

Hypothesis

A cooperative model for protonmotive heme-copper oxidases. The role of heme *a* in the proton pump of cytochrome *c* oxidase

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Abstract Oxido-reductions of metal centers in cytochrome *c* oxidase are linked to p*K* shifts of acidic groups in the enzyme (redox Bohr effects). The linkage at heme *a* results in proton uptake from the inner space upon reduction and proton release in the external space upon oxidation of the metal. The relationship of this process to the features of the proton pump in cytochrome *c* oxidase and its atomic structure revealed by X-ray crystallography to 2.8–2.3 Å resolution is examined. A mechanism for the proton pump of cytochrome *c* oxidase, based on cooperative coupling at heme *a*, is proposed.

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Key words: Cytochrome *c* oxidase; Proton pump; Cooperative coupling

1. Introduction

Heme-copper oxidases [1] catalyze the transfer of electrons from cytochrome *c* or quinol (in some prokaryotic oxidases) to dioxygen and convert redox energy in a transmembrane electrochemical proton gradient, Δp [2–4]. The latter derives, in the first place, directly from the membrane anisotropy of reduction of O₂ to H₂O, whereby electrons are donated by cytochrome *c* (or quinol) at the outer P side of the membrane and protons are taken up from the inner N aqueous phase [3]. In addition, electron flow in protonmotive oxidases is associated with proton pumping from the N to the P aqueous phase [5].

All protonmotive heme-copper oxidases have a heme-copper (Cu_B) center where the reductive chemistry of oxygen takes place. This binuclear center is associated with subunit I, which also has a low potential heme (heme *a* or *b* type). Cytochrome *c* oxidases have an additional bimetallic Cu center (Cu_A) which serves as the entry of electrons and is associated with subunit II. Subunits I and II are both essential for proton pumping [4]. In heme-copper oxidases there is a conserved third subunit and supernumerary subunits whose number varies from one (in prokaryotic oxidases) to up to 10 in mammalian oxidases [6]. Functional analysis, advanced spectroscopy, molecular genetics (see Fig. 1), mutational analysis and, more recently, crystallographic structures have begun to provide the necessary information to solve, at the atomic level, the mechanism of electron and proton transfer in heme-copper oxidases and in particular in cytochrome *c* oxidase [7].

This paper is aimed at examining new information arising

from these investigations and at putting forward, on this basis, a model of the proton pump in cytochrome *c* oxidase with cooperative coupling at heme *a*.

2. Cooperative proton transfer (redox Bohr effects) in cytochrome *c* oxidase

Based on the principle of cooperative thermodynamic linkage of solute binding at separate sites in allosteric proteins [8] and the observation of linkage between electron transfer at the metals and protolytic events (p*K* shifts) in cytochromes [9], Papa et al. in 1973 proposed a cooperative model for proton pumping in respiratory enzymes [3,10]. By analogy with the cooperative linkage phenomena in hemoglobin, known as the Bohr effect [8], the redox linkage in cytochromes and the derived cooperative model for proton pumping were denominated redox Bohr effects and vectorial Bohr mechanism respectively. The H⁺/e[−] linkage in cytochromes is likely to arise from modification of the coordination bonds of metal centers associated with change in their valence state. In the vectorial Bohr mechanism the cooperative events are conceived to be extended over the transmembrane span of the protein so as to promote proton uptake from the N aqueous phase and release to the P aqueous phase [3].

Analysis in our laboratory of the pH dependence of H⁺ transfer associated with redox transitions of the metal centers in isolated cytochrome *c* oxidase (COX) from bovine heart mitochondria allowed us to characterize the Bohr effects in this enzyme [11]. The experimental H⁺/COX linkage ratios measured, in the pH range 6–9, in the reduced/oxidized/reduced transitions of the soluble oxidase, could be best fitted with a function involving a minimum of four protolytic groups, each undergoing a p*K* increase in the oxidized/reduced transition. The four p*K* shifts were attributed to the individual metal centers from their correspondence with those obtained by best fit analysis of H⁺/COX ratios measured in the CN[−]-liganded oxidase (heme *a*₃ blocked in the oxidized state) and in the CO-liganded oxidase (heme *a*₃ and Cu_B blocked in the reduced state). Cu_A is irrelevant in this respect since its *E*_m is pH-independent. The H⁺/e[−] linkage ratios for the redox Bohr effects linked to hemes *a* and *a*₃ and Cu_B calculated from the p*K*_{ox} and p*K*_{red} of the respective linkage groups are presented in Fig. 2. The assignment of p*K* shifts of the four protolytic groups to individual metal centers does not exclude the possibility that the p*K* of a single group can also be influenced by cooperative interaction of different metals [12]. It can, however, be noted that the p*K*_{ox} and p*K*_{red} for the group linked to heme *a* and the H⁺/e[−] linkage ratios did not differ much when measured in the CN[−]-liganded and CO-liganded oxidase. The

