THE PROTONMOTIVE FORCE AS AN INTERMEDIATE IN ELECTRON TRANSPORT-LINKED PHOSPHORYLATION: PROBLEMS AND PROSPECTS.

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I. INTRODUCTION AND OVERVIEW

An important and unresolved general problem in cellular biochemistry concerns the question, in a metabolic pathway such as A --> B --> C --> D, of whether (i.e. to what extent) the intermediary metabolites such as B and C become free as a "pool" or, for whatever reason, are passed "directly" between the enzymes which catalyse their interconversion. The latter type of organisation is often referred to as "microcompartmentation" (Jones 1988), and Paul Srere (1987) has coined the term "metabolon" to describe such an "integrated" pathway. Many other contributors to this volume consider this question with respect to enzymes that are classically (although almost certainly erroneously; see e.g. Clegg 1984, Welch & Clegg 1987)) considered "soluble". I have been greatly influenced, for many years, by Paul's very incisive and thoughtful work in this area (cf. e.g. Srere & Mosbach 1974, Kell 1979), and it is a great privilege and pleasure to be able to contribute to this Festschrift in his honour. My aim in the present chapter is to consider this question in relation to the classically membrane-associated processes of oxidative and photosynthetic phosphorylation. Whilst, as in many other cases, the area of membrane protein bioenergetics seems to have been turned into a purely molecular biological discipline, it is my contention that one cannot hope to understand the workings of these devices in vitro if one does not even know what their true substrates, products and (more particularly) dynamic organisation are in vivo.

As is of course well known, the chemiosmotic hypothesis of oxidative and photosynthetic phosphorylation, proposed by Peter Mitchell (Mitchell 1961), has passed during the past 30 years via the status of a theory to have become the more-or-less widely accepted paradigm for the description of the energetic events linking exergonic membranous processes such as electron transport to the otherwise endergonic processes of ATP synthesis, active substrate transport and so on.

In spite (and even perhaps because) of its success, the chemiosmotic hypothesis continues to attract criticism, of various levels of sophistication, and it is worthwhile from time to time to (re-)examine the conceptual basis for the widespread belief in its veracity. Thus my purposes here will be: (a) to review the salient features of the overall chemiosmotic coupling hypothesis of electron transport phosphorylation, and the role of the protonmotive force therein; (b) to stress some of its crucial predictions; (c) to review some of the "key" experiments which have been done to test these predictions, and which have led to the widespread adoption of the chemiosmotic coupling hypothesis; (d) to show that the existing data obtained from these experiments are better interpreted as showing that energy coupling in electron transport phosphorylation (ETP) in vivo does not in fact proceed via a protonmotive force of any significant magnitude; (e) to develop some predictions by which the chemiosmotic hypothesis might be falsified (sensu Popper); (f) to describe the results of experiments designed to test these predictions, which indicate that any coupling intermediate is not shared between all redox chains and ATP synthases within a given energy coupling membrane vesicle; and, (g) to outline some of the general types of coupling mechanism which are at least consistent with the available data and which might provide novel approaches to the study of membrane bioenergetics. Finally, I shall outline one such novel approach, the development and application of nonlinear dielectric spectroscopy to membrane bioenergetics, with which this laboratory is presently concerned.

II. THE PRINCIPLES OF THE CHEMIOSMOTIC COUPLING HYPOTHESIS

The 1961 version of the chemiosmotic coupling hypothesis (Mitchell 1961) had 4 major proposals: that the activities of (i) redox chains and (ii) the ATP synthase enzymes were coupled to the translocation of protons across the membrane in which they were embedded, (iii) that such membranes contain translocases that facilitate electroneutral exchange of anions and cations with hydroxide ions and protons, respectively, and (iv) that the energy coupling membrane in which the systems of (i) - (iii) were embedded was relatively ion-impermeable. Each of these more general proposals has been shown to be true beyond reasonable doubt.

