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Review

Allosteric interactions and proton conducting pathways in proton pumping aa_3 oxidases: Heme *a* as a key coupling element ☆

Nazzareno Capitanio [a], Luigi Leonardo Palese [c], Giuseppe Capitanio [c], Pietro Luca Martino [c], Oliver-Matthias H. Richter [d], Bernd Ludwig [d], Sergio Papa [b,c,*]

[a] Department of Biomedical Science, University of Foggia, Foggia, Italy
[b] Institute of Biomembranes and Bioenergetics, CNR, Bari, Italy
[c] Department of Basic Medical Sciences, Section of Medical Biochemistry, University of Bari 'Aldo Moro', Bari, Italy
[d] Molecular Genetics, Institute of Biochemistry, Biocenter, Goethe University, Frankfurt am Main, Germany

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ABSTRACT

In this paper allosteric interactions in protonmotive heme aa_3 terminal oxidases of the respiratory chain are dealt with. The different lines of evidence supporting the key role of H^+/e^- coupling (redox Bohr effect) at the low spin heme *a* in the proton pump of the bovine oxidase are summarized. Results are presented showing that the I-R54M mutation in *P. denitrificans* aa_3 oxidase, which decreases by more than 200 mV the E_m of heme *a*, inhibits proton pumping. Mutational aminoacid replacement in proton channels, at the negative (N) side of membrane-inserted prokaryotic aa_3 oxidases, as well as Zn^{2+} binding at this site in the bovine oxidase, uncouples proton pumping. This effect appears to result from alteration of the structural/functional device, closer to the positive, opposite (P) surface, which separates pumped protons from those consumed in the reduction of O_2 to 2 H_2O. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

The molecular/atomic mechanism of energy-transfer, proton pumping heme aa_3 terminal oxidases of prokaryotic and eukaryotic respiratory chain in coupling membranes has been, and still is a matter of intensive investigation in different laboratories, and remains to be fully understood.

Cytochrome *c* aa_3 oxidase (COX) has four redox centers: a binuclear Cu_A, titrating as a one electron transfer center, bound to the conserved subunit II, in a hydrophilic, periplasmically oriented domain at the outer (P) side of the membrane embedded COX, a low and high spin heme *a* and a_3 respectively and Cu_B, all bound to the conserved hydrophobic subunit I, in a location close to the outer (P) surface [1,2]. In prokaryotic and eukaryotic COX the concerted flow of four electrons from ferrocytochrome *c* to dioxygen via Cu_A, heme *a*, heme a_3 and Cu_B is coupled to pumping of up to four H^+ from the inner (N) to the outer (P) space separated by the membrane [3,4], in addition to the consumption of four H^+ from the N space in the reduction of O_2 to 2 H_2O [5,6] (Fig. 1). Since there are no hydrogen carriers in the oxidase, proton translocation has to involve cooperative thermodynamic linkage between electron transfer by the Fe and Cu at the redox centers and proton transfer by acid/base groups in the enzyme [6,12,13]. Moreover, proton conducting pathways in the protein have to assure proton input from the inner (N) aqueous phase and their transfer to the binuclear a_3–Cu_B oxygen reduction center, as well as to the protonmotive coupling centers, all located close to the outer (P) side of the membrane and, from these, proton (and H_2O) release into the P space [1,2].

COX exhibits a complex network of cooperative and anti-cooperative thermodynamic linkages between oxido-reduction of the metals and among these and acid/base groups in the enzyme, like metal ligands, heme porphyrin substituents and aminoacid residues in the protein [3,13–15]. Direct electrostatic charge neutralization can, in the first instance, account for thermodynamic electron–proton coupling in redox proteins [16]. Redox transitions of the metal centers, ligand binding, and protonation/deprotonation of aminoacid residues at a given site in the protein can, however, induce conformational changes in the tertiary/quaternary structure of the protein, affecting the proton transfer capacity of aminoacid(s) at distant sites in protonmotive redox proteins [6,12,13,17,18]. Heme aa_3 oxidases offer different examples of this type of allosteric, heterotropic, interactions [10,19–23]. Thermodynamic H^+/e^- linkage at Cu_A, hemes *a* and a_3 and Cu_B is manifested by the pH dependence of their midpoint potential [24]. By analogy with the alkaline

Abbreviations: COX, purified cytochrome *c* oxidase; COV, cytochrome *c* oxidase reconstituted in phospholipid vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylendiaminetetracetic acid; CCCP, carbonyl cyanide 3-chloro-phenylhydrazone

☆ This article is part of a Special Issue entitled: Respiratory Oxidases.

* Corresponding author at: Institute of Biomembranes and Bioenergetics, CNR at Department of Basic Medical Sciences, Section of Medical Biochemistry, University of Bari 'Aldo Moro', Policlinico, P.zza G. Cesare, 70124 Bari, Italy. Tel.: +39 080 5448541; fax: +39 080 5448538.
E-mail address: s.papa@biochem.uniba.it (S. Papa).

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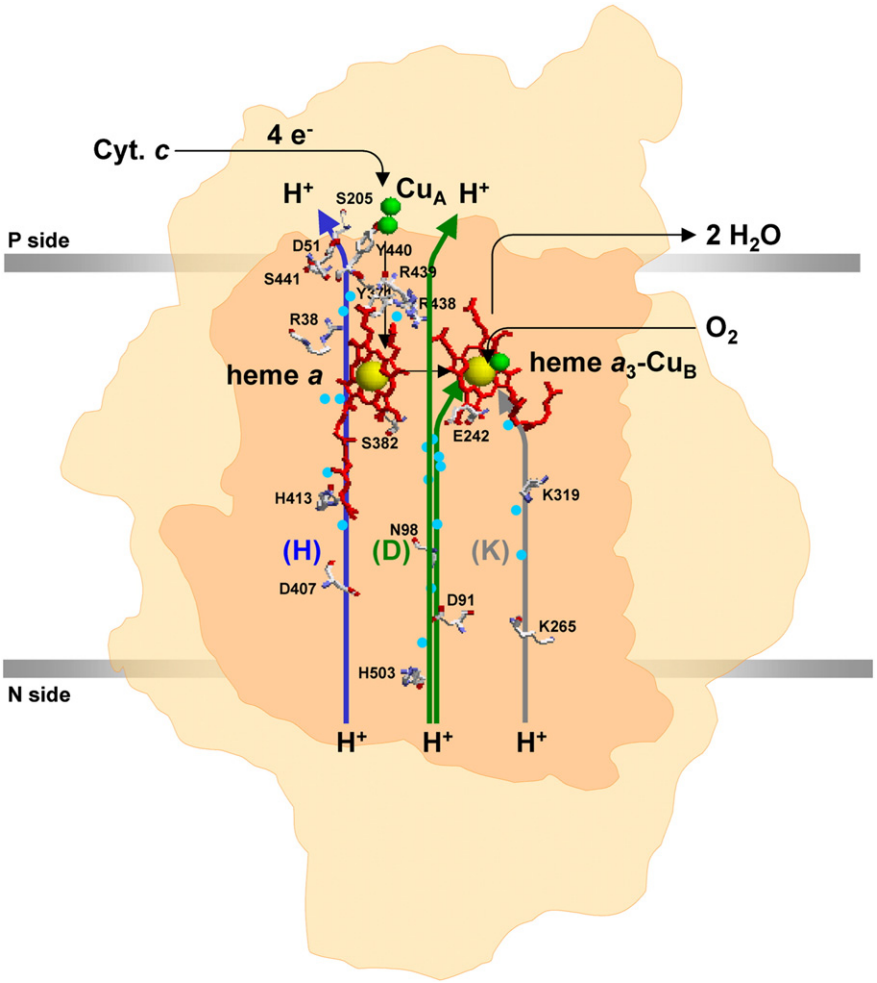

