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Purification of hydroxylamine oxidase from *Thiosphaera pantotropha*

Identification of electron acceptors that couple heterotrophic nitrification to aerobic denitrification

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Thiosphaera pantotropha, a Gram-negative heterotrophic nitrifying bacterium, expresses a soluble 20 kDa monomeric periplasmic hydroxylamine oxidase that differs markedly from the hydroxylamine oxidase found in autotrophic bacteria. This enzyme can use the periplasmic redox proteins, cytochrome c_{551} and pseudoazurin as electron acceptors, both of which can also donate electrons to denitrification enzymes. A model of electron transfer is proposed, that suggests a coupling of nitrification to denitrification and provides a mechanism by which nitrification can play a role in dissipating reductant.

Nitrification; Denitrification; Hydroxylamine oxidase; *Thiosphaera pantotropha*

1. INTRODUCTION

A number of heterotrophic bacteria have recently been shown to combine nitrification and denitrification [1,2]. In this process ammonia is completely dehydrogenated via sequential oxidation to hydroxylamine and nitrite (nitrification) followed by sequential reduction to nitric oxide, nitrous oxide and dinitrogen (denitrification). Since the oxidation of ammonia to hydroxylamine requires molecular oxygen, the conversion to nitrogen by this route is obligately aerobic and depends on the ability of the organism to denitrify aerobically.

The function of nitrification in autotrophic bacteria is ultimately to provide ATP and NADH for growth and maintenance reactions, including the reductive fixation of carbon dioxide. The model most favoured for the electron transfer reactions associated with ammonia oxidation in such bacteria is shown in Fig. 1 [3] and derives mainly from studies with *Nitrosomonas europaea*.

The role of nitrification in heterotrophic bacteria, such as *Thiosphaera pantotropha*, is presently unclear. It does not provide the organism with the capacity for autotrophic growth. Furthermore, cultures that are actively nitrifying have lower than expected growth yields [1]. It has been suggested that in heterotrophic bacteria nitrification may play an important role in the dissipation of excess reducing power generated during oxidative metabolism of reduced carbon substrates [1]. The mechanism by which an oxidative process can serve as a sink for electrons requires some clarification, but it may be intimately linked to denitrification.

In this communication, a partial purification of the hydroxylamine oxidase which catalyses oxidation of hydroxylamine to nitrite is presented. The properties of the *T. pantotropha* enzyme suggest that a new class of hydroxyamine oxidase should be recognised. An electron transfer model for nitrification coupled to denitrification is also presented which explains: the inability of ammonia oxidation to provide energy for autotrophic growth; the lower growth yields of actively nitrifying cultures and the role of nitrification in the dissipation of reductant.

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2. MATERIALS AND METHODS

Thiosphaera pantotropha strain LMD82 was obtained from Dr. L.A. Robertson, Delft and was cultured in the medium previously described [4], using acetate and ammonia as carbon and nitrogen sources, respectively. Aerobic cultures were grown at 37°C in 250 ml conical flasks, containing 50 ml growth media and shaken at 250 rpm. 10-l cultures were grown in 20-l bottles continuously agitated and sparged with air. Bacteria were harvested and fractionated into cytoplasm, periplasm and membranes using lysozyme digestion as previously described [5]. For purification of HAO, lysozyme-free periplasms were prepared by osmotic shock using the method previously described [6]. Periplasms were fractionated using a DEAE-CL6B anion-exchange column equilibrated with 5 mM Tris-HCl (pH 8.0) and developed using a 0–500 mM gradient of NaCl in the same buffer. Cytochrome fractions were identified from the absorbance at 410 nm of the column eluate and were tested as electron acceptors for HAO without further purification. Cytochrome c_{551} was purified to homogeneity as previously described [7]. Pseudoazurin could be monitored from the absorbance at 590 nm and was purified as previously described [8]. Hydroxylamine oxidase eluted from the anion exchange

