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An Analytical Appraisal of Energy Transduction Mechanisms

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Abstract

The nature of mechanisms and energy profiles for reactions in biological systems is examined. Simple molecular and complex "phase" intermediates are described. The conceptual problems in energy transduction, related to "phase" intermediates are made evident by reference to different facets of the problem—stereochemical changes, charge fluxes, E° changes, and changes in the activities of H_2O and H^+.

The problem to which I have been asked to address myself is the mechanism of energy transduction in biology. Before inspecting the proposed solutions to the problem it is as well to have in our minds what it is that we are looking for. In other words what is the sense in which the word *mechanism* is being used. Reaction pathways can be broken down to individual steps—elementary reactions—and conventional mechanisms are descriptions of the way in which these step processes take place. Very much of our thinking about such steps is based on the consideration of gas phase reactions such as

$$H^* + HH \rightleftharpoons H + H^*H$$

where * identifies one hydrogen atom in the exchange. From the considerations of such gas reactions a mechanism has come to mean a molecular sequence with a *molecular* intermediate here H*HH, and an associated energy profile with identifiable molecular species at the minima, Fig. 1. Starting from this point of view biochemists have assumed that mechanisms of biological reactions can also be written down as energy contour diagrams in which they see a sequence of *atom movements in small (substrate) molecules*. Is this a true or necessary view of such reactions? What happens when we turn to larger assemblies of atoms not in the gas phase?

Consider a problem involving a condensed phase—for example the evaporation of an NaCl molecule, a well defined species in the gas phase, from a crystal of sodium chloride. The equilibrium vapour pressure is a measure of the relative free energy of NaCl in the gas phase and its free energy per mole in the lattice. In the gas phase the molecular entity is NaCl and we can ignore interactions between molecules although molecular concentration adjusts the free energy through the entropy. In the condensed phase NaCl no longer exists (i.e. can not be identified) as a molecule and the energy per mole of an infinite crystal of sodium chloride is the sum to infinity of all the Na^+ and Cl^- interactions in the whole of the solid phase. How does this fact, that long-range interactions dominate the crystal, alter the

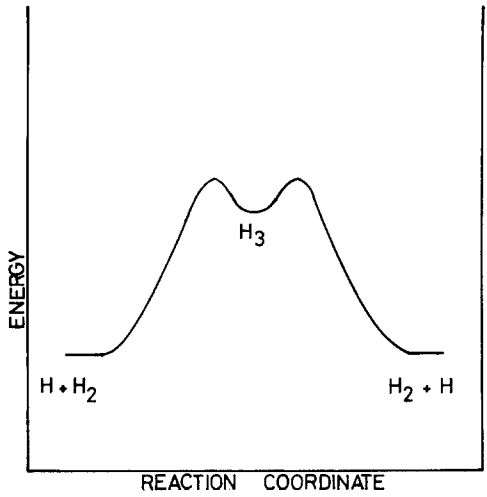

Figure 1. The energy profile for hydrogen exchange in the gas phase—a molecular intermediate.

picture of a contour diagram, Fig. 1, for a reaction—say that of the evaporation of an NaCl molecule from sodium chloride crystal surface. Clearly a potential energy diagram of the type of Fig. 1, see Fig. 2, can be drawn but while the right-hand side gives the energy of a defect lattice plus an NaCl molecule, the left-hand side gives the cooperative energy of a whole phase per mole and its description requires the whole lattice to be described (an infinite number of "molecules"). Thus a molecular mechanism for this reaction, requiring an intermediate to be drawn on a molecular scale, is impossible as the intermediate involves some degree of incipient NaCl molecule formation on the surface plus a cooperative change in the whole lattice.

Organic chemists do not often meet this lattice problem and they

define reactions in terms of molecules. Their systems usually approximate to gas phase reactions and their solvents can be treated as inert matrices. They are then entitled to write contour diagrams invoking simple chemical intermediates, Fig. 1. Does such a mechanistic view apply to biological systems or is the situation closer to that of a continuous phase problem?

