

The Third Datta Lecture

The mechanism of proton translocation in respiration and photosynthesis

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Redox-linked proton pump; Electron gating; Reorganization energy; Intrinsic uncoupling

1. THE KEY PROCESSES OF BIOENERGETICS

Life is a chemical phenomenon and, as such, much more complex and sophisticated than any man-made chemical system. Through milliards (American English, billions) of years of biological evolution, natural selection has led to elegant chemical solutions to the survival problems of living organisms on Earth. Superb examples of this are the key processes of bioenergetics, respiration and photosynthesis. Aerobic organisms derive most of the free energy necessary for life processes, such as growth, transport or locomotion, from the combustion of carbohydrates and fats with the dioxygen of air, leading to the formation of CO_2 and H_2O . This could, however, not go on indefinitely, were it not for the fact that green plants in photosynthesis use the energy of the sun to resynthesize carbohydrate from CO_2 and H_2O with

the concomitant liberation of O_2 . Thus, respiration and photosynthesis together form a continuous cycle driven by the sun (fig.1), and this represents an ideal energy system which Man should try to imitate for his survival.

Unraveling the secrets of photosynthesis and respiration is consequently an urgent challenge to the biochemist, but also an extremely demanding one. It is stated in a famous textbook of molecular biology [1] that "how light quanta are collected by the photosynthetic apparatus, or how a membrane protein pumps ions, increasingly have become the important biochemical questions that only chemically oriented scientists (as opposed to

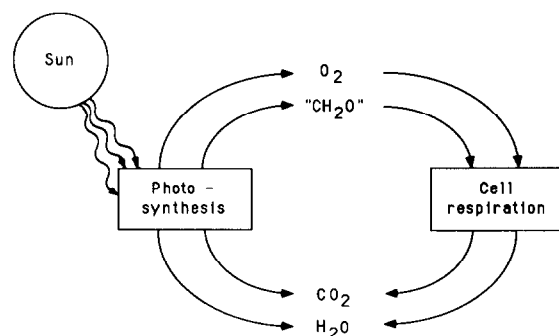


Fig.1. The carbon and oxygen cycles in the biosphere.

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Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine

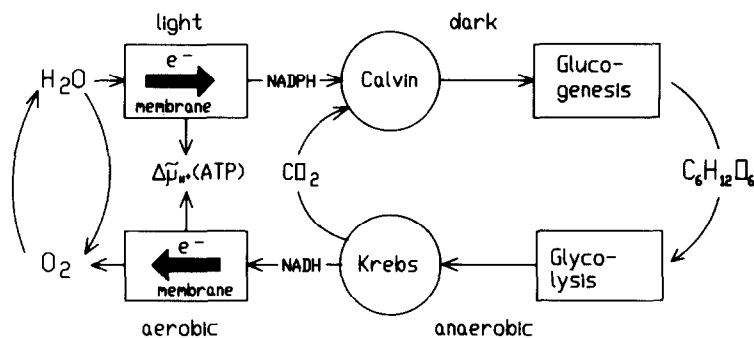


Fig.2. Respiration and photosynthesis as antiparallel processes.

biologically oriented investigators) will be able to answer". These are the two problem areas worked on in the Göteborg biochemistry and biophysics laboratory, the first one by my colleague, Professor Tore Vänngård, and his collaborators, the latter by my own research group.

There are good reasons for studying problems of

respiration and photosynthesis in the same laboratory, because these two processes are very similar in design. In fact, they can be said to be antiparallel (fig.2). For the purposes of this lecture it should, in particular, be noted that in both processes there are complex, membrane-bound electron-transport chains. In respiration, the

