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Review

A cooperative model for proton pumping in cytochrome *c* oxidase☆

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Abstract

In this paper, the mechanism of proton pumping in cytochrome *c* oxidase is examined. Data on cooperative linkage of vectorial proton translocation to oxido-reduction of Cu_A and heme *a* in the CO-inhibited, liposome-reconstituted bovine cytochrome *c* oxidase are reviewed. Results on proton translocation associated to single-turnover oxido-reduction of the four metal centers in the unliganded, membrane-reconstituted oxidase are also presented. On the basis of these results, X-ray crystallographic structures and spectrometric data for a proton pumping model in cytochrome *c* oxidase is proposed.

This model, which is specifically derived from data available for the bovine cytochrome *c* oxidase, is intended to illustrate the essential features of cooperative coupling of proton translocation at the low potential redox site. Variants will have to be introduced for those members of the heme copper oxidase family which differ in the redox components of the low potential site and in the amino acid network connected to this site.

The model we present describes in detail steps of cooperative coupling of proton pumping at the low potential Cu_A-heme *a* site in the bovine enzyme. It is then outlined how this cooperative proton transfer can be thermodynamically and kinetically coupled to the chemistry of oxygen reduction to water at the high potential Cu_B-heme a_3 center, so as to result in proton pumping, in the turning-over enzyme, against a transmembrane electrochemical proton gradient of some 250 mV.

Keywords: Cytochrome *c* oxidase; Proton pumping; Cooperativity; Mitochondria

1. Introduction

Cytochrome *c* oxidase of mitochondria and bacteria, like NADH-ubiquinone oxidoreductase and ubiquinol-cytocrome *c* oxidoreductase (bc_1 complex), is capable, in the native membrane or when inserted in the purified state, in a phospholipid membrane, to convert the free energy made available by downhill electron flow into uphill transmembrane electrochemical proton gradient ($\Delta\mu H^+$ or protonmotive force, PMF) [1].

In the 1960s, when Mitchell put forward the chemiosmotic hypothesis, he proposed that hydrogen conduction in one direction from the inner (negative, N) to the outer (positive, P) space and electron transfer in the opposite direction across the membrane by the prosthetic groups of the redox systems (protonmotive redox loops), resulted directly in transmembrane uphill proton translocation from the N to the P aqueous bulk phase [2]. Mitchell's [3] hypothesis did not attribute much of a role to the apoproteins of the redox complexes, except a passive involvement as scaffolds holding the redox prosthetic groups in the proper orientation in the membrane and possibly providing passive proton-conduction pathways (proton wells) connecting them with the aqueous phases. Detailed mechanisms, involving directly protonmotive primary catalysis at the redox centers, are today generally used to explain proton pumping in the bc_1 complex (ubiquinone cycle) and cytochrome *c* oxidase (see related papers in this volume). So far, less attention has been paid to the mechanism of proton pumping in NADH-ubiquinone oxidoreductase. On the other hand, the X-ray crystallographic structures, now available for both cytochrome *c* oxidase [4–8] and bc_1

☆ This paper is dedicated to the memory of Gerald Babcock, who definitively contributed to the present knowledge of membrane-bound redox enzymes.

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complex III [9–11], show proton transfer pathways in the apoprotein subunits of these complexes, calling for more than a simple passive role of the protein in vectorial proton translocation.

There is also functional, mechanistic evidence showing cooperative interactions [12] in redox enzymes [13,14]. Various cytochromes exhibit pH dependence of the midpoint redox potential [15,16]. It was proposed [17,18] that this proton-electron linkage in cytochromes can be considered as analogous to the Bohr effect in haemoglobin, where oxygen binding at the heme is linked to binding of protons to different sites in the protein [19]. The pH dependence of the redox potential of *b* and *a* cytochromes of respiratory chains, in particular, reveals a linkage between oxidoreduction of the hemes and proton binding/dissociation in these membrane-associated hemoproteins [16–18]. Based on the principles of cooperative linkage of solute binding at separate sites in allosteric proteins [12,19,20], Papa et al. [17,18] proposed, in the 1970s, a model based on cooperative H^+/e^- linkage in cytochromes (redox Bohr effect) for proton pumping in respiratory chains (vectorial Bohr mechanism).

The H^+/e^- linkage in cytochromes can arise from electrostatic effects and/or modification of the coordination bonds of metal centers caused by the change in their valence state. The linkage might involve p*K* shifts and exchange of axial ligands [21], porphyrin substituents and connected residues [22] and conformational propagation of primary effects over long distances in the protein, as in the oxygen Bohr effect of haemoglobin [19,20,23], thus promoting proton uptake from the inner (N) aqueous phase and release to the outer (P) aqueous phase [18].

2. Proton pumping cytochrome *c* oxidase

Cytochrome *c* oxidase belongs to the superfamily of the terminal heme-copper oxidases of prokaryotic and eukaryotic respiratory chains [24,25]. These enzymes conserve the free energy, made available in the reduction of O_2 to H_2O by ferrocytochrome *c* (or a quinol), as a PMF. The generation of PMF results, in all the members of the family, first from consumption of protons from the N aqueous phase in the process of reduction of O_2 to H_2O by electrons delivered by cytochrome *c* (or quinol) located at the P side of the membrane [3,18]. In addition to this protonmotive activity, cytochrome *c* oxidase and some other heme copper oxidases exhibit net proton pumping from the N to the P aqueous space, coupled to electron flow from the reductant to O_2 [26]. The efficiency of the proton pump appears, however, to be variable, being practically zero under certain conditions [27,28].

Cytochrome *c* oxidase has four redox centers: a binuclear Cu_A center, titrating as a one-electron redox entity, which is bound to subunit II, a low and high potential heme *a* and a_3, respectively, and a Cu_B, these being all bound to subunit I [24]. Cu_A is the entry port of the electrons delivered by cytochrome *c*, heme a_3 and Cu_B constitute the binuclear center where dioxygen is reduced to H_2O [24]. Functional and crystallographic data provide evidence showing a pathway for electron transfer from Cu_A to heme *a* and from this to the a_3-Cu_B binuclear center [4–8]. There is structural [4,6] and functional [28] evidence indicating that under certain conditions, electrons can also flow directly from Cu_A to the binuclear center, with practical suppression of proton pumping [27,28].

Studies of the mechanism of proton pumping in heme-copper oxidases have resulted, from time to time, in proposals that this process should be coupled to oxido-reduction of Cu_A [29], heme *a* [30–33] and/or the binuclear center [5,21,34]. In the last case, proton pumping from the N to the P aqueous space is envisaged to be directly coupled to specific steps in the oxygen reduction chemistry at the heme a_3-Cu_B binuclear center, where protons are also consumed in the protonation of intermediates of the oxygen reduction to H_2O (see related papers in this volume).

Proton transfer initiated by redox events at the catalytic centers, which are buried in the protein at discrete distances from the surfaces exposed to the water bulk phases, has to extend to the N and P phase through proton input and proton output pathways. Directional intraprotein proton pathways in heme-copper oxidases, as well as in other enzymes in which catalysis involves protons, have in fact been identified by X-ray crystallographic analysis of these proteins [4–8,35].

