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New Concepts

Proton Translocation by Cytochrome *c* Oxidase: A Rejoinder to Recent Criticism[†]

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ABSTRACT: Ten years ago, intermediate reaction steps in the catalytic cycle of cytochrome *c* oxidase were titrated with phosphorylation potential in isolated mitochondria, and the results were interpreted as evidence for thermodynamic linkage of proton translocation exclusively to the oxidative reaction steps of the catalytic cycle [Wikström, M. (1989) *Nature 338*, 776−778]. Michel has recently argued that this work was flawed, and proposed a mechanism in which one of the four steps of proton translocation is linked to the reductive phase of the catalytic cycle [Michel, H. (1999) *Biochemistry 38*, 15129−15140]. Here, the original data are scrutinized and related to information that has accumulated since this work was published. The analysis shows that the main conclusions from this work still hold. Michel's mechanism of proton translocation is briefly discussed, and found to be at odds with some experimental observations.

Cytochrome *c* oxidase catalyzes the respiratory reduction of O_2 to water, and couples this intrinsically exergonic reaction to translocation of four protons across the mitochondrial or bacterial membrane (Figure 1A). Reduction of O_2 takes place at the enzyme's binuclear $Fe_{a3}-Cu_B$[1] center, which accepts electrons from the low-spin Fe_a nearby. Fe_a, in turn, accepts electrons from the bimetallic Cu_A site on the **p**ositively charged side (*P*-side)[1] of the membrane. Cu_A is the electron acceptor from cytochrome *c* on that side. The four protons required to complete the O_2 reduction chemistry are taken up from the **n**egatively charged side (*N*-side) of the membrane. Thus, during turnover, a total of 8 electrical charge equivalents (q) are translocated across the membrane per O_2 reduced (see refs *1* and *2* for reviews). The X-ray structures of two cytochrome *c* oxidases are known (*3*, *4*).

In the catalytic cycle (Figure 1B), the reduced binuclear site (R) reacts with O_2 to form an oxygen adduct (Compound A). If there is no electron supply from Fe_a, a relatively stable state called P_M is formed next (*1*, *2*). However, if electrons are available in Fe_a and Cu_A, Compound A dissipates to a state called P_R with simultaneous transfer of one electron from Fe_a into the binuclear site (*5*, *6*), followed by the intermediate F. Transfer of the fourth electron yields the ferric/cupric forms of the site, viz., H and, finally, O (*1*, *2*). After two-electron reduction of O to R, a new cycle can begin. Figure 1B is a brief outline of the cycle (see *1*, *2*, *7*, and *8* and references cited therein). Note that proton movements have been omitted for clarity (but see below).

THE P AND F STATES OF THE BINUCLEAR HEME a_3-Cu_B CENTER

In 1981 it was discovered that the proton pump of cytochrome *c* oxidase could be reversed at high protonmotive force in isolated mitochondria, and that this apparently drove the catalytic cycle backward, revealing intermediate states

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[1] Abbreviations: EXAFS, extended X-ray absorption fine structure; Fe_a, low-spin heme *a*; Fe_{a3}, heme a_3 in the O_2-binding site; ΔG_p, phosphorylation potential, i.e., free energy change for the synthesis of ATP from ADP and P_i; *N*-side, negatively charged side of membrane; *P*-side, positively charged side of membrane; P_i, inorganic phosphate.