The name "molecule" is sensibly applied to a condensed system of a small number of atoms linked by relatively apolar bonds. It has no ultimate justification in the description of highly polar liquids or solids, e.g. sodium chloride crystals, or in a solid where the energy

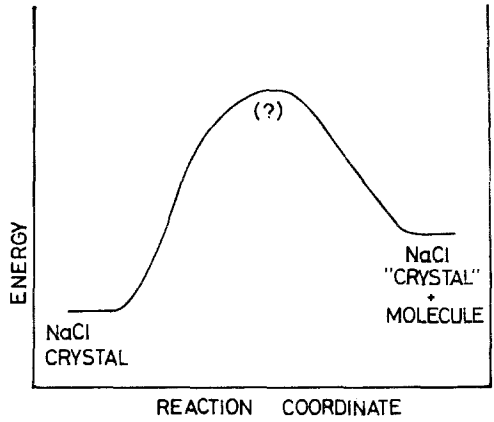

Figure 2. The energy profile for the evaporation of a NaCl molecule from a crystal of sodium chloride. No energy minimum is shown for it is quite uncertain how this should be represented even though it is known to occur. This is a case of a "phase" intermediate.

states result from any strong *cooperative* interaction over three dimensions e.g. diamond or sodium metal. What about a liquid such as water? Clearly a molecular description, H_2O, is a useful approximation for some properties but equally clearly there is considerable long-range interaction over a large number of neighbours. Evaporation of a water molecule is therefore a problem requiring consideration of large numbers of water molecules. It follows that mechanisms of reactions and their energy profiles in water cannot be fully described by the same treatments as apply to the hydrogen exchange reaction in the gas phase or to reactions of organic molecules in hydrocarbon solvents for energy is distributed into the *bulk* medium. How then should we look upon the reactions of proteins in water? Examples illustrate the type of description of the protein that must be invoked.

An obvious starting example is the reaction of myoglobin with

oxygen in water for the crystal structure of the protein is known and NMR allows us to follow some of its properties in solution.

$$Mb + O_2 \rightleftharpoons MbO_2$$

Before describing the intermediates we must describe the final states $Mb + O_2$ and MbO_2. O_2 is a gas molecule of no polarity and we can feel justified in treating it as a small separate molecule. Mb is a large partly polar unit cross-linked by hydrogen-bonds and ionic-links and interacting strongly with the bulk liquid. Any alteration that is made at one point in the Mb unit must alter to some degree (1) the whole of the interactions in the chain, (2) the solvent interaction. Thus a molecular description localized upon the active site may be quite inappropriate even when comparing reactant and product and therefore to the intermediates of the reaction with oxygen. In fact such molecular mechanisms could only be said to be a valid approximation if the major part (say 90%) of the energetics of the reaction were strongly localized. We see that energy profile diagrams of enzyme reactions can be misleading if they are taken to refer to the interaction of substrate and *localized* enzyme regions for in principle a very large part of an enzyme can be involved in a reaction path. The recognition of this feature of proteins is being developed slowly as the significance of remote protein sidechains upon enzyme reactions is felt.

Cooperativity in haemoglobin can be used as an example of increased protein complexity. The binding of the α and β chains together alters the oxygen affinity or redox potentials of the active site iron of the α and β chains separately by a very general rearrangement of the protein which includes minor movements of even α-helical regions. Oxygen binding to the $\alpha_2\beta_2$ unit stimulates further rearrangement which is cooperative or anti-cooperative with proton binding (at several points), carbon dioxide binding, and anion binding in the effector site—very many anions have some effect. No description of interaction between haem units which keeps small regions of the protein in mind can be of value in the discussion of the energetics for a general problem of long-range and short-range interactions over the whole of four protein sub-units is involved. Points of interest in the mechanism of oxygen uptake, such as changes at the iron and the salt-bridges are worthy of special note but it is quite improper to partition energy between the bits.

