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Review

Cytochrome *c* oxidase: 25 years of the elusive proton pump

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Abstract

Since its discovery [Nature 266 (1977) 271] [1], the function of cytochrome *c* oxidase (and other haem-copper oxidases) as a redox-driven proton pump has been subject of both intense research and controversy, and is one of the key unsolved issues of bioenergetics and of biochemistry more generally. Despite the fact that the mechanism of proton translocation is not yet fully understood on the molecular level, many important details and principles have been learned. In the hope of accelerating progress, some of these will be reviewed here, together with a brief presentation of a novel proton pump mechanism, and of the emergence of a molecular basis for control of its efficiency.

Keywords: Haem-copper oxidase; Proton pump; Chemiosmotic theory

1. Introduction

The notion of cytochrome *c* oxidase as a biomolecular nanomachine that transforms redox energy into protonmotive force across a biological membrane was very intriguing to Jerry Babcock. As an eminent chemist and spectroscopist, he saw early on that solving the mechanism would require a combination of structural and dynamic information, much of which he provided in his own research (see Refs. [2–5]). With his untimely death, the field not only lost one of its key protagonists, but also a unique warm-hearted personality whose attitude towards science was humble and unpretentious, and towards fellow scientists fair and humorous, spiced with exceptional intellectual sharpness.

The function of cytochrome *c* oxidase as a proton pump is sometimes loosely regarded as a mechanistic detail of the more general chemiosmotic principle of linking redox reactions to formation of protonmotive force (pmf). It is often considered on an equal basis with the photosynthetic reaction centre of bacteria, Photosystem II in plants, and the *bc*-type complexes, all being "proton pumps" in a very general sense of the term. However, there is a fundamental difference that makes redox-linked proton-pumping by the haem-copper oxidases unique in biology. All the aforementioned systems that generate pmf by a redox reaction, except cytochrome oxidase (and possibly Complex I), can be fully described at the schematic level as versions of Mitchell's oxidoreduction loop [6]. They are all examples of the conceptual theme of vectorially orientated metabolism that was the basis for formulating the chemiosmotic theory. One characteristic of such redox loops is the effective translocation of a single proton (charge) across the coupling membrane per transferred electron in the redox reaction. Another characteristic is that the proton is not translocated as such across the osmotic barrier. Yet, protons may nevertheless have to be transferred by proton-conducting pathways (Mitchell's proton "wells"; Ref. [7]) from the membrane surface to "meet" the electron and thus to complete the redox reaction within the membrane phase, as for example during the reduction of quinone in the bacterial reaction centre [8]. It is noteworthy that such proton transfers always concern protons that themselves take part in (are substrates or products of) the redox reaction.

These principles actually also apply to the haem-copper oxidases, as originally suggested by Mitchell and Moyle [9]. The reduction of O_2 to water at the haem a_3-Cu_B centre within the membrane domain is indeed vectorially orientated: the electrons derive from cytochrome *c* on the *P*-side of the membrane and the substrate protons from the *N*-side. The two oppositely charged particles "meet" with O_2 within the membrane, producing water and annihilating the charges, which results in net translocation of one electrical charge across the membrane per electron, in accordance with the oxidoreduction loop principle (Fig. 1). It is clear,

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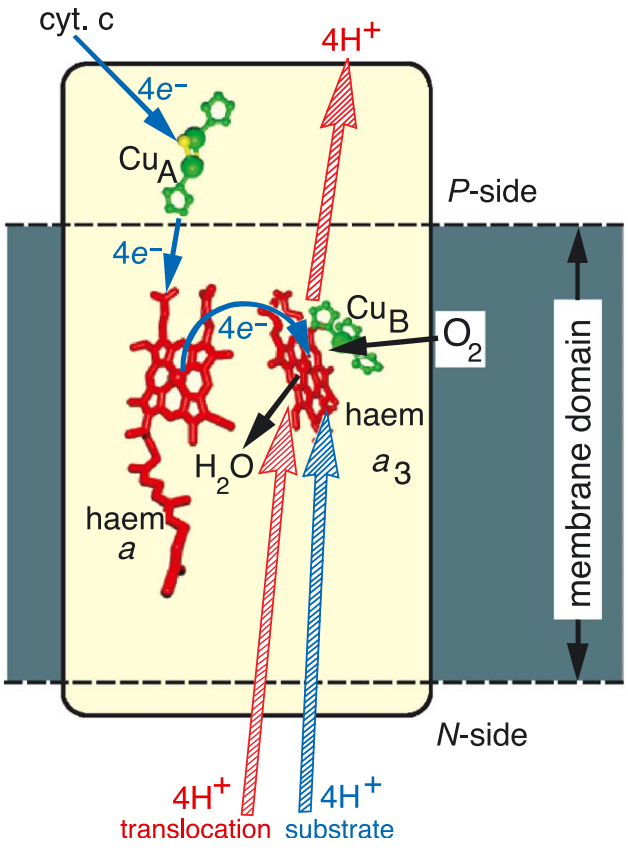

