Uncoupler titrations in co-reconstituted systems do not discriminate between localized and delocalized mechanisms of photophosphorylation

Hitchens and I have recently reintroduced various types of double inhibitor titrations of redox-linked phosphorylation as a means by which one might assess the extent to which the so-called 'high-energy' intermediate of this process exhibits pool behaviour (Hitchens & Kell, 1982*a*,*b*, 1983*a*,*b*). Although to gain a proper overview it is fruitful to consider all such approaches together, I here confine myself to the dual inhibitor titrations in which phosphorylation is titrated with an uncoupler under conditions in which a partially inhibitory titre of an inhibitor of the ATP synthase ('energy transfer' inhibitor) either is or is not present.

The principle of this particular experimental approach (e.g. Hitchens & Kell, 1983*b*; Kell & Hitchens, 1983; Ferguson, 1985; Kell & Westerhoff, 1985) is that if a delocalized protonmotive force (p.m.f.) drives phosphorylation, and uncouplers act by decreasing the p.m.f., the steady-state p.m.f. in the absence of uncoupler will probably be greater, and certainly not lower, when a partially inhibitory titre of ATP synthase inhibitor is present. Thus a delocalized model would predict that if the rate of phosphorylation depends solely on the magnitude of the p.m.f., the ability of uncouplers to decrease the p.m.f. in absolute terms (and hence the uncoupler potency) will be the same or lower when the partially inhibitory titre of ATP synthase is present. In contrast, a localized model permits, but does not require, uncouplers to act more potently under the latter conditions, and such behaviour may be observed in bacterial chromatophores (Hitchens & Kell, 1982*b*, 1983*a*,*b*). The converse, however, is not necessarily true, since special kinetic and experimental conditions must be fulfilled if one is to have the possibility even in principle of obtaining a 'localized' finding.

In a recent paper in this Journal, Van der Bend and others (1985) attempted to make a distinction of the above type, and concluded that it was indeed possible so to do. However, the conditions used by these workers do not in fact permit a clear conclusion to be drawn, for reasons which I wish to outline in the following.

The strategy of Van der Bend *et al.* (1985) was to use a co-reconstituted liposomal system containing bacteriorhodopsin plus a yeast F_0F_1-ATPase preparation. These workers chose to assume that this system would necessarily exhibit delocalized behaviour, although no basis in fact for any such assumption exists (Kell & Westerhoff, 1985). In particular, it is absolutely necessary to reiterate (Kell & Morris, 1981; Kell & Westerhoff, 1985; Casey, 1984) that the turnover number of the F_0F_1 in these coreconstituted systems is exceedingly low; the phosphorylation rate of 100 nmol·(min·mg of protein)$^{-1}$ observed (Van der Bend *et al.*, 1985) is approx. 2% of the turnover number *in vivo* (Van der Bend *et al.*, 1984). This may be due, *inter alia*, to the loss of so-called protoneural proteins (which are expected to stain poorly with Coomassie Blue); those remaining could easily account for the remnant 2% activity (Kell & Westerhoff, 1985), incorrect or incomplete reconstitution, energy-transducing and localized collisions (which might not occur *in vivo*) between complexes, or (in chemiosmotic coupling terms) for the fact that the system is incapable of generating a p.m.f. sufficient to produce a normal turnover number. Which of these explanations is most significant does not matter for our present purposes, but the low rates obtained are evidently inconsistent with the assumption that these co-reconstituted systems should be expected to exhibit delocalized behaviour. In spite of this, Van der Bend *et al.* (1985) chose to assume a turnover time of 1 ms. However, the data given by these authors allow us to calculate not only the actual turnover time but the concentrations of ATP synthases in their photophosphorylation assay. This latter is also crucial.

From the molar ratios of protein complexes (140 bacteriorhodopsin : 2 F_0F_1 per phospholipid vesicle) and molecular masses given (Van der Bend *et al.*, 1985) we may calculate that 21% of the protein by weight was F_0F_1, so that a phosphorylation rate of 100 nmol·(min·mg of protein)$^{-1}$ is equivalent to a turnover number of 3.3 s^{-1} or a turnover time of 300 ms, some two orders of magnitude different from that assumed. In the co-reconstitution method employed, 10 mg of phospholipid/ml are mixed with 1 mg of bacteriorhodopsin/ml (Van der Bend *et al.*, 1984), their molar concentrations therefore being 12.9 mM and 38 μM respectively. In the photophosphorylation assay, the phospholipid concentrations is '1–2 mg/ml' (Van der Bend *et al.*, 1984). If we take 1.5 mg/ml, the bacteriorhodopsin concentration = 5.8 μM so that the F_0F_1 concentration (from the molar ratios above) = 82 nM. This number is consistent with the molar ratio of F_0F_1 added during the co-reconstitution, allowing for a small loss of F_0F_1 (Van der Bend *et al.*, 1984). Thus the concentration of F_0F_1 enzymes present was less than 100 nM.

In the uncoupler titrations of Van der Bend *et al.* (1985), even the lowest titre added (their Fig. 2) was apparently 100 nM, which is therefore in excess of the molar concentration of F_0F_1. However, in order to discriminate localized and delocalized coupling, substoichiometric uncouplers are required (Hitchens & Kell, 1983*a*,*b*), i.e. uncouplers in which the effective dose for full uncoupling is less than the number of target enzymes present. [To obtain a clear-cut 'localized' result even with such substoichiometric uncouplers it is also necessary that the uncoupling step itself, and not diffusion between the localized coupling sites, is the (most) rate-determining step (Hitchens & Kell, 1982*b*, 1983*a*,*b*).]

