion to N₂O by \( k_{\text{cat}} \). In Eq. (2) the regeneration of reduced enzyme by the ascorbate/PMS/cytochrome c system is described; the rate of this reaction is assumed to be non-rate limiting and hence does not appear in the overall rate reaction (Eq. (4)). Substrate inhibition is described in Eq. (3) by the (equilibrium) inhibition constant \( K_i \); inhibition is proposed to be due to binding of NO to the oxidized enzyme. The simplest rate equation based on these assumptions is:

\[
v = k_{\text{cat}} \cdot e / \left( 1 + K_2 \left[ \frac{1}{[\text{NO}]} + K_1 / [\text{NO}]^2 \right] + [\text{NO}] / K_i \right)
\]

(4)

in which all constants should be regarded as apparent constants. A satisfactory fit of the steady-state kinetic data shown in Fig. 6 both for the low and the high concentrations of NO using Eq. (4) was obtained with the following set of values: \( K_i = 13.5 \, \mu\text{M} \); \( K_1 = 6.0 \, \mu\text{M} \); \( K_2 = 0.55 \, \mu\text{M} \) and \( k_{\text{cat}} \cdot e = 12 \) (arbitrary units).

4. Discussion

In this paper a new procedure for the purification of NO reductase from \textit{Pa. denitrificans} has been described. Because the enzyme is an integral membrane protein detergents are required for solubilization and purification. NO reductase could be extracted from highly purified membranes with the detergent lauryl maltoside while retaining about 60% of its activity. Quite pure enzyme in about 10% yield was obtained after application of anion exchange chromatography followed by chromatography on ceramic hydroxyapatite. With respect to previous purification protocols of the enzyme from \textit{Pa. denitrificans}, the procedure developed here is simpler, yields more protein [19] and more pure protein [17,18] enabling detailed — e.g., spectroscopic and kinetic — studies.

The purified NO reductase from \textit{Pa. denitrificans} consists of two subunits just like the enzymes from \textit{P. stutzeri} and \textit{Th. pantotropha} [16,19]. According to DNA sequence analysis the enzymes from \textit{P. stutzeri} and [22] \textit{Pa. denitrificans} [23] are encoded by two
structural genes *norC* and *norB* proposed to carry haem c and haem b, respectively. The molecular masses for the cytochrome c and cytochrome b subunits from *Pa. denitrificans*, calculated from the DNA sequence, are about 17 kDa and 52.5 kDa, respectively.

Two main bands with apparent molecular masses of 37 kDa and 17 kDa are observed in SDS gel electrophoresis pattern of the purified NO reductase (Fig. 1) corresponding to the cytochrome b and cytochrome c subunits, respectively. The discrepancy between the molecular mass of cytochrome b determined by SDS gel electrophoresis and from the DNA sequence of the structural gene is approximately the same as found for the enzyme from *P. stutzeri* and is likely to be due to the hydrophobic character of the cytochrome b subunit [21,42]. Likewise, the effect of boiling on the enzyme from *Pa. denitrificans* or incubation at elevated temperatures (e.g., 37°C or 65°C) prior to SDS electrophoresis is similar as described for the *P. stutzeri* enzyme, i.e., resulting in the appearance of higher molecular weight bands (for example at 65 kDa (cf. [21,42])) or even to complete aggregation of cytochrome b upon boiling, preventing its migration into the gel (data not shown). The gel pattern further shows a minor band with molecular mass of 22 kDa which could not be removed by additional chromatography.

Analysis of the prosthetic groups indicates a stoichiometry of two haems b per haem c, contrast to a previous report for the enzyme from *P. stutzeri* [21] in which a one to one ratio was calculated, but in agreement with [19]. Further, one non-haem iron per haem c was determined to be present in NO reductase and no copper or haem a. The detection of stoichiometric amounts non-haem iron has not been reported before. although superstoichiometric amounts of non-haem iron (≈ 3 mol Fe/mol enzyme [21]) were detected in the NO reductase from *P. stutzeri*. In general, NO reductase preparations from *Pa. denitrificans* in which treatment with EDTA or CDTA followed by gel filtration was omitted, contained 2–3 mol Fe/mol enzyme (data not shown).