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BSUB	MKFKWD	EFFVTGDPLI	LGAQVSIALS	TIAIFVLTY	FKKWKWLSE	WITTVDHKKL	GIMYIISAVI	MLFRGGVDGL	MMRAQLALP-	-----	85
ECOL	MFGKLSL	DAVPFHEPIV	MVTIAGIILG	GLALVGLITY	FGKWTYLWKE	WLTSDVHKRL	GIMYIIVAIV	MLLRGFADAI	MMRSQQALAS	AG-----	89
PDEN			MAD	AAVHGHDH	DTRGFTTRWF	MST--NHKDI	GILYLFRTAGI	VGLISVCFTV	YMRMELQHPG	VQYMCLEGAR	71
BEEF					MFINRWL	FST--NHKDI	GILYLLFGAW	AGMVGTLALS	LIRAEGLQPG	T--L-----	47
								I		+	
BSUB	-----NNSF	LDSNHYNEIF	THGTMIIIF	MAMFELI-GL	INVVVPLQIG	ARDVAFPPYN	NLSWTFVVG	AMLFNISFVI	GG-----SPN	AGWTSYMPPLA	173
ECOL	-----EAGF	LPPHHYDQIF	TAHGVMILFF	VAMPEVI-GL	MNLVVPLQIG	ARDVAFPPFN	NLSWTFVVG	VILVNVSLGV	GE-----FAQ	TGWLAYPPPLS	177
PDEN	LIADASAECT	PNGHLWNVMI	TYHGVLMFF	VVLPALFGCF	GVYFMPLHIG	APDMAPPRLN	NLSWMMYVCG	VALGVASLLA	PGGNDQMGSG	VGVVLYPPPLS	171
BEEF	-----L	GDDQIYNVVV	TAHAFVMIFF	MVMPIIMIGF	GNLWVPLMIG	APDMAPPRMN	NLSWLLPPS	FLLLLASMV	EA-----GAG	TGWTVYPPPLA	133
		+		II				III		+	
BSUB	-SNDMSGPG	ENYYLLGLQI	AGIGTMTGT	NFMVTILKMR	TGQMTLMRMP	MFTWTLTITM	VIIVFAFEPVL	TVALALISFD	RLFGAHEFTL	EAGGMPMLWA	272
ECOL	-GIEYSRGVG	VDYWIWSLQ	SGIGTTLTGT	NFFVTILKMR	APGMMFMKMP	VFTWASLCAN	VLIIASFEIL	TVTVALITLD	RYLGTHFFTN	DMGGNMMYI	276
PDEN	T--TEA-GYS	MDLAIFAVHV	SCASSILGAI	NIITTLNMR	APGMMFLKVP	LEFASVFITA	WLILLSLEVL	AGAITMLMD	RNFGTQFFDP	AGGGDFVLYQ	268
BEEF	GNLAHA-GAS	VDLTIFSLHL	ACVSSILGAI	NEITTLNMR	PPAMSQYQTP	LEFVSWMITA	VLLLSLEVL	AAGITMLMD	RNLNTTFFDP	AGGGDFVLYQ	232
		+		IV				V		+	VI
BSUB	NLFWIWHGPE	VYIVILPAFG	IFSEIISFFA	-RKQLFGYKA	MVGSIIAISV	LSEIIVWTHF	FTMGNSASVN	SFSITMTAI	SIEPTGVKIFN	WLFTMYKGRI	371
ECOL	NLIWAWHGPE	VYIILPLVFG	VFSEIAATFS	-RKRLFGYTS	LVWATVCITV	LSEIIVWLHFF	FTMGAGANVN	AFEGITMTII	AIPTGVKIFN	WLFTMYQGR	375
PDEN	HILWFFGHPE	VYIILPLCFG	ITSHVISTFA	-KKPIFGYLP	MVLAMAAIGI	LCFVWVAHMM	YTACMSLTQQ	AYEMLATMTI	AVPTGVKIFN	WLATMWGGSI	367
BEEF	HLPWFEGHPE	VYIILPLCFG	MISHVITYYS	GKREPFYMG	MVWAMMSIGF	LCFVWVAHMM	FTVGMVDVTR	AYETSATMTI	AIPTGVKIFN	WLATLHGGNI	332
		VI			VII				VIII		
BSUB	SFTTPMLWAL	AFIPNEVIGG	VTGVMLAMAA	ADYQYHNTYF	LVSHFFHVL	ACTVFACFAG	FIFWYPKMFG	HKLNERIGKW	FEWIFMIGFN	ICFFPQYELG	471
ECOL	VFHSAMLWTI	GFIVTFSVGG	MTGVLLAVPG	ADFVLHNSLF	LIAHFFHNVII	GGVVFCCFAG	MTYWPWPKAFG	FKLNETWGKR	AFWFIIIGFF	VAFMELYALG	475
PDEN	RCGMPPRMID	Y--PVEFAYW	NNISSIGAYI	SFASFLFFIG	IVFYTLFAGK	RVNVPNYW--	YYYWIGKMSG	ROYPEWAGQL	HEWMMFIGSN	LIFEPQHLG	467
BEEF	KWSPAMWAL	GFIFLETVGG	LTGIVLANSS	LDIVLHDTYY	VVAHFFHVL	MCAVFAIMGG	FVHWFPLESG	YTLDNTWAKI	HFAIMFVGVN	MTFEPQHLG	432
		IX			X					XI	
BSUB	LQGMPPRIYT	YGPNDGWTTL	NIISTVGAFM	MGVGLILCY	NIYYSFRYST	---REISGD	SW---GVGRT	LDWATSSAIP	PHYNFAVLPE	VKSQDAFLHM	564
ECOL	FMGMTRRLSQ	Q-IDPQFHTM	LMIAASGAVL	IAGILCLVI	QMYVSIRDRT	---QNRDLTD	PW---GGRT	LEWATSSP-P	PFYNFAVVP	VHERDAFWM	567
PDEN	RCGMPPRMID	Y--PVEFAYW	NNISSIGAYI	SFASFLFFIG	IVFYTLFAGK	RVNVPNYW--	---NEHDT	LEWTLPS-PP	PEHTFETLPK	REDWDRAH	558
BEEF	LSGMPPRYSD	Y--PDAYTMW	NTISSMGSFI	SLTAVMLMVF	IIWEAFASKR	EVL-----	---TVDLTTN	LEWLNGC-PP	PYHTFEEPTY	VNLK-----	514
		XI		XII							
BSUB	K-EEKTELYP	ESKFKKIHPM	SNSGRPFMS	-VAFGLAGFG	LVFEW--YWM	GVVGLIGVLL	CMVLRSEFYD	NGYISVDEI	KETERKISE-	-----	658
ECOL	KEGEAYKKP	D-HYEEIHPM	KNSGAGIVIA	AFST-IFGFA	MIW--HIWWL	ATVGAGMII	TIWVKSFD	VDYVVPVAE	EKLENQHFE	ITKAGLKN	663
PDEN	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	
BEEF	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	

Fig. 1. Sequence alignment of heme-copper oxidase subunit I. Ecol: *Escherichia coli* bo₃ quinol oxidase; Bsub: *Bacillus subtilis* aa₃-600 quinol oxidase; Pden: *P. denitrificans* cytochrome *c* oxidase chain Iβ; Beef: beef heart cytochrome *c* oxidase. Invariant or functionally conserved residues are white in black and dark gray boxes respectively. Transmembrane α-helices are gray-boxed with the signs – and + for the N and P side respectively. Data are taken from the Webnet Cytochrome Oxidase Home Page (<http://www-bioc.rice.edu/~graham/CcO.html>).

same appears to apply for Cu_B when pKs and H⁺/e[−] linkage ratios are calculated from the H⁺/COX ratios of CN[−]-liganded and CN[−]-liganded minus CO-liganded oxidase. It appears, then, that cooperative interactions of redox metals, when occurring, do not significantly affect the pK values arising from individual linkages. The finding of H⁺/e[−] linkage ratios lower than 4 H⁺/COX shows that the principle of electroneutrality advocated by Rich [12] for electron delivery to the metal centers in the oxidase does not apply in all conditions.