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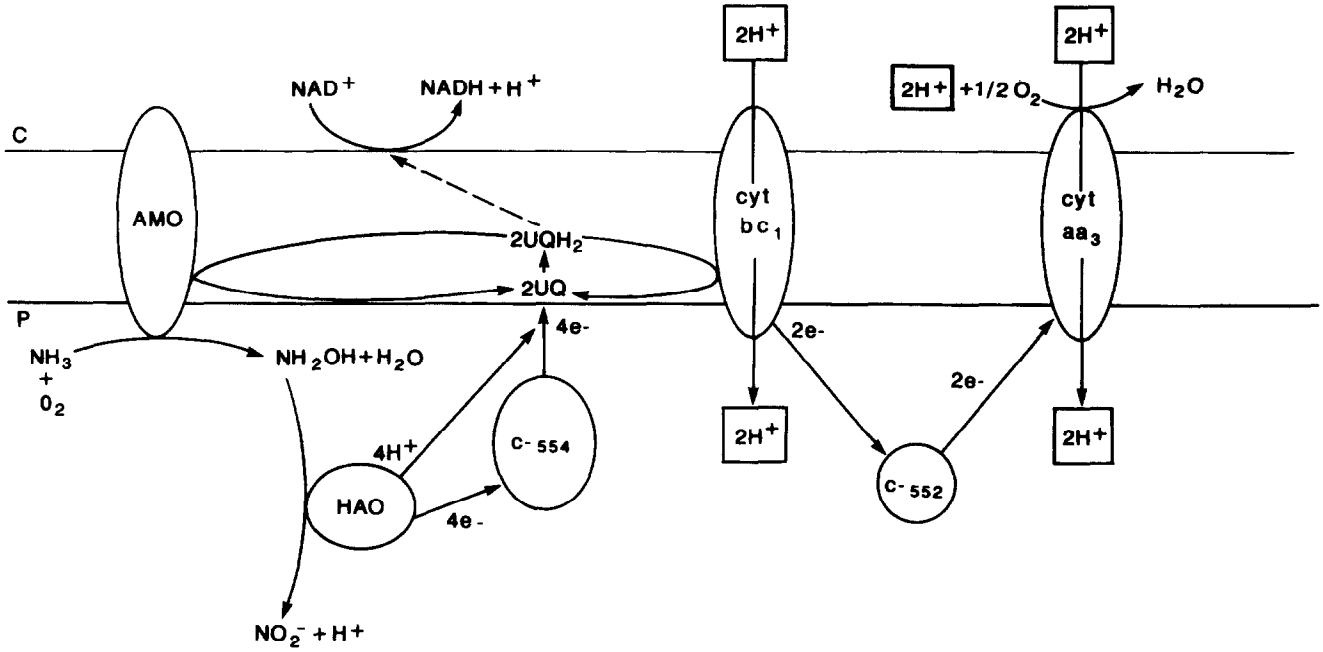

Fig. 1. The electron transfer pathways involved in nitrification in *Nitrosomonas europaea*. The nitrification pathway shown is based on that proposed by Wood [3]. The scheme depicts the oxidation of one molecule of ubiquinol, by the ammonia monooxygenase (AMO), which is consumed in the oxidation of ammonia to hydroxylamine. The hydroxylamine is oxidised to nitrite by the hydroxylamine oxidase, generating four electrons and five protons. The four electrons generated pass to cytochrome c_{554} and then, with four protons, are used to reduce two molecules of ubiquinone to two molecules of ubiquinol. One molecule of ubiquinol is oxidised by the cytochrome bc_1 complex, with the two electrons proceeding via two molecules of cytochrome c_{552} to the cytochrome aa_3 oxidase to reduce one atom of oxygen to water. The second ubiquinol molecule is used to refurbish the AMO with reductant. A small proportion of electrons (approximately two for every twenty ammonia molecules oxidised) are used to reduce NAD^+. The boxed protons will contribute to the generation of a proton electrochemical gradient across the cytoplasmic membrane which drives ATP synthesis and reverse electron transfer for the reduction of NAD^+. C = cytoplasm; P = periplasm.

column at around 80 mM NaCl and was contaminated by cytochrome c_{551}. The cytochrome was removed by chromatagraphing the fractions on a Sephacryl S-100 gel exclusion column. SDS-PAGE analysis was carried out using the discontinuous method of Laemmli [9].

HAO was assayed spectrophotometrically using either horse-heart cytochrome *c*, *T. pantotropha* cytochromes, pseudoazurin or ferricyanide as electron acceptors . For the cytochrome assays an increase of absorption at 550 nm was used to measure the reduction of the cytochrome. Reduction of peudoazurin and ferricyanide was monitored as a decrease in absorption at 590 nm and 400 nm, respectively. Reactions were carried out in 1 ml cuvettes at 25°C. Samples containing HAO and electron acceptor were mixed in the cuvette in 10 mM Tris-HCl, pH 7.8, and the reactions were initiated by addition of hydroxylamine. Malate dehydrogenase and nitrite were assayed as decribed previously [10,11].

