Chapter

Cytochrome *c* Oxidase: Insight into Functions from Studies of the Yeast *S. cerevisiae* Homologue

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Different members of the superfamily of haem-copper oxidases have diverse substrates, cofactors and protein subunits. Nevertheless, all appear to catalyse oxygen reduction with a structurally-similar catalytic binuclear centre. This is housed in a common subunit I together with a low spin haem electron donor to the catalytic centre. All also appear to be coupled to additional proton translocation. However, the possible pathways for movement of protons through the protein structures are not universally conserved. Hence, although the basic mechanism of coupling is likely to follow similar principles, the detailed atomic mechanisms are likely to be different between the A and the B/C subgroups, and have even been suggested to differ between vertebrate mitochondrial cytochrome *c* oxidases and bacterial quinol and cytochrome *c* oxidases within the same A1 subgroup. Here, studies of the yeast mitochondrial homologue are reviewed that shed light on roles of different possible proton transfer pathways and some of the additional subunits found in mitochondrial enzymes.

1. Introduction: Catalysis, Coupling and Efficiency

Aerobic respiration in eukaryotic mitochondria requires an oxidase that reduces the oxygen which provides a terminal sink in order to maintain electron flow through the respiratory electron transfer chain. In mammals, the yeast *S. cerevisiae* (hereafter referred to only as yeast) and many other eukaryotes, this oxygen-reducing function is catalyzed solely by cytochrome *c* oxidase (C*c*O), a large multiprotein complex that spans the inner mitochondrial membrane. Electrons from reduced cytochrome *c* are transferred to a buried catalytic site, the binuclear centre (BNC), formed from a haem A (haem a_3) and a copper atom (Cu_B). It uses four electrons from cytochrome *c* in the intermembrane space (IMS), together with four protons from the mitochondrial matrix, to reduce molecular oxygen to two waters. As a result of the vectorial nature of the reaction, part of the energy released by the exergonic oxygen reduction reaction can be conserved in the transmembrane electrochemical proton gradient, as envisaged in the original chemiosmotic proposals of Mitchell [Mitchell, 1966].

The thermodynamic efficiency of energy conservation for this vectorial reaction may be estimated for typical physiological conditions. The standard potential E'_o for O_2/H_20 at pH 7 is +815 mV *vs* SHE [Wood, 1988]. However, this refers to conditions with liquid water at 25C and pH 7 and an O_2 fugacity of 1 atmosphere. Given Henry's Law, the 21% content of oxygen in air and the fact that its concentration can be far lower in tissues distant from the blood supply (perhaps as low as 2% [Carreau *et al.*, 2011]), this means that the operative E'_h is 10–25 mV lower. Assuming that the reductant source cytochrome c^{2+}/c^{3+} ratio in aerobic tissues is around 1, the reductant E'_h would correspond to its E_{m7} of +260 mV. Hence the overall $\Delta E'_h$ for the four-electron reduction of oxygen to

water by cytochrome c^{2+} within cells at pH 7 is 545-530 mV ($\Delta G' \sim$-50 kcal.mol^{-1}). With a typical transmembrane PMF of ~200mV (mostly $\Delta\varphi$; positive outside), this means that only 35-40% of the available energy would be conserved from the four electrogenic PCET reactions due to their vectorial nature.

However, each full catalytic cycle also causes an additional four protons to be translocated from the matrix to the IMS [Wikström, 1977], hence increasing the fraction of energy that can be stored in the PMF to ~75% when operating against a large PMF. This means that, even with a high PMF of 200mV, the overall operative $\Delta G'$ of the coupled energy-conserving reaction is ~-12 kcal.mol^{-1}. Hence, the reaction is still operating far from equilibrium with 25% of the energy lost as heat. The reaction is essentially irreversible (for $\Delta G \sim$-12 kcal.mol^{-1}, ratio of forward/reverse reaction rate constants ~ 10^9) and it is not possible to drive oxygen generation by reversed electron flow, even with a high PMF, as has been confirmed experimentally [Wikström, 1988]. In environments such as exercising muscle, where the phosphate potential and the PMF may well drop considerably, the efficiency would be much lower, though the throughput rate and power output might increase [Rich, 1982].