In myoglobin and haemoglobin then there are ramifications at many remote groups of the proteins due to the movement of the FG part of the back-bone chain with oxygenation. In chymotrypsin there is a chain of interacting residues glu-his-ser and it is their interaction, and with other groups, which produces the unexplained anomolous serine reactivity; in carboxypeptidase the movement of the distant

tyrosine assists attack; in ribonuclease the role of the distant arginine in the attack is not readily described; in lysozyme the anomalous pK_a of a carboxylate group and the role of tryptophans is uncertain, and so on. In all these cases a general bulk description of the protein may be necessary if all features of the energy profile are to be appreciated. In order to avoid discussion of cooperative energies covering the whole protein it is conventional to try to reduce these problems to molecular dimensions by referring to pictorial concepts such as "induced fit", and "anomalous environment". Perhaps it would be better to aim at clarifying the energetics of such processes before great store is placed on the validity of these concepts. For example many of the above enzymes bind *charged* substrates. The charge on the substrate usually binds far from the attacking groups but might it not alter the whole protein both through bond and through space? Is that an "induced fit" or an "anomalous environment"? As we turn from the above, usually extracellular, proteins to intracellular proteins it becomes apparent, as in haemoglobin, that the protein reflects almost any change of its environment—salts, pH, phosphate effectors, etc., far from the active site. Other *intracellular* proteins respond to large numbers of so-called effectors, e.g. glutamate synthatase responds to about fifty known effectors. There are general as well as specific allosteric factors. Are we being driven to realize that each *soluble* intracellular protein is reflecting the solution conditions of the whole solution phase of the cell—is it an osmometer, an electroscope, and a selective reagent for a large number of chemicals? An energy profile, Fig. 1, must then come to reflect the state of the cell as a whole as well as the molecular events recorded by looking at the substrate, intermediate, and product, for the activation energy will be changing with cell conditions.

With the above conceptual problems in mind I am able to state why I think that there is so much confusion about the mechanism of energy transduction. When a biochemist wishes to write an intermediate for this "reaction" he is tempted to postulate a molecular equation (as in the gas phase)

$$E + S \rightleftharpoons ES \rightarrow \text{products}$$

Useful though such molecular descriptions are we need to remember their limitations and to note that we have no proof that *energy transduction* can be written with any form of molecular ES complex as an intermediate for although the final reactants and products can be reasonably well written in molecular form.

$$\text{energy source: } h\nu + \text{chlorophyll} \rightarrow \text{energy},$$
$$\text{or } O_2 + 2RH_2 \rightarrow 2R + 2H_2O + \text{energy};$$
$$\text{energy sink: energy} + P + ADP \rightarrow ATP,$$

there is much evidence now that molecular intermediates cannot be so drawn for the energy transduction itself although there may be many simultaneous local changes which can be so drawn. We may have to be content with a more general extended view over a relatively large bulk of material, a *phase intermediate*, much as is true for the evaporation of NaCl molecules from a sodium chloride lattice

$$\begin{matrix}\text{perfect sodium}\\ \text{chloride } \textit{crystal}\end{matrix} \rightleftharpoons \text{NaCl } \textit{molecule} + \begin{matrix}\text{defective sodium}\\ \text{chloride } \textit{crystal}\end{matrix}$$

Energy can be so spread out as to belong to a phase.

Now the nature of the evidence about intermediates in such a situation is bound to be very complicated and the more so when the phase is very complicated. Firstly the appearance of a "phase" intermediate as opposed to a "molecular" intermediate could be observed in two ways in the course of reaction:

(a) *General* redistribution of charge instead of very *local* charge changes.
(b) *General* redistribution of atomic positions instead of very *local* changes in atom positions.

There may or may not be changes in covalent bonds so typical of the ES complexes.

As changes of charge and atomic position are often easily followed we can tackle reactions of such complicated units as mitochondria and chloroplasts, or their fragments, immediately we have defined the phase to which we are referring. (In the case of haemoglobin we refer to the total $\alpha_2\beta_2$ subunit and ignore the water in the first instance.)

