Further insights into the structure of the alternative oxidase: from plants to parasites

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Abstract
The AOX (alternative oxidase) is a non-proton motive ubiquinol–oxygen oxidoreductase that couples the oxidation of ubiquinol with the complete reduction of water. Although it has long been recognized that it is ubiquitous among the plant kingdom, it has only recently become apparent that it is also widely found in other organisms including some human parasites. In this paper, we review experimental studies that have contributed to our current understanding of its structure, with particular reference to the catalytic site. Furthermore, we propose a model for the ubiquinol-binding site which identifies a hydrophobic pocket in other helices II and III, leading from a proposed membrane-binding domain to the catalytic domain.

Introduction
Mitochondria from all higher plants studied to date possess, in addition to the conventional cytochrome c oxidase, a second terminal oxidase (for reviews, see [1–3]). This AOX (alternative oxidase) couples the oxidation of ubiquinol with the reduction of molecular oxygen to water in a manner insensitive to inhibitors of the cytochrome oxidase pathway. The protein is located on the inner surface of the inner mitochondrial membrane on the substrate-side of the bc1 complex. The fact that the protein is non-proton motive [4], and therefore does not contribute to energy conservation, has caused the function of AOX to be the subject of much debate over the years. Although proposed roles of AOX in maintaining tricarboxylic acid cycle turnover while under a high cytosolic phosphorylation potential and in defence against oxidative stress remain to be established conclusively, it is generally accepted that its expression is stress-induced. A still more attractive argument is that the non-protonmotive nature of AOX allows flexible control of the respiratory system and phosphorylation potential in order to maintain growth rate and energy charge homoeostasis in the fluctuating and stressful environment to which plants are exposed [5]. In this respect, it has been suggested that induction of AOX activity itself may act as one of the early responses in a general stress response and hence may play a key role as an initiator of cellular reprogramming [6,7].

The occurrence of AOX is not restricted to plants, where it is ubiquitous, it is also found in some fungi and protists. When certain phytopathogenic fungi, for example, are exposed to commercial fungicidal compounds that are targeted to the mitochondrial bc1 complex, a plant-like AOX is expressed that confers a degree of fungicide resistance on these pathogens (see e.g. [5]). Furthermore, respiratory activity of the blood parasite Trypanosoma brucei relies solely on a terminal oxidase that is functionally very similar to the plant AOX [8] and, since it is absent from the human host, is a potential target for chemotherapeutic agents. In addition to T. brucei, AOX homologues have also been identified in other human parasites such as Trypanosoma vivax [9], Cryptosporidium parvum [10,11], Blastocystis hominis (K. Hamblin, A.L. Moore and M. van der Giezen, unpublished work), in addition to numerous α-proteobacteria, such as Novosphingobium aromaticivorans [12], and cyanobacteria. More recently, AOX-encoding genes have been detected in animals such as molluscs, nematodes and chordates [13], but as indicated above, not in mammals. The presence of AOX homologues in α-proteobacteria and cyanobacteria suggests that present day plant AOXs may well have evolved from prokaryotes and radiated into the α-proteobacterial and cyanobacterial lineages prior to events that gave rise to mitochondria and chloroplasts [1]. Indeed there is good evidence to suggest that AOXs are structurally related to rubredyrthin [14], a protein that itself has been detected in anaerobic organisms at the earliest branches of the evolutionary tree. This suggests that AOX-like proteins may have been formed very early on in an evolutionary time scale and have remained highly conserved throughout the development and divergence of the plant and protist kingdoms.

General structure of AOX
Structural studies of the AOX have been severely hampered over the years by the difficulties encountered in purification of a stable enzyme from any plant source to any degree of purity or stability. Nevertheless, early studies by Siedow and co-workers suggested that its size was approx. 27 kDa [15], which was later increased to 32 kDa after the isolation of the Sauromatum guttatum cDNA encoding Aox1 [16]. Compelling evidence that iron is required for full catalytic activity arose from the elegant studies of Minagawa et al. [17], who showed that AOX induction in the yeast Pichia anomala,
after treatment with antimycin A, was severely inhibited when Fe²⁺ was removed from the growth medium through the inclusion of o-phenanthroline. However, further direct confirmation of this metal requirement had to await advances in protein purification protocols. Affourtit and Moore [18] reported such a protocol for the preparation of pure AOX protein from *Arum maculatum* mitochondria, which, not only was highly active but, importantly, exhibited an exceptional stability upon storage. The purified protein was potently inhibited by metal chelators, with activity being fully restored by the subsequent addition of ferric iron in agreement with the notion that iron is essential for AOX catalysis.