The presence of this irreversible terminal step means that the upstream complexes of the respiratory chain can, provided that they have the necessary catalytic capacity, operate much closer to equilibrium and, therefore, operate closer to maximum efficiency. The fact that reversed electron transfer from reduced cytochrome *c* or ubiquinol to NAD^+ can occur readily in coupled mitochondria [Chance and Hollunger, 1961] suggests that this is the case. It also means that modulation of C*c*O activity, rather than activities of those complexes operating close to equilibrium, would most influence the overall throughput of the whole respiratory chain. This may well be one major reason why mitochondrial C*c*Os, in contrast to the other major respiratory complexes, possess a variety of isoforms of supernumerary subunits and ligand and phosphorylation sites that can influence activity allosterically [Kadenbach and Hüttemann, 2015], as discussed below.

2. Core Structural and Functional Variations in the HCO Superfamily

Mitochondrial C*c*Os are members of a haem-copper oxidase (HCO) superfamily, homologues of which are found throughout eukarya, bacteria and archaea [García-Horsman *et al*., 1994; Sousa *et al*., 2012; Hemp and Gennis, 2008]. The superfamily has been divided into A, B and C subgroups, and includes both CcOs and quinol oxidases (QOs). All have a homologous core subunit I which houses the catalytic BNC for oxygen reduction, formed by a high spin haem and a histidine-coordinated copper that has a catalytically-active tyrosine covalently linked to one of its histidine ligands [Tsukihara *et al*., 1996]. Electron donation into the BNC is *via* a *bis*-histidine-coordinated low spin haem that is also housed in subunit I. Variations occur in haem types (A, B and O), the sequence location of the catalytic tyrosine and the immediate donor to the low spin haem (Cu_B and/or bound haem(s)C in the C*c*Os; direct from quinol substrate in the QOs). Further variations occur in the number and types of additional subunits and the distribution of domains between them. Despite these differences, it seems likely that the basic BNC-catalysed mechanism of oxygen reduction is essentially the same in all HCOs. This involves a four-electron transfer to dioxygen in a single step after binding that follows reduction of the two BNC metals [Wikström *et al*., 2015; Rich, 2017]. This converts the dioxygen into two oxides without formation of kinetically-detectable, potentially damaging, reactive oxygen species. It seems that all HCOs share the same vectorial arrangement of delivery of reductant and substrate protons into the BNC described above that results in conservation of some of the energy of the exergonic oxygen reduction (in the case of QOs, the movement of the

electron towards the N phase is replaced at least in part by expulsion of the positive quinol proton towards the P phase).

It also seems likely that all HCOs are coupled to the additional translocation of protons across the membrane that increases the efficiency of energy conservation, though coupling stoichiometry may be lower [Han *et al*., 2011], or perhaps is more readily decreased by an opposing PMF [Wikström *et al*., 2015]. However, different HCOs possess different possible pathways that could conduct protons within the protein structures [Hemp and Gennis, 2008]. Specifically, all A-type HCOs have two very similar structures, the D and K channels [Konstantinov *et al*., 1997]. The D channel connects the N phase to a buried glutamic acid (A1-type, which includes all eukaryotic mitochondrial CcOs) or tyrosine (A2-type) that is roughly 10Å from both haem edges. The K channel connects the N phase to the catalytic tyrosine of the BNC. All A-type C*c*Os also have a third, H, channel, though with some variations (Figure 1) (and in A-type QOs the H structure is virtually absent [Wikström *et al*., 2015]. The H channel potentially connects the N and P phases, running past the formyl and farnesyl substituents on the D- and A-pyrrole ring of the low spin haem [Tsukihara *et al*., 1996]. There is ample evidence from kinetic analyses and mutation effects that the D channel conducts translocated protons in bacterial A-type C*c*Os [Wikström *et al*., 2015]. However, it has been suggested from structural and functional data that the H channel fulfills this role in mammalian mitochondrial C*c*Os (reviewed in [Yoshikawa and Shimada, 2015]). Particularly surprising is the evident lack of either a functional D or H channel in the B- and C-type HCOs, despite their protonmotive ability. However, they do possess the equivalent of a K channel (though lacking the central lysine). These differences have led to the conclusion that protonmotive coupling in the B- and C-type HCOs should utilise the variant K channel for both substrate and translocated protons though, to date, no comprehensive model has emerged. Hence, a current issue is that, despite the likelihood that the oxygen chemistry has a common mechanism in all HCOs, coupling to proton translocations across the membrane may be achieved with quite different pathways and mechanisms in different subgroups of HCOs and even within the same A1 subgroup.

