The purification of ammonia monooxygenase from *Paracoccus denitrificans*

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Abstract The heterotrophic nitrifier *Paracoccus denitrificans* expresses a membrane-associated ammonia monooxygenase. The active enzyme has been solubilized in the detergent dodecyl-β-D-maltoside and purified by standard chromatographic techniques. This is the first purification of an ammonia monooxygenase. The enzyme consists of two subunits with molecular masses of 38 and 46 kDa. The purified enzyme is a quinol oxidase, is inhibited by light and a variety of chelating agents and is activated by cupric ions. These properties indicate that this enzyme has similarities to a family of enzymes including the ammonia monooxygenase from *Nitrosomonas europaea* and the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath).

Key words: Ammonia monooxygenase; Particulate methane monooxygenase; Heterotrophic nitrification; *Paracoccus denitrificans*

1. Introduction

Nitrification is the bacterial process by which ammonia is oxidised to nitrite and nitrate. Autotrophic nitrifiers use either the oxidation of ammonia to nitrite or the oxidation of nitrite to nitrate as an energy source to support growth. Nitrification is also employed during heterotrophic growth of some bacteria although in this case the process dissipates rather than conserves energy [1,2].

In the heterotrophic nitrifier *Paracoccus denitrificans* GB17 (also known as *Thiosphaera pantotropha*) and the autotrophic nitrifier *Nitrosomonas europaea* ammonia is converted to nitrite via the intermediate hydroxylamine. The hydroxylamine oxidases (HAOs) of the two species are distinct; in *P. denitrificans* HAO is a 20 kDa monomer containing non-haem prosthetic groups [2], whereas HAO from *N. europaea* is a multimer of 66 kDa subunits each containing 8 haems [3]. The other enzyme of the pathway, ammonia monooxygenase (AMO), is the least well characterised enzyme of the nitrogen cycle and the only one which until now has eluded purification. Studies of AMO from *N. europaea* have been hampered by the instability of the enzyme once isolated from the cell [4,5]. However, a number of the inhibitors of the enzyme have been identified [6] and the genes have been sequenced [7,8]. AMO from *N. europaea* has been suggested to be homologous to the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* [9,10]. Both enzymes are thought to contain copper [5,11]. Whole-cell EPR spectroscopy of *M. capsulatus* has indicated that pMMO may contain a novel trinuclear copper centre [11]. Recently, a purification of pMMO was reported which indicated that the enzyme may contain both copper and iron [12].

Since the HAO from the heterotrophic nitrifier *P. denitrificans* is entirely distinct from the enzyme of the autotrophic nitrifier *N. europaea* we have endeavoured to investigate the properties of AMO from *P. denitrificans*.

2. Materials and methods

P. denitrificans 1222 and *P. denitrificans* 2621 (a cytochrome bb_3 quinol oxidase deficient strain obtained from Dr. R. van Spanning, Vrije Universiteit, Amsterdam [13]) were grown in liquid culture in a minimal medium [14] containing 30 mM sodium acetate as carbon and energy source. 50 ml cultures were incubated in 250 ml conical flasks shaken at 200 rpm at 37°C with oxygen as electron acceptor.

A cosmid, pLCC5, containing the genes for heterotrophic nitrification from *P. denitrificans* 1222 (Crossman et al., manuscript in preparation) was transferred into *P. denitrificans* 2621 from *Escherichia coli* DH5α by conjugation employing *E. coli* JM803 (pRK2013) as the helper strain. *E. coli* DH5α (pLCC5) was grown with 12.5 μg/ml tetracycline, *E. coli* JM803 (pRK2013) was grown with 200 μg/ml kanamycin and *P. denitrificans* 2621 was grown with 100 μg/ml rifampicin. The three strains were grown aerobically in LB to mid-exponential phase, harvested by centrifugation, washed and resuspended in LB and placed on a 0.45 μm nitrocellulose filters on an L agar plate. After incubation at 30°C for 20 h the mating was resuspended in LB and dilutions were plated onto L agar plates containing 100 μg/ml rifampicin and 1 μg/ml tetracycline. The selected strain, *P. denitrificans* 2621 (pLCC5), was used for purification of ammonia monooxygenase.

