A revised model of the active site of alternative oxidase

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Abstract The plant mitochondrial protein alternative oxidase catalyses dioxygen dependent ubiquinol oxidation to yield ubiquinone and water. A structure of this protein has previously been proposed based on an assumed structural homology to the di-iron carboxylate family of proteins. However, these authors suggested the protein has a very different topology than the known structures of di-iron carboxylate proteins. We have re-examined this model and based on comparison of recent sequences and structural data on di-iron carboxylate proteins we present a new model of the alternative oxidase which allows prediction of active site residues and a possible membrane binding motif.

Key words: Alternative oxidase; Fatty acid desaturase; Plant mitochondrion; Homology modeling; Membrane protein

1. Introduction

The di-iron carboxylate protein family has progressed from being considered an exotic member of the metalloproteins to becoming recognised as an important family of redox-active iron proteins. The number of di-iron carboxylate proteins discovered has grown in recent years and their functions as catalysts of a number of important redox reactions has been revealed. The best-studied di-iron carboxylate proteins are the R2 subunit of ribonucleotide reductase (RNR R2) [1] and the hydroxylase subunit of the soluble methane monooxygenase (MMOH) [2–7]. The reaction mechanism and molecular structure of these proteins have been studied in great detail. The active site consists of a binuclear Fe centre coordinated by two histidines and four carboxylate residues (Fig. 1A). Most of these enzymes catalyse dioxygen dependent redox reactions involving highly reactive ferryl-oxo intermediates and consequently can perform powerful chemistry including the generation of an organic radical (RNR R2) and the hydroxylation of methane to form methanol (MMOH). Other di-iron proteins of this family are the ferritins [8], stearoyl-acyl carrier protein Δ^9-desaturase (Δ^9-desaturase) [9,10] and rubrerythrin [11,12], for which structural data are available. Sequence comparisons suggest some additional hydrocarbon hydroxylases to be members of this group [13].

The di-iron carboxylate proteins can be divided into two groups (in SCOP classification termed 'RNR R2-like proteins' and 'ferritin-like proteins'), which all share a central four-helix bundle providing the carboxylate dominated first ligand sphere to the Fe centre. Among the RNR R2-like proteins, virtually no sequence homology exists, with the only similarity being the occurrence of an EXXH motif on both the second and fourth helices of the bundle. The first and third helices provide the terminal carboxylate ligands and the only true characteristic of these helices is the presence of one carboxylate residue per helix. As can be seen in Fig. 1A, the two histidines both coordinate the Fe centre from the same side and the two glutamates in the EXXH motifs (E115 and E238) serve as the bridging ligands. This arrangement requires that helices 2 and 4 lie anti-parallel next to each other (Fig. 1B).

The spacing of the different ligands always follows a specific pattern characteristic to these enzymes (Fig. 2). In the four-helix bundle, the helices are arranged in two pairs. Helices 1 and 2 form one pair and helices 3 and 4 form another pair. Within these pairs the spacing between the two co-ordinating carboxylates is always around 30 amino acids. The spacing between the two helix pairs is more variable with rubrerythrin having the shortest spacing and RNR R2 from *Escherichia coli* having the longest. In rubrerythrin the connection between the helix pairs consist of just a β-strand while in RNR R2 the connecting sequence consist of two α-helices and two β-strands making up the tip of the heart-shaped structure.

The mechanism of O_2 activation is probably similar in the di-iron carboxylate proteins [13]. The starting point for the reaction is the reduced, di-ferrous form of the enzyme. The metal centre reacts readily with O_2 and this results in the formation of a di-ferric-peroxide intermediate. This peroxide intermediate has been detected in MMOH [14], RNR R2 [15,16] Δ^9-desaturase [17] and a ferritin [18]. The next step in the reaction is the formation of the ferryl species, which has only been detected in MMOH [19] where it is the methane oxidising species. In RNR R2 one proton and one electron are supplied externally via a specific proton/electron transfer pathway [20–23] to generate intermediate X, an Fe(III)-Fe(IV) species [24–28] which is the precursor to the di-ferric tyrosyl-radical species.

