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Review

Cooperativity and flexibility of the protonmotive activity of mitochondrial respiratory chain

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Abstract

Functional and structural data are reviewed which provide evidence that proton pumping in cytochrome *c* oxidase is associated with extended allosteric cooperativity involving the four redox centers in the enzyme . Data are also summarized showing that the H^+/e^- stoichiometry for proton pumping in the cytochrome span of the mitochondrial respiratory chain is flexible. The ΔpH component of the bulk-phase membrane electrochemical proton gradient exerts a decoupling effect on the proton pump of both the bc_1 complex and cytochrome *c* oxidase. A slip in the pumping efficiency of the latter is also caused by high electron pressure. The mechanistic and physiological implications of proton-pump slips are examined. The easiness with which bulk phase ΔpH causes, at least above a threshold level, decoupling of proton pumping indicates that for active oxidative phosphorylation efficient protonic coupling between redox complexes and ATP synthase takes place at the membrane surface, likely in cristae, without significant formation of delocalized $\Delta\mu H^+$. A role of slips in modulating oxygen free radical production by the respiratory chain and the mitochondrial pathway of apoptosis is discussed.
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Keywords: Respiratory chain; Proton pumps; Cytochrome c oxidase; bc_1 complex

1. Introduction

Forty-five years after the chemiosmotic hypothesis was put forward by Mitchell [1] no one will doubt that oxidative phosphorylation in energy-transfer membranes is mediated by cyclic proton flow, a protonic current, between redox complexes of the respiratory chain and the F_0F_1 ATP synthase, each capable, separately, of coupling proton translocation to chemical catalysis [2]. However, there are three main questions which still remain to be solved. (i) What is the molecular/atomic mechanism by which proton translocation is coupled to redox catalysis in respiratory chain complexes? In this respect, the rotary-motor of the F_0F_1 complex provides a satisfactory mechanism for coupling of proton translocation to hydro–anhydro catalysis in this complex [3]. The same level of mechanistic resolution has not yet been reached for the three protonmotive complexes of the respiratory chain, complex I, III and IV. (ii) Is the H^+/e^- stoichiometry in these complexes fixed or flexible? This feature is obviously related to the molecular mechanism, with important physiological implication. (iii) Is protonic coupling in oxidative phosphorylation mediated by delocalized bulk-phase membrane $\Delta\mu H^+$, or does it take place, at least under active phosphorylation conditions, through fast, membrane-localized cyclic proton flow between respiratory chain complexes and ATP synthase without significant generation of bulk phase $\Delta\mu H^+$?

In what follows observations will be presented which contribute answers to the aforementioned questions and provide further insight into the physiology of the respiratory chain.

2. Allosteric cooperativity in the protonmotive respiratory chain complexes

The proposal that allosteric cooperativity could be responsible for proton pumping in redox enzymes, as an alternative to

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the direct ligand conduction mechanism of Mitchell [4], was first postulated in the early 1970s ([5,6] see in [7]).

By analogy with the well known Bohr effect in hemoglobin [8], i.e., allosteric linkage [9] between oxygen-binding at the heme-iron and proton transfer at distant residues in the protein, and taking into account the pH dependence of the E_m of respiratory chain cytochromes (*b* cytochrome in particular) [10], it was proposed that allosteric H^+/e^- coupling in respiratory chain redox enzymes can result in proton pumping if reduction of the metal prosthetic center causes pK increase of a residue in the protein in proton connection with the inner (N) side of the membrane, with H^+ uptake from this aqueous phase, and oxidation of the metal causes decrease of the pK of this, or another residue in protonic connection with the first, with proton release in the outer (P) aqueous phase [6,11]. Since then the mechanism of redox proton pumps has been the subject of intensive work and dispute [12]. As far as complex III (bc_1 complex) is concerned, the ubiquinone cycle [13], which represents a sophisticated version of the direct ligand conduction principle of Mitchell, can apparently rationalise a body of experimental observations and is largely accepted [12]. However, alternative mechanisms, which involve cooperative coupling at *b* cytochromes alone [14] or in combination with the protonmotive features of protein stabilized semiquinone/quinol species (Q-gated proton pump) [15], appear equally, if not better, apt to explain the protonmotive activity of complex III. Not much is known of the mechanism of proton pumping in complex I (NADH-ubiquinone oxidoreductase). Some observations would point to an involvement of conformational changes in the proton pumping activity of this complex [16], for which a Q-gated pump mechanism has also been proposed [17].