P. denitrificans 2621 (pLCC5) was grown in 100 ml LB+1 μg/ml tetracycline culture in a 250 ml conical flask at 37°C shaken at 200 rpm. This 100 ml culture was grown to a turbidity of $A_{650} = 1.0$ and subsequently used as an inoculum for 8 l of minimal medium+1 μg/ml tetracycline in a 20 l vessel. This culture was aerated by pumping with air at 2.5 l/min and maintained at 30°C. The culture was harvested at $A_{650} = 0.4$ by centrifugation at $5000 \times g$ at 4°C for 30 min and the pellet was resuspended in 40 ml 10 mM Tris-HCl (pH 8.0). The cells were broken by sonication at 4°C.

Chromatographic purification of AMO was performed by anion exchange using DEAE Sepharose CL6B and by gel exclusion using Sephadex G25-M PD10 columns, both obtained from Pharmacia. All chromatographic steps (and subsequent storage of purified enzyme) were executed in the dark at 4°C.

Protein samples were tested for purity by SDS-PAGE followed by silver staining using a kit obtained from Sigma. Protein concentration was assayed with bicinchoninic acid.

3. Results and discussion

3.1. Development of an ammonia monooxygenase assay

The activity of AMO in *P. denitrificans* was followed by measuring the change in oxygen uptake rate after the addition of 5 mM NH_4Cl to a suspension of cells or a cell-free extract. A mixture of NADH, diaphorase and duroquinone was used as an electron donor. This mixture gives rise to production of duroquinol in situ since reduction of duroquinone by NADH is catalysed by diaphorase. Oxygen disappearance from a sus-

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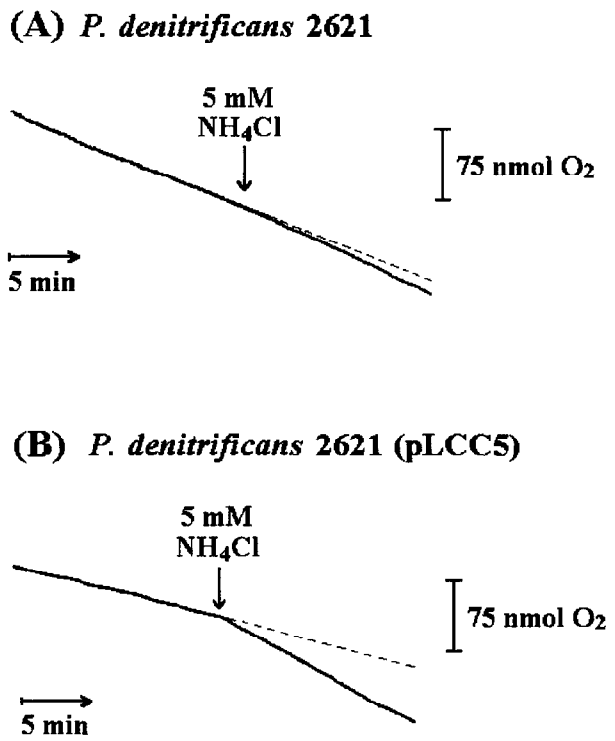

Fig. 1. Assays of AMO in *P. denitrificans* 2621. The effect of 5 mM NH_4Cl on the rate of oxygen uptake by sonicated extracts of (A) *P. denitrificans* 2621 and (B) *P. denitrificans* 2621 (pLCC5). Oxygen uptake was followed in a Clark electrode. The assay was performed in 10 mM Tris-HCl (pH 8.0) using a mixture of 0.5 mM NADH, 0.6 units diaphorase and 0.5 mM duroquinone as electron donor. The electrode traces are shown as solid lines. The projected trace had NH_4Cl not been added is a dotted line.