Fig. 1. View perpendicular to the membrane of the silhouette (pale yellow) of the 13 subunit monomer of bovine cytochrome *c* oxidase with the location in subunit I (deep yellow) of heme *a*, heme a_3–Cu_B and residues contributing to proton conducting pathways. The figure was created using the RasMol 2.6 program and the PDB ID: 2DYR atomic coordinates of bovine cytochrome *c* oxidase. The proton pathways are drawn as colored arrows: the gray, the green and the blue arrows indicate the K, D and H channels respectively. The electron transfer pathway is indicated by thin black arrows. The K pathway mediates the conduction from the N space to the a_3–Cu_B binuclear center of two of the four scalar protons consumed in the reduction of O_2 to two H_2O in the O→MV transition (see Fig. 6). The other two scalar protons for H_2O formation are conducted from the N space to the binuclear center by the D pathway (P_M→F and F→O transitions). The D pathway appears to be involved, at least in the prokaryotic oxidase, also in the translocation of the four pumped protons from the N space to the coupling site of the proton pump [7–9]. An additional H pathway identified in the bovine enzyme could, however, transfer at least two of the pumped protons [10,11].

and oxidation Bohr effect in hemoglobin, the thermodynamic H^+/e^- linkage in membrane bound cytochromes is denominated redox Bohr effect [6,11,12].

The current models of proton pumping in the oxidase can be grouped in two types: (i) those in which the allosteric cooperative coupling is conceived to be exclusively associated with the oxygen-reduction chemistry at the a_3–Cu_B binuclear center [8,9,20] (ii) those in which proton transfer coupled to electron transfer at heme *a* (and Cu_A) also plays a key role in the pump [3,25–31].

Mutational analysis (for a review see [32]) and X-ray crystallographic structures [1,2] have identified in both prokaryotic [1] and bovine heart COX [2] two conserved proton translocation pathways in subunit I of the enzyme, the K and the D pathways (Fig. 1). The K pathway mediates, from the N space to the a_3–Cu_B center, the conduction of two of the four scalar protons consumed in the reduction of O_2 to $2H_2O$ [1,2,33,34]. The other two protons consumed in the H_2O formation are conducted from the N space to the binuclear center by the D pathway [1,2,20,33,34]. The D pathway appears to be involved, at least in the prokaryotic COX, also in the translocation of the pumped protons from the N side to the coupling center [1,20,33,34]. The X-ray crystallographic structure of the bovine COX shows also an additional H proton pathway, with a water channel extending from the N space to the environment of heme *a*, and a hydrogen bond pathway from this to the P space [2,29]. This third pathway does not seem to be equally defined in prokaryotic COX [1,32,35]. By a sophisticated approach in HeLa cells, Yoshikawa et al. [36] have produced a hybrid COX in which 70–80% of the endogenous COX subunit I was replaced by a bovine subunit I, with different site-directed mutations of critical residues in the H pathway. All of these resulted, individually, in complete suppression of proton pumping [36]. On the other hand site-directed mutations in prokaryotic COX of residues in subunit I, putatively involved in the H pathway, do not result in suppression of proton pumping [35,37]. The question arises, whether the H pathway, specifically evolved in mammalian COX, thus providing an efficient proton pump pathway, preventing consumption of "pumped" protons for reduction of O_2 to water. It could, alternatively, be possible that the H and the D pathways contribute to different phases of proton pumping in COX (see [11]). The role of heme *a* in the proton pumping activity of aa_3 oxidases and the nature of the molecular structural/functional barrier which prevents consumption of the pumped protons in the reduction of O_2 to water are addressed in this paper.

2. The role of heme *a* in proton pumping

A key role of the low-spin heme *a* in the proton pump of COX is consistently supported by different, converging observations essentially observed in the bovine enzyme [2,11,25–31].

In anaerobic redox titration of the CO-ligated bovine COX the low-spin heme *a* and Cu_A exhibit perfectly superimposed pH dependence with a decrease of the midpoint redox potential for both of 15–20 mV/pH unit in the range 6.0–8.5 [31]. In this pH range oxidation of heme *a* and Cu_A was experimentally shown to result in the release, and, on reduction of these cofactors, in the uptake of around 1 H^+/COX [31]. The same H^+/COX ratio is measured in the CO-ligated *P. denitrificans* COX (Fig. 2, see also [38]). This ratio of electron/proton coupling at only one of these two centers, let say heme *a*, would be inconsistent with the decrease of the respective E_m of only 15–20 mV per pH unit increase [39,40]. This apparent difficulty [40] is however solved by the superimposed pH dependence of heme *a* and Cu_A redox potentials [31,41]. This implies that oxido-reduction of the two metals shares cooperative linkage with p*K* shifts of two or more acid/base groups whose overall balance results in the observed H^+ exchange [31,41].

An implication of interactive coupling of the oxidoreduction of both Cu_A and heme *a* with p*K* shifts in a common cluster of protolytic groups is that, whilst one electron reduction of Cu_A or heme *a* is sufficient to produce maximal protonation of the cluster, release of the proton from the cluster will take place only when both heme *a* and Cu_A are oxidized (see for details ref. [41]).

The phenomenological quantitative agreement between the pH dependence of the redox potential of heme *a* (and Cu_A) with the H^+ transfer associated with oxido-reduction of these two centers, and the equivalence of this coupling number with the maximal stoichiometry of proton pumping by the oxidase, amounting up to ≈1 H^+ per electron transferred from cytochrome *c* to oxygen, are essential prerequisites for a role of heme *a* in the proton pump mechanism [11].