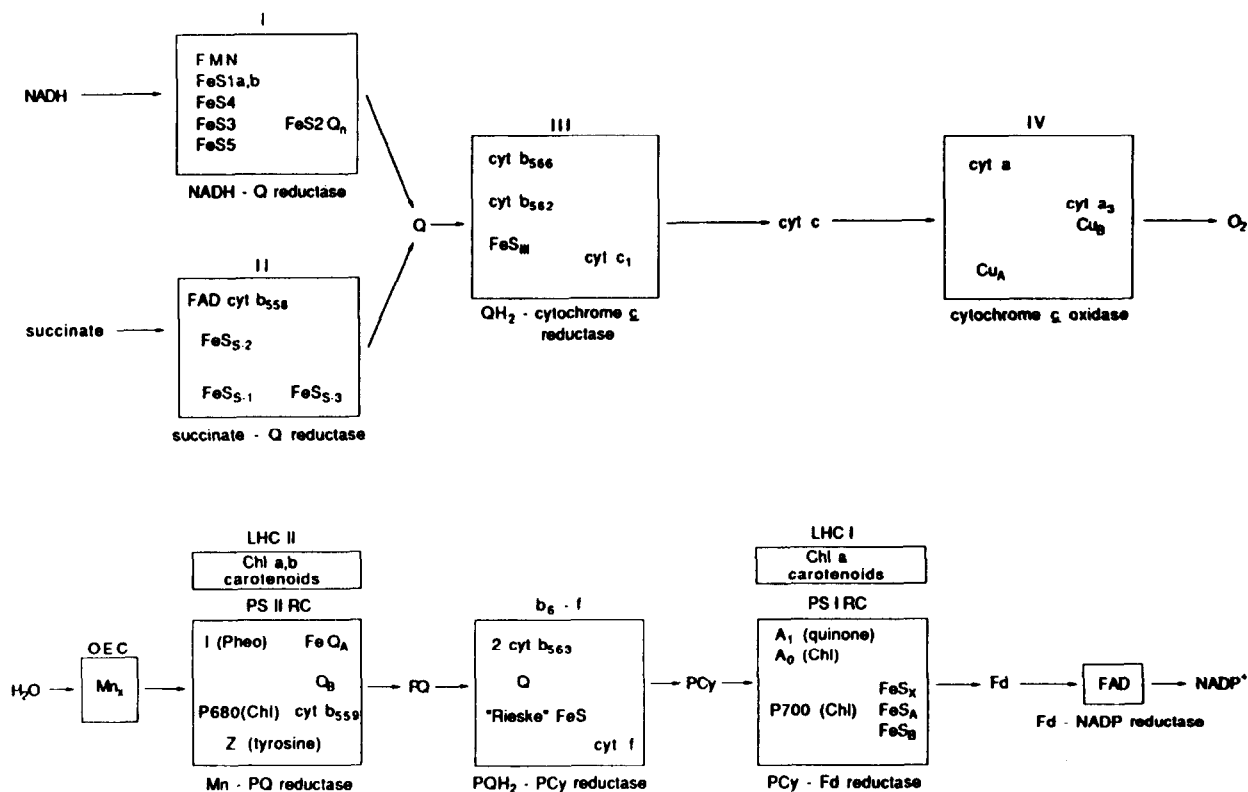


Fig.3. The electron-transport chains of respiration and photosynthesis. *Abbreviations:* Q, ubiquinone; PQ, plastoquinone; cyt, cytochrome; FeS, iron-sulphur cluster; PCy, plastocyanin; Fd, ferredoxin; OEC, oxygen-evolving complex; LHC, light-harvesting complex; PS, photosystem; RC, reaction centre.

NADH formed in the anaerobic part of the process is reoxidised by O_2 in the respiratory chain with a considerable release of free energy. In photosynthesis, the electron transfer is not a spontaneous reaction. Instead, the absorption of light energy in the reaction centres of both photosystems I and II leads to a charge separation. The electron released in photosystem I ends up in NADPH, which serves as the reductant in CO_2 fixation in the dark reaction, whereas the electron from photosystem II fills the positive hole in the other photosystem, its own hole being filled by the oxidation of H_2O .

The electron-transfer reactions in respiration and photosynthesis lead to the translocation of protons across the inner mitochondrial and thylakoid membrane, respectively, so that the free energy released is temporarily stored in the form of an electrochemical potential across the membrane. This potential is then used to drive the synthesis of ATP, the immediate source of free energy in most endergonic reactions of living cells, as first suggested by Peter Mitchell [2].

The electron-transfer complexes of the electron-transport chains in respiration and photosynthesis are compared in fig.3. It can be seen that, also on the molecular level, there are great similarities between the two processes. For example, in both cases electron transfer between complexes is mediated by a quinone at one point and by a small, H_2O -soluble metalloprotein at another. In particular, the composition of complex III in respiration is nearly identical with that of the cytochrome b_6f complex in photosynthesis. How the electron-transfer reactions in the two electron-transport chains can be coupled to the translocation of protons across the membranes will be the topic of this lecture.

2. THE MECHANISM OF PROTON TRANSLOCATION

In fact, we do not know the mechanism of proton translocation in complete detail for any part of the electron-transport chains. Mitchell's original suggestion was the redox loop, defined as a system which "translocates hydrogen groups one way and electrons the other way", so that "it thus gives a net translocation of protons" [2]. Obviously, such a system could result only in an H^+/e^- stoichiometry of 1. There is, however, firm experimental evidence that in complex I and IV of

respiration the stoichiometry is actually $2H^+/e^-$ [3,4]. A lower stoichiometry would indeed be wasteful, because the energy available at these sites exceeds the electrochemical potential ($\Delta\mu H^+$) by a factor >2 [3-5]. For example, for cytochrome oxidase (complex IV) the available driving force, i.e. the difference in reduction potential between O_2 and cytochrome c , is 0.5 V, whereas the proton-motive force (Δp), i.e. $\Delta\mu H^+$ expressed in volts, is 0.22 V [5]. It was consequently gratifying when Wikström [6] established in 1977 that cytochrome oxidase is a redox-linked proton pump, a term which he later introduced together with Krab [5].

The bc_1 (complex III) and b_6f complexes are so similar that they undoubtedly operate by the same mechanism. This is generally believed to be some sort of Q-cycle [3], a type of mechanism first proposed by Mitchell [7]. There is, however, no consensus on the details of the cycle at the molecular level. One possible version [8] is illustrated in fig.4. Despite the fact that the H carrier, QH_2 , is freely diffusible across the membrane, a vectorial H^+ reaction is achieved, because the NADH(Mn)-Q-reductase can only reduce Q on the matrix (stroma) side of the membrane, whereas QH_2 can only donate electrons to cytochrome c (plastocyanin) via the FeS centre, on the cytosol (thylakoid lumen) side. There are two electrons involved, but one cycles via the b cytochromes, so that only one is transported along the chain. Thus, $2H^+/e^-$ are released on the cytosol side, but there is only

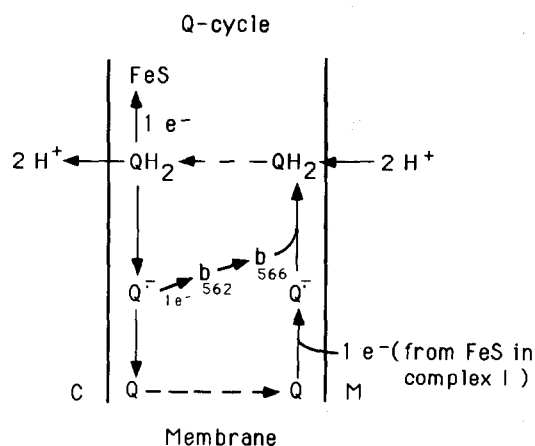


Fig.4. The Q-cycle. C and M represent the cytosol and matrix side, respectively, of the inner mitochondrial membrane. Adapted from [8].

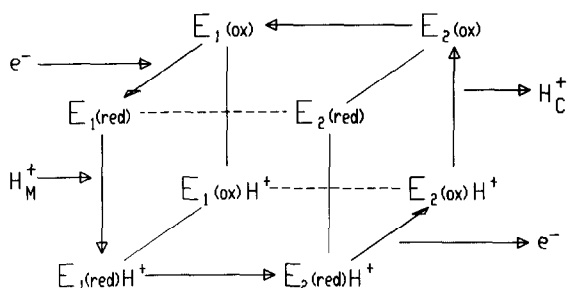


Fig.5. The cubic scheme for a redox-linked proton pump [5].

