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Review

The generation of proton electrochemical potential gradient by cytochrome *c* oxidase

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Abstract

Cytochrome *c* oxidase, the terminal oxidase of mitochondria and some bacteria, catalyzes the four electron reduction of oxygen, and generates a proton electrochemical potential gradient ($\Delta\mu_H$). The recently determined structures of the bacterial and the bovine enzymes, together with studies of site directed mutants of a bacterial cytochrome *c* oxidase and a closely related ubiquinol oxidase, have greatly advanced our understanding of the mechanism by which oxygen reduction is coupled to the generation of $\Delta\mu_H$. Two different mechanisms contribute to the generation of $\Delta\mu_H$: protons that are consumed by the reduction of oxygen, are taken exclusively from the mitochondrial matrix ('consumed' protons), while other protons are translocated by the enzyme across the membrane ('pumped' protons). It is suggested that both proton consumption and proton pumping are driven by the electrostatic charging of the enzyme reaction center by the reducing electrons. Proton consumption is suggested to result from the electrostatically driven ejection of hydroxyls into the matrix that is catalyzed by a tyrosine residue in the reaction center. Proton pumping is suggested to result from the electrostatically driven translocation of a glutamate residue near the reaction center, and is assisted by secondary acceptors that release the translocated protons. © 1998 Elsevier Science B.V.

Keywords: Cytochrome *c* oxidase; Ubiquinol oxidase; Proton transport; Electron transport; Hydroxyl transport

Contents

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1. Introduction

Cytochrome *c* oxidase, the terminal oxidase of the mitochondrial electron transport chain, belongs to the superfamily of heme-copper oxidases (cytochrome *c* oxidases (COX), and ubiquinol oxidases (UQOX)), which utilize the free energy extracted from the four electron reduction of molecular oxygen to water (Eq. (1)) to generate a proton electrochemical potential gradient, $\Delta\mu_H$, across the membrane (reviewed in [1–4]).

$$4e^- + O_2 + 4H^+ \rightarrow 2H_2O \quad (1)$$

The mammalian COX has 13 subunits, but only one subunit (subunit I) is conserved in all heme-copper oxidases. Subunit I is an integral membrane protein with 12 conserved transmembrane helices (I–XII) and contains three redox centers. Oxygen reduction occurs at a binuclear metal center (Fe–Cu), where the oxygen intermediates are bound between the metals. The iron is in the center of a high-spin heme with an axial histidine ligand (usually heme A in COX, and usually heme O in UQOX), and the copper (Cu_B) is ligated by three histidines. A low-spin heme (usually heme A in COX, and usually heme B in UQOX) that is ligated by two histidines is also located in subunit I, close to the high-spin heme. The low-spin heme does not interact with oxygen, but mediates electron transfer to the binuclear center. Most heme-copper oxidases also have a separate subunit, subunit II, which binds and oxidizes the electron donor. In cytochrome *c* oxidases subunit II contains another bi-metal (Cu) redox center, Cu_A. Cytochrome *c* binds to subunit II and reduces Cu_A, which in turn, reduces heme a, which forwards the electron to the binuclear center (reviewed in Ref. [5]). In UQOX, subunit II, which does not contain Cu, binds and oxidizes reduced ubiquinol, releasing the protons on the outer surface and delivering the electrons to subunit I [6]. Most of the proton pumping heme-copper oxidases also contain a highly conserved subunit III, but its precise function has not been elucidated as yet.

The reduction of oxygen at the binuclear center proceeds in several discrete steps: first, a two electron reduction of the binuclear center of the oxidized enzyme (O) produces the partially reduced enzyme (R); then, binding of oxygen produces the peroxy intermediate (P), which is followed by reduction (third electron), protonation and the formation of the ferryl intermediate (F); finally, reduction by the fourth electron and protonation results in the formation of two hydroxyls, regeneration of the oxidized enzyme, and the release of water [1–4,7]. It is now clear that most, if not all, heme-copper oxidases have two distinct mechanisms for coupling oxygen reduction to the generation of $\Delta\mu_H$. One process, with a stoichiometry of $1H^+/e^-$ ($4H^+/O_2$, Eq. (1)), is ubiquitous in the heme-copper oxidase superfamily, and results from the consumption of protons, which are taken from the mitochondrial matrix, as first suggested by Mitchell [8]. These protons are now referred to as 'substrate' or 'consumed' protons. The other process, which involves proton translocation across the membrane, is referred to as proton 'pumping', or 'vectorial' proton transport. Wikstrom showed that the stoichiometry of proton pumping is also $4H^+/O_2$ [9], but the coupling of vectorial proton transport and electron transport is not complete (cf. Refs. [10–13]), and very likely, the mechanism of proton pumping is not as uniform within the superfamily as the mechanism of proton consumption (cf. Refs. [14,15]).

The three dimensional structure of a four subunit bacterial COX (*P. denitrificans*) [16,17] and a 13 subunit mammalian COX (Bovine) have been determined recently [18,19].

Here, we review briefly studies of proton movements associated with the reduction of oxygen by COX and UQOX, and their interpretation within the current models of COX function. We suggest a new model that is based on the recently determined crystal structure of the bacterial and mammalian COX, and

on qualitative considerations of the electrostatics of oxygen reduction and H^+/OH^- binding and release.

2. Proton consumption and the generation of $\Delta\mu_H$

It was originally suggested by Peter Mitchell that the generation of $\Delta\mu_H$ by COX is an 'electronmotive' process [8]. Accordingly, the protein was assumed to transport *electrons* across the membrane osmotic barrier from the donor, cytochrome *c*, on the cytoplasmic surface of the mitochondrial inner membrane, to an oxygen reduction site on the matrix surface of the inner membrane. The movement of the electrons across the membrane, driven by the redox free energy of reaction [1], would thus generate $\Delta\Psi$ (negative inside) directly, and the consumption of protons at the inner surface would increase the matrix pH, thus generating ΔpH ($pH_i > pH_o$). It is now apparent that a large fraction of $\Delta\Psi$ generation in the bc_1 complex (ubiquinol:cytochrome *c* reductase) is due to electron transfer within cytochrome *b*, from a ubiquinol binding site, Q_{out}, on the outer surface to another ubiquinol binding site, Q_{in} on the inner membrane surface, mediated by the two hemes b_H and b_L [20]. Similarly, in the bacterial photosynthetic reaction center, $\Delta\Psi$ is generated largely by the light driven transport of electrons across the membrane from cyt C2 on the outer surface via the chlorophylls and pheophytin to Q_B on the inner surface [21].

