The biochemical characterization of a novel non-haem-iron hydroxylamine oxidase from *Paracoccus denitrificans* GB17

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The characterization of the hydroxylamine oxidase from the heterotrophic nitrifier *Paracoccus denitrificans* GB17 indicates the enzyme to be entirely distinct from the hydroxylamine oxidase from the autotrophic nitrifier *Nitrosomonas europaea*. Hydroxylamine oxidase from *P. denitrificans* contains three to five non-haem, non-iron–sulphur iron atoms as prosthetic groups, predominantly co-ordinated by carboxylate ligands. The interaction of the enzyme with the electron-accepting proteins cytochrome c_{550} and pseudoazurin is mainly hydrophobic. The catalytic mechanism of hydroxylamine oxidase from *P. denitrificans* is different from the enzyme from *N. europaea* because the production of nitrite by the former requires molecular oxygen. Under anaerobic conditions the enzyme makes nitrous oxide as a sole product.

INTRODUCTION

Paracoccus denitrificans GB17 (formerly *Thiosphaera pantotropha*; [1]) is an α-proteo bacterium capable of heterotrophic nitrification [2]. The biochemistry and genetics of nitrification have been studied in detail only in *Nitrosomonas europaea*, an autotrophic nitrifier. In *N. europaea* the enzyme that catalyses the oxidation of hydroxylamine to nitrite, hydroxylamine oxidase, is a multimer with a subunit molecular mass of 63 kDa with seven *c*-haems and one *P*-460 haem in each subunit [3,4]. The electron acceptor from this enzyme *in vitro* is a tetra-haem *c*-type cytochrome, c_{554}. Cytochrome c_{554} is considered to donate electrons into the electron transport chain at the level of the quinone pool [5]. For each turnover of hydroxylamine oxidase one molecule of hydroxylamine is oxidized to nitrite, with the associated four electrons being transferred to the quinone pool. Half of the resultant quinols transfer electrons to the ammonia mono-oxygenase so that one molecule of hydroxylamine is generated from each molecule of ammonia. The other half of the quinols transfer electrons either via the cytochrome bc_1 complex to an oxidase so that a protonmotive force is generated, or via reverse electron flow to the NADH dehydrogenase so that NADH is generated from NAD^+ [5].

Heterotrophic nitrifiers, unlike autotrophic nitrifiers, are incapable of using nitrification to support growth. It has therefore been proposed that heterotrophic nitrification is not linked to the generation of a protonmotive force and that the electron acceptors for hydroxylamine oxidase in heterotrophic nitrifiers have more positive potentials than the cytochrome bc_1 complex [6]. Hydroxylamine oxidase from *P. denitrificans* GB17 has been isolated from the periplasm and found to have different properties from the enzyme from *N. europaea* [6]. The enzyme from *P. denitrificans* has a molecular mass of 20 kDa and contains no haem, indicating that it belongs to a distinct class of hydroxylamine oxidases. A non-haem hydroxylamine oxidase has been partly purified from the Gram-positive heterotrophic nitrifier *Arthrobacter globiformis* [7], indicating that the non-haem-iron hydroxylamine oxidase might be widespread among heterotrophic nitrifiers. In this paper a characterization of the enzyme from *P. denitrificans* is presented.

MATERIALS AND METHODS

P. denitrificans GB17 was grown in oxic batch culture in minimal medium [8] with 30 mM potassium acetate as electron donor and carbon source, and 10 mM NH_4^+ as nitrogen source.

The procedures used for the purification of hydroxylamine oxidase were as described in [6]. The purity of hydroxylamine oxidase was determined with SDS/PAGE. Protein bands were revealed on SDS/PAGE by silver staining, with a kit obtained from the Sigma Chemical Company. Protein concentration was estimated with a modified Lowry kit from the Sigma Chemical Company. For further analysis of the properties of hydroxylamine oxidase the purified enzyme preparation was dialysed for 24 h against 10 mM Tris/HCl (pH 8), by using Spectra/Por dialysis tubing with a molecular mass cut-off of 3.5 kDa.

