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Minireview

Plasticity of proton pathways in haem–copper oxygen reductases

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Abstract Oxygen reductases are the final enzymes in the aerobic respiratory chains catalysing the reduction of dioxygen to water, with the concomitant translocation of protons through the bacterial cytoplasmatic or mitochondrial membranes. Most of these enzymes belong to the family of haem–copper oxygen reductases. Intraprotein proton-conducting pathways are needed for the chemical reaction and for the translocated protons. Based on sequence and structural analyses, and site-directed mutagenesis, two proton channels were established for the mitochondrial-like oxygen reductases. However, the amino acid residues forming these channels are not conserved among the family members. Most importantly, many oxygen reductases do not contain ionisable amino acid residues in the putative proton pathways nor in alternative positions. The diversity of channels in haem–copper oxygen reductases exemplifies the plasticity of proton pathways that occurred throughout evolution, and strongly suggests a substantial role for water as the main proton carrier. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Haem–copper oxygen reductases; Proton translocation; Proton channels

1. Introduction

Oxygen reductases are the terminal enzyme complexes in aerobic respiratory chains catalysing the reduction of dioxygen to water. The majority of these reductases belong to the haem–copper superfamily, which are characterised by the location of a low-spin haem and a binuclear centre harbouring a high-spin haem and a copper atom in their subunit I. These enzymes are able to oxidise peripheral or periplasmatic electron donors such as cytochromes, high potential iron–sulphur proteins and probably copper proteins, and membrane-bound electron donors like quinols. In contrast to quinol oxidising enzymes, cytochrome oxidases contain a mixed valence dinuclear copper centre (Cu_A) in their subunit II. The cbb_3 oxidases are reported to be cytochrome oxidases, which have one monohaemic and one dihaemic subunit instead of subunit II.

During the catalytic cycle, a charge separation of four charges is observed since the electrons and protons for the reaction are taken from opposite sides of the membrane. This contributes to the establishment of the electrochemical gradient, used for the synthesis of ATP. Furthermore, the catalytic reaction is exergonic and the generated energy is conserved by proton translocation across the membrane, further contributing to the electrochemical gradient (up to four protons per oxygen molecule reduced).

Oxygen reductases were originally identified in the 1920s by Warburg, and since then much attention has been dedicated to their study with remarkable landmarks, such as the discovery of proton pumping [1], the structure determination of the enzymes from the bovine heart [2], and the bacterium *Paracoccus denitrificans* [3]. Despite all this progress several questions still have not been fully answered. 1. Which are the catalytic intermediates? 2. Which is the proton/electron stoichiometry? 3. Which are the proton channel elements and the mechanism of proton translocation? 4. Which are the trigger and the gating mechanisms? In the present review we contribute to these key points by discussing the diversity of proton channel elements.

2. Establishment of haem–copper oxygen reductases families

As subunit I contains the catalytic centre – the haem–copper binuclear site – it is the only strictly common subunit to all haem–copper oxygen reductases. This implies that the amino acid residues binding its prosthetic groups, the binuclear site and the low-spin haem (six histidyls: HisI-94, HisI-276, HisI-325, HisI-326, HisI-411 and HisI-413, *P. denitrificans* numbering is used, unless otherwise stated) are strictly conserved in all haem–copper oxygen reductases. From inspections of the amino acid sequences of all oxygen reductases present in protein databases and by structure prediction analyses based on the determined crystallographic structures [2–4], it may be anticipated that the same general spatial arrangement is common for all haem–copper oxygen reductases. Also it can be observed that, besides the histidyl ligands to the metal centres, only three other amino acid residues are strictly conserved (ValI-279, TrpI-272 and ArgI-474) [5]. These residues were considered to be critical for the key processes of oxygen diffusion, proton pumping and electron transfer (e.g. [6–11]).