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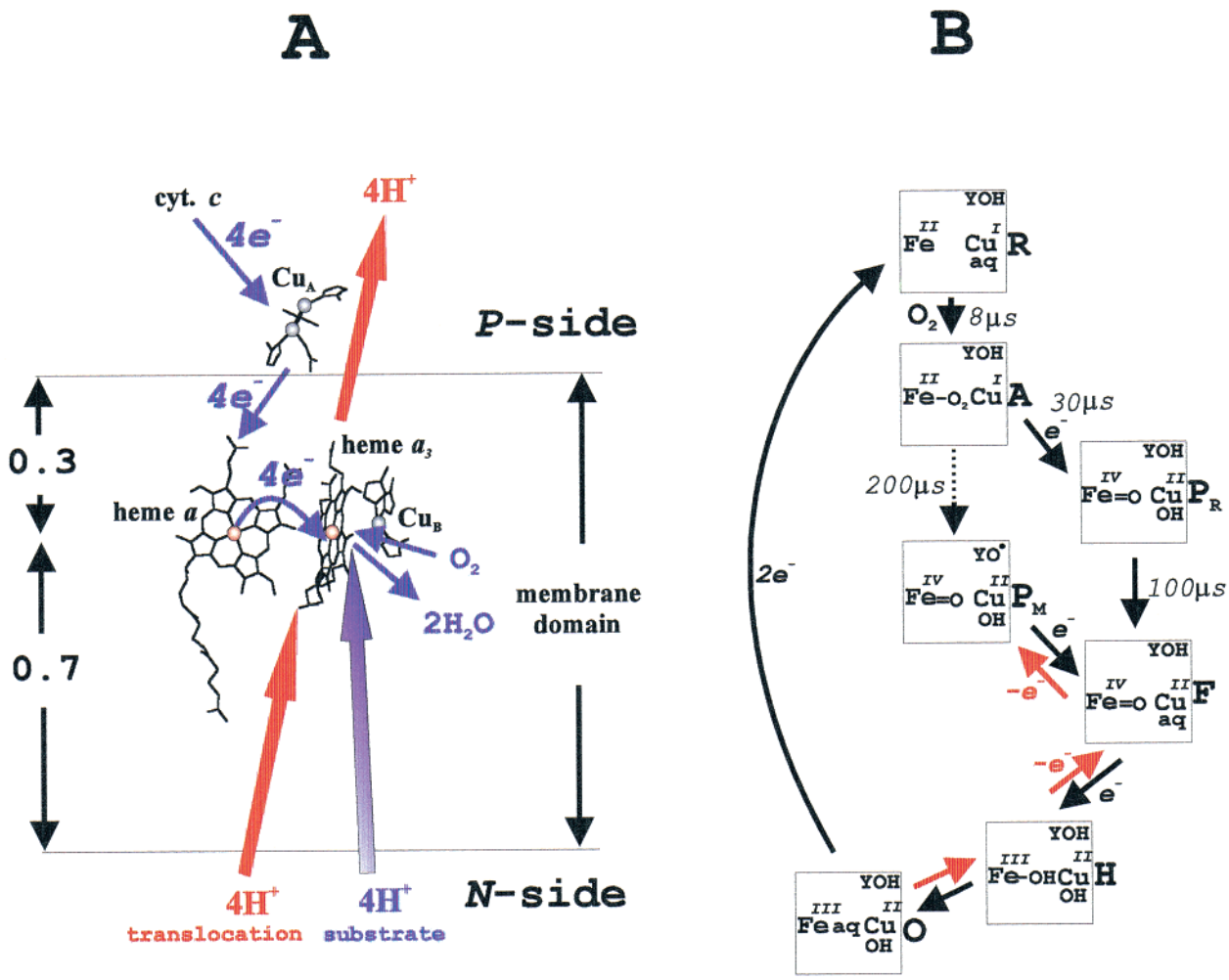

FIGURE 1: Cytochrome *c* oxidase. (A) Overall reaction in the membrane (modified from ref *23*). Electron transfer from cytochrome *c* occurs across ca. 0.3 and uptake of substrate protons (blue) across ca. 0.7 of the dielectric (*23*). A full O_2 turnover is further linked to translocation of four protons across the membrane. (B) Simplified catalytic cycle. The boxes depict the binuclear center with the nearby tyrosine (YOH) which can form the neutral radical (YO*). Only the distal ligand of heme a_3 (Fe) and one of the ligands of Cu_B are shown. No proton movements are shown. The red arrows indicate the path taken during reversal of the cycle at high protonmotive force. The O and H states are indistinguishable by optical spectroscopy, whereas P_M and P_R have the same spectra (for details, see text).

of the binuclear heme a_3−Cu_B center in its reaction with dioxygen (*9*, Figure 1B, red arrows). Two such states, identified by optical spectroscopy, were called F and P, and were shown to be associated with one- and two-electron oxidation of the ground state (state O) ferric−cupric binuclear center (plus water), respectively (*9*). Backflux of one or two electrons from the center into cytochrome *c* was indeed directly demonstrated in such conditions (*10*). In view of these results and plausible mechanisms of O_2 reduction, it seemed reasonable at the time to suggest that the F state might correspond to an oxo**f**erryl site, and that P might have a ferric **p**eroxide structure. It was early recognized, however, that the optical spectrum of P is atypical for ferric heme, and the possibility of an Fe[IV] structure was discussed (*11*). Subsequently, both the F and P states were indeed shown to be intermediates in the forward reaction (Figure 1B; *1*, *2*, *6*), as suggested, and Raman studies confirmed the oxoferryl heme structure for F (see *1*). Thus, part of the O_2 reduction mechanism had been revealed. The structure of the more enigmatic P state has been elucidated only more recently. Weng and Baker (*12*) had already pointed out that the similar Soret band of P and F suggests the same oxidation state for Fe_{a3}, i.e., Fe[IV]═O, but the dramatic difference in the α-band made us reluctant to accept this for a long time, in particular since the optical spectrum of P_R is the same as that for P_M (*6*). Weng and Baker (*12*), and others, proposed that the additional oxidizing equivalent in P_M, relative to F and P_R, may reside in the protein, perhaps in a nearby tyrosine, and this is the main current view (*7*, *8*, *13*). In addition, the optical spectral difference between P and F now finds a plausible explanation by weak and strong H-bonding to the oxo group, respectively, from a hydroxo or aquo ligand of Cu_B (Figure 1B; *13*, *14*).