1q⁺/e⁻ stoichiometry, i.e. a charge stoichiometry of 1 [3,4].

The electron-transfer events coupled to proton translocation in NADH-Q reductase and cytochrome oxidase are not known. Any redox-linked proton pump must, however, follow certain basic principles, as summarized in the cubic reaction scheme of Wikström and Krab [5]. This scheme (fig.5) is quite general and can be modified to describe any biological energy transducer. In its general form, it had, in fact, been proposed one year earlier by Wyman in the Proceedings of the Accademia Nazionale dei Lincei here in Rome [9].

One important feature of the cubic scheme is the existence of separate input and output states for H⁺ and e⁻. In this way, the electron transfer from donor to acceptor cannot be completed unless the transducer undergoes a transition from the input to output state. If these states, in addition, correspond to alternating access [10] of the proton-translocating group to the two sides of the membrane, then the electron-transfer reaction will drive proton pumping. For the pump not to slip, however, the input-output transitions must have a high degree of specificity, such that the rate constants for the transitions along the horizontal arrows in fig.5 are much higher than those for the transitions along the dashed lines. In other words, the transition from the input to output state should be rapid only when the transducer is reduced and protonated, whereas the return should be rapid only when it is oxidised and unprotonated.

3. CYTOCHROME OXIDASE AS A REDOX-LINKED PROTON PUMP

3.1. A hypothetical pump cycle

In this lecture, I will illustrate the use of these

general principles by applying them to a specific proton pump, cytochrome oxidase. Before doing so, however, I will describe a hypothetical pump cycle (fig.6) which, I believe, is closer to cytochrome oxidase in design compared to the cubic scheme but not quite so complicated. The pump in this cycle contains two metal centres, a and b, which can exist in the four redox states: a⁺b⁺, a⁰b⁺, a⁺b⁰ and a⁰b⁰. It also has two conformational states, E₁ and E₂, providing the alternating access to protons on the two sides of the membrane. This pump design suggests an easy way to ensure that the electron-transfer reaction can start only in the E₁ state but be completed in the E₂ state alone, since the donor can only react with E₁(a⁺b⁺) and the acceptor with E₂(a⁺b⁰). If, in addition, the conformational transition is coupled to the internal electron transfer (fig.6), and requires protonation to be rapid, then proton pumping will obviously follow.

It has often been stated that a thermodynamic linkage [11] between the binding of e⁻ (reduction) and H⁺ ('membrane Bohr effect') is a requirement for a redox-linked proton pump. Such a linkage would have definite kinetic advantages but is not a thermodynamic necessity. In the extreme case, the protonation could be an uphill reaction (fig.7) but would still occur, if the activation energy for the conformational transition were much lower in the protonated compared to the unprotonated state ('transition-state mechanism') [12].

The transition-state mechanism may appear extremely unlikely, since it would involve the pump operating at a rate which is only a small fraction of

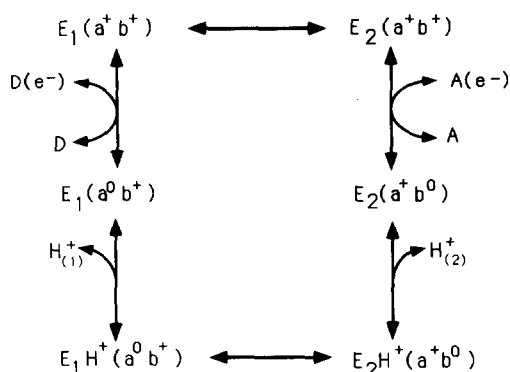


Fig.6. Hypothetical reaction cycle for a redox-linked proton pump. For explanation, see text.

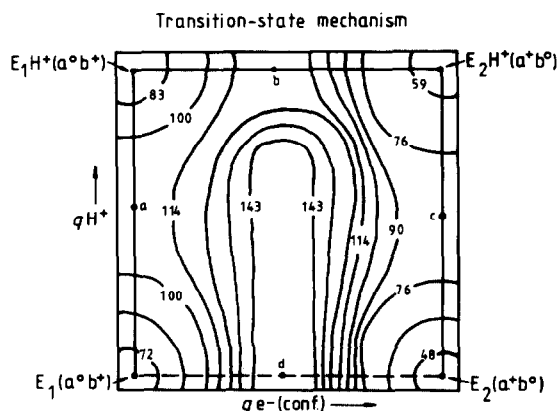


Fig. 7. Potential-energy map for the transition-state mechanism.

the optimal electron-transfer rate achieved at low pH. This is, however, exactly how cytochrome oxidase behaves. The value of k_{cat} increases from about 200 s^{-1} at pH 7.4 to $>10^3 \text{ s}^{-1}$ below pH 5, whereas k_{cat}/K_m remains constant [13–15]. Simulations of the kinetic results show that protonation of three groups with apparent pK_a values of 4.5, 6.8 and 7.8 increases the activity [14]. The highest pK_a has been suggested [13] to be associated with the binding of OH^- to oxidised cytochrome a_3 [16], whereas the two lower values are probably related to the proton pump.

3.2. The redox centres and the catalytic reaction

Since I have so far described some kinetic results with cytochrome oxidase, I believe that it is time for me to discuss this enzyme more closely (fig.8). It spans the membrane, as all ion pumps must do, in this case, the inner mitochondrial membrane, protruding more on the cytosol than on the matrix side. There are four redox-active metal centres, cytochromes a and a_3 , Cu_A and Cu_B , as well as one zinc and one magnesium ion [17], and possibly a third, non-redox-active copper ion [18]. The ligands of the redox-active centres have been established with reasonable certainty by a variety of spectroscopic techniques [6,19–21]. The distances between all centres are $>15 \text{ \AA}$ except that between cytochrome a_3 and Cu_B , which together form a binuclear unit [19].

