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Uncoupled and coupled electron transfer reactions

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This paper concerns an NMR study of cytochromes *c* in an effort to understand the coupling of redox state changes with protein conformation and proton movement. The objective is to find a model which will allow us to understand electron/proton diffusion coupling as seen in cytochrome oxidase and envisaged in one description of energy transduction.

We have studied the mitochondrial cytochromes *c* from several sources in great detail and we have also had available to us a variety of chemically modified and site-specifically modified proteins. The method we have used is NMR and we have analysed both proton and carbon spectra. We have also had great help from Dr. Gary Brayer (University of British Columbia) who provided us with the coordinates of crystal structures as he obtained them. The point of doing an NMR study together with a crystallographic study is to uncover the influence of chemical changes on features of structure and mobility of the protein in solution. Examples are the effect of protonation of a protein or of change of oxidation state of iron, i.e., electron transfer. A major problem associated with structural change generally which we wish to understand is the coupling of proton movements in the proteins with the electron transfer reactions or with the binding and redox reactions of dioxygen held by the iron of haem. Such changes are seen in proton gates or pumps. The basic feature of such a pump or gate is controlled proton diffusion after energisation by a redox (or other) reaction and it is central to the mechanism of energy conservation [1]. In this paper I shall show that cytochrome *c* is a very useful model protein for the study of such coupling devices.

To appreciate the problems involved, I clarify first the differences between electron and proton diffusions, Table I. The major difference is that an electron can hop, centre to centre, some 15 Å, while proton movement in a lattice is likely to be less than 1.0 Å. Thus, protons need a set of closely related lattice points, e.g., columns of water molecules, while electrons can move 'through' the protein lattice via widely dispersed points. The electronic hop wires of biology are well recognised and the haem iron of some cytochromes provides one such type of hop centre. The proton electrolytic wire is not well known, but theory suggests that it will be based on the making and breaking of H-bonds using a rotating group, e.g., an H_3O^+ donor molecule, and an H_2O acceptor, where both waters rotate after each proton hop, i.e., a rotational/hop diffusion [2].

Before tackling coupling we need therefore to appreciate the central requirements for electron transfer which are generally discussed under the heading of Marcus theory. Apart from indicating that the hop distance must be less than 20 Å, that the medium may be important and that the driving force must be taken into consideration, the theory draws attention to the fact that electron transfer will be impeded if the metal ion hop centre is involved in considerable structure changes from one oxidation state to another. Small structural changes are however essential to 'pre-equilibrate' the redox states and have associated relaxation energies. We must therefore examine the most robust electron transfer proteins to see to what degree the simplest of proteins undergo conformation changes on redox state

TABLE I

Proton and electron transport

	Protons	Electrons
Hop distance (Å)	0.5 to 1.0 Å	<15 Å
Site	bases	metal ions, aromatics
Activation energy	pK_a Change relaxation, *R* rotational diffusion	redox energy, ΔE relaxation, *R*

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change. It is essential that such studies be conducted not just in crystals but also in solution since crystal forces could trap structures and electron transfer can be followed only in solution. The best characterised *e*-transfer proteins are the blue copper proteins azurin and plastocyanin. Here, careful crystallographic and NMR studies show effectively no change in the protein lattice with redox state change. It is essential to notice that these proteins are robust β-barrels and that the copper ion in each is in a constrained entatic state which one could say is 'designed' to minimalise conformational change in the redox step [3]. This is not a rack-protein like haemoglobin, where action at the iron and the protein conformation are strained mutually – for example, when dioxygen is bound.

The next example I have taken is cytochrome b_5. The protein undergoes redox reaction, Fe(II) to Fe(III), in crystals without cracking the crystals. Using NMR spectroscopy and especially paramagnetic shift analysis [4] we have shown that the redox state change is associated with virtually no change in the protein lattice in solution [5]. To a large degree this is expected for two reasons: (a) the metal is a low-spin iron atom whence the iron changes size on redox reaction to but a small degree comparable with the changes in the iron hexacyanide anions; (b) the protein, though helical around the haem, is held together in a tight fold by a β-sheet. I stress: *no hydrogen bond changes are seen on redox state change*. Perhaps the purpose of a β-sheet is to prevent structural change.

The above examples suggest that certain metals are locked in metalloproteins which have evolved so as to act as effective components of wires without serious coupling to the protein lattice, the latter only serving to protect the metal and to build through its surfaces a specific wiring diagram. The presence of β-sheets or indeed of any other cross-linking between remote parts of sequences, e.g., -S–S- bridges, we take to be helpful in constraining the protein – i.e., there is no coupling to the lattice from the redox switch. Generalising, we might expect this rule to apply to all uncoupled electron transfer proteins, and indeed to other β-sheet-dominated domains, in enzymes for example, and here we note that all electron transfer Fe_n/S_n proteins are based on cross-linked β-sheets. This leaves only the cytochromes of the electron transfer chains to be discussed i.e., cytochromes *a*, *b* and *c*. (In passing we observe that β-sheet structures with or without other kinds of secondary structure associated are admirably suitable for recognition, e.g., in enzymes, but their mobility reduces their value in mechanical devices [6].)