It was demonstrated early on by Hinkle and Mitchell that energization of mitochondria by ATP shifts the redox potential of heme a (when equilibrated with cytochrome *c*) by about 50 mV, and it was concluded that only part of the potential is generated directly by electron transport [22]. It was suggested and later demonstrated (cf. Ref. [23]) that the consumed protons are taken up from the mitochondrial matrix, presumably through a 'channel', a 'proton well' or a 'proton wire' (see below), and thus account for the formation of ΔpH and part of the $\Delta\Psi$ that is generated by proton consumption (cf. Ref. [7]).

Studies with site-directed mutants of subunit I of the *E. coli* (EC) UQOX and the *R. sphaeroides* (RS) COX (reviewed in Ref. [24]), determined the identity of the liganding histidines of the hemes and Cu_B, and together with the hydropathy model of subunit I, suggested that both heme a and the binuclear center are located close to the outer surface and are not in the middle of the hydrophobic core as suggested previously. This conclusion was confirmed decisively by the recently determined crystal structures of the *P. denitrificans* (PD) COX [16,17], and the bovine heart COX [18,19], which show that both redox centers are close (~ 13 Å) to the outer membrane surface. The distance from the binuclear center to the inner surface of subunit I is well over 30 Å. [1]

The region of the protein close to the outer membrane surface, just above the redox centers is rich in polar residues and water molecules, while the hydrophobic nonpolar region of the protein, which is at the same depth as the hydrophobic core of the lipid bilayer, lies *between* the binuclear center and the inner surface [17,19]. Since the generation of transmembrane $\Delta\Psi$ requires a transfer of charge across this low dielectric barrier, the contribution of electron transfer from subunit II to the binuclear center should account for only a small fraction of the total transmembrane potential. Time-resolved, electrometric measurements of the generation of $\Delta\Psi$ by reconstituted bovine COX showed that 20% of the total potential was generated simultaneously with electron transfer from Cu_A to heme a on the transition from F to O [25]. Similar studies with the RS COX found that 30% of the total potential was associated with electron transfer to heme a [26]. The interpretation of these interesting measurements must await further studies.

To explain the uptake of protons from the mitochondrial matrix into the binuclear center, it must be assumed that there is a driving force for protons (e.g., $\Delta\Psi$), generated by the transfer of electrons into the binuclear center. Since each electron arriving at the binuclear center would generate, transiently, an excess negative charge at the binuclear center, it can be assumed that this electrostatic charging drives the uptake of protons into the center, where the charges are neutralized [27–29]. The standard redox potential of the first and second electron transport steps into the binuclear center is only about 350 mV, but in the third and fourth steps it can be as high as 1200 mV

[1] In this review all the distances between atoms and residues in COX were obtained from the bovine structure (PDB:#10CC). Accordingly, the numbering of COX residues in this review is that of the bovine COX, unless indicated otherwise.

and 1050 mV, respectively [1]. It was estimated that a single electron transfer into a protein embedded redox center would contribute more than 1350 mV in electrostatic energy [29]. Therefore, progress of the reaction forward requires a lowering of the electrostatic potential, which could be accomplished either by bringing a proton into the binuclear center, by removing a negative charge from the binuclear center, or by reorientation of the charges and dipoles that surround the binuclear center to lower the charging potential. Indeed, it has been demonstrated that the rate of delivery of electrons to the binuclear center during the reduction of the fully oxidized COX (i.e., the first two electrons) decreases at high pH, which is compatible with a requirement for a balancing proton uptake [30]. It was also demonstrated that the rate of the reverse electron transfer (i.e., from the reduced heme a_3 to oxidized heme a), which is associated with proton release, is also strongly pH dependent, increasing an order of magnitude at high pH [31,32]. The results of the latter studies were best fitted by a model in which the released proton is removed from a proton binding group that is sufficiently close to the binuclear center (5–10 Å) so that its pK decreases upon oxidation of the binuclear center from a pK of 9.7 to 8.5 [32].

Measurements of the extent of proton binding to isolated COX showed that the reduction of COX by cytochrome *c*, in the absence of oxygen, is associated with a net uptake of up to 2.4 protons [7,27–29]. Presumably, two of these are the consumed protons taken up in the reduction of the binuclear center. The reduction of oxygen is associated with the net uptake of two additional protons. The latter two proton consumption steps were shown to be associated with the third and fourth electron transfer steps: the transition from P to F (third proton), and from F to O (fourth proton) [7,27,28,33]. These results are compatible with the assumption that each electron that reaches the binuclear center must be followed by the uptake of a proton (i.e., proton consumption), before the next electron can be delivered to the binuclear center.

