

Review-Hypothesis

Chemiosmotic coupling in cytochrome oxidase

Possible protonmotive O loop and O cycle mechanisms

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Using the principle of specific vectorial ligand conduction, we outline directly coupled protonmotive O loop and O cycle mechanisms of cytochrome oxidase action that are analogous to protonmotive Q loop and Q cycle mechanisms of QH_2 dehydrogenase action. We discuss these directly coupled mechanisms in the light of available experimental knowledge, and suggest that they may stimulate useful new research initiatives designed to elucidate the osmochemistry of protonmotive oxygen reduction in cytochrome oxidase.

<i>Cytochrome oxidase</i>	<i>Protonmotive O loop</i>	<i>Protonmotive O cycle</i>	<i>Vectorial ligand conduction</i>
	<i>Osmochemistry</i>	<i>Catalytic mechanism</i>	

1. INTRODUCTION

Wikström and Casey [1] recently showed that the main basis on which some of us questioned the acceptability of the evidence for proton translocation by cytochrome oxidase in mitochondria [2,3] was erroneous. Consequently, as explained in detail elsewhere, we now agree with the widely adopted conclusion that cytochrome oxidase translocates protons with a $\leftarrow\text{H}^+/\text{e}^-$ ratio of 1. This brings us to the crucial question of the mechanism of proton translocation [4–12].

Somewhat surprisingly, it has generally been taken for granted that no hydrogen carrier is available in cytochrome oxidase to perform the trans-osmotic-barrier hydrogen-conducting function required in a directly coupled protonmotive redox loop or redox cycle system (see e.g. [6], p. 11), and this has led to the view that cytochrome oxidase must be equipped with a redox-linked proton pump of the indirectly coupled type, as defined by Wikström and Krab [4–6]. The main purpose of the present paper is therefore to consider whether,

by analogy with the action of the substrate ubiquinone as the conductor of hydrogen in protonmotive Q loop and Q cycle mechanisms in QH_2 dehydrogenases [13–15], the substrate oxygen may function as the conductor of hydrogen in a protonmotive O loop or O cycle mechanism in cytochrome oxidase. A special case of directly coupled oxygen-mediated proton translocation with $\leftarrow\text{H}^+/\text{e}^- = 0.5$ was recently suggested by Baum and colleagues [16] in a discussion of the involvement of hydrogen peroxide in the mechanism of action of cytochrome oxidase. The present paper pursues that initiative towards some of its logical conclusions, and provides a more general and explicit outline of some possible directly coupled chemiosmotic mechanisms with $\leftarrow\text{H}^+/\text{e}^- = 1$.

2. POSSIBLE DIRECTLY COUPLED CHEMIOSMOTIC MECHANISMS

2.1. General conceptual principles

Fig.1 shows conceivable protonmotive O loop (F1,C1) and protonmotive O cycle (F2–F4,C2,C3)