Cytochrome c binds to the enzyme on the cytosol side of the membrane. It donates electrons one at a time, the primary acceptor being either Cu_A or cytochrome a . These centres are in rapid

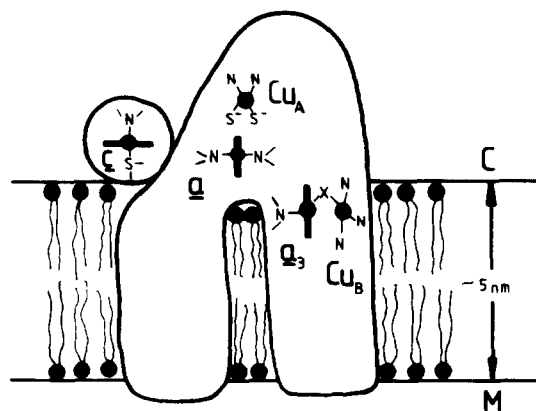
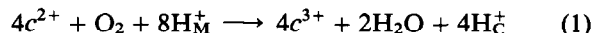


Fig. 8. A model for the cytochrome oxidase monomer and cytochrome c in the inner mitochondrial membrane.

redox equilibrium, so that they are reduced synchronously on the stopped-flow time scale [21,22]. Because the rate of product dissociation is also high [21,23], both Cu_A and cytochrome a are rapidly reduced. Not until this has occurred does intramolecular electron transfer to the binuclear site appear to take place [24,25], as Eraldo Antonini and I showed together with our co-workers in our very first cytochrome oxidase experiments carried out here in Rome in 1970. The intramolecular electron transfer has been considered to provide the main rate limit on k_{cat} , the catalytic constant [21,23], but we will soon see that this is not quite true. Dioxygen reacts with the reduced binuclear site.

The total reaction catalyzed by cytochrome oxidase is:



The four scalar protons consumed during dioxygen reduction are taken up from the matrix side [26]. In addition, one proton per electron is translocated to the cytosol side, so that the effective H^+/e^- stoichiometry is 2.

3.3. Kinetic tests of a reaction cycle

I shall now describe a pump cycle (fig.9) based on the principles discussed earlier and incorporating the kinetic information, which I have just briefly summarized. In doing so, I will use a notation which has become common in the field in recent years. The redox centres, in the order of cytochrome a , Cu_A , Cu_B and cytochrome a_3 , are

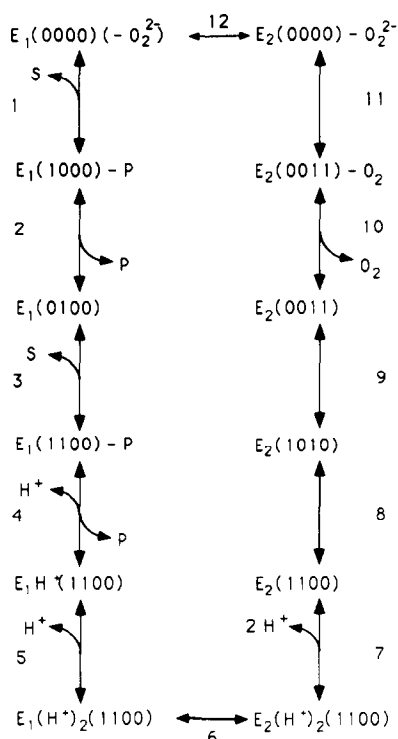


Fig.9. A reaction cycle for cytochrome oxidase as a redox-linked proton pump. S denotes ferro- and P, ferricytochrome *c*.

represented by digits; 0 representing an oxidised and 1 a reduced centre. In order not to make the scheme too large, two consecutive reactions have been combined in steps 1–4, 7 and 11; this is, in fact, kinetically justified for steps 1, 3 and 11, because in these a very rapid reaction follows a slow one.

I will now point out some important features of this hypothetical mechanism. For the enzyme to switch rapidly from the E_1 to the E_2 state, both cytochrome *a* and Cu_A must be reduced, and the enzyme must be doubly protonated (step 6). One of the proton-translocating groups has a high pK_a in E_1 and a low value in E_2 , whereas the second one has a low pK_a in both states. Such a situation could account for the apparent pK_a values derived from the pH dependence of k_{cat} . It would also have the consequence that the degree of protonation in step 5 is low at physiological pH, so that step 6 would be slow, as predicted in the transition-state mechanism [12]. It will be shown that the pH dependence of cytochrome oxidase kinetics is largely due to this step.

It is suggested that a complete enzyme turnover consists of two consecutive two-electron cycles, with oxygen intermediates bound at the binuclear site in the second cycle. One would expect that the oxidation of Cu_A and cytochrome *a* would be much more rapid in the second cycle, because of the higher driving force for electron transfer. This is about 0.1 V anaerobically [27] but increases to 0.6 V in the presence of O_2 [28] (table 1). It has been possible to measure the rate of oxidation of Cu_A and cytochrome *a* directly in flash-photolysis experiments with the CO compounds of the mixed-valence state [27,29] and fully reduced oxidase [30,31]. Surprisingly the rate constants are very close to each other in both cycles (table 1). This probably has two explanations. First, the reorganisational barrier to electron transfer, which is a major determinant of the free energy of activation, is probably much larger in the second cycle, since in this the O–O bond in dioxygen must be broken and two molecules of water formed. Second, the redox equilibrium between cytochrome *a* and Cu_A , which is rapid in E_1 [22], must be slow in E_2 , since cytochrome *a* would otherwise be rapidly oxidised via Cu_A . Thus, the rate of cytochrome *a* oxidation is mainly limited by this redox equilibrium in both cycles.

The key reaction of the pump cycle is the transition from the input to output state for both e^- and H^+ in step 6. Before discussing how this accomplishes the required gating of electron flow, however, let me describe some results on the pH dependence of cytochrome oxidase kinetics, supporting the overall reaction cycle. These have been simulated on the assumption that all rate constants are pH-independent and that the only significant effect of pH on the kinetics is derived from

Table 1

Driving forces (ΔG°) and rate constants (k) for the oxidation of Cu_A and cytochrome *a* in E_2^a

Centre oxidised	1st cycle		2nd cycle	
	ΔG° (kJ·mol ⁻¹)	k (s ⁻¹)	ΔG° (kJ·mol ⁻¹)	k (s ⁻¹)
Cu_A	–8.7	1.4×10^4	–62	1.0×10^4
<i>a</i> (via Cu_A)	–3.9, –8.7	400	–3.9, –52	800

^a From [27,28] and Oliveberg, M., Brzezinski, P. and Malmström, B.G., unpublished data

changes in the rate of protonation of the acid-base group with a low pK_a in both states (step 5).