Much more work has been devoted to complex IV (cytochrome *c* oxidase) as well as to other members of the protonmotive heme-copper oxidase family, but the molecular mechanism of proton pumping remains still elusive [18]. The mitochondrial aa_3 cytochrome *c* oxidase is the more extensively studied enzyme of the family. It has four redox centers : a binuclear Cu_A, titrating as one electron redox center, bound to subunit II, heme *a*, heme a_3 and Cu_B, all these bound to subunit I [19] (Fig. 1). Cytochrome *c*, at the P side of the membrane delivers electrons to Cu_A, heme *a* transfers electrons from Cu_A to the heme a_3 Cu_B binuclear center, where O_2 is reduced to H_2O with proton consumption from the N space. This catalytic process results per se in transmembrane $\Delta\mu H^+$ [6,13,20]. In addition, up to four protons are pumped from the N to the P space in the reduction of one O_2 molecule to $2H_2O$ [20,21]. In the catalytic cycle of the oxidase most of the free energy is released in the aerobic oxidation of heme a_3Cu_B, thus different groups have developed models in which proton pumping is directly coupled to the oxygen reduction steps at the binuclear center [18,22–24], neglecting a role of the low potential heme *a* and Cu_A, which was, on the other hand, advocated by others [22,25–31].

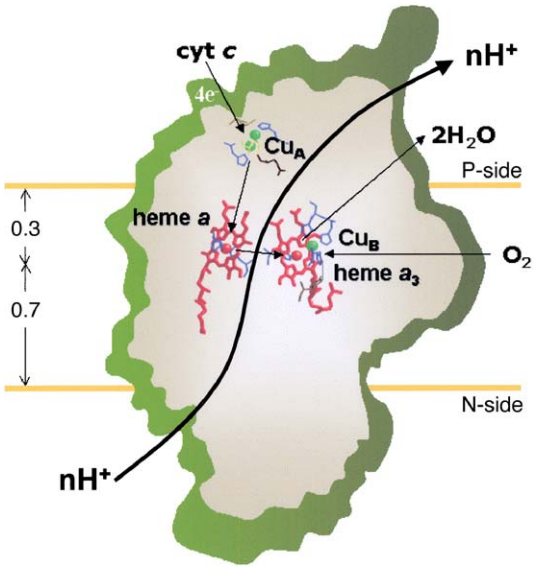

Fig. 1. Structure of cytochrome *c* oxidase with location of the four redox centers relative to the plane of the coupling membrane as shown by X-ray crystallographic structures [19].

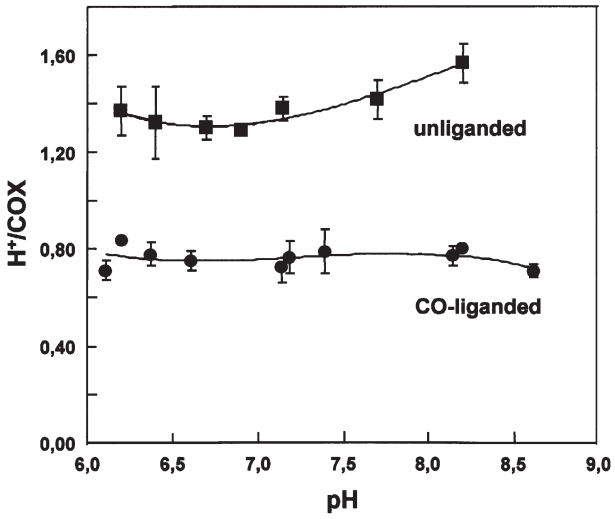

Fig. 2. pH dependence of proton release/uptake associated to oxido-reduction of metal centers induced by ferricyanide/ferrocyanide in anaerobic, soluble bovine heart cytochrome *c* oxidase. Black squares: H^+/COX ratios associated with oxido-reduction of hemes $a+a_3$, Cu_A and Cu_B in the unliganded COX. Black circles: H^+/COX ratios associated with oxido-reduction of heme *a* and Cu_A in the CO-liganded COX, heme a_3 and Cu_B clamped in the reduced state. For details see Refs. [33,34].

A role in proton pumping of the low potential centers of the oxidase, which are separate from the oxygen reduction site, has to involve cooperative H^+/e^- linkage. Functional and structural data on the bovine heart cytochrome *c* oxidase, provided by spectrometric and electrometric analysis and X-ray crystallography (see in [29,36]), provide in fact overwhelming evidence that proton pumping in the oxidase is associated with extended allosteric cooperativity, with involvement of all the four redox centers [32,34,37].

The E_m of Cu_A, heme *a*, heme a_3 and Cu_B is pH dependent [10,33,34]. There are pH dependent cooperative interactions of the metal centers [34]. X-ray crystallography and spectrometric analysis show redox coupled structural changes in subunit I of bovine heart cytochrome *c* oxidase. Reduction of heme *a* results