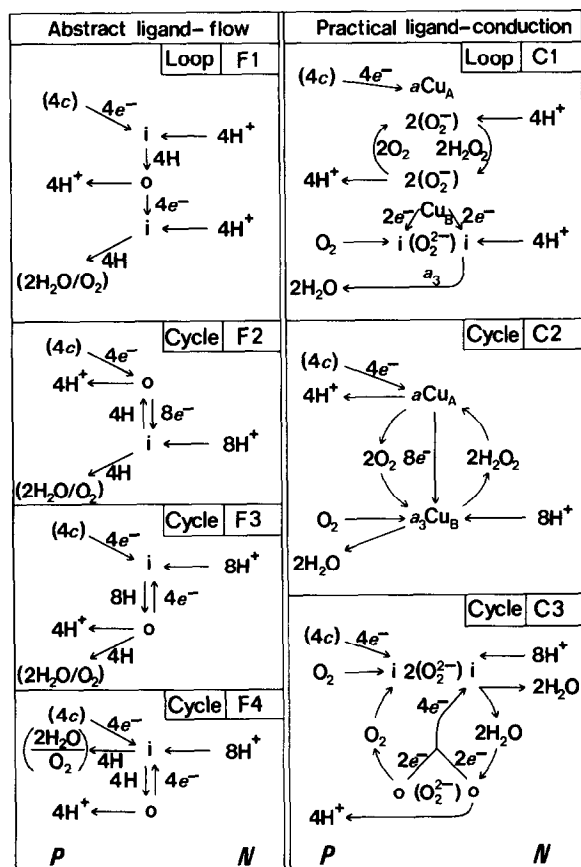


Fig.1. Abstract ligand-flow and practical ligand-conduction diagrams of hypothetical O loop and O cycle mechanisms in cytochrome oxidase. Symbols *P* and *N* represent the aqueous domains at protonically positive and negative potentials, respectively. Four ferrocycytochrome *c* molecules that donate $4e^-$ to the oxidase are shown as (4c), and the oxidant couple that accepts 4 H from the oxidase is shown as $(2H_2O/O_2)$. Symbols *i* and *o* represent protonic input and output centres, respectively; and the haem and copper centres of the oxidase are shown as *a*, a_3 and Cu_A , Cu_B , respectively.

Other explanations are in the text.

diagrams for cytochrome oxidase with $\leftarrow H^+/e^- = 1$. To devise these diagrams, we have used the conceptual principles and formality previously applied to Q loops and cycles (see [13–15]), bearing in mind that, during redox activity, cytochrome oxidase not only contains 4 electron-accepting centres (haem *a*, Cu_A , haem a_3 , Cu_B) that participate in electron conduction, but it also contains (electron + proton)-accepting components of the $2H_2O/O_2$ system (see [17], p. 40) that may participate in

hydrogen conduction. The 10 possible couples from the $2H_2O/O_2$ system that could conceivably act as hydrogen conductors are listed in table 1 with their midpoint potentials in aqueous media at pH 7. It should be noted, incidentally, that the midpoints involving O_2 have been adjusted to a standard state of 1.0 molal O_2 , in place of the conventional standard state of 1.2 mM (corresponding to the fugacity at NTP), so that they represent the redox potentials at equal molalities or probabilities of the non-aqueous components of these couples.

To facilitate exploration, we have used 2 kinds of diagram: abstract ligand-flow and practical ligand-conduction. Diagrams F1–F4 show abstract net ligand-flow patterns for protonmotive O loop and O cycle mechanisms; and these have been used to suggest conceivable practical implementations by means of appropriate ligand conductors, such as those shown in the ligand-conduction diagrams C1–C3. In the diagrams, and henceforth in the text, haem *a* and haem a_3 are abbreviated to *a* and a_3 , respectively.

The left and right sides of the diagrams correspond, respectively, to the *P* and *N* aqueous domains on opposite sides of the osmotic barrier domain through which the ligand-conducting pathways are supposed to pass in the cytochrome oxidase molecules. The symbols *i* and *o* represent centres of protonic input and output, respectively, at the junctions between pathways conducting electrons (e^-) and hydrogens ($H = e^- + H^+$) from the electron source at 4 cytochrome *c* haem centres (4c), which donate electrons successively through

Table 1

Possible hydrogen-conducting redox couples from the $2H_2O/O_2$ system

Redox couple	E_m at pH 7 ^a	Redox couple	E_m at pH 7 ^a
HO_2/O_2	– 28	H_2O_2/HO_2	+ 800
H_2O_2/O_2	+ 386	$2H_2O/O_2$	+ 873
$H_3O_2^b/O_2$	+ 391	$2H_2O/HO_2$	+ 1167
H_3O_2/H_2O_2	+ 400	$2H_2O/H_2O_2$	+ 1360
H_3O_2/HO_2	+ 600	$2H_2O/H_3O_2$	+ 2300

^a The E_m values (given in mV) are adjusted for standard state oxygen concentration of 1.0 molal using conventional E_m data from [17]