2.1. The consumed protons 'wire' (K-channel)

Attempts to identify the consumed proton pathway(s) from the matrix surface to the binuclear center have been based on studies of site directed mutants of the EC UQOX [34,35] and the RS COX [36], and on the examination of the crystal structures of the PD and bovine COX [16–19]. Both crystals appear to show segments of a hydrogen-bonded chain, linking side-chain residues of helix VI and helix VIII, leading from the matrix surface to the binuclear center. Fig. 1 shows the proposed consumed proton pathway in the bovine COX [19]. It begins on the matrix side (bottom of Fig. 1) with a lysine (K265), which is on the helix VI–VII loop, through several bound water molecules and polar residues on the C-terminus extension (T 489, T490, and N491) to a histidine (H256) on helix VI. From there another water molecule may connect to a serine (S255) on helix VI which is connected through bound water to a lysine (K319) on helix VIII. Above K319 there is a gap in the hydrogen-bonded chain. The channel may resume with T316 which is connected by a bound water molecule to the hydroxyl group of the hydroxyfarnesylethyl group of heme a_3 which is hydrogen bonded to a tyrosine (Y244) on helix VI. This tyrosine is

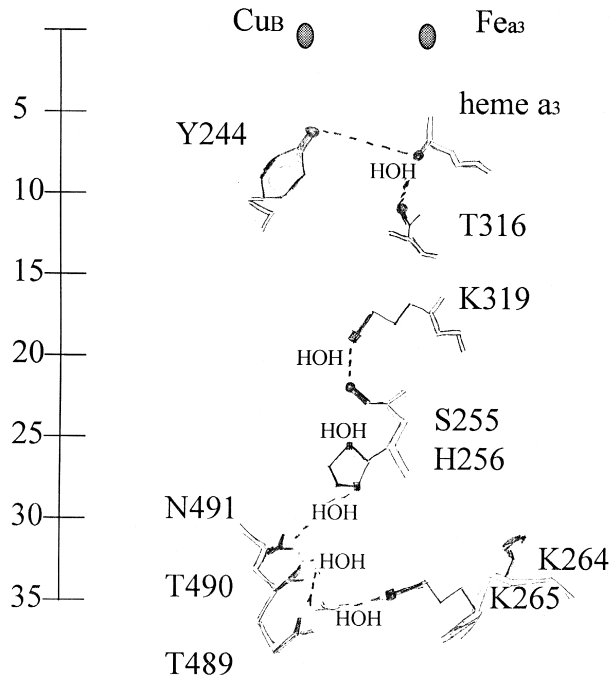

Fig. 1. The proposed consumed proton pathway in bovine COX. A schematic diagram that is based on the proposal of Tsukihara et al. [19], and drawn after the PDB structure (10 CC). The scale on the left shows the approximate distance in angstroms between different residues along the pathway.

very close (6.2 Å) to the binuclear center and may be covalently linked to H240, which is one of the Cu_B ligands [17]. Except for the polar residues of the C-terminus, all the residues on helix VI and VIII which appear to be part of this pathway in the bovine COX are conserved in most of the members of the heme-copper oxidase superfamily. This lysine containing channel is now referred to as the K-channel [26].

However, recent studies of site-directed mutants in EC UQOX and RS COX, of several K-channel residues, appear to be incompatible with the suggestion that the K-channel is the only consumed proton channel in subunit I. These results are summarized briefly below.

2.1.1. Tyrosine 244 (Y288 in RS COX and EC UQOX)

Mutations of this conserved tyrosine in helix VI, which is very close to the binuclear center, completely inactivated both COX and UQOX [24,34,35]. However, it was found that the inactive Y244 mutants did not incorporate Cu_B into the enzyme, and this defect must be the direct cause of enzyme inactivation [34,35]. Before the crystal structure was known, these results were interpreted as indicating that Y244 is a ligand of Cu_B [34]. The crystal structures show that Y244 is not a ligand of Cu_B [16–19]. Nevertheless, if the side-chain of Y244 is covalently linked to the Cu_B ligand H240, as recent crystallographic data suggest [17], than the compound residue His-Tyr can be considered a ligand of Cu_B. It is also possible that the tyrosine phenol group is ionized in COX, under most conditions, and is needed to balance the Cu_B charge (see below). We suggest that Y244 is the protonatable residue identified by Adelroth et al. as participating in balancing the charges of the binuclear center in oxidation/reduction reactions [31,32]. Its intrinsic pK ($\sim$10.0), is close to that determined experimentally (8.5 to 9.7). The tyrosine pK is expected to be raised in the low dielectric environment of the protein interior, but also lowered by the positive charges of the binuclear metals ((Fe_{a_3} is 6.1 Å away and Cu_B is 6.2 Å away). In addition to the charges on the metals, any other ionic species or dipoles in the binuclear center (e.g., OH^-, water, and the various oxygen reduction intermediates) would have great effect on the pK of Y244, and hence the charge of this residue. This suggests that Y244 has a critical role in controlling oxygen reduction steps and proton consumption in the binuclear center, and very likely is an important component of the reaction center (see below).

2.1.2. Threonine 316 (T359 in RS COX and EC UQOX)

Mutation of this conserved threonine on helix VIII to alanine, inhibited enzyme turnover moderately in both COX and UQOX, but had only minor effects on the binuclear center; mutation to serine inhibited activity only slightly [35,36]. T316 is connected by a hydrogen bond to a fixed water molecule which is connected (through the heme a_3 hydroxyl) to Y244. However, the pathway from T316 outward is not clear and it is likely that additional water molecules participate in this pathway ([17,19]; Fig. 1). Therefore, it is possible that in the T316A mutant another water molecule substitutes for the threonine hydroxyl, and hence the modest inhibition of electron transport in this mutant. The T359A mutation in UQOX had no effect on the kinetics of electron transfer and accelerated the rate of proton uptake in Flow-Flash experiments (i.e., flash induced dissociation of CO from the fully reduced enzyme followed by oxygen binding, reduction, and additional electron transfer to the oxygen) [37]. Also in RS COX this mutation had no effect on the kinetics of electron and proton uptake in Flow-Flash experiments (here associated with two electron transfer to the binuclear center) [38]. This mutation also did not affect $\Delta\Psi$ generation by a single electron transfer (from F to O) [26].