In bovine COX, non-resonant Raman spectroscopy revealed a redox-sensitive peak, assigned to a transition in the stretching vibrational mode of the C=C or C=N bond in the imidazole ring of one of the two axial histidine ligands of the low spin heme *a* [42]. The X-ray

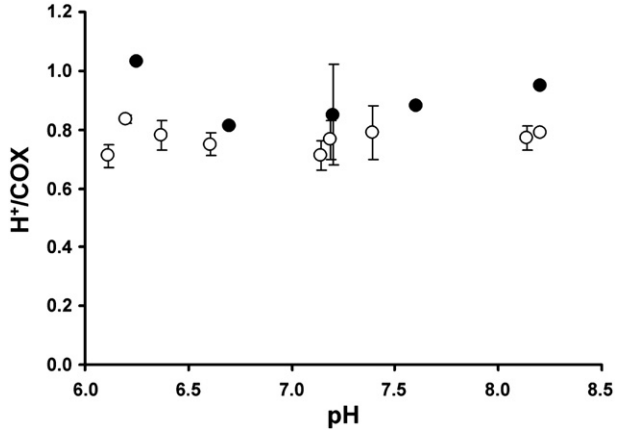

Fig. 2. pH dependence of H^+/COX ratio for proton release associated with anaerobic oxidation of heme *a* and Cu_A by ferricyanide in soluble CO-inhibited bovine heart and *P. denitrificans* COX. Empty circles: H^+/COX ratios associated with oxidation of heme *a* and Cu_A in the CO-ligated bovine COX (data from ref. [31]). Full circles: H^+/COX ratios associated with oxidation of heme *a* and Cu_A in the CO-ligated *P. denitrificans* COX. In CO-inhibited COX heme a_3 and Cu_B are clamped in the reduced state. For both bovine and *P. denitrificans* COX, the same experimental conditions were used (see ref. [31]). 3.5 μM purified cytochrome *c* oxidase was suspended in 0.15 M KCl and 0.1 mM EDTA and supplemented with 3.5 μM cytochrome *c*, 0.2 mg/ml broken beef heart mitochondria and 0.5 μg of rotenone/ml. The suspension was bubbled first with nitrogen and then with pure CO. Addition of 2 mM succinate to CO-saturated COX solution covered by a layer of deareated mineral oil caused formation of the fully reduced CO-liganded cytochrome *c* oxidase in 10–15 min. The oxidation of COX was obtained adding to the fully reduced CO-liganded cytochrome *c* oxidase, supplemented with 0.1 μM antymicin A plus 0.3 μM myxothiazol, an anaerobic amount of ferricyanide stoichiometric with the sum of the reduced metal centers, these amounted in the CO-ligated oxidase to two times the amount of COX reduced plus cytochrome *c*. The H^+/COX ratios refer to the proton release following COX oxidation and are given as μM changes. For further details see [31].

crystallographic structures of the oxidized and fully reduced bovine oxidase, obtained by the Yoshikawa group [29], reveals that reduction of the oxidase results in a few degrees of rotation of the imidazole plane of I-H378 on the axis perpendicular to the porphyrin plane. Resonance Raman spectroscopy shows that oxido-reduction of heme *a* is associated with structural perturbation in its environment, a change, in particular, in the strength of the hydrogen bond between the porphyrin formyl-carboxyl and a basic residue in the protein [26], likely the conserved I-R38 (bovine numbering) in subunit I [29]. X-ray crystallograghic analysis also shows that reduction of the oxidase causes the loss of a hydrogen bond between the OH group of the hydroxyfarnesyl substituent of heme *a* and I-S382, with movement of the serine and the hydrocarbon chain of the hydroxyfarnesyl group in the putative H proton channel [29]. A conformational wave, induced by reduction of the oxidase, reaches the outer cytosolic surface of subunit I in contact with subunit II. A segment of subunit I from I-G49 to I-N55 moves towards the surface with the carboxylic group of I-D51 becoming exposed to the aqueous phase [29].

The membrane sidedness of Bohr protons linked to oxido-reduction of the metal centers in liposome reconstituted purified bovine COX has been thoroughly analysed by Papa et al. [11] (see also refs. [25,43]). The results of the measurements in Papa et al. [11] show, directly, that Bohr protons coupled to anaerobic oxido-reduction of heme *a* (and Cu_A) exhibit membrane vectoriality, i.e. protons are taken up from the N space upon reduction of these centers and released in the P space upon their oxidation, just as expected for their involvement in proton pumping. Redox Bohr protons coupled to anaerobic oxido-reduction of heme a_3 do not, on the contrary, exhibit vectorial nature: protons are taken up upon reduction and released upon oxidation only in the outer P space [11].

3. The impact on electron flow and proton translocation of the I-R54M mutation in *P. denitrificans* heme *a* environment

The impact of the I-R54M point mutation on electron transfer and proton translocation was studied separately by Kannt et al. [44] and by Jasaitis et al. [45]. Kannt et al. [44] found that the I-R54M mutation in *P. denitrificans* caused a blue shift of the heme *a* α-band by 15 nm, similar to that observed by Callahan and Babcock to be caused by alkaline pHs [46], and a marked decrease of the midpoint redox potential. In this mutant the site of heme *a* was also partially occupied by heme *o*, which has even a lower midpoint redox potential [44]. The changes caused in the low-spin heme by the I-R54M mutation are likely to be due to the loss of the hydrogen bond between I-R54, located in the proximity of the heme [2,29] and the formyl substituent in the porphyrin ring of the heme *a* (see also [47]). The hydrogen bond is thus essential for the specific binding in subunit I of the low spin heme *a*, appearance of its characteristic spectrum and adjustment of the redox potential to a value which favors sequential $Cu_A \rightarrow$ heme $a \rightarrow a_3Cu_B$ electron transfer. Investigation on the I-R54M mutant by Jasaitis et al. [45] confirmed the dramatic impact on the midpoint redox potential of the low-spin heme *a*. The changes in the low-spin heme *a* caused by the I-R54M mutation resulted in a decrease of the COX turnover to a small percentage of that exhibited by the wild type COX. Two alternative explanations were put forward for the residual electron transfer activity in the I-R54M mutant. According to Kannt et al. [44] electron transfer from Cu_A to the a_3–Cu_B binuclear center could essentially occur in the I-R54M mutant COX directly, bypassing heme *a*. Jasaitis et al. [45] proposed, instead, that the residual electron transfer from Cu_A to the binuclear center still took place via heme *a*.

