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# Crystallization and preliminary X-ray analysis of nitrous oxide reductase from *Paracoccus pantotrophus*

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Nitrous oxide reductase is a periplasmic respiratory protein with a novel copper catalytic centre; it catalyses the terminal step, reduction of nitrous oxide to nitrogen, of the bacterial denitrification process. Nitrous oxide reductase from *Paracoccus pantotrophus* has been crystallized by the hanging-drop method. A prerequisite for crystallization was the oxidation of the enzyme with potassium ferricyanide in order to obtain homogenous oxidation states of the copper centres. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 116.4$, $b = 118.3$, $c = 267.0$ Å. Two homodimers, of approximate molecular weight 67 kDa per subunit, probably constitute the asymmetric unit and give a Matthews coefficient, $V_m$, of 3.4 $\text{Å}^3\ \text{Da}^{-1}$ and a solvent content of 59% by volume. The crystals diffract X-rays to 3.0 Å resolution on an in-house source and are suitable for structure determination.

## 1. Introduction

Denitrification is the part of the biological nitrogen cycle in which nitrate is reduced *via* nitrite, nitric oxide and nitrous oxide to nitrogen gas (Berks *et al.*, 1995; Zumft, 1997; Ferguson, 1998). Each of these reduction reactions is catalysed by an enzyme that receives electrons from the respiratory chain apparatus of the bacterial cytoplasmic membrane. Of these enzymes, nitrous oxide reductase is the least understood. It has been purified from the periplasm of several denitrifying bacteria (Zumft, 1997) and is almost always a homodimer with an estimated eight Cu atoms but no other metal centres. Four of these Cu atoms form two copper *A* centres, one on each monomer, which are clearly very similar to the binuclear copper *A* centre of the mitochondrial cytochrome $aa_3$ oxidase (Zumft, 1997). On the basis of this known structure and a variety of other evidence (Zumft, 1997), this $Cu_A$ centre is not the catalytic site. The other ($Cu_Z$) site has been deduced to comprise two other Cu atoms which are thought to constitute a novel arrangement (Farrar *et al.*, 1991). The mechanism of nitrous oxide reduction and the presumed role of copper presents an intriguing problem. This is mainly because there are no known inorganic complexes of copper with nitrous oxide and nitrous oxide is very inert, not least in terms of its reactivity as a ligand to transition metals. Indeed, few complexes of nitrous oxide with transition metals have been documented.

Low-resolution (~20 Å) solution studies of nitrous oxide reductase from *Alcaligenes xylosoxidans* revealed the shape of the molecule and confirmed its dimerization in solution (Ferretti *et al.*, 1999). No high-resolution three-dimensional structure has been published so far for a nitrous oxide reductase; thus, a crystal structure can be anticipated to provide insight into a novel active site that can bind and reduce the inert nitrous oxide gas. To this end, we have purified and crystallized nitrous oxide reductase from *P. pantotrophus* (formerly *Thiosphaera pantotropha*) and the crystal structure is being determined.

## 2. Materials and methods

### 2.1. Protein purification

Nitrous oxide reductase from *P. pantotrophus* was purified from a soluble fraction released from cells that had been grown anaerobically as described by Berks *et al.* (1993). The volume of cell culture ranged from 60 to 200 l. The purification was performed under aerobic conditions. The periplasmic extract (between 300 ml and 1 l) was loaded onto a 300 ml DEAE CL-6B (Pharmacia) column at 277 K which had been pre-equilibrated with 50 m*M* Tris–HCl, 20% glycerol pH 8.0. Nitrous oxide reductase was eluted with a linear gradient of 0–1 *M* NaCl that was applied over five column volumes of the Tris–HCl buffer. Fractions containing nitrous oxide reductase were identified by SDS–PAGE (Laemmli, 1970), which also showed that there were significant impurities in the enzyme preparation. In particular, the abundant cytochrome $cd_1$ nitrite reductase of this organism was a significant contaminant. The fractions with the highest nitrous oxide reductase

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content were pooled and subsequently loaded onto a phenyl Superose (Pharmacia) column which had been previously equilibrated with 40% ammonium sulfate, 0.1 *M* Tris–HCl pH 8.0. This column was eluted with a gradient of 40–0% ammonium sulfate applied over three to five column volumes of 0.1 *M* Tris–HCl and was optimized to achieve the greatest separation of nitrous oxide reductase and cytochrome $cd_1$. The fractions with the highest nitrous oxide reductase content were again identified by SDS–PAGE. These fractions were pooled, concentrated using a Centricon (30 kDa cutoff) concentrator and transferred to 50 m*M* Tris–HCl pH 8.0 using a PD10 column. Material thus obtained was loaded onto a Mono Q 16/10 column which had previously been equilibrated with 50 m*M* Tris–HCl pH 8.0. Nitrous oxide reductase was eluted by applying a linear 0–0.5 *M* gradient of NaCl to the column. Nitrous oxide reductase eluted at approximately 0.15 *M* NaCl; SDS–PAGE of the principal fractions containing nitrous oxide reductase showed that the protein had been purified to homogeneity. In particular, the significant traces of cytochrome $cd_1$ nitrite reductase that had been found in previous reports of purification were completely absent. To speed up the purification profile, it was found that the DEAE CL-6B could be replaced by DEAE FF and the phenyl Superose could be replaced by a SOURCE Phe column. The typical yield of pure protein was 5 mg per 20 l of original culture.