pension of sonicated *P. denitrificans* 1222 was followed in a Clark O_2 electrode. The assay was performed in the presence of the cytochrome bc_1 complex inhibitor myxothiazol to inhibit electron flow via the cytochrome bc_1 complex to the cytochrome *c* oxidases [13]. In spite of this inhibition no change in the rate of oxygen uptake on adding NH_4Cl was detectable (data not shown). Therefore, the experiment was repeated using *P. denitrificans* 2621 a strain deficient in the cytochrome bb_3 oxidase (which takes electrons from the quinol pool) [13]. In this strain there is a much lower background rate of oxygen uptake since, in the presence of myxothiazol, electron transport to the cytochrome bc_1 complex-dependent respiratory oxidases is inhibited and there is no alternative quinol oxidase. Fig. 1A shows that addition of NH_4Cl caused a small increase in the uptake of oxygen, indicating that extracts of *P. denitrificans* produce an ammonia oxidising enzyme which utilizes oxygen.

A cosmid, pLCC5, which contains the genes required for nitrification has been isolated from a *P. denitrificans* 1222 library (Crossman et al., manuscript in preparation). The rate of ammonia stimulated oxygen uptake by extracts of *P. denitrificans* 2621 (pLCC5) was 4-fold higher than that in *P. denitrificans* 2621, presumably because of a gene dosage effect (Fig. 1B). Therefore, this strain was used for the subsequent analysis of AMO from *P. denitrificans*. If duroquinone was omitted from the reaction mixture then there was no detectable AMO activity in sonicated extracts of *P. denitrificans* 2621 (pLCC5) indicating that duroquinol is the electron donor to AMO in the assay.

The stimulation of oxygen uptake by the addition of ammonia was measured in whole cells of *P. denitrificans* 2621 (pLCC5) and in extracts generated by sonication. In sonicated extracts AMO activity was 25% of that in whole cells (Table 1). The two assays are not equivalent, however, since duroquinol is the non-physiological electron donor to AMO in sonicated extracts whereas the assay in whole cells employs acetate to donate electrons into the physiological electron transport pathway which may be subject to respiratory control (ubiquinol is the likely electron donor to AMO). In spite of this we can say that the AMO activity from *P. denitrificans* is more stable after cell breakage compared to *N. europaea* in which active extracts can be obtained only in the presence of stabilizing agents such as bovine serum albumin, spermine and Mg^{2+} [4] and are further stabilized by Cu^{2+} [5].

3.2. *Purification of the AMO from* P. denitrificans

The sonicated extract from 8 l of *P. denitrificans* 2621 (pLCC5) was separated into particulate material and water soluble material by centrifugation at 30 000×*g* for 30 min at 4°C. The AMO activity was found in the particulate fraction (Table 1) indicating a membraneous location for the enzyme, as is the case for AMO from *N. europaea*. Solubilization trials were performed using the detergents Triton X-100, CHAPS, cholate and dodecyl-β-D-maltoside. It was found that the AMO was solubilized in an active form only in dodecyl-β-D-maltoside. The particulate fraction of *P. denitrificans* 2621 (pLCC5) was resuspended in 10 mM Tris-HCl (pH 8.0) to a concentration of 7.5 mg/ml protein and dodecyl-β-D-maltoside was added as a solid to concentrations of 0.5, 1.0, 1.5 and 2.0%. The suspensions were mixed at 4°C in the dark for 1 h and the solubilized material was separated from insoluble material by centrifugation at 30 000×*g* at 4°C for 30 min. The AMO activity in the solubilized fraction was

Table 1
Purification of AMO from *P. denitrificans*

	Total AMO activity (nmol O_2/min)	Protein content (mg)	Specific activity (nmol O_2/min per mg)
Whole cells[a]	9600	800	12
Sonicated extract	2400	800	3.0
Particulate fraction	2400	120	20
Particulate protein solubilized in dodecyl-β-D-maltoside	800	39	10
DEAE-CL6B column 1	1700	3.6	470
DEAE-CL6B column 2	1600	2.5	640
Gel exclusion	1600	0.2	8000

The purification of the ammonia oxygenase activity from 8 l of *P. denitrificans* 2621 (pLCC5) as described in the text. Enzyme activity was assayed as described in the legend for Fig. 1.
[a]The assay of ammonia monooxygenase activity in whole cells was as described in the text.