Among the other proteins known to contain a di-iron site, none are capable of activating molecular oxygen. Instead the metal centre is used either for O_2 transport, as in hemerythrin [29] or for phosphoryl transfer reactions, as in purple acid-phosphatase [30]. Like the ferritins, hemerythrin is a four-helix bundle but the order and direction of the helices is different from the RNR R2-like proteins [31–33]. Furthermore, in hemerythrin the ligands to the Fe centre are five His and two bridging carboxylate residues. Even though hemerythrin is a four-helix bundle the spacing of the Fe ligands is different compared to the RNR R2-like proteins. The first helix in the bundle provides one ligand while the other three helices provide two ligands each to the Fe centre. The spacing of the ligands on these three last helices are: helix 2: HXXXE, helix 3: HXXXH, and helix 4: HXXXXD.

In purple acid-phosphatase the di-iron centre is coordinated

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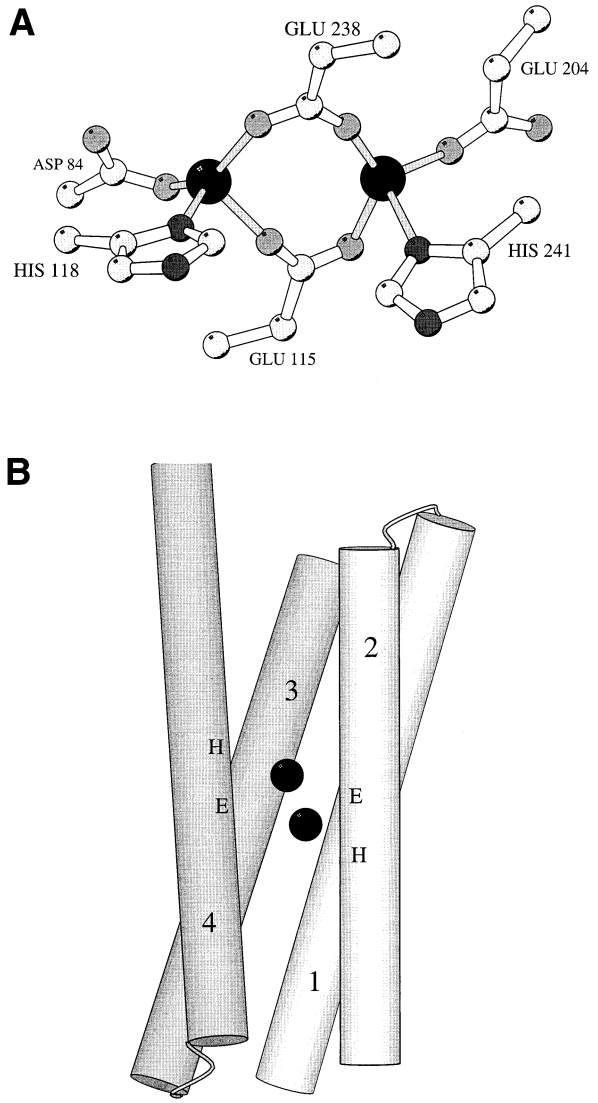

Fig. 1. A: The di-ferrous form of the iron centre in RNR R2. B: Arrangement of the helices in the four-helix bundle of the R2-type of di-iron proteins. The E and H of the two EXXH motifs are indicated. The helix numbers are placed in the N-terminal end of each helix. These figures were drawn using Molscript [54].

by 3 His, two Asp, one Asn and one Tyr [34]. The ligands to the di-iron site are provided by loops emerging from 2 β-sheets and the structure of this protein is not related to the RNR R2-like proteins.

Other metal ions can be used for the purpose of activating coordinated waters or hydroxides, eg. manganese in arginase [35]. In the crystal structure of this protein a di-Mn centre with a coordination highly similar to the RNR R2-like proteins was found [36]. The Mn-centre is coordinated by four carboxylates and two His, but this is an enzyme with α/β structure and the Mn ligands are provided by loops between a central β-sheet and the surrounding helices.

