IS THE TRANSMEMBRANE ELECTROCHEMICAL POTENTIAL A COMPETENT INTERMEDIATE IN MEMBRANE ASSOCIATED ATP SYNTHESIS?

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There is no doubt that the chemiosmotic hypothesis for oxidative and photosynthetic phosphorylation(Mitchell,1966)has given a powerful impulse to the study and the understanding of membrane associated electron transfer and its coupling to ATP formation.The hypothesis has been of particular importance in emphasizing the vectorial aspects of oxidoreduction reactions in energy conserving membranes and the relevance of protonic activity and electrostatic potential differences for the coupling of energy transducing enzymes.Many basic features of the hypothesis have been experimentally substantiated;such features include the asymmetric distribution of redox carriers across the membranes,the formation of transmembrane differences in pH and/or in electrostatic potential by the various redox complexes and by the ATPase,the possibility of driving ATP synthesis by artificially imposed ΔpH or diffusion potentials of ions,or by a combination of these two forces,and the possibility of coupling chemiosmotically in reconstituted vesicles heterologous energy transducing complexes(Boyer et al.,1977; Ferguson,Sorgato,1982).

In the last decade the quantitative aspects of the hypothesis have been subjected to an intensive scrutiny.These studies,aimed to verify whether the thermodynamic and kinetic expectations of chemiosmotic coupling were met in different systems,have generally been much less in agreement with chemiosmosis than the qualitative enzymatic and structural studies.Studies in various systems have often disclosed severe quantitative deviations (with the noticeable exception of higher plant chloroplasts in continuous light (Portis, McCarty, 1974))from the expected behaviour of a chemiosmotic system.It is the purpose of this paper to briefly review these observations, grouping in three main types of anomalies,and to try to derive a minimum model for a modified chemiosmotic coupling mechanism which would be capable of explaining the kinetic and thermodynamic behaviour of energy transducing membranes.

The simplest possible version of the chemiosmotic model visualizes the membrane in a fluid moasaic structure in which the various enzyme complexes are freely diffusable and energetically coupled through the circulation of protons. No barrier for the diffusion of protons is assumed between the aqueous bulk compartments,facing both sides of the membrane,and the proton releasing or accepting sites of the proton translocating enzymes. Thus these sites must be considered in rapid equilibrium with the proton activity of the aqueous phase facing them. Quantitative considerations of the diffusion rate of protons in aqueous enviroments in fact demonstrate

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conclusively,that,in the time range for the occurrence of the redox or/of the ATP forming reactions,no substantial proton activity difference can be maintained between points in space at a distance corresponding to the size of a bacterium or an intracytoplasmic organelle(Mitchell,1981).It follows that the thermodynamic force seen by the energy transducing enzymes and driving ,for example ATP synthesis or reversed electron transfer,is measured by the bulk-bulk electrochemical potential difference of protons. For ATP synthesis therefore,the maximal(positive)free energy change measured when no further net ATP formation can take place (state 4) can never exceed the value of $\Delta\bar{\mu}_{H^+}$ multiplied by a stoichiometric coefficient,which is related to the number of protons translocated per molecule of ATP formed:

eq.1) $$\Delta G_{ATP} \leq n_{H^+} \Delta\bar{\mu}_{H^+}$$

In eq.1 the "less than" symbol is meant to indicate that the ATP synthetase enzyme can be intrinsically uncoupled and that some free energy loss can occur in the coupling of $\Delta\bar{\mu}_{H^+}$ to ATP formation within the complex itself (Baccarini Melandri et al.,1977;Rottenberg,1973).These losses should be however relatively constant so that the force($\Delta G_{ATP}/\Delta\bar{\mu}_{H^+}$ in state 4) should be approximatively constant when $\Delta\bar{\mu}_{H^+}$ is varied.

From a kinetic stand point,both the redox complexes and the ATP synthase are considered as independent units in the coupling membrane,whose interaction is only mediated by the protonic activity;since proton concentrations are obviously involved in the kinetics of any proton translocating system, the rate of all energy transducing complexes will be a function,inter alia,of the proton activity on the two sides of the membrane.In other words,for a given set of experimental conditions(external pH constant, ionic and substrate concentrations constant)the rate of catalysis should be a single-valued function of the proton electrochemical potential difference,being the state of all the other enzyme complexes in the membrane immaterial.Thus,for example,the rate of ATP synthesis should be determined by the ATP,ADP ,P_i and Mg^{++} concentrations,and by the extent of the proton gradient(or the rate of respiration by the substrate and oxygen concentrations and by $\Delta\bar{\mu}_{H^+}$):

eq.2) $$V_{ATP} = f\ (\Delta\bar{\mu}_{H^+}, \Delta G_{ATP})$$; at constant external pH

These quantitative expectations stem out from the simplest version of the chemiosmotic model.We will refer to it as to the "delocalized"chemiosmotic coupling since the two bulk phases are considered in electrochemical equilibrium for protons and high potential protons are supposed to be available with an identical probability to all sites facing the same bulk phase.

