Volume 226, number 1, 1–7 FEB 05376 December 1987

Discussion Letter

The mechanism of cytochrome oxidase and other reaction centres for electron/proton pumping

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Received 31 August 1987; revised version received 19 October 1987

The functional significance of the metal centres of cytochrome oxidase is deduced from the ways in which the centres are bound into its peptides. To this end use is made of structural knowledge of other metalloproteins for dioxygen binding, haemocyanin and haemoglobin, and for electron transfer, cytochromes *b* and azurin. The order and manner in which the motions of helical sections of the oxidase are linked to proton pumping are suggested and a comparison is made with other proton pumps, for example that of ATP synthetases.

Cytochrome oxidase; Copper; Iron; Proton pumping; Helix dynamics; Dioxygen

1. INTRODUCTION

There is a vast literature on the chemistry and biochemistry of cytochrome oxidase associated with some of the greatest names in biochemistry. I do not have the space to summarise this work but see the paper by Beinert [7]. It often appears that the very sensitivity of the protein to the modes of handling it causes serious problems for the investigating scientist. In this article I wish to try to strip away the many mysteries with a summary of almost casual character. The summary will start from the known properties of molecular oxygen and its binding to two oxygen carriers, haemoglobin and haemocyanin.

2. HAEMOGLOBIN

The binding of dioxygen to haemoglobin is understood through the effects of spin-state changes of iron, high-spin to low-spin, possibly concomitant with valence-state changes after oxygen binding, and with effects of hydrogen-bonding from the bound O_2. The overall result is some change of coordinates of the iron and the porphyrin relative to those of the protein backbone and of changes of the iron-attached histidine which together cause a relay of effects in the protein. Many of these points were made [1] independent of detailed knowledge of the transformation as shown by X-ray crystallography but only the latter has allowed a full and refined knowledge of the haemoglobin trigger to be obtained [2,3]. In essence the oxygen binding is allosteric, i.e. it allows energy transfer by mechanical means, through the relative motion of helices in particular. This type of movement will be a constant theme in the present paper.

3. HAEMOCYANIN

Haemocyanin is another reversible allosteric dioxygen-carrier protein which is of known struc-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

ture. In this case the binding of dioxygen is to a bimetal centre when the Cu(I) ions change to Cu(II) and the oxygen is held as O_2^{2-}. There is no difficulty in understanding such reversible chemistry which has now been followed in many model complexes of several metals. In passing, note that the single electron transfer to dioxygen to give superoxide is also reversible and a simple reaction at a metal centre. It is easier to retain the peroxide anion than the superoxide radical anion at metal centres and perhaps this explains its appearance in the haemocyanin and haemerythrin molecules. Returning to the haemocyanin protein it is clear that the oxygenation causes a massive change in the size of the copper ions from radii of around 1.0 to 0.6 Å [3]. These ions are held by three histidines [4] and inorganic chemistry leaves us in little doubt that the valence change of copper will cause changes not only in bond distances from copper to these histidines, but also in bond angles.

Of course the dioxygen itself on binding alters its bond angles to the copper ions and its internal bond length O–O as it passes to O_2^{2-}. The effect on the protein is known to cause an allosteric switch. We observe that the protein, like haemoglobin, is made of a series of crossed helices [4]. (The third oxygen carrier, haemerythrin, is also made in this way.) We shall therefore take it that the effect of the dioxygen binding at the copper site of the protein is to cause a relative shift in the helices, so producing the allosteric change. There is a further observation concerning haemocyanin which may be of general interest. Limpet haemocyanins can make ion channels in membranes [5].