The membrane vectoriality of the redox Bohr effects was then analyzed by measurement of proton transfer associated with oxido-reduction of the metal centers in the purified bovine heart cytochrome *c* oxidase, in the unliganded and CO-liganded state, incorporated in liposomes (COV) [13,14]. The results showed that the proton transfer resulting from the redox Bohr effects linked to heme *a* and Cu_B in the bovine heart cytochrome *c* oxidase displays membrane vectorial asymmetry, i.e. protons are taken up from the inner N aqueous space, upon reduction, and released in the external P space, upon oxidation of the metals. This direction of the proton uptake and release is just what is expected from a vectorial Bohr mechanism [3]. The two groups whose pKs change upon oxido-reduction of heme *a*₃ apparently exchange protons only with the external aqueous phase. This would exclude a primary role of these two groups in proton pump-

ing. It has to be recalled that the group linked to heme *a* which can transfer up to 0.9 H⁺/e[−] could by itself provide the major contribution to the proton pump. The group linked to Cu_B can, with a maximum H⁺/e[−] ratio of 0.3 at pHs below 7, but less than 0.2 at pHs 7.4–7.6, have only a small contribution to the proton pump. The kinetics of the translocation of the Bohr protons and the relationship of these partial steps with the overall process of proton pumping, associated with reduction of dioxygen to water, remain to be elucidated. It may be recalled, in this respect, that kinetic analysis of proton release during flash-induced aerobic oxidation of cytochrome *c* oxidase incorporated in liposomes showed a rapid release of 1.2 or more H⁺/COX at pH 7.5 with a rate constant of 9 × 10² s^{−1}, which is kinetically competent with the catalytic process [15].

3. Features of the proton pump in cytochrome *c* oxidase

A problem of particular interest is whether the H⁺/e[−] stoichiometry of the pump in cytochrome *c* oxidase is fixed or variable [5,16–18] and in the latter case, which are the factors modulating the stoichiometry. The observed non-linearity of the steady-state relationship between respiratory rate and Δ*p* is taken by some authors as evidence of slip in proton pumps [19], by others as due to non-ohmic increases of membrane proton conductance at high Δ*p* (leak) [20].

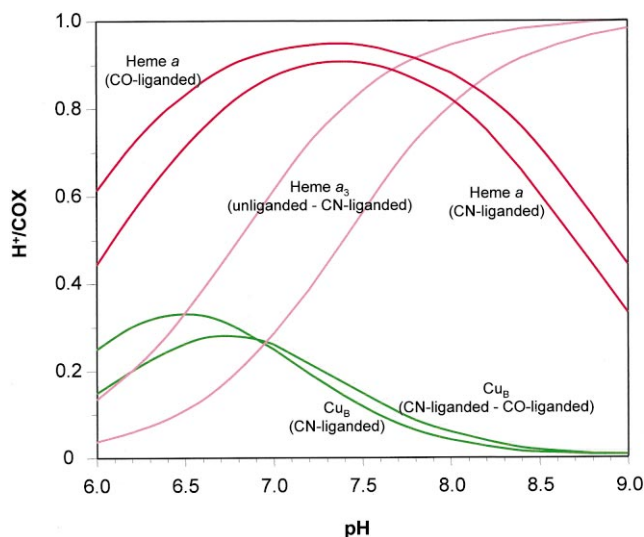


Fig. 2. Redox Bohr effects in bovine heart cytochrome *c* oxidase. pH dependence of the H^+/e^- linkage ratios for redox Bohr effects. The H^+/e^- linkage ratios for heme *a* (red curves) were calculated from the best fit analysis of the H^+/COX ratios measured in the CN^- -liganded oxidase, giving pK_{ox} of 6.1 ± 0.2 and pK_{red} of 8.7 ± 0.1 , and the best fit analysis of the H^+/COX ratios measured in the CO-liganded oxidase giving pK_{ox} of 5.8 ± 0.1 and pK_{red} of 8.9 ± 0.1 . The H^+/e^- linkage ratios for Cu_B (green curves) were calculated from the best fit analysis of the H^+/COX ratios measured in the CN^- -liganded oxidase giving pK_{ox} of 6.2 ± 0.2 and pK_{red} of 6.8 ± 0.1 and the best fit analysis of the difference between the H^+/COX ratios in the CN^- -liganded oxidase minus the H^+/COX ratios in the CO-liganded oxidase, giving pK_{ox} of 6.5 ± 0.2 and pK_{red} of 7.0 ± 0.1 . The H^+/e^- linkage ratios for heme a_3 (pink curves) were obtained from the best fit analysis of the difference between the H^+/COX ratios measured in the unliganded oxidase and the H^+/COX ratios in the CN^- -liganded oxidase giving two groups linked to heme a_3 with pK_{ox} values of 6.8 ± 0.2 and 7.4 ± 0.2 and pK_{red} values ≥ 12 respectively. For other details see [11].

Papa et al. have carried out an extensive study of the influence of kinetic and thermodynamic factors on the H^+/e^- stoichiometry of cytochrome *c* oxidase in mitochondria and in the isolated-reconstituted state (COV) [17,18,21]. The results of the investigations, based on measurement of the rates of electron flow and proton ejection at level flow, showed that the H^+/e^- ratio of proton pumping by the oxidase varies, both in intact mitochondria and in the isolated-reconstituted oxidase, from around zero, at extremes of low and high rates, to about one at intermediate rates (see also [14]). It was also found that the H^+/e^- ratios for proton ejection driven by ferrocyanide oxidation in COV increase with pH, up to about one at a pH around 8 and then decline at higher pHs [21]. When compared with the pH dependence of redox Bohr effects in the soluble oxidase, the pH dependence of the H^+/e^- ratio for proton pumping is similar to that observed for the H^+/e^- ratio of the Bohr effect linked to oxido-reduction of heme *a* (Fig. 2) [14].