The crystal structures of bovine and prokaryotic cytochrome *c* oxidases reveal in subunit I possible proton-conducting pathways which start at the N side of the membrane [4–8]. Two of these, denominated D and K pathways, respectively, can apparently conduct H^+ from the N aqueous space to the binuclear heme a_3-Cu_B center, which is located in the protein 30 Å away from the N surface. A third one, denominated H pathway, initially identified in the bovine enzyme [4], can conduct H^+ from the N space to heme *a* also located 30 Å away from the N surface. Extensive amino acid sequence comparison and structural alignment of a large number of heme-copper oxidases, as well as site-directed mutagenesis studies show, however, that some of the protonable residues, thought to be critical for H^+ conduction in the D, K, and H pathways, are not conserved in some heme-copper oxidases which are fully functional [35,36]. On the other hand, cavities are seen in these proton pathways which can be occupied by water molecules [4–8]. This water, bound to hydrophilic residues or peptide backbone amide/carboxyl groups, can contribute efficient H^+ transfer [37]. Proton-conduction pathways might, in fact, require a less stringent amino acid specificity than electron transfer pathways and a search for critical protonable residues by sequence comparison and/or site-directed mutagenesis could sometimes turn out to be useless if not misleading.

3. Coupling at heme *a* and Cu_A

Protonmotive coupling at heme *a* and/or Cu_A, which are distant from the a_3-Cu_B binuclear center and not involved in oxygen binding and reduction, requires indirect, cooperative linkage between oxido-reduction of these centers and proton transfer by acid–base groups in the enzyme. Measurement of proton release and uptake, associated with the oxidation and reduction, respectively of heme *a* and Cu_A, in the soluble CO-inhibited purified bovine cytochrome *c* oxidase (COX) (a_3 and Cu_B are clamped in the reduced state) has shown that oxido-reduction of these centers is coupled to net H^+ transfer, with an H^+/COX ratio varying between 0.7 and 0.9 in the pH range 6.0–8.5 [38] (Fig. 1). This redox-coupled H^+ transfer was initially attributed to heme *a*, as the available data indicated the E'm of Cu_A to be pH-independent [39]. Electron/proton coupling at heme *a* only, with an H^+/COX coupling ratio of 0.7–0.9, was however in apparent conflict with previous measurements of the pH dependence of the Em of heme *a*, which in the CO-inhibited COX was reported to amount to not more than −20 mV per pH unit increase in the same pH range [39]. A solution to this apparent conflict was, however, provided by the finding that Cu_A exhibits a pH dependence which is completely superimposed on that of heme *a*, the E'm of both centers decreasing by 16 mV/pH unit increase [38]. This shows that oxido-reduction of both heme *a* and Cu_A is linked to p*K* shifts of two or more common acid–base groups, whose overall balance in the soluble enzyme results in the observed net H^+ release upon oxidation of the two centers and proton uptake upon their reduction.

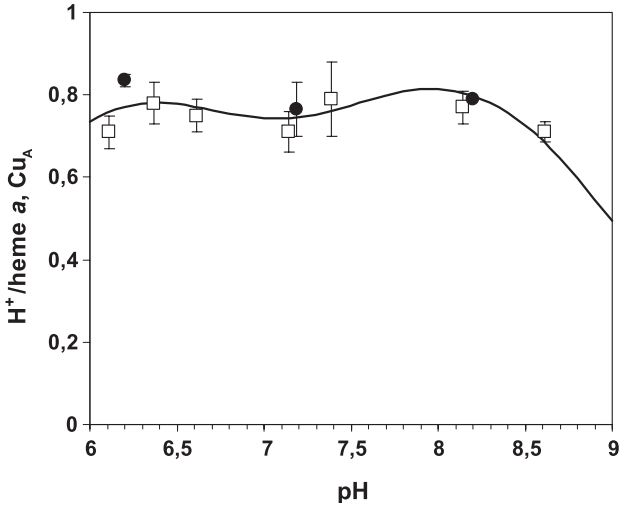

Fig. 1. Redox Bohr effect associated with heme *a* and Cu_A in CO-inhibited, soluble cytochrome *c* oxidase: pH dependence of the H^+ release associated with oxidation by ferricyanide of heme a^{2+} and Cu_A^+ reduced by traces of succinate-cytochrome *c* oxidoreductase (white squares) or hexaammineruthenium(II) (black circles). The curve represents the best fit obtained using the equation: H^+/heme *a*, $Cu_A = \Sigma_i|(1/(1+10^{pH-pKi,\ ox})) - (1/(1+10^{pH-pKi,\ red}))|$, with two acid–base groups (p*K*ox–p*K*red of 5.4–6.9 and 7.3–9.0). Reproduced with permission from Fig. 4 of Ref. [38].

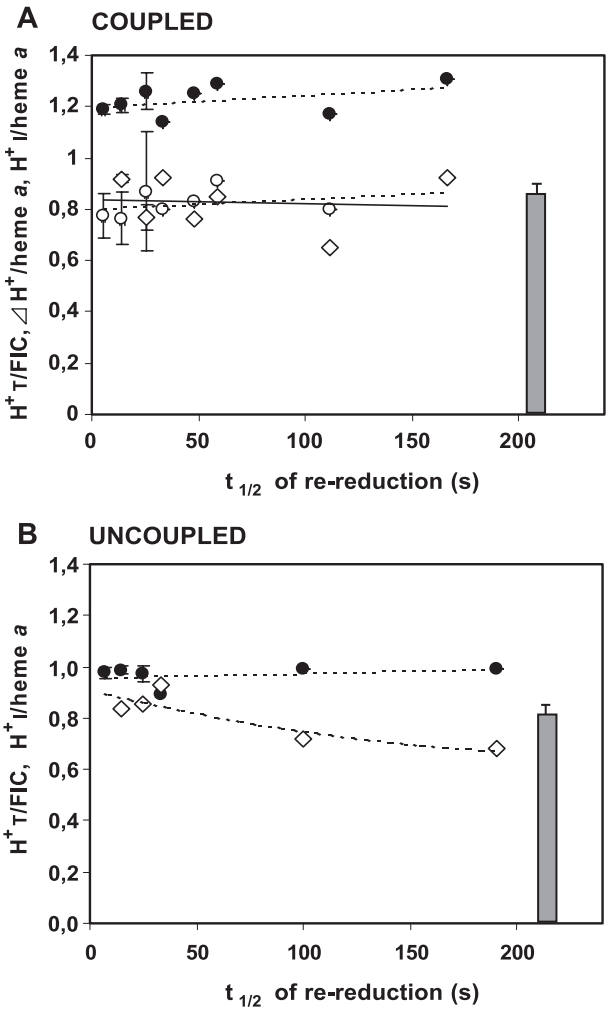

Fig. 2. Analysis of vectorial H^+ transfer linked to redox transition of heme *a* and Cu_A in CO-liganded COV. The metal centers of COX were reduced by traces of succinate-cytochrome *c* oxidoreductase in anaerobic COV suspension saturated with CO. The reduction rate was varied by changing the concentration of added malonate, oxidation of heme *a* and Cu_A was effected by the addition of ferricyanide. The experiments were performed in the absence (A) or in the presence (B) of 3 μM CCCP. Black circles, H_T^+/FIC, i.e., the ratio between the total amount of H^+ release following the oxidation/reduction cycle of heme *a* and Cu_A and the amount of oxidizing equivalents added as ferricyanide; white diamonds, H_I^+/heme *a*, i.e., the ratio between the initial proton release associated with the rapid oxidation of heme *a* and Cu_A elicited by the addition of ferricyanide and the amount of oxidized heme *a* (an equal amount of Cu_A^+ was oxidized). White circles, ΔH^+/heme *a*, i.e., the ratio between the extra H^+ release with respect to the amount of ferricyanide added and the amount of heme *a* undergoing oxido-reduction. The columns indicate the H^+/heme *a* ratio obtained in the presence of antimycin A *plus* myxothiazol and refer to the overall H^+ release associated with the permanent oxidation of heme *a*. Reproduced with permission from Ref. [40], where the experimental details are described.

