The Diversity of Bacterial Redox Proteins

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Consideration of a phlorin structure for haem P-460 of hydroxylamine oxidoreductase and its implications regarding reaction mechanism

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Introduction

The aerobic, lithotrophic, autotrophic bacteria exemplified by *Nitrosomonas europaea* derive energy from the activities of hydroxylamine oxidoreductase (HAO; $\text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{HNO}_2 + 4\text{e}^- + 4\text{H}^+$) [1] and ammonia monoxygenase (AMO; $\text{NH}_3 + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O}$) [2] which are co-dependent for generation of substrate or electrons, respectively [3-5]. Electrons pass from HAO to the tetra-haem cytochrome c-554 [6,7] and then to AMO or, by way of the monohaem cytochrome c-552, to an aa3-Cu,,-type terminal oxidase [8], a nitrite- [9,10] or NO-reductase [11], a cytochrome c peroxidase [12] or an NAD reductase. HAO will also rapidly oxidize hydrazine to dinitrogen, a reaction which is experimentally useful and may be reflected in the production of dinitrogen in the bacterial anaerobic oxidation of ammonia [1,13]. The periplasmic cytochrome P-460 [14], also found in the methylotrophs [15], oxidizes hydroxylamine too but its significance to cellular energy economy is unknown.

**HAO**

HAO, one of the most complex haemoproteins known, consists of 63 kDa subunits [16] each containing 7 c-haems [17] and one residue of a novel haem P-460 [18]. The haems have midpoint potentials (and $g_{\text{max, resting}}$) of $+288 \ (3.0)$, $+11 \ (3.4/2.7)$, $-10 \ (3.4/2.7)$, $-162 \ (3.0)$, $-192 \ (7.7)$, $-260 \ (7.7)$ (P-460), $-265 \ (3.0)$, and $-412 \text{ mV} \ (3.0)$ [19-22] and behave as one-electron redox centres [20]. The range of midpoint potentials observed in HAO for c-haems, all with bis-(His) coordination, demonstrates the influence of the protein environment. From the crystallographic structure, c-haems 1 and 2, c-haems 3 and 5, and c-haems 6, 7 and haem P-460 are parallel (but not stacked), with a 4 Å interplanar distance [23] leading to their designation as ‘dihaem or trihaem clusters’; only c-haem 8 appears to be non-interacting. Magnetic spectroscopy suggests that two pairs are coupled: the two c haems with a weak magnetic interaction and an $E_{\text{m7}}$ near 0 mV are probably the number 1/2 or number 3/4 pair; and the P-460 haem is ferromagnetically exchange-coupled to one c-haem [24] (see below). The four other c-haems have optical properties and $g$-values typical of isolated low-spin c-haem centres. In the presence of hydroxylamine the three highest-potential haems of HAO are reduced [7].

**Haem P-460**

Haem P-460, a component of the active site [20,25-27], is named for the optical band (presumably a Soret) at 463 nm in the spectrum of dithionite-reduced HAO [28,29]. The Soret band of oxidized P-460 is weak and underlies the intense Soret bands of ferric HAO, but may be inferred from optical changes which occur upon treatment of the enzyme with cyanide [30], alkyl hydrazines [31], or hydrogen peroxide [25]. During electrochemical titrations a weak broad optical band at 740 nm associated with haem P-460, which is absent in ferric HAO, appears after the 0 mV haems have been reduced [32] and disappears as the P-460 haem is...
The disappearance of this band has also been kinetically linked to the reduction of haem P-460 [32]. Since haem P-460 is low spin in fully oxidized HA0 but converts to a high-spin haem prior to its reduction, the 740 nm band is clearly associated with the high-spin haem. The appearance of the 740 nm band does not follow Nernstian behaviour, however, and the potential at which it appears depends on the direction of the spectropotentiometric titration suggesting that the appearance of the 740 nm band reflects the spin state transition for the P-460 haem rather than reduction of a specific c-haem.

Elucidation of the structure of haem P-460 was initiated by finding that the P-460 chromophore in a crosslinked di-peptide produced by proteolysis of HA0 was attached to one polypeptide chain through a c-haem-binding motif (Cys-Xaa-Xaa-Cys-His) and the sequence of the second peptide contained a residue not assignable to a specific amino acid [17]. Subsequent analysis by NMR and mass spectroscopy of a smaller chromo-peptide identified a protoporphyrin IX covalently attached through a meso carbon to a ring carbon of a tyrosine [27]. This is the only known biologically derived crosslinking of a tyrosine to a haem although one can be non-biologically induced by treatment of myoglobin with H2O2 [33]. Protein- and gene-sequencing has shown that Tyr-467, Cys-229 and Cys-232 are the amino acids involved in haem P-460 linkage [17,27,34].