The 1966 version of the hypothesis (Mitchell 1966), however, laid much more emphasis on the primacy of the protonmotive force (pmf) as the energetic intermediate between electron transport and phosphorylation. If redox chains (and ATP hydrolases) pump protons across membranes, and given that the diffusion of protons is one of the fastest chemical reactions known, then the result of this pumping of protons is the setting up of an electrochemical potential difference for protons (the protonmotive force) between the aqueous phases that the coupling membrane serves to separate. This protonmotive force (pmf) should be created at a rate, and possess a magnitude, sufficient to account for ATP synthesis. It also follows (i) that no "extra" coupling proteins are necessary for energy coupling in ETP: only the generators and consumers of the pmf are necessary, provided that the coupling membrane in which they are embedded is sufficiently ion-tight, and (ii) that because the pmf is a macroscopic thermodynamic variable, the pmf generated by any redox chain in a given vesicle should be able to be utilised by any H^+-ATP synthase in that membrane.

III. PHOSPHORYLATION DRIVEN BY AN ARTIFICIAL PMF

Another important prediction of the chemiosmotic coupling hypothesis is that, if the pmf is the energy coupling intermediate in ETP, an artificially applied pmf should drive phosphorylation at a rate greater than or equal to the in vivo rate. As reviewed elsewhere (Kell 1986, Kell 1988a), this type of experiment has been performed many times since it was pioneered by Jagendorf and Uribe (1966). In virtually all cases using bacteria, mitochondria and thylakoids (there is one striking exception (van Walraven et al 1985, 1990)), both in purified energy coupling membranes and in proteoliposomes containing only the F_oF_1-H^+-ATP synthase as protein component, the results show (a) that phosphorylation can be driven at something near the in vivo rate by an artifical pmf if its magnitude is sufficiently high, but (b) that there is a threshold pmf, usually of some 150 mV, below which no phosphorylation is induced (even if thermodynamically favourable and in the presence of an ATP "trap"). Thus (Kell 1986, 1988a), whilst the results provide a most useful set of boundary conditions, the standard prediction, in vacuo as it were, is meaningless if we wish to know whether the pmf is an energy coupling intermediate of ETP in vivo. For this we need to know what the magnitude of the pmf as generated by electron transport is: if less than 150 mV then it is obviously hard to argue that the pmf is an intermediate, since the artifical-pmf experiments show that one can not for instance take refuge in the the idea of an arbitrarily large -->H^+/ATP ratio.

IV. VALUES OF THE PMF OBTAINED WHEN MEASURED DIRECTLY

In mitochondria and thylakoids, the membrane potential and/or pmf (as measured with microelectrodes) are energetically insignificant, and in E. coli are claimed even by their supporters to be less than 150 mV (see an extensive list of references in Kell 1988a). In each case it has been shown that the microelectrode is definitely inside the vesicle of interest. Obviously such data might most simply be interpreted as sounding the death knell for the idea that the pmf has an energetic role in ETP (Ferguson 1985), but astonishingly enough they have not. Instead, most workers have adopted the techniques of ion- and acid-base distribution (see Kell 1979, Nicholls 1982, Azzone et al 1984) for estimating the values of the pmf. Even here, despite the well-recognised problems of energy-dependent and -independent ion binding etc., which mean that a virtually arbitrary value for the pmf may be adopted depending upon which "corrections" one chooses to apply to the data at hand, it is found in almost all cases that the pmf so estimated is still less than the 150 mV demonstrably necessary for energy coupling to ATP synthesis, and certainly less than the 180-200 mV required to obtain the state 3 rate (Kell 1988a).