The redox potential of copper in the copper sites of haemocyanin is high and it has not been found to be possible to oxidise the copper(I) to copper(II) directly, except in the presence of high-potential reagents such as dioxygen or nitric oxide. The copper redox potential in haemocyanin is undoubtedly in excess of 0.5 V. This condition arises from the ligands and the geometry of the site. The ligands are neutral imidazoles (histidines) and the site is close to a trigonal structure of three imidazoles having either water or just space in the fourth corner of a tetrahedron [4]. Both the stereochemistry and the particular ligands force copper to a high redox potential, as shown clearly in model complexes [6]. Note that there could also be a bond-length control imposed by the protein so that this is really a protein for binding copper(I) directly when the Cu(II) state would be strained. The outside (and inside) of cells contains sufficient chloride to maintain $Cu(I)/Cl^-$ complexing and in such states the redox potential of free copper is itself near 0.5 V. It may well be that haemocyanin is a copper(I) and not really a copper(II) protein because of bond-length constraints at the three histidine sites – contrast the coordination sphere in carbonic anhydrase and observe the parallel with the type I copper sites of 'blue' electron transfer proteins [4].

In the two proteins it is asserted generally that the protein does not relax easily with change in oxygenation/deoxygenation so that strain is induced in both metal coordination chemistry and protein structure in some or all of the states of the molecules, the so-called entatic states. This interactive binding allows modulation of the function of haemoglobin and haemocyanin by added reagents which bind at remote centres from the iron or the copper, e.g. H^+, Ca^{2+} and certain phosphates. The modulation is known to be affected by an adjustment in the helices in haemoglobin. Here we are looking at cooperative thermodynamic equilibrium states [1,2]. Cooperative dynamics are also possible.

4. ELECTRON TRANSFER SITES OF CYTOCHROME OXIDASES [7]

Cytochrome oxidase itself contains two metal atoms associated with the dioxygen binding and at least two further metal atoms involved in electron transfer to the dioxygen eventually. There is a necessity to describe electron transfer sites as well as dioxygen-binding sites. There are two copper sites with which we start the analysis. Of the two sites one is rather like a copper, type I, blue site in that it possesses the typical spectral features (EPR and absorption) and has a redox potential of around 0.4 V. This copper (copper A) is probably held in an inflexible site much as is the copper blue atom of azurin. The copper(II) in such proteins is in a strained (entatic) state where the protein does not adjust itself to the oxidation state of the metal and once again we can say that it, in parallel with all other blue sites, is designed for Cu(I). This copper is then a typical fast electron transfer site with

no capability for binding extra ligands. The evidence for this is far from complete but the sequence analogies with other copper blue proteins for the site and the sequence itself suggest that the subunit II containing the site is a β-sheet protein. Elsewhere we have shown empirically that β-sheet proteins are not allosteric and are not able to transfer energy by mechanical means from site to site [8]. It is then part of an electron depository and serves a condenser-like electronic function.

There is a second electron transfer site in cytochrome oxidase which is close in space to the first copper and is a bis-imidazole cytochrome *a* site of redox potential about 0.4 V. Now we know many of the properties of such electron transfer sites in proteins. Typical parallels amongst six-coordinate low-spin haem proteins show that they are all α-helical (all known cytochromes *b* and *c*), all low-spin and all undergo rapid electron transfer. In the case of cytochromes *c* the electron transfer processes cause little running change in the helices since they are cross-linked to the haem through thio-ether bridges. The cytochromes *b* are very different and it has been pointed out for many years that proteins containing several iron protoporphyrins, e.g. the *b* cytochromes of the particle III of the electron transfer chain, are cooperatively linked, probably via α-helical structures. I include a further note about such cytochromes later. Here we shall take it that cytochrome *a* of cytochrome oxidase resembles such a cytochrome *b*. Very intriguingly Fee [9] has reported recently that in some bacterial proteins the complement of a cytochrome oxidase is $b \cdot Cu_A \cdot a_3 \cdot Cu_B$ and not $a \cdot Cu_A \cdot a_3 \cdot Cu_B$. We shall take it that electron transfer changes can cause running changes in the haem-linked helices of this type of low-spin haem, i.e. haem *a* or *b*. The site of cytochrome *a* is on a helix of subunit I of cytochrome oxidase and we note that cytochrome a_3 is also in subunit I so that the relationship of haem *a* and haem a_3 is not unlike that of the two cytochromes *b* of the bc_1 and b_6f systems of mitochondrial particle III and chloroplasts (see fig.1).