The vectorial nature of H^+/e^- coupling at heme *a* and Cu_A was analysed in our laboratory by following pH changes in the external bulk phase associated with oxido-reduction of heme *a* and Cu_A in the CO-liganded COX reconstituted in liposomes [40]. In this experiment, the oxidase was brought into the reduced CO-inhibited state

by succinate oxidation, mediated by a trace of broken mitochondria and cytochrome *c*. The oxidation of Cu_A and heme *a*, induced by the addition of a stoichiometric amount of ferricyanide, was accompanied by initial, fast H^+ release, which continued, as expected, during the re-reduction of the metal centers by succinate oxidation, accordingly to the following reactions:

[1] $(Fe_a^{2+}Cu_A^+)nH^+ + 2[Fe(CN)_6]^{3-}$

$\rightarrow (Fe_a^{3+}Cu_A^{2+}) + 2[Fe(CN)_6]^{4-} + nH^+_{out}$

(fast oxidative phase)

[2] succinate $+ (Fe_a^{3+}Cu_A^{2+}) + nH^+_{in}$

$\rightarrow$ fumarate $+ (Fe_a^{2+}Cu_A^+)nH^+ + 2H^+_{out}$

(slow re-reduction phase)

[1] + [2] succinate $+ 2[Fe(CN)_6]^{3-} + nH^+_{in}$

$\rightarrow$ fumarate $+ 2H^+_{out} + nH^+_{out}$

Practically the same initial acidification, but suppression of the second slow acidification phase, was observed when oxidation by ferricyanide of reduced COX centers was effected in the presence of myxothiazol and antimycin A, which blocked further succinate oxidation [40]. In Fig. 2, the H^+ release/ferricyanide ratios, measured in this series of experiments, in which the rate of succinate oxidation was progressively inhibited by increasing amounts of malonate, are presented [40]. The final reduction of added ferricyanide by succinate should have resulted in the 1:1 stoichiometric H^+ release in the external medium. An extra acidification was, however, observed at all the malonate concentrations, which exceeded the amount of ferricyanide added, the ratio H^+ release/ferricyanide added amounting to 1.20–1.25. This extra acidification, referred to the extent of heme *a* (and Cu_A) oxidized, gave an H^+/COX release ratio of 0.75–0.85. The same H^+/COX release ratio of around 0.80 could be calculated from the initial rapid acidification associated with the oxidation of heme *a* and Cu_A. These results thus show that the transient oxidation of heme *a* and Cu_A results in rapid H^+ release; i.e., the Bohr protons associated with the oxidation of heme *a* and Cu_A are released from the oxidase in the external (P) space. If the redox Bohr protons released in the external phase were then taken up by the oxidase from the same space upon re-reduction of heme *a* and Cu_A by succinate, no net excess of H^+ release, with respect to the ferricyanide added,

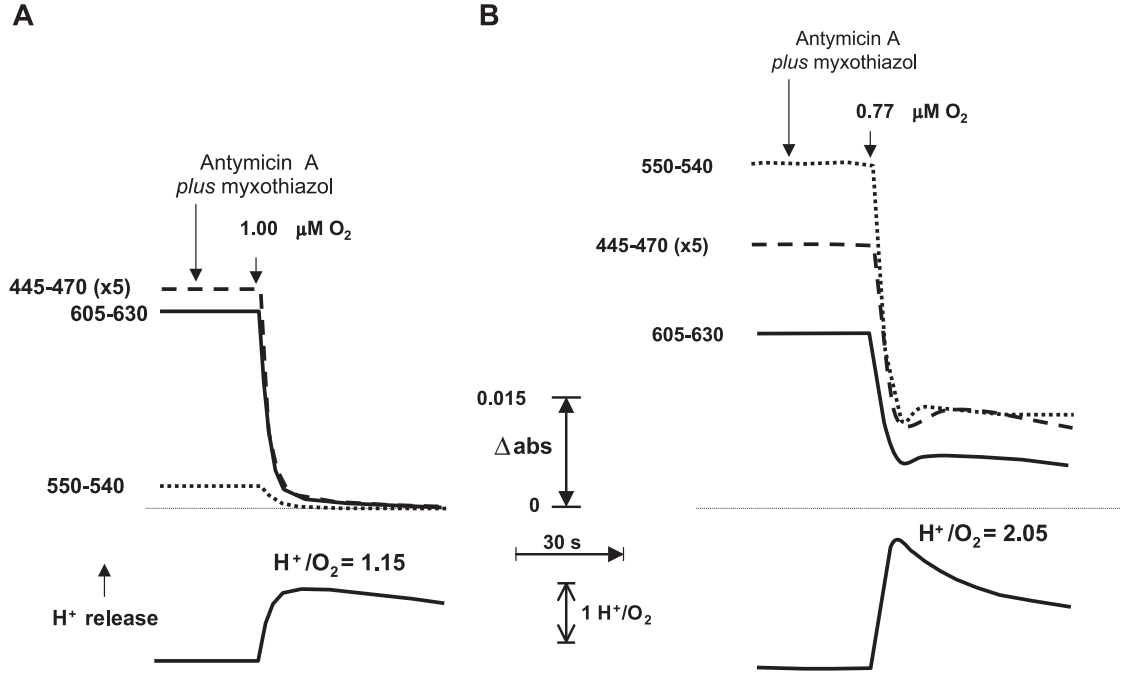

Fig. 3. Measurement of proton release associated with single-turnover aerobic oxidation-reduction of unliganded COV. 1.1 µM (exp. A) and 0.80 µM (exp. B) membrane-reconstituted COX were suspended in 150 mM KCl, pH 7.4 supplemented with 3 µg valinomycin/ml, 0.1 mg protein/ml of broken mitochondria and 0.15 µM (A) or 2.5 µM (B) cytochrome *c*. Simultaneous measurement of redox transitions of cytochrome *c*, hemes *a* and a_3 and pH changes were recorded by a diode-array spectrophotometer and a fast responding pH electrode, respectively. Anaerobiosis with reduction of cytochrome *c* and cytochrome *c* oxidase was attained by the addition of 10 mM succinate. 1 µM antimycin A and 3 µM myxothiazol were then added to block succinate oxidation and rapid aerobic oxidation of the reduced redox centers was obtained by the addition of O_2 slightly substoichiometric with respect to the amount of the reduced COX centers. The extent of the re-reduction by ferrocytochrome *c* of the metal centers of COX, following the initial rapid oxidative phase, amounted to around 2% and 25% in (A) and (B), respectively. In these experiments, the right side-out orientation of the oxidase in the liposomal membrane was $\geq$95%, the respiratory control index $\geq$20. The intrinsic passive proton conduction of COV, measured upon generation with valinomycin of a positive K^+ gradient in COV, was $\leq$2% of that measured in the presence of valinomycin plus 3 µM CCCP. For other details, see Refs. [28,40].

should have been left when these centers were fully re-reduced. Reuptake of the Bohr protons did, instead, occur with a considerable delay after re-reduction of the redox centers (see Fig. 5 in Ref. [40]). These observations show that the Bohr protons associated with re-reduction of heme *a* and Cu_A are taken up from the inner aqueous space. This was confirmed by the observation that in the presence of CCCP, at the completion of the re-reduction of heme *a* and Cu_A, no extra-acidification, with respect to the ferricyanide added, was observed (Fig. 2B).