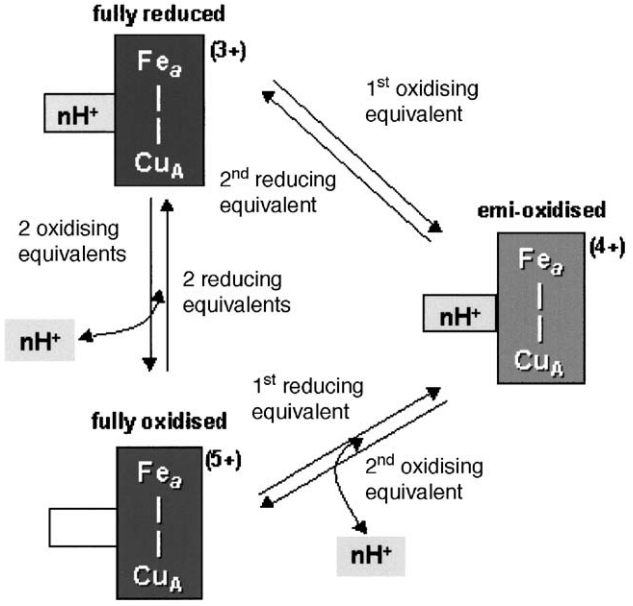

Fig. 3. Model of interactive proton release/uptake coupled with oxidation/reduction of Cu_A and heme a in cytochrome *c* oxidase. Reproduced with permission from Ref. [36].

in a conformational wave which starts with a small movement of the I-His378 heme ligand [35], rupture of a hydrogen bond between the hydroxyfarnesil substituent of the heme and porphyrin I-Ser382, and extends some 10–15 Å in the protein reaching the P surface where the carboxylic group of I-Asp51 moves towards the surface becoming exposed to the aqueous phase [29,30]. This redox-linked conformational change can be responsible for the observed pH dependence of the E_m of heme *a* and proton release/uptake coupled with oxidation/reduction of the heme-Fe, respectively [33] (Fig. 2). Experiments on liposome reconstituted, CO-liganded bovine heart cytochrome *c* oxidase provide evidence showing that protons are released upon oxidation of heme *a* and Cu_A in the external P space and are then taken up, upon reduction, from the inner N space [36]. Furthermore, it was found that heme *a* shares with Cu_A cooperative H^+/e^- linkage with a common acid/base cluster (C_1), this explaining the attenuated pH dependence, of some 20 mV/pH unit, of the E_m of both metals [33]. The interactive H^+/e^- coupling of heme *a* and Cu_A implies that, while one electron reduction of Cu_A/heme *a* is sufficient to produce maximal protonation of the cluster ($\approx 1H^+$/oxidase molecule), release of the proton from the cluster will take place only when both heme *a* and Cu_A are oxidized [32,36] (Fig. 3). Therefore, at the steady state one electron at the time has to pass through Cu_A and heme *a* so as to result in the translocation of 1 H^+/e^-. Anaerobic oxidation by ferricyanide of heme a_3 Fe^{2+}–Cu_B^{1+} also results in the release of $\approx 1H^+$/oxidase molecule [34]. This shows that oxido-reduction of the two metals of the binuclear center is cooperatively linked to deprotonation/protonation of a second acid/base cluster (C_2). Cooperative H^+/e^- coupling at the two clusters, acting in series, will constitute the gate of the pump [32,37] (Fig. 4). Upon electron transfer, one at a time, from cytochrome *c* to the binuclear center, a proton will be