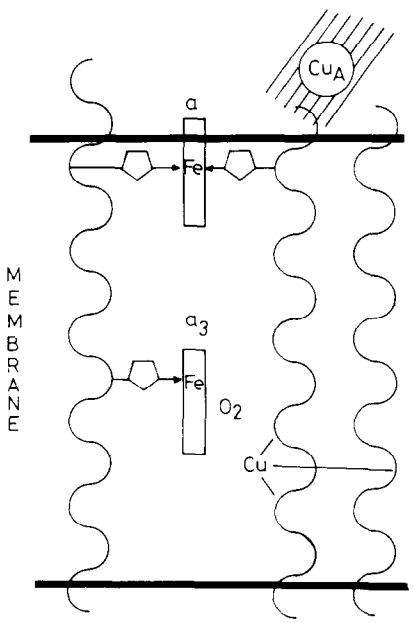


Fig.1. A sketch of cytochrome oxidase based on the work of Wikström [10] and others [7]. All the helices shown belong to subunit I. There are additional helices running through the membrane to support a proton channel. Copper A is in a β-sheet protein.

5. CYTOCHROME a_3

We shall use the model of Wikström [10] for the description of the sites of the haem a_3 and copper B sites which are some 10 Å into the membrane (fig.1). This positioning is based on many probe studies not summarised here and the model incorporates several earlier ideas [7] but has some important novelty (fig.1). Firstly, it uses the now conventional way of identifying the appropriate subunit connections to the metal ions. Subunit II has the above copper(A) while subunit I has the three other metal ions. (The metals of haem a_3 and copper(B) are then placed in the membrane helices. The metal Cu(A) is outside the membrane in a presumed β-sheet structure and rather close to haem *a*.) In the model of Wikström the cytochrome a_3 iron and the Cu_B are brought within 5 Å of one another so that a molecule of dioxygen can bind between them. There is much EXAFS and other spectroscopic evidence for this structural feature. At this point clear resemblances emerge between (i) the disposition of haem a_3 and its immediate trans-membrane helix binding to the iron through a single imidazole and the parallel elements of the haemoglobin structure [11] and (ii) the Cu_B site and either of the two copper sites of

haemocyanin. In other words haem a_3:Cu_B can be said to be half haemoglobin and half haemocyanin. Note that both oxygen carriers are cooperative and in both helix/helix movements follow dioxygen binding. Little imagination is needed to see that the very nature of the sites of haem a_3 and Cu_B will lead to forced helix/helix changes on binding dioxygen. Both the iron, which is caused to switch spin state (cf. haemoglobin) and valence state, and the copper, which is caused to switch valence state (cf. haemocyanin) by oxygen binding and conversion to peroxide, undergo large changes in size, ligand field energy and stereochemistry. This change is assisted by the change in the dioxygen, O_2, to the peroxide structure. Note that further changes due to reduction of the total $Fe \cdot O_2 \cdot Cu$ site, eventually giving two molecules of water, will cause further geometric switchings. Thus, in cytochrome oxidase we expect strain to be induced at the metal centres as the protein goes through a series of states. This strain will fluctuate at each electron transfer step and will be relayed into the helices as in the two oxygen carriers but observe that there is now a long sequence of states which cannot be described in any way by a two-state allosteric switch. In fact, several intermediates, even high-spin Fe(III), are seen [7].