We have investigated the impact of the I-R54M mutational replacement on the electron transfer and proton pumping activity of *P. denitrificans* COX. Reduction of the detergent-solubilized I-R54M oxidase by ascorbate at a [cytochrome *c*]/[[oxidase] molar ratio around 1, shows that full reduction of cytochrome *c*, Cu_A and heme

a_3 can be reached whilst no reduction of heme *a* is detectable (Fig. 3A). Stopped flow spectrophotometric analysis of the oxidation by O_2 of the solubilized I-R54M mutant *P. denitrificans* oxidase is shown in Fig. 3B. The enzyme was pre-reduced in anaerobiosis by ascorbate, under conditions in which, whilst heme a_3 and Cu_A were completely reduced, no detectable reduction of heme *a* occurred (see Fig. 3A). Upon oxygenation, heme a_3 of the reduced binuclear center was converted in its oxidized form in about 200 ms. The oxidation of the binuclear center was synchronous with oxidation of Cu_A, both exhibiting a $t_{1/2}$ of 15–16 ms. It can be noted that these rates of oxidation of the binuclear center and Cu_A, observed in the I-R54M mutant, were much lower than those observed in the wild type oxidase in which both processes, as well as the oxidation of heme *a* (completely reduced by ascorbate in this case) were almost completed in the dead time of the instrument (1.25 ms, not shown). When electrons were delivered by ferrocytochrome *c* to the solubilized I-R54M oxidase treated with CN^-, which clamps heme a_3 in the oxidized state, whilst Cu_A was completely reduced, the reduction of heme *a* was negligible also when Cu_A was completely reduced. It can be noted that in the membrane reconstituted *P. denitrificans* COX the electrical potential generated by electron flow from Cu_A to heme *a* in the wild type COX, was absent in the I-R54M mutant COX [44]. The slow rate of Cu_A reduction could be a consequence of the altered heme *a* environment. Cu_A has, in fact, been found to share with heme *a* H^+/e^- cooperative linkage at a common acid/base residue cluster [31].

These observations make it likely that in the I-R54M mutant direct electron flow from Cu_A to the a_3–Cu_B binuclear center can take place, beside eventual residual electron flow via heme *a*.

When the purified *P. denitrificans* I-R54M mutant COX was incorporated in liposomes. Aerobic oxidation of ascorbate plus cytochrome *c* generated a steady-state transmembrane $\Delta\psi$ of the same magnitude as that observed in the wild-type COX vesicles (W-COV) (Fig. 4A). In the respiring steady-state ΔpH collapse by nigericin was replaced, both in W-COV and I-R54M-COV, by an increase in $\Delta\psi$, which was however slower and slightly smaller in the mutant enzyme. It should be recalled that the steady state $\Delta\mu H^+$ set up in respiring COV can be essentially maintained by the membrane anisotropy of the reduction of O_2 to H_2O, whereby electrons are donated to the oxidase by cytochrome *c* at the outer (P) side of the membrane and protons are taken up from the inner (N) aqueous space.

The H^+/e^- stoichiometry of proton pumping in COV, activated by electron delivery from ferrocytochrome *c* to the oxidized enzyme, either by the reductant pulse method or under level flow conditions, was then measured. Using the reductant pulse method, addition of 6.5–8 μM ferrocytochrome *c*, in the amount sufficient to generate 3–4 reduction–oxidation cycles of the aerobic reconstituted COX, generated, in the presence of valinomycin plus K^+, rapid proton ejection into the medium, which in W-COV exhibited an H^+/e^- around 0.7 (±0.09) (Fig. 4B). In the I-R54M-COV ferrocytochrome *c* addition still generated proton pumping, but the H^+/e^- ratio was around 0.3 (±0.05) (Fig. 4B). The H^+/e^- ratio measured for proton consumption in the presence of CCCP associated with the reduction of oxygen to H_2O amounted to 0.83 (±0.08) in W-COV and 0.84 (±0.08) in I-R54M-COV. This shows that, besides proton pumping, ferrocytochrome *c* addition to COV induces some scalar proton release, which subtracts from the proton consumption in the reduction of O_2 to H_2O. Correction for this acidification process, of the proton ejection elicited by ferrocytochrome *c* in the coupled state leaves an H^+/e^- ratio for proton pumping of 0.5 in W-COV but only of 0.2 in I-R54M-COV, which, in the latter case, can be associated with residual electron flow via heme *a*.

Under level-flow conditions (Fig. 4C), the H^+/e^- ratio in W-COV decreased linearly from around 0.7 to 0.1 as the rate of electron flow was raised by increasing the concentration of cytochrome *c*. In I-R54M-COV the H^+/e^- ratio amounted to not more than 0.25 at low respiratory rates and decreased to zero as the respiratory rate