V. REDOX-LINKED PROTON TRANSLOCATION

If energy coupling is via a "delocalised" (bulk-to-bulk- phase) pmf, then one should expect to be able to see its formation via the pumping of protons into a bulk aqueous phase. These redox-linked proton translocation ("oxygen pulse") experiments were pioneered by Mitchell and Moyle (1967) in mitochondria, and have been widely repeated in a variety of systems. The results show that the observable (rate and) extent of proton translocation to the bulk aqueous phase is highly correlated with the ionic permeability of the membrane (via uniport), in that when this is greatly enhanced over its natural value by the addition of valinomycin (in the presence of K^+) or of moderately high concentrations of the membrane-permeant (and chaotropic) thiocyanate ion the apparent -->H^+/O ratio increases dramatically. The chemiosmotic explanation is that the uniport activity serves to dissipate a substantial membrane potential rapidly set up following the initiation of electrogenic proton pumping, which would otherwise cause "slip" in the proton pumps or lead to a rapid return of pumped protons before the pH electrode in the outer aqueous phase could respond.

If the above explanation is true, then doubling the size of the oxygen pulse should lower the apparent -->H^+/O ratio in the absence of added uniporters still further (since the membrane potential should now be maximal). However, it is in fact unchanged (see Hitchens & Kell 1984, Kell 1988a and full references to the primary literature therein). Further, if the amount of O_2 added per cell is diminished, the maximum membrane potential which could be created (given a knowledge of the membrane capacitance, which is available (see e.g. Kell & Harris 1985, Harris et al. 1987, Kell 1987, Pethig & Kell 1987)) may be made arbitrarily small (<kT), so that the measured -->H^+/O ratio should be the same whether uniporters are added or not. Again, this prediction is not borne out (Hitchens & Kell 1984, Kell 1988a), indicating that essentially all observable protons are ejected in symport or antiport with an anion or cation, are thus

electroneutral, and therefore do not contribute to the generation of a substantial membrane potential.

VI. CO-RECONSTITUTED SYSTEMS; A SHORTAGE IN THE IMPERIAL COUTURE

Thus we have seen that whilst the classically-recognised proton pumps in the "prokaryotic-type" membranes of mitochondria, thylakoids and bacteria pump protons, they do not seem to be able to create a pmf of a magnitude sufficient to drive ATP synthesis. Nonetheless, it is assumed that if chemiosmotic coupling is operative, all that is needed for phosphorylation is the co-reconstiution of a "primary" and "secondary" (i.e. ATP synthase) proton pump in an ion-tight vesicle. Experiments that attempted to illustrate this were pioneered by Racker and Stoeckenius (1974), who showed that such co-reconstituted systems could in fact make ATP, and have been confirmed in numerous other systems. It was argued (in a fashion that I find quite mystical, since any energy coupling observed in these systems might be going via any routes), that this somehow demonstrated the veracity of chemiosmotic coupling. However, the turnover number of the ATP synthases in these early systems was less than 0.01% of the in vivo rate (Kell & Westerhoff 1985), and the "world record" to date (van der Bend et al. 1984) is approximately 2% (Kell 1988a). Thus, especially given that these "purified" preparations, especially of the ATP synthase, are never >98% pure, one could better argue the opposite: that the co-reconstitution experiments show that for successful coupling one requires proteins additional to the generally-recognised primary and secondary proton pumps, and that chemiosmotic coupling is not therefore accounting for the (limited) phosphorylation observed. Since the turnover number of the ATP synthase is not increased by increasing the proportion of primary proton pump (Hauska et al 1980, van der Bend et al 1984), it is not realistic to argue that the cause of the negligible rate of phosphorylation in the in vitro system is an energetically insignificant pmf per se. There is also substantial genetic and other evidence for the existence of these extra, "protoneural" proteins (Kell et al 1981, Kell & Westerhoff 1985, Kell 1988a). I discuss one interesting set of studies.

VII. PROTONOEURAL PROTEINS AS TARGETS FOR INHIBITORY DRUGS

If proteins of the type alluded to above are important parts of energy-coupling reactions, it is reasonable not only that there should be genetic evidence for their existence and role, which there is (see Kell et al. 1981, Kell & Westerhoff 1985), but that they might also be the targets of appropriate drugs or reagents whose inhibitory action could not otherwise be easily explained. We have argued at some length (Kell et al. 1981, Kell & Westerhoff 1985) that many "natural" antibiotics, the membrane-active bacteriocins, are exactly such drugs.