3. Yeast C*c*O – a Link Between Mammalian and A1 Bacterial C*c*Os

Yeast mitochondrial C*c*O offers an experimentally-amenable model system to explore possible functional variations in mitochondrial C*c*Os [Maréchal *et al*., 2012]. Its core subunits have extensive sequence identity/similarity with mammalian subunits (for example, its subunit I has 59% sequence identity with bovine C*c*O subunit I). Homology modelling of structure confirms that it has D and K channels that are very similar to those present in other A1-type bacterial and mammalian C*c*Os. It also has a clear H channel, though with some differences. Figure 1 compares H channel residues in bovine and yeast mitochondrial C*c*Os together with those in the *P. denitrificans* A1-type C*c*O. All three have hydrophilic residues in the lower part of their structures that in bovine C*c*O is suggested to house water-accessible domains [Yoshikawa and Shimada, 2015], including an equivalent of residue S382, which undergoes potentially important conformational alterations [Yano *et al*., 2016; Liu *et al*., 2011]. Also conserved in all three are residues R38 and Y371 which interact with haem *a*. However, H413 that is in the centre of the lower part of the structure in mammalian and bacterial C*c*Os is replaced by a glutamine in yeast C*c*O.

Much more poorly conserved are residues that form the upper H-bonding network part of the bovine H channel. In particular, neither yeast nor *P. denitrificans* C*c*Os retain the proposed ‘gate’ formed by S440-Y441 amide link, or the conformationally-flexible D51 at the P phase exit (though silent human polymorphisms D51/G and D51/Y have anyway been reported [mitomap, 2017]). The principal residues that form the bovine Na^{+}/Ca^{++} binding site are also only partly conserved in yeast and *P.*

denitrificans C*c*Os, possibly explaining the closer similarity of yeast C*c*O with bacterial rather than bovine C*c*O in terms of ease of dissociation of bound of Ca^{++} [Maréchal *et al*., 2013]. Nevertheless, there is still a substantial potential H-bonding network of residues within this region in all three C*c*Os.