The iron content of purified hydroxylamine oxidase was estimated colorimetrically with 3-(2-pyridyl)-5,6-bis[2-(5-furylsulphonic acid)]-1,2,4-triazine (also known as Ferene S). Samples (0.5 ml) of protein were added to 0.2 ml of 1.4 M HCl and denatured by heating at 80 °C for 10 min. After cooling, 0.4 ml of 10 % (w/v) trichloroacetic acid was added. The mixture was centrifuged at 4 °C for 15 min at 12000 ***g*** to remove precipitated protein. A 1 ml aliquot of the supernatant was taken and reduced with ascorbate; then 0.2 ml of 5 mM Ferene S (dissolved in 2.5 M sodium acetate) was added and the blue colour of the Fe^{II}–Ferene S complex was developed for 5 min. The intensity of the signal was measured at 600 nm. Fe^{III} EDTA solutions were used as standards.

UV/visible spectroscopy of protein samples was performed in the range 220–800 nm with a Hitachi U3000 spectrophotometer. Spectra were obtained at room temperature. Assays of hydroxylamine oxidase activity were performed as described previously [6] with horse heart cytochrome *c* (obtained from Sigma), cytochrome c_{550} from *P. denitrificans* [9] and pseudoazurin from *P. denitrificans* [10] as electron acceptors.

Nitrite was assayed colorimetrically [11]. Nitrous oxide was assayed by gas chromatography. Assays were performed in cuvettes fitted with gas-tight rubber septa. Samples of gas were withdrawn with a Hamilton syringe and injected into the sample loop of a Unicam 610 gas chromatograph. The gases were

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separated at 60 °C on a Hayesep Q column and nitrous oxide was detected by electron capture.

Molecular mass was determined by electrospray mass spectrometry. Spectra were measured on a VG Platform single-quadrupole atmospheric-pressure mass spectrometer equipped with a VG electrospray interface. Samples (20 μl) of purified protein at a concentration of 1 mg/ml were injected into the electrospray source via a loop injector at a flow rate of 2 μl/min.

RESULTS

Kinetic analysis

Hydroxylamine oxidase was assayed routinely under pseudo-first-order conditions with 30 nM enzyme, 30 μM cytochrome c_{550} as an electron acceptor and 1 mM NH_2OH as substrate in 10 mM Tris/HCl (pH 8). Under these conditions k_{cat} was 32 min^{-1}. The effects of ionic strength on hydroxylamine oxidase activity with horse heart cytochrome *c*, cytochrome c_{550} and pseudoazurin (each at a concentration of 30 μM) were examined with NaCl. Hydroxylamine oxidase activity with horse heart cytochrome *c* as an electron acceptor was inhibited by NaCl such that the rate with 25 mM NaCl was half that with no added salt (Figure 1A). However, with pseudoazurin (Figure 1B) and cytochrome c_{550} (Figure 1C) there was no such loss of hydroxylamine oxidase activity on adding low concentrations of NaCl. Enzyme activity with these electron acceptors was only inhibited on addition of NaCl to concentrations over 100 mM. Hydroxylamine oxidase activity with cytochrome c_{550} as electron acceptor was enhanced by NaCl concentrations between 5 and 50 mM. These results indicate that the interaction of hydroxylamine oxidase with horse heart cytochrome *c* is predominantly electrostatic whereas the interactions of hydroxylamine oxidase with cytochrome c_{550} and pseudoazurin are predominantly hydrophobic.

