The alternative oxidases: simple oxidoreductase proteins with complex functions

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Abstract
The alternative oxidases are membrane-bound monotopic terminal electron transport proteins found in all plants and in some agrochemically important fungi and parasites including *Trypanosoma brucei*, which is the causative agent of trypanosomiasis. They are integral membrane proteins and reduce oxygen to water in a four electron process. The recent elucidation of the crystal structure of the trypanosomal alternative oxidase at 2.85 Å (1 Å = 0.1 nm) has revealed salient structural features necessary for its function. In the present review we compare the primary and secondary ligation spheres of the alternative oxidases with other di-iron carboxylate proteins and propose a mechanism for the reduction of oxygen to water.

Introduction
It is generally recognized that the distribution of the AOX (alternative oxidase) is substantially wider than previously thought [1]. In addition to being ubiquitous among the plant kingdom, it is also common among some agrochemically important fungi and parasites including *Chalara fraxinea* (U.K. die-back Ash disease) and *Magnaporthe grisea* (rice blast fungus) and protists [1,2]. The AOX is also widespread among human parasites such as *Trypanosoma brucei* (the causative agent of African sleeping sickness) [3,4], intestinal parasites such as *Cryptosporidium parvum* and *Blastozyctis hominis* [5,6] and opportunistic human pathogens such as *Candida albicans* [7].

Prior to the publication of the structure of any AOX, sequence analysis, extensive site-directed mutagenesis, EPR and spectroscopic studies [8–18] predicted that it was an integral (∼32 kDa) interfacial membrane protein that interacted with a single leaflet of the lipid bilayer, which contained a non-haem di-iron carboxylate active site [10] comparable with that observed in other di-iron proteins such as RNR R2 (ribonucleotide reductase R2 subunit) and MMO (methane mono-oxygenase). Interestingly, the recombinant protein self-assembles when expressed in *Schizosaccharomyces pombe* or *Escherichia coli* without the requirement of any specific co-factors (apart from iron) and attaches itself into the membrane to become a fully viable member of the electron transport chain [19].

Non-haem di-iron-containing enzymes are a ubiquitous and diverse superfamily of metalloenzymes [20]. They can be divided into different subfamilies with a wide range of distinct catalytic functions, such as peroxidation, oxidation, hydroxylation, desaturation, NO reduction and even aging-related disorders and utilize a wide variety of substrates (see [21]). Despite their diverse range of activities, however, the majority of the enzymes in this di-iron family share very common structural elements. These include a common fold involving a four-helix bundle, a bridging carboxylate group across the di-iron centre, the presence of common ligands, such as the widespread motif comprising two histidine residues and four carboxylate groups and importantly the possession of a common catalytic function, namely the activation of molecular oxygen [20,21]. Given these common structural characteristics and yet diverse chemical reactivities, the question arises as to whether changes in chemical reactivity are a reflection of the type or position of the amino-acid residues in the primary and secondary ligation spheres.

The AOX is one of the newest members of the di-iron group of proteins whose structure has recently been elucidated [1,22] and in the present review we compare the structure of this protein with that of other di-iron proteins. We believe that both a structural and mechanistic understanding of this protein is important not only to direct and inform future rational phytopathogenic or anti-parasitic drug design, but also because the structural simplicity and stability of this family of proteins make them an ideal choice for structure–function correlation studies. Furthermore, we also believe that since the AOX is a naturally occurring oxidoreductase containing a minimal four-helical scaffold structure which lacks spectral features that complicate other such respiratory chain proteins, such studies should reveal salient structural features that not only are necessary for its oxidoreductase function, but also will inform how minor variations in the geometry or ligation site of the active site of such proteins result in such a diverse range of functions.
**Figure 1 | Structure of TAO and comparison of its active site with other di-iron carboxylate proteins**

(A) Cartoon representation of the dimeric structure of TAO in which helices are visualized as cylinders roughly parallel to the membrane. (B) Surface representation of the TAO dimer showing the hydrophobic and hydrophilic surfaces. Colours are according to the following hydrophobicity scale: red, high hydrophobicity; white, low hydrophobicity (http://www.pymolwiki.org/index.php/Color_h). Conserved basic residues located on the interface between TAO and the membrane (Arg^{106}, Arg^{180}, Arg^{203}, and Arg^{106*}) are coloured blue. (C) Di-iron active sites of TAO (green), (D) ruberythrin (cyan), (E) \(\alpha\)-subunit of MMO (yellow) and (F) ribonucleotide reductase R2 subunit (orange). Oxygen and nitrogen atoms are coloured red and blue respectively. Di-iron, hydroxo and \(\mu\)-oxo atoms are shown as spheres. Coordinate bonds to di-iron centres are shown by continuous lines. The non-protein ligand acetate in (E) is shown as dark yellow sticks. Wat stands for water molecule. This Figure was generated using PDB code 3VV9.