The simultaneous quantitative evaluation of the thermodynamic and kinetic parameters of photosynthetic or oxidative phosphorylation have demonstrated remarkably large deviations from this expected behaviour. These anomalies can be order into three groups and will be discussed briefly below.

Anomaly 1:The Force Ratio in State 4 is not Constant Different Values of $\Delta\bar{\mu}_{H^+}$.

If an energy transducing system is allowed to synthetize ATP until no net ATP formation is observed,the maximal free energy change for ATP formation is can be evaluated and compared with the extent of $\Delta\bar{\mu}_{H^+}$.Under these conditions ,according to eq.1,the force ratio should be the minimal estimate for the H^+/ATP stoichiometry.In early experiments in mitochondria these ratios were generally found to be higher than the value of 2 proposed originally by Mitchell(Azzone et al.,1977;Van Dam et al.,1978);these discrepancy is not related to the polarity of the energy transducing membrane utilized in the experiments,since it was observed both in mitochondria,where the H^+-P_i symport mechanism or the electrogenic ADP-ATP exchange proposed for the translocation of phosphorylation substrates could cause complications in the correct analysis of these parameters,and in chloroplasts(Avron,1979)or bacterial membrane fragments(Kell et al.,1978;Baccarini Melandri et al.,1977)where F_1 faces the external phase of the vesicles.
The most obvious inconsistency with eq.1,however,was the observation that $\Delta G_{ATP}/\Delta\bar{\mu}_{H^+}$ varied with different values of $\Delta\bar{\mu}_{H^+}$ and increased with decreasing $\Delta\bar{\mu}_{H^+}$.Moreover,the force ratio was found to change when conditions where set to alter the electrostatic versus the concetration components of $\Delta\bar{\mu}_{H^+}$ (Wilson, Forman, 1982). In this type of experiments possible interference by the adenylate kinase equilibrium should be carefully taken into account,especially when low values of ΔG_{ATP} are measured.There is no doubt,nevertheless,that the bulk to bulk $\Delta\bar{\mu}_{H^+}$ is not a single-valued relation with ΔG_{ATP} as expected for a fully reversible ATPase in equilibrium with aqueous protons.

Anomaly 2:There is a Limited Correlation between $\Delta\bar{\mu}_{H^+}$ and the Rates of Electron Transfer or of ATP Synthesis.

The kinetic behaviour of ATP synthase should be a single-valued function of $\Delta\bar{\mu}_{H^+}$(at least at constant pH for the outside compartment).In many systems,however,(except for chloroplast in continuos light)it was observed that large variations of the rate of ATP formation could be caused by the inhibition of the rate of electron transfer without a corresponding significant decrease in the value of $\Delta\bar{\mu}_{H^+}$.This observation,originally reported for chromatophores of photosynthetic bacteria(Baccarini et al.,1977) has been extended to many bacterial and mammalian respiratory systems(Zoratti et al.,1982;Mandolino et al.,1983; Decker, Lang, 1978).As a complementary aspect of this phenomenon,it was observed in a pioneer work by Padan and Rottenberg(1973)that the stimulation of respiration by ADP and P_i was accompanied by a decrease in $\Delta\bar{\mu}_{H^+}$ markedly smaller than that needed to promote a comparable stimulation by uncouplers(Zoratti et al., 1983).In general this type of observation indicates a coupling between