Haem–copper oxygen reductases catalyse the reduction of O_2 to H_2O:

$$O_2 + 4e + 4H^+ \rightarrow 2H_2O.$$

In this reaction, at least, four protons are involved. As the

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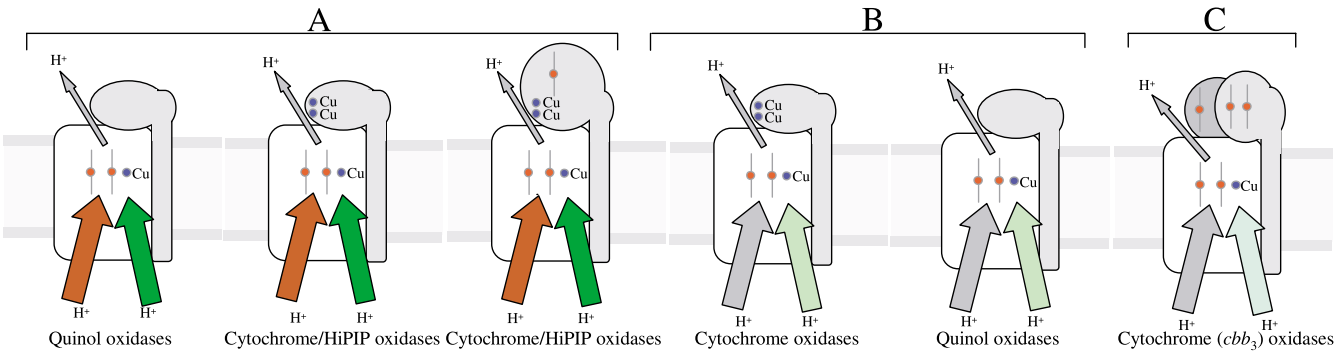

Fig. 1. Schematic representation of the main examples of proteins among the three families of haem–copper oxygen reductase: type A, B and C. The families were established based on their proton pathways [5], which are here schematically represented by arrows. Arrow's colour: Red: D channel – GluI-278 (A1-type)/TyrI-256 (*R. marinus*, A2-type), AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193. Dark green: K channel – LysI-354, ThrI-351, SerI-291 and TyrI-280. Green: K channel (alternative) – ThrI-312, SerI-309, TyrI-244 and TyrI-237 (*T. thermophilus*, ba_3 oxidase). Light green: K channel (alternative) – SerI-355, TyrI-295 (*B. japonicum*, cbb_3 oxidase). Grey: Unknown channel.

catalytic site is well imbedded in the protein (approximately 13 Å from the positive side of the membrane and 30 Å from the negative side), and not freely permeable to protons, intraprotein channels for proton conduction must exist. Furthermore, these channels have to be kinetically competent in order to ensure an efficient supply of protons for water formation, compatible with the enzyme turnover, and avoid the appearance of reactive oxygen radical species. On the other hand, the same or other channels are also required to couple oxygen reduction with proton-pumping activity. Most studies on haem–copper oxygen reductases have been performed on reductases from purple bacteria or mitochondria, which have a quite similar subunit I. However, with studies of an increasing number of oxygen reductases from phylogenetically distant organisms, the apparent overall conserved elements of these reductases were shown not to be conserved at all.

It was based on their intraprotein proton channels that we recently classified haem–copper oxygen reductases into three families named A, B and C [5] (Table 1 and Fig. 1).

3. Type A family: oxygen reductases with D and K channels

The type A family is divided into two subfamilies: A1 and A2. The A1 subfamily comprises the haem–copper oxygen reductases having the D and K channels first established for the mitochondrial-like enzymes (Table 1). Besides AspI-124 (D), the D channel is composed of the residues AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193 and GluI-278. The K channel leads directly to the binuclear site and is composed of the residues LysI-354 (K), ThrI-351, SerI-291 and TyrI-280 (e.g. [6,12]). This last tyrosyl is covalently bound to one of the histidyl ligands of Cu_B (HisI-276), and is proposed to intervene in the catalytic cycle forming a transient tyrosyl radical and allowing the efficient heterolytic splitting of the O_2 molecule [13–15]. In both the bovine and the *P. denitrificans* enzymes, the D channel appears to be composed of two parts: a hydrophilic one, starting at the surface of the protein facing the negative side of the membrane and ending at GluI-278, and a hydrophobic one, leading from the glutamate residue to the binuclear centre [2,3]. The proton conductivity from the glutamyl onwards to that site has to be assured by water molecules [9,16,17].