The crystal structure confirms a crosslinked Tyr-467 and adds that the crosslink is intersubunit and involves the C3 ring carbon of the tyrosine. Most significantly, the fact that the tyrosyl ring is above and thus perpendicular to the plane of the porphyrin raises an important point not raised in the crystal structure paper. If the porphyrin ring structure is of protoporphyrin IX, then the substituent (whether a histidine or a tyrosine) of the meso position should be in the equatorial plane pointing directly away from the Fe centre. The orientation of Tyr-467 is thus more consistent with the C5 meso (CHC) haem position involved in the covalent linkage as a tetrahedral sp3 centre having both axial and equatorial bonds in addition to the two bonds to adjacent pyrrole rings. The haem moiety of haem P-460 would then be a dihydroporphyrin unsaturated at the C5 meso position (a phlorin, [35]). This would represent an Fe-containing tetrapyrrole structure not previously observed in nature.

Several features of a phlorin-like haem P-460 could have interesting mechanistic consequences. First, Tyr-467 would be held close to the metal centre and could participate in catalysis more easily either by direct interaction with the substrate or by acting as the conduit for electron flow out of the substrate rather than through the Fe-centre. Secondly, the tyrosyl-haem P-460 structure would be isoelectronic with a free c-haem and unbound Tyr-467; the three structures in Figure 1 would be equivalent. A potentially significant consequence of the linkage would be the movement of electron density from the tyrosine into the porphyrin ring system resulting in a pseudo-oxidized tyrosine and a pseudo-reduced porphyrin. In addition, the ring system and the -OH of Tyr-467 would not be in conjugation with the porphyrin ring system as it would be if the haem was protoporphyrin IX.

How might the properties of a phlorin-like haem P-460 bear on a reaction involving the removal of four electrons from NH2OH, presumably generating the nitrosonium cation (NO+*) which would condense spontaneously with water to form nitrite? A conventional mechanism would predict all chemistry to occur at the Fe centre of haem P-460 with all intermediates remaining bound to the Fe [36] (Figure 2A); although oxidation of NH2OH in this manner is energetically simple, it would be unusual for a cytochrome. c-Cytochromes commonly undergo redox changes (i.e. act as electron transfer catalysts) but catalysis involving the removal of multiple electrons and protons from substrate is unprecedented. Instead, oxidations carried out by cytochromes have always involved O2 or H2O2 mediated by the generation of an activated Fe(IV) = O intermediate. Clearly, for a dehydrogenation-type reaction to occur at the Fe centre of haem P-460, the structure of haem P-460 must play a pivotal role in allowing the reaction. What is counterintuitive to this scheme
is the distribution of electron density resulting from the phlorin-like porphyrin structure; why should a reduced porphyrin ring be more suitable for the oxidation of substrate?

Could the oxidation take place by a mechanism not centred at the Fe? In principle, oxidations can occur in two additional fundamentally different ways. In the molybdopterin enzyme, sulphite oxidase, an activated oxygen species is generated as the oxidant but it arises from the oxidation of water by two electrons to an oxomolybdo species before insertion into the substrate [37]. This type of mechanism is shown in Figure 2(B). It seems highly unlikely that HAO would utilize this mechanism since an oxidant as strong as ferryl oxygen should not be required to oxidize NH$_3$OH (or the HNO intermediate), there is no known precedent for this reaction with ferric haemoproteins, and the potential of the electrons generated by formation of the ferryl oxygen intermediate would be of too high a potential to be of benefit to the organism. The other oxidative class of reactions are 2e$^-$ dehydrogenations catalysed by conjugated ring systems such as NAD$^+$, FAD, $o$-quinones, etc. but not metals. Two mechanisms for oxidation of NH$_3$OH by HAO take advantage of the fact that Tyr-467 could, in theory, fulfil the role of a conjugated ring system. A mechanism similar to oxidation of methyamine by the pyrroloquinoline quinone (PQQ)-dependent enzyme, methyamine dehydrogenase [38], could be written for HAO if the $-$OH of Tyr-467 were in conjugation with the porphyrin ring system (Figure 2C). Here NH$_3$OH would react with Tyr-467 instead of the Fe centre to form a Schiff base intermediate; protoporphyrin IX would fulfil the role of the second O in an orthoquinone-like cofactor. However, since Tyr-467 of the putative dihydro-porphyrin haem P-460 is not in conjugation with the

**Figure 2**

Hypothetical mechanisms for oxidation of hydroxylamine by HAO

(A) Oxidation through the Fe centre. (B) Oxidation of water. (C) Formation and oxidation of Schiff base (PPIX, protoporphyrin IX). (D) Hydride-transfer.