3. RESULTS AND DISCUSSION

3.1. *Partial purification of the hydroxylamine oxidase*

Cells of *T. pantotropha* were fractionated into periplasm, membranes and cytoplasm. Hydroxylamine oxidase activity could be identified using either the horse heart cytochrome *c* or ferricyanide assay and was located exclusively in the periplasmic fraction (Fig. 2; data shown for horse heart cytochrome *c* assay). The integrity of the fractionations was established by assaying for malate dehydrogenase which fractionated into the cytoplasm (Fig. 2). These results clearly demonstrate that the HAO of *T. pantotropha* is a periplasmic enzyme, like its autotrophic counterpart [12]

Fractionation of the periplasmic fractions using anion exchange and gel-exclusion chromatography re-

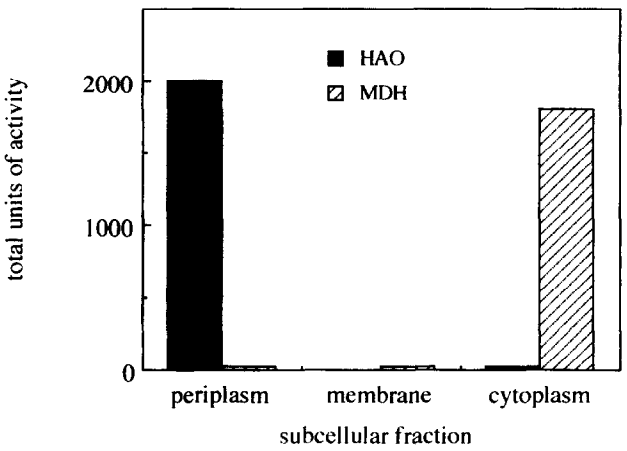

Fig. 2. The subcellular distribution of hydroxylamine oxidase activity in *Thiosphaera pantotropha*. One litre of aerobically grown cells was fractionated. The figures represent total units of activity in each subcellular fraction. Malate dehydrogenase (MDH) was assayed as a cytoplasmic marker. HAO, one activity unit = 1 μmol ferri cytochrome *c* reduced per minute. MDH, one activity unit = 0.1 μmol of NADH oxidised per minute.

sulted in fractions containing HAO activity that were not contaminated by cytochrome (as judged by the complete lack of haem-type spectral signals in the visible absorption spectrum). Although not analysed quantitatively, the oxidation of hydroxylamine by the partially purified *T. pantotropha* HAO resulted in the accumulation of nitrite. The enzyme could also utilise hydrazine as an alternative substrate, a property it shares with the autotrophic enzyme.

Analysis of these HAO fractions using a calibrated gel-exclusion column indicated that the enzyme had an apparent molecular weight of 20 kDa. SDS-PAGE analysis revealed the presence of a major polypeptide with a molecular weight of 20 kDa (Fig. 3). N-terminal analysis of this band revealed no significant homology with any other protein sequence currently in the data bases, including peptide sequences of the *N. europaea* hydroxylamine oxidase [13]. The 20-kDa band was electroeluted and dialysed extensively against Tris-HCl (5 mM pH 8.0). The electroeluted sample could catalyse hydroxylamine dependent horse heart cytochrome *c* reduction, but this was dependent on the presence of ferric ions in the assay mixture. This suggests that the enzyme could possess a non-haem ferric iron centre that is lost when the 20-kDa polypeptide is subjected to SDS-PAGE, but that can be reconstituted by addition of ferric ions to the refolded enzyme. Accordingly, preliminary EPR spectroscopic analysis on the partially purifed HAO sample, from the gel exclusion step, indicates the presence of a non-haem iron redox centre (Farrar and Thomson, personal communication). Taken together, these results suggest that the HAO may be a non-haem iron monomeric 20-kDa enzyme. This contrasts markedly with the HAO purified from *N. europaea*, which is a multimer of a 60-kDa polypeptide that binds 7 c-haems and a haem P-460 [13,14]. On this basis it is suggested that a second class of HAO should be recognised.

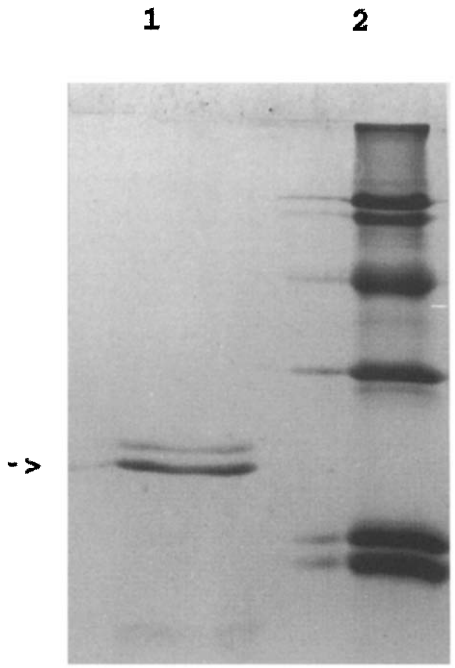

Fig. 3. SDS-PAGE of the partially purified hydroxylamine oxidase sample that was obtained from the gel filtration chromatography step. The gel was stained with Coomassie blue-R. Standard markers are as follows: ovotransferin 76–78,000 Da; albumin 66,250 Da; ovalbumin 42,700 Da; carbonic anhydrase 30,000 Da; myoglobin 17,200 Da; cytochrome *c* 12,300 Da. Lane 1, hydroxylamine sample; lane 2, standards. The arrow indicates the polypeptide that was electroeluted and shown to possess hydroxylamine oxidase activity.