Fig. 1. Overall function of cytochrome *c* oxidase. The blue arrows depict the chemical reaction of O_2 reduction to water and its orientation with respect to the positively charged *P*- and the negatively charged *N*-side of the membrane. The red arrows depict proton translocation (pumping) coupled to this chemistry. Reprinted from Ref. [34] with permission from Elsevier. The programme VMD was used for the molecular graphics [48].

therefore, that Mitchell's notion of vectorially orientated redox reactions in primary biological energy transduction is still a highly successful concept that has proved to be true for all such reactions studied to date. Yet, it is not sufficient to describe the protonmotive function of the haem-copper oxidases.

2. The life of a proton pump in relation to orthodox chemiosmotic theory

From the above it is clear that the discovery that cytochrome oxidase is a true proton pump that translocates two electrical charges per transferred electron was unexpected (Fig. 1), and it is not surprising that this discovery led to strong opposition [10,11]. The mechanistic problem of how to translocate two electrical charges per electron, rather than one, is not trivial. The first schematic attempts to explain this were presented already in 1978 (Fig. 2; Refs. [12,13]). In today's terms, these may be described as follows: electron transfer from cytochrome *c* to haem *a* (via Cu_A) is linked to uptake of a proton from the *N*-side into an undefined "pump site" within the membrane domain. Subsequent transfer of the electron to the binuclear haem a_3-Cu_B site is linked to release of the proton from the pump site to the *P*-side of the membrane, followed by uptake of the substrate proton from the *N*-side into the binuclear site to produce water.

Note that this scheme could also be drawn as two sequential Mitchellian redox loops, assuming that haem *a* is formally a hydrogen acceptor [14], which is supported by the pH-dependence of its redox potential [15]. This view created much discussion about the nature of electron–proton coupling: would the chemiosmotic concept accept a more indirect, non-covalent linkage between, say, the electron reducing the haem, and the proton taken up in response to this, but being bound to a nearby amino acid residue, to a ligand of haem iron [16], or even a porphyrin substituent such as the propionate group [17]? To me, it was initially surprising that Mitchell did not accept such coupling, which in his terminology was deemed "indirect" or "conformational", even for the cases exemplified above, where there would surely be strong electrostatic interactions between the two oppositely charged particles. However, there is one important aspect of this particular description that does not conform to the original redox loop concept, unless it is broadened far beyond its origins.

In an orthodox redox loop the vectoriality of the reaction is assured by placing proton uptake and release sites at a safe distance from one another (even at the opposite sides of the membrane, although that is not absolutely required), and by establishing contact between them via pure electron transfer and diffusion of a neutral hydrogenated redox carrier, such as ubiquinone, in the opposite direction. However, with a structurally fixed redox site, such as haem *a*, diffusion of its hydrogenated (reduced) form would obviously be out of the question. Therefore, the type of scheme shown in Fig. 2 implicitly

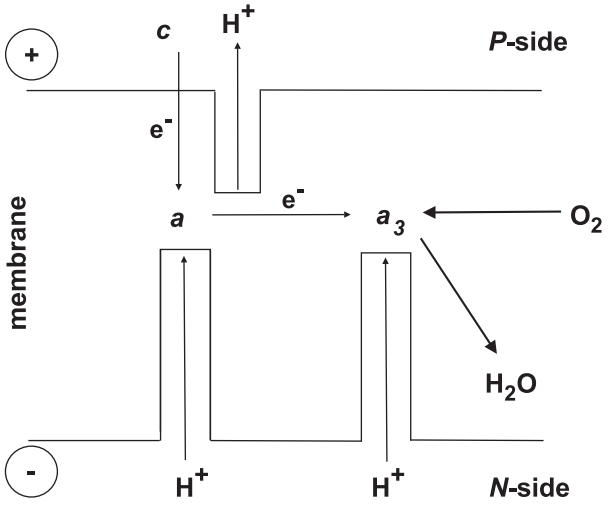

Fig. 2. Schematic view of proton translocation by cytochrome *c* oxidase according to Artzatbanov et al. [13] and Wikström et al. [12] (for details, see the text).

requires that the protonatable pump site would have to attain two "conformations", one in strict protonic contact with the *N*-side of the membrane, and another with the *P*-side, as discussed in some detail at the time [15,18]. The molecular meaning of such "conformations" obviously includes many different possibilities, and is certainly not restricted to large conformational changes, although such changes cannot be excluded either [19]. For example, subtle "gating" of proton transfer pathways would be sufficient.