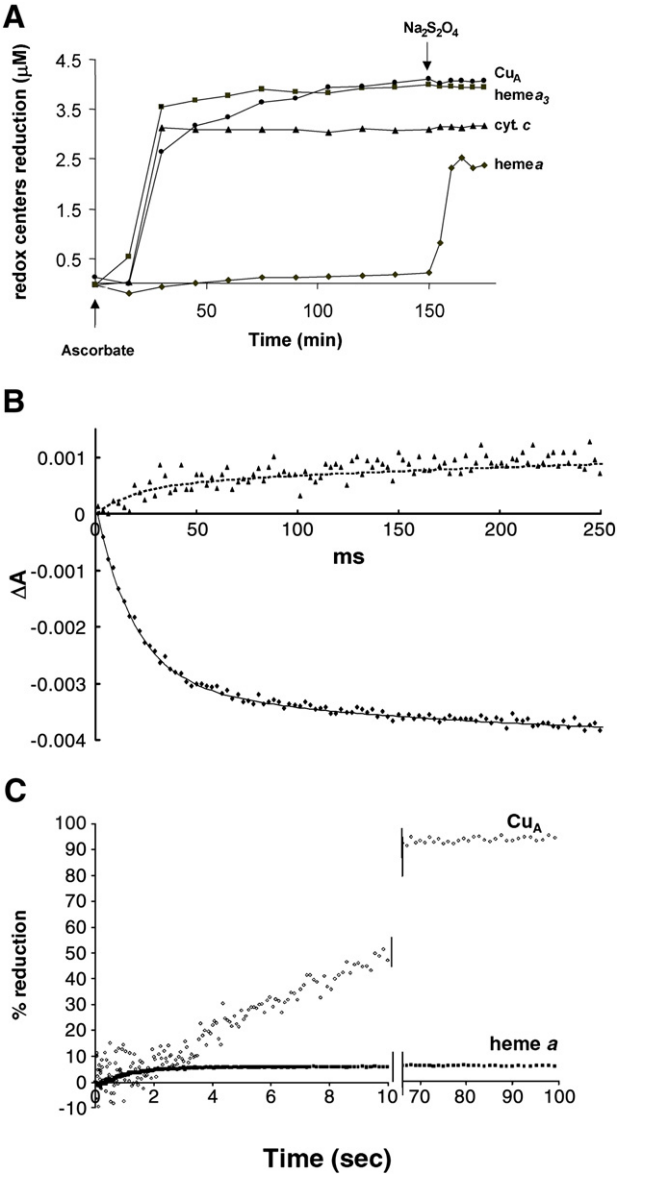

Fig. 3. Panel A. Reduction and oxidation of metal centers in the I-R54M mutant of *P. denitrificans* COX. Redox centers of the oxidase were reduced by the addition of 5 mM ascorbate to an aerobic suspension of the soluble enzyme supplemented with cytochrome *c* in a gas-tight system. Where indicated dithionite was added as saturated solution. The differential wavelength couples selected to follow the kinetics of the redox centers were 550–540 nm for cytochrome *c* ($\Delta\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), 800–710 nm for Cu_A ($\Delta\varepsilon = 1\ mM^{-1}\ cm^{-1}$). The specific contribution of heme *a* and a_3 was estimated by deconvolution of the absorbances at 592–630 nm and 612–630 nm. The R54M mutant oxidase exhibited the following extinction coefficients for the reduced minus oxidized spectra: $\Delta\varepsilon_{592-630} = 12.85\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{612-630} = 1.16\ mM^{-1}\ cm^{-1}$ for heme *a*; $\Delta\varepsilon_{592-630} = 1.32\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{612-630} = 3.23\ mM^{-1}\ cm^{-1}$ for heme a_3. Panel B. Oxidation kinetics of reduced I-R54M mutant *P. denitrificans* COX. Pre-steady state oxidation kinetics of soluble R54M mutant COX induced by oxygenation of the anaerobically reduced enzyme was analysed by a stopped flow spectrophotometer. The figure shows the observed absorbance changes (average of 5 independent traces) at the wavelength couples 612–630 nm (black diamonds) and 800–730 nm (black triangles), the latter after correction for the cytochrome *c* contribution at this wavelength couple. The single exponential fit of the experimental data are also reported as solid line (at the 612–630 wavelength couple) and dashed line (at the 800–730 nm wavelength couple). Oxidation was initiated by mixing the ascorbate reduced oxidase suspension with air equilibrated medium (mixing ratio 1:1). Panel C. Reduction kinetics of the KCN-inhibited R54M mutant *P. denitrificans* COX. Kinetic analysis was carried out with the stopped-flow spectrophotometer as in panel B. For reduction, KCN-inhibited I-R54M mutant soluble oxidase was mixed at 1:1 ratio with 25 mM ascorbate plus 1.5 μM cytochrome *c* containing medium.

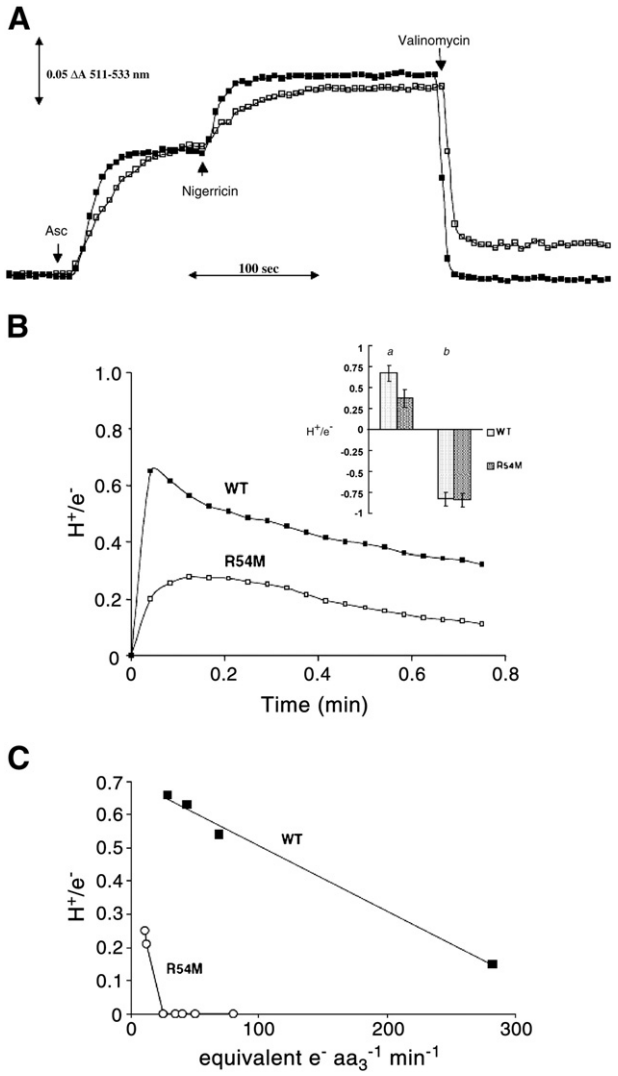

Fig. 4. Panel A. Membrane potential generation in wild type and I-R54M mutant *P. denitrificans* COX vesicles. Membrane potential generation in COX vesicles of wild type (filled squares) and R54M mutant (open squares) was measured spectrophotometrically at 25 °C using the ΔΨ-sensitive probe safranine as reported in [48]. COX vesicles were prepared essentially as reported in [49]. The $t_{1/2}$ for membrane potential generation were 14.5 s and 23 s for wild type and R54M mutant, respectively. The $t_{1/2}$ for the ΔΨ increment caused by nigericin were 9.7 s and 23 s respectively. Panel B. Proton translocation generated by ferrocytochrome *c* pulses in wild type and I-R54M mutant *P. denitrificans* COX vesicles. Proton translocation was measured at 25 °C by pulsing the aerobic cytochrome *c* oxidase vesicle (0.5 μM) with 6.5–8 μM reduced cytochrome *c*. The proton/electron ratios are the average of 8 and 7 independent experiments for the W-COV (solid squares) and R54M-COV (open squares) respectively. COX vesicles were suspended as in [4] and pH was monitored with a fast-responding glass combination electrode. Proton consumption in the uncoupled state was measured by pulsing the same amount of reduced cytochrome *c*, in the presence of CCCP. Inset: a) H^+/e^- ratios measured under coupled conditions in the reductant pulse experiments. b) H^+/e^- ratios for proton consumption measured in the presence of CCCP. The values reported are the average of 8 and 7 experiments for the W-COV and the R54M-COV respectively in each conditions and bars represent SD. Panel C. Rate dependence of the proton pumping activity in COX vesicles. Level-flow H^+/e^- ratios were measured in WT-COV (solid square) and R54M-COV (open circles) essentially as described in [4]. The reaction was started by adding different concentrations (1.5–6 μM) of ferricytochrome *c*, in the presence of 25 mM ascorbate.

was slightly enhanced. It can be noted that at any turnover rate the H^+/e^- ratio in the mutant was much lower, if not zero, as compared to the H^+/e^- ratios measured at the same rates in the wild COX. A rate-dependent drop of the H^+/e^- ratio for proton pumping has, previously, been observed in the case of the bovine heart cytochrome *c* oxidase [4]. It has been attributed to promotion, at high electron transfer rate, of direct electron flow from Cu_A to the binuclear a_3–Cu_B center thus bypassing heme *a* [4,13,50].

The above measurements, clearly, show that the I-R54M mutation in the *P. denitrificans* COX causes strong inhibition of proton pumping. Two causes can, in principle be conceived for the inhibition of proton pumping. One is that in the I-R54M mutation electrons flow directly from Cu_A to heme a_3 thus bypassing heme *a* (and heme *o*), whose redox turnover could be, not only thermodynamically but also kinetically, impaired in the mutant. The other is that the alteration of the environment of heme *a*, caused by I-R54M replacement, destroys cooperative H^+/e^- linkage at this site. Both effects can contribute to the inhibition of proton pumping. Their relative impact can vary depending on the actual reduction pressure exerted on the oxidase (see Fig. 4, panels B and C). Jasaitis et al. [45] showed that an oxygen pulse of the fully reduced I-R54M mutant oxidase resulted in an H^+ release close to that in the wild type enzyme. It can be noted that the oxygen pulse technique involved pre-reduction of COX in the presence of a large reduction input potential (15 μM cytochrome *c* plus 5 mM ascorbate). Under these conditions the oxidation–rereduction cycle might take place in a condition overcoming the thermodynamic/kinetic limitations caused by the I-R54M mutation.

