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c-Cytochromes of the ammonia-oxidizing chemolithoautotrophic bacteria

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The major enzymes associated with the oxidation of ammonia by chemolithoauthotrophic bacteria are studied, including some cytochromes *c*, are described and details of their properties are given.

Nitrosomonas is an obligately lithotrophic, autotrophic and aerobic bacterium which produces energy for growth from the following reactions [1,2]:

$NH_3 + O_2 + 2e^- \rightarrow NH_2OH$ (ammonia monoxygenase, "AMO");

$NH_2OH + H_2O$

$\rightarrow 4e^- + 4H^+ + HNO_2$ (hydroxylamine oxidoreductase, "HAO");

and

$\frac{1}{2}O_2 + 2e^- + 2H^+ \rightarrow H_2O$ (an aa_3 type terminal oxidase).

HAO, which is soluble and periplasmic, catalyzes a proton-yielding reaction which, in concert with proton-pumping from the cytoplasmic-side (and proton-utilizing reaction) of the terminal oxidase, is presumed to contribute to energy conservation. AMO is membrane-associated.

The HAO reaction yields four electrons. Two electrons must be returned to the AMO reaction to regenerate hydroxylamine. The other two electrons are most often consumed in the terminal oxidase reaction. For some fraction of the turnovers of AMO, two electrons are used to reduce pyridine nucleotide for biosynthetic reactions. Under low oxygen concentrations, a significant fraction of the electrons usually destined for the terminal oxidase are consumed by a nitrite-reducing enzyme. A consequence of the multiplicity of pathways of electron transport is the requirement for a variety of redox-active species and a mechanism for sorting electrons into the appropriate pathways. Cytochrome *c*-554 [3] is apparently the electron acceptor from HAO. Other possible electron-transfer components found in the organism include the soluble, periplasmic mono-heme cytochrome *c*-552, ubiquinone, some intrinsic membrane *c*-cytochrome(s) and cytochrome aa_3; few or no *b*-type cytochromes are present [4].

The two *c*-cytochromes involved in this pathway which have been most completely characterized are unique multi-heme proteins. They are HAO [5], in which hemes have both a catalytic and electron transfer role, and cytochrome *c*-554 [6], in which *c*-hemes have an electron-transfer role.

Hydroxylamine oxidoreductase

This enzyme contains many *c*-hemes, several of which are reduced upon addition of substrate. Because of the complexity of subunit structure and the large number of hemes it has been difficult to elucidate the number of hemes per subunit of HAO. By determining the amino acid sequence of all trypsin- and chymotrypsin-generated heme-peptides, the subunit of this protein has now been shown to contain 7 *c*-hemes [7]. The enzyme is probably an aggregate of 2 or 3 of the 63 kDa [8] subunits. HAO also contains a covalently-bound pigment absorbing at 460 or 450 nm in the dithionite-reduced or dithionite-reduced + CO forms, respectively. Treatment of ferric HAO with H_2O_2 results in loss of optical absorbance at 460 nm (when reduced) but not absorbance due to *c*-hemes; the P-460 ring is apparently destroyed. H_2O_2-treated HAO lacks both the ability to catalyse the oxidation of NH_2OH (in the presence of

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Abbreviations: AMO, ammonia monoxygenase; HAO, hydroxylamine oxidoreductase.

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substrate amounts of electron acceptor) and the substrate-reducibility of *c* hemes of the enzyme [9]. Thus, during turnover of HAO, electrons are thought to pass $NH_2OH \rightarrow$ P-460-containing active site → *c*-hemes of HAO → cytochrome *c*-554. Spectroscopic data suggest that P-460 is a heme-like iron macrocycle [10]. Studies with model compounds have suggested that the axial ligand of the iron of ferrous P-460 is a strong anion.

The *c*-hemes and P-460 have been characterized on the basis of approximate number, α-band absorption maxima and midpoint oxidation-reduction potential at pH 7 as follows [11]: 1 *c*-553 (+295 mV), 1 *c*-553 (+10 mV), 2 *c*-559 (0 mV), 2 *c*-553 (−190 mV), P-460 (−260 mV), 1 *c*-553 (−390 mV). EPR spectra of HAO reveal a rich collection of species and interactions [12,13]. They cannot yet be unambiguously assigned to the hemes mentioned above. The spectrum of resting HAO contains signals characteristic of low-spin *c*-hemes at $g = 3.03$ and 2.98 in the ratio 3:2 and unusual signals at apparent g values of 3.4 and 2.7 which are thought to be spin-coupled [14]. Equilibrium redox titration reveals (a) unusual electronic interactions between the highest potential heme(s) and the $g = 3.03$ hemes (which shift EPR properties upon reduction of high-potential hemes), (b) coupling between P-460 and one of the *c*-hemes and (c) coupling which renders the lowest potential *c*-heme EPR-silent. A titration is most readily described in four steps. *Step 1*: reduction of hemes *c*-553 (+295 mV) leads to disappearance of all $g = 3.03$ spins and an *increase* in EPR absorbance at $g = 2.98$, suggesting that one or more $g = 3.03$ center(s) have been reduced and another shifted to 2.98. *Step 2*: reduction of hemes *c*-553 (+10 mV) and *c*-559 (0 mV) results in disappearance of the 2.98 species and disappearance of the spin-coupled pair ($g = 3.4$ and 2.7). *Step 3*: Reduction of the *c*-553 (−190 mV) hemes results in loss of the $g = 2.98$ signal; simultaneously, the complex signal in the $g = 6$ region appears in the spectrum for the first time. The latter is thought to arise from P-460, since it disappears at the redox potential at which P-460 is reduced. Because these complex $g = 6$ EPR signals are not seen in the spectrum of oxidized HAO we hypothesize that P-460 is coupled to another paramagnetic center (presumably one of the *c*-553 (−190 mV) hemes) in resting HAO. *Step 4*: After reduction of P-460 and *c*-553 (−190 mV) but prior to reduction of the lowest potential *c* heme all EPR absorbance typical of low spin *c*-heme has disappeared from the spectrum; thus, the lowest potential *c*-heme is EPR-silent and may be spin-coupled.