Proton pumping in cytochrome *c* oxidase at the steady state was found to be decoupled by the ΔpH component of Δp [18]. This decoupling should not be confused with the Δp -dependent membrane leak. It has, in fact, been found that promotion of the steady-state proton leak in valinomycin and K^+ supplemented COV by the protonophore CCCP, which decreases the ΔpH , alleviates ΔpH -dependent slip in COV and increases the H^+/e^- ratio of the pump [18].

4. A cooperative proton pump and role of heme *a* in cytochrome *c* oxidase

It is generally accepted that electron flow in the oxidase coupled to proton pumping follows the sequence $cyt.c \rightarrow Cu_A \rightarrow heme\ a \rightarrow heme\ a_3-Cu_B$ [4] (Fig. 3). O_2 reduction to H_2O at the binuclear heme a_3-Cu_B center is considered by different groups to be directly coupled to proton pumping [4,12,22,23]. The crystallographic structures of *Paracoccus denitrificans* [23] and bovine heart cytochrome *c* oxidase [24] show a hydrogen-bond network of residues in subunits I and II which can provide an efficient electron transfer pathway from Cu_A to heme *a* (see Fig. 3). Two hydrogen-bond networks of subunits I and II residues can also be identified in both the bovine heart [24] and *P. denitrificans* [25] oxidase crystals (see Fig. 3), which could serve as direct electron transfer pathways from Cu_A to the heme a_3-Cu_B center [24]. Electron transfer from Cu_A to the heme a_3-Cu_B via heme *a* will be associated, through the cooperative linkage discussed above, to proton pumping. Electron transfer from Cu_A to the heme a_3-Cu_B , bypassing heme *a*, will result in decoupling of the pump. The distance from the lower Cu_A to the heme *a* Fe is $2.6\ \text{\AA}$ shorter than that to the heme a_3 Fe [23,24]. Thus the rate of electron transfer from Cu_A to heme *a* would be much faster than to heme a_3 as is, indeed, indicated by kinetic data [26] and calculated electron-tunneling pathway [27]. It is, however, conceivable that under conditions of high electron pressure on Cu_A and high transmembrane $\Delta\mu H^+$, which would exert inhibitory back pressure on proton coupled electron transfer from Cu_A to heme a_3-Cu_B via heme *a*, without obviously affecting the decoupled direct electron transfer to the binuclear center, the latter becomes significant. This could explain the observed decoupling of the proton pump in the oxidase observed at high rates of electron flow and the decoupling effect of transmembrane ΔpH reported above. There are, however, other possible mechanisms of slippage in the proton pump (see below and [16]). The crystal structure of cytochrome *c* oxidase can direct mutational analysis to verify the proposed occurrence of the two pathways for electron transfer from Cu_A to the heme a_3-Cu_B center.

Let us now examine in more detail the putative steps and residues involved in the proton pump of cytochrome *c* oxidase. The results on the redox Bohr effects in cytochrome *c* oxidase indicate that the transfer via heme *a* of each electron from Cu_A to the heme a_3-Cu_B center can be associated with the uptake of up to one proton from the N aqueous phase and its translocation towards the P side of the membrane. It may be recalled that involvement of a protolytic group linked to heme *a* in the proton pump of cytochrome *c* oxidase had already been proposed by Artzabanov et al. [28] and Wikström [29]. A role in proton pumping was also advocated for the formyl substituent of heme *a*, hydrogen-bonded to a protolytic residue in the protein [30]. Rousseau et al. [31] presented resonance Raman spectroscopic evidence for the presence of H_2O molecules near heme *a* and proposed that these H_2O molecules could shuttle protons from the N aqueous phase to protolytic group(s) whose pK is governed by the redox state of heme *a*. X-ray crystallography shows, in fact, H_2O molecules near heme *a* [25,32]. The crystal structure of subunit I of the *P. denitrificans* oxidase shows hydrogen bonds between R54 and the formyl of heme *a* [23] (Fig. 3A). In the bovine oxidase, in addition to the hydrogen bond between the