the redox and the ATP forming enzymes tighter than that existing between these energy transducers and the bulk-to-bulk $\Delta\bar{\mu}_{H^+}$. This kinetic situation can be interpreted as evidence for a coupling mechanism alternative, or at least parallel, to the "delocalized" chemiosmotic one, or for the existence of mutual regulatory controls between redox and ATP forming complexes. This second interpretation, on the other hand, appears unreconcileable with the observation discussed in Anomaly 1, since kinetic controls cannot affect, in principle, equilibrium states.
In bacterial photosynthesis the light-induced and cyclic nature of the electron transfer chain allows for control of the rate of redox reactions by using trains of single turnover flashes fired at variable frequency. Coupling this technique with the sensitive luciferin-luciferase assay for ATP, Venturoli and Melandri(1982) were able to demonstrate that the amount of ATP formed per flash was strictly proportional to the number of photosynthetic units still operative when electron transfer was progressively inhibited by antimycin A. At high ADP/ATP concentration ratios, ATP could be formed, although with a yield per flash of about half of that maximally observed,already after a single turnover of the electron transfer chain, when no ΔpH was yet formed and a membrane potential of about 70 mV was produced (Melandri et al.,1980). This observation is in agreement with the high force ratio observed at low $\Delta\bar{\mu}_{H^+}$ in respiratory systems (Anomaly 1). Moreover, it could be demonstrated that no ATP formation took place in preilluminated membranes which maintained a high and slowly decreasing $\Delta\psi$, unless one additional turnover of the electron transfer chain was elicited by a single turnover flash. Under those specific conditions, one flash alone was unable to drive ATP formation per se and the photophosphorylation was dependent upon the preenergization of the membrane. Under these conditions the decrease in the ATP yield accurately followed the decay of the membrane potential; both parameters were destabilized by K^+ and valinomycin (Melandri et al.,1980). This observation, if one compares the single turnover behaviour of bacterial chromatophores to that in steady state of respiratory systems, indicate that both a competent $\Delta\bar{\mu}_{H^+}$ and electron transport are conditions required for ATP formation.

Anomaly 3: The Response of Energy Transducing Systems to Double Inhibition of ATPase and of the Electron Transfer Chain.

In their work on oxidative phosphorylation in mitochondria, Baum et al.(1971) pioneered the use of double inhibitor titration of electron transfer reactions and of ATPase as an approach for the study of the interaction between energy transducers. In the chemiosmotic model, the coupling between two enzyme complexes is mediated by the "delocalized" protons, so that if one of the two transducers is kinetically limiting the overall rate, the inhibition of the other complex should not influence the overall velocity of the process. It was, on the contrary found that this was not the case: when, for example, NAD^+ reduction from succinate was driven by ATP hydrolysis, the sensitivity to rotenone inhibition was increased rather then decreased following partial

inhibition of ATPase with oligomycin. This observation was subsequently generalized to many direct or reverse processes of mitochondrial (Westerhoff et al.,1983; Baum et al.,1971) or bacterial respiration (Kell et al.,1979; Parsonage, Ferguson, 1982). In photosynthetic systems the double inhibition approach has been extensively utilized in chromatophores by Hitchens and Kell (1982). Using many combinations of inhibitors, they also obtained evidence for a direct kinetic interaction between ATP synthase and electron transport. With an analogous rationale Venturoli and Melandri (1982) observed that the degree of DCCD inhibition of ATP synthesis was not affected by varying the spacing in time of single turnover flashes by two orders of magnitude. The synergistic inhibition generally observed in dual inhibitor titrations again points to a direct functional interaction between energy transducing complexes not mediated by a "delocalized" intermediate. This approach in principle does not require the evaluation of $\Delta\bar{\mu}_{H^+}$, a measure always open to theoretical and experimental criticisms. The possible non-linear behaviour of the kinetics of the energy transducers versus the driving thermodynamic forces could complicate the interpretation of such results. An other possible source of uncertainty is a possible rapid exchange of the inhibitor between the complexes and / or an energy dependent binding, which could delocalize, or respectively alter the effectiveness of the inhibitor. In the authors' opinion nevertheless, this experimental approach, only recently reintroduced in the study of energy transduction, should be pursued further and preferibly substantiated by parallel measurements of the proton gradient.

Devising a Minimal Hypothesis

The observations discussed in the preceeding sessions are irreconciliable with a fully "delocalized" chemiosmotic model. Any model capable of predicting the features of coupling in energy transducing membranes should therefore incorporate Anomalies 1-3 as well as the data supporting the "delocalized" hypothesis. We shall try to propose such a model and to discuss briefly how it can explain the "localized" coupling phenomena described above.
In the classic chemiosmotic hypothesis the various energy transducing complexes are considered to be freely diffusable: no restriction is imposed upon the random distribution of these complexes in the membrane except the obvious one related to the parallel orientation of all complexes which are perpendicular to the membrane plane (as far as proton translocation is concerned).
We shall propose, on the contrary, that the various complexes are laterally ordered structurally or functionally so that a close protonic interaction between them can occur. We propose, moreover, that the proton domain in which the coupling takes place is separated from the bulk phases by diffusion barriers,so that the leakage of protons from the domain into the aqueous phase is a rare event,at least in the time range in which the redox or ATP-forming reactions occur. The coupling membrane is considered to be composed of single coupling units, e.g. including a single electron transfer