**AOX crystal structure**

We were able to recently solve the structure of the AOX in the absence and presence of AOX-specific inhibitors at 2.6 and 2.3 \(\AA\) (1 \(\AA\) = 0.1 nm) respectively [PDB codes 3VV9 for TAO (trypanosomal AOX), 3VVA for TAO-AF2779OH complex and 3W54 for TAO-collatochlorin] [22]. This structure represents a major research milestone, for it is not only the first structure of any AOX irrespective of species to be crystallized, but also the first membrane-bound di-iron protein and the last of the respiratory chain oxidoreductases to be solved. Crystal structures indicate that the AOX is a homodimer with each monomer comprising six long \(\alpha\)-helices and four short \(\alpha\)-helices [22] (Figure 1A). The four long \(\alpha\)-helices (2, 3, 5 and 6) are arranged in an antiparallel fashion and form a four-helix bundle which acts as a scaffold to bind the two iron atoms. Within the homodimer, the two monomers are related by a two-fold axis perpendicular to the membrane surface and the dimer interface is built...
around α-helices 2, 3 and 4 of one monomer and α-helices 2°, 3° and 4° of its neighbour (Figure 1B). A large hydrophobic face is visible on one side of the dimer surface which is formed by α-helices 1 and 4 plus the C-terminal region of α-helix 2 and the N-terminal region of α-helix 5 (Figure 1B). Similar to other monotopic proteins such as the yeast NADH dehydrogenase (Ndi1) [23] and prostaglandin H₂ synthase [24], this hydrophobic region undoubtedly anchors the protein to the inner surface of the inner mitochondrial membrane via a series of conserved arginine residues which probably interact with the phospholipid headgroups (Figure 1B).

Nature of the active site
The active site is buried deep within the AOX molecule in a hydrophobic environment. Under oxidized conditions the iron atoms within the active site of the AOX are ligated by a single hydroxo-bridge and four glutamate residues, but, unusually for di-iron proteins, the two highly conserved histidine residues are too far away (>4 Å) from the di-iron centre to act as ligands. Evidence in favour of a hydroxo-bridge (rather than an oxo-bridge) include the lack of absorbance above 340 nm, the result that a mixed-valence EPR signal is seen only at very low temperatures and the small value of the exchange coupling constant ($-J$) [16,17]. In addition to the hydroxo-bridge, the iron atoms are bridged by Glu162 and Glu265, whereas Glu213 and Glu215 act as bidentate ligands to Fe1 and Fe2 respectively. The Fe1–Fe2 distances are 3.3 Å, which are compatible with a differic state rather than a diferrous state in which the distances are ~4.2 Å [21,25]. Such a primary ligation sphere gives rise to a five-co-ordinated di-iron centre with a distorted square pyramidal geometry (Figure 1C) similar to that observed in the reduced form of the castor acyl-ACP (acyl carrier protein) desaturase [25]. The redox-active tyrosine residue (Tyr220), which is highly conserved across all AOXs and is critical for activity, is within 4 Å of the active site (Figure 1C). Figures 1(C)–1(F) compare the primary ligation spheres of the AOX with other di-iron centres under oxidized conditions and it is obvious from such a comparison that the AOXs are unusual inasmuch that all of the other centres are ligated by at least one (such as ruberythrin; Figure 1D), if not two (such as MMO and RNR R2) histidine residues. AOX does share some features common to other di-iron proteins such as the hydroxo-bridge being on the same side of the di-iron axis (compare with MMO)