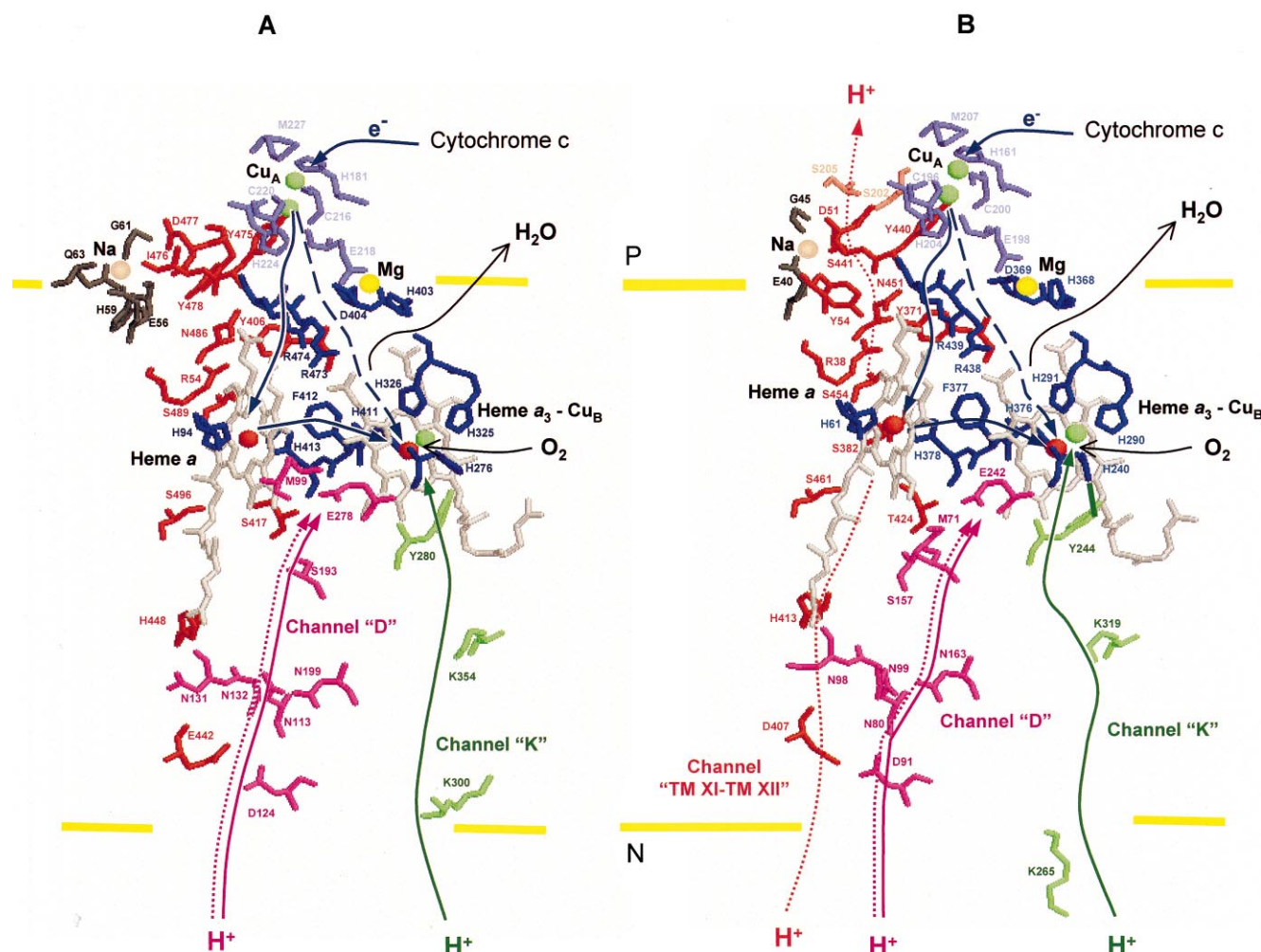


Fig. 3. View parallel to the membrane of the location in subunit I of cytochrome *c* oxidase of heme *a*, heme *a*₃-Cu_B and protolytic residues contributing to proton conducting pathways. Data from the PDB coordinates of the crystal structure of *P. denitrificans* (A) [23,25] and bovine heart (B) [24,32] cytochrome *c* oxidase, shown using the Ras Mol 2.6 program. The conserved protolytic residues contributing to the D channel, shown in magenta, are going from the N surface to the interior of the protein D124, N113, N131, N132, N199 in the *P. denitrificans* oxidase (Fig. 3A). These residues are followed by a cavity, lined with hydrophobic residues like S193, which can be filled by water molecules leading to E278 [23,35]. The corresponding D channel in the bovine oxidase, also in magenta, is made up of D91, N80, N98, N99, N163, S157, E242 [24] (B). The conserved protolytic residues of the K channel, shown in green, are K300, K354, Y280 intercalated by water-filled cavities in *P. denitrificans* oxidase [23,35] (A). The corresponding K channel in the bovine oxidase, also in green, is made up of K265, K319, Y244 (B). The protolytic residues of the TMXI-TMXII channel in the bovine oxidase [24], shown in orange, are D407, H413, S461, possibly intercalated by water-filled cavities (B). The corresponding proton pathways in the *P. denitrificans* oxidase, also shown in orange, are E442, H448, S496, drawn from [23] (A). The D51 proton release pathway in the bovine oxidase, shown in orange, is made up of D51, the peptide bond Y440–S441, Y54, R38 [32]. The corresponding proton release pathway drawn for *P. denitrificans*, from [23], also shown in orange, is made up of D477, the peptide bond Y475–I476, N486 and R54 (A). Coupled electron transfer pathway is indicated by solid blue arrows; decoupled electron transfer from Cu_A to the binuclear center by a dashed blue arrow. For other details see text.

conserved R38 and the heme *a* formyl, S382 can be hydrogen-bonded to the OH group of the farnesyl [24] (Fig. 3B).

X-ray crystallography reveals proton conduction pathways by which heme *a* and its surrounding residues can be connected to the N and the P aqueous phases [23,24,32]. Two distinct proton conduction pathways have been identified in the crystal structure of subunit I of cytochrome *c* oxidase which are remarkably similar in the *P. denitrificans* [23] and bovine heart enzyme [24] (Fig. 3). One, the D channel, leads, through conserved protolytic residues [33] and H₂O-filled cavities [32,34,35], from a conserved aspartic residue at the N surface (D91 in *Beef*, D124 in *Pden*) [33] to a conserved glutamic residue (E242 in *Beef*, E278 in *Pden*) located in the central part of transmembrane helix VI [23,24,32] (see Fig.

3). The other pathway, the K channel, starts with a conserved lysine at the N surface (K265 in *Beef*, K300 in *Pden*) [24,36] and leads through conserved protolytic residues and water-filled cavities [23–25] to a conserved tyrosine (Y244 in *Beef*, Y280 in *Pden*) which is covalently bound to a Cu_B histidine ligand (H240 in *Beef*, H276 in *Pden*) [25,32] (see Fig. 3). The K channel was hence proposed to conduct chemical protons from the N phase to the heme a₃-Cu_B center where they are consumed in the reduction of O₂ to H₂O. The D channel was, on the other hand, proposed to translocate pumped protons from the N space to the central glutamic residue (E242 in *Beef*, E278 in *Pden*) and from this to the Cu_B histidine ligands (H290 or H291 in *Beef*, H325 or H326 in *Pden*) whose redox-linked shuttling on Cu_B is proposed to represent the critical