Arthrobacter globiformis is the only other heterotrophic bacterium from which a purification of hydroxylamine oxidase has been attempted [15]. In this study cell-free extracts were fractionated with ammonium sulphate and the HAO activity did not co-fractionate with cytochrome [15]. It seems possible that the *A. globiformis* enzyme is a similar species to that identified in *T. pantotropha*. In the case of the Gram-positive *A. globiformis*, the enzyme may be anchored to the outer face of the cytoplasmic membrane.

3.2. *Electron acceptors for the hydroxylamine oxidase*

Fractionation of the *T. pantotropha* periplasm revealed a number of *c*-type cytochromes which included those that have previously been recognised to be associated with the periplasmic nitrate reductase [5], cytochrome cd_1 [8], cytochrome *c* peroxidase (Richardson and Ferguson, unpublished), and cytochrome *c'* (Moir, Richardson and Ferguson, unpublished) and a number of as yet unassigned cytochromes *c*. All of the cytochrome-containing fractions were tested as electron acceptors for the HAO. Only one species, cytochrome c_{551}, could serve as electron acceptor. Cytochrome c_{551} is a small (14 kDa) class 1 type cytochrome that can serve in electron transfer to denitrification enzymes [7,8,16]. The rate of cytochrome c_{551} reduction was a hyperbolic function of cytochrome concentration and an apparent K_m of 10 μM could be calculated. *T. pantotropha* can also express a cupredoxin, pseudoazurin [8]. This could also serve as an electron acceptor for the HAO. Again, a hyperbolic relationship of rate of reduction to pseudoazurin concentration could be established and an apparent K_m of 33 μM calculated. V_{max} was 0.129 $\mu mol \cdot min^{-1} \cdot mgHAO^{-1}$ for cytochrome c_{551} and 0.99 $\mu mol \cdot min^{-1} \cdot mgHAO^{-1}$ for pseudoazurin.

3.3. *A model of electron transport that couples nitrification to denitrification in T. pantotropha*

Both cytochrome c_{551} and pseudoazurin are soluble redox proteins that can donate electrons to the denitrification enzymes [7,8,16]. However, they cannot mediate electron transfer to the quinone pool. This suggests a mechanism, shown in Fig. 4A, in which electrons originating from ubiquinol pass via AMO and HAO into the cytochrome c_{551}/pseudoazurin pool. The reaction also generates nitrite, which together with the reduced cyto-