4. Proton translocation associated to single-turnover oxido-reduction of cytochrome *c* oxidase vesicles

Proton translocation associated to single-turnover oxido-reduction of the four metal centers in the unliganded, membrane-reconstituted bovine cytochrome *c* oxidase vesicles (COV) has also been analysed. Nilsson et al. [41,42] and Verkhovsky et al. [43] reported that aerobic oxidation of fully reduced COV results in the release in the external (P) phase of 1–2 H^+/COX. What both groups showed in the experiments they presented was, in fact, an H^+/COX release ratio of 1.2–1.3 [41–43]. Verkhovsky et al. [43] also showed that re-reduction of COV resulted in a significant additional H^+ release.

We have carried out a study of the H^+ release associated to aerobic and anaerobic ferricyanide-induced oxidation and reduction of COV (Fig. 3). Aerobic oxidation of the fully reduced COV resulted in the release of 1.22 ± 0.1 H^+/COX (Figs. 3 and 4), thus confirming the ratio determined by the previous authors [41–43]. Our analysis reveals, in addition, new relevant aspects of the H^+ release associated to single-turnover oxidation/reduction of the oxidase. The aerobic H^+/COX release from coupled COV was practically the same as that observed upon anaerobic ferricyanide oxidation of the fully reduced COV, either in the coupled (H^+/COX ratio 1.41 ± 0.18) or uncoupled state (1.28 ± 0.06) (Fig. 4). Furthermore, the H^+/COX release ratio for COV oxidation was practically the same as that measured for the reversible H^+ release associated to (anaerobic) ferricyanide oxidation of the fully reduced unliganded, soluble oxidase (H^+/COX ratio 1.40 ± 0.06 [44]). As reported elsewhere, this H^+

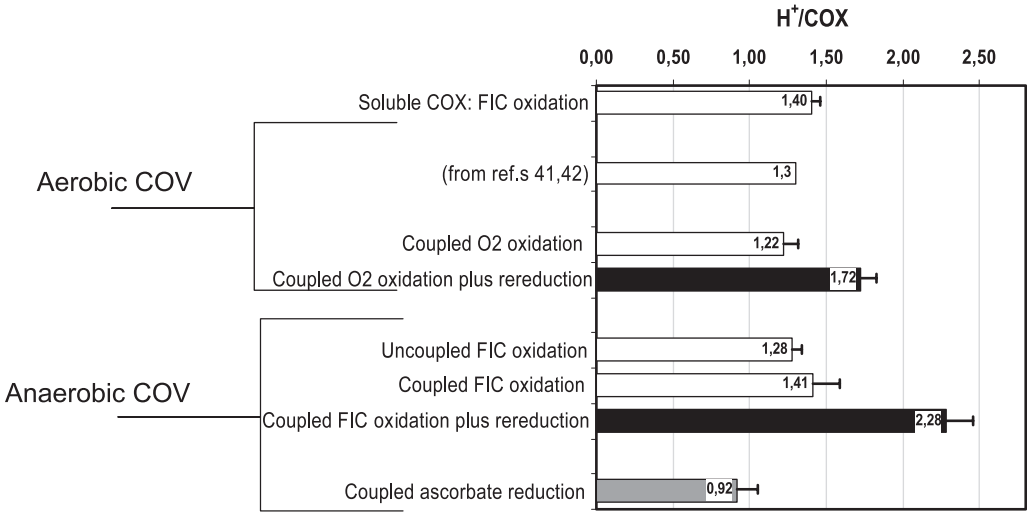

Fig. 4. Proton release associated with single-turnover aerobic and anaerobic ferricyanide induced oxidation-rereduction of unliganded COV. For experimental details see Fig. 3. Anaerobic reduction of COX was attained by succinate oxidation. *Aerobic oxidation-rereduction:* aerobic oxidation of the reduced redox centers in cytochrome *c* oxidase was obtained by the addition of O_2 slightly substoichiometric with respect to the amount of the reduced COX centers. Re-reduction by the excess of succinate of the oxidized metal centers in the oxidase was slowed down by the addition of malonate. The extent of the re-reduction of metal centers of COX following the initial oxidative phase was modulated by changing the amount of added cytochrome *c*. The empty column refer to the measured H^+/COX ratio associated with the oxidative phase (practical complete oxidation of heme *a* and a_3). For comparison, the H^+/COX release ratio measured under these conditions by Nilsson et al. [41,42] is also presented. The filled column refers to the overall proton release associated with full oxidation of COX metal centers and partial (25% on the basis of heme a_3) re-reduction. Verkhovsky et al. [43] have presented a significantly larger proton release in the re-reduction of COX. The reason of this discrepancy is not clear. *Anaerobic ferricyanide oxidation:* anaerobic reduction of COX was attained by succinate oxidation. Rapid and permanent anaerobic oxidation of reduced COV and ferrocytochrome *c* (0.5 μM) was produced by the addition of ferricyanide slightly in excess with respect to the sum of the reduced metal centers in the presence of 1 μM of antimycin A and 3 μM myxothiazol. The experiment was carried out in the absence and in the presence of 3 μM CCCP or with soluble COX. The H^+/COX ratios relative to the proton release associated to the oxidation phase are shown as empty columns. When antimycin A and myxothiazol were omitted, the ferricyanide-induced oxidation of the metal centers was followed by re-reduction sustained by the excess of succinate. This resulted in further acidification synchronous with the re-reduction phase. The filled column shows the H^+/COX ratio relative to the proton release associated with the overall oxidation-reduction cycle corrected for the scalar proton release due to the oxidation of succinate ($1H^+$/equivalent of ferricyanide added). The grey column shows the proton release associated with reduction of COX metal centers attained by ascorbate addition to COV, a few minutes after COX complete oxidation by ferricyanide in the presence of antimycin A and myxothiazol; the H^+/COX ratio was corrected for the scalar proton release due to the oxidation of ascorbate. The measured H^+/COX release ratios are indicated within the columns together with the S.E.M. deviation ($n = 4–10$). See text for further explanation.

release in the case of soluble COX can represent the sum of the Bohr effects linked to Cu_A and heme *a* and protonation/deprotonation reactions linked to oxido-reduction of heme a_3-Cu_B [44].

These findings indicate that in a single-turnover experiment, in which the fully reduced enzyme is oxidized and then re-reduced by ferrocytochrome *c*, the H^+ release associated to the oxidation phase of the four metal centers by oxygen or by ferricyanide does not represent proton pumping. It appears, in fact, to correspond to the direct deprotonation of acidic groups in the oxidase linked to oxidation of the metal centers (see Figs. 2 and 4 in Ref. [44]). Our measurements show that upon re-reduction of the enzyme oxidized by O_2, an extra H^+/COX release of 0.50 ± 0.2, in addition to that measured in the oxidative phase, was observed. Re-reduction of the four metals centers, obtained after their (anaerobic) ferricyanide oxidation, also resulted in an extra H^+/COX release of 0.87 ± 0.15 in addition to that measured in the oxidation phase. An H^+/COX release ratio of 0.92 ± 0.13 was observed upon full reduction of COV kept for minutes in the oxidized anaerobic state (cf. Refs. [43,45]).

