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Hypothesis

A mechano-chemical model for energy transduction in cytochrome *c* oxidase: the work of a Maxwell's god

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Abstract **Cytochrome c_3 has a central role in the energetics of *Desulfovibrio* sp., where it performs an electroprotonic energy transduction step. This process uses a network of cooperativities, largely based on anti-Coulomb components, resulting from a mechano-chemical energy coupling mechanism. This mechanism provides a model coherent with the data available for the redox chemistry of haem *a* of cytochrome *c* oxidase and its link to the activation of protons. A crucial feature of the model is an anti-Coulomb effect that sets the stage for a molecular ratchet, ensuring vectoriality for the redox-driven localised movement of protons across the membrane, against an electrochemical gradient.**

Key words: Energy transduction; Cooperativity; Redox-Bohr effect; Cytochrome *c* oxidase; Cytochrome c_3

1. Introduction

The molecular basis for the fine regulation of several types of cooperativity (i.e. efficient coupling of energetic processes) is well established [1–3]. In particular, the complex network of homo- and heterotropic cooperativities present in haemoglobin (i.e. cooperativities between functional centres binding identical or different molecules/particles [4]) makes it a paradigm for studies on cooperativity [1,5]. In haemoglobin, the cooperative effect between the affinity of the different haems for oxygen and their antagonistic dependence on the protonation of acid/base centres (Bohr effect) is governed by an equilibrium between two structural states, linked by a mechano-chemical coupling mechanism: the tense, T (structure of low energy, with low affinity for oxygen and high affinity for protons), and the relaxed, R (structure of high energy, with inverse affinities).

Comparatively, less is known about the structural basis for electron/electron and electron/proton cooperativities [6–10] and its relevance to energy transduction processes, leading to oxidative phosphorylation. Electroprotonic energy transduction mechanisms must involve a thermodynamic link (cooperativity) [4] between redox and protolytic centres (i.e. affinity for electrons, E_m, and protons, pK_a, such that in mV $\Delta E_{m,acid/base} = \Delta pK_a^{redox} = pK_a^{red} - pK_a^{ox} \neq 0$). By analogy with haemoglobin, this coupling between redox and protolytic centres is called redox-Bohr effect, rB [4,7,11]. Thus, in order to understand cooperativity at molecular and structural levels, the thermodynamic parameters for the functional centres and their cooperativities must be assessed and related to relevant conformation features [9,10]. This is usually difficult to achieve for transmembrane enzymes, e.g. those of oxygen respiratory chains. However, mechanisms established for smaller proteins can provide models to test their relevance in explaining the molecular and structural bases that control energy transduction in more complex systems [12,13]. The characterisation of small and soluble proteins has demonstrated that the interactions between a redox and a protolytic centre can generate either positive cooperativity, rB^+ (i.e. $\Delta pK_a^{redox} > 0$), when dominated by direct electrostatic interactions [7] (Fig. 1A), or negative cooperativity, rB^- (i.e. $\Delta pK_a^{redox} < 0$ [12]), when dominated by mechano-chemical components [8] that oppose the electrostatic contribution. This negative rB can be described adapting the haemoglobin structural switch model [1] (Fig. 1B).

The detailed structural and thermodynamic characterisation of the redox and protolytic centres of tetrahaem cytochrome c_3 from *Desulfovibrio* sp., Cytc_3, including their pairwise cooperative effects, show how the interactions between charged residues control its functional mechanism [6–10]. The molecular basis for this control is a redox-linked conformational change [9,10], resulting in cooperativity effects with marked deviations from Coulomb's law [8]. In particular, this mechano-chemical coupling controls an anti-Coulomb positive cooperativity between two haems that leads to a concerted two-electron step [7,14], but can also reinforce the electrostatic effect of some positive rBs [8]. These effects govern the redox-linked activation of protons performed by Cytc_3, which is coupled to (and stimulates) hydrogenase activity [7]. Another example of an antielectrostatic effect is that controlled by the haem reduction-linked structural change in *Metylophilus methylotrophus* cytochrome *c*″, which results in acidification of a haem propionic substituent [15].