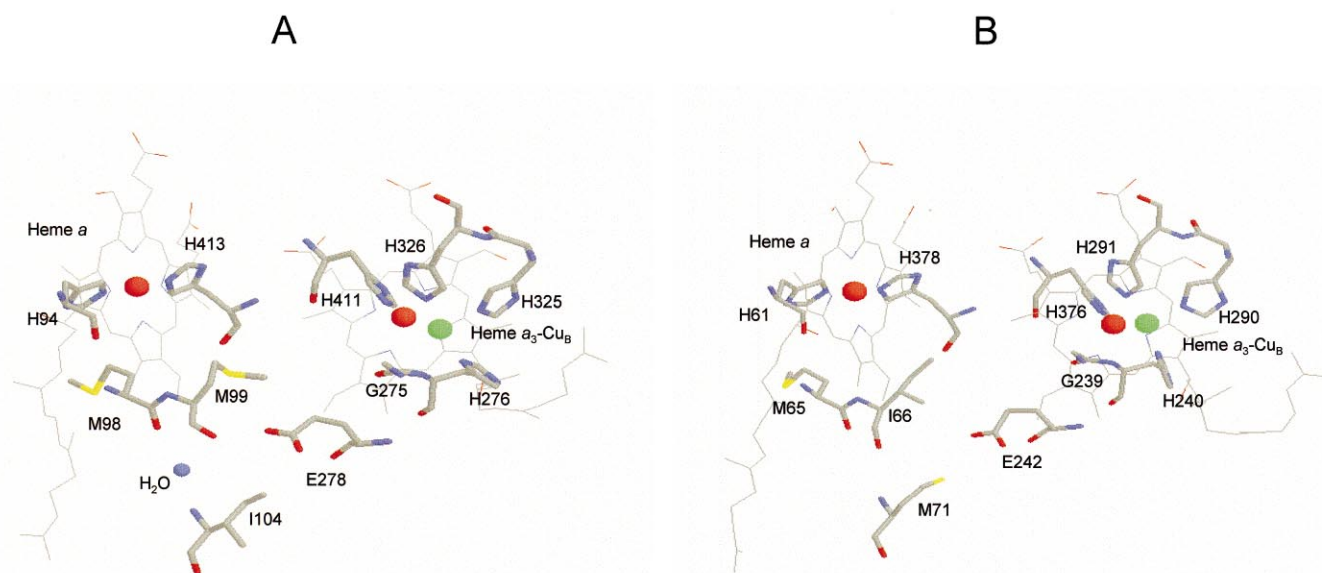


Fig. 4. Views parallel to the membrane of heme *a*, heme *a*₃-Cu_B and surrounding amino acid residues in cytochrome *c* oxidase. Data from PBD coordinates of the crystal structure of *P. denitrificans* (A) [23,25] and bovine heart (B) [24,32] cytochrome *c* oxidase, drawn using the Ras Mol 2.6 program. For other details see text.

step in the model of proton pumping denominated the ‘histidine cycle’ [22,23,34].

More recent observations seem to require these conclusions to be, at least in part, revised. (i) Mutational analyses by Konstantinov et al. [37,38] and others [39] have provided evidence indicating that whilst the K channel is only involved in the uptake of chemical protons in the transition from the ‘oxidized’ to the ‘peroxy’ intermediate of the binuclear center (the eu-oxidase half of the redox cycle) (see Fig. 5), the D channel would transfer both pumped protons and chemical protons consumed in the reduction of H₂O₂ to 2 H₂O at the binuclear center (P→F and F→O steps, the peroxidase half of the cycle) (see Fig. 5). (ii) The latter point seems to be consistent with the observation that mutational replacement of conserved protolytic residues in the D channel suppresses proton pumping but also causes inhibition of electron flow [33]. (iii) The recent X-ray analysis at 2.3–2.5 Å resolution of the bovine enzyme shows the three histidine ligands to Cu_B to be bound to the metal in both the oxidized and the reduced oxidase crystal, as well as in the azide-liganded form ([32], see also [25]). This structural information, as well as the functional characterization of the protolytic group apparently linked to Cu_B (see Fig. 2 and text), indicating transition of one of the Cu_B-bound histidines between the imidazole/imidazolium state, but not imidazolate (pK_a ≈ 14), with a maximum H⁺/e[−] coupling number of 0.3 [11], do not support the ‘histidine cycle’ model of the proton pump, at least in the version based on redox-linked binding change at Cu_B of one of the three histidines between Cu_B-bound imidazolate, imidazole and free imidazolium [22,23,34]. E242 (*Beef*, E278 in *Pden*) could, however, be in protonic connection with the heme *a*₃ propionates [23,40], or with the same oxygen reduction intermediates (P or F compounds at the binuclear center) (see Fig. 5). This glutamic residue seems to deserve particular attention. Its mutational replacement by a cysteine blocks proton pumping and causes inhibition of electron flow with

accumulation of the peroxy intermediate [41]. Infrared spectroscopy [42], molecular dynamics [35] and statistical-mechanical potential of mean force [34] indicate that E242 (*Beef*, E278 in *Pden*) may be connected by bound water molecules to the binuclear center. The crystal structure of subunit I of bovine heart oxidase (Fig. 4) shows that the conserved E242 (facing a cavity delimited by helices VI, II and X) can, on one side, establish a protonic connection, through protolytic residues of helix II and bound water molecules, with H61, ligand to the heme *a* Fe, or with protonatable groups of the heme *a* porphyrin. FTIR spectroscopy results indicate that the ionization state or the position of E278 (*Pden*) is coupled to electron transfer to/from heme *a* [43]. Mutation in *Saccharomyces cerevisiae* of the leucine corresponding to I66, located in the bovine oxidase between E242 and heme *a* (see Fig. 4), has been found to lower the heme *a* redox potential and its pH dependence [44]. On the other side, E242 is close to the backbone of H240 and could be connected by water molecules to the binuclear center [34,35]. On the basis of new crystallographic data on the bovine oxidase Yoshikawa (personal communication), revising a previous statement [32], proposes that E242 can transfer protons to monoatomic oxygen reduction intermediates bound at the heme *a*₃ Fe, through hydrogen bonds involving the peptide carbonyl of G239 and Cu_B-bound OH[−]/H₂O. Increased resolution of the *P. denitrificans* oxidase crystal shows that the carboxylic group of E278 is hydrogen-bonded to the backbone carbonyl oxygen of M99, which is located further towards the P side, closer to heme *a* (Figs. 3A and 4) [25]. The carboxylic group of the corresponding E242 is found in the crystal of the bovine oxidase to be hydrogen-bonded, both in the oxidized and in the reduced state, to the sulfur atom of M71 which is located below E242 in the D channel [32] (Figs. 3B and 4). These observations substantiate a structural and functional connection of E242 (*Beef*, E278 in *Pden*) with heme *a* and its surrounding residues, as well as a protonic connection of this glutamic with the binuclear