4. Aminoacid replacement or Zn^{2+} binding at the N side of proton channels decouples proton pumping

It has been reported that I-N131D (*P. denitrificans* numbering, N98 bovine numbering) mutational replacement, at the N side of the D pathway suppresses proton pumping without inhibition of the rate of dioxygen reduction [21,51,52]. This finding has been taken as an evidence that pumped protons are exclusively translocated by the D pathway, which transfers 2 of the 4 protons consumed in the reduction of O_2 to 2 H_2O (namely in the P→F and F→O steps of the dioxygen reduction chemistry) [21,51,52] (see however [53]). Since direct inhibition of H^+ translocation in the Asn/Asp mutation should inhibit not only the transfer of pumped protons in the D pathway but also that of chemical protons, a conformational change induced by the aminoacid replacement has to be involved in the decoupling of proton pumping. Various lines of evidence indicate that the N131D replacement at the N side of the D channel alters the environment of E278 (*P. denitrificans* numbering, E242 bovine numbering), at the inner end of the D pathway, normally assuring alternative transfer of pumped and chemical protons [1,9,20–23,54,55].

We have analysed the impact of the I-N131D replacement in the *P. denitrificans* COX on the redox Bohr effects of the four metal centers (Table 1). In the unligated mutated COX the number of protons released upon oxidation of the four redox centers (hemes *a* and a_3, Cu_A and Cu_B) were practically the same as those measured in the wild type COX, except a small increase at alkaline pH. In the CN-liganded oxidase, in which heme a_3 is clamped in the oxidized state and only Cu_A, heme *a* and Cu_B can undergo oxido-reduction, the I-N131D replacement had no effect at all on the number of Bohr protons. These results seem to exclude the possibility that the decoupling of the pump caused by the I-N131D replacement is due to suppression of the cooperative H^+/e^- linkage at heme *a* and Cu_A.

Papa et al. [49,58] have recently studied the effect of the binding of exogenous Zn^{2+} at the N side of purified bovine COX, reconstituted in liposomes, on electron and proton transfer. Extended X-ray absorption spectroscopy EXAFS [58], and X-ray crystallographic analysis of Yoshikawa et al. [19], show that exogenous Zn^{2+}, bound to the N surface of COX is coordinated to a cluster of residues at the entrance of the D pathway close to the conserved I-D91 (bovine numbering) (Fig. 5). This Zn^{2+} binding resulted in 50% decoupling of the proton pump of COX without significant effect on the rate of oxygen reduction [49]. Analysis of the pH dependence of the inhibition by bound Zn^{2+} of proton release in the oxidative and reductive phases of the catalytic cycle of COX indicated that Zn^{2+} suppresses 2 of the 4 proton pumping steps in the cycle, namely those taking place when 2 OH^- produced in the reduction of O_2 at the binuclear center are protonated to 2

Table 1
Statistical analysis of H^+ release linked to anaerobic ferricyanide oxidation of the metal centers of soluble *P. denitrificans* cytochrome *c* oxidase in wild type and N131D variant. The values, mean ± S.E.M. of COX oxidation and H^+ release are given as μM changes. Site-directed mutagenesis (N131D variant) and purification of *P. denitrificans* COX were performed as in [23]. The CN-ligated oxidase (wild type or N131D mutant) was prepared as in [49]. The concentration of aa_3 was determined using an extinction coefficient at 445–480 nm of 181 mM^{-1} cm^{-1} for heme aa_3 [56]. The concentration of aa_3 in the CN-inhibited cytochrome *c* oxidase was determined using a $\Delta\varepsilon$ at 605–630 nm of 18.7 mM^{-1} cm^{-1} [56,57]. All measurements in the unligated and CN-ligated cytochrome *c* oxidase were performed as detailed in [49]. 1.5–2 μM aa_3 was suspended in 150 mM KCl, 0.01% n-dodecyl-β-D-maltoside and supplemented with 2 μM cytochrome *c*, 2 mM EDTA, 0.1 μM riboflavin, 30 μg/ml SOD and 3000 U/ml catalase. The anaerobic reduction of cytochrome *c* oxidase (and cytochrome *c*) was achieved by the photoactivated EDTA/riboflavin system as in [49]. Rapid oxidation of 2 μM cytochrome *c* oxidase (and 2 μM cytochrome *c*) was produced by the addition of an amount of anaerobic ferricyanide stoichiometric with respect to the sum of the reduced metal centers, these amounted in the unligated COX, to four times the amount of COX reduced plus cytochrome *c* (i.e. 10 μM) and in the CN-ligated COX, to three times the amount of COX reduced plus cytochrome *c* (i.e. 8 μM). The H^+/COX ratios reported were calculated dividing the amount of proton release by the amount of COX oxidized.

Experimental conditions	pH	H^+/COX	
		Wild type	N131D
Unligated	6.4	1.68 ± 0.09 (4)	1.61 ± 0.05 (5)
	7.3	1.45 ± 0.06 (4)	1.38 ± 0.02 (4)
	8.3	2.24 ± 0.01 (4)	2.45 ± 0.08 (4)
CN-ligated	7.3	0.98 ± 0.08 (4)	0.98 ± 0.06 (7)

H_2O (O → MV transition in the COX catalytic cycle, see Figs. 6 and 7 C) [49]. Analysis of the effect of Zn^{2+} on the redox Bohr protons in COX [49] indicated that this decoupling effect could be associated with a conformational alteration of an acid/base cluster linked to heme a_3 which normally prevents annihilation of pumped protons in the formation of H_2O.

In another study [59,60] site directed mutagenesis of the conserved I-K304L in subunit I of *B. subtilis* aa_3 quinol oxidase provided interesting information on the use of proton channels in the translocation of pumped and chemical protons. X-ray crystallographic analysis shows that the corresponding I-K300 in *P. denitrificans* [1] and I-K265 in the bovine enzyme [2] protrude at the N surface of the oxidase in a position which is close to the residues located in the K proton channel, but distant from the residues located at the entrance of the D proton pathway (Fig. 5). The I-K304L mutant in the *B. subtilis* oxidase did not alter significantly the respiratory rate, but strongly depressed proton pumping [59,60]. It is possible that the I-K304L replacement depressed the entry of the chemical proton in the K channel and, at the same time, induced, through an indirect effect, or by a short-circuit, consumption in the reduction of O_2 to 2 H_2O of the pumped protons in the D pathway.