Although His165 and His269 appear to be too distant from Fe1 and Fe2 under oxidized conditions to act as ligands, Figure 2 shows that they are within hydrogen-bond distances of Glu213, Gln161, Gln162, Glu215 and Asp265 (Figure 2A). Gln161 and Asp265, which are highly conserved across all AOX sequences, are in the centre of this secondary ligation sphere and extend the hydrogen network to also include Trp47, Tyr246 and Tyr237. Apart from Trp47, both Tyr246 and Tyr237 are also very highly conserved and Trp47, in addition to playing a key role in the stabilization of the active site, may also be critical for electron and proton transfer, since mutagenesis of this residue results in 100% inhibition (M.S. Albury and A.L. Moore, unpublished work). In RNR R2, Trp48 plays a key electron transfer role in the catalytic cycle of this important di-iron protein [26] (Figure 2B) and it is remarkable how similar in terms of ligand position and components of the PCET (proton-coupled electron transfer) network the two functionally dissimilar proteins are (Figure 2C). Such a finding adds further credence to the notion that in the AOXs Trp47 may equally have a role in electron transfer, since the PCET network is so highly conserved. Although both AOX and RNR R2 possess a redox-active tyrosine residue, their positions within the ligation sphere differ. For instance, Tyr220 (AOX) is located on α-helix 5 (equivalent to helix 3 in RNR R2) in a position where it could be hydrogen-bonded to Glu123 or Fe1 and spatially is in a position similar to that of Tyr179 of RNR R2 with respect to its distance from Fe1. Tyr179, however, is located on helix 2 (equivalent to helix 3 in AOX), but in both cases the tyrosine residues are within 5 Å of the di-iron centre [1,10].

As indicated above, a key research objective within our groups is to determine whether modification of the ligation spheres affects catalytic activity. Indeed, there is evidence in the literature to suggest that minor modifications of the active site can elicit profound changes in enzyme reactivity. For instance, Guy et al. [27] reported that a single amino acid substitution in the active site within the castor bean desaturase enzyme (from threonine to aspartate) switched reactivity from desaturation to oxidation. More recently DeGrado and co-workers [28] described the rational reprogramming of a designed de novo di-iron protein that exhibited a dramatic switch of function through a single active-site mutation. With respect to whether it is possible to modify AOX activity through modification of the active site, we mutated Thr24 (positioned adjacent to the ligand Glu123, the effect of which resulted in a considerable change in both oxygen affinity and catalytic activity [29]). We have suggested previously that this change in affinity was unlikely to have a direct effect upon AOX, but was more likely to be due to subtle secondary-structure rearrangements that affect iron-ligating residues such as Glu123. In this respect, it is worth noting that mutation of the equivalent carboxylate ligand in RNR R2 causes accumulation of a peroxodi-iron intermediate [30]. Such a species is part of the catalytic cycle of several other di-iron proteins and has indeed been proposed as a possible AOX reaction intermediate [10,31] and such mutants should prove helpful in the elucidation of the catalytic cycle.

Catalytic cycle for the reduction of oxygen to water
It is well established that many di-iron proteins, including stearoyl-ACP Δ⁹-desaturase [32], MMO [33] and ruberythrin [34], are capable of fully reducing oxygen to water and not peroxide as a side reaction to their main respective catalytic activities, and the AOX is no exception to performing such a function. Oxygen activation is a key step in this reductive process [10,31], but, before AOX can react with oxygen,
Figure 2 | Route of proton-coupled electron transfer pathway through the AOX and RNR R2

(A) In TAO, Asn161 is located in the centre of the PCET pathway and forms hydrogen bonds (broken lines) with Tyr246, Asp265 and His165, which in turn is within hydrogen-bonding distance of Fe1. Asp265 forms a hydrogen-bond network with Trp65 and Trp247. Docking modelling suggests that ubiquinol forms a PCET network with Tyr220, Glu123 and Fe1. (B) PCET pathway in RNR R2 in which Asp237 forms hydrogen bonds with His118 and Fe1, whereas Tyr122 is hydrogen-bonded through Asp124 to Fe1. (C) Overlay of the proposed PCET pathway of TAO (green residues) with RNR R2 (grey residues). (D) Location of the primary (green residues) and secondary (orange residues) ligation sphere of TAO, indicating hydrogen-bond distances between Trp247, Asp265 and Fe1. Location of a hydrophobic cavity predicted by CAVER protein analysis software [36] is indicated in green, whereas the predicted position of ubiquinol and its hydrogen-bond distance to Tyr220 is indicated in yellow. The hydrogen-bond network is depicted by broken lines. This Figure was generated using PDB code 3VV9.