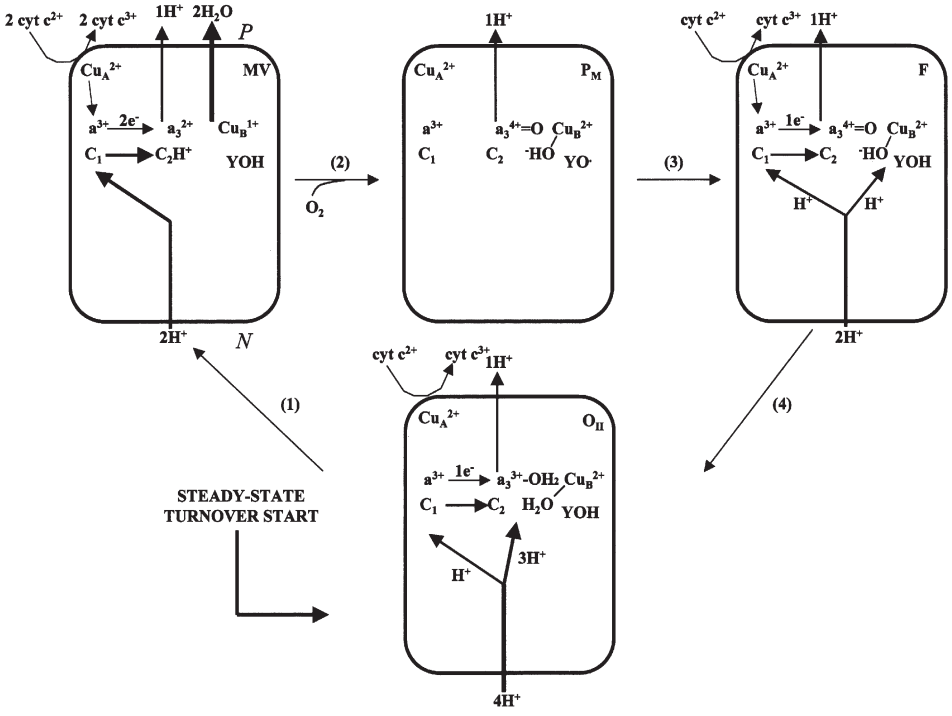

Fig. 4. Model of the catalytic cycle of reduction of O_2 to H_2O by ferrocytochrome *c* and proton pumping in cytochrome *c* oxidase in the coupling membrane at the respiratory steady-state. *P*: outer aqueous space, *N*: inner aqueous space. The steady-state turnover of the oxidase starts with electron delivery from ferrocytochrome *c* to the fully oxidized COX. Upon transfer of the first two electrons to the heme a_3/Cu_B center the MV state is generated (step 1). Molecular oxygen reacting with the MV state is reductively cleaved generating the P_M state (step 2). Transfer of the third electron converts the P_M to the F state (step 3) which is finally converted to the O state upon delivery of the fourth electron (step 4). At pHs ≤6.5, proton consumption in the production of the two water molecules at the binuclear center takes place, as shown in the model, in the oxidative phase of the catalytic cycle of the oxidase. For further details, see Ref. [37].

taken up from the N space by the C_1 cluster upon reduction of Cu_A/heme *a*. When the electron moves further to the binuclear center the proton leaves C_1, whose pK decreases and, rather than flowing back towards the N space, is captured by the C_2 cluster, whose pK simultaneously increases. Upon aerobic oxidation of the binuclear center and H_2O production the proton is finally released in the outer P space [32,37] (Fig. 4). During electron flow from cytochrome *c* to oxygen the proton pump will slip under conditions in which the couple Cu_A/heme *a* remains half-reduced [32,36,37].

Other mechanisms have also been envisaged which could cause slip in the proton pump of the oxidase. These include: direct electron flow from Cu_A to the binuclear center, bypassing heme *a* [21,34]; impairment of the translocation of protons from the N space to the heme *a* pocket by a glutamate switch [28] or decay of a metastable oxidized intermediate of the oxidase, involved in proton pumping [23].

The functional model proposed here, whilst based on specific structural and functional properties of mammalian cytochrome *c* oxidase, is intended to outline general features of cooperative H^+/e^- coupling in protonmotive heme-copper oxidases, including bacterial enzymes with different, but functionally equivalent redox centers and critical residues [38].

3. Slipping in the proton pump results in flexible H^+/e^- stoichiometry

Evidence of intrinsic uncoupling (slips) of proton pumps comes first from observations on the flux/force (respiratory rate/$\Delta\mu H^+$) relationship in steady-state respiring mitochondria [39,40].

A systematic study of the respiratory chain proton pump in intact mitochondria and in isolated-reconstituted complex III and complex IV revealed that the H^+/e^- stoichiometry varies under the influence of the rate of electron flow and the ΔpH component of the transmembrane $\Delta\mu H^+$ [21,41–44]. Results from this study, summarized in Fig. 5 show that the H^+/e^- stoichiometry for succinate oxidation in intact mitochondria first increases, up to around 3 under level flow conditions, upon enhancement of the respiratory rate, then drops as the rate is further raised [41]. Under steady-state conditions, the H^+/e^- ratio was significantly lower than at level flow. Whilst the H^+/e^- ratio for proton pumping associated with electron flow from succinate to ferricyanide was independent of the rate of electron flow, the H^+/e^- ratio for cytochrome *c* oxidation exhibited a bell-shaped rate-dependence, as observed for succinate oxidation, varying from around one at intermediate rates to around zero at low and high rates (Fig. 5A) [41]. It should be recalled here that previous investigations, which happened to deal with conditions in which there was no sign of proton pumping by cytochrome *c* oxidase [45], as well as related observations, had raised doubts on the existence of a proton pump in the oxidase [45] (see also [46]). The occurrence of a rate-dependent proton pump in cytochrome *c* oxidase was verified in our laboratory in experiments with purified oxidase reconstituted in liposomes, which showed bell-shaped rate dependence of the H^+/e^- ratio for cytochrome *c* oxidation, which, under level flow conditions, approached the value of 1 at intermediate rates [21,42].