Early AOX sequence data allowed Siedow et al. [19] to provide the first initial clues to the overall structure of the AOX. They postulated that AOX contained a non-haem di-iron carboxylate at its active site comparable with that observed in MMO (methane mono-oxygenase). This model was based on the presence of conserved metal-binding motifs that are typical of this protein family and the fact that the protein neither absorbed light above 340 nm nor possessed standard EPR signals. With the advent of increasing AOX sequence data and structural information on the soluble non-haem di-iron carboxylate proteins, the original AOX structural model was later modified based on the soluble di-iron protein, steroyl Δ⁹-desaturase [20]. Although no crystallographic structure is currently available, AOX is considered to be a monotopic integral membrane protein [20,21], associating with one leaflet of the bi-layer in a manner similar to that proposed for PGH₂ (prostaglandin H₂) synthase and squalene synthase, two proteins whose monotopic structural nature has been confirmed by X-ray crystallography (see [20,21]).

**Catalytic site**

In non-haem di-iron enzymes, such as MMO and R2 [RNR (ribonucleotide reductase) subunit 2], protein-derived coordination of the metal atoms is primarily provided by amino acid residues that all reside within a four-helix bundle (see [2]). The requirement of such a tertiary structural motif, as well as the necessary spacing between the iron-ligating amino acids, imposes considerable constraints on a possible overall fold of AOX and, consequently, on its membrane topology [20,21]. Most of the information on the identification of iron-ligating amino acids has arisen from the use of microbial expression systems. Most of the trypanosomal and *Arabidopsis* investigations have been done using an *Escherichia coli* system deficient in haem synthesis [22], whereas we have expressed AOX genes in *Schizosaccharomyces pombe* [23] (which lacks endogenous AOX activity [24]). In this system, AOX is targeted to mitochondria, and after import and cleavage of the targeting signal, iron is incorporated and the protein is successfully inserted into the leaflet of the inner mitochondrial membrane to become a functional member of the respiratory chain. Site-directed mutagenesis studies using either system have unequivocally demonstrated that the glutamic acid and histidine residues indicated in Figure 1 act as iron-binding ligands, although controversy still surrounds whether the glutamate residue in helix III is Glu²⁶⁸ or Glu²⁷⁰, as mutagenesis of either residue results in an inactive protein [25–28].

Until recently, the only direct evidence that AOX is an iron-containing protein arose from the studies of Berthold et al. [29] who, using a truncated but active AtAOX1a (*Arabidopsis thaliana* AOX1a) protein expressed in *E. coli* membranes, detected an EPR signal characteristic of a mixed-valent Fe(II)/Fe(III) binuclear iron centre, suggesting that the iron centre is hydroxo-bridged. Further confirmation that AOX contains an antiferromagnetic-coupled binuclear iron centre was obtained more recently by Moore et al. [30], who in a series of X-band EPR experiments repeatedly observed signals of approximately \( g = 16 \) in parallel mode typical of two exchange-coupled diferrous centres (\( S = 4 \)) in both a purified AOX sample, in intact *Arum* mitochondria and importantly in the TAO (trypanosomal AOX) expressed in *E. coli* that had been fully reduced, confirming the presence of a di-iron centre. Importantly, the signal could be removed either on oxidation of the sample or on addition of the AOX specific inhibitor octyl gallate, confirming that it was physiologically relevant. To our knowledge, these results represent the first EPR signals from AOX present in its native membrane environment.