5. Conclusions

The structural and functional features of the Bohr H^+ at heme *a* (and Cu_A) observed in the bovine COX, and the present observations on the I-R54M replacement in the *P. denitrificans* COX, qualify the cooperative H^+/e^- linkage at the low spin heme *a* as an essential element of the proton pump both in the bovine and prokaryotic COX. An essential role of the H^+/e^- coupling at heme *a* is independent of whether H^+ conduction from the N space to the environment of heme *a* is operated by the H or the D proton pathway. Papa et al. already proposed in 1998 [28,60] a switch mechanism of the conserved I-E242 at the inner end of the D pathway, which was conceived to move, during electron transfer at the low spin heme (see also [61]) between two positions, one in which it transfers pumped protons to a C_1 cluster associated with the low spin heme *a* the other in which it transfers scalar protons to the binuclear site. This is similar to the I-E242 switch which has been proposed to ensure alternating transfer of protons in the D pathway to a yet unidentified proton pumping site and to the binuclear site for consumption in the reduction of O_2 to 2 H_2O [1,9,20–23,54,55].

Fig. 6 shows a schematic model of the mechanism of proton pumping in COX at the respiratory steady-state [7,49,62,63]. The scheme is drawn to emphasize the role of cooperative linkage between electron transfer by the low spin heme *a* and proton transfer by the acid/base residue cluster (C_1) at the heme environment. It is assumed that redox-coupled transfer of pumped protons through the C_1 cluster is an essential step in proton pumping. It should be recalled here that according to schemes proposed by other authors [7,63,64], pumped protons are directly transferred from the input channel, to an acid/base residue cluster in the environment of the a_3–Cu_B binuclear site. There are, however, observations indicating the existence of an extended hydrogen-bond network connecting acid/base residue clusters at heme *a* and a_3–Cu_B binuclear site [64]. The schemes show in red the overall transfer of pumped protons, in the various phases of the catalytic cycle, without a specification of the pathway used (H or D).

The cycle in scheme of Fig. 6 (see also Fig. 7) starts with the fully oxidized enzyme in which both the metals of the Fe_{a3}–Cu_B binuclear center bind either H_2O or OH^-, depending on the actual pH [65]. At physiological alkaline pH's of the N space (pH ≥ 7.4), which more closely matches the physiological condition [66] two OH^- are proposed to be

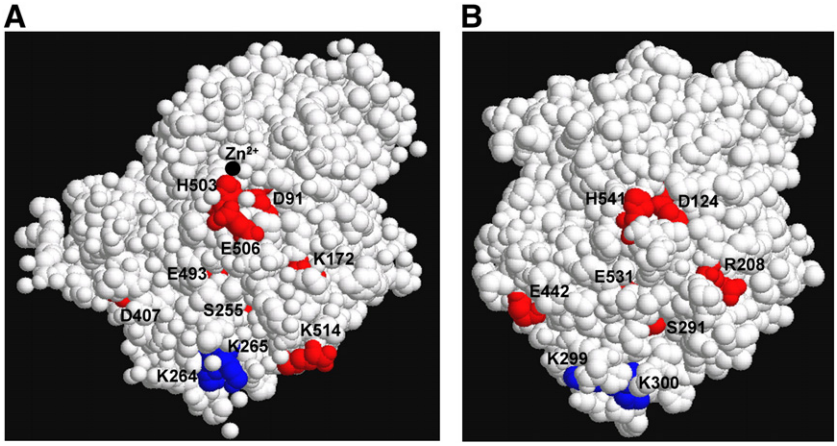

Fig. 5. Space-fill view of the mass of subunit I of bovine heart (A) and *P. denitrificans* (B) cytochrome *c* oxidase exposed at the N surface of the membrane. In different colors and with their respective numbering the conserved protolytic residues emerging at the N surface of subunit I located at the entrance of the D and K channel are shown. The binding site of Zn^{2+} in the bovine oxidase is shown near the entrance of the D channel. The pictures were elaborated with the RasMol 2.6 program using the PDB ID: 2DYR and PDB ID: 1AR1 atomic coordinates of bovine and *P. denitrificans* COX respectively.

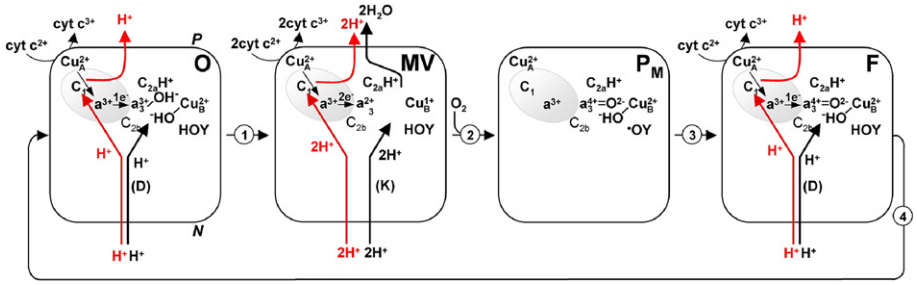

Fig. 6. Scheme of the proton pumping steps in the oxygen reduction catalytic cycle of COX at the steady-state respiring condition. P, outer aqueous space; N, inner aqueous space. O, MV, P_M and F represent the catalytic intermediates of the oxygen reduction cycle at the heme a_3–Cu_B and the nearby Y244 reaction site [7,49]. Red and black arrows indicate pumped and chemical proton transfer respectively through proton channels in the transitions from O to MV, P_M to F and F to O respectively. C_1 indicates a cluster of acid–base groups whose pKs are linked to redox transitions of Cu_A/heme *a*. C_{2a} and C_{2b} indicate clusters of acid–base groups whose pKs are linked to redox transitions of heme a_3 and Cu_B respectively. The cycle refers to a condition of alkaline pH. See [49] and text for further details.

transiently bound at the oxidized heme a_3–Cu_B binuclear site. At lower pH the OH^- groups could be already protonated in the oxidative phase of the cycle [65]. Upon transfer of two electrons from ferrocytochrome *c* to heme a_3/Cu_B, one at a time via Cu_A/heme *a*, with generation of the mixed valence (MV) intermediate, the two OH^- are protonated to 2 H_2O by 2 H^+, taken up from the N space by the K proton conducting pathway [7,34]. At the same time of H_2O formation 2 H^+ are pumped from the N to the P space. O_2, after binding at the reduced binuclear center, undergoes reductive cleavage [7,67,68]. Two electrons come from the oxidation of Fe^{2+}_{a3} to Fe^{4+}_{a3}, one from Cu^{1+}_B and the fourth, together with a chemical proton, from a tyrosine residue, with generation of the PM intermediate. The transfer of the third electron via Cu_A/heme *a* to the binuclear site, conversion of the PM to the F intermediate, results in the uptake of two H^+ from the N space. One H^+, together with the electron converts the tyrosine radical to protonated tyrosine, the other H^+ is pumped into the P space [7]. The transfer of the fourth electron via Cu_A/heme *a* to the binuclear center, which converts the F to the O intermediate is associated with the uptake of 2 H^+ from the N space. One is the fourth chemical proton utilized in the conversion of $Fe^{4+}_{a3} = O$ to Fe^{3+}_{a3}-OH^-, which, like the third, is conducted by the D pathway, the other is the fourth pumped H^+ [11], see also refs. [69–71].