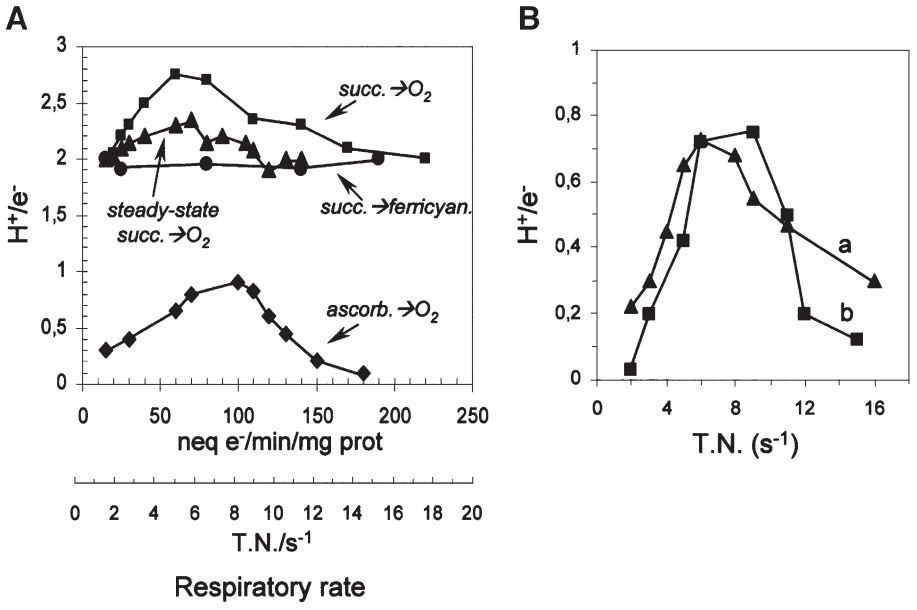

Fig. 5. Dependence of the H^+/e^- ratio on the rate of electron flow in the cytochrome system in intact mitochondria and in isolated liposome reconstituted cytochrome *c* oxidase. (A) Rat liver mitochondria; the electron flow rate from succinate to oxygen, at level flow or respiring steady-state, or to ferrycianide in the presence of KCN, was adjusted with malonate. The respiratory rate with ascorbate was adjusted varying the concentration of TMPD. For level flow measurements, the H^+/e^- ratios were obtained from the initial rates elicited by the addition of the substrates. The H^+/e^- ratios in the respiring steady-state were obtained from the rates of respiration and proton translocation measured at the aerobic steady state. For details, see Ref. [41]. (B) (a) Cytochrome c oxidase liposomes (COV) were supplemented with cytochrome *c* and ascorbate plus TMPD. The respiratory rate was varied changing the concentration of ferricytochrome *c*. (b) The oxidase vesicles were supplemented with cytochrome *c*, duroquinol, and a trace of soluble cytochrome *c* reductase whose concentration was changed to vary the overall respiratory rate. Valinomycin (plus K^+) was present to collapse aerobic $\Delta\psi$. The points of all the curves represent the mean of six or more experiments. Reproduced with permission from Ref. [21].

Experiments on purified liposome-reconstituted complex III and complex IV have shown that the depression of the H^+/e^- stoichiometry observed at the steady-state for succinate oxidation in mitochondria is due to a slip effected on the proton pump of both complexes by the ΔpH component of the transmembrane $\Delta\mu H^+$. The results for the bc_1-liposomes summarized in Fig. 6 show that the H^+/e^- ratio for proton pumping in this complex, which under level flow conditions was 1 independent of the rate of electron flow (only the vectorial proton was measured), dropped to one third at the steady-state [43]. The depression of the H^+/e^- ratio was due to a decoupling effect, a slip, directly exerted on the pump by the ΔpH component of the protonmotive force (valinomycin was present to collapse $\Delta\psi$). In fact, promotion of the steady-state proton leak by the protonophore CCCP decreased ΔpH and increased at the same time the steady-state H^+/e^- ratio [43]. An inverse linear relationship was also found between the H^+/e^- ratio and the steady-state ΔpH, when this was changed with different effectors [43].

An interesting beneficial effect was exerted on the steady state H^+/e^- ratio for proton pumping in bc_1 vesicles by submicromolar concentrations of arachidonic acid (Fig. 7) [44]. This effect, which was additive with the rescue effect induced by lowering the ΔpH with low concentrations of CCCP, was duplicated by azide. The positive effect of weak acids on the proton pump of the *bc1* complex is similar to that observed for bacteriorhodopsin, where proton pumping was rescued by azide in a defective Asp96 Asn mutant [47]. Apparently, certain weak acids can promote the activity of proton pumps by facilitating proton input in the entry mouth of the pump, in particular at high pHs in the N space.

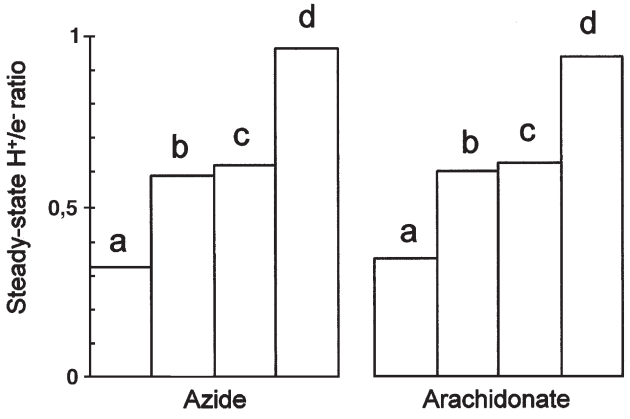

Fig. 7. Full reactivation of the steady-state proton pump of bc1 vesicles by a combination of anions and CCCP. (a) control, (b) 6 nM CCCP, (c) 50 μM azide or 0.25 μM arachidonate, (d) azide or arachidonate *plus* CCCP. Reproduced with permission from Ref. [44].