Mechanism of turnover of HAO

As NH_2OH is oxidized to nitrite, electrons pass to 2 or 3 of the highest potential *c* hemes at a rate commensurate with turnover rates of HAO in vitro or in vivo [14]. The reaction is complex, involving removal of 4 electrons and 3 protons and the net addition of one atom of oxygen from water. Some of the likely *N*-oxide intermediates (e.g., NO^-, NO, NO^+) are typical ligands to ferrous heme centers; thus one might expect to find a ferrous center in the active site during catalysis. Significantly, although P-460 is part of the active site, optical measurements during reduction of the *c*-hemes by substrate reveal no evidence of reduction of P-460; i.e., P-460 remains in the ferric state throughout catalysis. EPR and Mössbauer spectra show that P-460 is a high-spin center and therefore pentacoordinate. P-460 is apparently coupled to a heme *c*-553 and may have a strongly anionic axial ligand. We suggest that P-460 and the active site *c*-heme are connected by a bridging anionic ligand. The *c*-heme has a normal, histidine ligand on the face opposite the P-460 whereas the P-460 has no ligand on the face opposite the *c*-heme:

(his – *c* heme – A^- – P460).

Substrate binding to this bi-heme site could occur in one of two ways: (a) NH_2OH could bind to P-460 on the face opposite the *c*-heme (that is, substrate could bind only to P-460):

(his – *c* heme – A^- – P-460-NH_2OH)

or (b) NH_2OH could displace the anionic ligand and bind as a bridging ligand between the two hemes [15]:

(his – *c* heme – NH_2O^- – P-460).

The latter alternative allows for a more satisfactory explanation of the known and reasonably-expected properties of the active site. For instance, as mentioned above, P-460 apparently remains ferric during catalysis; however, the first observable event during stopped-flow studies of the reaction is the reduction of a *c*-heme. We hypothesize that the *c*-heme of the active site is reduced to the ferrous state and can then effectively bind and stabilize the *N*-oxide intermediates of the reaction. In the ferric form, P-460 has the capability of serving as a hard acid to stabilize anionic intermediates. Protons may be removed during the reaction by the concerted effect of P-460 and the bridging anionic (basic) ligand. The net addition of OH^- from H_2O to the last intermediate, NO^+, is also thought to be facilitated by ferric P-460 and the bridging anionic ligand. During catalysis the high-potential *c*-hemes of HAO function as acceptors of electrons from the active site *c*-heme.

One of the several lines of evidence supporting this hypothesis is the inhibition of the reaction by cyanide [16]. Cyanide binds to ferric HAO, almost certainly to the iron of the P-460 center. The affinity of the CN^--enzyme for substrate is essentially unchanged, but the overall reduction of the high-potential *c*-hemes occurs about 100-fold slower. The same number of *c*-hemes are reduced in the presence or absence of CN^-. These observations are readily interpreted in terms of model (b), above; CN^- binds to P-460 on the face opposite to the *c*-heme, forcing it into a low-spin ferric state and reducing the effective positive charge on the iron by one. Substrate can still bind across the bi heme site, but the P-460 would now be much less effective as an acid catalyst. These observations are much more difficult to rationalize using model (a). Model (a) also does not provide for a ferrous component of the active site.

If this model is correct, HAO shares with cytochrome *c*-peroxidase and possibly the hexaheme nitrite reductase (Ref. 17; see Ref. 23) in having a substrate-binding and reactive *c*-heme. There is no reason to think that *c*-hemes are employed in these enzymes because of special catalytic properties resulting from covalent binding of the heme. Rather, the *c*-heme and P-460 are likely to be covalently bound simply because the *c*-cytochromes are in the periplasm where loss of the heme to the solvent would be irreversible [18]. It is possible that, even in the *c*-cytochromes, which are intrinsic membrane proteins, the heme is always in a protein domain which projects into the periplasm, as has been shown for cytochrome *c*-550 of *B. subtilis* [19].