The lack of proton pumping in the MV → P_M conversion might be consistent with the X-ray crystallographic analysis of bovine heart cytochrome *c* oxidase [10] showing that binding of CO or NO to Fe^{2+}_{a3}, likely also of O_2, induces a conformational change in helix X of subunit I, which would prevent free exchange of H^+ between the N space and the environment of heme *a*. On the basis of the crystallographic structures of the bovine heart oxidase Muramoto et al. [10] have proposed that upon reduction of Fe_{a3} and Cu_B 4 pumped protons are conducted from the N space, by the water channel of the H pathway in the open state, to the hydrogen-bond network at the environment of heme *a* (which includes I-R38, the formyl and propionate substituents of heme *a*, cluster C_1?). Upon O_2 binding at the binuclear site, with its reductive cleavage and formation of the P intermediate, the

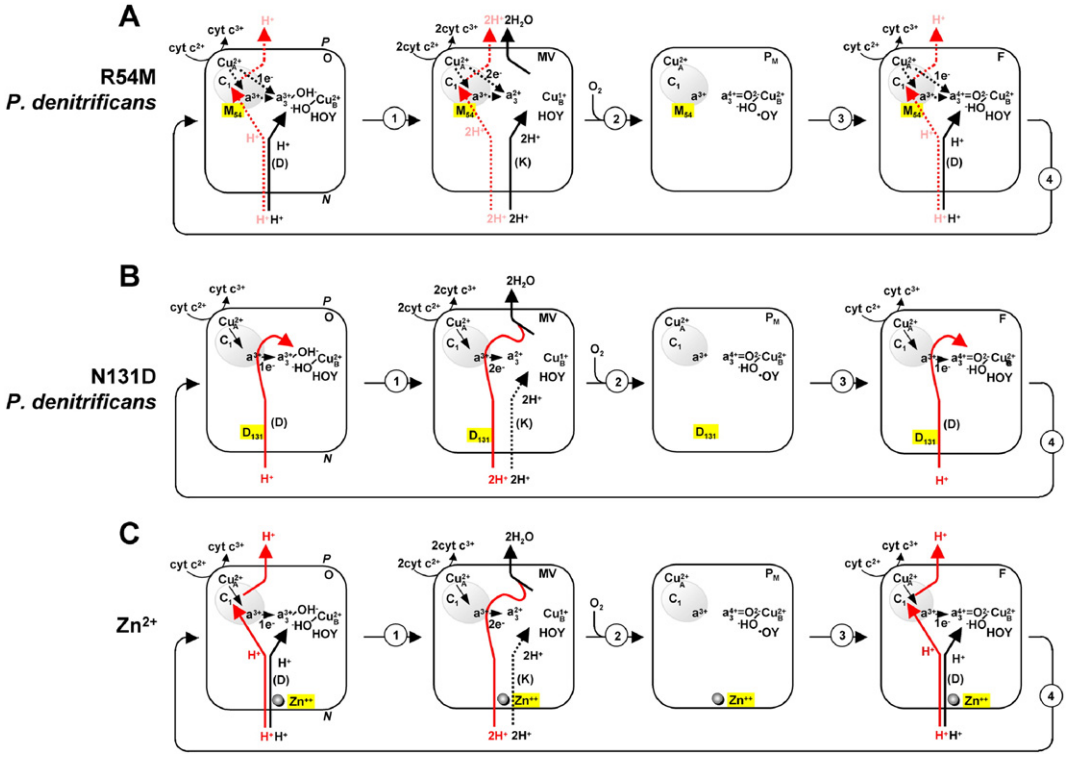

Fig. 7. Models of the protonmotive catalytic cycle of reduction of O_2 to 2 H_2O by ferrocytochrome *c* in COX in the coupling membrane at the respiratory steady-state. The cycles refer the conditions and specifications given in Fig. 6. Panel A refers to the catalytic cycle in the I-R54M mutant *P. denitrificans* cytochrome *c* oxidase; panel B refers to the catalytic cycle in the I-N131D mutant *P. denitrificans* COX; panel C refers to the catalytic cycle in the bovine heart cytochrome *c* oxidase in the presence of exogenous Zn^{2+} bound at the N surface of COX. In panel A red dot lines show depressed H^+ pumping; black dot lines indicate alternative routes of electron flow from Cu_A to the a_3–Cu_B binuclear center. In panels B and C the dotted black lines for the K channel mean replacement of H^+ translocation in the D channel. For further details see the text.

water channel of the H pathway in the bovine oxidase was proposed to acquire a closed state preventing further H^+ translocation. We would prefer a mechanism in which 2 pumped protons are translocated by the water channel of the H pathway in the O→MV step and the other 2 by the D pathway up to I-E242, followed in both cases by further transfer to a pump site above the hemes [11].

Fig. 7A presents a scheme of the possible mechanism by which the I-R54M mutational replacement in the *P. denitrificans* COX (R38 in bovine COX) results in depression of proton pumping. This aminoacid replacement, with the large decrease in the E_m of heme *a*, results in depression of proton pumping. This can be due to alteration of the H^+/e^- coupling function of the C_1 cluster as well as to direct electron flow from Cu_A to the a_3–Cu_B binuclear site.

The scheme in Fig. 7B depicts that the N131D mutational replacement at the N side of the D channel in the *P. denitrificans* COX (N98 in bovine COX) induces a conformational change in the enzyme, eliminating the gate function of the pump (at E278). In the wild enzyme, this assures alternative transfer of pumped and scalar protons preventing the consumption of all the protons transferred by the D pathway in the reduction of O_2 to 2 H_2O [1,20,23,54,55] (see also [60]).

The scheme in Fig. 7C refers to the decoupling effect exerted by Zn^{2+} binding at the N entry of the D channel [49,58]. In this case the Zn^{2+} binding can induce an allosteric conformational change in the enzyme, which alters a structural/functional barrier (likely at the heme a_3 environment) normally preventing consumption in the reduction of O_2 to 2 H_2O of the 2 H^+ pumped in the reduction of the oxidized enzyme to its mixed valence state. In conclusion the structural/functional devices, which assure separate translocation of scalar and pumped proton, can, under certain critical conditions, be circumvented with loss of the proton pumping capacity. A critical feature of proton pumping aa_3 oxidases is, in fact, the conservation of the structural/functional barrier, which prevents wasting pumped protons in the reduction of O_2 to water. Loss of this barrier can easily result in suppression of proton pumping, without inhibition, however, of the rate of O_2 reduction to water and the direct generation of $\Delta\mu H^+$ associated with the membrane anisotropy of this process as originally proposed by Mitchell [5] (see also [6]).

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