2.1.3. Lysine 319 (K362 in RS COX and EC UQOX)

Mutation of this conserved lysine on helix VIII, further away from the binuclear center (about 18 Å, Fig. 1), inhibited very strongly the turnover rate of both COX and UQOX [35,36]. The K319M mutation in COX also affected the Fe–histidine bond of heme a_3 and weakened the binding of CO [36]. In UQOX the K319M mutation affected the extent of reversal of electron transfer, after the dissociation of CO from the reduced binuclear center [37]. Since the extent of this reaction appears to depend on the extent of dissociation of a proton from a protonatable group near the binuclear center [31,32], tentatively identified here as Y244, it suggests that K319 lowers the pK of Y244. A likely explanation for the effects on

Fe_{a_3} [36], and apparently, on the pK of Y244, is that K319 is charged (see below). Its effect on Fe_{a_3} (18 Å away) could be mediated by its electrostatic effect on Y244 which is only 13 Å away. In Flow-Flash experiments with the K319M (K362M) mutants of both COX and UQOX there was no inhibition of the rates of electron transport or the rates of proton uptake [35,38]. The K319M mutation modulated slightly the kinetics of $\Delta\Psi$ generation by the fourth electron transfer (F to O) but did not inhibit it significantly [26]. The most significant effect of the K319M mutation on COX partial reactions is the inhibition of the reduction of heme a_3 [26,36,38–40].

These experiments with the T316 and K319 mutants were considered to be incompatible with the suggestion that all four of the consumed protons are taken in through the K-channel (Fig. 1). It was therefore suggested that only the first two consumed protons, which are associated with enzyme reduction (which controls the rate of enzyme turnover), are taken up by the K-channel, while the other two consumed protons, which are associated with oxygen reduction, are taken in through the vectorial proton channel (D-channel, see below) [26]. Since the T316A mutants have little effect on enzyme function anyway, this conclusion is based largely on the effect of the K319M substitution (but see also discussion of the E242 and D90 mutants below).

The validity of this conclusion depends on the assumption that the K319 side-chain is essential for proton transport through the K channel. However, the proposed pathway for consumed protons through the K-channel also raises serious questions regarding the mechanism of proton transfer along this pathway. In all the proton transport pathways that have been studied previously (e.g., bacteriorhodopsin [41], bacterial reaction center [42], ATP synthethase [43], lac permease [44], and including the pumped proton pathway in COX (see below)), acidic residues (e.g., aspartate and/or glutamate) were found to be critical for function. As discussed in more detail below, acidic residues play a dual function in proton transport: at the surface, the negative surface charge serves to concentrate protons at the pathway gate, while inside the protein, acidic residues first bind and then deliver the proton forward ('charge relay'). The intrinsic low pK of these residues insures, not only that they are able to release the protons at physiological pH, but, more importantly, that the dissociation rate is sufficiently fast [45].

In the putative consumed proton pathway (Fig. 1) there are no acidic residues, but there are two conserved lysines in this pathway: one inside the hydrophobic core of the protein (K319) and one on the surface (K265), both of which can be expected to be positively charged. The K319 which is inside the hydrophobic core of subunit I is close to (8 Å) an acidic residue on subunit II (E62) which may stabilize its charge (i.e., higher pK_a). Since lysine has a high intrinsic pK (10.5), the rate of proton dissociation from these groups should be very slow [45]; Moreover, the transient charging of the binuclear center should increase the pK of K319, further reducing the rate at which a proton can dissociate from K319. Most enzymes of the heme-copper oxidases superfamily have one or two additional positive residues next to K265 (K264 in the bovine COX, Fig. 1). Inspection of the inner surface of the bovine COX reveals that the area surrounding the gate of the putative consumed proton pathway has a strong positive potential. These observations suggest that the probability of finding a proton at the gate is very low, and that proton transport in the channel is very slow.

2.2. An alternative hypothesis: the hydrogen-bonded pathway from the inner surface to the binuclear center is a hydroxyl 'wire'

Much of the discussion concerning proton transport in membranes and proteins is focused on the movement of the positively charged proton (or hydronium). However, because of the reversible dissociation of water to protons and hydroxyl anions, it is very difficult to distinguish between proton movements and hydroxyl anion movements. In fact, all of the observations, discussed above, regarding proton binding or release, could be the result of hydroxyl release or binding, respectively.

The mechanism of proton transfer by a hydrogen-bonded chain of water molecules in ice (Grotthuss Mechanism), has its exact equivalent in OH^- transport [46]. This mechanism consists of two steps: charge transfer and bond exchange (i.e., 'hop/turn' mechanism [47–49]). In proton transport, a positive charge ('defect') moves rapidly from a H_3O^+ bound at one end of a hydrogen bonded chain of water

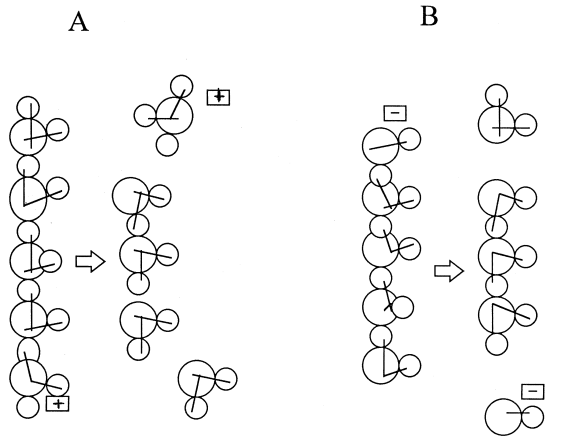

Fig. 2. A schematic diagram showing the sequence of events in proton conduction (A) and hydroxyl conduction (B) by a hydrogen bonded chain of water molecules. Large circle = oxygen atom, small circle = hydrogen atom, covalent bonds are shown as lines. Charge hoping (from bottom to top in A, and from top to bottom in B, is followed by bond turns (from bottom to top in A, and from top to bottom in B).

molecules (Fig. 2A, bottom) to the other end of the chain; the resulting hydrogen radical at the donor side *attacks* the oxygen of the next water molecule, and this process of hydrogen atom transfer ('bonding defect') continues until H_3O^+ is produced at the other end of the chain (Fig. 2A). In hydroxyl transport, the electron leaves the hydroxyl anion (Fig. 2B, top), and moves along the hydrogen-bonded chain of water molecules to the other end; the resulting hydroxyl radical on the donor side abstracts an hydrogen atom *from* the hydrogen-bonded chain to form water and the process of hydrogen abstraction is continued until a negatively charged hydroxyl anion is left at the other end of the chain (Fig. 2B) [47]. The probability of either event depends on the probability of finding a proton or hydroxyl at the right position and the electrostatic forces that act on these charges.