These anti-Coulomb effects are examples of how misleading it may be to consider that functional mechanistic models for

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Abbreviations: COX, cytochrome *c* oxidase; Cyt*c*3, cytochrome c_3; P- and N-side (phase), positively and negatively charged sides (phases) of the membrane; $E_{m,i}$, midpoint reduction potential at a particular pH or protonation state; rB, redox-Bohr effect; $\Delta pK_a^{redox} = pK_a^{red} - pK_a^{ox}$

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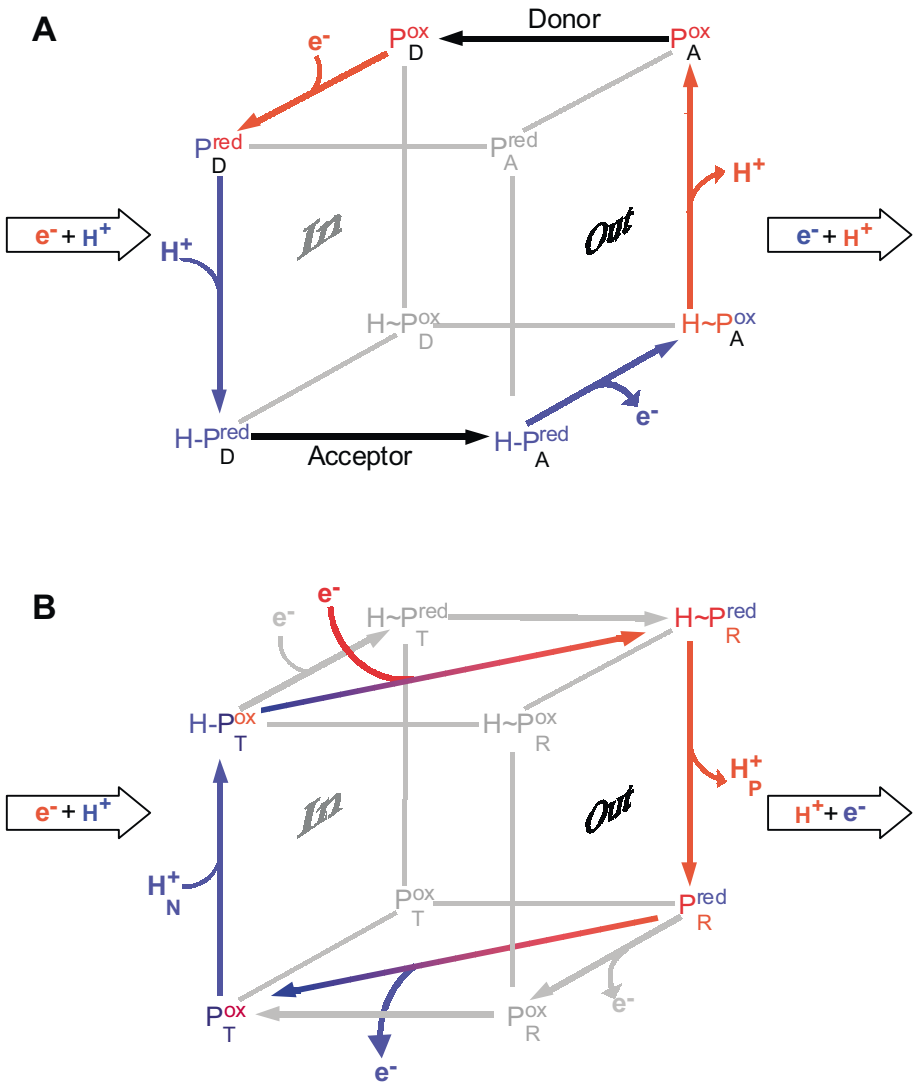