The optical spectrum of dithionite reduced NO reductase shows the presence of one low-spin haem c with an absorbance maximum at 551 nm and of (at least) one low-spin haem b absorbing at 558 nm. The EPR spectrum of oxidized NO reductase shows three resonances $g_{z,y,x} = 2.99, 2.28$ and 1.46 belonging to a low-spin haem c center and one resonance at $g_z = 3.53$ of a highly anisotropic haem b center. Quantitation of the EPR signals indicates that the amount of the haem c represented by the $g_z = 2.99$ signal is the same as that determined with the pyridine haemochrome method (see Table 2), whereas the amount of haem b determined from the $g_z = 3.53$ signal is about 0.71 of the amount of haem c. It has been shown that the single-peak quantitation procedure applied to strain deformed low-spin haem signals such as performed here to quantitate the asymmetric $g_z = 3.53$ resonance can easily result in an underestimation of the amount by 20–35% [52]. We therefore conclude that the $g_z = 3.53$ peak represents one low-spin haem b per NO reductase.

The CO-difference spectrum of dithionite reduced NO reductase is very similar to that of the cytochrome *bo* quinol oxidase from *E. coli* [43] and the *bc*-type oxidases from *Rhodobacter* species [31,32]. The effect of CO on the spectrum strongly suggests the presence of a high-spin and/or five-coordinated haem b species. The same conclusion may be drawn from the type of shifts obtained in the optical spectrum of oxidized NO reductase upon addition of NO. The presence of a high-spin ferric haem b in the oxidized NO reductase may also be inferred from the absorbance at 590 nm, since a similar absorbance band is present in high-spin metmyoglobin hydroxide [43].

The chemically determined stoichiometry of haem c/haem b/non-haem Fe = 1:2:1. There is good indication from the optical and EPR spectra that one of the b-haems is in the high-spin state, the other one in the low-spin state. Furthermore, the high-spin ferric haem b and the non-haem iron are apparently EPR silent. One possibility is that the non-haem iron is in the ferrous state and therefore either diamagnetic in case of a low-spin system or very difficult to detect in case of a $S = 2$ system. EPR silence of the ferric high-spin haem cannot be explained easily. However, in view of the similarities between NO reductase and various members of the haem-copper oxidase family regarding the primary structure [24,25] and regarding haem content, and in order to explain the absence of stoichiometric amounts of high-spin haem and non-haem iron from the EPR spectrum — as in haem-copper oxidases [53] — we propose that in the
oxidized NO reductase the non-haem iron center is: (i) in the ferric state, (ii) is high-spin, and (iii) antiferromagnetically coupled to high-spin haem b via an oxo or hydroxo bridge; i.e., a Fe$^{3+}$-O(H)-Fe$^{3+}$ bridge analogous to the Fe$^{3+}$-O(H)-Cu$^{2+}$ bridge present the cytochrome $a_3$ quinol oxidase from $B$. subtilis [54,55] (see Fig. 7). Analogous to the situation in oxidases [33–35], we propose that NO reduction chemistry occurs at this dinuclear center. It is further proposed that electrons enter the NO reductase via haem c, in the 17 kDa subunit, which can be regarded as the functional counterpart of Cu in cytochrome-$c$ oxidase.

Note that antiferromagnetic coupling of two $S = 5/2$ spins leads to a diamagnetic ($S = 0$) groundstate, whereas ferromagnetic coupling results in a $S = 5$ groundstate. In the latter case a signal at approx. $g = 20$ might be present in the EPR spectrum. Such a signal has not been observed so far, but its intensity might be so low that it could only be detected by parallel-mode EPR spectroscopy. Evidence favouring antiferromagnetic coupling may come from the properties of a compound recently synthesized which may serve as a model for the dinuclear center in NO reductases [56]. This model compound is an oxo-bridged high-spin haem/high-spin non-haem iron complex with a pronounced Fe$^{3+}$-O-Fe$^{3+}$ bending around the O-atom of 157.3° and shows antiferromagnetic coupling [56].

The EPR spectrum of oxidized NO reductase provides some indication for the presence of high-spin haem iron ($g = 5.8–6.2$) and of high-spin non-haem iron ($g = 2.009$). Although the amount represented by both signals is substoichiometric and it cannot be ruled out that these signals are due to impurities and/or denatured NO reductase, the possibility exists that they are derived from an ‘uncoupled’ dinuclear center particularly because their concentration is about the same. Uncoupling may also occur upon partial reduction of the enzyme, just as in cytochrome oxidase [57] where the Cu$_B$ becomes reduced and EPR silent concomitant with the appearance of the haem $a_3$ signal, possibly explaining why in the EPR spectrum of NO reductase from $P$. stutzeri the high-spin haem signal accounts for about one spin per enzyme molecule [20] and the $g = 2.009$ signal is not observed because the non-haem iron center is in the ferrous state.