Cytochrome *c*-554

This cytochrome, previously thought to be soluble and periplasmic, is in fact an extrinsic membrane protein. After removal of the cytochrome at high salt concentrations, the amount which was washed off rebinds to a high-affinity membrane binding site, presumably by ionic interactions [21]. Additional added cytochrome *c*-554 binds with lower affinity. This is unusual, since most *c*-cytochromes appear to be either intrinsic proteins or soluble periplasmic proteins. Alternatively, some extrinsic *c*-cytochromes which are presently considered to be intrinsic proteins may not have been experimentally challenged with a high enough salt concentration.

The four hemes of cytochrome *c*-554 are all magnetically coupled, resulting in a highly unusual frequency-dependent EPR spectrum with a major feature at $g = 3.3$ as measured at the X-band [6]. Mössbauer spectra show that 75% of the hemes are low spin and 25% high spin. An increased fraction becomes high-spin as the pH is decreased. The values of E_0' are as follows: two hemes at $+47$ mV (a high-spin and low-spin pair), one heme at -147 mV and one at -276 mV (Arciero, Collins, Haladjian, Bianco and Hooper, unpublished data). Cytochrome *c*-554 and HAO interact to form a kinetically competent, electrostatically stabilized complex. Kinetics of electron transfer from HAO to cytochrome *c*-554 during turnover of hydroxylamine account for in vivo rates of turnover of HAO. Further, cytochrome *c*-554 is a two-electron acceptor with the electrons residing, at equilibrium, on the high-potential pair of *c*-hemes [21]. It is possible that, as a two-electron mediator, this cytochrome may participate in the 'sorting' of electrons to AMO and the terminal oxidase. Ferrous cytochrome *c*-554 could also readily donate electrons to a two-electron acceptor such as ubiquinone. The role of the two low-potential hemes, the significance of the high-spin heme (since high spin hemes are commonly involved in ligand binding) and the significance and structural basis for the electronic coupling of the hemes are unknown.

Other *c*-cytochromes of *Nitrosomonas*

The periplasm also contains a mono-heme cytochrome *c*-552, which is able to accept electrons from cytochrome *c*-554 [3]. Several electron transport components are found in nearly integer quantities: HAO (per P-460), cytochrome *c*-554, ubiquinone, cytochrome *c*-552, cytochrome aa_3 [4,21]. This suggests a role for these components in a major electron transport pathway. There is an additional integer amount of *c*-cytochrome in the membrane, though it is not known how many species are represented. The ferrous form of a membrane species will bind CO. Several soluble (presumably periplasmic) *c*-cytochromes are present in relatively small amounts. They include cytochrome c_{co}-550, cytochrome c_{co}-552, cytochrome *c*-553 and cytochrome *c*-peroxidase.

Aspects of evolution of HAO

Interestingly, HAO shares many properties with the 'cytochrome *c*-552' respiratory nitrite reductases from *E. coli*, *Wolinella*, *Desulfovibrio* and *Vibrio* [17]. The latter catalyze the reaction

$HNO_2 + 6e^- + 6H^+ \rightarrow NH_3 + 2H_2O$;

the reaction catalyzed by HAO is a shortened version of the reversal of that reaction. Whereas each subunit of the nitrite reductase is a hexaheme protein (56, 57, 63 or 66 kDa), the hepta-*c*-heme subunit of HAO has a mass of 63 kDa. In addition, HAO and the cytochrome *c*-552 nitrite reductase are remarkably similar in amino acid composition [5,17,22]. These similarities suggest that the two enzymes might have a common evolutionary ancestor. Would this putative ancestral enzyme have functioned in the oxidative or reductive direction? The early atmosphere contained ammonia but lacked dioxygen and, presumably, nitrite. Thus there would have been no evolutionary pressure towards a nitrite-reducing enzyme. Large amounts of nitrite would most likely have appeared in the biosphere after the advent of oxygenic photosynthesis and then aerobic nitrification. Thus, the ancestral enzyme might have participated in the latter oxidative process. Alternatively, nitrite might have been first produced in the biosphere by an anaerobic nitrifying enzyme (i.e., by an organism which derived electrons from ammonia by the water-utilizing reversal of the reaction catalysed by the present hexaheme cytochrome *c*-552 nitrite reductase). In this evolutionary scenario the latter enzyme would have evolved into HAO. The ammonia-to-hydroxylamine portion of the ancestral enzyme would have been replaced by a new enzyme (AMO) which catalysed a more efficient oxygen-utilizing reaction.

Multiplicity of HAO genes

By reaction of genomic digests with a 29-base polynucleotide probe coding for the sequence of amino acids near one of the heme-binding sites of HAO, we find that the genome of *Nitrosomonas* has multiple copies of genes for HAO [22]. This is seen in separate experiments utilizing ten different restriction enzymes for the genomic digestion. Plasmids were not detected. Thus there appear to be three identical copies of a 9-amino-acid region of HAO in the genome of *Nitrosomonas*. It is not known whether the coding sequence for the entire protein is duplicated. The function of this duplication is not known. HAO is obviously central to the energy metabolism of *Nitrosomonas* and accounts for approx. 5% of the total soluble protein. It is possible that cells have three identical copies of the gene to facilitate rapid transcription or that three slightly different forms of the enzyme have three different biochemical roles.

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