The effects of pH on hydroxylamine oxidase activity with horse heart cytochrome *c*, cytochrome c_{550} and pseudoazurin (each at a concentration of 30 μM) were examined by performing the hydroxylamine oxidase assay in a variety of buffers over the pH range 5–12. The buffers used for these assays were sodium acetate, Mes, sodium phosphate, Tris, sodium borate, Tricine and 3-(cyclohexylamino)propane-1-sulphonic acid (CAPS). Concentrated HCl or NaOH was used to adjust the pH of the buffers. Tris/HCl as buffer gave an activity approx. 20 % higher than the other buffers at any given pH. Marked differences in the effect of pH were seen with each of the electron acceptors (Figure 2). With horse heart cytochrome *c*, maximum hydroxylamine oxidase activity was observed at pH 8.5, whereas hydroxylamine oxidase activity continued to increase to pH 10 with pseudoazurin and to pH 11 with cytochrome c_{550}. With all three electron acceptors there was virtually no hydroxylamine oxidase activity below pH 6. The decrease in activity of hydroxylamine oxidase above pH 8.5 with horse heart cytochrome *c* as electron acceptor is consistent with the finding that the interaction is primarily hydrophilic. Horse heart cytochrome *c* can transfer electrons with redox partners via a lysine-rich patch; the deprotonation of this above pH 8.5 might be the cause of the loss of activity. The effects of pH on the activity of hydroxylamine oxidase with pseudoazurin and cytochrome c_{550} as electron acceptors might be due to the pK_a of amino acid residues important in either the activity of hydroxylamine oxidase itself or in the interactions between the enzyme and the electron acceptors.

Cyanide was found to be an inhibitor of hydroxylamine oxidase. Hydroxylamine oxidase (30 nM) was used in assays with 30 μM cytochrome c_{550} as electron acceptor. With 1 mM cyanide, turnover of the enzyme seemed to be necessary to cause

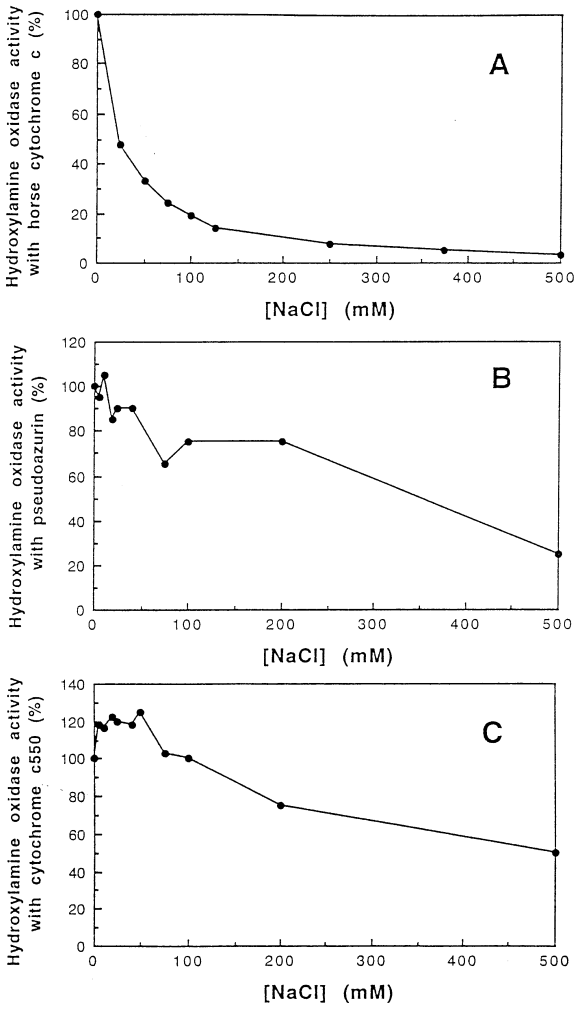

Figure 1 The effects of NaCl on hydroxylamine oxidase activity

Hydroxylamine oxidase activity was measured in 10 mM Tris/HCl (pH 8) with 1 mM hydroxylamine and the purified enzyme at a concentration of 30 nM. The electron acceptors were used at concentrations of 30 μM: (**A**) horse heart cytochrome *c*; (**B**) pseudoazurin; (**C**) cytochrome c_{550}. The rate of reduction of the electron acceptors was measured at 550 nm for the cytochromes and 590 nm for pseudoazurin. Results are expressed as the percentage of the activity without added NaCl.