Finally, it may be of historical interest to note that after accepting the proton-pumping function of cytochrome *c* oxidase, Mitchell suggested a mechanistic model that did indeed allow electron–proton coupling between a metal site (Cu_B) and its ligand (OH^- or H_2O), as well as a "conformational change" (in the coordination shell of the copper) that brought the protonatable ligand in protonic contact with either side of the membrane [20]. This model, therefore, conformed fully with the original principles of a redox-linked proton pump [14–16,18].

3. Protonic linkage to oxidoreduction of haem *a*

The scheme discussed above (Fig. 2) suggests a priori that the redox state of haem *a* may exhibit a pH-dependence that, moreover, should be exerted both from the inside and from the outside of the membrane, depending on the state of the system. After reinterpretation [21] of the contributions of haems *a* and a_3 in redox potential titrations, it was evident that both haem groups shows a pH-dependent redox potential as long as the companion haem is oxidised [15]. More interestingly, most of the pH-dependence of haem *a* was shown to be exerted from the *N*-side of the membrane [13], and some from the *P*-side [22,23]. However, redox changes in haem *a* following a jump of pH in the *N*-phase were found to be extremely slow [22] relative to turnover of the enzyme. Moreover, the pH-dependence was lost almost completely when the binuclear site was held reduced; the remaining small pH-dependence observed was now exerted from the *P*-side of the membrane. Together, these observations can hardly be taken as strong evidence for the idea that haem *a* has a key role in the pump mechanism, although they do not dismiss that possibility either (see below). Papa et al. [24] and Michel [25] later proposed detailed mechanisms related to the scheme in Fig. 2, where haem *a* has such a role.

4. Fundamental properties of a redox-linked proton pump

In a redox-linked proton pump mechanism it is not an obligatory requirement that the pK_a of the proton-binding group is dependent on the redox state, as may be envisioned using a cubic reaction scheme (Fig. 3; Ref. [15]). Above all,

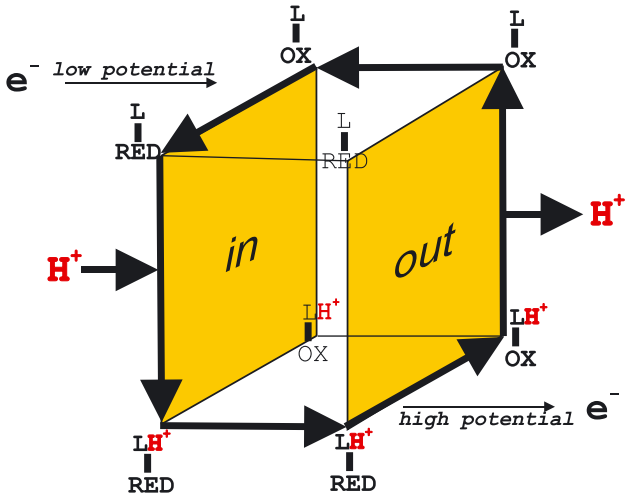

Fig. 3. Cubic scheme of the functioning of a redox-linked proton pump (cf. Refs. [15,18]). Ox and Red denote the oxidised and reduced states, respectively, of a redox cofactor, and L is a protonatable "pump site" linked to the redox element. The states on the left surface (denoted "in") are in protonic equilibrium with the *N*-side of the membrane, while the states on the right surface (marked "out") are in protonic equilibrium with the aqueous *P*-side. Thick arrows denote a possible cycle of fully coupled redox-linked proton translocation. Thin lines between "in" and "out" states show transitions that, if they occur, can lead to leaks or decoupling of the pump (see the text).