In all energy-conserving reactions there is the added complication that we have to consider at first a multiphase system, one membrane and two aqueous phases at least. It is a general truth that those phases which are *in equilibrium* will rapidly return to balance reflecting changes initially brought about in one of them so that all three phases should be examined, apparently. Fortunately biological phases are not in equilibrium so that there can be "effective" or "semi-effective" discontinuity between action in one and action in any other phase. The reactions which give rise to ATP formation do not equilibrate with the aqueous phase of the cell and in the first instance we shall treat the membrane phase in which ATP is produced separately from the aqueous phase. (*Note.* Chemiosmosis does not allow us to do this.)

The Membrane Phase: Charge Distribution

The mitochondrial and chloroplast inner membranes or the regions of the outer membranes of bacteria which carry out either oxidative phosphorylation or photophosphorylation are composed of

a large group of chemicals including lipids, quinones, electron-transfer proteins, ATP-ases. What do we know of the charge distribution amongst these chemicals? As yet the chemicals are so ill-defined that we can hardly make any quantitative statement about their absolute nature. However if we go from a state in which oxidative phosphorylation is occurring, state III, to one, state IV, in which it is not, then it is obvious that there is a *relatively large switch* to higher oxidation, i.e. increase in positive charge. The charge redistribution is gross and involves upwards of twenty proteins and many small molecules. It is our desire to find a connection between the changes in these charged entities and the ATP-ase(s) which are apparently unaffected in the first instance by this charge redistribution in that they contain no redox centres. It is also clear that in these reactions there is no need for us to discuss the original source of the change of charge for no matter which substrates provide the reducing or oxidizing equivalents the oxidation in the phase is that of bound hydrogen or metal ions in the first instance. *The substrates are irrelevant.* Moreover there is no experimental reason why we should try to analyse particular oxidative phosphorylation stages—I, II or III in mitochondria or the stages of photophosphorylation—for we have only discovered *one* type of ATP-ase protein which must respond to all the stages. Finally as the membrane phase is surrounded by aqueous phases there is no reason to doubt that membrane charge, surface potentials, will change with the degree of oxidation of the membrane and it is bound to do so asymmetrically as the reactions are dislocated[1] or translocated.[2] Inevitably there is a connection with phases surrounding the membrane but I shall maintain that this is secondary to the major events of oxidative phosphorylation and is not necessarily brought into *equilibrium* with the membrane phase processes. (This is in strict contrast with chemi-osmotic theory.)

Changes in Atomic Positions

There is a danger that changes in atomic position, conformation changes will be over interpreted. The limits of our knowledge are (1) mitochondrial and chloroplast membranes alter their properties *as a whole* from the energized to the de-energized state. (2) *All* redox enzymes cycle through conformational states with the changes of oxidation state no matter which membrane state we are discussing. This is known for cytochromes a_3, b, c, cc^1, flavoproteins, ferredoxins, rubredoxin. In some cases the evidence is from redox potential data, E° changes, and in others it is from structural or spectroscopic studies. It is also self-evident that conformational changes and redistribution of charge are inevitably linked in protein systems because of the nature of proteins as polar molecules, see earlier discussion of NaCl,

myoglobin, and haemoglobin. The evidence for a phase mechanism of initial oxidative energy distribution rather than an active site mechanism is strong, on the basis of these conformational and charge fluctuations, although we cannot indicate the way in which the energy is partitioned in the different parts of the membrane phase.

Sources of Charge

The obvious source of charge is the removal of electrons by oxygen. There is then a flow of negative charge from the hydrogen of substrates into the space of accumulated positively charged centres. The change in net charge causes the switch of the mitochondrial state; the flux of charge in the energized state carries out oxidative phosphorylation. The flux is not of substrate hydrogen but of electrons, though in