inhibition of the enzyme activity. Cyanide (1 mM) was incubated with the enzyme for 10 min before the assay was initiated, and the initial rate of hydroxylamine oxidase activity was the same as in the absence of the inhibitor, but inhibition was observed with $t_{\frac{1}{2}} = 10$ s. If the enzyme plus hydroxylamine or the enzyme plus cytochrome c_{550} was preincubated with 1 mM cyanide the initial rate of activity was the same as with the uninhibited enzyme, inhibition subsequently occurring during the course of the assay. Also, when the enzyme was reduced with dithionite and pre-incubated with 1 mM cyanide before the beginning of the assay the initial hydroxylamine oxidase activity was the same as the uninhibited rate but inhibition occurred during the course of the assay. These results imply that the cyanide-sensitive form of the enzyme exists only during turnover.

Under aerobic assay conditions 1.5–2.0 mol of cytochrome c_{550} was reduced per mol of hydroxylamine. Nitrite was the major reaction product [1.5–2.0 mol of nitrite produced per mol of cytochrome c_{550} reduced] but N_2O was also detected at low

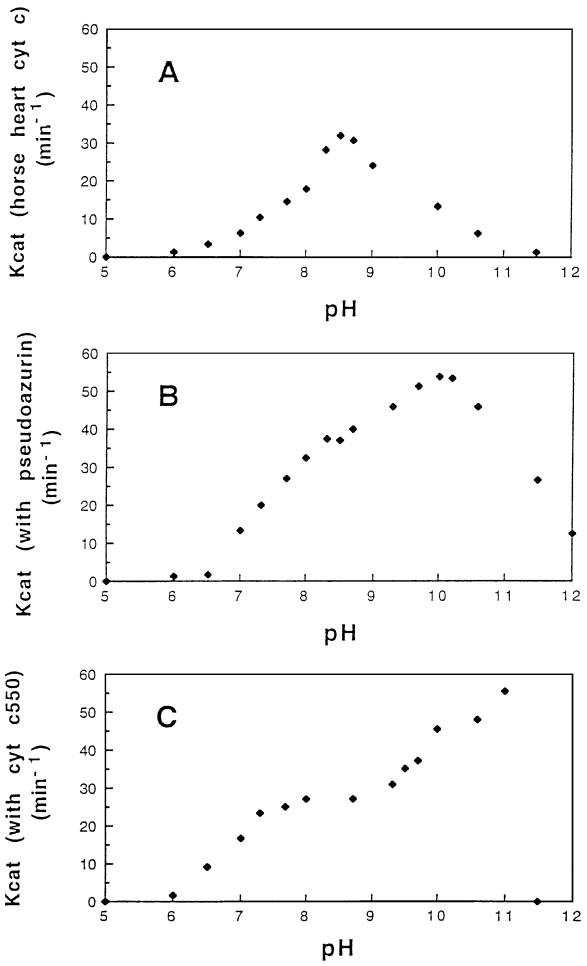

Figure 2 The effects of pH on hydroxylamine oxidase activity

Hydroxylamine oxidase activity was measured with 1 mM hydroxylamine and the purified enzyme at a concentration of 30 nM. The buffers used all had a concentration of 10 mM. Buffers used were Na acetate (pH 5–6), Mes (pH 6–7), sodium phosphate (pH 6.5–8), Tricine (pH 8–9), sodium borate (pH 8.5–10) and 3-(cyclohexylamino)propane-1-sulphonic acid (pH 10–12). Concentrated HCl or 10 M NaOH was used to adjust the pH of the buffers, as appropriate. The electron acceptors for the assays were used at concentrations of 30 μM: (**A**) horse heart cytochrome *c*; (**B**) pseudoazurin; (**C**) cytochrome c_{550}. The rate of reduction of the electron acceptors was measured at 550 nm for the cytochromes and 590 nm for pseudoazurin.