Experiments on cytochrome *c* oxidase showed that also in this complex the proton pumping activity was decoupled by the steady-state transmembrane ΔpH (Fig. 8). Also in this case the

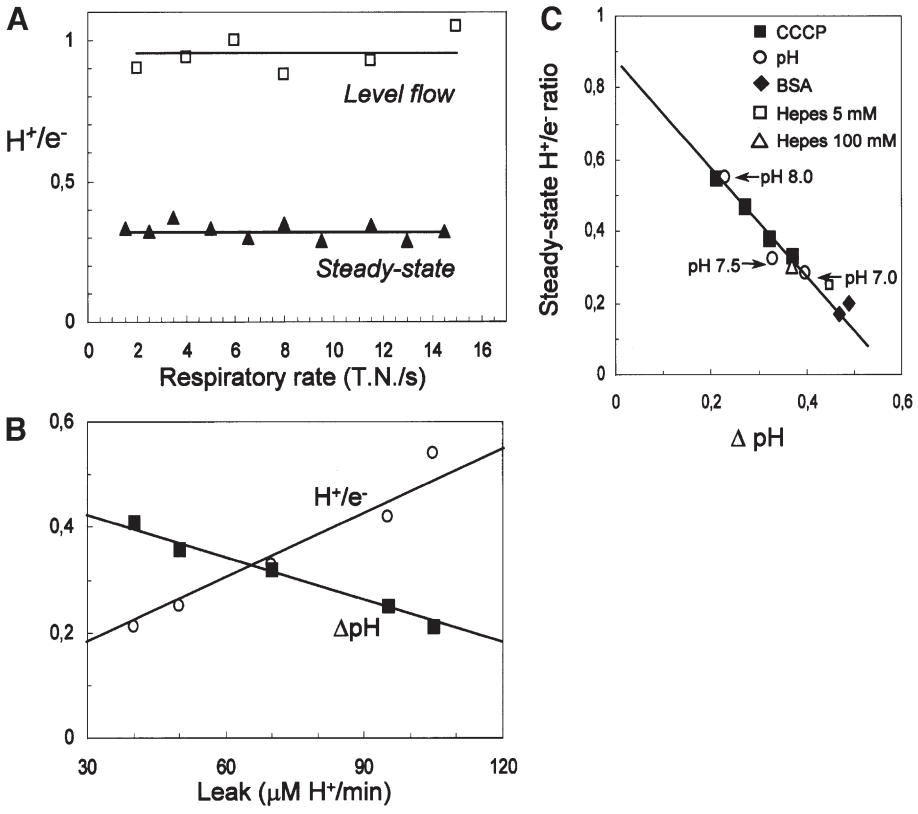

Fig. 6. H^+/e^- ratio dependence on electron transfer rate and transmembrane ΔpH in bc1 reconstituted liposomal vesicles. (A) Level flow and steady-state H^+/e^- ratio measured at different electron transfer rates [43]. (B) Effect of membrane proton leak (induced by nM CCCP concentrations) on steady-state H^+/e^- ratio and transmembrane ΔpH [43]. (C) Steady-state H^+/e^- ratios are plotted as function of transmembrane ΔpH value. This was modulated by introducing in the medium low concentrations of CCCP (1 to 6 nM), 0.5% BSA, or by preparing vesicles with different internal buffer capacity and different intraliposomal pH (7.0–8.0) [43].

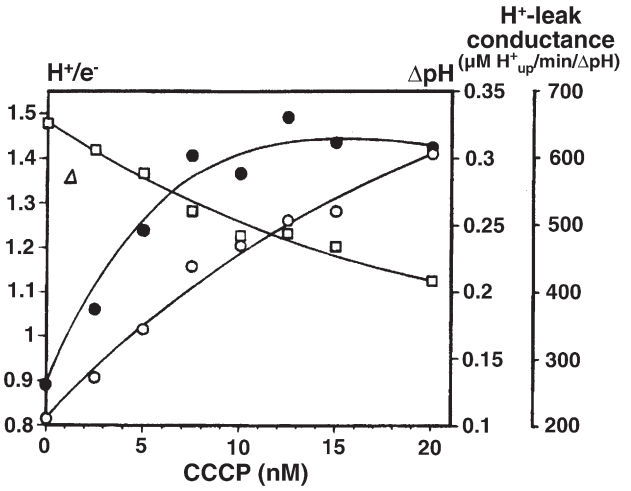

Fig. 8. Effect of transmembrane ΔpH on the steady-state H^+/e^- ratio in cytochrome *c* oxidase vesicles. Effect of CCCP on proton leak, ΔpH, and H^+/e^- ratios measured under respiring steady-state conditions in cytochrome *c* oxidase vesicles. Reproduced with permission from Ref. [21].

slip in the pump could be rescued by decreasing the ΔpH with CCCP-induced proton leak. These observations on complex III and IV clearly show that in the proton pumping activity of the respiratory chain complexes there is an inverse relationship between proton leaks in the coupling membrane and slips in the pump. ΔpH dissipation by proton leaks enhances the efficiency of the pump by preventing ΔpH-induced slip in the pump.