DEPENDENCE OF F AND P ON PHOSPHORYLATION POTENTIAL AND pH

The Equilibrium Approximation. Phosphorylation potential (ΔG_p) titrations of the interconversions between the O and F and the F and P states at high redox potential in intact mitochondria suggested that these transitions are linked to the hydrolysis of 0.70−0.75 and 0.9−1 molecules of ATP, respectively (*15*). Since the oxidase reaction yields 2 ATP molecules overall per O_2 reduced, it was concluded that all proton-pumping events must be coupled to the P→F and F→O steps, as they appeared to be linked to 80−88% of all ATP synthesized. Michel recently criticized this work (*8*),

claiming that it provides no evidence for such linkage. The assumption of equilibrium between ΔG_p and the partial reactions of cytochrome oxidase is obvious in this work, and naturally includes equilibration with the intermediary protonmotive force since a chemiosmotic principle of coupling is assumed. Of course, true equilibrium will not be established in such an open system, but an equilibrium *approximation* can be made for the case where net flux through the system is much slower than backward and forward rates of individual processes. This approximation holds reasonably well for tightly coupled mitochondria, because the rate of oligomycin-sensitive ATP hydrolysis (<0.2 s^{-1}; see, e.g., *16*), which is a measure of the protonic leaks through the membrane, is much slower than the rate by which ATP hydrolysis affects cytochrome *c* oxidase (>3.5 s^{-1}; *10*, *17*). Titrations of this kind have indeed yielded linear and reversible results in well-coupled mitochondria (e.g., *15*, *18*, *19*), supporting the equilibrium approximation. Michel (*8*) cited a general statement in a review article (*20*) as the only argument against its validity.

Conversion to Number of Translocated Protons. Since hydrolysis of extramitochondrial ATP is likely to be coupled to translocation of 4 charge equivalents across the inner membrane (3 protons pumped by the H^+-ATPase and 1 additional charge due to electrogenic ATP/ADP exchange), the slopes of the titrations with ΔG_p indicated linkage of the F/O and P/F reaction steps to translocation of 2.8−3.0 and 3.6−4.0 charge equivalents (q), respectively (*15*). At the time of this work, it was not known which reactions of the catalytic cycle would be associated with uptake of the *substrate* protons to form water (or bound hydroxide) at the binuclear center. Yet, this information is crucial in order to interpret the ΔG_p titrations in terms of the number of *pumped* protons. *However, the results from the titrations with ΔG_p were already alone strongly indicative of thermodynamic coupling of all proton translocation to the P→F and F→O steps*. Today, it is known that reduction of the binuclear center is linked to translocation of *two* electrical charges across the dielectric (two electrons from the *P*-side and two protons taken up from the *N*-side; see below), which amounts to 25% of all charge translocation by the enzyme. Therefore, the finding that ca. 80−88% of the ATP formed in the catalytic cycle is coupled to the P→F and F→O reaction steps must mean that all proton pumping is linked to these steps. Deduction of the number of pumped protons was much more difficult, however, because it depended on the interpretation of the dependences of the F/O and P/F equilibria on pH at high ΔG_p. This pH dependence was expected to reveal the number of net substrate protons taken up in the respective reaction. Subtraction of this from the total number of charges translocated would then yield the number of pumped protons.

The F/O Equilibrium. At high ΔG_p, the pH dependence of the F/O reaction showed a slope of 2 decades per pH unit below pH 7.2, but strongly bent off toward zero slope above this pH (*21*). If the pH inside the mitochondria would rise significantly above the extramitochondrial pH of 7.2 in the titration with ΔG_p (*15*), as suggested by Michel (*8*), it would indeed be incorrect to subtract one substrate proton from the overall number of 2.8−3.0 to obtain the number of pumped protons (as done in *15*), because the flat pH dependence above pH 7.2 indicates little or no net proton consumption. However, according to present knowledge, the F→O reaction step must be associated with the uptake of one proton from the *N*-side to form water at the binuclear site (*22*). Together with the electron transfer, this accounts for translocation of about one electrical charge across the dielectric. Therefore, the results in (*15*) must in any case mean that 1.8−2.0 q are translocated due to proton pumping in this step, as concluded at the time. The lack of *net* proton uptake at pH >7.2 may then mean that the uptake of the substrate proton is compensated for by release of another proton from the enzyme in these conditions, which might be part of a proton translocation step (see *14*). Incidentally, the conclusion that 1.8−2.0 q are translocated due to proton pumping coupled to the F→O reaction would also mean that relaxation of the recently observed metastable O state (*23*) can be reversed at high protonmotive force.