It has been suggested that proton transport in proton pumping proteins may depend on a similar type of mechanism in which a hydrogen-bonded chain of amino-acid residues (HBC) forms a 'proton wire' into and across the hydrophobic core of the protein [48,49]. However, little attention has been paid to the possible existence of an 'hydroxyl wire' in these proteins (but see Refs. [47,50]).

The proton concentration near the matrix surface of the inner membrane is very low (about 10^{-8} M). Moreover, under physiological conditions, because of $\Delta\mu_H$, the probability of OH^- residing in the K-channel is many orders of magnitude higher than the probability of a proton residing in the channel. Since a Fe_{a_3}-bound hydroxyl signal was detected after the four electron reduction of molecular oxygen [51], and a Cu_B-bound hydroxyl (or water) signal was detected in the oxidized enzyme [52], it is clear that at least in the oxidized enzyme, hydroxyl transfer is an event with very high probability.

The positive charges along the K-channel (Fig. 1), and the positive potential surrounding the putative gate to the channel, should also favor hydroxyl transport by the K-channel. Moreover, if the direct driving force for proton consumption is indeed the transient excess negative charge of the binuclear center [27–32], then this force would be much stronger in repelling an adjacent negative charge than in attracting a distant proton, because of the strong dependence of the potential on distance (Coulomb's law).

When an hydroxyl moves down a hydrogen-bonded wire, only the electron actually moves down the wire, while the hydrogen radical moves in the opposite direction (Fig. 2B); therefore, the donor need not be an hydroxyl anion (OH^-). Other enolates, for example tyrosinate (YO^-), could also send an electron down the wire and extract a hydrogen radical from the wire to form the neutral species (YOH). The rate of this reaction should depend on the pK of the phenol moiety.

The proposed hydroxyl wire, which delivers electrons from the binuclear center to the inner surface, thus extends electron transport all the way from the outer surface to the inner surface, as originally envisioned by Mitchell [8], except, that the water is formed from oxygen at the core of the enzyme and not at the surface, and hydroxyl is ejected to the matrix instead of proton consumption.

According to this model, when membrane-embedded COX reduces O_2, the substrates of the reactions are four electrons from the outer surface, molecular oxygen, and four water molecules from the inner surface, and the products are four hydroxyl on the inner surface, and two molecules of water (Eq. (2)):

$$4e^-(\text{out}) + O_2 + 4H_2O(\text{in}) \rightarrow 4OH^-(\text{in}) + 2H_2O \quad (2)$$

and the net reaction is:

$$4e^- + O_2 + 2H_2O \rightarrow 4OH^- \quad (3)$$

Eq. (3) simply depicts the reduction of oxygen at high pH, in contrast to the conventional description, Eq. (1), which depicts the reduction of oxygen at low pH.

An interesting observation that may be related to this mechanism is provided by the studies of the involvement of water in oxygen reduction. According to Eq. (1), oxygen reduction by COX produces water, while according to Eq. (2) water molecules are first consumed and then produced along the reaction pathway. Studies of the involvement of water in COX-catalyzed reduction of oxygen have led to the conclusion that water molecules are taken up by the enzyme in an early step of enzyme reduction and are released in a later step [53], which is compatible with Eq. (2).

The hypothesis that the process of proton consumption involves hydroxyl ejection from the binuclear center and not proton uptake into the center does not require any major modification of the conventional description of the mechanism of oxygen reduction [1–4]. Nevertheless, a detailed molecular modeling, with special emphasis on the electrostatics of the process is instructive and may provide new insights into the mechanism of both oxygen reduction and proton pumping.

We consider, qualitatively, the electrostatics of oxygen reduction, in a model of a 'reaction center' that include the binuclear center, a tyrosinate and the oxygen intermediates. The reaction center is assumed to be electroneutral in all stable states. When the reaction center becomes transiently charged by injection of an electron, the electrostatic potential could drive electron ejection from the tyrosinate anion (Y244) down the hydroxyl wire. This would result in OH^- formation (from water) on the inner surface of COX and in protonation of the tyrosinate. Before the next electron (i.e., OH^-) can be ejected, the tyrosine must dissociate and deliver its proton to the anionic (oxygenous) species at the binuclear center. The rate of hydroxyl ejection will depend strongly on the pK of the tyrosine phenol moiety. If the pK is too high (relative to the pK of the anionic species), the tyrosine would not dissociate and hydroxyl ejection would be blocked, if the pK is too low, the electron would be held too strongly by the tyrosinate and hydroxyl ejection would be slow.

In the fully oxidized, 'pulsed', state (state I (OX), in Fig. 3), there is one positive charge on heme a_3, and two positive charges on Cu_B. We propose that these charges are neutralized by two hydroxyls (the products of the oxygen reduction) and the tyrosinate anion YO^- (Y244 on helix VI). The two hydroxyls could be hydrogen-bonded so that the binuclear center is magnetically coupled.

The transfer of the first electron to Cu_B is coupled to the ejection of the tyrosinate electron through the hydroxyl wire and results in its protonation to YOH. If the actual pK of the OH^- (intrinsic pK 14) is higher than that of the tyrosine (intrinsic pK 10), the proton will be transferred to an hydroxyl to form water (state II). Because the standard redox potential of the reduction step is low (0.35 V [1]), and the free energy of transferring negative charge into the binuclear center is probably much higher [28], it is not possible to populate a state having a negatively charged binuclear center. Therefore, the rate of the first electron reduction, should depend on the rate of hydroxyl ejection.

In state II each metal carries one positive charge which is balanced by one hydroxyl and the tyrosinate.