Tissue-specific slip in the proton pumping activity of cytochrome *c* oxidase has been reported to be effected by high ATP/ADP ratio in the reconstituted heart and skeletal muscle isozyme [40,48] and by palmitate in the kidney and liver isozyme [40,49].

4. Mechanistic and physiological implications of slips in proton pumping

In principle a flexible H^+/e^- stoichiometry, i.e. slips in proton pumps, can conceptually be more easily accommodated with indirect allosteric cooperative mechanisms rather than with direct mechanisms. However, possible decoupling mechanisms in direct protonmotive models have been conceived, like in the case of the ubiquinone-cycle in the *bc1* complex [50]. Possible decoupling events in the proton pump of cytochrome *c* oxidase have been previously discussed in Section 2 of this paper.

A second important mechanistic implication of the easiness with which the generation of a delocalized bulk-phase ΔpH causes, at least above a certain threshold level, decoupling of proton pumping is that optimal coupling conditions for active oxidative phosphorylation are met when membrane localized cyclic proton flow, between redox $\Delta\mu H^+$ generators and $\Delta\mu H^+$ utilizing F_0F_1 ATP synthase, takes place without significant generation of delocalized bulk-phase $\Delta\mu H^+$. Respiratory chain complexes have been found to be associated to form supercomplexes [51]. The ATP synthase forms also a supercomplex with the adenine nucleotide translocator and the phosphate carrier [52]. Dimeric ATP synthase appears to be involved in generating inner-mitochondrial cristae and to be localized in their invagination [53,54]. Communication of the intracristal space, which has a high cytochrome *c* content [55], with the space between the inner and the outer mitochondrial membranes is limited by a narrow junction [56]. Thus at cristae membrane surface efficient protonic coupling between respiratory complexes and ATP synthase could take place, without significant formation of delocalized $\Delta\mu H^+$ (Fig. 9). Oligomycin titration shows that under conditions of active ATP synthesis in respiratory state 3, there is an excess of ATP synthase capacity [57]. This indicates that during ATP synthesis, protons pumped by the redox complexes can be directly channelled back by the synthase, thus preventing their diffusion to/from the bulk phases. When the rate of $\Delta\mu H^+$ utilization by the ATP synthase decreases below that of $\Delta\mu H^+$ generation by the redox pump, till to the extreme condition of state 4, (provided that state 4 can be attained under in vivo physiological conditions), delocalized $\Delta\mu H^+$ arises. Under these conditions, the redox proton pump starts to slip, resulting in a decrease of the H^+/e^- stoichiometry. Slip in the pump will not take place, on the other hand, for the reasons outlined above, in state 3, thus no variability change in the P/O ratio will be observed in this state. Attenuation of delocalized $\Delta\mu H^+$ by slips can, on the other hand, have an important physiological role since delocalized $\Delta\mu H^+$ promotes oxygen superoxide formation by the respiratory chain (with

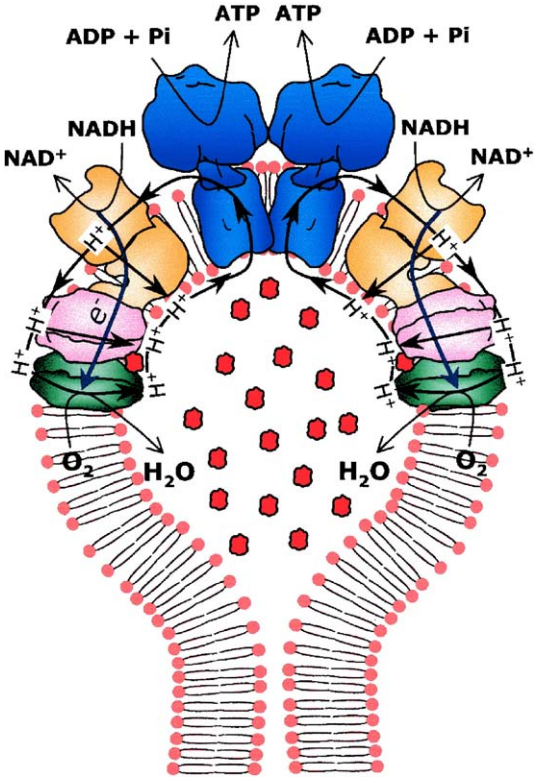

Fig. 9. Possible localized protonic coupling between redox complexes and ATP synthase at inner membrane cristae. F_0F_1 ATP synthase in bleu; complex I in yellow; complex III in pink, complex IV in green; cytochrome *c* in red.