chain and an associated ATPase, each one at least partially isolated from the others and from the bulk phases by diffusion barriers for protons. In this way the protonic coupling will not occur by means of the circulation of protons through the bulk phases, but rather preferentially within the protonic domain of a single coupling unit. In this sense the coupling is still chemiosmotic, but the phosphorylating unit no longer coincides with the whole coupling membrane. The energy transducing membrane is composed by a mosaic of coupling units which are at least partially independent.
In the model any dissipation is considered to occur both through leaks within a single coupling unit (this kind of energy dissipation within a protonic pump has been proposed previously as a slip of the pump (Pietrobon et al. 1981,1982)) and through leaks between the two bulk phases. According to this model the bulk-to-bulk $\Delta\bar{\mu}_{H^+}$ results from the slow diffusion of protons from the localized protonic domain to the bulk phases. Analogously, the charge separation phenomena within the various coupling units will form an array of dipoles oriented across the membrane (Skulachev 1982). The resulting electric field will not be completely equipotential along the planes parallel to the membrane, but will be slowly delocalized by the diffusion of protons in the bulk phases and compensated by ion redistribution. The resulting voltage profile will be a function of the physical spacing between the coupling units, the electrical conductivity and the exact location of the proton diffusion barriers,and the conductivity of the bulk-to-bulk leaks (cf. Zimanyi,Garab 1982).
The proton electrochemical potential difference within the domain of a single coupling unit (in the following indicated as $\Delta\zeta_{Hi}$) is not in equilibrium and is, in general, greater than the bulk phase potential difference $\Delta\bar{\mu}_{H^+}$. Again, the actual difference between these two parameters will depend upon the conductivity of the diffusion barriers as compared with membrane leaks. The more the conductivity of the membrane leaks exceeds that of the diffusion barrier, the more the different coupling units will behave independently from one another. The meaning of the model therefore becomes clear: a proton released by the primary pump into the domain will have a much greater probability of being utilized by the secondary pump associated with that domain, than to diffuse into the bulk phase and to be utilized by other secondary pumps or dissipated through the bulk-to-bulk leaks.
Using these assumptions the anomalies can be qualitatively interpreted (for a quantitative interpretation cf. Westerhoff et al. in preparation). In double inhibitor titration experiments (Anomaly 3) the inhibition of either the primary or the secondary pump of one unit will result in the effective block of the entire unit, since the efficiency of proton utilization by the other units will be much smaller. For these experiments the most clear cut results have to be expected with an "all or none" inhibitor, i.e. for inhibitors whose residence time on the accepting site is at least as long as the turnover time of the target complex. Likewise, when the number of primary pumps is decreased by an electron transfer inhibitor (Anomaly 2),

the rate of ATP synthesis will follow the pattern of that inhibition, while the value of $\Delta\bar{\mu}_{H^+}$ will have only a limited relevance for the actual rate of phosphorylation. More complex is the analysis of the behaviour of the model as far as $\Delta G_{ATP}/\Delta\bar{\mu}_{H^+}$ vs $\Delta\bar{\mu}_{H^+}$ is concerned (Anomaly 1). The inhibition of a primary pump will not markedly alter the value of $\Delta\lambda_{Hi}$ in the uninhibited units, but will affect the value of $\Delta\bar{\mu}_{H^+}$ by altering the ratio between the active units and the bulk-to-bulk leaks. A similar effect will be obtained by increasing the leaks with a protonophore (which could, however, also affect the conductivity of the diffusion barriers of the coupling units and therefore perturb the $\Delta\lambda_{Hi}$ vs. $\Delta\bar{\mu}_{H^+}$ relationship). The result will be a preferential decrease in $\Delta\bar{\mu}_{H^+}$ accompanied by a less marked one of $\Delta\lambda_{Hi}$, and consequently of ΔG_{ATP} which is in equilibrium with the latter. The value of $\Delta G_{ATP}/\Delta\bar{\mu}_{H^+}$ will therefore be higher at low $\Delta\bar{\mu}_{H^+}$s as experimentally observed.

The exact nature of the diffusion barrier has been left intentionally undefined in the model. We shall notice, however, that any hydrophilic structure in physical contact with the aqueous phases (e.g. cf. Kell 1979) seems to be kinetically inadeguate to delay the diffusion of protons for a time interval of the order of magnitude of the turnover of the energy transducer complex (several milliseconds). The barrier must be therefore part of more complex structures of the coupling membrane. Experimental evidence for the existence of such structures and of their lability during "in vitro" storage or following mechanical or themal stress is beginning to emerge (Auslânder, Junge, 1974; Theg et al.,1982; Hong, Junge, 1983).

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