^b H_3O_2 (hydrated hydroxide radical) is equivalent to OH

the *P* surface of the oxidase molecules, to the hydrogen sink ($2\text{H}_2\text{O}/\text{O}_2$) in the aqueous *P* domain, to which hydrogen is conducted as water from the catalytic domain in the oxidase molecules. The protonic input and output centres, *i* and *o*, are respectively connected to the aqueous *N* and *P* domains by proton-conducting pathways or wells in the polypeptide system of the oxidase. The lateral splitting of the *i* and *o* centres in diagrams C1 and C3 is intended to represent electron transfer events that happen sequentially in time, but do not necessarily involve significant movements of the ligands in space. The diagrams are meant to show the topological connectedness of the centres and domains by the ligand-conduction pathways in the oxidase during the reduction of one molecule of oxygen. They are not meant to represent the actual spatial locations of the redox centres in the oxidase molecules.

The successful operation of such directly coupled systems depends, of course, on the kinetic competence of the ligand conductors, and on the specification of the flow stoichiometry by the local osmochemistry at one or more of the redox centres, as discussed below.

2.2. Protonmotive O loop mechanisms

In the redox loop type of mechanism, owing to the linearity of the redox flow (fig.1,F1), the protonmotive stoichiometry is set by the ligand-conducting specificity and topological arrangement of the conductors of electrons, protons, and hydrogens, linked through the *i* and *o* centres. The stoichiometry does not otherwise depend on the detailed osmochemistry at the *i* and *o* centres. However, the O loop system is mechanistically more complex than a conventional redox loop, because the reductant component of the hydrogen-carrying couple (e.g. H_2O_2) is an intermediate in the reduction of the terminal oxidant (O_2) as well as the carrier of hydrogen to that oxidant. Thus, the O loop mechanism suggested in fig.1,C1 requires the oxidation of $2\text{H}_2\text{O}_2$ to 2O_2 by the reduction of O_2 (via H_2O_2) to $2\text{H}_2\text{O}$. This partially dismutational process (supposed in fig.1,C1 to be catalysed across Cu_B) would require the relatively tight complexation of the peroxide anion, O_2^{2-} , or its protonated counterpart, H_2O_2 , at centre *i*, between a_3 and Cu_B . Otherwise, as indicated by the 2 (successive) two electron transfers, it would not

be possible for the first stage of reduction of O_2 at centre *i* (producing bound O_2^{2-} or bound H_2O_2 as intermediate) to drive the oxidation of H_2O_2 at centre *o*, on the other side of Cu_B .

Our suggestion in fig.1,C1 that *i* and *o* redox centres may be situated on either side of Cu_B is consistent with the conclusion, drawn from optical absorption, EPR and MCD studies, that ligands, such as NO and CN^- [18], or NO and N_3^- [19,20], or CO and H_2O [21] may be simultaneously bound at separate centres neighbouring Cu_B , and that electrons may be conducted between these centres, presumably via Cu_B ([21,22] and see [23,24]). It is also consistent with the inference that, on photolysis of the CO compound of the oxidase, the CO migrates from a site between a_3 and Cu_B to a pocket neighbouring Cu_B [25-27].

It is a fundamental, and potentially diagnostic, attribute of the protonmotive O loop flow system (fig.1,F1) that the low- and high-potential electron-conducting pathways (expected to work at E_h values around 300 and 520 mV, respectively) must be connected by a hydrogen-conducting, and not by an electron-conducting, pathway. Thus, according to the mechanism suggested in fig.1,C1, there should be no transfer of electrons, as such, from the $a\text{-Cu}_\text{A}$ to the $\text{Cu}_\text{B}\text{-}a_3$ centres, but the reducing equivalents should be transferred exclusively as hydrogen, conducted by the $\text{H}_2\text{O}_2/\text{O}_2$ couple. This couple has been selected from table 1 because (in aqueous media) it has a midpoint potential of 386 mV, intermediate between 300 and 520 mV, and it appears to be a promising candidate for the kinetically competent transfer of hydrogen between the $a\text{-Cu}_\text{A}$ and $\text{Cu}_\text{B}\text{-}a_3$ centres in the oxidase, on the assumption that the relative tightness of binding of H_2O_2 and O_2 at the relevant *i* and *o* centres would adjust the local midpoint redox potential to around 300 and 520 mV, respectively. As indicated in fig.1,C1, the superoxide ion (O_2^-) would also have to be appropriately bound at these *i* and *o* centres to equalise the redox potentials of the successive one-electron transfers during reversible peroxide formation.