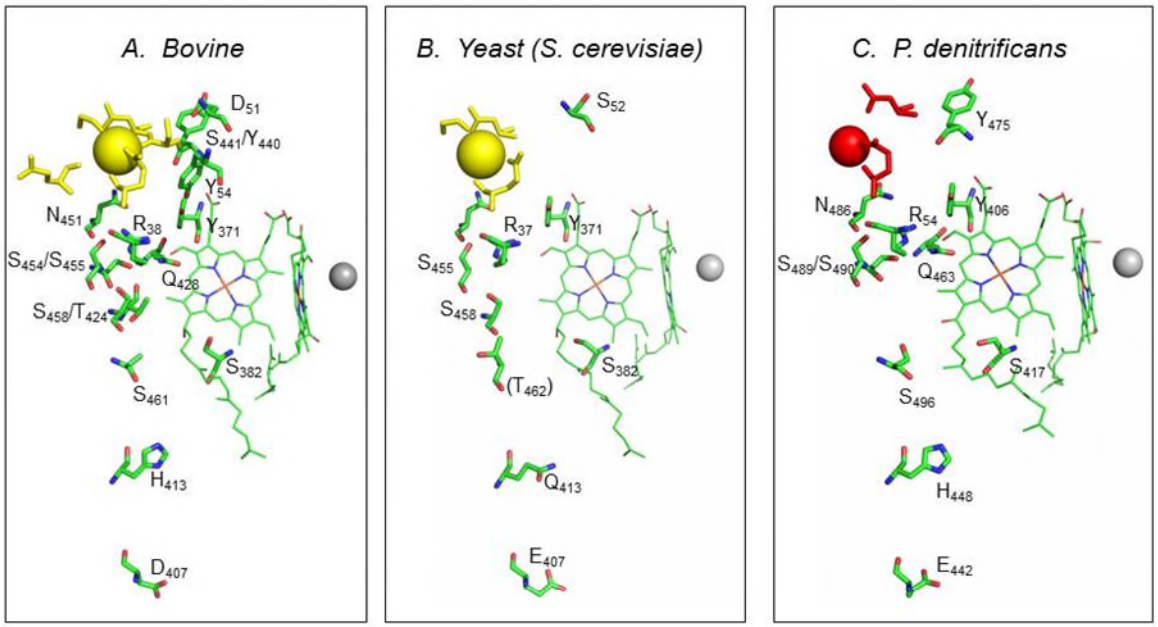

Figure 1. Comparison of residues within the H channel of bovine, yeast and *Paracoccus denitrificans* C*c*Os. Figures were constructed from PDB files of oxidised forms of C*c*Os: 5B1A (bovine, 1.8Å [Yano *et al*., 2016]); homology modelled yeast structure [Maréchal *et al*., 2012] (with an assumed sodium rather than Ca^{++} ion); 3HB3 (P. denitrificans, 2.25Å [Koepke *et al*., 2009]. In panels B and C, only residues with sequence identity/similarity to bovine H channel residues are shown (yeast T_{462} is one residue from the sequence-aligned residue A_{461}). Residues interacting with the sodium ion are shown in yellow (bovine and yeast); those interacting with the calcium ion (*P. denitrificans*) are shown in red.

3.1. *The H channel and its possible roles*

Particularly useful in the yeast system is the availability of technology for site-directed mutagenesis of the core, mitochondrially-encoded subunits [Meunier *et al*., 2012]. An extensive set of mutations have been introduced into the K, D and H channel regions of subunit I. Initial studies on effects on coupling ratio of a mutant with four mutations in the H channel were performed using vesicle-reconstituted enzyme. The data suggested that, like bacterial CcOs, coupling efficiency was not perturbed by such H channel interference [Dodia, 2014]. Further more precise studies of coupling efficiencies in enzyme with mutations of single residues in all three channels were made by measurements of 'classical' H^+/O ratios determined from state 3/4 transitions induced by ADP additions to intact respiring mitochondria [Chance and Williams, 1955]. These investigations confirmed this finding and also indicated that mutations in the D channel have effects similar to those found in bacterial C*c*Os, including uncoupling of proton translocation from oxygen catalysis in a D channel mutant whose bacterial equivalent has the same effect [Maréchal *et al*., 2014; Maréchal *et al*., 2017]. As a result, an alternative possible 'dielectric channel' role of the H channel in yeast mitochondrial and bacterial A1 C*c*Os has been suggested [Rich and Maréchal, 2013]. The structure of a dielectric channel should be similar to proton-conducting channels, being composed of charged or polarisable amino acids, water molecules [Nagle and Tristram-Nagle, 1983; DeCoursey, 2003] and other moieties. However, the structure is unable to support a continuous current of protons through the