Fig. 1. Mechanisms for rBs: A: Positive ($\Delta pK_a^{redox} > 0$). The energy transducting cycle starts with protein, P, in the oxidised and unprotonated state bound to the donor, P_D^{ox}. It can receive a low redox potential electron (high reducing energy, since the $E_{m,base}$ is low). Once reduced, the pK_a of the coupled protolytic centre increases and it can recruit a proton to form the common intermediate H-P^{red}, which then binds to the acceptor (H-P_A^{red}). Reoxidation by the acceptor (with a high $E_{m,acid}$) results in the acidification (energisation, i.e. low pK_a^{ox}) of the protolytic group (H$\sim P_A^{ox}$), which can then give its proton to the acceptor. This electroprotonic energy transduction mechanism can be achieved by direct electrostatic interactions and depends only on the stepwise specific recognition (i.e. binding) of the oxidised and deprotonated (reduced and protonated) state of the transduction protein to the relevant state of the donor (acceptor), as in the proton activation mechanism performed by Cyt*c*3 [7]. B: Negative (rB^-, $\Delta pK_a^{redox} < 0$). The rB^- effect involves a redox-linked conformation switch between the structures of low and high energy (T→R, i.e. a T-state with high affinity for protons, high pK_a^{ox}, and low affinity for electrons, low $E_{m,acid}$, and an R-state, with inverse affinities). Starting in the T-state with the protein in the oxidised, but already protonated state (H-T^{ox}), recruitment of an energised electron (low E_m) triggers the structural modification, acidifying the acid (H$\sim P_R^{red}$, where the tilde represents the acidification of the common intermediate of this mechanism), which becomes primed to eject the proton. Concomitantly, the redox potential is lowered by the direct electrostatic effect operative within the R-states (output face of the cube), and the electron can be transferred to the next redox centre. State-dependent protein recognition (A) and unfavourable overall thermodynamics (B) select out the steps represented by thin grey lines, which would short-circuit the energy transduction mechanism. Grey arrows represent the coupled redox/conformation change steps, also shown as the combined step represented by blue/red shading arrows. Blue (deenergised) and red (energised) are used for comparative purposes. Subscripts T and R indicate the conformational states of the protein. N and P subscripts (B) represent protons recruited (ejected) from (towards) the negatively (positively) charged side of the membrane (see text and Section 2.1).

proteins must involve only direct electrostatic effects [12]. Indeed, conformational changes can induce redistribution of charges within the protein [5] and elicit effects that oppose direct electrostatic interactions [12,13].

2. Cytochrome *c* oxidase (COX)

COX is a terminal oxidase of oxygen respiration ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). It contains four redox centres: Cu_A, haem *a* (Fe_a), and the active site, a binuclear centre with haem a_3 (Fe_{a3}) and Cu_B [16–18]. Electrons supplied to Cu_A by reduced cytochrome *c* are sequentially transferred through Fe_a to the active site.

Wikström's work showing that, coupled to the reductase activity, COX translocates protons across a membrane against a protonic electrochemical potential to activate ATP synthase

[19], and his proposal for a 'redox-linked proton pump' mechanism ([16] and references therein) generated an enormous amount of work to seek the primary mechanism for this important function. A further boost to this effort came from the availability of the first X-ray crystal structures [20,21]. Nevertheless, this objective has not yet been accomplished [18] and the explanation for several experimental observations remains debatable [12,13,17,18,22].

One of the aspects that has remained problematic for longest is the redox chemistry of the low-spin haem *a*: Having the highest redox potential (cf. legend of [23]), this centre is reduced at the very beginning of enzyme reduction, but its redox potential has a deceptively small pH dependence, as reported for the unliganded and the CN- and CO-liganded enzyme [24–27]. In this context, the recent awareness that, in contrast to general expectation [18], proton(s) may be translocated during the reductive phase of the catalytic cycle [28,29] and the controversy generated thereafter [22,30] support the need to invoke an unorthodox mechanism. Collectively, these observations suggest the involvement of a rB^- [12,13], in contradiction with the current models used to explain the proton activation mechanism of COX [31], which include only direct electrostatic effects, even though this generalisation has been disputed [32].