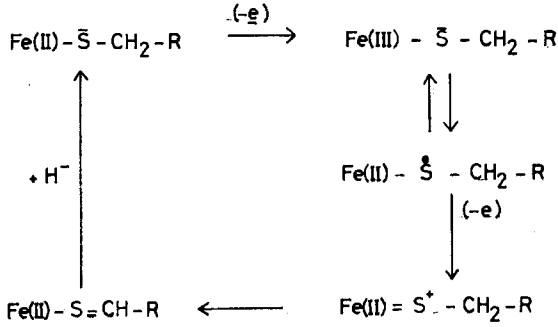

Figure 3. A suggestion as to the all-important role of the iron–sulphur proteins. They are the fundamental catalysts of the reaction $H^+ \rightleftharpoons H^- - 2e$. It could be that copper and molybdenum can carry out this reaction too for all these sulphides are active as inorganic hydrogenation catalysts. Electron transfer could be mediated by a similar series of steps.

different regions of the path of the electron, coenzyme and enzyme protons are first picked up and then rejected. These protons are those that are bound by such coenzymes as NADH, reduced flavin, and reduced quinones and by protein –SH and imidazole. The catalysis of the oxidation of these moieties is brought about by iron–sulphur and possibly copper–sulphur and molybdenum–sulphur proteins. A possible mechanism for this step can be drawn from our knowledge of the iron–sulphur protein structures and involves a metal–sulphide double bond, Fig. 3. Loss of the metal–sulphur proteins will cause oxidative phosphorylation to disappear. As the electrons flow through the membrane phase protons are cycling on to the coenzyme (and protein?) carriers, reduction step, and are leaving the membrane for the aqueous phase as H_3O^+, oxidation step, Fig. 4. In this sense the electron flux can act as a pump for lowering the water activity in the membrane. In 1956 I pointed out that this cycle was a possible way of

generating ATP by generating protons in an organic phase.[3] As we have pointed out subsequently there is a compulsory, concomitant, conformational change.[1,4]

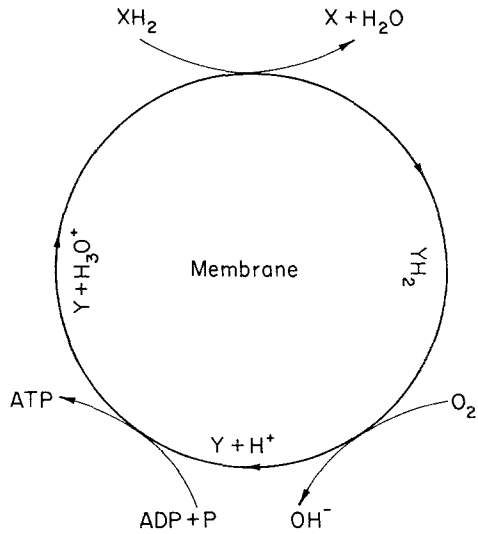

Figure 4. The dehydration cycle in oxidative phosphorylation.

The Problem of Standard State E° Values

In 1969 I drew attention to the fact that all the E° values of redox carriers must be expected to change in the different states of mitochondria.[1,4] (An interesting side effect is that this undermined cross-over theory.) The evidence for such changes was then slight. Subsequently Slater and coworkers[5] and Chance and coworkers[6] claimed that specific changes occurred but these authors have gone on and attempted to connect the E° changes directly with coupling. I fail to see any justification for this for I am convinced that *all* the very many proteins of the cytochrome chain will prove to show such changes. All the changes will be kinetically competent if judged by rate data—they have to be for the reaction is coupled—but in the absence of a *demonstrated physical or chemical connection* between one or another of the carriers the direct relevance to coupling is minimal. However this switch of E° is a direct pointer to the changed ambient conditions of the membrane phase—its changed conformation and charge, its changed effective H^+ activity, its changed surface potential and so on. All are changes of condition of a phase.

Uncoupling Problem

It is my opinion that uncoupling will not prove to be one type of reaction. This follows from the nature of the connection with

"phase-intermediates". Removal of charge from the membrane, e.g. by phenolates, or prevention of conformational change can uncouple—compare the effect of organic phosphates, protons, CO_2, salts on the Hill coefficient for oxygen uptake by haemoglobin.