We have not been able to find experimental evidence to discriminate between the practically universal view that electrons are transferred, as such, from the $a\text{-Cu}_\text{A}$ to the $a_3\text{-Cu}_\text{B}$ centres [6,9,11,12] and the alternative possibility, suggested by diagrams F1 and C1 in fig.1, that hydrogen is

transferred between these centres. The observation that redox contact between the a -Cu_A centres and the a_3 -Cu_B centres is almost completely suppressed, either when the oxidase is in the oxidised resting state [28–32], or when the oxygen concentration is below 0.1 μ M [33,34], may be as convincingly attributed to loss or modification of the putative hydrogen-carrying couple as to the conventionally invoked change to a conformational state of low electron-conducting activity. The peculiar requirement for reduction of a to 'open' the oxidase for access and binding of cyanide to the oxidised a_3 -Cu_B domain also appears to be as compatible with the O loop mechanism, involving the circulation of the members of the H₂O₂/O₂ couple in a domain or pocket between the a -Cu_A and Cu_B- a_3 centres (fig.1,C1), as it is with the conventional type of mechanism [35,36]. Work by Moroney et al. [37], on the effect of electric membrane potential on the redox poise of a and a_3 in cytochrome oxidase incorporated in liposomes, indicated that the transfer of reducing equivalents was sensitive to the pH gradient rather than to the electric membrane potential. They assumed that they were observing electron transfer between a and a_3 , but their experimental results seem to be at least as consistent with the transfer of hydrogen. Indications [38,39] that the midpoint potentials of a and a_3 are both dependent on pH_N are in accord with the hydrogen-conducting requirement of the O loop mechanism.

The protonmotive O loop scheme of fig.1,C1 is not, of course, the only possible implementation of the O loop flow system that might provide the basis for explaining how cytochrome oxidase works. For example, a_3 and Cu_B might be transposed, or a different hydrogen-carrying couple might be employed. Our main object in suggesting the mechanism of fig.1,C1 is to illustrate the general potentialities of the directly coupled O loop type of mechanism in the context of experimental research on cytochrome oxidase.

2.3. Protonmotive O cycle mechanisms

Protonmotive redox cycles differ from protonmotive redox loops in that they contain 2 branch points in the redox chain, joined by a pathway that enables reducing equivalents to be fed back up the redox chain and recycled. As indicated in fig.1, there are 2 main classes of cycle in which the feed-

back pathway conducts either hydrogens (F2) or electrons (F3). There are also hybrid types of cycle (e.g. F4) that have Q cycle analogues.