Fig.10 shows plots of cytochrome *c* oxidation together with cytochrome *a* reduction and reoxidation in stopped-flow experiments with ferrocyanochrome *c* as the only reducing substrate. In the simulations, experimental rate constants have been used for 6 of the 12 steps in the cycle. In addition, all monomolecular steps have been given pH-independent rate constants with values $>10^3 \text{ s}^{-1}$ to account for the high values of k_{cat} at low pH. The critical step is the binding of H^+ to the acid-base group with a low pK_a in step 5 (fig.9). A pK_a of 6.4 for this group was found to give the best fit to the cytochrome *c* curve at pH 7.4 (curve c), giving a k_{off} for H^+ in step 5 of $4 \times 10^3 \text{ s}^{-1}$, if k_{on} is diffusion limited ($10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$). The same rate constants were then used for the simulations of all cytochrome *c* and cytochrome *a* curves. This severe restriction in the choice of rate constants explains

why the fit between the experimental and simulated curves is only semi-quantitative. All qualitative features are, however, well reproduced, for example, a maximum rate of cytochrome *c* oxidation at pH 6.4 or a minimum degree of cytochrome *a* reduction at pH 5.4.

The same rate constants have also been used to simulate kinetic results from experiments in which cytochrome *c* was kept partially reduced with ascorbate and TMPD (fig.11) [23]. We see an initial rapid oxidation of cytochrome *c* with the concurrent reduction of cytochrome *a* and Cu_A . This is followed by a certain amount of reoxidation of cytochrome *a* and Cu_A to a constant steady-state level. The steady-state phase lasts until all dioxygen has been consumed, when all redox centres become rapidly reduced. Again, the fit between the experimental and simulated traces is only semi-quantitative, but the duration of the steady state was always well reproduced at pH 7.0 as well

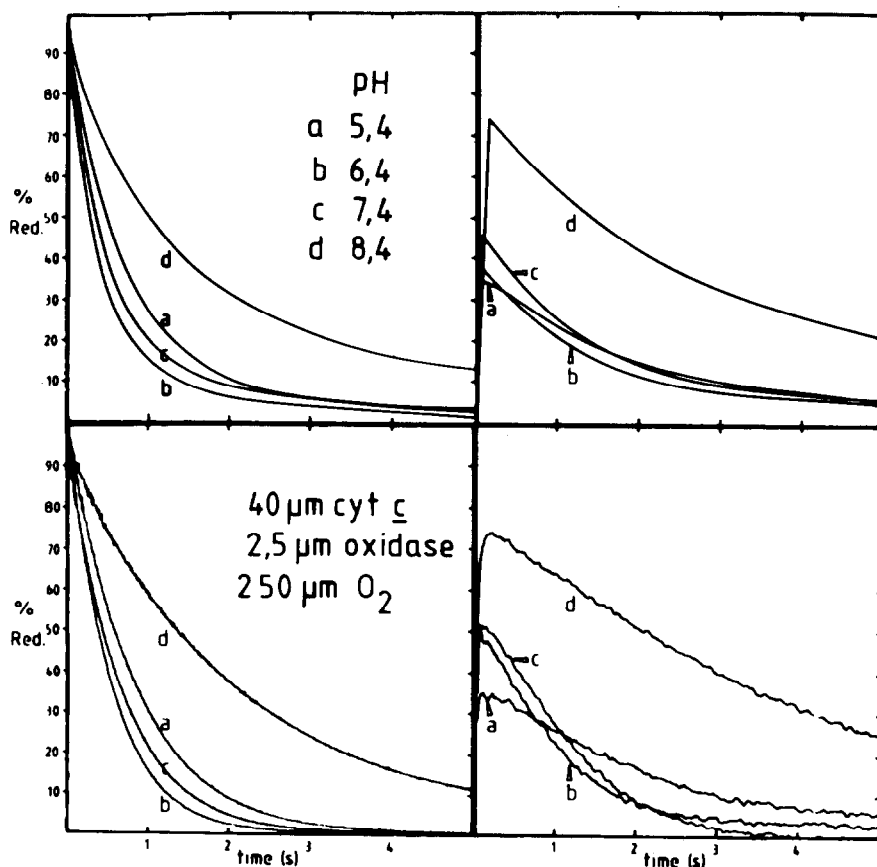


Fig.10. Aerobic kinetics of cytochrome *c* and cytochrome *a*. Adapted from [23].