The ATP-ase Problem

There is the distinct possibility that all *membrane* ATP-ases can be run forwards or backwards, i.e. to ATP degradation or formation, in a highly selective way. ATP formation in a membrane can be coupled to salt *gradients*, to *oxidative* or to *photo-energy*. ATP hydrolysis can be coupled to *reduction*, *light production*, or reversed salt *gradients*. However ATP hydrolysis uncoupled to energy conserving processes does *NOT* normally occur in membranes. A catalyst by itself cannot direct reactions in this way and therefore the ATP-ase must be switched on or off in a controlled way. In mitochondria or chloroplasts there are two situations (a) a situation in which the ATP-ase governs an *equilibrium in the membrane phase which is unrelated to the ATP equilibrium in the aqueous phase.* (b) a situation in which the ATP-ase is switched off. Uncoupling is a chemical effect operating on the link between the phase intermediate and the ATP-ase.

The Switched-on Problem

The reaction of concern in the membrane phase is

$$[ADP]_0 + [P]_0 \rightleftharpoons [ATP]_0 + [H_2O]_0$$

We know that $[ADP]_0$, $[P]_0$ and $[ATP]_0$ relate closely to aqueous phase concentrations for the energy calculation ratios used by Slater balance $[ATP]_w/[ADP]_w.[P]_w$ against the flux of redox equivalents. As the system does not equilibrate in the aqueous phase we know therefore that $[H_2O]_w$ cannot equilibrate with the

$$[ATP]_0/[ADP]_0[P]_0$$

quotient. Thus the ATP-ase cannot have access to bulk water. The bulk components of the organic and aqueous phases are not connected.

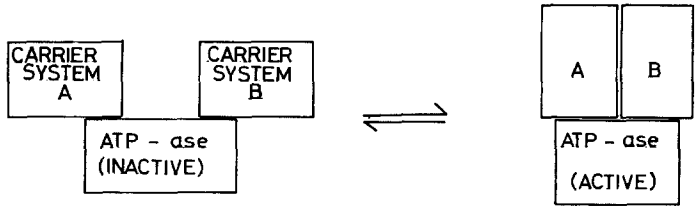

Figure 5. The switch for the ATP-ase.

The above diagram, Fig. 5, is an attempt to illustrate the switched-off $\rightleftharpoons$ switched-on relationship indicating that gross conformation changes in carriers and ATP-ase which affect the membrane.

The Charge Flux Problem

A major mystery in the energy conservation steps is the nature of the charge carriers, electrons or protons, and the sites, traps, which they visit. Soon the detailed structures of four or five electron-transfer type proteins will be known, cytochromes *c* and b_5, flavodoxin, high potential iron protein (HIPIP), and rubredoxin. We know already that minor or major conformation changes occur on redox change in very many (all?) such proteins so that there is an inevitable conformational and charge flux in the coupled state. For many of the carriers tunnelling of electrons cannot be the only charge-carrying process for this cannot be coupled. Again NADH, flavin, quinone, must undergo protonic changes. There is then a mobile pool of charge in the membrane as well as one associated with the fixed proteins so that there is a phase potential—a proton potential—connected directly with the redox potential. This potential does *not* equilibrate rapidly with the aqueous phase as H^+ is not freely mobile across the phase boundary. *This potential must not be linked to osmosis which is inevitably linked to the aqueous water activity*. The iron–sulphur proteins could be the essential $H^+ \rightleftharpoons H^- - 2e$ catalysts of the membrane which are absent from, and out of contact with, the aqueous phase.

A Specific Example

We shall consider the series of proteins associated with succinate dehydrogenase

$$\text{Succinate} \rightarrow \text{Flavin } Fe_4S_4 \rightarrow Fe_4S_4 \rightarrow \text{cyt. } b \rightarrow \text{cyt. } c \text{ cyt } c_1$$

Succinate is a source of hydride to the flavin Fe_4S_4 unit. This unit passes electrons to the iron sulphur protein Fe_4S_4 at a potential around 0·0 volts. Thus this step of the reaction is very like that in many simpler dehydrogenases, e.g. in detoxification

$$\text{NADH} \rightarrow \text{Flavin} \rightarrow Fe_2S_2 \rightarrow \text{cyt. P450}$$