Most cytochromes are based on a different structural principle from the above in that they contain little other secondary structure than multiple α-helices and in this they are related to the α-helical proteins such as all dioxygen carriers, e.g., haemoglobin, and to calcium triggers, e.g., calmodulin. Immediately in view of the allosteric nature of these latter proteins there is the suggestion that helices do not constrain protein structures greatly, unlike β-sheets, and that coupling of changes in helices with the binding of ions (Ca^{2+}), molecules (O_2) or electrons could be, quite generally, the basis of coupling between two types of event in different parts of a protein. The simplest electron transfer protein which is helical is cytochrome *c*, although on one side of the haem it is cross-linked through thio-ether links (Fig. 1) and, naturally enough, cross-linking restricts mobility.

Let us now see the way in which we can find out from studies of cytochrome *c* (mitochondrial) using NMR how proton and redox states could be coupled. We have proved, using NMR, that all the most obvious features of the structures of oxidised and reduced states of mitochondrial cytochromes *c* from horse, yeast (four mutants) and tuna are the same [7]. (We are in complete agreement with the studies by Englander and his colleagues [8] concerning the horse protein.) We have checked these findings using as a basis of our discussion the known crystal structures of cytochromes *c* in one or the other oxidation state but we note that redox reactions in the crystals crack the crystals [9]. In order to refine the differences between oxidised and reduced states in solution we have gone on to use the most sensitive methods for NMR structure analysis, paramagnetic shift probes together with NOE data. These structural tools allow use to follow not only structure itself but changes in hydrogen-bonding strengths [7,8]. (Note that H-bond pattern changes are the essential prerequisite for proton movements by rotational/hop diffusion in lattices and, again generally speaking, are expected to be associated with coupling, i.e., protein conformation changes.)

The immediate point here is that the structure of cytochrome *c* does change from one oxidation state to another. The difference represents coupling of conformational states to oxidation states and therefore it gives a model for energy transfer into the protein lattice well beyond that required for the electron transfer process itself. Reference to Fig. 1 and Fig. 2 shows that there is a sequence region from 38 to 85 which is most involved in the structure change. The region excludes the cross-linked portion of the protein (at sequence positions 14 and 17), the two long helices comprising residues 1–12 and 87–100, which are tightly held to one another and to the cross links, and the region from 18 (His ligand) to around 35–38. After residue 38 the protein is a series of not very well defined helices linked by some (tight) bends and more open strands, including one which holds Met-80 and which is bound to the iron in both oxidation states. The two propionates of the haem lie in this zone and are *buried* within the protein which distinguishes cytochrome *c* from cytochrome b_5. The

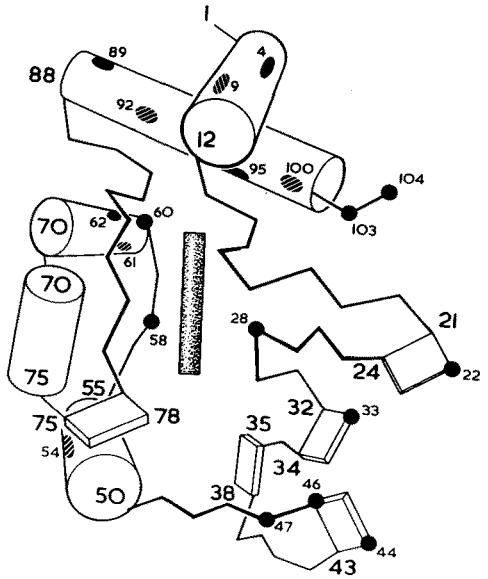

Fig. 1. The outline structure of cytochrome *c*. Sites of amino substitutions which have been studied or are under study are shaded.

redox conformational change has been described in crystals as involving the region around one water molecule, WAT 166, which is H-bonded to or close to Thr-78, Met-80, Tyr-67, Asn-52 and propionate-7 of the haem [9,10]. The water molecule, the haem and several of these residues have been shown to undergo movements, but the H-bond changes are not well-defined in such studies. Our NMR data show further changes in that there are small secondary structure adjustments