The experiments on CO-liganded COV showed that reduction of Cu_A and heme *a* is linked to proton uptake from the inner (N) space, thus the reductive release in the P space of pumped protons has to be associated to net electron flow from ferrocytochrome *c*, via Cu_A heme *a*, to heme a_3-Cu_B. Our measurements could have underestimated the H^+/COX release ratio for proton pumping associated to the reduction phase of the binuclear center. Further work is in progress to verify this ratio. It should also be taken into account that estimation of the H^+/e^- ratio for proton pumping associated to electron flow from heme *a* to the binuclear center would require knowledge of the space from which the scalar protons taken up by the oxidase upon reduction of heme a_3 and Cu_B derive [44].

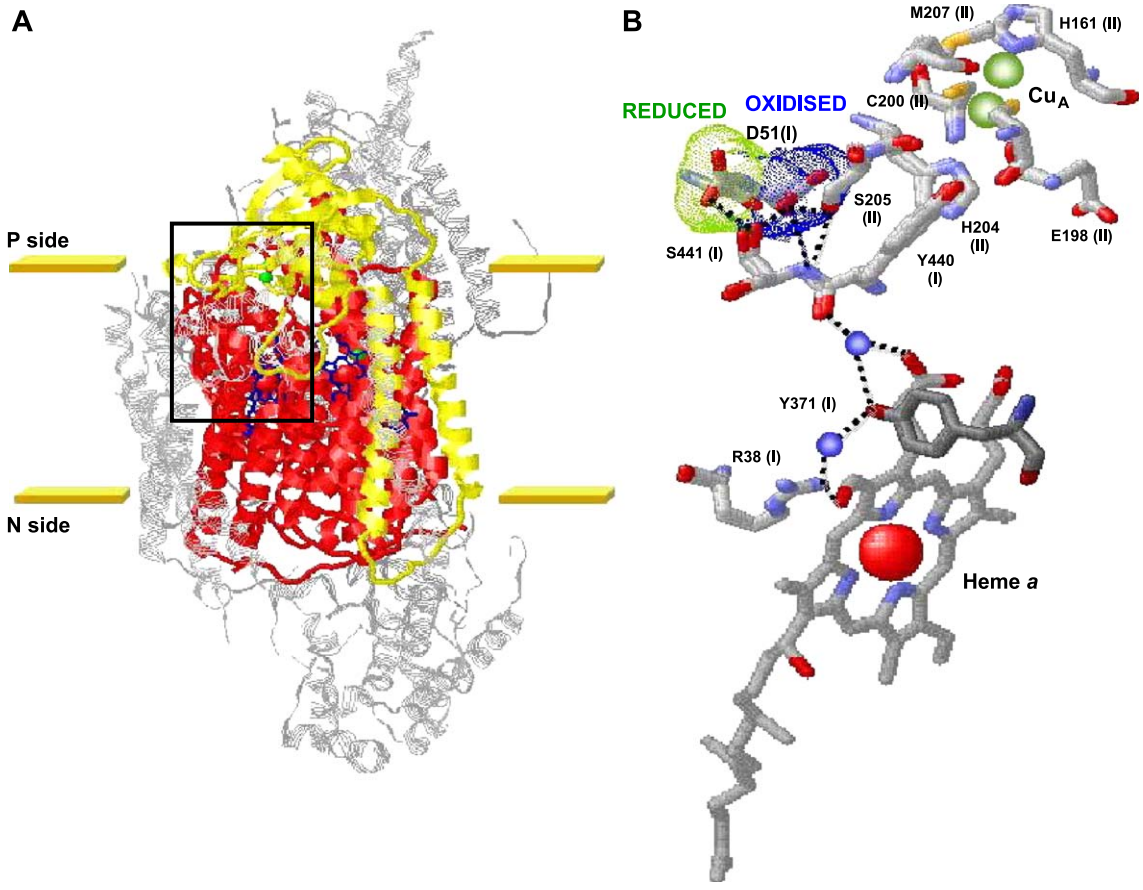

Fig. 5. Redox-linked conformational change in bovine cytochrome *c* oxidase. (A) View parallel to the membrane of the 13 subunits bovine heart cytochrome *c* oxidase. Subunits I and II are shown in colour (red and yellow, respectively), all the others in gray. The picture was elaborated with the Rasmol 2.6 program using the 2OCC atomic coordinates from the PDB data bank of the monomer of cytochrome *c* oxidase. The black frame defines a detail of the structure enlarged in B where selected residues in subunits I and II of bovine cytochrome *c* oxidase are shown. The picture was elaborated by superimposing the 2OCC (2.35 Å resolution) and 1OCR (2.30 Å resolution) atomic coordinates from the PDB data bank of the oxidized and reduced crystal structure of bovine cytochrome *c* oxidase, respectively (“What if” program) and re-drawn by the Rasmol 2.6 program. The major redox-linked conformational change of D51 (see text and Ref. [8]) is evidenced by dotting in colour the Wan der Waals’ atomic radii. The stick structure of other residues shows only minor position changes of their atomic coordinates. The dotted lines show possible hydrogen bond networks [8,46].

5. A mechanism for cooperative proton pumping in cytochrome *c* oxidase

X-ray crystallographic analysis of the bovine heart cytochrome *c* oxidase has revealed that the reduction of the enzyme results in a significant conformational change in a region of subunit I which connects the heme *a* environment to the P surface. On reduction, a segment of this subunit from G49 to N55 moves towards the P surface by 4 Å with the carboxylic group of D51 becoming accessible to the P aqueous phase. In the oxidized state, the carboxylic group of D51 is buried in the protein and connected by a proton pathway to the guanidinium group of R38, which is hydrogen-bonded to the formyl substituent of heme *a* ([8]; see also Ref. [22]) (Fig. 5). In the reduced state of heme *a* R38 would be protonated [8,22]. Yoshikawa et al. [8,46] have proposed that starting from the reduced enzyme oxidation of heme *a* decreases the proton affinity of R38. At the same time, D51 turns inside the protein and gets hydrogen-bonded to the peptide amide of S441 in the network connected to R38. The proton donated by R38 translocates via the Y440-S441 peptide bond and protonates D51. The unidirectionality of the proton transfer through the peptide bond would be given by the higher stability of the keto form relative to the enol form of the peptide bond [46,47]. On re-reduction, D51 moves out at the protein surface and releases a proton in the P space, whilst R38 takes up a proton from a channel in protonic equilibrium with the inner (N) aqueous space. The channel by which protons reach R38 in the heme pocket (or OH^- leaves this space) is an open issue. Yoshikawa et al. [8] have proposed that this channel is provided by the H pathway they have identified in the crystal structure of the bovine enzyme. It has, however, to be taken into account that the specific residues considered as constituents of the output D51 channel, as well as those of the H pathway, are conserved in animal, but not in plant and bacterial protonmotive heme-copper oxidases [25,35]. The heme *a* pocket could also exchange H^+ (or OH^-) with the N space by the D pathway [33]. This pathway is generally thought to conduct H^+ to the a_3-Cu_B site [4–8]. There are, however observations indicating that protonable residues at the inner side of the D channel, in particular a glutammic residue present in bovine [4] (E242) and some prokaryotic oxidases [5,7], or equivalent residues replacing E242 in other oxidases [25,35], can switch between two positions/states [7,48]: one could be involved in proton pumping, coupled to heme *a* [33] or the binuclear site [7], the other in the translocation of protons consumed in the reduction of O_2 to H_2O [7,33].