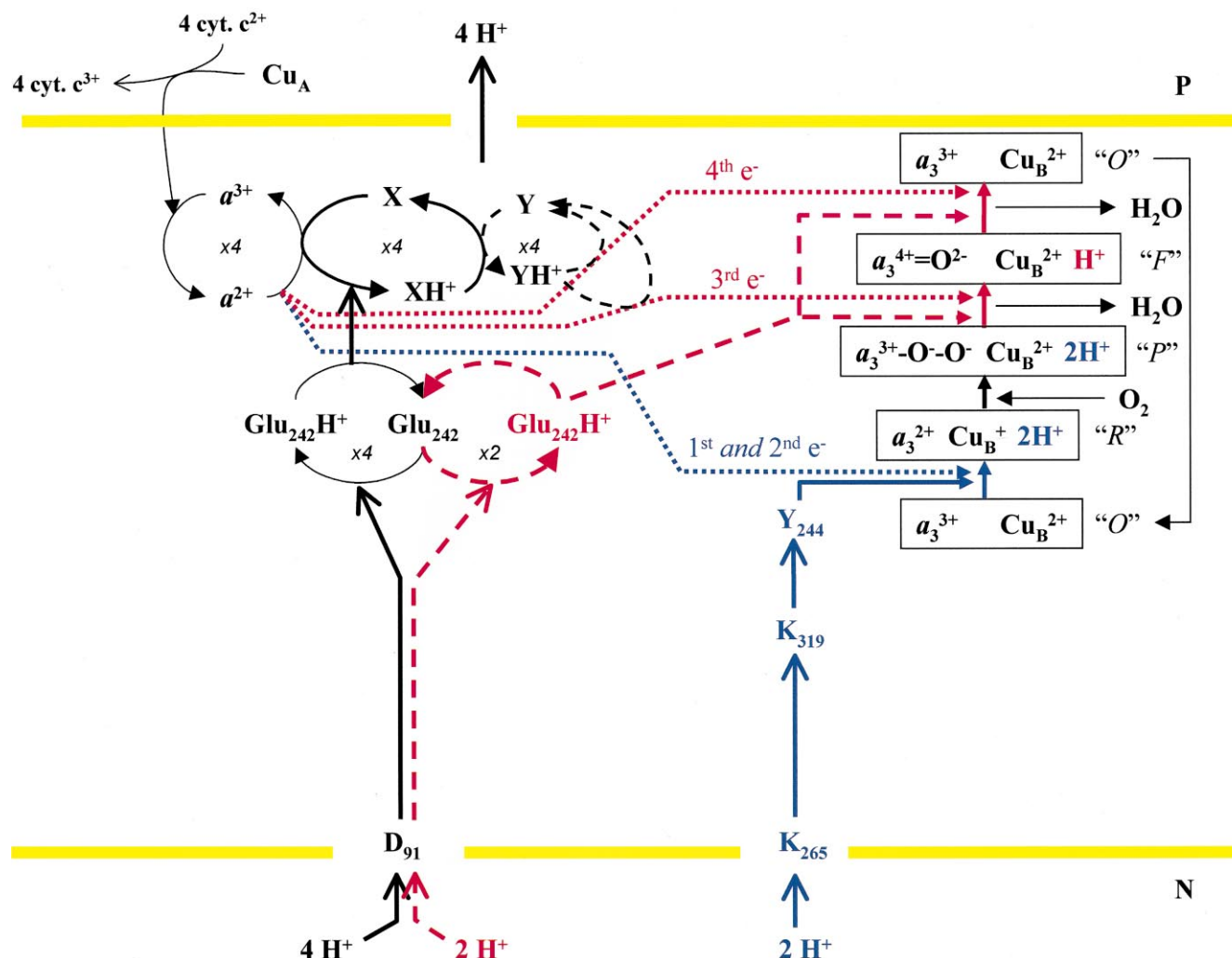


Fig. 5. A cooperative proton pump model in cytochrome *c* oxidase with the involvement of the vectorial Bohr effect linked to heme *a* and proton conduction pathways identified by mutational analysis of heme-copper oxidases and X-ray crystals of *P. denitrificans* and bovine heart oxidases. Solid black lines denote the pathway of pumped protons, dashed red (channel D) and solid blue lines (channel K) denote pathways of chemical protons consumed in the chemistry of O_2 reduction to H_2O . Dashed black line shows possible cooperative coupling, involving a Y proton trap, between the utilization of the third and fourth electrons and the output pathway of pumped protons. Dotted lines show the transfer of the first and second (in blue) and the third and fourth (in red) electrons from heme *a* to the binuclear center in the reduction of O_2 to H_2O . Yellow lines denote the inner (N) and outer (P) surfaces of the membrane. The P intermediate is shown as a O–O peroxy compound. Evidence has, however, been presented that the O–O bond can be broken at this stage with generation of a hydrogen-bonded oxoferryl or ferriyl-oxene form [51]. For details see text.

center or with the same intermediates of oxygen reduction (P and/or F) (Fig. 5).

Whilst the D channel might constitute the conduction pathway for protons from the N phase to the heme *a* environment, the recent X-crystallographic analysis of the bovine oxidase by Yoshikawa et al. [32] indicates the existence of a pathway for proton release from heme *a* to the P phase. These authors found that D51, in the G49–N55 segment located at the P side of subunit I, moves upon reduction of the oxidase by 4.5 Å from a buried position to one in which its carboxylic group becomes exposed to the P aqueous phase (Fig. 3B). In the oxidized state D51 would be in protonic connection with the N aqueous phase through hydrogen-bonded protolytic residues interacting with heme *a*: these are the peptide bond Y440–S441, Y54, R38. Yoshikawa et al. [32] propose that D51, moving upon reduction of the oxidase (in particular heme *a* which interacts with the hydrogen bond network mentioned above) from inside to outside, undergoes a p*K* decrease

and releases pumped protons in the P phase. Upon reoxidation of the enzyme, D51 moves back to the interior of the protein and is reprotated by N protons through the peptide bond Y440–S441. This bond, with the NH group hydrogen-bonded to D51 and the CO group to Y54, will act as unidirectional proton transfer path, since the equilibrium of the two tautomers, –CO–NH– and –C(OH)=N–, is largely poised towards the former [32]. It has, however, been pointed out that the kinetics of this tautomerization is debatable [45].