it is not absolutely necessary that the reduced and protonated species (LH^+-red) has much occupancy during turnover. In such a case it is of particular importance that the reduced unprotonated site (L-red) cannot switch from the state with proton contact towards the inside to the state with contact to the outside, because that will decouple the pump mechanism. The same is obviously true in the opposite direction for the oxidised protonated form of the pump (LH^+-ox). This is the requirement of kinetic coupling, which, if strong, can override strong requirements of thermodynamic linkage. Such kinetic coupling thus means that certain key transitions in the mechanism are kinetically forbidden. There are two more important aspects to the balance between the extreme modes of kinetic and thermodynamic proton–electron coupling. The weaker the thermodynamic linkage, the poorer will be the kinetic performance of the pump, because the occupancies of the reduced protonated (LH^+-red) and oxidised deprotonated (L-ox) forms may be low. The case of weak thermodynamic coupling also maximises the requirement of gating. For example, at a high occupancy of the reduced unprotonated form of the pump, relative to the protonated form, switching the former to the output conformation (or opening the proton gate towards the *P*-side) must be effectively prevented. Such control of efficiency is clearly less critical when thermodynamic coupling is strong, but becomes critical again for any pump mechanism operating close to thermodynamic equilibrium (see below).

It is important to keep in mind that the cytochrome oxidase proton pump reaction is reversible [26]. The overall

driving force of 500 meV per electron for the redox reaction (cytochrome *c* operates at 300 mV; O_2/H_2O at 800 mV) is opposed by 440 meV per electron from the protonmotive force of 220 mV and the coupling of two translocated charges per electron. Thus, at high pmf there is an overall driving force of only ca. 60 meV per electron for the reaction. This scenario of operating the transducer relatively close to equilibrium between the driving and the driven reactions is vastly different from the situation in the light-driven photosynthetic reaction centres, or in bacteriorhodopsin, where a substantial amount of energy is wasted as heat. The high expenditure of energy assures the virtual absence of non-productive back reactions (leaks) in the coupled process. In a transducer such as cytochrome oxidase, which operates much closer to equilibrium, there is a much higher risk of unproductive leak reactions. Again, this can easily be envisioned from a scheme such as Fig. 3, where it is obvious that back-pressure from a high protonmotive force will have the tendency to increase the probability of leak pathways. This, in turn, puts high demands on the effectivity of kinetic gating.

It is also possible that proton–electronic coupling only appears to be weak, because of intrinsic opposing factors, such as negative cooperative effects due to redox-linked structural changes [27]. Although there is little evidence for such conformational changes at present, this possibility must also be kept in mind.

In summary, a redox-linked proton pump may work even though there is only weak experimental evidence for thermodynamic proton–electron coupling. To compensate for this, the mechanism must ensure high specificity (kinetic gating) of the inward/outward transitions, and a high rate constant for these transitions to overcome low occupancy. Such specificity becomes particularly important at high protonmotive force. Hence, schemes such as the one in Fig. 2 are not excluded by the observed weak protonic coupling of haem *a*. In addition to the early analysis of the issue of thermodynamic versus kinetic coupling in a redox-driven proton pump already cited, this issue was also raised later [28,29]. Most notably, Bo G. Malmström [29] based his "transition state" mechanism on the very notion of poor thermodynamic and strong kinetic coupling in cytochrome oxidase.

5. The role of the substrate proton in proton pumping

In 1994 we proposed that the uptake of the substrate proton into the binuclear site is an essential component of the proton-pumping mechanism in that it may facilitate electrostatically the release of pumped protons from the membrane [30]. This idea was brought forward independently by Peter Rich [31], who formulated it as a requirement of electroneutrality at the binuclear haem-copper centre, which is still considered a valid concept. Supporting experimental data have been published, including the observation that electron transfer from haem *a* to the binuclear site is limited by proton uptake [32]. In our work, we added the purely hypothetical notion of a key role of a histidine residue in carrying out translocation of 2 H^+ per transferred electron [30]. The latter seemed a necessary postulate at the time because equilibrium titrations of the P, F and O intermediates of the binuclear centre with protonmotive force had suggested that all four proton translocation steps occurred within the oxidative phase of the catalytic cycle (Ref. [33]; see Fig. 4). However, this notion had to be revised on the basis of new unexpected findings, as reviewed below. Yet, the key role of the substrate proton in the proton pump mechanism has been retained.