Haem–copper oxygen reductases from subfamily A2 have all the residues of the D and K channels with the exception of the helix VI glutamyl (GluI-278) at the hydrophobic end of the D channel (Table 1). This residue had been considered a key element for proton transfer, since most of the mutations at this position prevented the enzyme from completing the catalytic cycle and consequently impaired the proton-pumping activity (e.g. [6,8,9,11]). However, *Rhodothermus marinus* and *Thermus thermophilus* caa_3 oxidases are examples of A2-type members, which were shown to reduce O_2 completely to water and to pump protons [18–20]. Based on a homology model performed for the *R. marinus* oxidase, it was suggested that a tyrosine residue, in a position equivalent to PheI-274, i.e. one helix turn below the glutamate residue in helix VI, whose hydroxyl group occupies the spatial place of the carboxyl group of the glutamyl, and a consecutive seryl were relevant for proton transfer [19,21]. This so-called YS motif is the fingerprint of the members of type A2 subfamily, which also

Table 1
Classification of haem–copper oxygen reductases on the basis of the proton channels on subunit I [5]

Oxygen reductase type	Channels					
	'D'[a]		'K'[b]			
	E/Y	Others	K/T	T/S	S/Y	Y
A1 (e.g. cytochrome oxidases caa_3 and aa_3, quinol oxidase bo_3)	E	+	K	T	S	Y
A2 (e.g. cytochrome oxidases caa_3 and aa_3)	Y	+	K	T	S	Y
B (cytochrome/quinol oxidases)	–	–	T	S	Y	Y
C (cytochrome oxidase)	Y	–	–	S	Y	–

[a]GluI-278, TyrI-256 (*R. marinus*), others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193.
[b]LysI-354, ThrI-351, SerI-291 and TyrI-280. This tyrosyl is covalently bound to one of the histidyl ligands of Cu_B (HisI-276).