The steady-state kinetics of NO reduction by the NO reductase can be described with a simple model in which two molecules of NO bind sequentially, yielding the quadratic relation between rate and NO at low concentrations of NO Fig. 6. At higher NO concentrations substrate inhibition is observed suggested to be due to binding of NO to the oxidized enzyme (Eq. (3)). Indeed, the optical spectrum in Fig. 3 shows that NO does bind to the oxidized enzyme thus providing the basis for inhibition. The value of $K_1$ (13.5 µM) is calculated from the slope of the Dixon plot in Fig. 6 and is accurate to about ±3 µM.

In contrast to the value $K_1$, the values for $K_2$ and $K_3$ (6.0 µM and 0.55 µM) are obtained by a multi-parameter simulation (see Fig. 6) and their (apparent) values are only accurate to about a factor of 2. Although optimal simulation of the kinetic traces consistently require that binding of the second molecule of NO is more firmly than that of the first molecule of NO, the factor of 10 in affinity determined by the particular simulation as shown in Fig. 6 should not be regarded as a definitive value (a value as low as 4 was sometimes calculated from the simulations of other traces). Furthermore, the fit to the data at low [NO] shows deviations from the
simple model and the possibility that binding of NO shows co-operativity cannot be ruled out. However, given the inaccuracies related to the rate determinations at low [NO], proposing more complex models to describe the steady-state kinetics is at present not realistic.

A few suggestions on the mechanism of bacterial NO reduction have been made. A very interesting proposal has been put forward by Ye et al. [4], inspired by the mechanism for the nitrosyl-catalysed reaction of NO with CO yielding N₂O + CO₂ [58], in which binding of two molecules of NO to a single non-haem iron center is proposed as a reaction intermediate. However, in view of all the evidence favouring a close structural similarity between cytochrome oxidases and NO reductase a tentative model for NO reduction involving both the non-haem iron and the haem iron may be more appropriate and is presented in Fig. 7. Accordingly, the reduction of the non-haem iron and the haem iron is followed by protonation of the bridging O(H)-ligand which leaves as water, whereafter the first molecule of NO is proposed to bind as a ligand bridging the two ferrous ions. When the second NO comes in, this bridge is broken and each iron atom now binds one molecule of NO. Due to the proximity of the two molecules of NO, the N–N bond can be formed giving the ‘N₂O₂’-intermediate. Two-electron reduction of one of the ‘O’ atoms of the ‘N₂O₂’-intermediate yields ‘O²⁻’ which bridges the two Fe³⁺-ions while N₂O is liberated. The model for NO reduction as presented is consistent with the optical, EPR and steady-state kinetic properties determined in this work though tentative, and it is realized that in the absence of detailed (pre)-steady-state kinetic measurements other alternatives would be consistent as well.

In conclusion, the study presented here on purified NO reductase from *Pa. denitrificans* provides direct chemical and spectroscopic evidence for the similarity between the cytochrome *b* subunit of NO reductase and subunit I of the haem-copper oxidases suggested previously on basis of the similarities between their primary structures [24,25]. Both enzymes contain a dinuclear centre consisting of a high-spin haem coupled antiferromagnetically to a non-haem iron center in NO reductase or to a copper center (Cu₉) in the oxidases. Further, the low-spin haem *b* center of NO reductase that gives the EPR signal at *g*₂ = 3.53 and the absorbance maximum at 558 nm in the optical spectrum is the functional equivalent of, e.g., haem a. The second subunit of NO reductase is a *c*-type cytochrome, similar in function to the *c*-cytochrome subunit of the *cb*-type oxidases and equivalent in function to Cu₉ (cf. Refs. [31,32]).

Despite all the similarities between NO reductase and cytochrome-*c* oxidase, the former enzyme is apparently not a proton pump and the reduction of NO to N₂O is not even electrogenic [3,5,37,38]. Whilst the absence of proton pumping by NO reductase could be due to the absence of a proton-pumping channel [27–29], a situation that can be obtained in cytochrome-*c* oxidase by making suitable mutations [59,60], non-electrogenicity of the NO reductase reaction is far more difficult to understand. The absence of electrogenicity implies that the pathway of the chemical protons in NO reductase and cytochrome-*c* oxidase would be opposite, this in spite of the fact that all transmembrane spanning α-helices are conserved as well as the number and the topology of the prosthetic groups. Experiments with purified NO reductase embedded in closed phospholipid vesicles might resolve the question whether or not the NO reductase reaction is electrogenic and whether or not the NO reductase is a proton pump just like the *cb*-type oxidases [61].

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