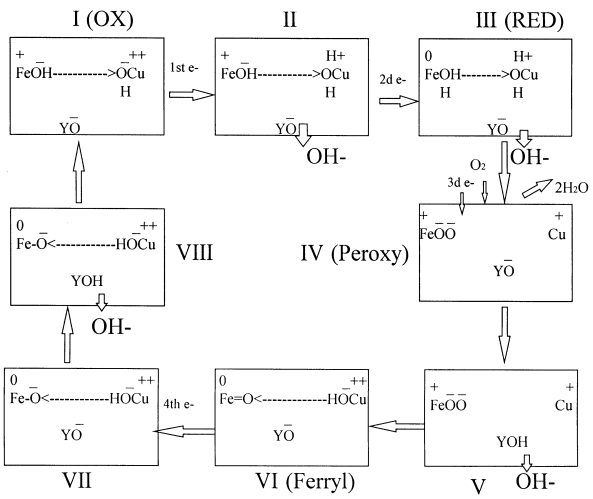

Fig. 3. A model of the catalytic cycle of oxygen reduction in heme-copper oxidases. The diagram shows the electronic events in the binuclear center which couple hydroxyl ejection to oxygen reduction. See text for further details.

The transfer of the second electron to the reaction center can proceed only if the tyrosinate has already formed. After hydroxyl ejection, the tyrosine would deliver the second consumed proton to the second hydroxyl to form the partially reduced enzyme (state III (RED)). The rate of the reduction of the binuclear center by the second electron should also depend on the rate of hydroxyl ejection because the low redox potential of this step (0.35 V [1]) is also not sufficient to populate a 'charged' state.

The oxygen reduction starts upon the binding of oxygen at the reduced binuclear center, which pushes the water out of the binding site. The binding of oxygen is followed by the redistribution of the charges and the formation of the peroxy anion, which is followed by the delivery of the third electron (State IV, PEROXY).

In this state there is an excess negative charge at the binuclear center. This state could be populated because the standard redox potential of this state is high [1]. Most likely, the charging of the reaction center would trigger a conformational transition that would further stabilize this state. This state could play an important role in proton pumping (see below). The conformational transition could affect the structure of the hydroxyl wire and/or the pK of the tyrosinate in a way that slows hydroxyl ejection and further stabilizes a 'charged state'. Nevertheless, eventually, the electron (i.e., hydroxyl) will be ejected, because of the excess negative charge, thus forming state V, in which YOH is reformed and the conformation has returned to the ground state. Next, YOH will donate its proton to the peroxy anion, which will split forming hydroxyl and the ferryl oxygen intermediate, which are hydrogen bonded (state VI (Ferryl)). Next the fourth electron reaches the binuclear center and reduces the Ferryl species to form the $Fe–O^-$ species (state VII). Because the redox potential of the fourth electron reduction is also high [1], this state can be populated. This charged state can also be stabilized by a conformational transition (see below). Eventually, the excess electron would leave, again forming the protonated YOH species (state VIII). Now YOH will transfer its proton to the $Fe–O^-$ species to form the second hydroxyl and the ferric species and the binuclear center has returned to its fully oxidized state (state I (OX)).

This model for the generation of $\Delta\mu_H$ by hydroxyl ejection is compatible with all the experimental results that are normally interpreted as proton consumption, as discussed above. Although the details of the oxygen intermediate hydrogenation steps (Fig. 3) have been modified slightly from the conventional description (cf. Refs. [1,4]), to better fit this model, these modifications are not an essential element of the model.

The model explains well the properties of the oxidized 'Pulsed' (Fast) enzyme [3,54]: one needs hydroxyl anions at the binuclear center for fast reduction of the enzyme, while at same time, high external pH inhibits the reduction [30]. Any treatment of the enzyme that allows the hydroxyls to exchange with other anions would result in a 'slow' enzyme.

The model also explains well the properties of the K319 mutants. Assuming that K319 is positively charged (see above), it would lower the pK of Y244, which may determine the rate of dissociation of YOH. This explains why in the K362M mutants the second electron transfer rate (i.e., reduction of Fe_{a_3}) is strongly inhibited. In contrast, the oxidative electron transport steps are not inhibited because the formation of the charged state is not dependent on the rate of hydroxyl ejection. Also, since the later steps occur in the 'charged' enzyme, it is possible that the structure of the hydroxyl pathway (Fig. 1) is different in this state. In fact, since it appears that there are many unresolved water molecules in this pore [19], it is not entirely unlikely that the hydroxyl wire is composed of a chain of water molecules, and is not identical with the proposed chain of alternating water and polar residues (Fig. 1).

3. Proton pumping in cytochrome c oxidase

It is well-established now that in addition to the four 'consumed' ('substrate') protons ($1H^+/e^-$), up to four additional protons ($1H^+/e^-$) are pumped across the membrane ('vectorial' protons) during the reduction of molecular oxygen [1–4,7,8]. There is strong evidence that proton pumping is only associated with the transfer of the third and fourth electrons to the binuclear center, i.e., with the transitions P → F and F → O, which suggests that the stoichiometry of each of these two steps is $2H^+/e^-$ [1,4,7,55–57].

Several models have been proposed in the past to account for the 'vectorial' proton pumping, but until recently there was little supporting evidence for any of these models (reviewed in Ref. [58]). Most current models are based on the idea that one of the ligated metal redox centers can drive vectorial proton transfer by redox-coupled ligand exchange accompanied by changes in the orientation and protonation states of the ligand [59]. The implicit assumption in these models is that the redox free energy is converted directly by the redox metal ligand to proton binding, translocation, and dissociation. Although it is clear that electrostatic energy must be considered in any model that couples electron and proton transfer steps, these forces are considered only in a very short range, in the immediate vicinity of the metals, the coupling ligand, and the oxygen intermediates. Earlier versions of this model postulated a direct role for a particular histidine ligand of heme a_3 [60,61], and more recently, Cu_B ([62,16], reviewed in Ref. [4]), in the generation of $\Delta\mu_H$. Other models, which suggest less direct interaction between the redox reactions and proton pumping, including electrostatic and conformational models, have been discussed extensively [29,58,63–66].