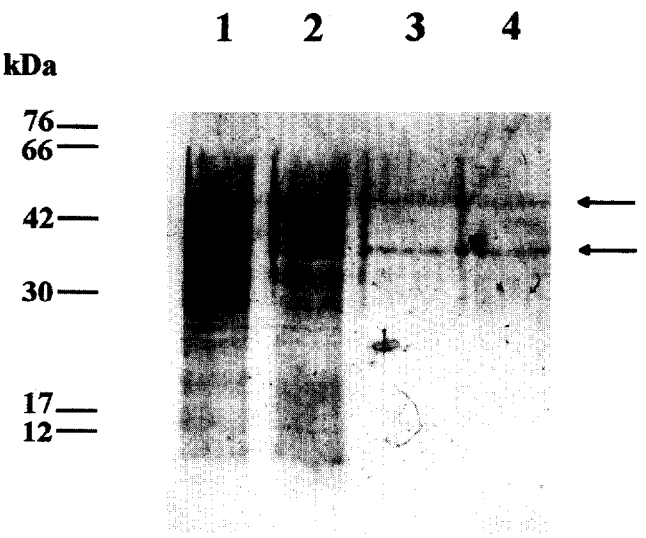

Fig. 2. SDS-PAGE of purified AMO from *P. denitrificans* 2621 (pLCC5). A PD-10 gel exclusion column equilibrated with 10 mM Tris-HCl (pH 8.0)+0.02% dodecyl-β-D-maltoside was loaded with 2.5 ml of partially purified AMO after two anion exchange columns. Lanes 1–4 of a silver-stained 12% SDS-PAGE contain material eluted with 2.5–4.5, 4.5–6.5, 6.5–8.5 and 8.5–10.5 ml of equilibration buffer. Mobilities of molecular weight standards are shown to the left of the gel. The two subunits of purified AMO, in lanes 3 and 4, are marked by arrows to the right of the gel.

assayed. Most activity was retained with 1.0 and 1.5% dodecyl-β-D-maltoside compared to lower or higher concentrations (Table 2). 50% of the activity from unsolubilized membranes was retained with 1.0 or 1.5% dodecyl-β-D-maltoside. Presumably at low concentrations of dodecyl-β-D-maltoside there was insufficient detergent to remove lipid and hence solubilize the activity, whereas at high concentrations the detergent adversely affected the enzyme conformation thus inactivating it. There have not been any reports of successful detergent solubilization of AMO from *N. europaea*, again indicating the greater stability of the enzyme from *P. denitrificans*.

Membrane protein, solubilized with 1% dodecyl-β-D-maltoside, was loaded onto a column (1 cm × 10 cm) containing DEAE Sepharose CL6B equilibrated with 10 mM Tris-HCl (pH 8.0) + 0.02% dodecyl-β-D-maltoside and the AMO activity was found to bind to this resin. The column was developed with a gradient of 0–250 mM NaCl in 10 mM Tris-HCl (pH 8.0) + 0.02% dodecyl-β-D-maltoside. The peak of AMO activity eluted with 50 mM NaCl. There is a 2-fold activation of the enzyme following this anion exchange column (Table 1) possibly indicating the removal of some unknown inhibitory substance. The fractions containing enzyme activity were pooled, diluted 2-fold with 10 mM Tris-HCl (pH 9.0), and loaded onto a column (1 cm × 10 cm) containing DEAE Sepharose CL6B equilibrated with 10 mM Tris-HCl (pH 9.0) + 0.02% dodecyl-β-D-maltoside. Again the activity was retained on the column until the column was developed with a gradient of 0–250 mM NaCl in Tris-HCl (pH 9.0) + 0.02% dodecyl-β-D-maltoside. The peak of activity eluted with 140 mM NaCl at this higher pH. The enzyme was purified further using gel exclusion chromatography on PD10 columns containing the resin G25-M and with a void volume of 2.5 ml. Volumes of 2.5 ml were loaded onto the PD10 column equilibrated with 10 mM Tris-HCl (pH 8.0) + 0.02% dodecyl-β-D-maltoside and developed with 10 mM Tris-HCl (pH 8.0) + 0.02% dodecyl-β-D-maltoside. Although the majority of the protein eluted after 2.5 to 6.5 ml, AMO activity eluted after 6.5 to 10.5 ml. The specific activities of AMO during the purification are shown in Table 1. The AMO has been purified 2500-fold. SDS-PAGE of fractions collected from the PD10 column show that the purified AMO contains two bands of molecular masses 38 and 46 kDa (Fig. 2).