It is in this scenario that a new mechanism is discussed [12,13]. It takes into consideration cooperativity mechanisms known to operate in small proteins and, in particular, the verification that they can use a redox-linked conformational modification to control, and even override direct electrostatic interactions [8]. The experimental data cited above for Fe_a are fully compatible with a mechanism that includes a rB^- as the basis to elicit anticooperativity between an incoming electron and a previously protonated group. Indeed, as shown in Fig. 1B, inclusion of a redox-linked conformational change explains how reduction of Fe_a can energise an acid group (i.e. $pK_a^{red} < pK_a^{ox}$) to eject its proton. Furthermore, the pH dependence of E_m^{Fea}, much smaller than the 60 mV/pH unit expected from one direct rB^+ group, is easily explained by including a protolytic centre with a rB^- [12]: As shown in Fig. 2, reliable experimental data obtained for CN-COX [26] can easily be simulated with the simultaneous implication of rB effects of opposite sign, considering the interaction with two protolytic centres (1 and 2), such that $pK_{a1}^{ox} < pK_{a2}^{red} < pK_{a2}^{ox} < pK_{a1}^{red}$ [13]. Furthermore, the inversion of the midpoint reduction potentials of haems Fe_a and Fe_{a3}, between the first electron input to Fe_a (from Cu_A) and its output (to Fe_{a3}) [23], is consistent with the inclusion of the above combination of rBs for Fe_a (Fig. 2), linked to the well-established rB^+ affecting Fe_{a3} [23,25,31–36]. In fact, as previously shown for the unliganded enzyme [23] in blue upon electron transfer from Fe_a to Fe_{a3}, the E_m of Fe_a decreases from 390 to 220 mV, and that of Fe_{a3} increases from 200 to 390 mV. The drop of 170 mV for the E_m^{Fea} is sufficient to accommodate the redox interaction effect due to the reduction of Fe_{a3} (−35 mV [25]), leaving 135 mV for the ΔE_m^{Fea} caused by its rBs. Indeed, both protonation of the rB^+_{Fea} and deprotonation of the rB^-_{Fea} groups contribute to the observed decrease of E_m^{Fea} (cf. Fig. 1). On the contrary, due to the mandatory reciprocity of cooperativities [14], if this drop