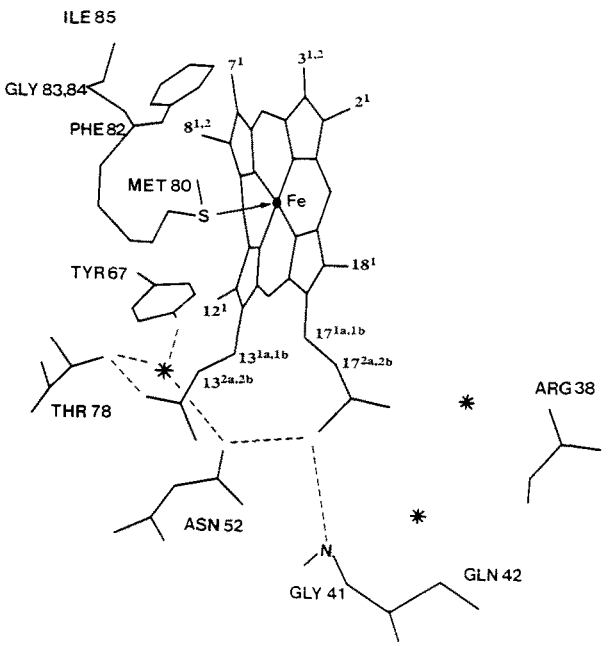

Fig. 2. The H-bond network of cytochrome *c* through from the sequence region 38–41 via 52, (59) 78, 67, 80, heme propionates (13 and 17), and water molecules (*). The network passes through the interior of the protein and is broken, and remade in a new way, by redox reaction.

throughout the regions from 38 to 85, including alterations in the short helices. However on oxidation state change there are several large shifts, $\geqslant 1.0$ ppm, not due to the paramagnetic effect, and specifically in the N*H* of Gly-41, Ala-43, Asp-60, Lys-79 and Met-80 and of side-chains of Thr-78(OH), and Arg-38 $CH_{(\epsilon)}$ with little change in their α-CH protons. This must mean, for example, that the H-bond from Gly-41 to propionate-6 of the haem (Fig. 1) makes and breaks during the redox reaction. A very little thought shows that this is effectively a proton/electron coupled-diffusion change. It involves proton movements associated with carboxylate groups, the propionates, inside a protein matrix. We also know that the redox switch changes access of protons from solvent water to this region, since the H/D exchange of protons from one internal group in this region, Tryp-59 ring N*H*, is 100-times faster in the oxidised state. Here are all the characteristics of a rate of proton movement controlled and gated by a redox reaction. The other smaller adjustments in the body of the protein are in the nearby helices 50–75 and in their side-chains. The units apparently required for coupling are buried anions to feel the change of charge and helical secondary structures.

Remembering now that the essential ideas of proton diffusion and ionisation in protein media in membranes are at the heart of energy transduction we have in cytochrome *c* a potential model system which is readily linked to the model we have put forward for many other redox-linked proton changes [1]. We are particularly interested in the proton activities of proton pumps which require both energisation of protons and gating of their movements. It requires little imagination to utilise the oxidation-reduction reactions of a centre, be it a haem or not, to generate pK_a changes in a protein, e.g., the haem Bohr effect. In the reactions of many cytochromes we have often examined coupled changes of pK_a values of haem propionates [10]. In membranes, the associated uptake and loss of a proton, energisation of protons in membranes, is one required step of a proton pump. However, this is not sufficient. A second necessary step is a conformational change to gate the proton movement. Using the structural changes in cytochrome *c* we picture such gating as follows. (1) In the reduced state an H-bond network allows the protonation of a group (haem propionate?) from one direction. (2) Oxidation occurs so as to break this network and generate a new H-bond network which allows the proton to move only in a new direction. The proton leaves and the first H-bond network is remade on reduction. The redox reaction is then coupled to (gated) diffusion control of *energised* protons in membranes. This is the model of energy tranduction put forward in 1961 [1]. Energisation of protons by redox reactions in membranes is now known in quinone and haem electron transfer reactions. The coupling model requires mecha-

nical changes (not necessarily large) and helical proteins which are common in membranes are ideally suited for the construction of these devices. Subsequent binding of the energised protons to further mechanical devices can drive other mechanical/chemical devices, e.g., ATP-synthases. Again buried anions are likely to be important. Coupling redox and ATP reactions by such diffusion controls is at the heart of so-called localised energy transduction and here again we have considered that it is buried carboxylate groups in the membrane that are associated with ion binding. The carboxylates themselves are now in helical structures in membranes and coupling is to be seen in terms of changes in membrane helices linked to devices like the hinge bending of phosphoglycerate kinase [11].

In the context of the work on cytochromes c' reported in this volume there is a further remark. This protein is a four-helical bundle, like cytochrome b_5, but it has no β-sheet. Like cytochrome c it has a conformation which is sensitive to changes, especially changes such as cyanide-binding to the iron. Cytochrome c' actually binds CO and NO. The possibility arises that cytochrome c' is a sensor for these molecules and in this respect would resemble the NO-heme protein sensor recently found in smooth muscle. It must then have a partner protein to which it binds and relays information (see Ref. 12 (this issue) for details).

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