The "orthodox" view (e.g. Konisky 1982, Cramer et al. 1983, Davidson et al. 1984, Bourdineaud et al. 1990, Pattus et al. 1990, Parker et al. 1990, Jakes et al. 1990), which, no doubt as a consequence of the presently pervasive reductionism, is based largely on the results of experiments other than those carried out actually to study the mode of action of these compounds on sensitive cells, is that such bacteriocins, of which the colicins are the most well-known, act by forming relatively non-specific, possibly voltage-gated, ion channels in the bacterial cytoplasmic membrane, leading to the efflux of ions and hence to cell death. Certainly numerous studies have shown that they can exhibit such ionophorous activity in vitro (by which

I mean in black lipid membranes (BLM)). However, so can many other **non-cytotoxic** molecules such as cytochrome c (Kimelberg & Papahadjopoulos 1971, Lee & Kim 1989), lysozyme (Kim & Kim 1989) and even small artifical polypeptides that are not long enough to cross a bilayer membrane, such as Ac-(Ser-Val-Lys-Val)$_n$-NHCH$_3$ (n = 1-3) (Ono et al. 1990). Given that the specificity of the membrane-active bacteriocins is extremely narrow, their potency as bacteriocins very much greater than as ionophores, that they require functional, inner-membrane target proteins if they are to be bactericidal, and that known ionophores cannot reproduce their physiological effects on sensitive cells (Kell et al. 1981, Kell & Westerhoff 1985), it really is quite unrealistic (not to say naive) to extrapolate the findings with BLM to the situation in vivo. The most sensible alternative, which has the merit of accounting for the data observed, is that the membrane-active bacteriocins are cytotoxic because they interact with proteins involved in energy coupling but which are not substrate carriers, redox-linked proton pumps or ATP synthases.

VIII. DOUBLE-INHIBITOR TITRATIONS

If energy coupling in electron transport-linked phosphorylation occurs via a protonmotive force (or indeed via any other macroscopic thermodynamic variable (Welch & Kell 1986)), then the free energy released by any individual redox pump may be used by any H^+-ATP synthase in the same vesicle. Whether this is true or not may be assessed via the so-called double-inhibitor titration techniques, in which the efficiency of inhibition of the overall phosphorylation process by a particular specific inhibitor is assessed with and without partial inhibition (or uncoupling) by another inhibitor. The special benefit of this approach, given the controversies as to what values are realistic, is that it does not require that one knows the value of the putative pmf existing under the conditions of measurement. Whilst the interpretation of these types of data has become rather sophisticated (Caplan & Pietrobon 1987, Westerhoff & Kell 1988), we would stress two crucial types of data, which have been observed in every coupled system studied and which cannot be interpreted in terms of the existence of a "delocalised" coupling intermediate, however convoluted the counterarguments raised: (i) the finding with pairs of electron transport and ATP synthase inhibitors that partial inhibition by one does not affect the normalised titration curve of the other, **and vice versa**, and (ii) the finding that partial inhibition with an ATP synthase inhibitor decreases the titre of uncoupler needed for complete uncoupling. Thus, again we see that whatever the coupling intermediate is, it cannot be a "delocalised" one such as the pmf. It is also worth pointing out that this general type of approach may be used (Kell & Westerhoff 1989, Welch & Keleti 1989), in concert with the Metabolic Control Analysis (see Kell & Westerhoff 1986, Kell et al 1989), to provide good evidence for (or against) the existence of metabolons in all kinds of metabolic pathways.