Consequently, several structural motifs exist which can accommodate a di-nuclear metal centre, but all of the proteins with known structures containing the EXXH motifs have the same fold of the central four-helix bundle. No other known protein folds containing a di-metal centre has an EXXH motif in the ligation sphere of the metal site.

2. Alternative oxidase

In the mitochondria of plants, fungi, yeast, and trypanosomes the respiratory chain has an additional protein, in addition to cytochrome *c* oxidase, that can act as the terminal electron acceptor [37]. The substrates of this 'alternative oxidase' (AOX) are ubiquinol and dioxygen, which are converted to ubiquinone and water respectively [38,39]. Respiration can thus be uncoupled from proton pumping across the matrix membrane. This enzyme was discovered as it retains catalytic activity in the presence of cyanide, a potent cytochrome *c* oxidase inhibitor [40].

Due to its strong association to the mitochondrial inner membrane, purification of the alternative oxidase has been troublesome and pure preparations of this protein have not yet been produced. Instead the biochemical work has been performed on partially purified enzyme or mitochondrial membrane fractions. There is evidence that the alternative oxidase activity requires iron [41] but the preparations of the enzyme produced to date do not show any EPR signal or absorbance above 350 nm [42]. This is unusual for a Fe protein but similar to MMOH [43] and Siedow et al. suggested in a previous modelling study that the alternative oxidase contains a di-iron centre [44,45]. In the alignments of the alternative oxidase sequences known at the time this proposal was made, three EXXH motifs were found to be conserved [44,46]. Two of these motifs were suggested to be involved in forming the di-iron centre. From proteolysis studies it was shown that the N and C-termini of the protein are on the matrix side of the membrane [47] and from hydropathy plots, two hydrophobic regions proposed to span the membrane were found [37,48]. Since the EXXH motif nearest the N-terminus is lying between the two proposed transmembrane regions, this EXXH motif was predicted to lie in the intermembrane space, hence it could not be involved in forming

	Helix 1	Helix 2		Helix 3	Helix 4
R2 *E. coli*	---D---	---E--H---		---E---	---E--H---
	84 < 30 >	115 118	< 85 >	204 < 33 >	238 241
R2 *S.typhi.*	---D---	---E--H---		---E---	---E--H---
	67 < 30 >	98 101	< 56 >	158 < 33 >	192 195
R2 mouse	---D---	---E--H---		---E---	---E--H---
	139 < 30 >	170 173	< 59 >	233 < 33 >	267 270
MMO	---E---	---E--H---		---E---	---E--H---
	114 < 29 >	144 147	< 61 >	209 < 33 >	243 246
Δ^9 desaturase	---E---	---E--H---		---E---	---E--H---
	105 < 37 >	143 146	< 49 >	196 < 32 >	229 232
Bacterioferritin	---E---	---E--H---		---E---	---E--H---
	18 < 32 >	51 54	< 39 >	94 < 32 >	127 130
Rubrerythrin	---E---	---E--(H)---		---E--E--	---E--H---
	20 < 32 >	53 56	< 37 >	94 97 < 30 >	128 131

Fig. 2. Sequential spacing of the Fe ligands in di-iron carboxylate proteins. This list includes all proteins of this class for which an X-ray crystal structure is available. R2 *E. coli*: RNR R2 (NrdB) from *Escherichia coli* [55]. R2 *S. typhi*: RNR R2 (NrdF) from *Salmonella typhimurium* [56]. R2 mouse: RNR R2 from mouse [57]. MMO *M. capsu*: the α-subunit of the methane monooxygenase hydroxylase from *Methylococcus capsulatus* (Bath) [58]. The crystal structure of the MMO hydroxylase from *Methylosinum trichosporium* (OB3b) [59] has also been determined and the ligand numbering and spacing is identical to the *M. capsulatus* enzyme. Δ^9 desaturase: stearoyl-acyl carrier protein Δ^9-desaturase from castor (*Ricinus communis*) [10]. Bacterioferritin: bacterioferritin from *E. coli* [60]. Rubrerythrin: rubrerythrin from *Desulfovibrio vulgaris* (Hildenborough) [12].