Regardless of the channel by which H^+ (or OH^-) can move between the heme a pocket and the N space a central role in cooperative coupling of proton pumping at

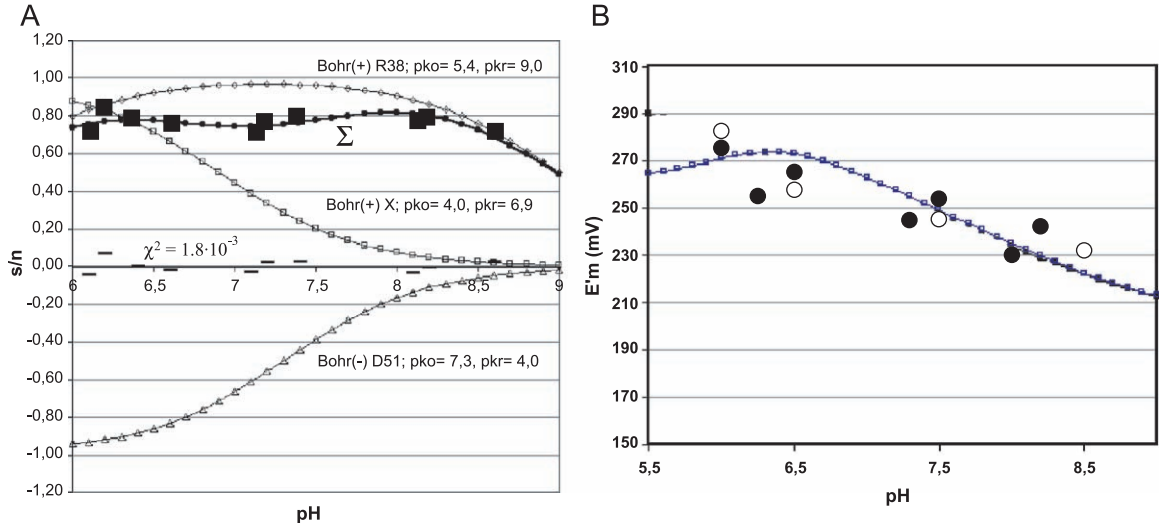

Fig. 6. Redox Bohr effect associated with heme *a* and Cu_A in CO-inhibited, soluble cytochrome *c* oxidase: effect of pH. The number of groups used in the fitting procedure (see equation in the legend of Fig. 1) for the pH dependence of H^+ release associated with oxidation of heme a^{2+} and Cu_A^+ in CO-inhibited COX (A) was three and the p*K*'s (p*K*ox–p*K*red) obtained were: 4.0–6.9, 5.4–9.0 (both Bohr positive) and 7.3–4.0 (Bohr negative). The residual of the experimental points with respect to the best fit is shown together with the value of X^2. The contribution of each protolytic group is also shown as thin lines. (B) pH dependence of the E'm of heme *a* (filled circles) and Cu_A (empty circles). The solid curve is the result of mathematical simulation using the equation:

$$E'\,m = Eo + 0.059/n\left(\log\frac{[H^+]^x + Kred_1[H^+]^{x-1} + Kred_1Kred_2[H^+]^{x-2}\ldots[Kred_1Kred_2Kred_3\ldots Kred_n]}{[H^+]^x + Kox_1[H^+]^{x-1} + Kox_1Kox_2[H^+]^{x-2}\ldots[Kox_1Kox_2Kox_3\ldots Kox_n]}\right)$$

for common linkage to oxido-reduction of both heme *a* and Cu_A ($n=2$) of three protolytic groups whose p*K*ox and p*K*red are those obtained from the simulation of the pH dependence of measured redox-linked proton transfer in CO-COX (A). It should be noted that the experimental values of the s/n (H^+/COX ratio) and Cu_A and heme *a* E′ m can equally best-fitted with equations incorporating more Bohr groups.

the low potential site can be played by water located near the formyl group of heme *a*. Evidence for the location of this H_2O was initially provided by resonance Raman spectroscopy by Rousseau et al. [31,49].

As mentioned above, in the membrane-reconstituted CO-inhibited COX, the protons released in the P space, upon oxidation of heme *a* and Cu_A, are taken back on their re-reduction from the N space [40]. This proton transfer would seem opposite to that predicted for the redox-linked movement of D51 seen in the COX crystals [8]. In the oxido-reduction of heme *a*, this negative Bohr effect of D51 can however be associated with positive Bohr effects [14,50]. Mathematical analysis of the pH dependence of proton transfer coupled to oxido-reduction of heme *a* and Cu_A and of the Em of the two redox centers in the CO-liganded soluble COX, results in a best-fit of the experimental points by an equation representing the case in which both heme *a* and Cu_A share coupling with a negative Bohr effect (D51?) and a minimum of two positive Bohr effects (Fig. 6). It seems significant in this context that S205 of subunit II, which is not too distant from the binuclear Cu_A center, is in the crystal of the oxidized COX hydrogen bonded to D51 [8].

Putting together this wealth of structural and functional data, a tentative, molecular mechanism for proton pumping is proposed. It should, however, be kept in mind that the details of this mechanism specifically apply to the bovine enzyme.

Fig. 7 describes how the Bohr effects linked to heme *a* and Cu_A can operate the proton pump in the turning-over oxidase in the membrane upon transfer of each electron from ferrocytochrome *c*, via Cu_A and heme *a*, to the binuclear center. In the oxidized state before the arrival of the electron, two protonatable groups, in the hydrogen-bond network connecting D51 at the P surface to the heme *a* environment are in the deprotonated state (positive Bohr effects). R38 and a propionate of heme *a* could be components of the network. In the oxidized state, D51 is buried in the protein in the protonated state (negative Bohr effect). Single-electron reduction of Cu_A (or heme *a*) by ferrocytochrome *c* is associated with the transfer of a