The O cycle ligand-conduction scheme shown in fig.1,C2 is developed from the flow diagram F2. At first sight, this scheme might appear to be mechanistically impossible, because the 4-electron reduction of a -Cu_A (centre o) by cytochrome c is compounded with the 8-electron oxidation of this centre by the a_3 -Cu_B centre, and with the oxidation of 2 H₂O₂ to 2 O₂. However, we do not assume that these processes are coupled chemically at the a -Cu_A centre. Instead, we assume that the reduction of 3 O₂ at the a_3 -Cu_B centre (centre i) obligatorily produces 2 H₂O and 2 H₂O₂. This might, for example, be achieved by the cooperatively coupled reduction of 2 pairs of O₂ molecules to 2 pairs of H₂O₂ molecules along parallel internal and external pathways associated with the a_3 -Cu_B centre, followed by dismutation of the 2 H₂O₂ in the external pathway, to give O₂ and 2 H₂O, and the conduction of the 2 H₂O₂ in the internal pathway to the a -Cu_A centre. In that way, the protonmotive stoichiometry would be maintained at the a -Cu_A centre, provided that H₂O₂ could not escape from the conduction pathway between a_3 -Cu_B and a -Cu_A. But, it is a special point of interest that, if H₂O₂ could escape from this pathway, the conservation of H₂O₂ in the pathway, and the tightness of coupling of proton translocation to electron transfer, would depend on the extent to which the conduction of electrons to the a -Cu_A centre by cytochrome c was rate limiting. It might possibly be relevant that Kramer and Perelstein [40] observed that, in rat liver mitochondria, the P/O ratio declined continuously when the rate of electron transfer was decreased by oxygen limitation, but remained constant when the electron transfer rate was decreased over the same range by reductant (succinate) limitation.

The O cycle ligand-conduction scheme shown in fig.1,C3 is developed from the flow diagram F4. It corresponds to a Q cycle running backwards with reversed P/N polarity, and with two-electron transfers substituted for one-electron transfers. It would depend on the principle that the successive electron transfers at centre i must occur at low and high redox potentials, respectively (from cytochrome c , and perhaps from a - a_3 acting as an electron-conducting doublet), while the successive

electron transfers at centre o must occur at an intermediate redox potential. Thus, the peroxide anion O_2^{2-} would have to be relatively tightly bound (as such or in the protonated state) at centre o. We have not indicated the dispositions of the haem and copper centres because, bearing in mind that this system might involve a dimeric oxidase, there appear to be several possibilities. The very fast electron transfer from cytochrome *c* to O_2 observed by Hill and Greenwood [41,42] in flow-flash spectrophotometry of aerobic fully reduced cytochrome oxidase in the presence of CO, and the synchronous oxidation of a_3 and reduction of Cu_A observed by Boelens and colleagues [26] on photolysis of the CO compound of anaerobic mixed-valence oxidase, suggest that Cu_A and a_3 might act at centre i, and *a* and Cu_B at centre o. But it is difficult to assess the feasibility and relative merits of such O cycle schemes at the present early stage of development and evaluation of our thesis. As with the O loop scheme, our main aim is to illustrate the general potentialities of the directly coupled O cycle type of mechanism in the context of experimental research on cytochrome oxidase.

An interesting attribute to be expected of the type of O cycle shown in fig.1,C3 would obviously be the reductant-linked oxidation of the second electron donor (i.e. the component donating electrons to O_2^{2-}) at centre i. But, by analogy with oxidant-linked reduction (of cytochrome *b*) at centre o of the Q cycle [13], the reductant-linked oxidation at centre i of this putative O cycle might not be readily observable without inhibition (as by antimycin in the Q cycle) of electron acceptance from centre o. Could it be that the well known phenomenon, commonly called haem/haem interaction [6], or metal site cooperativity [43], might be better described as reductant-linked oxidation?

As Kojima and Palmer [34] have nicely pointed out, "there is a striking inconsistency between the equilibrium (midpoint) potentials obtained by potentiometry and those implicit in stopped flow data of the reaction of cytochrome c^{2+} with oxidase ([33,44]). These latter data imply that the midpoint potential of cytochrome *a* is no more than 30 mV more positive than cytochrome *c* and that the midpoint potential of *a* and Cu_A ... are equal. By contrast, potentiometry shows that the midpoint potential of cytochrome *a* is at least

70–100 mV more positive than cytochrome *c* and that Cu_A has a potential 60 mV more negative than cytochrome *a*." It would be inappropriate to pursue this matter in more detail here, but perhaps the foregoing will suffice to indicate that it may be particularly rewarding to explore inconsistencies between equilibrium and kinetic data with O cycle schemes, and also with O loop schemes, in mind. These considerations might also apply to the question of control of oxidase turnover.