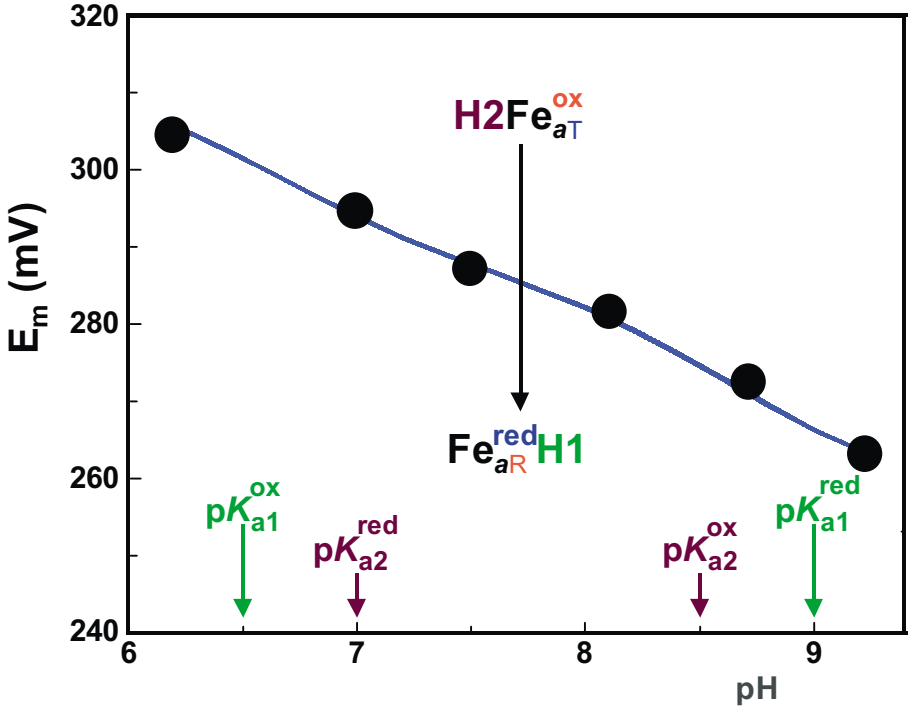

Fig. 2. Simulation of the pH dependence of the Fe_a E_m in CN-liganded COX. The blue curve was calculated for the rB of two protolytic centres, adapting equation 4 of [27] to use the following microscopic pK_as: $pK_{a1}^{ox} = 6.5$ and $pK_{a1}^{red} = 9.0$, rB_1^+, and $pK_{a2}^{ox} = 8.5$ and $pK_{a2}^{red} = 7.0$, rB_2^-. Even without trying to optimise the pK_as, an excellent simulation of previously published experimental points (dots taken from [26]) is obtained, particularly when compared with a linear slope (cf. figure 3 of [26] and the data points obtained for the enzyme in other conditions [24,25,27]). It should be noted that upon reduction at intermediate pH values, centre 2 ejects a proton and group 1 recruits another proton. Also, that although compatible with the values used, this solution is not unique. In particular, similar simulations can be obtained including more protolytic groups, each one accounting for part of the total effect. In fact, the global rB_1^+ can result from two complementary flanking groups (1A and 1B, see text). Indeed, the same curve is obtained with: (i) $pK_{a1A}^{ox} = pK_{a1}^{ox}$; (ii) $pK_{a1}^{red} = pK_{a1B}^{red}$; (iii) $pK_{a1A}^{red} = pK_{a1B}^{ox}$; and (iv) maintaining the pK_as for the rB_2^-. Colour code, subscripts, and superscripts used are the same as those of Fig. 1.

was due to the Fe_a/Fe_{a3} redox interaction [23], the reciprocal redox effect due to the reoxidation of Fe_a would contribute with 170 mV (not 35 mV) to the 190 mV increase of E_m^{Fea3}. This would leave only 20 mV for rB^+_{Fea3} ($\Delta pK_a^{redox} = 0.34$ pH units, instead of the previously obtained 1.5 [25]), which is obviously too small a value for a rB^+_{Fea3} to be functional.

The conformational change governing the rB^- is also coherent with the low electron transfer rate observed for this output step [37].

2.1. A minimal model

The anti-Coulomb contribution used to explain these widely accepted data involves an antagonist effect between the reduction of Fe_a and the protonation of a coupled protolytic centre (cf. Fig. 1B). Also, this mechanistic feature can be used as the core of a minimal model coherent with the proton activation mechanism of COX, which prompts ATP synthase [12]. Fig. 3 illustrates how such a model can link the rB^- of Fe_a to the functional activation of protons previously recruited from the N-side (negatively charged side) of the membrane. Mechanochemical energy coupling governs the rB^- of a specific protolytic group, which underlies a molecular ratchet mechanism that sets the stage to ensure vectoriality for the redox-linked movement of protons (see Fig. 1B, where the cubane paradigm for energy transduction is used (cf. [16] and references therein), but changing the generally accepted order of steps to the thermodynamic requirements of the rB^-). When Fe_a^{ox} is reduced by Cu_A^{red}, the rB^- group (high pK_a^{ox}, thus previously protonated by recruiting a proton from the N-side) is acidified (low pK_a^{red}) by the redox-linked structural energisation. The resulting acidified state, $H \sim P_R^{red}$ (Figs. 1B and 3), functions as the common intermediate state, mandatory for biological energy coupling processes. In this energised state, the proton is ready to be ejected towards the P-side (positively charged side of the membrane) and assist ATP synthesis. When Fe_a is reoxidised, the enzyme returns to the T-state and the protolytic group can be reloaded. The electron follows to the binuclear centre, where the highly exergonic reduction of oxygen ensures the turnover of the proton activation cycle.

It is noteworthy that the mandatory redox-linked conformation switch of the negative rB can enforce directionality by alternating the link/break communication between the entry and the exit sides of the proton channel, a mechanism whose importance has often been stressed [38,39]. Also, although for the sake of clarity only the rB^- (rB_2^- of Fig. 2) effect in Fe_a is included in the above description of the minimal model (Fig.