The next point to observe is that the helical structure crosses a membrane and its active site is placed in the centre of the membrane (fig.1). This is the first *active* site of a protein to be so placed (but see particle III below). Now membrane helices generally are disposed so that two types of movement are possible. The first is a simple movement of a helix through the membrane caused by binding to sites on one side or the other of the membrane. This up and down slip of a single helix has been discussed in signalling within the protein glycophorin and in the EGF receptor [8,12]. The second movement is a rotational/translation movement of two or more helices relative to one another. This type of motion has been described in calmodulins [3,8], in haemoglobins [1,2] and in many membrane proteins [8]. Particularly interesting here are such helix movements postulated in channel proteins for ion movements. The ideas are general to channels for ions which are connected to pumps. As an example the reversible proton-pump/ATP synthetase has a channel, F_0, which has been described by several authors as a set of parallel helices (see, for example [8,13]). It is very probable that the conformations of the helices are dependent upon their states of protonation and that the mechanical coupling of these states to states of the F_1 head of the F_0F_1 complex leads to proton pumping. We now look at the potential helix movements in cytochrome oxidase.

Without further ado it is possible to devise a working hypothesis for the connectivities between oxygen binding to haem $a_3 \cdot Cu_B$ to adjustments in electron transfer rates from haem a and Cu_A and to proton pumping. The two critical statements are

(i) Electron transfer rates are governed in part by the *helical* connections betwen haem $a_3 \cdot Cu_B$ and haem a, the connecting helix between a and a_3 moving up or down in the membrane and undergoing some rotational relaxation with respect to the other helices in the bundle (see fig.1).

(ii) Proton pumping is due to cyclic changes of a set of helices holding haem a_3, copper Cu_B and haem a, as the metals cycle through oxidation states following uptake of oxygen and the ensuing final release of water. This is dynamic allosteric change at each step almost exactly as in a mechanical water pump or bicycle tyre pump. The several helices then provide a proton channel. It is quite clear that protons are involved in the reaction $O_2 + 4\bar{e} + 4H^+ \longrightarrow 2H_2O$ but these are not the only protons involved although all these four protons come from one side of the membrane.

In this scheme the copper A is required only as a reservoir or condenser to assist a four-electron process. The dynamics of this and the other centres are all based strictly on very reasonable analogies. The exact description of the electron transfers, and the chemical and stereochemical states of the cooperative system will be very difficult to obtain and matching of trapped states with kinetic entities even more troublesome. The machine is both a proton pump and an electron pump and one movement should drive the other. Oxygen escapes with the electrons and some protons as H_2O. There is a sense in which the operation is a compulsory kinetic scheme and in table 1 I include enzymes which are known or are thought to have this com-

Table 1

Helical enzymes

Enzyme	Comment
Cytochrome P-450	Haem-protein; compulsory order of substrate binding and redox reactions
Citrate synthetase	Compulsory order of substrate binding
Cytochrome oxidase (?)	Haem-protein; compulsory link of e^-/H^+ reactions
Cytochromes *b* (?) (particle III)	Haem-protein; compulsory link of e^-/H^+ reactions
Peroxidases	Haem-protein; very little substrate specificity
Catalases	Haem-protein; simple metal ion reaction

N.B. It appears that the tetrapyrrole unit is peculiarly adapted to a helical fold (see haem, pheophytin and chlorophyll proteins) and that this allows membrane incorporation. Note too that in the reactions of very small substrates, e.g. O_2 and H_2O_2, the site selectivity is almost entirely the packing of hydrophobic groups around the haem so that the protein side-chain chemistry is hardly involved in the reactions in contrast with most other enzymes which are based on β-sheets

pulsory connection. They are all helical enzymes. All other enzymes of known crystal structure (more than fifty) are based on β-sheets close to active sites and do not show compulsory order kinetics or series of conformational states.

It is now necessary to propose additional points of binding and release of protons which cycle with the electron-transfer dioxygen reaction. The fact that the central bunch of helices which carry the metal ions moves means that they move relative to the other membrane helices of the complex cytochrome oxidase and we may suppose that some part of the proton movement is associated with channels made by these structures. It has not yet proved possible to define any ion channel but the above description has a parallel in the electron/proton coupled channel of particle III.