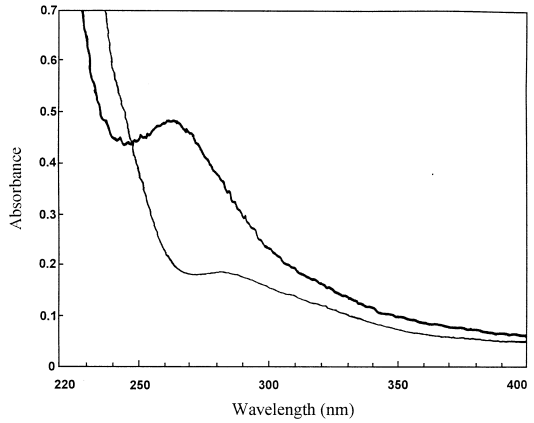

Figure 3 UV/visible spectroscopy of hydroxylamine oxidase

Absorption spectroscopy was performed with a Hitachi U3000 spectrometer. The bold line represents the spectrum of oxidized hydroxylamine oxidase (protein concentration 4 μM, Fe concentration 20 μM). The fainter line represents the absorption spectrum of the same protein sample after reduction with sodium dithionite. Dithionite was added to the sample after making it anaerobic by purging with nitrogen. Spectra were measured at room temperature.

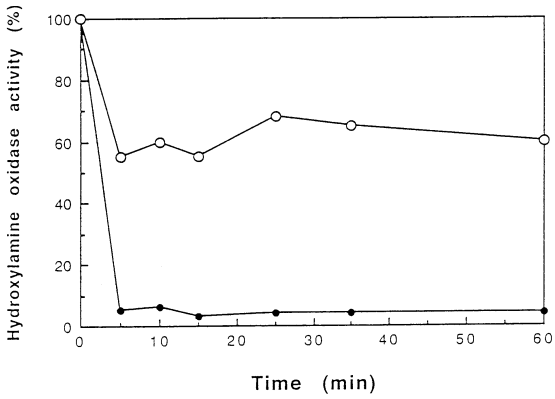

Figure 4 The effect of EDTA on hydroxylamine oxidase activity

Hydroxylamine oxidase (5 μM) was treated with 1 mM EDTA in 10 mM Tris/HCl at pH 8 (●) and pH 10 (○). Aliquots were taken and assayed for hydroxylamine oxidase activity for 1 h. Results are expressed as the percentage of the activity before treatment with EDTA.

levels (0.1 mol per mol of cytochrome c_{550} reduced). Under anaerobic conditions no nitrite was produced but nitrous oxide could again be detected in the headspace, though at non-stoichiometric levels (0.1 mol of cytochrome c_{550} reduced per mol). This result indicated that with cytochrome c_{550} as electron acceptor the production of nitrite by *P. denitrificans* hydroxylamine oxidase is an oxygen-dependent reaction.

Analysis of metal centres

The purified hydroxylamine oxidase [dialysed against 10 mM Tris/HCl (pH 8)] was colourless; UV/visible spectroscopy indicated no visible absorbances but revealed an intense absorption band at 260 nm (Figure 3). This band was redox-active. Careful titration with sodium dithionite caused the disappearance of the band at 260 nm, leaving a much weaker signal at 280 nm, presumably the absorption by aromatic amino acid residues within the protein.

The absorption band at 260 nm was found to be removed by dialysis against 10 mM Tris/HCl (pH 8)/1 mM EDTA for 24 h to leave a much smaller absorbance at 280 nm (as seen in the dithionite-reduced form of the enzyme; Figure 3). This treatment with EDTA caused complete inactivation of hydroxylamine oxidase. Treatment of the enzyme after inactivation by EDTA with Fe^{III} or Fe^{II} ions caused a return of hydroxylamine oxidase activity and the absorption band at 260 nm, indicating that the enzyme has one or more labile non-haem iron atoms as its prosthetic group(s).