protein structure by the Grotthuss mechanism [Grotthuss, 2006]. This mechanism requires charge relocation along an H-bonded chain to a gated protonation sink, followed by molecular reorientations so that protons can be repetitively taken from the source and delivered to the sink. A dielectric channel can also relocate charge – in the case of the H channel this would occur in particular in response to charge change caused by reduction/oxidation of the low spin haem A – again by H-bonding changes as in the first part of the Grotthuss mechanism, or by charge/dipole movements more generally. However, unlike proton-conducting channels, the changes are not linked to the consumption of a proton at a source and delivery to a gated sink to create a net proton transfer with each redox cycle. Instead, any charge relocation (including any redox-linked protonation) occurs reversibly with each redox cycle, so that there is no resulting net transfer of a proton or other charged species. Hence, instead of facilitating a current of protons from source to sink, its function is to influence thermodynamic and kinetic properties of the buried electron transfer event by fully reversible charge relocations. The dielectric well effect could result in a reversible redox-linked protonation change within the channel if an appropriate protonatable group is present, akin to the redox Bohr proton effect [Papa *et al*., 1979; Papa, 2005]. However, its physical basis can instead involve quite different groups whose positions or polarities change in concert to compensate the charge change. The induced changes can also be spatially extensive in that the charge rearrangements can be transmitted through the channel to one or both aqueous surfaces where remote protonation or ionic redistributions can occur. Whether the H channel fulfills such a function in mammalian C*c*Os in addition to, or instead of, providing a conduit for a current translocated protons remains to be established. It might be noted that a similar dielectric role has been suggested for the K channel in order to partially charge-compensate transiently-charged BNC intermediates [Jünemann *et al*., 1997; Lepp *et al*., 2008], hence facilitating their formation by lowering the free energy required. Interestingly, it has been noted that the H channel is absent in the A1-type QOs [Wikström *et al*., 2015]. In this case, the electron donor is an electroneutral quinol from within the membrane and the system instead has a proton-conducting half channel to conduct protons released by quinol oxidation into the P phase [Abramson *et al*., 2000], in addition to well-conserved D and K channels.

3.2. *Supernumerary and Additional Subunits*

The mitochondrial CcOs are considerably larger than their bacterial counterparts because they have additional supernumerary subunits. Human and bovine CcOs have 10 such additional subunits [Kadenbach and Hüttemann, 2015] and yeast CcO has eight, all of which are homologous to human/bovine subunits, though with low sequence identities in some cases [Maréchal *et al*., 2012]. These additional subunits do not have direct roles in proton/electron transfer reactions. Some can occur as tissue- or environment-dependent isoforms. In yeast, only Cox5 (homologous to mammalian subunit IV) has isoforms but in mammalian CcOs isoforms of subunits IV, VIa, VIb, VIc, VIIa, VIIb and VIII have been found. Several subunits house sites that may become phosphorylated [Hüttemann *et al*., 2012], acetylated [Liko *et al*., 2016] or can bind nucleotides [Kadenbach and Hüttemann, 2015; Arnold, 2012]. Specific functions of some of these isoforms, their post-translational modifications and ligand binding sites have been suggested, though the supporting data in many cases remain inconsistent. Only one bacterial homologue of these supernumerary subunits has been reported, the *ctFm* gene product in the cyanobacterium *Synechocystis*, which has 50% and 20% sequence identity with yeast Cox5 and bovine subunit IV, respectively [Alge *et al*., 1999].

In BN-PAGE gels of digitonin-extracted membranes, CcO can be found as monomers, dimers or in supercomplexes with complexes I and III [Wittig and Schägger, 2009; Stuart, 2008; Dudkina *et al*., 2008; Cui *et al*., 2014]. Bovine CcO crystallises as a homodimer with the dimer interface formed primarily by subunits VIb and III, with smaller contributions from Vb and Via [Tsukihara *et al*.,

1996]. How important the dimer is in the natural membrane remains unclear. It has recently been suggested that propensity to form dimers may be influenced by isoforms of subunits VIa and VIIa in mammalian cells types [Cogliati *et al*., 2016]. In the known supercomplex structures, bovine and yeast CcOs are attached as monomers. However, even when incorporated into supercomplexes, the dimer-forming surface is sufficiently exposed that it could still allow dimerisation [Letts *et al*., 2016] (Figure 2).