(A) \[ \text{Fe-NH}_3\text{OH} \rightarrow \text{[Fe-HNO]} + 2e^- + 2H^+ \]
\[ \text{[FeHNO]} \rightarrow \text{[Fe-NO]} \rightarrow \text{[Fe-NO']} + 2e^- + H^+ \]
\[ \text{[Fe-NO']} + H_2O \rightarrow \text{HNO}_3 + H^+ \]

(B) \[ \text{NH}_3\text{OH} \rightarrow \text{H-NO} + 2e^- + 2H^+ \]
\[ \text{Fe-H}_2\text{O} \rightarrow \text{[Fe(V)=O]} + 2e^- + 2H^+ \]
\[ \text{[Fe(V)=O]} + \text{H-NO} \rightarrow \text{H}_2\text{O-NO} \]
\[ \text{NH}_3\text{OH} + H_2O \rightarrow \text{HNO}_3 + 4e^- + 4H^+ \]
porphyrin ring system, this mechanism is unlikely. Rather, the dihydro-porphyrin structure of haem P-460 is compatible with a hydride transfer mechanism. The phlorin-like haem P-460, in which redistribution of electron density leads to pseudo-oxidized and pseudo-reduced tyrosine and haem rings respectively, may be thought of as a structure in which the tyrosyl haem, rather than the substrate or oxygen, has been ‘activated’ to carry out the reaction. Even with substrate bound at the Fe this structure could help to direct the flow of electrons through Tyr-467 instead of through the Fe centre by promoting hydride transfer to a ketonized form of Tyr-467 (Figure 2D). A weakness of this hypothesis is that the linkage between the haem and Tyr-467 must break and re-form during the reaction cycle. In addition, the fate of the two hydride electrons must be dealt with. In this regard, the exchange-coupled, haem P-460/haem 6 di-haem pair could, in fact, act as a two-electron acceptor. In this hydride transfer mechanism, the two electrons could be delocalized over both haem centres with subsequent electron transfer to other redox centres occurring very rapidly.

Identification of c-haem coupled to haem P-460

In order to understand fully the interactions among c-haems of HAO as well as intra- and inter-molecular electron transfer involving HAO, assigning midpoint potentials to individual c-haems will ultimately be required. We have recently been able to assign a midpoint potential to the c-haem initially coupled to haem P-460 [32]. In fully oxidized HAO, haem P-460 is a low-spin S = 1/2 ferric centre but participates in a weak, ferromagnetic exchange-coupling with another low-spin ferric centre [24]. The coupled centre is characterized by an S = 1 integer spin signal at g = 7.7 observed in parallel-mode EPR. In fully reduced HAO, haem P-460 is a high-spin S = 2 centre [18]. In between these two extremes, the P-460 Fe exists in two other distinct electronic environments. The first is an EPR-silent form resulting from the disappearance of the g = 7.7 signal as a Nernstian redox centre with E'_0 of approximately −140 mV becomes reduced. The second is a high-spin ferric centre characterized by a g = 6 signal which appears as another Nernstian redox centre (with E'_0 of approximately −190 mV) becomes reduced. Because no EPR signals appear as the g = 7.7 signal disappears, we have concluded that the c-haem initially coupled to haem P-460 is not reduced at this point. Instead, we believe that another c-haem in close proximity to the coupled centre affects the coupling between haem P-460 and its c-haem partner. The effect on coupling could result from: (i) coupling changing from ferromagnetic to antiferromagnetic; (ii) haem P-460 becoming high spin with the resulting s = 2 or 3 centre becoming EPR-silent. The c-haem coupled to haem P-460 is thus assigned to a −190 mV redox centre. The crystal structure of HAO reveals that haem 6 is the only c-haem in close enough proximity to haem P-460 to qualify as the exchange-coupled partner [32].

Cytochrome P-460

A second protein in N. europaeae, cytochrome P-460, also appears to possess a haem P-460-like chromophore [39] but no c-haems. Its gene sequence shows no homology with HAO but does contain a single c-haem binding motif [40]. Sequence analysis of a haem-containing peptide has revealed that the haem in cytochrome P-460 is also cross-linked to a distant region of the polypeptide chain. However, the cross-link appears to be a lysine instead of a tyrosine [41]. While no structural information is available regarding details of the cross-link or position of the lysine relative to the porphyrin ring, we can note that tyrosine and lysine have similar pKₐ values.

Conclusions

Consideration and consequences of the haem P-460 of HAO being a phlorin-like haem have been discussed. General oxidation mechanisms have been reviewed and re-worked in the context of HAO. Although intriguing alternative mechanisms are proposed for HAO, it seems likely that oxidation of NH₂OH by HAO proceeds with all chemistry occurring at the Fe centre of haem P-460.

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