In the uncoupler titration experiments discussed, the only kinetic constant which we know is the turnover number of the ATP synthase. However, we may set the boundary conditions for this type of experiments by following a treatment given by Rich (1984) and elaborated by Ragan & Cottingham (1985) in a discussion of the chemical kinetics and pool behaviour of quinones. Rich (1984) considered the number N of electron acceptors which could react with a donor quinol during its lifetime t, and concluded that for values of N exceeding 10, even localized (electron) transfer would give behaviour indistinguishable from pool kinetics. In our case, we wish to know the relative numbers of uncoupler and ATP synthase molecules with which an energized quantum may interact, since they are in competition with each other. Notwithstanding that protein complexes have membrane diffusion coefficients some 10 times less than those of small molecules such as phospholipids and uncouplers (see, e.g., Kell, 1984; Kell & Harris, 1985), and

in the absence of knowledge of the kinetics of the individual steps in coupling and uncoupling, we can therefore state that if the number of competing acceptors (uncoupler molecules) exceeds the number of ATP synthase molecules then we always have the possibility of apparent pool behaviour. This will be exacerbated as the turnover number of the ATP synthases is decreased, such that if we need a ratio of 10 uncouplers to 1 ATP synthase to effect uncoupling of that synthase we will always obtain behaviour indistinguishable from pool behaviour, whatever the reality (Rich, 1984).

In our own photophosphorylation work (cited above), we confined ourselves to situations in which the titre for full uncoupling did not exceed approx. $\frac{1}{3}$ the number of redox chains or ATP synthases present. In the work of Van der Bend *et al.* (1985), the (extrapolated) titres for full uncoupling (which was not in fact obtained) corresponded to more than 30 times the number of F_0F_1 molecules present.

Thus the claim of Van der Bend *et al.* (1985), using superstoichimetric uncouplers and poorly coupled co-reconstituted systems, that the observation of apparent pool behaviour in their titration experiments not only might provide a means of distinguishing localized from delocalized coupling, but indicated delocalized coupling, is erroneous.

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Received 11 November 1985

A model system for delocalized chemiosmotic coupling exhibited the features thought diagnostic of localized coupling

Quite a few experimental observations (Westerhoff *et al.*, 1984; Ferguson, 1985) are not readily understandable in terms of the 'delocalized' chemiosmotic coupling model (Mitchell, 1981). Whereas it is appropriate to develop alternative views, the experimental observations should also be scrutinized both with respect to systematic experimental errors (e.g. Woelders *et al.*, 1985) and with respect to their interpretation. At issue here is the interpretation of the uncoupler–inhibitor (ui) and inhibitor–inhibitor (ii) titrations.

We shall discuss all the experiments as if dealing with oxidative phosphorylation: one inhibits some 50% of the electron-transfer chains (uil, iil) or 50% of the H^+-ATPases (ui2, ii2) and examines how this affects the potency by which (ui) an uncoupler or (ii1) an inhibitor of the H^+-ATPases, or (ii2) an inhibitor of the electron-transfer chains, inhibits oxidative phosphorylation. For delocalized coupling, Hitchens & Kell's (1982*a*) rule of thumb for ii experiments has been that the potency be reduced; for ui experiments, that the potency of the uncoupler not be increased. Baum *et al.* (1971, 1978), Hitchens & Kell (1982*a*,*b*, 1983*a*,*b*) and we (Westerhoff *et al.*, 1983*a*,*b*) found that experimental results in actual energy-coupling systems conflicted with these rules of thumb. This has been taken as evidence against delocalized coupling.

We (Van der Bend *et al.*, 1985) undertook checking the operational validity of these rules of thumb in an experimental model system which we assumed to have delocalized chemiosmotic coupling: bacteriorhodopsin and yeast H^+-ATPase reconstituted into liposomes (Van der Bend *et al.*, 1985). In experiments of type ii, the inhibitors did not reduce each other's potency. The above rules of thumb would lead us to the conclusion that the model system for delocalized chemiosmotic coupling would operate in a localized fashion. By such *reductio ad absurdum* we proved that, for the experiments of the types ii and ui1, the rules of thumb are not by themselves sufficient to build a case against chemiosmotic coupling. The case ui2 was special in that the results obtained in our delocalized model system did conform to the rule of thumb: contrary to the observations in the more physiological systems, inhibition of H^+-ATPases did not increase the potency of the uncoupler. Consequently, the rule of thumb for the experiment of type ui2 was the only one not compromised by our experimental results, and we granted that this type of experiment might still discriminate between delocalized and localized coupling.

Bacteriorhodopsin, reconstituted with H^+-ATPase in liposomes, has been characterized experimentally [for a review, see Westerhoff & Van Dam (1986)]. The rate of ATP synthesis ($-J_p$) should depend on the activity of the H^+-ATPase (L_p), the activity of bacteriorhodopsin (L_ν) and the proton permeability of the membrane ($L_H{}^1$) as follows:

$$-J_p = A_\nu \cdot L_p \cdot L_\nu / (L_p + L_\nu + L_H{}^1)$$

(A_ν is a constant). Since the system is fairly leaky towards protons (cf. below), this equation predicts our experimental results if indeed our co-reconstituted system were delocalized. This reassures us that there is no inconsistency between our experimental results and our *a priori* assumption that, in the co-reconstituted system, energy coupling was delocalized.

The more striking conclusion that can be deduced from this equation is, however, that (in contrast with the contention by Dr. Kell) the proper rule of thumb for an experiment of type ui2 demands that, in a delocalized system, an inhibitor of the H^+-ATPases does not