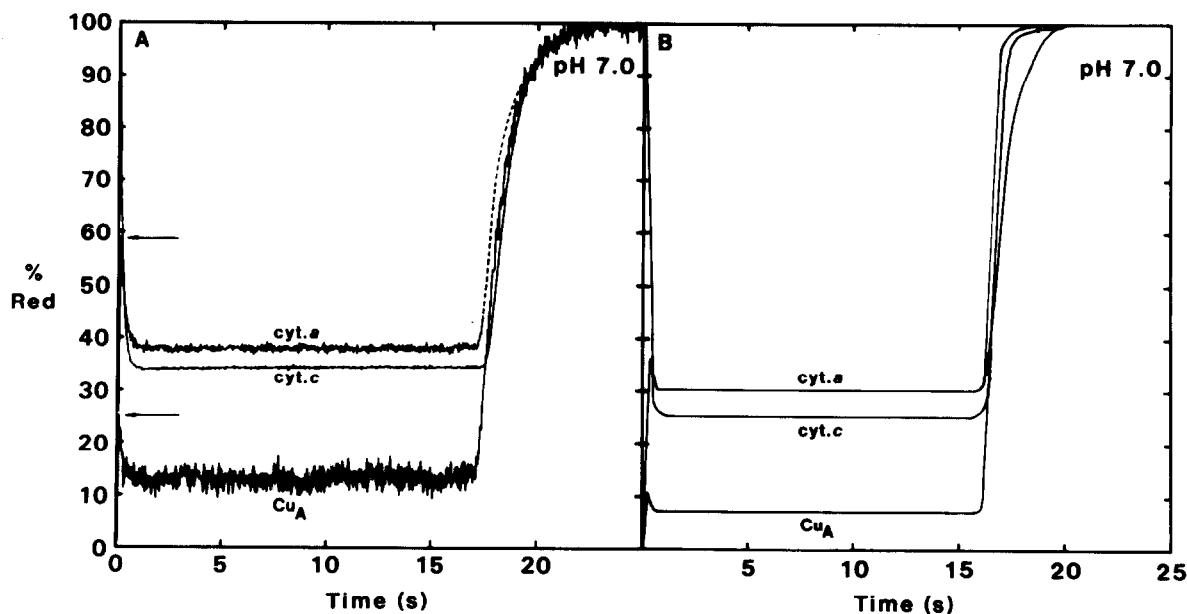


Fig.11. The aerobic kinetics of cytochrome *c*, cytochrome *a* and Cu_A in the presence of ascorbate and TMPD [23].

as pH 8.0 with three different concentrations of cytochrome *c*. Thus, the results from these two types of experiments clearly show that the pH dependence of the kinetics is largely due to the variation with pH of the rate of protonation of a group with a pK_a of 6.4.

3.4. Intrinsic uncoupling

Before I proceed to considering the molecular basis of electron gating in the oxidase, let me also show that under non-physiological conditions there can be an intrinsic uncoupling of the pump, i.e. some of the required specificities discussed earlier are not absolute. It has long been known [32] that the steady-state kinetic equation of cytochrome oxidase is nonhyperbolic, containing the sum of two Michaelis-Menten terms. This was originally interpreted as evidence for the existence of two active sites, but a more natural explanation, it would appear to me, is that cytochrome *c* can slowly reduce cytochrome *a* in the electron output state, as illustrated in fig.12 [33]. In E_1 , the reduction potential of cytochrome *a* is higher than that of cytochrome *c* [22,34], whereas it is lower than the cytochrome *c* potential in E_2 [27]. One would thus expect the reduction of cytochrome *a* to be faster in E_1 than in E_2 . However, when the rate of

reduction in E_2 becomes appreciable at high concentrations of cytochrome *c*, then the enzyme chooses to go this way, as the rate of the conformational transition back to E_1 can be predicted to be more rapid in $E_1(1000)$ than in $E_2(0000)$ [33]. This consequently accounts for the nonhyperbolic kinetics. The kinetic data can be nicely simulated, if it is assumed that the second-order rate constant for the reaction with cytochrome *c* is 100-times larger in E_1 as compared to E_2 [33]. There is thus an intrinsic uncoupling or 'slip' but, since this occurs only at very high concentrations of cytochrome *c*, it has no adverse physiological effects.

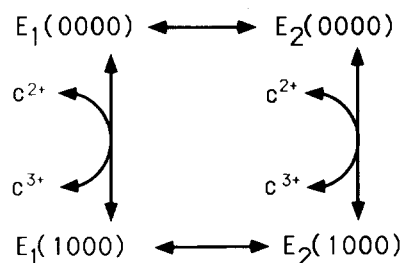


Fig.12. A conformational model for the nonhyperbolic steady-state kinetics of cytochrome oxidase.

There is also an intrinsic uncoupling with regard to H^+ , as shown by the stopped-flow results with enzyme in phospholipid vesicles in fig.13 [35]. It can be seen that the ratio of the molecular activity in the uncoupled and coupled states is 3.0 at pH 8.4 and 7.4 but decreases to 1.5 at pH 6.4 and becomes 1.1 at pH 5.4. This is exactly what should be expected if the protonated enzyme in E_2 can return to E_1 , when the pK_a of the group involved is 6.4. That the coupled rate is still appreciable at pH 8.4, where the degree of protonation is only 1%, can be ascribed to extrinsic uncoupling caused by proton leaks through the lipid bilayer. With 40 μM cytochrome *c*, the activity still increases on uncoupling at pH 5.4, because the electron-transfer rate now exceeds the intrinsic leak rate, which thus must be lower than $30\ s^{-1}$. Again, the intrinsic uncoupling has no adverse effect, as in mitochondria the outside pH is expected to be rather constant at 7.4. Should the outside pH actually fall below 7, an intrinsic uncoupling may, in fact, be favourable, as the scalar reaction will still continue with a concomitant consumption of protons from the inside [26].

3.5. Electron gating by reorganization energy

Now we are prepared to look more closely at the

conformational transition in step 6 and how this accomplishes an efficient gating of the electron flow. To do this, we have to consider the main factors that determine the rate of intramolecular electron transfer between two redox centres some distance apart in a protein [36]: (i) the driving force, i.e. the free-energy change (ΔG^0) for the reaction; (ii) the reorganisation energy (λ) due to different nuclear positions in the oxidised and reduced centres; (iii) the distance between the two centres; and (iv) the intervening medium. I believe that it is mainly changes in the first two factors which are involved in regulating the electron flow in redox-linked proton pumps.

To illustrate how the driving force and reorganisation energy influence the electron-transfer rate, let us look at the conventional profiles of free-energy surfaces for a donor(D)-acceptor(A) pair before and after the electron-transfer event (fig.14). First, we consider the case in which $\Delta G^0 = 0$ (fig.14A), so that λ determines the free energy of activation (ΔG^*). Since the nuclear coordinates in A/D are different from those in A^+/D^- , the nuclei have to move as the electron is being transferred. Nuclei are, however, heavy compared to the electron, so if the latter were to move, the nuclei could not keep up.