When the pH of the medium was raised to 8.3, the *total* number of charges translocated in the F→O step decreased to 2.2 q, as deduced from a titration of the F/O equilibrium with membrane potential (*15*). The simplest interpretation of this decrease by about 0.6−0.8 q is that at this high pH one proton may be abstracted from the binuclear site, deprotonating the aquo ligand of Fe_{a3} to hydroxo, which is not discernible by optical spectroscopy in the α-band. The lack of this proton-transfer event in the titration, across 0.7 of the dielectric (*23*), is expected to decrease the observed number of translocated charges by 0.7, as observed. Subtraction of the fractional charge translocated due to electron transfer in this reaction (0.3 q; Figure 1A) again brings the number of charges translocated due to proton pumping close to 2 for the F→O step.

The P/F Equilibrium. The pH dependence of the P/F transition at high ΔG_p was reported to be about 2 decades/pH unit (*21*). As pointed out by Michel (*8*), ΔG_p increases with pH, especially above pH 7 (*24*), and this was in fact originally thought to be the sole reason for the pH dependence (*9*). It was later found, however, that whether driven by reversal of the ATP synthase or by electron transfer and thus independently of ΔG_p, this equilibrium still exhibits an approximately 2 decades/pH unit dependence, and specifically on pH on the *N*-side of the membrane (*25*). In fact, there is no dependence on pH on the *P*-side after accounting for the pH dependence of ΔG_p (*19*). Moreover, careful inspection of the data points of log(*P*/*F*) versus pH at high ΔG_p (e.g., Figure 2A of ref *21*) reveals that above pH 7 the slope shows signs of being steeper than 2 decades/pH unit. In this experiment, the pHs on the *P*- and *N*-sides were titrated simultaneously due to the presence of nigericin. The pH dependence of ΔG_p has the effect of increasing the slope by up to 0.9−1 decade/pH unit, which is probably why in this experiment the pH dependence seemed steeper than when the protonmotive force was generated by electron transfer. For these reasons, the P→F transition was interpreted in (*15*) as being linked to the uptake of *two* protons from the *N*-side at high protonmotive force, *after* accounting approximately for the pH dependence of ΔG_p. As pointed out by Michel (*8*), this differs from the isolated enzyme in solution, which shows a net uptake of only about *one* proton in this step (*22*, *26*−*28*). The difference may arise from an effect of the protonmotive force, which may pull out an additional proton from the enzyme toward the *N*-side of the membrane (*29*), as described below.

Also based on the structure, Michel (*8*) disputed that the oxidation of F to P could be associated with the net release of *two* protons. The present structures only allow for one proton (*22, 26*−*28*): a water molecule in F becomes a hydroxo ligand of Cu_B in P_M (Figure 1B). The second proton abstraction is more intriguing, but finds a rational explanation from recent results. During reduction of Fe_{a3}, a proton is taken up from the *N*-side via the so-called K-channel (*7, 26*). Density functional calculations suggest that this proton, which is of key importance during scission of the O−O bond, becomes associated to the hydroxyethyl group of the side chain of Fe_{a3} (*30*). This proton is hydrogen-bonded to the tyrosine in the binuclear site, and will be transferred to the tyrosine oxygen on reduction of the tyrosine radical (*30*). Thus, in the backward reaction, when oxidation of the tyrosine in F yields the tyrosine radical in P_M (Figure 1B), this proton is liberated, and at high protonmotive force it is expected to be pulled back out via the K-channel toward the *N*-side. Therefore, the observed net uptake of *two* protons in the P→F transition *at high* ΔG_p is fully consistent with present knowledge, and actually predicted the presence of a second protonatable site in the binuclear center.

The titration of the P/F equilibrium with ΔG_p suggested translocation of a total of 3.6−4.0 q across the dielectric (*15*, cf. above). The net uptake of *two* protons in this step into the binuclear center accounts for ca. 1.4 q (see above), and the electron transfer for ca. 0.3 q (Figure 1B). Hence, these data indicate linkage of the P→F reaction step to pumping of 1.9−2.3 q, which is very close to the original conclusion in (*15*).