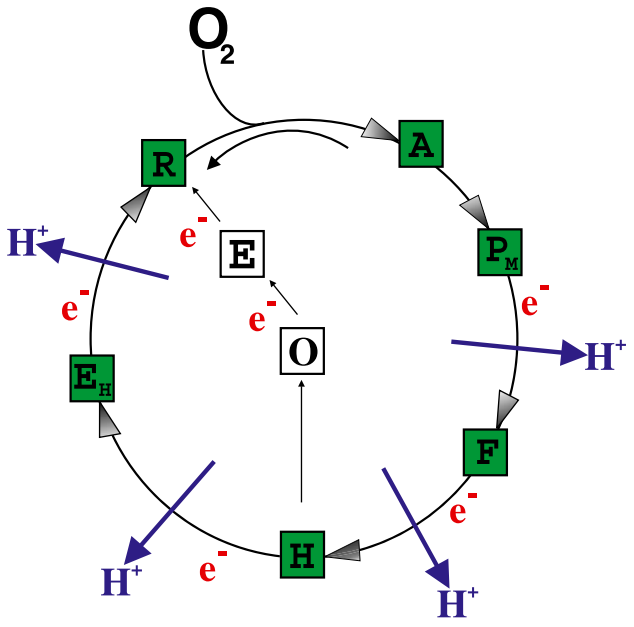

Fig. 4. Catalytic cycle of cytochrome *c* oxidase. The squares represent states of the binuclear haem a_3-Cu_B centre. The green squares represent the main cycle. The *oxidative reaction phase* starts at state R (reduced, Fe[II]Cu[I]), and continues with states A (oxygen adduct, Fe[II]-O_2 Cu[I]), P_M (Fe[IV]=O Cu[II] tyr-O·), F (Fe[IV]=O Cu[II] tyr-O^-), and H (Fe[III Cu[II]). In the absence of an electron donor the metastable oxidised H state may relax into state O (Fe[III Cu[II]), which may be reduced back to state R via state E (Fe[III] Cu[I]). Alternatively, during continuous turnover, the H state is reduced back to R via state E_H (Fe[III] Cu[I]). The *reductive reaction phase* of the catalytic cycle comprises reduction of states H or O back to R. Both the *oxidative* and the main *reductive* phase is coupled to translocation of two protons (blue arrows), one for each electron transfer reaction. However, after relaxation of H to O, the reductive phase is no longer linked to proton-pumping (see the text). Each electron transfer into the binuclear centre (marked with e^-) is linked to uptake of a substrate proton (not shown).

6. A water-gated mechanism of proton translocation

We have recently proposed a mechanism of proton translocation [34] that is, in effect, also related to the scheme in Fig. 2. Seven out of the eight protons taken up from the *N*-side of the membrane per cycle (Fig. 1) are transferred

into the enzyme by the same so-called D-pathway that leads to a conserved glutamic acid some 10 Å from the binuclear centre, whether these protons are destined to be pumped across the membrane, or consumed at the binuclear site [35]. This makes a "switch" necessary by which protons are directed in a controlled and redox-coupled fashion to either destiny. We proposed that three to four water molecules predicted to reside in the hydrophobic cavity beyond the glutamic acid [36] form a proton-conducting array, the orientation of which depends on the redox states of the electron donor, haem *a*, the acceptor, and the binuclear site. This is due to a redox state-dependent electric field between the two sites, which orientates the water dipoles between them. When the electron resides at haem *a*, the water array conducts a proton to be pumped from the glutamic acid to the hydrophilic domain above the haem groups via the Δ-propionate of haem a_3 [37]. This proton transfer allows electron transfer from haem *a* to the binuclear site, which, in turn, inverts the electric field between electron donor and acceptor so that the water array will now re-orientate to conduct protons from the glutamic acid to the binuclear site. Uptake of the second proton to this latter site to form water causes ejection of the first proton towards the *P*-side of the membrane by electrostatic repulsion. This mechanism requires that one proton is translocated for each electron transferred between haem *a* and the binuclear site, and this is consistent with the recently clarified properties of the main catalytic cycle (see below).

7. A side path from the main catalytic cycle

Flash-induced electron injection into oxidised resting cytochrome *c* oxidase with the aid of a photo-activatable reductant has shown unequivocally that fast reduction of haem *a* is not associated with measurable proton uptake, which takes place only on further transfer of the electron to the binuclear site (Ref. [38]; contrast Ref. [39]). Moreover, no proton pumping has been observed in such experiments [38,40], in which the oxidised "as isolated" enzyme is reduced by one or two electrons. These findings appear to contradict the mechanism proposed above.