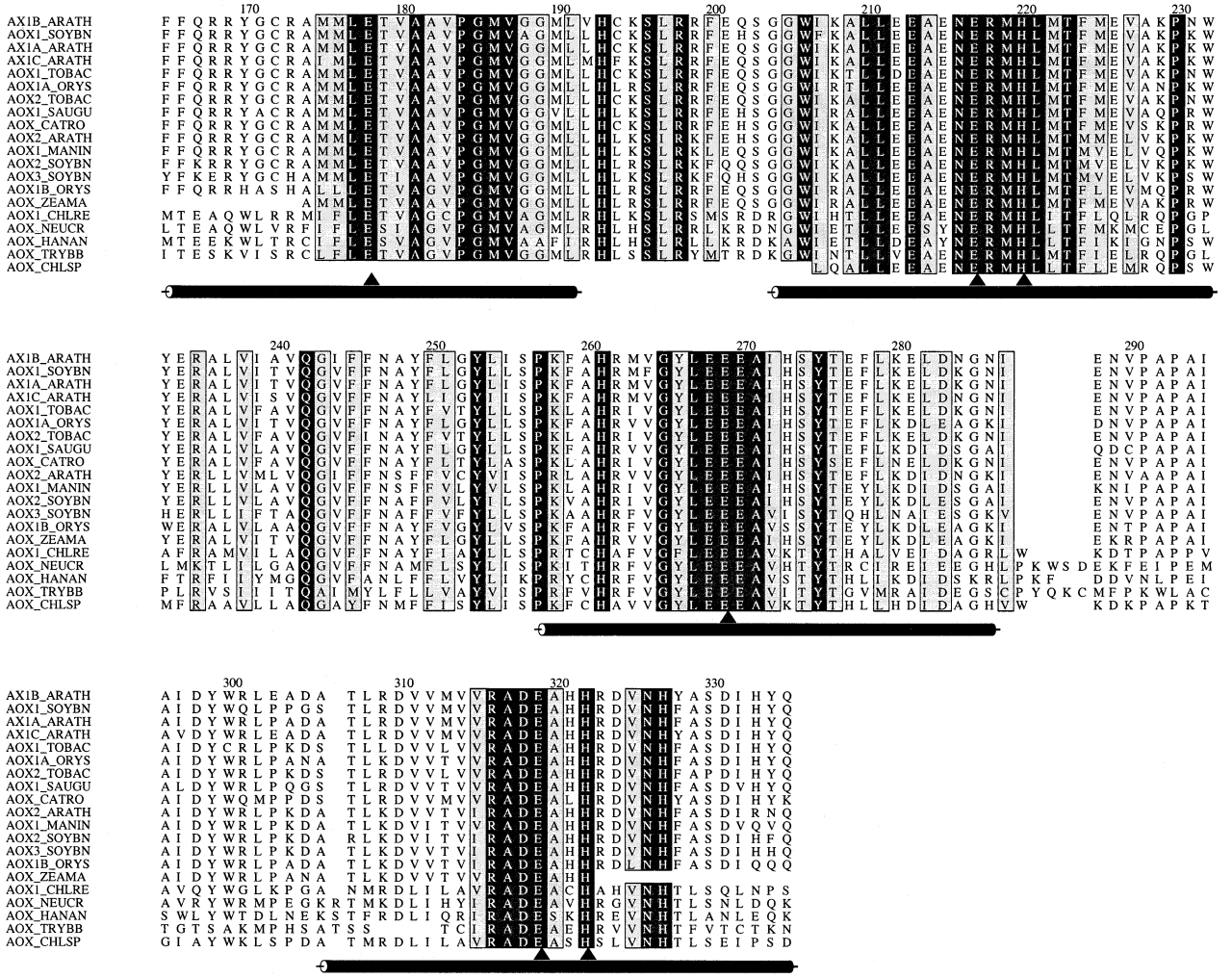

Fig. 3. Multiple sequence alignment of the most conserved region (residues 165–335, *S. guttatum* numbering) of the alternative oxidase sequences known to date. Numbering corresponds to the *S. guttatum* sequence. Arrows under the sequences correspond to the proposed Fe ligands. Black background corresponds to residues completely conserved between all species. Grey background corresponds to conservative substitutions. Abbreviations in gene names are as follows: AX and AOX: alternative oxidase; NEUCR: *Neurospora crassa*, ARATH: *Arabidopsis thaliana*; ORYS: *Oryza sativa*; CHLRE: *Chlamydomonas reinhardtii*; SAUGU: *Sauromatum guttatum*; CHLSP: *Chlamydomonas* sp.; SOYBN: soy bean (*Glycine max*); CATRO: *Catharanthus roseus*; TOBAC: tobacco plant (*Nicotiana tabacum*); HANAN: *Hansenula anomala*; TRYBB: *Trypanosoma brucei brucei*; MANIN: *Mangifera indica*; ZEAMA: *Zea mays*.

the Fe centre, since the C-terminal part would be on the matrix side. Assuming the existence of two transmembrane helices, the authors instead used the two EXXH motifs closest to the C-terminus to build a model of the di-iron site and the central four-helix bundle inspired by the structure of MMOH and RNR R2. However, the model that was presented is not in agreement with what is known about these proteins in regards to ligand spacing and order and direction of the helices in the bundle. The helices are unusually short and the EXXH motifs appear on the first and fourth helices instead of on the second and fourth helices in the bundle. Since this proposal was made the model has been the basis for planning expression experiments, mutagenesis experiments and interpretation of data and seems to have gained acceptance as a true structure of the AOX active site. We therefore feel the need to point out weak points in this model and present an alternative model for the structure of the AOX protein.

The Siedow model suggests a di-iron site residing in a topologically and therefore evolutionarily unrelated fold to the RNR R2-like family. This is problematic from an evolutionary perspective and our doubt is further supported by additional sequence data that have recently become available, which show that one of the EXXH motifs used to build the model is not evolutionarily conserved. Meanwhile, the EXXH motif considered unlikely due to its proposed positioning in the intermembrane space, is fully conserved (Fig. 3). Based on these observations we present a new model of the alternative oxidase. This model shows how AOX could accommodate a di-iron centre within an RNR R2-like fold and further suggests possible ways of how AOX could interact with the membrane and bind the ubiquinol substrate.

3. Results and discussion

3.1. Overall structure of AOX

As the previously proposed model of the active site of the alternative oxidase [44] appears incorrect, with one of the proposed iron ligands not being conserved, we present a new structural model where only conserved residues are modelled as Fe ligands and which is consistent with an evolution-