Treatment of the purified hydroxylamine oxidase with 1 mM EDTA led to almost complete inhibition of activity when performed in 10 mM Tris/HCl (pH 8) over a period of a few minutes. However, in 10 mM Tris/HCl (pH 10)/1 mM EDTA there was only partial inhibition of activity over the first few minutes and the remainder of the activity was retained over a period of hours (Figure 4). This can be rationalized because Fe^{III} iron will be ligated in the protein more strongly at high pH, when

the amino acid residues ligating the iron are less likely to be protonated.

Addition of 1 mM EDTA to hydroxylamine oxidase at pH 8 (without subsequent dialysis) did not cause complete inactivation (Figure 4). The residual activity was found to be less than 5 % of that of the uninhibited enzyme. Fe^{III} EDTA was found to have 3 % of the hydroxylamine oxidase activity of the hydroxylamine oxidase enzyme itself with cytochrome c_{550} as electron acceptor.

UV/visible spectra of Fe^{III} EDTA and Fe^{III} citrate gave absorption bands at 255 nm with a molar absorption coefficient per iron of 8000 $M^{-1} \cdot cm^{-1}$, whereas Fe^{III} complexes with a chelating agent in which the carboxylate groups of EDTA were replaced with benzimidazoles [*N*,*N*,*N*′,*N*′-tetrakis-(2-benzimidazolylmethyl)-1,2-ethanediamine, which was kindly supplied by Dr. P. S. Dobbin, University of East Anglia], did not give absorption bands in this region, indicating that the ligand formation that gives rise to the band at 260 nm in hydroxylamine oxidase is not from amino acids with imidazole or amine side chains.

The iron content of purified hydroxylamine oxidase before dialysis against 10 mM Tris/HCl (pH 8) was high, indicating an iron content of approx. 100 per molecule of enzyme. However, the iron content of purified hydroxylamine oxidase, dialysed against 10 mM Tris/HCl (pH 8) for 24 h, ranged from three to five atoms per protein molecule and there was no significant loss of activity on dialysis, indicating the presence of a large amount of adventitious iron in the non-dialysed samples. Given a molecular mass of hydroxylamine oxidase of 18506 ± 1 Da (obtained by electrospray mass spectrometry), a molar absorption coefficient of hydroxylamine oxidase for the band at 260 nm (ϵ_{260}) of 75–125 $mM^{-1} \cdot cm^{-1}$ per enzyme molecule was calculated. The variation in molar absorption coefficient was correlated with iron content of hydroxylamine oxidase and indicated that the ϵ_{260} per iron was 25 $mM^{-1} \cdot cm^{-1}$. This is 3-fold higher than the molar absorption coefficients for Fe^{III} EDTA and Fe^{III} citrate bands around this wavelength, but this difference would have been contributed to by the background absorption by the amino acid backbone, and scattering by the protein solution in the UV.

To examine the accessibility of the iron centres of hydroxylamine oxidase to chelation by exogenous ligands, the appearance of a visible charge-transfer band by treatment with thiocyanate (SCN^-) was examined in comparison with Fe^{III} EDTA. Thiocyanate was found to give rise to a charge-transfer band centred at 450 nm in Fe^{III} EDTA, with a molar absorption coefficient of 400 $M^{-1} \cdot cm^{-1}$, but no visible charge-transfer bands were observed after treatment of 10 μM hydroxylamine oxidase (containing 45 μM iron) with thiocyanate. This indicated that the iron centres of hydroxylamine oxidase are less accessible to ligand binding by this species than the hexadentate Fe^{III}–EDTA complex.