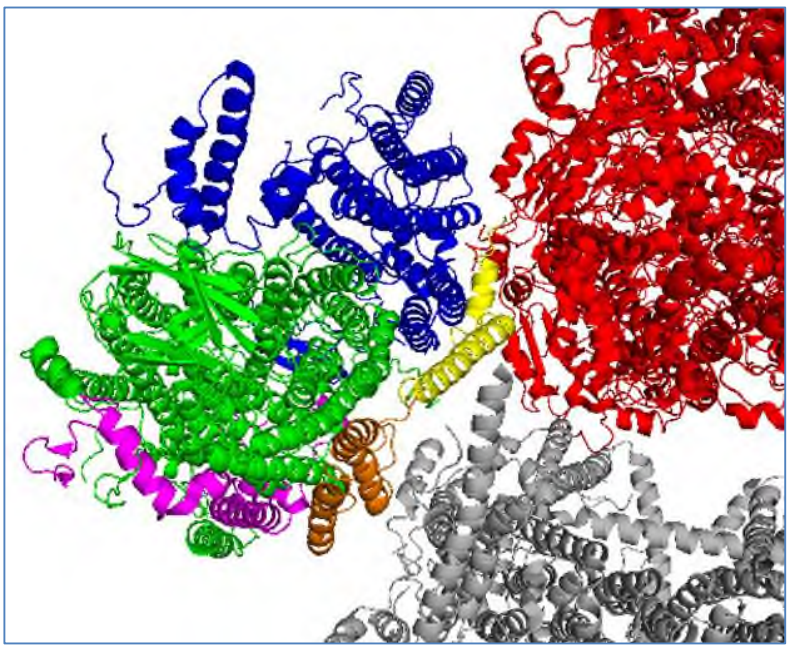

Figure 2. Relative positions of supernumerary subunits of ovine C*c*O involved in supercomplex interactions and dimerisation. The figure is drawn from coordinates of the tight form of the I/III_2/IV supercomplex in PDB 5J4Z [Letts *et al*., 2016]. Interaction regions of complex I and complex III and shown in grey and red, respectively. Subunits of C*c*O are colour-coded as: BROWN, subunits VIIc, VIII interacting with complex I; YELLOW, subunit VIIa (or VIIa2l) interacting with complex III; BLUE, subunits VIb, III, Vb, VIa that contribute to the C*c*O dimer interface in crystal forms; MAGENTA, subunit IV (homologue of yeast subunit Cox5); GREEN, all other C*c*O subunits.

Several subunits are involved in interactions with partner complexes to form supercomplexes in mammalian systems (Figure 2). In the mammalian I/III_2/IV supercomplex [Letts *et al*., 2016], subunit VIIa (or possibly the homologous subunit COX7a2l, also known as SCAF1 [Pérez-Pérez *et al*., 2016; Cogliati *et al*., 2016; Lapuente-Brun *et al*., 2013; Ikeda *et al*., 2013]) appears to interact with subunits UQCR11 and UQCR1 of complex III, and subunits VIIc and VIII interact with subunits ND5 and B12/NDUFB3 of complex I. However, it is not yet clear if the same interaction with complex III occurs in the yeast III_2/$IV_{1/2}$ supercomplex. Yeast Cox7 has weak (12-18%) sequence identity with all three isoforms of bovine subunit VIIa. It does not have the additional N-terminal extension characteristic of COX7a2l (Figure 3) that has been proposed to interact with complex III, and lacks an important histidine residue (H76 in the bovine VIIa2l sequence in Figure 3) [Cogliati *et al*., 2016]. No other Cox7 homologues are evident in the *S. cerevisiae* genome. In addition, previous EM-derived models of the yeast III_2/IV_2 supercomplex have suggested that Cox5, which is spatially distant from Cox7, provides the interface with complex III [Mileykovskaya *et al*., 2012].

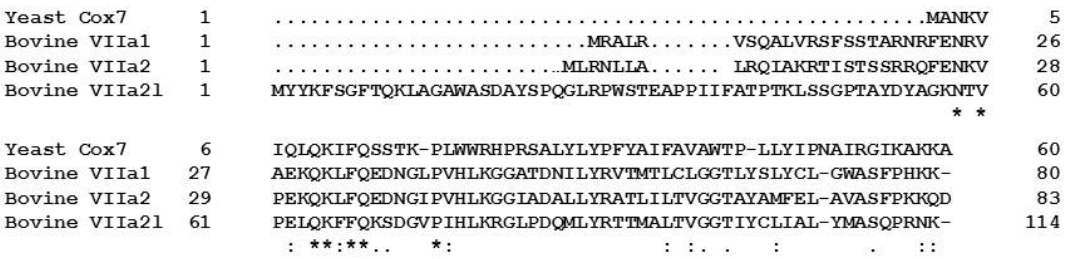

Fig. 3. Clustal alignment of yeast Cox7 with homologous bovine subunits VIIa1, VIIa2 and with VIIa2l.