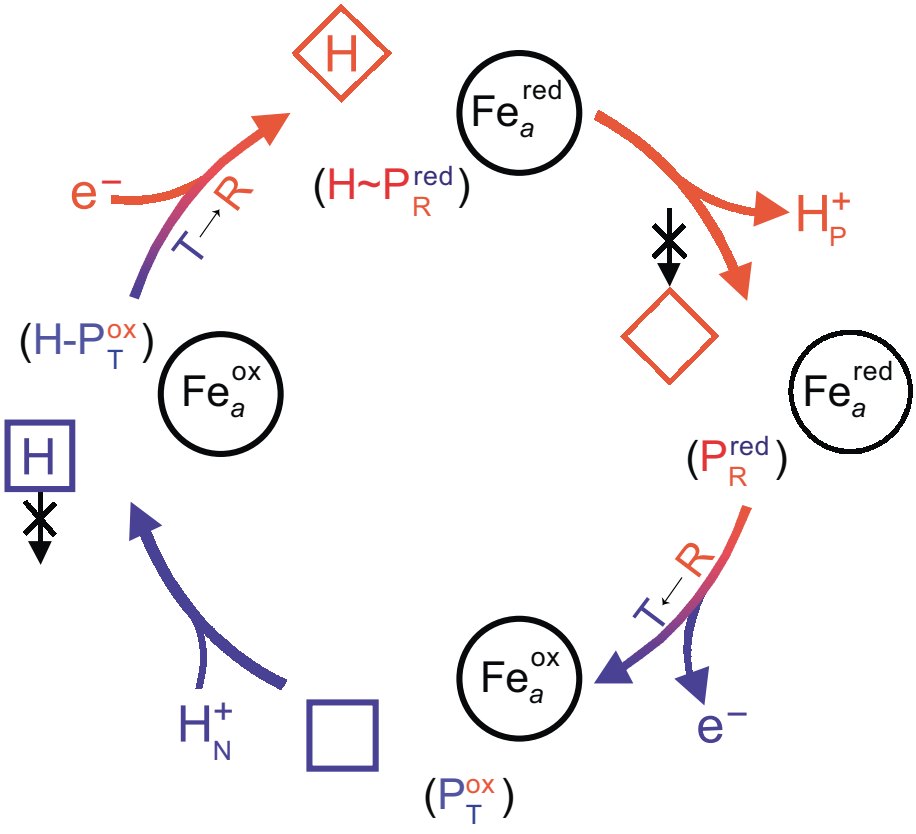

Fig. 3. Minimal model for proton activation by COX: A molecular ratchet mechanism using the rB^- protolytic group as a pawl. The functionality of the cycle implies that the starting state is protonated ($H\text{-}P_T^{ox}$), which prevalence depends on the (exogenous) initial experimental conditions that control its stability (cf. [29]). Thus, proton activation can obviously be restricted by experimental conditions. The T to R redox-linked conformational change of the protolytic group is represented by the blue and red squares, respectively. The crucial functional states of Fig. 1B are shown in parentheses; the colour code, subscripts, and superscripts used in the previous figures is maintained; H_N^+ and H_P^+ represent protons recruited from the N-side and ejected towards the P-side of the membrane, respectively. Again, it should be stressed that, although making the schematic description of the model more complex, inclusion of the flanking protolytic centres with positive rBs with Fe_a (rB^+_{1A} and rB^+_{1B}, see text) will contribute to reinforce the directional aspects of the model. The black arrows with crosses represent inefficient movement of protons due to the relevant pK_as of the flanking protolytic groups (valves) at the entry (rB^+_{1B}) and exit (rB^+_{1A}) channels (see text and legend of Fig. 2), which were not included in the figure for the sake of clarity. Colour code, subscripts, and superscripts used are the same as those of Fig. 1.

3), the rB^+ effect required to explain the redox chemistry of Fe_a (Fig. 2) can contribute to the vectoriality of the proton movement. To explain this point it is necessary to further analyse the fit of Fig. 2 (see legend): The pK_as of the rB_1^+ can result from the combined effects of two microscopic rB_1^+s, one with more acidic pK_as, rB_{1A}^+, and another with more basic pK_as, rB_{1B}^+, with the value of $pK_{a1A}^{red} \approx pK_{a1B}^{ox}$ and optimised to work at the relevant 'local pHs' [44]. In this situation, $pK_{a1B}^{ox} < pK_{a2}^{ox}$ and $pK_{a1A}^{red} > pK_{a2}^{red}$ will contribute to block the movement of protons back to the N-side (see black arrows with crosses in Fig. 3).