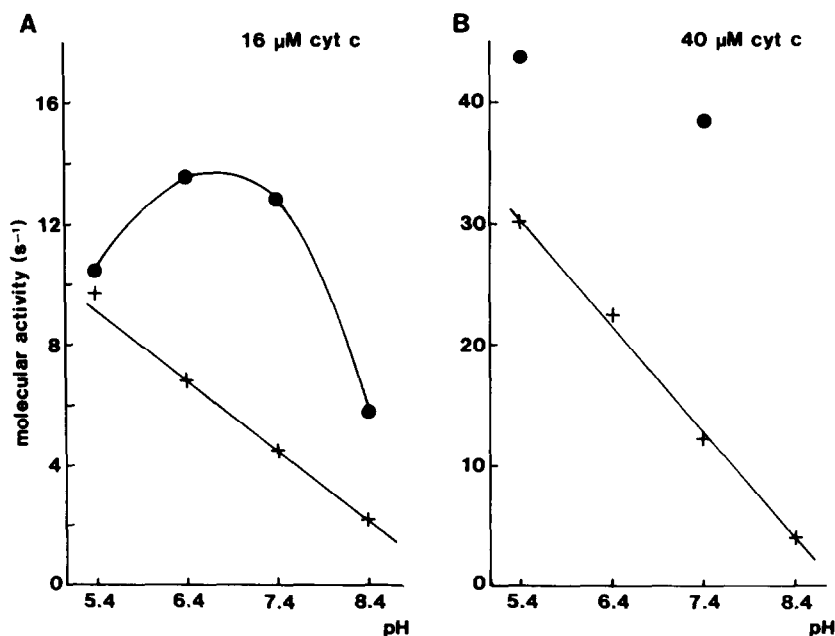


Fig.13. The rate of cytochrome *c* oxidation catalysed by cytochrome oxidase in coupled and uncoupled phospholipid vesicles [35].

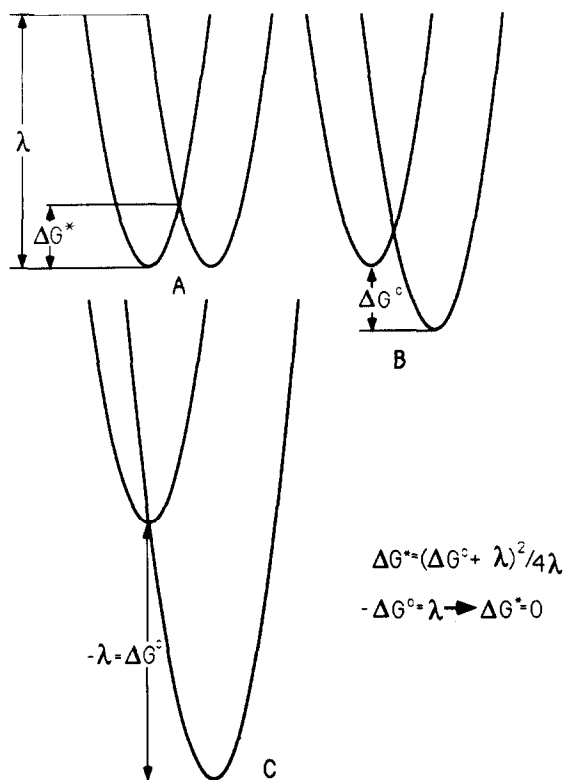


Fig.14. Profiles of free-energy surfaces for electron transfer in a D/A pair at constant λ for three different values of ΔG° . For explanation, see text.

Therefore, the nuclei are required to move first to a point along the nuclear coordinate, where the potential-energy curve for A/D crosses that of A^+/D^- , at which point the electron transfer can take place. The resulting ΔG^* is lowered if $\Delta G^\circ < 0$ (fig.14B) and becomes zero if $-\Delta G^\circ = \lambda$ (fig.14C).

My suggestion, on the basis of results that I will soon present, is that there is a small driving force and large reorganisation energy associated with the internal electron transfer from the initial acceptors to the binuclear centre in the E_1 conformation (fig.15). The reorganizational barrier is in the binuclear site, because cytochrome *a* and Cu_A , being related to cytochrome *c* and blue copper proteins, respectively, are expected to be good electron-transfer agents [37]. Reduction of cytochrome *a* and Cu_A , combined with protonation of the enzyme, results in a conformational transition to the E_2 state (fig.15B). In an allosteric fashion, this alters the structure of the cytochrome

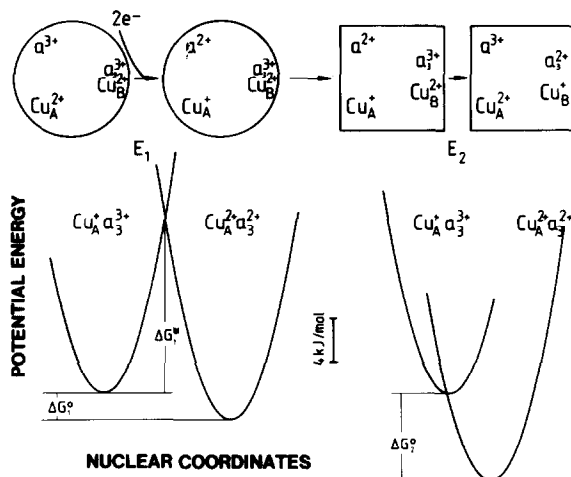


Fig.15. Schematic illustration of the mechanism of electron gating in cytochrome oxidase [27]. For explanation, see text.

a_3 - Cu_B site in such a way that λ is decreased by about 1 V at the same time as $-\Delta G^\circ$ increases by about 0.2 V. One can then predict [36,38] that the electron-transfer rate would be increased by a factor 10^6 in E_2 compared to E_1 .

Let me now present some of the evidence for this mechanism of electron gating. Perhaps the most important support is derived from a study [27] of the temperature and pH dependence of the internal electron transfer with the oxidase in the mixed-valence state, i.e. an enzyme form in which cytochrome *a* and Cu_A are both oxidised, whereas the cytochrome a_3 - Cu_B site is stabilized in the reduced state by CO binding. When the CO is photodissociated with a laser flash, there is a small back-flow of electrons from cytochrome a_3 - Cu_B to Cu_A [27,29]. From the measured relaxation time and equilibrium constant, the rate for electron transfer in the normal direction from Cu_A to the binuclear site was calculated to be $1.4 \times 10^4 \text{ s}^{-1}$. This is much larger than k_{cat} , which under the conditions used is only 50 s^{-1} . The large rate constant suggests that the enzyme is in the electron-output state, E_2 , and that it is the transition from E_1 to E_2 that imposes the limit on k_{cat} , as indicated earlier. This interpretation is supported by the equilibrium constants, including that for a second phase in which an electron is transferred to cytochrome *a*, which give values for the reduction potentials of cytochrome *a* and a_3 of 220 and 350 mV, respectively. The cytochrome *a* potential is thus lower

than that of cytochrome *c*, showing that the enzyme is not in the electron-input state.