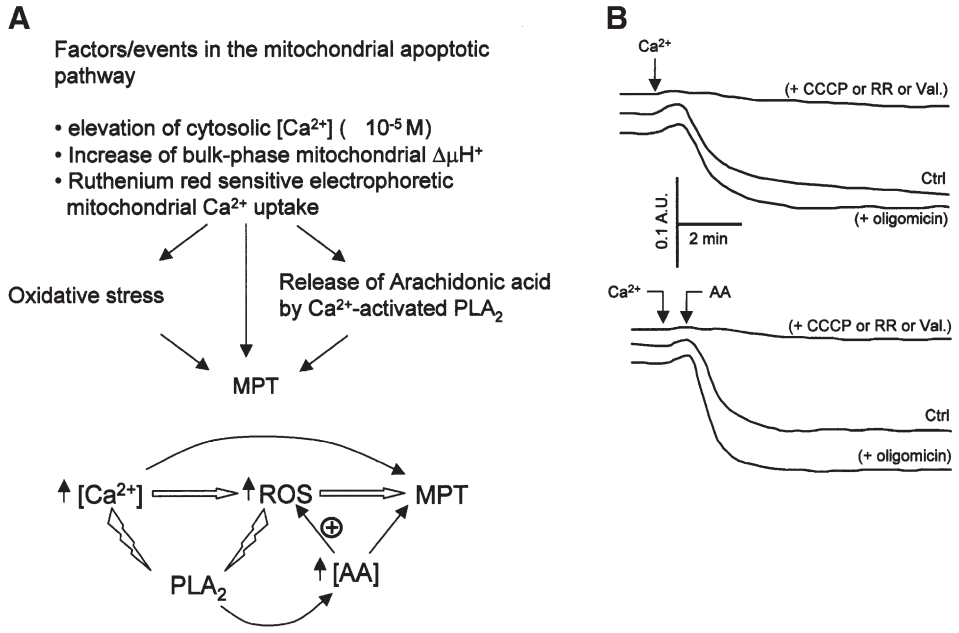

Fig. 10. (A) Scheme on the sequence of factors/events involved in the mitochondrial pathway of apoptosis. For details, see text. (B) Ruthenium red sensitive mitochondrial Ca^{2+} uptake leading to inner membrane permeability increase (MPT). Effect of arachidonic acid. Traces refer to absorbance decrease measured at 540 nm, showing mitochondria swelling. Upper traces: swelling was induced by the addition to mitochondrial suspension of 100 μM Ca^{2+}, in the absence or in the presence of 1 μg/ml oligomycin, 0.25 μM CCCP, 1 μg/ml valinomycin or 5 μM Ruthenium Red. Lower traces: swelling was triggered by the addition of 10 μM arachidonic acid in the presence of 30 μM Ca^{2+}. Abbreviations: ruthenium red, RR; arachidonic acid, AA; mitochondrial phospholipase(s) A_2, PLA_2. For details, see text and Ref. [63].

further generation of other free-radicals) [40,58] and, in higher eukaryotes, the mitochondrial pathway of apoptosis.

Under conditions leading to elevated cytosolic Ca^{+2} concentrations ($\geq 10^{-5}$ M) [59] or direct Ca^{+2} translocation to mitochondria from adhering Ca^{+2} loaded organelles [60], upon generation in mitochondria of a delocalized $\Delta\mu H^+$ by the respiratory chain (and/or ATP hydrolysis) Ca^{+2} is electrophoretically taken up by the ruthenium-red sensitive uniporter (Fig. 10). At relatively high concentrations, Ca^{+2} taken up by mitochondria is per se sufficient to open the cyclosporine-sensitive, permeability transition pore (see the mitochondrial absorbance traces in Fig. 10). Elevation of cellular Ca^{+2} concentration results also in activation of cytosolic and mitochondrial phospholipase(s) A_2 [61,62], with release of fatty acids, in particular arachidonic acid. Arachidonic acid at micromolar concentrations can, together with mitochondrial Ca^{+2}, induce cyclosporin-sensitive or cyclosporin-insensitive permeabilization of the inner mitochondrial membrane (Fig. 10). It is interesting to note here that initial low concentrations of arachidonic acid enhance the coupling efficiency of the redox pump of the bc_1 complex (Fig. 7; Ref. [44]). This could represent a self-protective effect against slips in the proton pump. Above a threshold level additional effects start to be exerted by arachidonic acid: these will include dissipation of mitochondrial $\Delta\mu H^+$ [63–65], inhibition of complex I NADH-ubiquinone oxidoreductase [66], production of reactive oxygen species [66] opening of the MPT [63,67–71], with release of apoptogenic mitochondrial factors. Events which can all be interrelated with a detrimental amplifying effect.

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