IX. WHAT TYPES OF MECHANISM ARE THEREFORE POSSIBLE?

It is not necessary to be so heterodox, when constructing an hypothesis or a theory, that one ignores the vast amount of data which have been gathered in purported support of the chemiosmotic coupling hypothesis. Rather, as I have tried to show above, it is necessary, in suggesting an alternative viewpoint, to take them into account. Given the observable pumping of at least some (probably electroneutral) protons, the demonstrable inadequacy of the electron-transport-generated pmf when measured either directly or indirectly, the inability of co-

reconstituted systems to make ATP at anything approaching the in vivo rate, and the existence of one or more "localised" intermediates, it is only necessary to propose the following: that redox-linked proton pumping sets up some kind of membrane polarisation, that the energy is however stored within the primary proton pump, that it is released either by ion transport but otherwise for our purposes only when the (possibly) "random" motions of "protoneural" proteins and the ATP synthase lead to the transient formation of a "supercomplex" through which energy is transferred in a rapid and dispersionless fashion to the ATP synthase (Kell 1988a,b, Welch & Kell 1986), and that, according to this mechanism, energy coupling is non-thermal.

Predictions (for worthwhile experiments) which might follow from this point of view, in addition to those derived a posteriori above, include the following: (a) that gentle crosslinking of (non-leaky) membranes under conditions in which the individual components (primary and secondary proton pumps) are unharmed should inhibit coupling, (b) that uncouplers should not affect the efficiency of electroporation of state 4 mitochondria using 1 ms pulses (they should if there is a membrane potential, and a K^+-valinomycin diffusion potential provides a nice control to this effect) (Kell 1988a), (c) that **AC** fields of a modest magnitude insufficient to create the "threshold" DC membrane potential could drive phosphorylation, (d) that phosphorylating systems might absorb and emit non-faradaic AC electromagnetic radiation in a highly specific fashion, (e) that temperature-sensitive mutants might exist for the protoneural proteins (see Kell & Westerhoff 1985) (the original series apparently having been lost), and (f) that patch clamping could be used to show that, **as expected**, the redox-driven membrane potential is energetically negligible (2-4 mV was observed (Hamamoto et al. 1985), but astonishingly interpreted as energetically **significant** (!) in a BLM system (see Kell 1988a), despite the fact that kT is some 26 mV).

X. NONLINEAR DIELECTRIC SPECTROSCOPY; A NOVEL APPROACH TO THE STUDY OF MEMBRANE ENERGY-COUPLING SYSTEMS

In the concluding section of this brief overview, I draw attention to our own development of the use of nonlinear dielectric spectroscopy for the study of membranous enzymes. The idea behind this approach (Woodward & Kell 1990) is that if the turnover of energy-coupling membrane enzymes is linked to appropriate electrical charge displacements, such enzymes might be able to absorb **AC** (but not DC) electrical energy, and that this should be reflected, inter alia (see (c) above), in the generation of electrical fields at frequencies other than that of a stimulating, pure sinusoid (Kell et al. 1988, Westerhoff et al. 1988a,b, Astumian & Robertson 1989), under conditions in which the Langevin function (see Pethig & Kell 1987) is miniscule, i.e. in which the energy in the electrical field is very much less than kT. Indeed, our early experiments with S. cerevisiae (Woodward & Kell 1990 and unpublished) have shown that the membrane H^+-ATPase of this organism displays nonlinear dielectricity in a strongly field- and frequency-dependent fashion, opening up the possibiity of studying this type of behaviour in the systems catalysing oxidative and photosynthetic phosphorylation. The ability to differentiate enzyme molecules that are turning over from those that are not (Woodward & Kell 1990) also permits an entirely novel approach to the analysis of microcompartmentation based on inhibitor

titrations, and suggests that the substrate of the membrane H^+-ATPase of S. cerevisiae is not a free pool of ATP (Kell & Woodward 1991, Woodward & Kell 1991).

It may be concluded that whatever problems may beset the field of membrane bioenergetics, a dearth of important future experiments that could be directed towards the coupling problem is not one of them.

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