HYDROXO LIGANDS AT HEME a_3 AND Cu_B

Michel dismissed the possibility of a state of the binuclear center with hydroxo ligands at both Fe_{a3} and Cu_B, referring to structural data which show that the two OH^- groups would come too close to one another with unacceptable electrostatic repulsion (*8*). However, there are no X-ray structural data for the H-state of the catalytic cycle (Figure 1B), in which a hydroxo ligand at Fe_{a3} has been identified by resonance Raman spectroscopy (*1, 31, 32*). In this state, a hydroxo ligand at Cu_B need not lie close to the OH^- ligand of Fe_{a3}, especially if the copper has trigonal geometry with only two histidine ligands. Evidence for loss of one of the Cu_B histidine ligands upon reduction of the binuclear center and its reaction with CO was recently obtained by EXAFS studies (*33, 34*), and is one of the key features of a newly proposed model of proton translocation (*14*).

MICHEL'S MODEL

Michel (*8*) presented a detailed scheme of the catalytic cycle of cytochrome *c* oxidase. Although proton translocation events were included, there was no attempt to explain one fundamental aspect of any redox-linked proton pump, viz., that of the control of the protonic sidedness (or "gating"; see *1, 11, 35*). For example, it was postulated that reduction of Fe_a is linked to proton transfer all the way from the *N*-side of the membrane into a hydrophilic cluster in the propionate domain of the heme groups near the *P*-side. Subsequently, this proton is released (pumped) to the aqueous medium on the *P*-side. Since the hydrophilic cluster is connected protonically to the *P*-side of the membrane, it is not clear why reduction of Fe_a would not simply attract a proton from this side rather than from the *N*-side. The model also provides no explanation for how proton transfer takes place from the glutamic acid residue in the D-channel to either the heme propionate or the binuclear center, both some 12 Å away.

Michel's model (*8*) is also inconsistent with some experimental data. Zaslavsky et al. (*36*) showed that the injection of an electron into the enzyme with the binuclear center in the F state causes reduction of Fe_a in 50 μs, followed by its reoxidation by electron transfer into the binuclear center in 1−4 ms. Translocation of electrical charge was measured by time-resolved electrometry, and the amplitude of the fast phase was $^1/_4$ of the slower phases (*36*). According to Michel (*8*), reduction of Fe_a is coupled to proton uptake from the *N*-side into the hydrophilic cluster, i.e., equivalent of translocating one full charge across the dielectric. If so, the 4 times larger amplitude during reduction of the binuclear center by Fe_a (*36*) would mean that there is translocation of *four* charge equivalents in the F→O reaction. Proton uptake linked to reduction of Fe_a is a key feature of Michel's model, and reminiscent of an early proposal from 1978 (*37*), but it is refuted by this experiment. There is also no rational explanation in this model for its inherent inconsistency that while reduction of Fe_a generally causes proton uptake, this does not occur upon the first electron transfer to the oxidized enzyme.

The elegant study by Vygodina et al. (*38*) showed that steady-state cycling of the enzyme in a peroxidative mode, using H_2O_2 and a high-potential electron donor, leads to translocation of 4 protons per H_2O_2 reduced, which is not consistent with Michel's model. Obviously, this model also does not account for the thermodynamic coupling revealed by the data in (*15*), and discussed above.

The P_M state is unstable in Michel's scheme, and spontaneously converts into an F-like state (called F′) with pumping of one proton (*8*). To be compatible with catalysis, this would have to occur in less than 1 ms, which disagrees with the known relative stability of P_M. Michel (*8*) explained this discrepancy by proposing that an artifactually stable P_M state is formed specifically when the binuclear center is reduced by CO. However, the enzyme may be reduced without CO; CO may then be added to stabilize the reduced binuclear center, followed by excess ferricyanide to oxidize Fe_a and Cu_A. After mixing with O_2, flash photolysis of this "CO-mixed valence" state also yields P_M, as first shown by the pioneering work of Chance et al. (*39*). In this case, the lifetime of P_M is about 20 s at room temperature and pH 8 (J. E. Morgan and M. I. Verkhovsky, unpublished), which is $>10^4$ times too long to be compatible with Michel's model. Our recent finding (*23*) that there is no proton translocation upon reduction of the ground-state oxidized binuclear site (O) also contradicts this model. Michel (*8, 40*) has criticized the experiments in (*23*), but these objections are unfounded; the suggested multiple turnovers of the enzyme could not have occurred (*41*).

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