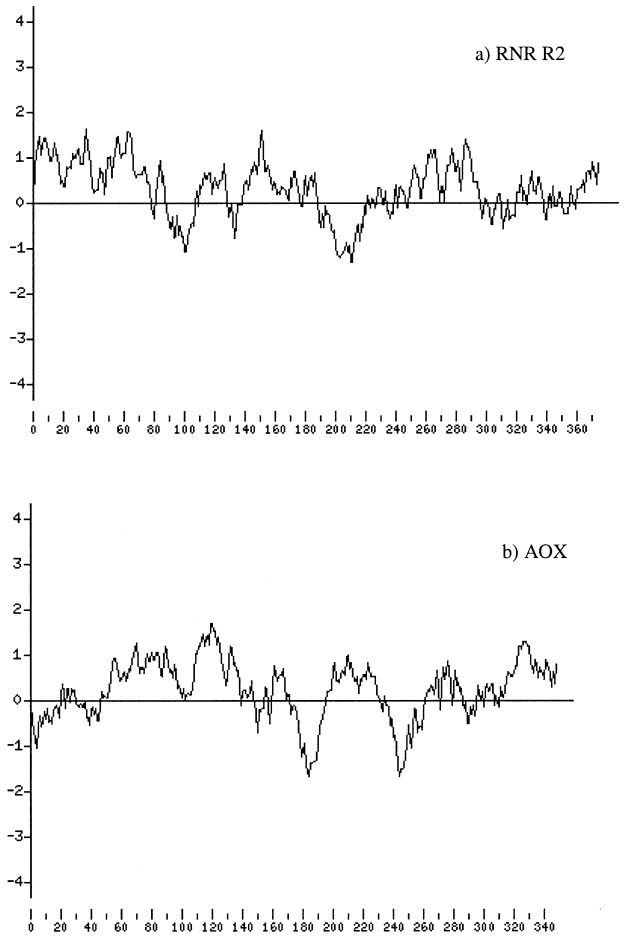

Fig. 4. Hydrophobicity plots of RNR R2 and alternative oxidase. In RNR R2 the two hydrophobic regions 85–115 and 190–230 correspond to helix C and helix E respectively. Helix C is involved in interactions forming the RNR R2 dimer and helix E is completely buried in the RNR R2 monomer surrounded by more amphiphilic helices.

ary relationship to the RNR R2-like proteins. After careful sequence comparison we decided to use the structure of the Δ^9-desaturase protein as a template for modelling the most conserved, central part of AOX. The main reason for selecting Δ^9-desaturase as a template was that the spacing of Fe ligands in the AOX sequence agrees best with that of the Δ^9-desaturase. The model, which was built using QUANTA (Molecular Simulations Inc.), consists of residues 165–335 (*Sauromatum guttatum* numbering) and covers the four-helix bundle plus the connecting region between helices 2 and 3. According to our prediction, residues 286–304 would be an insertion between helices 3 and 4, hence they were not built into the model. The assignment of the two hydrophobic regions in AOX as being transmembrane is not consistent with our model, we have therefore reevaluated the predictions of the transmembrane helices and found them not to be unambiguously predicted (using e.g. the Kyte-Doolittle method). Instead our model suggests that AOX could be an interfacial membrane protein, or that the hydrophobic helices could be involved in protein-protein interactions attaching the alternative oxidase to other membrane bound proteins. Alternatively, the hydrophobic helices could be buried, either in the core of the protein or in the dimer interface. In fact, a study conducted to map the topology of the alternative oxidase failed to conclude that the protein has a transmembrane region [47]. Comparison of hydropathy plots of the other di-iron carboxylate proteins show that helices which are completely buried within the protein can have a hydrophobicity similar to that found in the hydrophobic regions of alternative oxidase (Fig. 4). Another enlightening example of the failure to predict the structure of a membrane bound protein is that of prostaglandin H_2 synthase-1 [49]. This protein contains one stretch of sequence that was predicted to be a transmembrane helix [50] but when the crystal structure of this protein was determined it turned out that this region is not involved in membrane binding but instead is an integral part of the catalytic domain.

3.2. Model of the active site

We propose that the ligands to the Fe centre of AOX are E178, E217, H220, E269, E319 and H322 (Fig. 3). In analogy with the other di-iron proteins E217 and E319 are bridging carboxylates, while E178 and E269 are terminal ligands. This model gives spacing of sequence motifs that is more in agreement with the di-iron carboxylate proteins (Figs. 5 and 6) even though one major discrepancy still exists, the E269 residue, which gives a longer second helix pair than is normally found in di-iron proteins. One other possible Fe ligand is D283, which is conserved as D or E; however, the D283 residue is in a region of much lower conservation than E269 and is only four amino acids away from where insertions appear to have occurred making it less probable that this region is in a four-helix bundle.

On helix 3 there are three possible Fe ligands (i.e. E268, E269 or E270). Finding the correct Fe ligand among these three residues is not trivial but comparisons to the Δ^9-desaturase give some hints. On each of helices 1 and 3 of Δ^9-desaturase there are three hydrophilic residues (T104, E105 and E106 on helix 1 and Q/R195 E196 and R197 on helix 3) of which E105 and E196 are the Fe ligands. It seems that the structure can accommodate one hydrophilic residue on each side of the Fe ligand. We therefore assigned E269 as the Fe ligand of helix 3 in AOX but if E268 or E270 were to be the true Fe ligand, the main conclusions from our model would still be valid.