The molecular ratchet mechanism that confers vectoriality to the redox-linked movement of protons can be seen as a paradoxical effect, in the sense that a positively charged particle (bound to the rB_2^- group, the ratchet pawl) is ejected upon binding of a negatively charged one. This type of effects is often observed in life chemistry processes, where they are commonly described as the work of a Maxwell's demon [40]. The common characteristic of the processes generally considered as one of the many reincarnations of Maxwell's demon is that they proceed in apparent violation of the second law of thermodynamics. The stepwise choice of the electron transfer pathway and the conformational change that governs the thermodynamic link between the redox and the rB^- group are essential components to provide the pawl of the ratchet mechanism that warrants a productive catalytic cycle of COX. Thus, the structure of COX evolved towards a level of organisation such that the topology [41] and molecular choreography [3] of its functional centres can restrict alternative pathways, which could lead to eventually faster, but futile cycles [16] that would uncouple the proton energisation activity of the enzyme. Actually, since the work of this envisioned creature accomplishes essential steps for life chemistry, such as the proton activation of COX, it would be better described as a Maxwell's god [12].

3. Conclusions and perspectives

In contradistinction with the use of direct electrostatics, inclusion of an electron/proton antagonistic effect adequately describes the redox chemistry of COX Fe_a and its involvement in the productive activation of protons. Interestingly, the small conformational change required by this mechanism might not be detectable by X-ray crystallography, as is the case for anti-Coulomb interactions even in small proteins [10].

This deceptively simple, but counterintuitive mechanism is coherent with the experimental data available, fulfilling the first objective of a model. However, another objective in proposing a new model is its usefulness. Clearly, this model is amenable to further tests and improvements by supporting and explaining either existing data, mechanisms, and hypotheses, or data obtained by specifically designed experiments. One example is the observation that Fe_a senses the pH of the N-side [24]. Another is the comparison of the X-ray structures of oxidised and reduced bovine heart COX, showing that upon reduction the carboxylate group of Asp51 moves from the interior of the protein to a solvent exposed region on the P-side [42]. This redox-linked structural rearrangement is fully compatible with a rB^- effect. Curiously, this involves the rearrangement of an H-bond network, reminiscent of that used by *Desulfovibrio gigas* $Cytc_3$ to promote an antielectrostatic two-electron step [10]. Furthermore, this mechanism is phenomenologically equivalent to that used in the bacteriorhodopsin photocycle, where a mechano-chemical component prompts the ejection of a proton from a group protonated prior to photoisomerisation (see figure 1 of [43]). It should be stressed that this minimal model does not take into consideration all possible interactions involving haem *a* with other coupled protolytic groups and redox centres [25] and subsequent rereductions of Fe_a [44–46]. However, a complete quantitative description of this mechanism must await further thermodynamic, kinetic, and structural data for the unliganded and liganded enzyme.

In the light of this model, the proton motive force is achieved when activated protons reach the exit gate, primed to assist ATP synthesis [12]. Although using a novel mechanism and including a common chemical intermediate, this model is conceptually compatible with Williams' seminal proposal, which considers that the primary step to create the proton motive force is the energisation of protons localised in the transducer, acquiring and storing a high electrochemical potential that does not equilibrate with that of the P-phase [47]. This is in contrast with Mitchell's proposal [48] in which delocalised protons are conducted to the P-phase through spatially oriented proton wells, equilibrating with the electrochemical potential of the bulk phase (see [49] and references therein). According to the central feature of the chemiosmosis theory [48], the resulting electrochemical potential gradient across the membrane (which in Williams' proposal is a secondary result of the steady-state equilibrium [47]) is the coupling intermediary for oxidative phosphorylation. The new mechanism also brings to memory the structural [50] and chemical [51] coupling hypotheses. Ironically, it is difficult to envisage how the energised mechano-chemical common intermediate state ($H \sim P_R^{red}$) could be detected using the techniques available at the time when, on the basis of negative results, these mechanistic hypotheses were discarded.

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