DISCUSSION

The results presented here confirm that the hydroxylamine oxidase from *P. denitrificans* is distinct from the multi-haem hydroxylamine oxidase from *N. europaea* [6]. The enzyme from *P. denitrificans* is a 20 kDa monomer that contains no haem. The inhibition of the enzyme with EDTA and subsequent re-activation with Fe^{II} or Fe^{III} ions indicates that the enzyme contains labile iron that is necessary for the activity. The absence of visible absorbance bands characteristic of iron–sulphur clusters indicates the enzyme contains non-haem, non-iron–sulphur iron centres. A non-haem hydroxylamine oxidase has been partly purified from the heterotrophic nitrifier *A. globiformis* and found to be activated by Fe^{II} ions [7], indicating that it might be similar to hydroxylamine oxidase from *P. denitrificans*.

The absorption band at 255 nm in Fe^{III} EDTA is caused by the carboxylates rather than by the amine ligands because the chelator *N*,*N*,*N*′,*N*′-tetrakis-(2-benzimidazolylmethyl)-1,2-ethanediamine, in which carboxylate ligands are replaced by imidazoles, did not give rise to the 255 nm absorbance. The imidazole ligands of *N*,*N*,*N*′,*N*′-tetrakis-(2-benzimidazolylmethyl)-1,2-ethanediamine did not give rise to absorbances in the UV. Some other non-haem-iron proteins have previously been found to have ligation by sulphurous ligands and by tyrosine residues, both of which give rise to visible charge-transfer bands at approx. 500 nm; isopenicillin N synthase has sulphur ligation [12]; catechol dioxygenase has tyrosine ligation [13]. The presence of an absorption band at 260 nm and the absence of visible absorbances together imply that the non-haem iron atoms are ligated predominantly by carboxylates.

Measurements of the stoichiometry of iron in hydroxylamine oxidase from *P. denitrificans* indicate the presence of three to five iron atoms per molecule. The variability in measurement of iron content is due to the lability of the iron centres of the enzyme and the large amount of adventitious iron associated with the purified enzyme before dialysis.

Reactions of hydroxylamine with iron have been reported previously. Under strongly acidic conditions Fe^{III} ions are soluble and are reduced to Fe^{II} ions by hydroxylamine, with concomitant oxidation of hydroxylamine to nitrous oxide. Kinetic analysis of this reaction has not yielded consistent results. Different workers have reported the reaction to be first- or second-order with respect to Fe^{III} ions [14,15]. However, both sets of workers invoke a mechanism employing the reduction of two Fe^{III} ions to Fe^{II} to oxidize one molecule of hydroxylamine to nitroxyl (NOH). Two molecules of nitroxyl are then considered to react to give nitrous oxide and water as final products. The only nitrogenous product of this reaction is nitrous oxide. The pH dependence of this reaction indicates that the reactive species is uncharged hydroxylamine (NH_2OH), even though at the low pH of the acid reaction hydroxylamine is mainly in a protonated form (NH_3OH^+). (This reaction had to be performed in acid conditions because Fe^{III} ions are only freely soluble in aqueous solution at low pH). Under strongly alkaline conditions Fe^{III} EDTA acts as a catalyst of hydroxylamine oxidation by molecular oxygen. This reaction gives a stoichiometric mixture of nitrogenous products, nitrite and nitroxyl. In this reaction the reactive species is considered to be the deprotonated form of hydroxylamine, NH_2O^-, because the reaction is strongly inhibited below pH 11.5 [16].

The oxidation of hydroxylamine by hydroxylamine oxidase is similar to the acid reaction. The activity of hydroxylamine oxidase decreases from pH 8 to undetectable below pH 6, indicating that the reactive species is uncharged hydroxylamine (the pK_a of NH_3OH^+/NH_2OH is 8). Above pH 11.5, the active range for the alkaline reaction, hydroxylamine oxidase is not active. In the alkaline reaction the Fe^{III} ions act as catalysts and are not reduced overall; whereas in hydroxylamine oxidase Fe^{III} ions must be reduced to Fe^{II} ions by the hydroxylamine so that the enzyme acts as a cytochrome reductase.