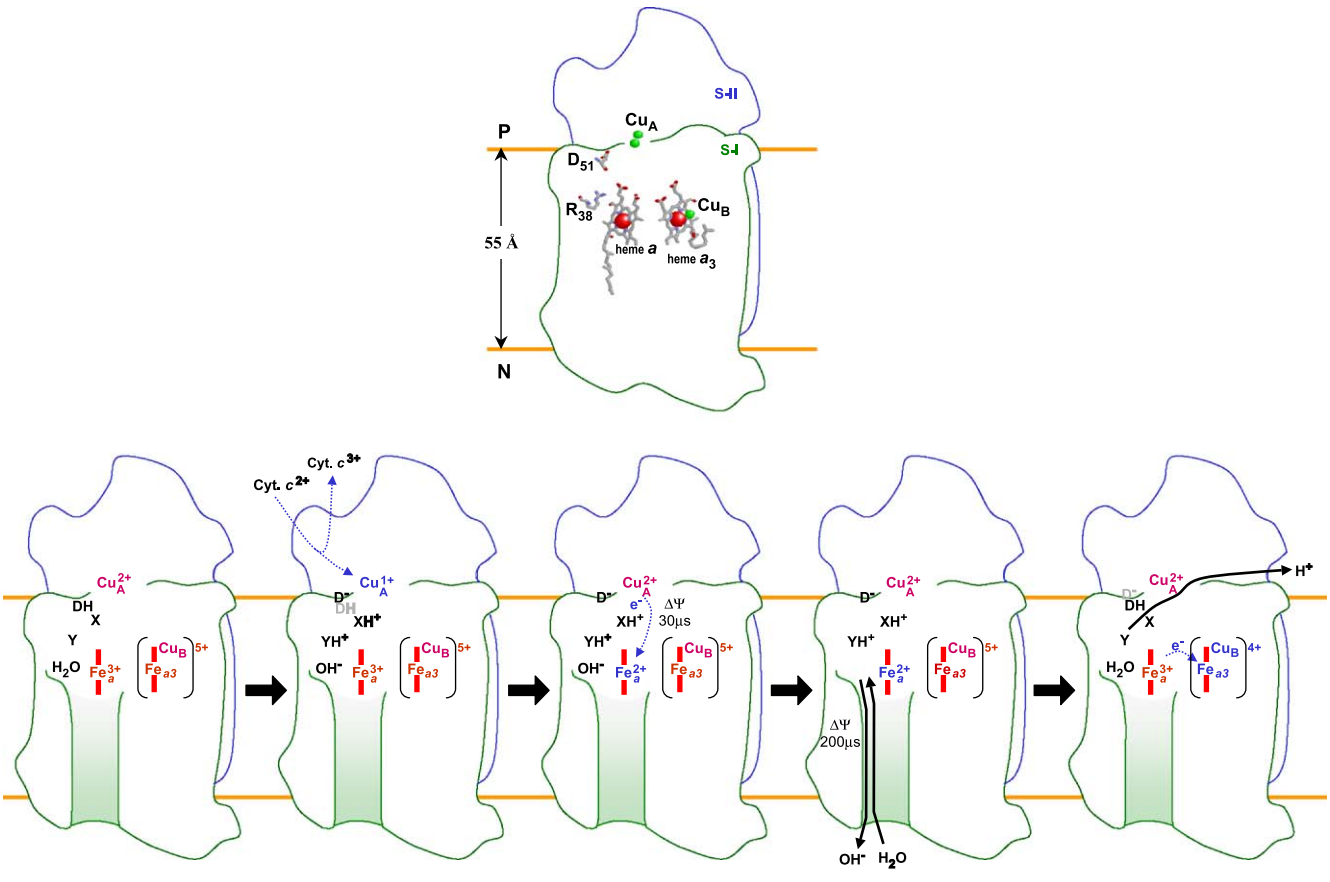

Fig. 7. Model for vectorial proton translocation coupled to single electron flow from ferrocytochrome *c* to the heme a_3-Cu_B binuclear center, via Cu_A-heme *a*, in the turning-over oxidase in the membrane. The upper sketch shows the position relative to the dielectric membrane barrier of the redox centers and residues proposed to be involved in proton pumping in subunit I and II of bovine cytochrome *c* oxidase (PDB entry 2OCC). The lower sketch illustrates the sequential steps of vectorial proton translocation associated with the successive steps of electron flow from ferrocytochrome *c* to Cu_A, heme *a* and the heme a_3-Cu_B center. It should be noted that the groups undergoing p*K* shifts in the network coupled to Cu_A and heme *a* could be more than the three indicated in the model. For description of the model see the text.

proton from the H_2O near the formyl of heme *a* to a nearby protonatable group *Y* (likely the guanidium group of R38). D51 carboxylate becomes exposed to the P space, the proton from the carboxylate is temporarily accepted by a protonatable group in the network, *X*. The protons contributed by H_2O and D51 move only by few angstroms, in opposite directions along the axis perpendicular to the plane of the membrane, thus reduction of Cu_A does not result in any significant charge translocation across the membrane. It has, in fact, been shown that the midpoint potential of Cu_A is not affected by transmembrane PMF [51,52]. The electron donated by ferrocytochrome rapidly moves from Cu_A at the P surface to heme *a* which is located in the middle of the membrane 20 Å away from the P surface, resulting in a fast rise of the membrane potential (time constant ≈ 30 μs, cf. Refs. [45,53,54]). This phase is followed by a slower (time constant ≈ 200 μs [45,53,54]) electrogenic exchange of OH^- with H_2O from the N phase.

Upon final transfer of the electron from heme *a* to the binuclear center, leaving both Cu_A and heme *a* oxidized, the two groups in the network are deprotonated. The carboxylate of D51 moves back inwards and picks up one of the protons released by the two groups, the other proton is ultimately released in the P aqueous phase. The transfer of each of the successive three electrons needed to reduce molecular oxygen to two water molecules is coupled to net pumping of one proton from the N to the P aqueous space.

As already pointed out, the mechanism proposed, whilst making use of the specific structural and functional data of the Cu_A aa_3 Cu_B bovine cytochrome *c* oxidase, is intended to outline the essential elements for cooperative coupling of the proton pump at the low potential redox centers of the heme-copper oxidases. D51 and connected residues [8], as well as residues in the H and D pathway supposedly involved in the bovine enzyme in proton connectivity of the heme *a* site with N aqueous space, can be replaced in other heme-copper oxidases by corresponding residues and/or structured water. In quinol heme copper oxidases, which lack the Cu_A center [25], heme *a* alone (or in association with quinone), maintains the essential properties to function