For crystallization, the purified enzyme was desalted in 50 m*M* imidazole–HCl pH 7.0 and the concentration of the protein was adjusted to between 3–8 mg ml$^{-1}$ either by diluting with the same buffer or concentrating with a Centricon concentrator. The UV–visible absorption spectrum (Fig. 1) of nitrous oxide reductase as prepared suggested that the protein was a mixture of the oxidized and reduced forms. The spectrum shows peaks at 480 and 540 nm, which correspond to the oxidized enzyme, but there was also a peak at 630 nm which arises from the reduced form of the enzyme. In order to obtain an enzyme that was homogeneous with respect to oxidation state, nitrous oxide reductase was oxidized prior to crystallization trials. This was achieved by adding 100 µl of 2 *M* potassium ferricyanide to 2 ml of enzyme solution. Immediately after mixing, the solution of oxidized nitrous oxide reductase was placed in a Centricon concentrator fitted with a 30 kDa cut-off membrane and the sample was centrifuged according to the manufacturers' instructions. When the volume in the Centricon had fallen to approximately 100 µl, a further 1.5 ml of 50 m*M* imidazole–HCl pH 7.0 was added. This treatment was continued for approximately 8 h, adding extra imidazole–HCl pH 7.0 buffer as appropriate, in order to ensure that ferricyanide and ferrocyanide were removed from the sample. At the end of this procedure, the nitrous oxide reductase was at a concentration of approximately 45 mg ml$^{-1}$.

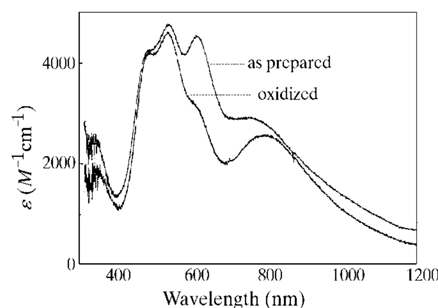


**Figure 1**
Visible spectra of *P. pantotrophus* nitrous oxide reductase. The enzyme was buffered in 50 m*M* imidazole–HCl pH 7.0 and was then oxidized with 50 m*M* potassium ferricyanide.

**Figure 2**
Crystals of nitrous oxide reductase from *P. pantotrophus*. The largest dimension is 0.6 mm.

### 2.2. Crystallization and X-ray analysis

Crystallization trials were performed using the hanging-drop vapour-diffusion method either immediately after concentrating the protein or several days later. A large number of conditions were screened, but the optimal conditions were at 295 K with crystallization buffer consisting of 36% PEG 8000, 0.1 *M* Tris–HCl pH 8.23 in the well. Initially, the hanging drop contained 2 µl of the crystallization buffer and 2 µl of the nitrous oxide reductase solution at 45 mg ml$^{-1}$ in 50 m*M* imidazole–HCl pH 7.0. Purple crystals were obtained within 2–3 weeks which were chunky and irregular (Fig. 2). No crystals were obtained if the oxidation step with potassium ferricyanide was omitted; reduction of the enzyme with sodium ascorbate or sodium dithionite also failed to produce crystals. For room-temperature crystallographic analysis the crystals could be mounted in a capillary. It was noted that addition of further crystallization buffer caused cracking of the crystals. In order to avoid radiation damage, frozen crystals were also required. This proved problematic because the crystals could not be transferred to a new buffer which included cryo-protectant. Freezing of crystals by direct exposure to liquid nitrogen or a 100 K nitrogen-gas stream caused cracking. However, it proved possible to maintain the integrity of the crystals by flash-freezing them in liquid propane. Preliminary X-ray experiments were performed in-house using a Rigaku rotating-anode generator operating at 60 kV, 70 mA with Cu $K\alpha$ radiation and a 30 cm MAR Research imaging-plate detector. Data were processed with the *HKL* suite of programs (Otwinowski & Minor, 1997).

## 3. Results and discussion

Crystals of nitrous oxide reductase reached maximum dimensions of approximately 0.6 × 0.4 × 0.4 mm. They belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 116.4, $b$ = 118.3, $c$ = 267.0 Å. A data set collected at 100 K contains 149 896 observations with 59 348 unique reflections and is 79.3% complete in the resolution range 30.0–3.0 Å, with $R_{\rm merge}$ = 0.133 and $I/\sigma(I)$ = 7.3. The completeness in the highest resolution shell between 3.11 and 3.0 Å was 69.0% and the $R_{\rm merge}$ value in this range was 0.462 [$I/\sigma(I)$ = 3.0]. A value for the Matthews coefficient (Matthews, 1968) of 3.4 Å$^3$ Da$^{-1}$ was calculated assuming two homodimers of approximate molecular mass 67 kDa per subunit in the asymmetric unit, which corresponds to 59% solvent content by volume. The self-rotation function failed to reveal the directions of the non-crystallographic rotation axes.

Nitrous oxide reductase crystals were suitable for flash-freezing using the conditions identified in the present work, but their fragile nature suggests that the identification of heavy-metal derivatives is unlikely. However, the multiple copper content of nitrous oxide reductase opens up the possibility of solving the structure using the multiwavelength anomalous dispersion

(MAD) technique combined with fourfold non-crystallographic molecular averaging in conjunction with the propane-frozen crystals. This work is in progress.

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