3.1. The identification of the pumped proton pathways

Studies with site-directed mutants of bacterial UQOX (EC) and COX (RS) have led to the identification of several subunit I residues that are important for proton pumping. The first acidic residue which was found to be essential for proton pumping in both enzymes is D91. D91 is located on loop II–III outside the membrane, on the inner (matrix) surface, at the entrance of what appears to be a pore [16,19]. Substitution of this residue (D135 in EC UQOX, and D132 in RS COX) with asparagine, lysine or alanine abolished proton pumping in both enzymes [67–69], and inhibited activity strongly ($\sim$95%) in COX [69]. However, the inhibition of activity in the UQOX mutants was very mild: $\sim$55% [67,68]. Glutamate could substitute for aspartate without loss of proton pumping [26,67,68,70]. In addition, in UQOX, two asparagines, N80 (N113) and N98 (N131), which are at the two ends of loop II–III, at the entrance of the pore, were also found to be important for proton pumping [68], suggesting that this site is the entrance ('funnel') to the vectorial proton pathway (D-channel). The essential asparagine residues may form a hydronium binding site [71]. Proton pumping could be restored to the UQOX D135N mutant by introducing negatively charged residues on loop II–III at two alternative positions on the loop [68]. In the COX D91A and D91N mutants, the loss of proton pumping is associated with a loss of respiratory control [69]. The addition of free fatty acids to the D91 mutant in the bacterial COX enhanced electron transport and restored respiratory control [72]. These findings suggest that D91 does not participate directly in the pumping process, but facilitates the transfer and binding of hydronium ions in the funnel site. The subunit I inner surface in the vicinity of the hydronium funnel in COX has a negative potential, contributed by acidic residues of the carboxyl terminus (E506 and E507), in addition to D91. This negatively charged surface would concentrate protons near the funnel.

Above the hydronium funnel, at the hydrophobic core of subunit I, on helix III and helix IV, there are several partially conserved asparagines, serines and threonines, some of which may form a hydrogen bonded proton wire, together with water molecules (D-channel) [4,16,18,26,73]. The pumped proton pathway from the inner surface into the membrane core in the bovine COX was suggested to include N98, S101, S108, and S115 on helix III and S157, S156, S149, T146 and S142 on helix IV [18]. In the PD COX the homologous residues from the inner side of helix III (Q131, S134) were also suggested to be part of the proton pathway. On helix IV residues S192, S193 (homologous to S156 and S157), N199, and T203 were suggested to participate in the pumped proton channel of the PD COX. However, recent studies with RS COX, in which the three helix IV residues S201, Q207 and T211 (corresponding to S193, N199 and T203 in PD COX) were mutated, did not provide support for a critical role of these residues in proton pumping [74]. A pathway for exit of pumped protons was also suggested to exist between helix XI and XII [18]. However, several substitutions of residue D407, which was suggested to be part of this pathway [18], had no effect on proton pumping either in RS COX or on EC UQOX [70,75].

3.2. The identification of E242 as the 'pumped protons' acceptor

Most models of proton pumping assume that there is a protonatable residue, an acceptor, in the hydrophobic core of the pump, which first binds the pumped protons. The search for the pumped protons acceptor(s) in COX and UQOX by site-directed mutagenesis has resulted in the recent identification of helix VI glutamate (E242), which is the only conserved acidic residue in the core of subunit I, as the most probable acceptor for pumped protons. In EC UQOX, a mutant homologous to E242A (E286A) was inactive [34,67]. The mutant homologous to E242Q was also shown recently to be inactive [70]; Similarly, in RS COX both mutants were inactive [24,76]. In EC COX, the E286D was active and translocated protons readily, but the E286C mutant, which was partially active, did not pump protons [70]. Studies with the inactive UQOX mutant homologous to E242A (E286A) show that the transfer of the third electron to the binuclear center in Flow-Flash experiments is accelerated, while proton uptake was impaired [77]. More recent studies with the same mutant reveal that oxygen binding and the rate of heme–heme electron transfer are unaffected, but the product of the reaction is the P (PEROXY state), and not the F (ferryl) state that is observed in the wild type [78]. This finding suggested that the step that is inhibited in the mutant is the delivery of (consumed) protons to the active site. The RS COX E286Q mutant did not generate the slow (proton associated) phase of $\Delta\Psi$ generation on the transition from the ferryl to the oxidized state [26]. Flow-Flash experiments with this mutant also show inhibition of proton uptake, impaired formation of the F (Ferryl) state and inhibition of electron transfer from Cu_A [79].

Recent modeling studies of water molecules in COX suggest that there are two water channels, one connecting the input proton channel (D-channel, discussed above) with E242, and another connecting E242 with the binuclear center [73]. A movement of E242 between the two channels may be necessary for proton transfer, since positively charged M91 mutants, which are suggested to fix E242 in one position, inhibited proton pumping and partially inhibited activity [73]. An FTIR study of the E242 (E286) mutants of UQOX, identified the carbonyl stretching frequency of this residue which appeared to be protonated [80]. The binding of CO to Cu_B resulted in a frequency shift which was interpreted as evidence for the existence of water array between E242 and a histidine ligand of Cu_B.

The findings described above were interpreted to suggest that the D-channel and E242 provide a pathway not only for pumped protons but also for the consumed protons during the oxidative half of the catalytic cycle [4,26,79]. However, it should be pointed out that the inhibition of activity, and specifically the transition from P to F, in E286 mutants is not necessarily the result of the inhibition of proton consumption. The steady-state absorption spectra of one of these mutants suggests that Fe_{a_3} is ligated in a low-spin complex [26], and this or other effects on the reaction center structure (cf. Ref. [81]) may be the direct cause of inactivation. In this respect it is interesting that the E286C mutant, which is capable of forming an anion, retains significant activity [70]. It should also be pointed out that the D91 (D135) mutants of UQOX retain substantial activity but not proton pumping [67,68]. Moreover, The D135N and D135E had the same activity (45% of wt), but the former did not pump protons, while the latter did, which suggest no relationship between activity and proton pumping in these mutants. These findings are not entirely compatible with the suggestion that the D-channel is a pathway for consumed protons.