It should be recalled that the oxidation of the metal centers of the oxidase (and of heme *a* in particular) is found to be associated with net proton release from the protein (Fig. 2) [11,12,15,46]. The role of the redox-linked movement of D51 is also compatible with proton release upon oxidation of the metal centers. In this case, the carboxyl of D51 in the deprotonated state would move, upon reduction, towards the P side leaving the proton on H_2O hydrogen-bonded to protolytic residues in the protein. Then a hydronium ion is released in

the P phase upon oxidation of the enzyme [32]. The protolytic residues of the D51 proton release pathway in the bovine oxidase are not all conserved in prokaryotic oxidases (see Fig. 1). A possible proton-conducting network of protolytic residues, analogous to the G49–N55 segment of the bovine oxidase can, however, be identified in the crystal structure of subunit I of *P. denitrificans* oxidase, which extends from the domain of heme *a* to the P surface (Fig. 3A). This network would be made up of R54, N486, the peptide bond Y475–I476 and D477.

In Fig. 5, a scheme, based on the information summarized, is presented which attempts to describe the steps of proton translocation associated with reduction of dioxygen to H₂O by ferrocycytochrome *c* in cytochrome *c* oxidase (bovine residue numbering is used). Upon delivery to the oxidase of the first electron (of the four which reduce O₂ to 2 H₂O), reduction of heme *a*, through enhancement of the p*K* of a critical residue of its environment in protonic connection with E242 (or of E242 itself), will result in the uptake, via channel D, of the first pumped proton from the N aqueous space. With the transfer of the first electron from heme *a* to the binuclear center the proton is released in the P aqueous phase directly, or transiently trapped by a second acceptor [12] at the P side of subunit I. The proton release pathway could be contributed by the output structure of D51 which undergoes the redox-linked conformational change described above. The same events apply to the transfer of the second electron via heme *a* to the binuclear center. Arrival of the first two electrons at the binuclear center is associated with uptake, via channel K, of two protons to neutralize the negative charges introduced there, followed, upon oxygen binding, by formation of the peroxy compound [37].

Arrival of the third and fourth electrons will result, upon two successive reductions of heme *a*, in the uptake, via channel D, of the third and fourth pumped protons. If the third and fourth pumped protons are transiently trapped, like the first two, by protolytic groups in the oxidase, up to this stage no transmembrane charge translocation will take place. Upon transfer of the third and fourth electrons to the binuclear center and their utilization in the P→F and F→O steps, the four pumped protons are released in the P aqueous phase with transmembrane translocation of four positive charges. In the absence of transient trapping one proton will be translocated, from the N to the P aqueous phase, for each electron transferred by heme *a* (from Cu_A to the binuclear center).

In the catalytic cycle of the oxidase, reductive cleavage of the peroxy and ferryl compound are the two steps associated with the largest Δ*G* [4]. Response of the redox equilibria of O, F and P intermediates to mitochondrial energization by ATP [47] as well as direct dynamic measurement of charge translocation in the oxidation of reduced cytochrome *c* oxidase by O₂ [48] and in the isolated P→F and F→O transitions [38], and of proton translocation in the peroxidase reaction [49] have provided evidence indicating that the P→F and F→O steps are each coupled to transmembrane translocation of 2 H⁺/e[−]. If this is indeed the case, one has to think that the P→F and F→O steps induce a conformational change and/or electrostatic forces, extending from the binuclear center domain to the proton exit pathway described (see Figs. 3 and 5), which result in the release of the trapped pumped protons in the P aqueous phase.

As already pointed out, evidence has been obtained indicat-

ing that the D channel, but not the K channel, can also be used for the transfer of the two chemical protons utilized in the P→F and F→O steps [36–38]. Upon transfer of the electrons from the heme *a* to the binuclear center, the p*K* of the group linked to heme *a* returns to the low p*K*₀ value; at the same time, strong negative charges are generated at the binuclear center by the arrival of the third and fourth electrons so that E242, rapidly reprotonated through channel D from the N phase, in the absence of proton delivery by the K channel, now transfers chemical protons to the binuclear center. It is possible that the transfer of the third and fourth electrons from heme *a* to the binuclear center and their utilization in P→F and F→O steps causes a movement of E242, with rupture of the hydrogen bond with M71 (E278 and M99 in *Pden*), or simply a rotation of the carboxylic group around this hydrogen bond, to a position in which it transfers the protons received from the D channel to the binuclear center ligands. This ‘glutamate switch’ might represent a critical step in the proton pump and its disturbance at high electron pressure and transmembrane Δ*pH*⁺ could also explain the decoupling effect observed under these conditions.

Michel [50] has developed a model in which each of the four reductions of heme *a* is coupled to the uptake of one proton via the D channel. The first proton is pumped in the P phase upon double reduction of O₂ to the P intermediate, the second and the third during the P→F transition, the fourth in the F→O transition.

We would like to stress that the scheme outlined in Fig. 5 is primarily intended to emphasize the role in the proton pump of cytochrome *c* oxidase of cooperative coupling at heme *a*. The specific pathways followed by the pumped and chemical protons in the polypeptide chain and the mechanism by which cooperative proton translocation, associated with oxido-reduction of heme *a*, is coupled to the oxygen-reduction chemistry at the binuclear center, remain to be defined. The critical role of E242 and of the D channel in proton pumping is supported by converging genetic, structural and mutational evidence. However, it could be possible that, at least in animal cytochrome *c* oxidase, the proton pump, always operated by cooperative coupling at heme *a*, involves another putative proton conduction pathway identified in the crystal structure of subunit I of the bovine heart oxidase which is contributed by protolytic residues of transmembrane helices XI and XII [31] (see Fig. 3B). Proton conduction along this pathway might be controlled by redox transition of heme *a* through hydrogen bonds between its porphyrin substituents and protolytic residues in subunit I [24,32]. The TM XI–TM XII pathway is also found in the *P. denitrificans* with the exception of one protolytic residue [25] (see Fig. 3A). It should, however, be recalled that the protolytic residues in this pathway are not conserved in other prokaryotic protonmotive heme-copper oxidases (see Fig. 1).

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