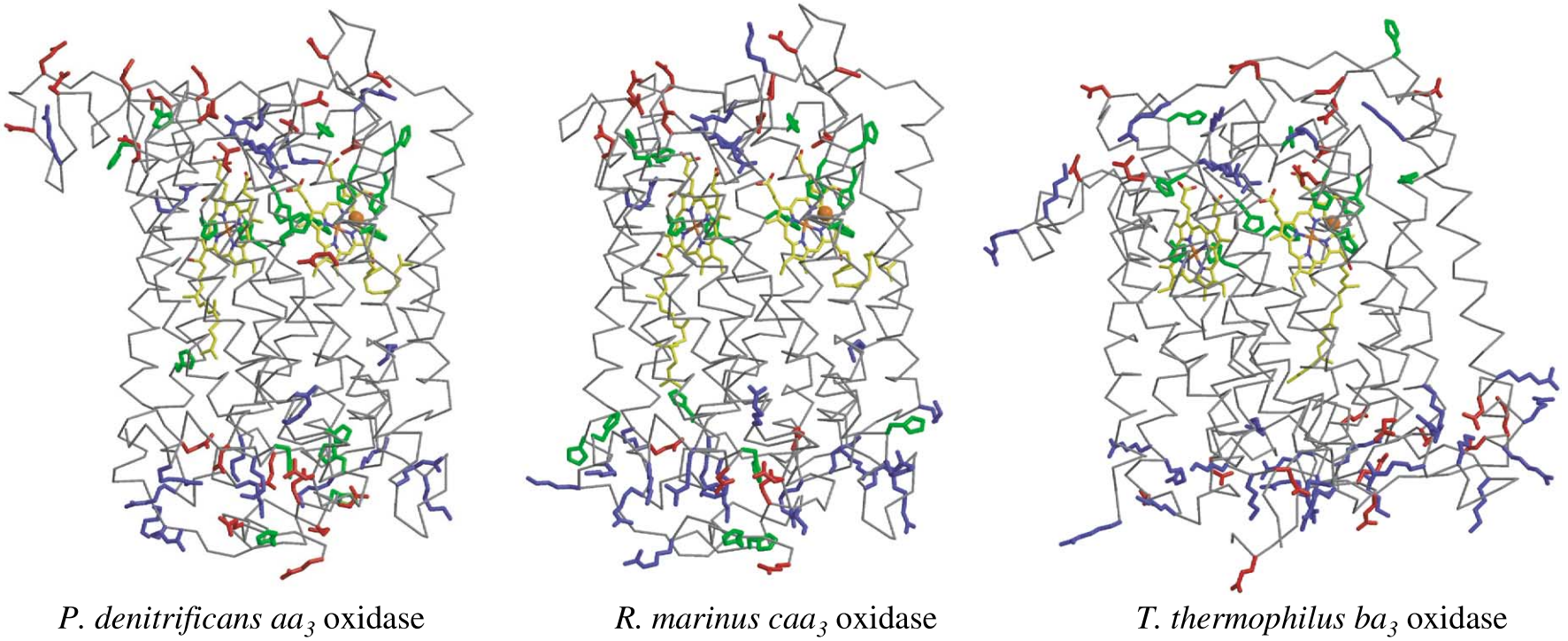

Fig. 2. Structures of subunit I from *P. denitrificans* (aa_3) [3] (left side) and *T. thermophilus* (ba_3) [4] (right side) haem–copper oxygen reductases and homology model of subunit I from the caa_3 haem–copper oxygen reductase from *R. marinus* [19] (centre). Possible protonable amino acid residues are shown (red, acidic residues; blue, basic residues; green, histidyls). It can be observed for the *R. marinus* (with exception of Lys332 of the K channel) and the *T. thermophilus* oxygen reductases that there is not any protonable amino acid residue between the protein surface facing the negative side and the catalytic site. The figure was prepared using XtalView [43] and Raster3D [44].

includes enzymes from mesophiles, such as those from cyanobacteria [5]. The functional role of this motif was proven by site-directed mutants of the *P. denitrificans* enzyme [22].

4. Type B family: oxygen reductases with D channel absent and alternative K

In the B-type enzymes, the residues forming the D and K channels in type A oxygen reductases are not conserved (Table 1). However, a K channel homologue seems to be present, with a threonine, a serine and a tyrosine residues replacing LysI-354, ThrI-351 and SerI-291, respectively. These enzymes also contain the tyrosyl covalently bound to the histidyl-coordinating Cu_B (TyrI-280). The type B members, such as the oxidases ba_3 from *T. thermophilus* [23], aa_3 from *Acidianus ambivalens* [24] and SoxABCD from *Sulfolobus acidocaldarius* [25], can pump protons. Thus, efficient proton channels have to be present, but their nature has yet to be established. The crystallographic structure of the ba_3 oxidase from *T. thermophilus* [4] suggests that, apart from the alternative K channel, there are two other possible proton channels: (i) one, named Q-pathway, is composed of residues GlnI-254, ThrI-396, LeuI-392, SerI-391, ThrI-394, ThrI-81, GlnI-388 and LeuI-387 (*T. thermophilus* numbering), which appears 'behind' the low-spin haem; (ii) another, involving residues GluI-17, TyrI-91, ThrI-21, SerI-109, GlnI-86, SerI-155, ThrI-156 and GlnI-82 (*T. thermophilus* numbering), is in a spatial position equivalent to that of the D channel in the type A oxygen reductases. The functionality of these channels remains to be supported by mutagenesis studies. Furthermore, inspection of the sequence alignment of the known type B oxygen reductases shows that none of those residues (or equivalent ones) is common to all of them [5]. However, a double mutant of the *P. denitrificans* aa_3 oxidase (PheI-274Thr, GluI-278Ala) showed that ThrI-156 from the *T. thermophilus* ba_3 oxidase may indeed be part of a proton channel [24]. For the aa_3 oxidase from *A. ambivalens* a putative pseudo D channel was suggested on the basis of a structural model [26]. Interestingly, the hydrophilic part of this channel ends at a glutamate residue (GluI-80, *A. ambivalens* numbering), located in helix II rather than in helix VI as in the type A1 enzymes; this proposal was corroborated by a double mutant I in *Rhodobacter sphaeroides* aa_3 oxidase mimicking the *A. ambivalens* enzyme, which is competent in proton pumping [27].

5. Type C family: oxygen reductases with D channel absent and alternative K only partly conserved

The type C family of haem–copper oxygen reductases is composed of the cbb_3 oxidases. These reductases seem to have only part of the alternative K channel conserved, with a seryl and a tyrosyl in the place of the *P. denitrificans* ThrI-351 and SerI-291 (Table 1). It is interesting that an equivalent to TyrI-280, the tyrosyl covalently bound to a copper histidyl ligand, is not present in these enzymes. None of the canonical residues of the D channel is present; there is a tyrosine residue in all cbb_3 oxidases, with the exception of the one from *Helicobacter pylori*, in the same sequence position as the tyrosyl of the YS motif of type A2 reductases that, as for these reductases, may be part of the proton pathway [5]. The members of this family are also proton pumps, as has first been shown using cells where the genes for other reductases had been disrupted [28,29], and later with *Bradyrhizobium japonicum* cbb_3 oxidase reconstituted in artificial liposomes [30].