3.3. A mechanism for proton pumping which is based on the electrostatics of the proposed hydroxyl ejection mechanism

The primary proton pumping residue is assumed to be E242. The central role of E242 (which is supported by the data discussed above) is explained as follows: E242 is the proton acceptor from the input channel (D-channel). In the charged states (IV and VII in Fig. 3), the conformation of the protein allows E242 to assume two positions, in one orientation (input) it can accept a proton from the D-channel, while in the second orientation (output), it can deliver the proton to an internal pathway that leads to several secondary acceptors (A^-). The movement of E244 between these two positions is electrostatically driven and determined by the charge of the two redox centers (i.e., heme a and the binuclear center) and the

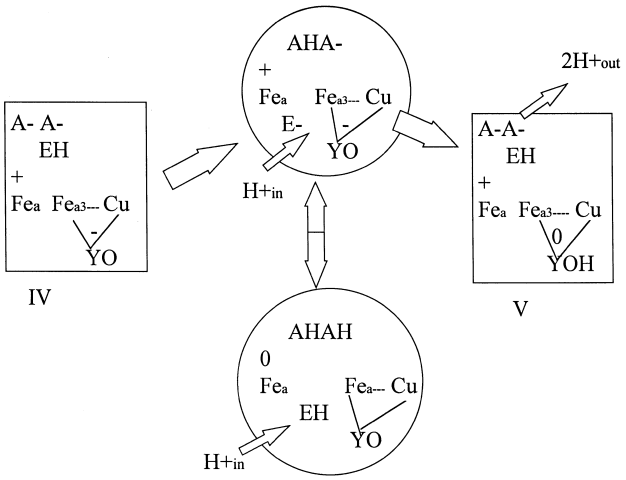

Fig. 4. A model of electrostatic coupling between the redox reactions and proton pumping during the transition from the peroxy state to the ferryl state. See text for further detail.

protonation state of E242 itself. Fig. 4 shows a scheme of this electrostatic model of proton pumping in COX. Pumping starts by the charging of the redox centers by the third or fourth electron (state IV or VII in Fig. 3). Before the charging, subunit I is in an electroneutral, ground state; E242 is in its output position and can exchange its proton with the secondary acceptors (A^-), which can release the protons to the outer surface. When the redox centers are charged (by the third or fourth electron) the pK_a of the secondary acceptors increase more than the pK of E242, which therefore delivers its proton to the secondary acceptors. The charging of the binuclear center induces a conformational transition to the 'charged' state (here depicted as a circle). In the charged state, there is no access from the secondary acceptors (A^-) to the outer surface, and E242 can assume two positions: input (connecting to the D-channel) and output (connecting to (A^-). E242 prefers the output position when it is protonated and the input position when it is charged. In the input position, its pK is higher (due to the negative charge of the binuclear center). In the output position, E242 is shielded from the negative charge of the binuclear center and the pK is lower. The swinging of E242 between these two positions is the only pathway for protons between the input and output channels. This design would result in shuttling protons, only in the charged states, from the input D-channel to the secondary acceptors. The stoichiometry would depend on the number of protons that can be bound by A^- (discussed below). We also suggest that the oxidation state of heme a, in addition to the charge of the binuclear center, modulates the pK_a of E242 and the secondary acceptors (A^-). Therefore, when heme a is reduced, the pK_a of A^- increase, which increases their ability to bind protons. Once the charged state has decayed (by the ejection of hydroxyl), the conformation has returned to the more stable, electroneutral conformation. Now that the binuclear center is neutral and heme a is positively charged, the pK_a of the secondary acceptors (A^-) are reduced, and they release their protons to the outer surface.

To account for a stoichiometry, $2H^+/e^-$, in each of the two transitions, we only need to assume that there are at least two secondary acceptors that participate in proton pumping, in addition to E242. It is possible that there are several secondary acceptors that can be protonated and deprotonated during the reaction cycle, thus participating, to various degrees in proton pumping.

To explain the strong coupling between proton pumping and electron transport, we suggest that the pK of Y244 is also modulated by E242 and vice versa. Thus, binding of the pumped proton to E242 would lower the pK of Y244 and enhance the rate of proton transfer from Y244 to the oxygen intermediate and hence the rate of the catalytic reactions in the binuclear center. In addition, the mechanical link between these two residues could link movement of one residue to that of the other.

The combined model of proton pumping and hydroxyl ejection (i.e., 'Proton consumption'), as described here, is centered on two key residues: E242 which pumps the protons, and Y244 which ejects the hydroxyls. The position of these key residues on opposite faces of helix VI, in the middle of the most conserved segment of subunit I, suggests that their interactions, electrical, quantum-mechanical, as well as mechanical, play a crucial role in the coupling of electron transport to proton transport.

There are several recent findings that are in agreement with this model. (i) The observation that the rate of proton binding after partial oxygen reduction (presumably ending with the ferryl intermediate) in the K-channel mutants of UQOX was 2–3 times faster than wt [37]. (ii) The findings that there are

several residues in COX in which the pK is modulated by the redox state of the redox centers (cf. Ref. [82]), and that one of these residues, which is modulated by the redox state of cytochrome *a*, appears to participate in proton pumping, with a pK shift from 6 to 9 [83]. (iii) The observation that substitution of M71 with either lysine or arginine in UQOX (M116) inhibited proton pumping, which was attributed to tethering of E242 (E286) in its position, preventing its movement during proton translocation [73]. This effect could also result from a lowering of the pK of E242 by the adjacent positive charge.

The mechanism of proton pumping described above is similar, in principle, to that proposed for the bacterial rhodopsins, in which a light-induced polarization (and isomerization) of the chromophore induces, independently: (a) a conformational transition between output and input conformation (Switch) and (b) pK changes that induce proton binding and release (Transport) [84].

Much remains to be learned about the exact mechanism of oxygen reduction by COX and UQOX and the coupled charge movements (protons and hydroxyls ions) into and out of the protein. Some of the predictions of the model suggested in this review could be subjected to experimental tests that may enhance our understanding of the mechanism of this complex process.

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