However, when reduction of the binuclear centre follows immediately after oxidation of the enzyme by O_2, the reductive phase was found to be coupled to pumping of two protons [40]. Thus, translocation of two protons was associated with both oxidation and re-reduction of the enzyme in contrast to the view that had evolved earlier, but in agreement with the water-gated mechanism proposed above [34]. Since reduction of the resting oxidised enzyme is not linked to proton-pumping, it was necessary to postulate two forms of the oxidised enzyme, and that the enzyme departs from its main catalytic cycle if left without reductant (Fig. 4). This proposal may well be related to the much earlier notion of "pulsed" and "resting" states of cytochrome oxidase, as described in considerable detail by Antonini, Brunori and Wilson and their collaborators [41–43]. This key observation suggests corollaries that may be tested independently. First, the redox potential of Cu_B (and possibly of haem a_3) should be considerably higher in the metastable H state (Fig. 4) than those measured for the relaxed O state. Secondly, the structure of the binuclear site should be different in H and O. Both these predictions have recently been supported experimentally (I. Belevich, M.I. Verkhovsky and M. Wikström, unpublished data).

8. Static and dynamic states of cytochrome *c oxidase*

It may be concluded that some of the discrepancies encountered earlier have been due to the different properties of cytochrome *c* oxidase in static and dynamic conditions. Thus, reduction of the relaxed oxidised enzyme is not coupled to proton translocation, whereas reduction that follows immediately after oxidation by O_2 is linked to proton-pumping. This could also mean that data obtained from redox potential titrations at equilibrium, including measurements of the pH-dependence of haem *a*, may not be directly applicable in turnover conditions. Indeed, such titrations show only a ca. 100 mV decrease of the midpoint potential of haems *a* and a_3 in mitochondria at a high protonmotive force [21], which is far too small to be consistent with linkage of the reduction of the binuclear centre to proton translocation. The same may be true for the redox potentials of haem a_3 and Cu_B which do not exceed 0.4 V in equilibrium titrations [15].

The slopes in static titrations of intermediates O, F and P (see Fig. 4) with phosphorylation potential in mitochondria indicated that three and four charge equivalents were translocated coupled to the $F \rightarrow O$ and $P \rightarrow F$ reactions, respectively [33]. This result was taken to suggest that all four proton translocation steps may be coupled to the oxidative phase of the catalytic cycle. In contrast, more recent dynamic measurements showed that the oxidative and reductive phases are each associated with pumping of two protons as long as the reductive phase follows immediately after oxidation [40]. In view of the present observations, it seems possible that protonic backflux through the pump, driven by the high protonmotive force (see above, Fig. 3), may have caused an overestimation of the number of charges translocated under near-equilibrium conditions.

It may well be that the difference between static and dynamic conditions is due to the enzyme accessing states higher in energy during turnover than at rest, which might include kinetic availability to such states that is absent at rest. It is possible, for example, that the production of water molecules as a product during turnover provides kinetic (protonic) access to such states, but that key domains within the protein "dry out" when the enzyme is not functioning [44].

9. Putative control of the proton-translocating efficiency

This new picture of the catalytic cycle (Fig. 4) might also identify a means by which the efficiency of the proton pump may be controlled. At limiting electron input from cytochrome *c* (for example at high protonmotive force), the catalytic cycle might be diverted via intermediate O, in which case two protons would be translocated per cycle instead of four. This would lower the protonmotive efficiency of cytochrome *c* oxidase by 25% (6 electrical charges translocated per O_2 reduced, instead of 8), and the overall efficiency of the entire respiratory chain from 5 translocated charges per electron [45] to 4.5, i.e. to 90% which is in good agreement with the theoretical optimal degree of coupling (91%) that achieves maximal power output of ATP synthesis [46]. However, it can be deduced from the observed ATP/O ratio and the H^+/ATP quotient of the ATP synthase that there is no significant loss of coupling efficiency of the pump during active (State 3) oxidative phosphorylation in mitochondria [35]. Therefore, a possible decrease of coupling efficiency would be expected to set in only at the very highest values of protonmotive force, i.e. in mitochondria at "static head" (State 4) where there is no net ATP synthesis. Another reason for control of the proton-pumping efficiency of cytochrome oxidase is perhaps more likely, viz. to avoid very high values of protonmotive force, which have been shown to be associated with maximal rates of production of reactive oxygen species by the mitochondrial respiratory chain [47].

It still cannot be entirely excluded at the present time, however, that the relaxed intermediate O is an artifact of some kind. However, the reductive phase of the catalytic cycle is not linked to proton translocation during anaerobic titrations of intact mitochondria (see above), which supports the idea that O may be a real physiologically relevant intermediate. Also the fact that the states O and H are found in the cytochrome *c* oxidases from both bovine heart and *Paracoccus denitrificans* (unpublished observations) speaks in favour of this possibility.

Acknowledgements

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