It was found [27] that the rate constant for the electron transfer from Cu_A to the binuclear site is almost independent of temperature. Application of Marcus theory to the results shows that ΔG^* is close to zero. Since the driving force is quite moderate, this demonstrates that λ is also small, in accordance with the hypothesis. With the use of an expression for the distance dependence of the electron-transfer rate [36], it was estimated that the Cu_A–cytochrome *a*₃ distance should be about 15 Å, in agreement with spectroscopic results [39].

The gating mechanism just described receives additional support from studies of the kinetics of cyanide inhibition and its pH dependence. From these it has been concluded that the binuclear site can exist in a closed or an open conformation [40]. The open form is characterized by a very high second-order rate constant for the binding of cyanide to oxidised cytochrome *a*₃ (about $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) [40], whereas this constant is small for oxidised, pulsed oxidase ($20 \text{ M}^{-1} \cdot \text{s}^{-1}$) [42] and even smaller for the resting enzyme. Both cytochrome *a* and Cu_A need to be reduced in order to convert the enzyme from the closed to the open state [43]. This two-electron requirement can be related to the observation, discussed earlier, that the internal electron transfer cannot take place in the one-electron reduced enzyme [24,25]. This makes it attractive to consider the open conformation to be identical with the electron-output state, E₂. Since cyanide binds to oxidised cytochrome *a*₃, the transition to the E₂ state must precede the internal electron transfer. We have also seen that this is rapid in E₂, so that it must be the conformational change, and not the electron transfer per se, which limits k_{cat} .

The suggestion just given is supported by the recent demonstration [44] that the rate of the conformational transition varies with pH in the same way as the catalytic activity does. It should be noted that large-scale conformational changes are uncommon in redox proteins, as they are counterproductive to rapid electron transfer [37]. In a proton pump, however, they have to occur because of the requirement of proton and electron gating [5,20,45]. This lends further attraction to the idea that the closed-open transition is operating in such gating. In addition, the changed cyanide reactivity

shows that reduction of cytochrome *a* and Cu_A alters the structure of the *oxidised* cytochrome *a*₃–Cu_B site, in agreement with the suggestion that the conformational change removes the reorganizational barrier in the electron acceptor, making the internal electron transfer rapid in the open, or E₂, state.

4. MOLECULAR MODELS AND PROTON PUMPING

The lack of a high-resolution X-ray structure makes it very difficult for one to design experiments illuminating the microscopic basis for the protonation requirement of the input-output transition, particularly if, as I believe, the coupling between electron transfer and proton translocation in cytochrome oxidase is of an indirect form. Holm et al. [46] have constructed structural models of subunits I and II, and their redox centres, on the basis of evolutionary conservation, predicted membrane topography and the known properties of the redox centres. The structure proposed for subunit I has the haem edges of cytochromes *a* and *a*₃ located very close to each other, which would seem to be inconsistent with the relatively low rate of electron transfer between these centres [27,31]. In addition, it would appear to be difficult to modulate the electron-transfer rate structurally, as required in a redox-linked proton pump. Because of this, Steve Mayo in Berkeley, in collaboration with Harry Gray and I, has constructed alternative models of subunit I, one of which is illustrated in fig.16. In this model, the haem groups of cytochromes *a* and *a*₃ are bound on opposite sides of a transmembrane helix, which increases the haem–haem distance. The model also suggests a structural basis for my proposed mechanism of electron gating. If cytochrome *a* becomes more compact when reduced, it could exercise a pull on cytochrome *a*₃, thereby changing the structure of the binuclear site. This idea is supported by the finding [47] that cytochrome *c*, which is rather similar to cytochrome *a*, has a smaller radius of gyration in the reduced form compared to the oxidised species, and by the observed effect of pressure on the aerobic steady state of cytochrome oxidase [48].

It is also easy to visualize a molecular basis for the demand that the enzyme must be doubly pro-



Fig.16. Structural details of a molecular model of subunit I of cytochrome oxidase, showing cytochrome *a* and *a*₃ bound on opposite sides of the same helix (courtesy of Dr Stephen L. Mayo).

tonated for the conformational transition to be rapid. The three-dimensional structure of a protein is to a large extent stabilised by electrostatic interactions, such as salt bridges and hydrogen bonds. The binding of protons to amino acid side chains can lead to the breaking as well as to the formation of such bonds. Thus, I would like to suggest that the protonation of two acid-base groups in the oxidase stabilises the E₂ state relative to E₁, which would also lead to an increase in the rate of the conformational transition, as required in the pump mechanism. Since I now appear to be rapidly departing from a solid experimental basis toward a more speculative realm, this would seem to be suitable point at which to close this lecture. Therefore, let me just make a few summarizing remarks.

A proton pump must have separate input and output states for electrons in order to couple the electron-transfer reaction to proton translocation. This requires efficient gating of the electron flow into and out of the pump redox site(s). Experience with model systems [39] suggests that this is most readily achieved by changes in the reorganisation energy brought about by redox-induced conformational transitions. I have tried to illustrate this with

experimental data for a specific proton pump, cytochrome oxidase. These data can be interpreted on the basis of a hypothetical reaction cycle. In this, reduction of the two primary electron acceptors, cytochrome *a* and Cu_A, triggers a conformational transition, which, in an allosteric fashion, is transmitted to the oxidised binuclear site, changing its structure. The structural change is such that the reorganisation energy for the internal electron transfer is drastically lowered. Thus, this transfer is now rapid. Since the conformational transition is rapid only when the enzyme is doubly protonated, efficient coupling between the electron-transfer reaction and proton translocation is achieved.

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