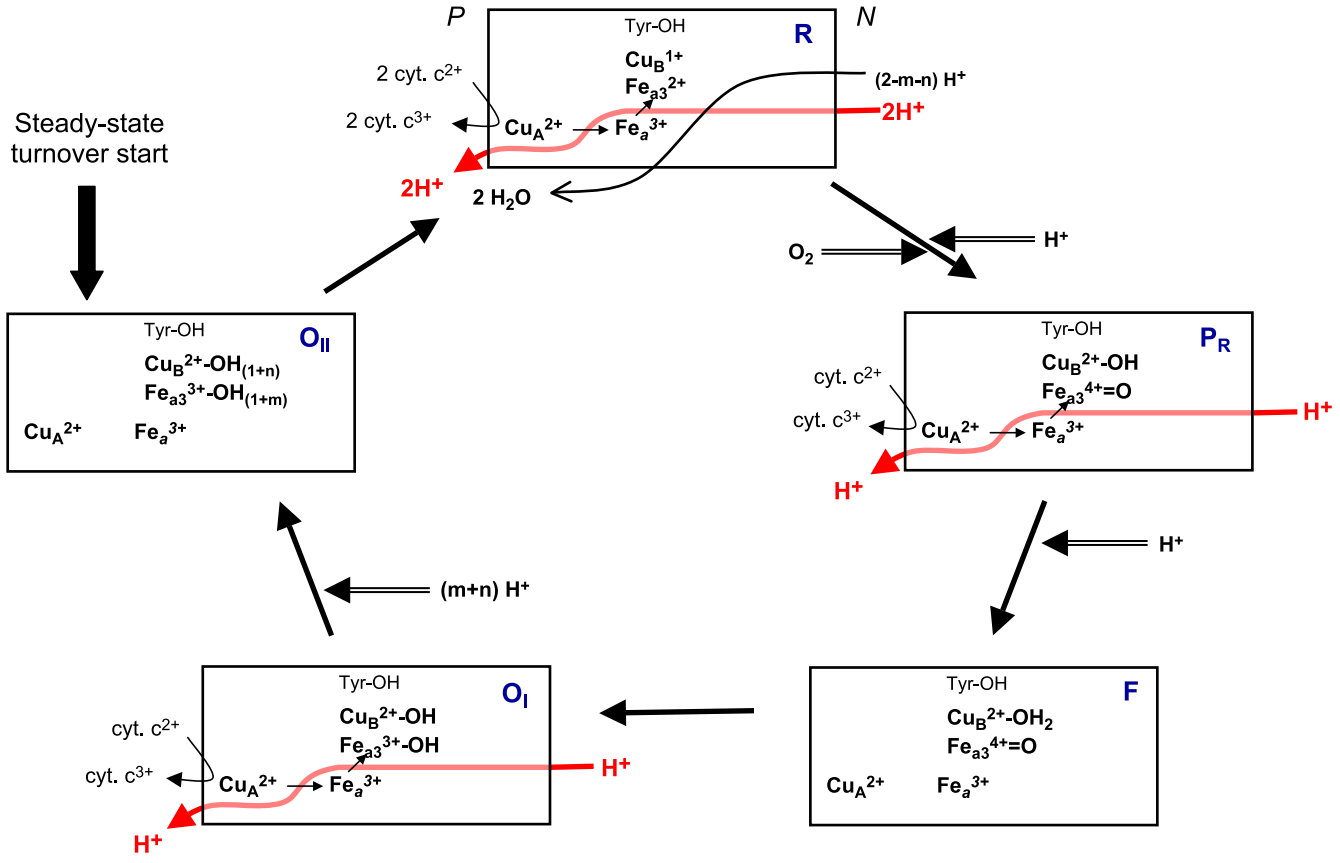

Fig. 8. Proposed catalytic cycle for reduction of O_2 to H_2O by ferrocytochrome *c* and proton pumping in the respiring steady-state cytochrome *c* oxidase. The four metal centers in cytochrome *c* oxidase are shown together with tyrosine 244 of subunit I (bovine numbering). The boldface letters refer to different redox states of the binuclear site, namely the fully reduced state (**R**), the 607 nm species (**P_R**), the oxoferryl 580 nm species (**F**) and the fully oxidized states (**O_I**) and (**O_{II}**) (see Ref. [44]). The steps of proton pumping from the N (right) side to the P (left) side coupled to electron transfer via Cu_A-heme *a* and proton consumption in the reduction of the oxygen-reduction intermediates at the heme a_3-Cu_B binuclear center are shown. For the number of protons consumed in the reduction of O_2 to $2H_2O$ in the oxidation and reduction phase of a turnover of the oxidase, respectively, see Ref. [44]. The R → F conversion could also be contributed by transient oxidation of Tyr 244 of subunit I to its tyrosil radical.

as the low potential redox site for cooperative proton translocation.

The model proposed describes the elementary steps of cooperative coupling of proton translocation to electron flow at the low redox potential site. It is however obvious that, in the respiring steady-state, once the first two electrons have reduced heme a_3 and Cu_B, transfer of the other two electrons, to complete a turnover of the enzyme, involves binding and reduction of O_2. It will, in fact, be the thermodynamic pulling effect of the aerobic oxidation of heme a_3 and Cu_B that provides the necessary energy to sustain net proton pumping against a steady-state PMF of some 250 mV.

The cooperative proton transfer in the network coupled to heme a and Cu_A might not involve, per se, large ΔG (cf. Ref. [55]). The situation might in a sense be analogous to that in the F_0F_1ATP synthase. In this enzyme, the energy provided by the PMF, generated by respiration is utilised for the removal from the catalytic site of ATP, thus driving net ATP production, whose synthesis from ADP and inorganic phosphate does not require, per se, significant energy expenditure [56]. It also has to be appreciated that the steps of the oxygen reduction chemistry take place at the binuclear center within microseconds and are much faster than electron flow from ferrocytochrome *c* to heme a_3 and Cu_B [57]. This provides an additional positive kinetic factor in the operation of the proton pump. Whilst the cooperative steps of proton translocation at heme *a* and Cu_A can be thermodynamically and kinetically coupled to the oxygen reduction chemistry at the binuclear center, the two processes are physically separated; otherwise, protons taken up from the inner space, rather than being pumped in the outer space, would be annihilated in the reduction of O_2 to $2H_2O$.

Time-resolved spectroscopic analysis has led to detailed characterization of the intermediate steps through which the reduction of O_2 takes place at the heme a_3-Cu_B binuclear center [57]. A scheme can be drawn (Fig. 8) which associates the steps of proton pumping in cytochrome *c* oxidase, turning-over at the respiratory steady-state, with those in the oxygen reduction chemistry. Under normal physiological conditions, a turn-over of cytochrome *c* oxidase in the membrane starts with the fully oxidized enzyme. Spectroscopic analysis shows in fact that in the respiring steady-state heme *a* and particularly heme a_3 are almost completely oxidized [58]. The transfer, via Cu_A and heme *a*, of the first two electrons from ferrocytochrome *c* to heme a_3 and Cu_B, completes the protonation from the inner space of two OH^- groups bound to the binuclear center to water molecules, which are then released in the outer bulk phase [44]. Transfer of each of the two electrons to heme a_3 and Cu_B is coupled to pumping of $\sim 1\ H^+$ from the N to the P space. O_2 can now diffuse to the binuclear center and in one step undergoes reductive cleavage of the double bond by three electrons donated by heme a_3 Fe and Cu_B and an electron delivered by cytochrome *c*. The transfer of this third electron donated by cytochrome *c* is associated with the pumping of a third proton. The P_R compound generated at this stage is converted to the F compound with consumption of a proton from the inner space [34]. Finally, the transfer of the fourth electron to the binuclear center converts the F compound to the O_I compound and a fourth proton is pumped. The O_I compound is then protonated to the O_{II} compound. At acidic pH values, two water molecules can already be formed and released in the outer bulk phase at this stage. At pH's ≥ 7.4, protonation of the two OH^- groups bound at the binuclear center, due to their p*K*'s, is only partially completed in the oxidative phase. Upon reduction of heme a_3 and Cu_B, protonation of the OH^- groups is completed and water is released in the outer space [44].

6. Conclusions

The model presented is intended to illustrate how cooperativity in aa_3 cytochrome *c* oxidase can contribute to a chemiosmotic mechanism for conversion of redox energy into a transmembrane PMF. It is also outlined how the cooperative steps of H^+ pumping at the low potential Cu_A-heme *a* site can be kinetically and thermodynamically coupled to the O_2 reduction chemistry at the high potential heme a_3-Cu_B binuclear site. Discussion of alternative mechanisms in which the oxygen reduction chemistry is envisaged to be directly involved in proton pumping is largely covered in other papers of the present volume, to which the interested reader is addressed. It can, on the other hand, be noted that the other two protonmotive enzymes of the respiratory mitochondrial chain have redox subunits, Fe–S proteins (in particular cluster II) in NADH-ubiquinone oxidoreductase [59], cytochrome *b* [18] and the Fe–S protein [60] in the bc_1 complex, which also exhibit redox Bohr effects. In these two complexes, cooperative proton coupling at the metal centers could be associated, also in series, with direct protonmotive protein-bound quinones [1]. Future work might consolidate extension of the ideas and approaches outlined here for cytochrome *c* oxidase to these other two complexes.

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