In all other R2-like di-iron proteins the Fe binding histidines make hydrogen bonds from the δ1 nitrogen (the nitrogen not involved in Fe binding). This is also true in our model of AOX where these hydrogen bonds are provided by residues D318 and N216 (see Fig. 7).

One disturbing property of the Siedow model, apart from the fold of the four-helix bundle, is the fact that Fe ligands are missing. There is no equivalent to E204 in R2 and the histidine corresponding to H115 is not evolutionarily conserved. In other di-iron proteins with one histidine missing, either no dinuclear metal centre is formed (as in RNR R2 H115A) or its structure is significantly perturbed (as in RNR R2 H241A)

	Helix 1		Helix 2		Helix 3		Helix 4
Alt ox. Siedow	--E--H--		--D--		--No ligands--		---E--H---
	270 273	< 9 >	283				319 322
Alt. ox. New	---E---		---E--H---		---E---		---E--H---
	178	< 38 >	217 220	< 47 >	268	< 50 >	319 322

Fig. 5. Comparison of the spacing in sequence motifs between the Siedow model and the model presented in this paper of alternative oxidase.

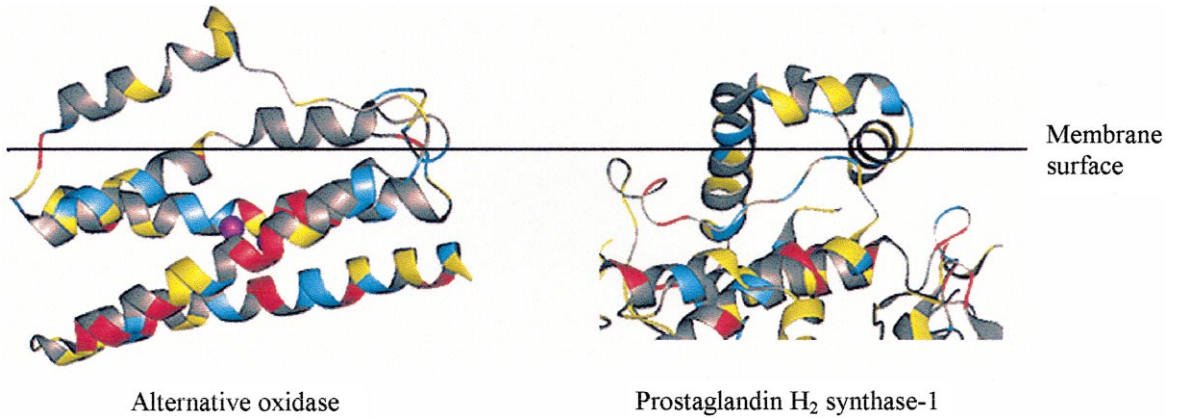

Fig. 6. Comparison of the membrane binding domain of prostaglandin H_2 synthase-1 with the proposed membrane binding domain of AOX. Grey residues are hydrophobic, yellow are hydrophilic uncharged, blue are positive, and red are negative. Note the rim of positively charged residues around the proposed membrane binding domain of AOX.

[51]. If one carboxylate and one histidine is missing the Fe centre is formed only transiently with no evidence for high-valent species being formed (as is the case in e.g. the ferritins) [52].

3.3. Ubiquinol binding site

One intriguing feature of this model is that when the sequence of the alternative oxidase is mapped onto the structure of the Δ^9-desaturase protein according to our proposed model, a hydrophobic crevice reaching down to the Fe centre is formed. This crevice is some 5 Å wide and 10 Å long and is lined by the small, conserved amino acids L177, A181, P184, G185, V187, L210, A/S214, G265, L268 and V/I272. A similar crevice is seen if RNR R2 is used as a template for model building. In this case the crevice is smaller and less hydrophobic and is lined by residues A181, P184, A/S214, H261, G265 and E268. These residues are all positioned one and two helix turns from the Fe ligands on helices 1, 2 and 3. This crevice could serve as a ubiquinol binding site and it is fairly close to two residues (corresponding to F259 and V263 in *S. guttatum* AOX) which have been proposed, from a mutagenesis screen, to be involved in ubiquinol binding in the *Arabidopsis thaliana* alternative oxidase [53].