Given that 16% of the total AMO activity from whole cells is retained in the purified enzyme and that the specific activity is increased 400-fold during purification from particulate fraction to purified enzyme, it is estimated that AMO comprises approx. 1.5% of total membrane protein in intact cells of *P. denitrificans* 2621 (pLCC5) grown under the conditions described herein. It has been estimated that pMMO comprised 36% of the total membrane protein of *M. capsulatus* (Bath) [12]. Although the enzyme from *M. capsulatus* is clearly present in larger quantities than the enzyme from *P. denitrificans* it is remarkable that the specific activity of the enzyme purification described in this paper is 700× higher than that for pMMO [12]. The implication that the purified pMMO from *M. capsulatus* (Bath) has a low specific activity raises questions about the functional importance of the spectroscopic information that has been presented [12].

3.3. Characterisation of the purified AMO

The purified AMO contains two polypeptides of molecular masses 38 and 46 kDa (Fig. 2). These are similar to the molecular masses of the two gene products AmoA and AmoB from the autotrophic nitrifier *N. europaea* [7,8] and the homologous gene products PmoA and PmoB from the methanotroph *M. capsulatus* (Bath) [9,10]. These gene products are presumed to form the AMO and pMMO, respectively, although it has been reported that the PmoA and PmoB subunits alone do not give an active pMMO [12].

AMO from *N. europaea* and pMMO from *M. capsulatus* are inhibited by acetylene, light and a range of copper chelating agents [6]. Both enzymes are believed to have copper at the active site [5,11]. The purified AMO from *P. denitrificans*

Table 2
Solubilization of AMO in dodecyl-β-D-maltoside

	Total AMO activity (nmol O_2/min)	Protein content (mg)	Specific activity (nmol O_2/min per mg)
Particulate fraction	120	6.0	20
Membranes in dodecyl-β-D-maltoside at:			
0.5%	20	3.8	5
1.0%	40	3.9	10
1.5%	40	4.1	10
2.0%	0	4.3	0

Solubilization conditions are described in the text.

was dialysed for 24 h against 10 mM Tris-HCl (pH 8.0) + 0.02% dodecyl-β-D-maltoside and was found to have lost its activity. The activity was recovered by adding 100 μM $CuCl_2$ to the enzyme preparation, indicating that the enzyme from *P. denitrificans* contains a labile copper centre. The effects of known inhibitors of AMO from *N. europaea* and pMMO from *M. capsulatus* (Bath) [6] on purified AMO from *P. denitrificans* indicated a broad similarity between the enzymes. 1 mM diethyldithiocarbamate and 0.5 mM allylthiourea completely inhibited *P. denitrificans* AMO. Ordinary laboratory lighting inhibited AMO over a period of 1 h. In contrast, however, acetylene which is commonly used as an inhibitor of AMO from *N. europaea* and pMMO from *M. capsulatus* is not an inhibitor of the AMO from *P. denitrificans* at a concentration of 1 mM. Presumably this inhibitor cannot enter the active site of the enzyme from *P. denitrificans*. This difference between the ammonia monooxygenases of *P. denitrificans* and *N. europaea* may allow identification of the relative importances of heterotrophic and autotrophic nitrification in the environment, although it is not yet clear if heterotrophic AMOs are likely to be as closely related to one another as are the AMOs of autotrophs and pMMOs of methanotrophs [10].

In conclusion, *P. denitrificans* has been shown to possess an ammonia monooxygenase activity and the enzyme responsible for this has been purified. Enzymological analysis indicates that this enzyme may belong to the family which includes AMO from the autotrophic nitrifiers and pMMO from methanotrophs. This purification opens the way for the study of an enzyme which has previously eluded biochemical characterisation.

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