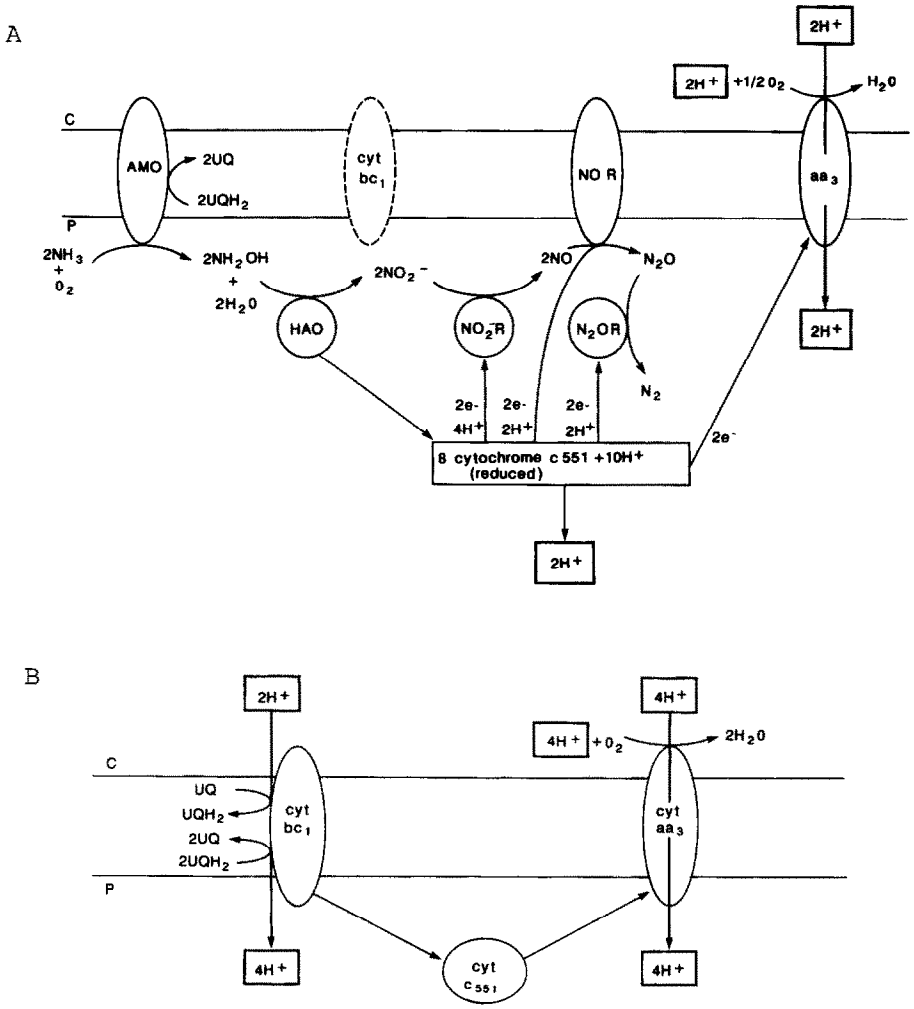

Fig. 4. A model for heterotrophic nitrification coupled to aerobic denitrification in *Thiosphaera pantotropha*. In this scheme the oxidation of two molecules of ubiquinol by two turnovers of the ammonia monooxygenase (AMO) is coupled to the generation of two molecules of hydroxylamine from ammonia. The two molecules of hydroxylamine are oxidised to two molecules of nitrite by the hydroxylamine oxidase (HAO) resulting in the generation of eight molecules of reduced cytochrome c_{551} and ten protons. Cytochrome c_{551} is also an electron donor to the denitrification enzymes and the cytochrome aa_3 oxidase. Thus the turnover of HAO provides the substrates required for complete conversion of two molecules of nitrite to dinitrogen: nitrite, six molecules of cytochrome c_{551} and eight protons. The remaining two molecules of cytochrome c_{551} can be used to reduce an atom of oxygen to water. The result of this process is that there is a net removal of two molecules of ubiquinol from the ubiquinol/ubiquinone pool accompanied by the translocation of only two protons across the cytoplasmic membrane. The consumption of two protons in the cytoplasm by oxygen reduction and the net production of two protons in the periplasm from hydroxylamine oxidation will also contribute to the generation of a proton electrochemical gradient. (B) In the absence of nitrification coupled to denitrification the complete oxidation of two molecules of ubiquinol via the cytochrome bc_1 complex and the cytochrome aa_3 oxidase would be predicted to be accompanied by the appearance of eight protons in the periplasmic compartment and depletion of ten protons in the cytoplasm. Thus the coupling of nitrification to denitrification by the mechanism shown in (A) can provide an effective mechanism for oxidising ubiquinol via a poorly coupled electron transport pathway that proceeds independently of the cytochrome bc_1 complex. C = cytoplasm; P = periplasm.

chrome c_{551}/pseudoazurin pool, results in both of the required substrates for denitrification being present. Thus, using the electrons generated from hydroxylamine oxidation, the nitrite can be sequentially reduced to NO, N_2O and N_2. Comparison of Fig. 4A and B shows the potential bioenergetic consequences of oxidising two molecules of UQH_2 by this route. In the complete absence of nitrification all of the electrons will pass via the protonmotive cytochrome bc_1 complex and cytochrome aa_3 oxidase and reduce a molecule of oxygen. The oxidation of two molecules of UQH_2 by AMO coupled to denitification will result in the protonmotive cytochrome bc_1 complex being by-passed and the reduction of only 0.5 O_2 by the cytochrome aa_3 oxidase. Thus this scheme of denitrification coupled to nitrification provides a mechanism by which reductant can be dissipated through the oxidation of UQH_2 via a relatively uncoupled electron transfer pathway. It therefore accounts also for the lower than expected growth yields observed in actively nitrifying cultures of *T. pantotropha*

[1]. Finally the model explains the inability of *T. pantotropha* to couple oxidation of ammonia to autotrophic growth, since it does not provide a route by which electrons generated from hydroxylamine oxidation can re-enter the Q-pool to provide substrate for the continued turnover of the AMO or reversed electron transfer to NAD^+.

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