3. EXPERIMENTAL EXPLORATION OF MECHANISTICS

The hypothetical directly coupled protonmotive O loop and O cycle mechanisms of cytochrome oxidase action described in this paper are distinguished chemically by their dependence on what one can aptly call the substrate-level Bohr effect (see [14,15]). Like Q loop and Q cycle systems, the putative O loop and O cycle systems contain both substrate-reducing protonic input centres (i) at which electronation of oxygen atoms makes these atoms go basic and become protonated, and substrate-oxidising protonic output centres (o) at which de-electronation of hydroxyl groups makes these groups go acidic and become deprotonated. Possession of the substrate-oxidising type of protonic output centre (rather than the indirectly linked type of acid-deprotonation centre) distinguishes the directly coupled protonmotive mechanisms fundamentally from their redox-linked proton-pump counterparts. Therefore, in research designed to explain how proton translocation is coupled to electron transfer in cytochrome oxidase, we suggest that it would be strategically wise to concentrate attention on the detailed events at the protonic output centre o. In particular, one should aim to determine whether centre o is a site of hydroxyl group oxidation, as it must indeed be in any O loop or O cycle mechanism.

As mentioned in section 2.2, experimental evidence has already been obtained for the existence of 2 ligand-binding sites neighbouring Cu_B , and for the conduction of electrons between these sites [18–24]. Thus, one of these sites can evidently act as an oxidation centre. It is a crucial question whether this site (see fig.1,C1) is connected to the aqueous *P* domain by a proton-conducting system or well, and whether it may act as a centre of

hydroxyl group oxidation and protolysis. It seems likely that answers to these and other related questions could be obtained without much difficulty by appropriate experiments on partial redox and protonic output and input processes catalysed by cytochrome oxidase incorporated in liposomes. By analogy with work on QH_2 dehydrogenases, it also seems possible that valuable mechanistic information on the protonmotive mechanism of cytochrome oxidase might be obtained by dual inhibitor experiments, designed to facilitate the separate study of events catalysed at the substrate-reducing centre(s) on the one hand, and at the (putative) substrate-oxidising centre on the other hand.

The reader may have thought it self evident from sections 2.2 and 2.3 that it is easier to be specific in formulating O loop mechanisms that may be operationally feasible than in formulating corresponding O cycle mechanisms, because the latter mechanisms are capable of being considerably more complex. For that reason, it may be advisable to devise experimental tests of the O loop type of mechanism in the first instance, and then to proceed to consider O cycle mechanisms in detail, if evidence should happen to emerge for the subtleties of redox behaviour that are known to be characteristic of such systems [13].

4. CONCLUSION AND PROSPECT

The conclusion has been widely accepted that cytochrome oxidase could not translocate protons by a directly coupled mechanism, but must be equipped with an indirectly coupled proton pump of the redox-linked type, "simply because the oxidase contains only formal electron carriers" (see [6], p.11). The experimental evidence available at present does not make it possible to determine whether the directly coupled chemiosmotic mechanisms of cytochrome oxidase action suggested in this paper may be any better than the indirectly coupled redox-linked proton pump models in providing a satisfactory answer to the question: How does cytochrome oxidase pump protons? However, by removing the restrictive misconception that cytochrome oxidase could not, in principle, translocate protons by a directly coupled type of mechanism, the O loop and O cycle ligand-conduction systems described here offer new and

relatively adventurous scope for experimental research programmes designed to investigate the osmochemistry of the protonmotive electron-transfer mechanism.

Since the concepts of vectorial metabolism and specific vectorial ligand conduction, on which the O loop and O cycle mechanisms logically depend, have been widely discussed (see e.g. [45]), it may seem surprising, in retrospect, that these mechanisms have not been explicitly considered before. Now that attention has been drawn to them in this paper, we hope that they may help to stimulate new experimental initiatives designed to find out how cytochrome oxidase actually works.

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