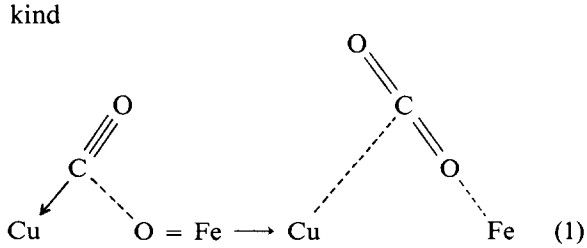
6. OTHER PROTON/ELECTRON PUMPS

It has frequently been postulated that the cytochrome bc_1 complex of mitochondria and the cytochrome c_6f complex of chloroplasts are also proton pumps. The similarity with the cytochrome aa_3 complex of the respiratory chain lies not only in the fact that each system has pairs of haem units but also that both systems pump protons while undergoing electron transfer. Further proven experimental similarities are that during turnover there are considerable switches in EPR characteristics of the haems showing that they all undergo series of conformational transitions. Several authors have stated that the haems *b* of the b_6f_1 and bc_1 complexes are in strained environments (entatic state) and that the strain is adjusted during reaction, paralleling the comments made above. To complete the connection between the two systems it is highly probable that the *b* haems (two in b_6f_1 and two in bc_1) are linked to parallel helical stretches of protein running through the membrane much as is stated to be the case for the haem a_3 (Cu) and the haem *a* in peptide I of the cytochrome oxidase (see fig.1). All the haems are bound by histidine ligands. Given these several known and proposed similarities between the system, it is natural to assume that the fundamental mechanism of coupled electron/proton transfer which they share has common features. The feature to which I wish to draw attention is that proton and electron movements can be readily coupled by the mechanical movement of helices in membranes. The coupling is fundamentally the same for all helical systems within proteins in that a combination of lateral and rotational movement about a fulcrum where side chains of helices meet is like a system of levers. Strain energy engendered, here at electron transfer steps but elsewhere on binding calcium (calmodulins) or ATP (ATP/proton or other cation pumps), is transmitted to movement of remote parts of the helical system so as to generate mechanical pumping and gating. (I do not include electrical gating which may require only the minimum of movements in proteins and is used for very fast switching.) A general hypothesis is 'Protein helices are the mechanical (elastic) coupling rods of biology'. Tension is readily developed in their structures. Note finally that the system as a whole is not allosteric but allodynamic for want of a better word.

7. CONSEQUENCES

There are certain additional pieces of evidence

concerning the reactions of cytochrome oxidase which lend weight to the above structural discussion. First, the reaction should be capable of going backwards in a chemical sense but not of necessity in a thermodynamic sense (see [10]). The concerted involvement of proton movement with electron transfer would seem to be totally reversible and it should then be possible to go from the fully reduced state to the Fe(III)·Cu(II) states in a controlled way. The movements of the helices should also be reversible. The formation of Fe(IV) and of O_2 involves the chemistry of the $2H_2O \longrightarrow O_2 + 4H^+$ reaction and here real reversibility is very doubtful. The difficult step is not likely to be the formation of FeO(IV) from water and Fe(III) since we know that this can be achieved in peroxidase. It is the combination of a further oxygen atom with FeO to give an O–O bond which is so difficult and could well meet a large (deliberately 'designed') thermodynamic and kinetic barrier. The parallel with haemoglobin chemistry is lost. A somewhat similar downhill reaction at the cytochrome oxidase reaction site is known. It is of CO with FeO (a_3) to give CO_2 and this reaction must be of the kind



(1)

(I thank Dr S. Chan for a description and discussion of this reaction [14].) A final parallel reaction system is known.