Several additional proteins have been identified that are associated with digitonin-extracted mitochondrial C*c*Os but were not retained in the bovine C*c*O crystals produced with harsher detergents used to prepare samples for atomic structure determination (Table 1). In *S. cerevisiae* two proteins, Rcf1 and Rcf2, are associated with C*c*O, complex III_2 and III_2/IV supercomplexes [Vukotic *et al*., 2012; Strogolova *et al*., 2012] and may also regulate C*c*O activity independently of supercomplex formation [Rydström Lundin *et al*., 2016; Hayashi *et al*., 2015]. Two orthologues of Rcf1 are found in mammals and are also associated with C*c*O. However, surprisingly, neither was present in the recently solved ovine I/III_2/IV supercomplex structure [Letts *et al*., 2016] though again a direct effect of the Rcf1 orthologue (Higd1a) on catalytic activity has been suggested [Hayashi *et al*., 2015].

Table 1. Additional weakly-bound proteins associated with yeast and mammalian C*c*Os.

Yeast subunit	Mammalian subunit	Possible function
-	SCAF-1 (COX7a2l)	Forms III/IV interface
Rcf1	Rcf1a (Higd1a), Rcf1b	Required for supercomplex formation; regulatory function
Rcf2	-	Amplification of Rcf1 effects
YPR010C-A	NDUFA4	Regulation and biogenesis
COX26	-	Associated with III/IV supercomplex
AAC2		ADP/ATP exchange across IMM

Interestingly, Higd1a is induced in hypoxic conditions and is proposed to bind to a site close to subunit IV and the H channel of subunit I [Hayashi *et al*., 2015]. This is in a relatively exposed region of C*c*O that is not involved in the dimer interface or in supercomplex interactions (Figure 2). Both bovine subunit IV and its yeast homologue Cox5 have isoforms related to oxygen levels, raising the possibility that binding of Rcf/Higd1 isoforms are influenced by subunit IV/Cox5 isoforms. Interestingly, subunit IV/Cox5 interacts with two helices of core subunit I that provide part of the H channel structure [Rich and Maréchal, 2013]. Hence, it may be speculated that changes in subunit IV/Cox5 isoforms and/or binding of further protein partners or modifications such as phosphorylation might induces changes to catalysis parameters through allosteric effects of the nearby H channel by influencing its properties as a dielectric channel. An additional subunit, Cox26, has recently been found to be part of the yeast III_2/IV supercomplex [Levchenco *et al*., 2016; Strecker *et al*., 2016]. A further subunit, NDUFA4, originally thought to be a subunit of complex I, has now clearly been shown to be a component of mammalian complex IV [Balsa *et al*., 2012] and also co-purifies with yeast III_2/IV supercomplex [Vukotic *et al*., 2012], though its function is unknown. Association of the ADP/ATP carrier (Aac2) has also been found to be associated with the yeast supercomplex [Dienhart

and Stuart, 2008]. For all of these more loosely-attached additional subunits, further work will be required to establish more firmly their roles in supramolecular structures, stability, allosteric control, assembly and/or interactions with as-yet unknown factors.

4. Concluding Remarks

In mitochondrial and bacterial forms of A1-type C*c*Os the pathways for protons through the protein must be made by one or more of the three identifiable hydrophilic networks of amino acids and associated waters. These may also provide a separate function as dielectric channels in some situations, a role which has not been widely recognised or tested to date. Further assessments of proton transfer *versus* dielectric roles of these channels may help to resolve the current discussions of whether conduction of translocated protons switches from the D channel in yeast mitochondrial and bacterial C*c*Os to the H channel in mammalian C*c*Os. Yeast C*c*O also offers a system to probe the possible control roles of its tightly bound 'supernumerary' subunits and more weakly associated factors, many of which have mammalian homologues, and the effects of incorporation into supercomplexes.

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