**Other important residues**

Site-directed mutagenesis studies have also identified key residues with respect to AOX activity other than those involved in ligating the di-iron centre [25–28]. Of particular interest is the finding that most of the AOX sequences possess only four highly conserved tyrosine residues, namely Tyr²⁵¹, Tyr²⁶⁴, Tyr²⁷⁵ and Tyr²⁹⁹. Both Albury et al. [27] and Nakamura et al. [31] found that Tyr²⁷⁵ was essential for catalytic activity, and we have suggested that this residue may be important for radical formation during catalysis [3].
Ty275 is seven residues after the iron-co-ordinating glutamate residue (Glu268) in helix III (Figure 1), which differs from the position of the reactive tyrosine residue in class Ia and 1b R2s. In these R2s, this reactive tyrosine is located seven residues after the iron-co-ordinating glutamate residue in helix II [32]. Berthold et al. [21] suggest that the location of Tyr275 upstream from Glu268 in AOXs makes it ideally positioned to hydrogen-bond to the terminal ligand Glu178 in helix I. In this sense, the structure of AOX is more similar to the structure of ruberythrin than to class 1a and 1b R2s. In contrast with class 1a and b, however, class 1c R2s such as Chlamydia trachomatis also lack the otherwise conserved radical tyrosine residue in helix II, which instead is replaced by a phenylalanine residue [33]. Rather than harbouring a reactive tyrosine residue, this class of di-iron proteins has been proposed to generate an active radical at the di-iron site itself. Close scrutiny of plant AOXs and TAOs reveals that a phenylalanine residue is also present at this very same position, namely seven residues upstream from the glutamate residue in helix II. Högbom et al. [33] have suggested that all the R2 sequences that lack the tyrosine residue display other MMO-like metal-site features, indicating a mechanistic relationship between these di-iron proteins. The question does arise, however, as to what advantage organisms within this class gain by not using a tyrosine residue for radical generation and stabilization, particularly since it has been used so successfully by other R2s. As suggested by Högbom et al. [33], this change in the normal structural solution could offer advantages for certain pathogens such as Chlamydia, since it would permit them to survive in the human host, as the tyrosyl radical is considered to be the key target for the antiproliferative effects of nitric oxide produced during the immune response [34]. Thus the replacement of tyrosine by a phenylalanine would make pathogens such as Chlamydia more resistant to nitric oxide. In this respect, it is interesting to note that AOX sequences in plants, trypanosomes and other parasitic organisms also contain a phenylalanine rather than a tyrosine residue at this position. It has long been known that AOX activity in plant mitochondria is resistant to nitric oxide [35] even though plants possess nitric oxide synthase activity [36], and hence it is interesting to speculate whether this insensitivity is due to the lack of a tyrosyl radical in this region of the protein.

If the spatial positioning of Tyr275 in helix III does facilitate the provision of a protein-derived electron during catalysis, then as previously pointed out [3] there is a requirement for an additional electron donor. We have suggested that Trp206, a universally conserved residue located at the C-terminal end of helix II, could be an electron donor [3], and site-directed mutagenesis has indeed confirmed (M.A. Albury and A.L. Moore, unpublished work) that replacement by tyrosine or phenylalanine results in an inactive enzyme. As it is in the equivalent place to that of the reactive tyrosine in ruberythrin [14], a long-lived tyrosyl radical should be detected by EPR; future experiments will reveal whether this is indeed the case.

With respect to the function of the other three conserved tyrosine residues, although Tyr266 and Tyr299 are both highly conserved across all organisms, mutation of either to alanine results in a highly inhibited enzyme ([31] and M.A. Albury and A.L. Moore, unpublished work), suggesting that these residues do play some role in electron transport. However, at present, it is difficult to rule out that the inhibitory effect is not due to any alteration of the native structure of the enzyme. The role of Tyr235, nonetheless, is becoming much clearer and is discussed below.

One other residue worthy of discussion, in the plant AOXs at least, is Thr179, which is located adjacent to the iron-ligating glutamate residue in helix I (see Figure 1). Although a T179A mutant retains partial activity, it does show a significant increase in the affinity of the AOX for oxygen [Km (app) changes from 18 to 5 μM] [37]. Again it is interesting to note that mutation of the Glu84 in the class I R2 protein (at the equivalent position to Glu178 in AOXs) causes accumulation of a peroxo-di-iron intermediate [38], and it will be important to determine whether similar spectroscopic signals can be detected in this mutant form of AOX.