6. The plasticity of proton pathways

The three haem–copper oxygen reductase families clearly illustrate the plasticity of the proton pathways among even a single functional family of proteins. The diversity of the proton channels in these enzymes is now so large and undefined that the search for common elements responsible for the channels efficiency becomes more difficult. Amino acid sequence comparisons and structural alignments clearly show that specific amino acids are not universally present. Also, it is immediately apparent that several enzymes, such as, for example, the ba_3 oxidase from *T. thermophilus* and the caa_3 oxidases from *R. marinus* and *T. thermophilus*, do not have protonable residues in between the protein surface at the negative side of the membrane and the metal centres (Fig. 2). However, fast kinetic studies performed for the caa_3 oxidase of *R. marinus* showed that the rates of proton uptake by this enzyme are similar to those reported for enzymes containing the protonable glutamate residue [31], indicating that its presence is not necessary for an efficient proton transfer. The absence of protonable residues can also be observed in many other distinct enzymes, which involve protons in their reaction and do not have their catalytic site exposed to the solvent. On the other hand, all these 'channels' have residues with hydrophilic side chains, which may establish, eventually together with the peptide backbone amide/carboxyl groups, a way to hold water chains in the proper orientation to allow kinetically efficient proton transfer.

In fact, proton transfer in channels such as those of gramicidin or voltage-gated channels, do not involve protonable residues (e.g. [32–34]). In this respect, it should be noted that mutations of the D channel non-ionisable residues in *P. denitrificans* aa_3 oxidase (e.g. [35]) led to a complete decoupling of the catalytic oxygen reduction and the proton-pumping activities, which may have resulted from a partial destruction of the hydrogen-bonding interactions mediated by water molecules along the D channel. Another important aspect generally not considered in detail is that proteins are highly flexible and dynamic and that water molecules may reside in hydrophobic protein cavities with a life-time similar to those encountered in hydrophilic protein cavities [36].

The absence of putative amino acid residues acting as gating elements at the end of the proton channels (e.g. *T. thermophilus* ba_3 and *R. marinus* and *T. thermophilus* caa_3 oxidases lack any ionisable residue between the protein surface and the metal centres) shows that the control of proton pumping must result from a complex combination of thermodynamic and/or dynamic properties of several amino acid residues/clusters, tightly controlled by the multiple redox and catalytic intermediates, which occur along the catalytic cycle. This control may be exerted by redox-linked or electrostatic-induced interactions such as redox–Bohr effects (which can be positive due to direct electrostatic interactions or negative due to redox-linked conformational changes) [37–39]. Such a network of interactions, strongly affecting the pK_a of amino acid residues that may reside at the protein surfaces, can assure the directionality of proton transfer, both by the modification of their proton affinities and by rearrangements of the water-mediated hydrogen networks. Indeed, although crystallo-

graphic data of catalytic intermediates of haem–copper oxygen reductases are still missing, some conformational alterations during the catalytic cycle have been proposed (e.g. [40–42]).

7. Conclusions

The presence of intraprotein proton pathways is not exclusive of haem–copper oxygen reductases. Their existence can be expected in all enzymes in which catalysis involves protons and which do not have their catalytic centres exposed to the solvent. In order to be efficient, a proton channel must (i) be kinetically competent, (ii) have a trigger mechanism to initiate the proton transfer, and (iii) possess a controlling mechanism that ensures the directionality of the transfer. The haem–copper oxygen reductase superfamily shows the diversity of solutions that proton channels can adopt. Furthermore, it is shown that these solutions do not need protonable amino acid residues for intraprotein proton conduction. The presence of protonable residues between the protein surface and the active centre is also not universal. Thus this observation confers an important role to water-mediated proton transfer and to amino acid residues with hydrogen-bonding capability. In this case, the trigger and the directionality of proton transfer may be due to redox-linked pK_a changes of residues at the protein surface, which may also induce conformational rearrangements of the hydrogen bond network, such as involving conformational changes of some amino acid side chains and/or of water molecules.

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