3.4. Membrane binding domain

In the model of AOX presented here one possible membrane binding domain becomes evident. The connecting sequence between the two helix pairs (residues 236–255) is highly hydrophobic and is one of the regions predicted to be transmembrane. This connecting sequence together with the C-terminal part of helix 1 (residues 180–190) forms a hydrophobic region that could be inserted into the membrane in a manner similar to prostaglandin H2 synthase-1 (Fig. 6). This region is lined by several conserved, positively charged residues (R/H173, H193, R198, R218, K/R235 and K/R258) that could interact with the phosphate groups on the phospholipid membrane. Furthermore, the crevice we propose to be the uboquinol binding site opens up towards this hydrophobic domain which would explain how ubiquinol can access the active site of the enzyme. This is also similar to the prostaglandin H_2 synthase-1 where a substrate binding channel leads from the membrane binding domain into the active site in the catalytic domain.

However, the part of the structure connecting helices 2 and 3 in the other di-iron carboxylate proteins is normally part of the dimer interface and this could also be the case for AOX since this protein is believed to be a dimer. Several other modes for attaching the protein to the membrane, such as protein-protein interactions or a membrane binding motif in the less conserved N-terminus of the protein, are also possible.

4. Conclusions

In the present study we propose a structural model of the membrane protein alternative oxidase. This protein was previously predicted to be a di-iron carboxylate protein and a

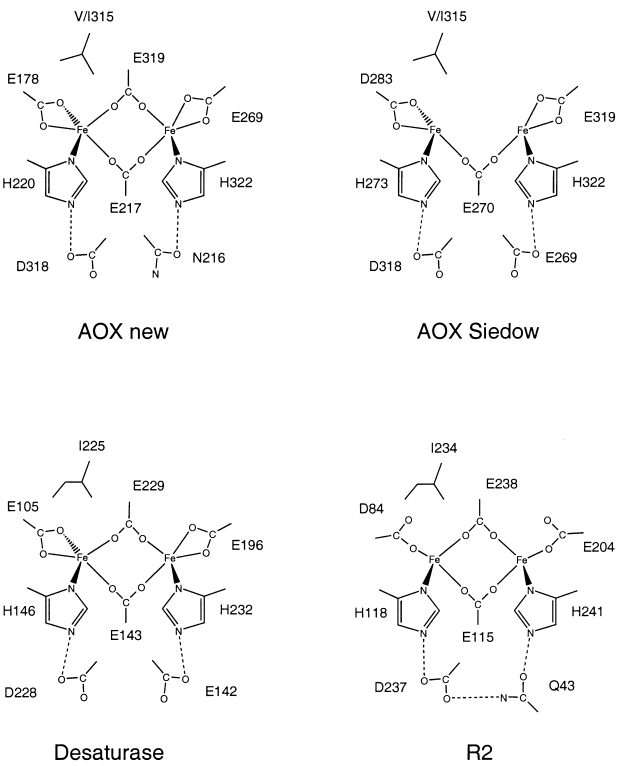

Fig. 7. Comparison of the Fe centres of the two AOX models and Δ^9-desaturase and RNR R2.

model of the active site of AOX was presented. However, this model was not in agreement with the structure of other di-iron carboxylate proteins since the order and length of the helices in the four-helix bundle was different. Our new model corresponds well with a common evolutionary origin of AOX and the di-iron carboxylate proteins since the size and order of the four helices in the bundle and the spacing of Fe ligands in the sequence is similar to di-iron carboxylate proteins. Our model is also consistent with recent sequence information on AOX since the residues we propose to be Fe ligands are completely conserved between all species. Furthermore, our model suggests that AOX is an interfacial membrane protein (or attached to the membrane via protein-protein interactions) without any transmembrane helices in analogy with prostaglandin H_2 synthase-1. This model can now be experimentally tested and used to guide mutagenesis studies to probe Fe binding and ubiquinol binding residues and allow for better planning of expression and purification efforts on this protein.

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