Cyanide exerts its inhibitory effect on hydroxylamine oxidase during the turnover of the enzyme. Two explanations can be offered for this phenomenon. First, the inhibition by cyanide might be possible only during turnover if the active site of the enzyme is open to cyanide binding only during the turnover of the enzyme. This possibility is supported by the finding that hydroxylamine oxidase is not accessible to the ligand SCN^- (whereas Fe^{III} EDTA is), i.e. the active site of hydroxylamine

oxidase is opened up to cyanide binding only once hydroxylamine and cytochrome *c* (or pseudoazurin) are present and the enzyme conformation has changed. The second explanation for the inhibition by cyanide during turnover is that the inhibitor binds to a redox state of the enzyme that is not the resting redox state or the dithionite-reduced state. The active site of the resting enzyme may be a Fe^{III}–Fe^{III} binuclear centre. After treatment with dithionite the active site becomes Fe^{II}–Fe^{II}. It is possible that cyanide does not bind to either of these forms and can bind only to a semi-reduced, i.e. Fe^{III}–Fe^{II}, binuclear centre existing only during turnover of the enzyme.

The possibility of a binuclear iron centre is enticing. This is in keeping with the proposed chemical mechanism of Fe^{III} ion reduction by hydroxylamine in which two Fe^{III} ions are involved in the oxidation of a molecule of hydroxylamine to nitroxyl. The invocation of a binuclear centre at the active site of hydroxylamine oxidase from *P. denitrificans* makes it analogous with the more complex enzyme from *N. europaea*, in which an integer-spin resonance has been assigned to a bi-haem centre consisting of the *P*-460 haem and one of the *c*-haems [17].

If the resting form of hydroxylamine oxidase has a Fe^{III}–Fe^{III} binuclear centre at the active site, the first reaction intermediate of hydroxylamine oxidase in its reaction with hydroxylamine is a Fe^{II}–Fe^{II} binuclear site bound to a molecule of nitroxyl. With cytochrome c_{550} as the electron acceptor the binuclear active site is reoxidized with reduction of two molecules of cytochrome c_{550}, possibly with electron transfer occurring via mononuclear non-haem-iron centres. Under anaerobic conditions the only measured nitrogenous product of hydroxylamine oxidase *in vitro* is nitrous oxide. It is possible that, as in the acidic reaction of Fe^{III} iron with hydroxylamine, the product nitroxyl leaves the centre and combines with a second nitroxyl to give nitrous oxide and water. Under aerobic conditions both nitrous oxide and nitrite are found as products. Presumably, therefore, the formation of nitrite is the result of an oxidation of nitroxyl to nitrite by molecular oxygen.

In *N. europaea*, nitrite is the product of hydroxylamine oxidase even in the absence of oxygen. Four electrons are passed to cytochrome c_{554} from hydroxylamine oxidase as hydroxylamine is oxidized to nitrite. Two of these electrons are used by the ammonia mono-oxygenase and the other two electrons are used in the electron transport chain, either to generate a protonmotive force via the cytochrome bc_1 complex to an oxidase or to generate NADH via reverse electron transfer to NADH dehydrogenase. Therefore more than two electrons from the hydroxylamine oxidase must reduce cytochrome c_{554} so that ATP and NADH can be produced as a result of autotrophic nitrification. If the product of hydroxylamine oxidase is nitroxyl and the reaction to nitrite involves reaction with oxygen, only two electrons are transferred to cytochrome per oxidation of hydroxylamine. This is not a problem in *P. denitrificans*, in which the hydroxylamine oxidase reaction is not used to conserve energy.

The requirement that the enzyme from *N. europaea* must perform a four-electron oxidation (i.e. transfer electrons from the active site to the other redox centres of the enzyme to avoid the release of nitroxyl), whereas that from *P. denitrificans* performs only a two-electron oxidation, is presumably a reason for the apparently greater complexity of the *N. europaea* enzyme than the *P. denitrificans* enzyme.

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