Now in this detoxification system the flavin/Fe_2S_2 is able to hold electrons until the substrates for P450 are bound i.e. O_2 and sterol. The electron transfer is triggered by the presence of substrates but equally substrate/substrate reaction is triggered by the presence of electrons in an overall four electron redox reaction

$$2e + O_2 + SH \xrightarrow{2H^+} H_2O + SOH$$

The mutual reaction is controlled by conformation changes and this must be the case in the succinate chain. The substrate for the Fe_4S_4/cyt. *b* system is a quinone present in large excess so that the electrons are passed next to the quinone which must pick up protons from the surrounding medium. This is a two-electron step. The hydroquinone diffuses or transfers protons to the interior of the system where it reacts with oxidized cytochrome *b*/cytochrome *c* and must give $2H^+$ and two electrons. Now there is a pool of potentially poised couples Flavin.Fe_4S_4, Fe_4S_4, cyt. *b*, cyt. *c*, cyt. c_1, and perhaps other Fe_4S_4 proteins, as well as the major redox buffer QH_2/Q, H^+. All these proteins change conformation on redox reaction and their phase interaction is controlled by their potential poising and vice versa, Fig. 6. Thus they all change E° values. In this coupled state electrons

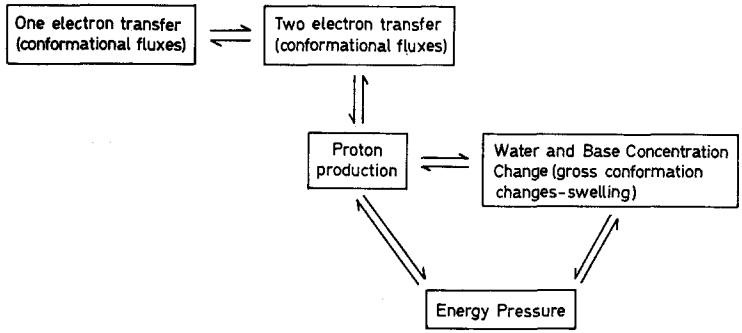

Figure 6. The relationship between the conformation changes in the chloroplast and mitochondrial membranes. Note all proteins change their conformation both with redox and proton flux and with changes of steady state concentration of charge.

flow, effectively two at a time, due to the controlled quinone reaction. It is necessary to remove H^+ from inside the membrane and to pick it up from the aqueous phase at each turn of the cycle, Fig. 4, and the internally generated H^+ can remove water from the interior of the membrane, Fig. 4. The water is replaced at a steady rate by $ADP + P \rightarrow ATP$ condensation. The system is just a logical extension from haemoglobin via mixed function oxidases (and in general multi-protein dehydrogenases coupled to oxygen), to dehydration by carrying out the dehydrogenation and dehydration steps in a non-aqueous phase.

Conclusion

My conclusion is as follows. Energy transduction has been examined with an incorrect view of mechanism taken over from small molecule chemistry. Energy transduction occurs through the generation of a charged/conformational store of energy which does not equilibrate

with the aqueous phase though it can equilibrate internally in the membrane phase with [ATP], [P], [ADP], H^-, H^+ and $[H_2O]_0$. This occurs in the switched-on geometry. The switched-on geometry is in steady state balance with the switched-off geometry through the supply of substrates. The switched-on geometry is asymmetric and it enters into charge exchange with and transport of ions from the bulk aqueous medium. This exchange is in competition with ATP formation and equilibrates more slowly than ATP formation. The examination of many external and internal probes, e.g. $[H^+]$, $[Ca^{2+}]$, $[K^+]$, $[Na^+]$, carotenoid absorption, fluorescence probes, etc., will reflect this phase intermediate. The energetics of the process are then best represented by a description of the local activity of water at the ATP-ase site. The water activity is driven to a low value by the flux of charge, probably protons, through the site. The only "molecular" intermediates are those of the ATP-ase. The experimental test of such a theory rests in the measurement of local H_2O or H^+ concentrations—a very difficult problem. In my opinion none of the present experimental approaches adequately tackle this problem.

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