The reaction centre of bacteria for light capture is now well described [15]. It is a series of electron transfer centres in a membrane and associated with a helical protein. Now here we are interested in the further connection of this kind of electron transfer with oxygen production as seen in photosystem II where there is a connection to proton pumping. We observe immediately that the metal ions involved are manganese and not those of the oxygen carriers. Here we need oxygen to escape. Once again we need to start from the fully reduced condition and work to O_2. The picture emerging today is that there is a four-manganese atom cluster and knowing the chemistry of manganese we may postulate that the O_2 is released from two manganese atoms in steps which parallel reaction scheme 1.

We draw the scheme

$$\begin{array}{ccccccc} O & & OH & & O & & O \\ Mn(IV) & \ldots & Mn(III) & \longrightarrow & Mn(IV) & \ldots & Mn(IV) \longrightarrow \\ & & & & O_2 & & \\ & & Mn(II) & \ldots & Mn(II) & & \end{array} \quad (2)$$

Now we observe that in the cycle of manganese states there is the same problem of stereochemical and size changes as for the copper oxidation states so that there is again a dynamic relationship between mechanical stress and states. Noting that the connecting electron transfer proteins in the membrane are known to be helical [15] we can again suppose that the system becomes an electron/proton pump through helix movements.

8. CONCLUSION

The essence of this paper is that electron transfer involving such ions as Mn(II), Fe(II) (in high-spin $\rightleftharpoons$ low-spin switches), and Cu(I) will demand conformational change [16]. This conformational change can be minimised by straining one or other of the electron transfer sites such that there is minor relaxation of a protein, for example in copper blue proteins, or by avoiding spin-state changes as in cytochromes *c*. Such sites have evolved to assist (simple) electron transfer. If the protein is allowed to relax with the electron transfer steps (changes in ionic radius, i.e. oxidation state or spin state, or changes in bonding stereochemistry) then the process can be mechanically coupled within an ordered matrix. The ordered matrices in biology suitable for this coupling are bunches of helices as in the three known dioxygen carriers, haemoglobin, haemocyanin and haemerythrin. The principle can be carried over to allosteric coupling between redox state changes and states of these proteins generated by binding H^+, Ca^{2+} or phosphates. For such an organised helical protein system which crosses a membrane then a corresponding series of dynamic allosteric properties is easily visualised (as in a mechanical water pump) such that electron and protein flow is coupled. This I believe is the basis of the coupling in cytochrome oxidase, the bc_1 and b_6f systems and the O_2-evolving devices of chloroplasts.

Finally, it is worth noting that all of the descriptions given above involve concepts of *localised* energy states, of metal ions (entatic states), of helices, and of protons in membranes. It is as well to remember the distinction between this approach to biological machinery and that of chemiosmosis. I refer to a recent review of Slater [13].

9. SUMMARY

The novel features of the proposed structure of cytochrome oxidase are:

(i) The Fe/Cu pair is a composite of a haemoglobin-like site (Fe) and a haemocyanin-like site (Cu) both of which are in subunit I.

(ii) The Fe/Cu oxygen-binding site shows the cooperative behaviour of a typical oxygen-carrier protein but interacts with cytochrome *a* which is also bound to subunit I.

(iii) The cooperativity derives from the fact that haem *a*, haem a_3 and this copper are all associated with helical rod stretches of protein which run in the membrane. The helical rods are the mechanical coupling rods of conventional machines.

(iv) The second copper site is for electron storage and rapid transport only. It has the characteristics of a blue copper site and this site belongs to a β-barrel peptide in subunit II, which is not mobile.

(v) Every electron transfer from haem *a* to the oxygen site causes helix/helix relative motion, rotation/translation, and the proton pumping of the oxidase is due to this motion. A model is available in calmodulin.

(vi) Proton pumping is then not directly connected to the redox energy and in this sense it is related to proton pumping in the bc_1 particle and indeed in the proton pumping reversible ATP synthetase.

ACKNOWLEDGEMENTS

I wish to thank many colleagues for exchanges of both data and ideas especially where acknowledgement may be inadequately covered by a very limited set of references.

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