the resting oxidized state must be reduced by ubiquinol to generate the diferrous centre. FTIR (Fourier-transform infrared) spectroscopy studies [18] suggest that the iron-ligating carboxylates are protonated during the generation of this state. Upon binding of oxygen to the diferrous centre (Figure 3), we have previously suggested that two short-lived intermediates are generated prior to the formation of the peroxodi-iron species [1], similar to that observed in other systems [35]. The first electron, which is transferred from the di-iron core, forms a superoxo species (Fe-O-O•) which is immediately reduced to a hydroperoxo intermediate following the transfer of a proton and electron from a nearby...
source that can form a stable radical (probably Tyr220 or a bound ubiquinol). Following these transfers, the di-iron core rearranges to form a peroxodi-iron species, losing water in the process. A second rearrangement follows with the bridging peroxy migrating between the irons, allowing for homolytic cleavage of the O-O bond and the formation of the diamond core. Transfer of protons from the carboxylates and reformation of the bidentate arrangement around the irons allows for the formation of the second molecule of water and regeneration of the resting state occurs following the transfer of a proton and electron from a second amino acid which is in close vicinity of the active site. The oxidation of two ubiquinol molecules completes the cycle by reducing the amino acid radicals and the iron atoms and re-protonating the carboxylates as indicated above and in Figure 3.

Evidence in favour of such a catalytic cycle include: (i) the generation of a mixed-valence Fe(II)/Fe(III) EPR signal observed following the introduction of molecular oxygen to a fully reduced sample [16]; (ii) the occurrence of a carboxylate shift following reduction of the oxodi-iron species to form the diferrous state [18]; and (iii) the location of Tyr220 in a catalytically active position (within 4 Å of the bimetallic active site) [22] and the fact that this residue is universally conserved across all AOX species and is essential for catalytic activity [1].

In the above scheme we suggest that, in addition to ubiquinol and tyrosine, the catalytic cycle may also include a second protein-derived radical (Figure 3) as is the case with RNR R2 [26]. We have previously suggested that, similar to RNR R2, a highly conserved tryptophan residue (Trp150) may play such a role since site-directed mutagenesis studies suggested that this residue was critical for activity [29,31]. The crystal structure reveals, however, that this residue is too far away from the di-iron centre to play a role in electron transport and is more than likely to be involved in π–π interactions with Phe276 [22]. As indicated in Figure 2, however, Trp247 is within the secondary ligation sphere and hence could act as an electron donor in a manner analogous to that described for RNR R2. What is not immediately obvious, however, is the route of the electron transfer pathway for the re-reduction of the Trp247 radical. It is conceivable that oxidation of Trp247 is merely part of a back-up mechanism to prevent the build-up of a highly reactive Fe(IV), thereby allowing a more stable Fe(III) species to exist when there is no bound ubiquinol. Alternatively, it is also possible that loosely bound ubiquinol serves as the re-reductant for both protein-based radicals as depicted in the catalytic cycle.

**Future directions**

Although recent major advances in our understanding of the structure of the AOX has allowed us to definitively characterize AOXs as monotopic membrane-bound ubiquinol oxidoreductases that possess a di-iron carboxylate active-centre, many important problems remain to be solved. Further high-resolution structures of the TAO protein under reducing conditions are required to understand at an atomic level whether carboxylate shifts occur as a result of conformational changes due to substrate binding. For instance, the extent to which two protein-based radicals are utilized and whether high-valence iron species are generated in the diamond core during the oxidation/reduction cycle. Furthermore, species-specific differences in the N-terminal tails and the presence or absence of regulatory motifs within the main sequence suggest that a more complete model of how activity affects function will have to await high-resolution structures of the plant and fungal enzymes. An interesting question from attempts to crystallize the plant
and fungal enzymes to date is whether difficulty in obtaining such structures derives from the dynamic properties of the N-terminal tail interaction. Indeed N-terminal truncated versions of the plant AOX lack activity, but they do form dimers (M.S. Albury and A.L. Moore, unpublished work), suggesting that the N-terminal tail is important for activity, but not dimerization.

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**References**


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