**Quinol-binding site**

In addition to a catalytic site for the reduction of oxygen to water, the AOX contains a binding site for its substrate ubiquinol. Hydroquinone-binding sites offer the greatest potential as targets for drug design and as such have attracted much attention. The structures of several enzymes that interact with quinone/quinol have been determined to atomic resolution [39–41]. Although these structures have not yet led to the elucidation of a universal structure of a quinone-binding site, several features that are considered to be important in quinone binding have been identified. For example, mapping of the quinone-binding site in the cytochrome bc1 complex has resulted in a model analogous to quinone sites in photosynthetic reaction centres in which the quinone-binding pocket is formed by the ends of two-transmembrane helices [41]. Aromatic residues have also been identified near quinone-binding regions where the aromatic ring may interact with the quinone head group in a parallel manner. Furthermore, Abramson et al. [41] identified a novel ubiquinone-binding domain that contains a cluster of polar residues exposed to the interior of the lipid bilayer that is not present in cytochrome c oxidase. A bioinformatics search of respiratory and photosynthetic complexes that react with quinones has resulted in the identification of a putative quinone-binding motif, consisting of an HR (His–Arg) pair and a triad element [42]. Residues, other than those highlighted in [42], that have been identified by biochemical means as being critical for quinone binding in SQP (succinate:ubiquinone oxidoreductase) include a serine, arginine and tyrosine [43–45]. Analysis of AOX inhibitor-resistant mutants has also uncovered functional characteristics of quinone-binding proteins [46].

Our own bioinformatics search has led us to propose a model for the ubiquinol-binding site in AOX (represented in Figure 2), which identifies a hydrophobic pocket, between helices II and III, leading from a proposed membrane-binding domain to the catalytic domain. We propose that this
crevice could act as a channel through which the substrate gains entry to the active site. Within this model, (i) we have confirmed that the His261/Arg262 dyad, part of a potential hydroquinone-binding motif [42], is almost totally conserved among plant AOX and other organisms; (ii) it is consistent with residues identified by inhibitor-resistant screening as being important in ubiquinol binding [46], these residues flank the HR pair (see Figure 2); (iii) the fully conserved Tyr253 is positioned in the hydrophobic pocket and is proximal to the HR pair and we have previously suggested that aromatic residues such as Tyr253 could be involved in quinone binding [47]; (iv) analysis of differences in sensitivity of TAO iso-enzymes to the inhibitor ascofuranone [9] has led us to identify two further residues (Gln242 and Asn247) of potential importance for ubiquinol binding; and (v) the postulated ubiquinone-binding domain contains polar and charged residues similar to the case of the structurally determined ubiquinol oxidase of E. coli [41] and the binding domain also contains a highly conserved serine residue, which, in complex II, appears to play a key role in ubiquinone binding [43,44].

Preliminary site-directed mutagenesis of this region in Saurornatatum guttatum AOX expressed in Schizosaccharomyces pombe indicates that mutation of Gln242, Tyr253, Ser256 and Arg262 results in considerable inhibition of oxidase activity, suggesting that these residues are essential for catalysis (M.A. Albury and A.L. Moore, unpublished work). The extent to which any of these mutations affect inhibitor-sensitivity will, however, have to await further analysis.

Conclusions

It may be evident from the above discussion that considerable progress has been made in our understanding of the molecular nature and organism diversity of the AOX. Nevertheless, further detailed knowledge will have to await a high-resolution crystallographic structure, which hopefully will not be too long in being generated, given that the plant and the trypanosomal protein can be purified to virtual homogeneity and in sufficient quantities to facilitate crystallization trials. Given the increased occurrence of AOX in medically relevant organisms, the protein is now considered to be a potential target for chemotherapeutic agents, and indeed one such compound, ascofuranone, is currently undergoing clinical trials [48]. Obtaining knowledge of the quinone-binding site and catalytic mechanism of the protein from both plants and pathogenic organisms will no doubt not only lead to a greater understanding of the structure and functional diversity of AOXs, but also to a more rational design of highly targeted antiparasitic drugs.

References


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