A critical appraisal of evidence for localized energy coupling

Kinetic studies on liposomes containing bacteriorhodopsin and ATP synthase

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In intact systems (chloroplasts, mitochondria and bacteria) many experiments have been reported which are indicative of localized coupling between ATP synthase and electron transfer complexes. We have carried out similar experiments with a system in which we may assume that specific interactions between the proton pumps are absent: reconstituted vesicles containing bacteriorhodopsin and yeast mitochondrial ATP synthase. The only experiment that gives results which differ from those previously published for intact systems concerns the effect of uncouplers on the rate of ATP synthesis at different levels of inhibition of the ATP synthase. We propose that this type of experiment may discriminate between localized and delocalized coupling.

The involvement of protons in free-energy transduction (Mitchell, 1961, 1966) is now widely accepted. However, evidence has accumulated that free-energy transduction cannot fully be explained by the classic formulation of the chemiosmotic hypothesis (for a review see Westerhoff *et al.*, 1984). Analysing this, Kell & Morris (1981), Dilly *et al.* (1982) and Westerhoff *et al.* (1984) introduced a localized form of chemiosmotic coupling: protons were thought to remain in a local domain when going from the primary proton pump (e.g. the respiratory chain or photosynthesis) to the secondary proton pump (e.g. ATP synthase or systems involved in solute transport) instead of going via the bulk phase (cf. Williams, 1978). Kell & Morris (1981) suggested that specific proteins would be involved in transferring the protons between the proton-pumping systems. Their model resembles the model of Dilly *et al.* (1982), who also found evidence that protons remain in the membrane phase, being transmitted via protein-defined routes. In the model of Westerhoff *et al.* (1984) barriers were postulated for protons between the local domains and the bulk phase, which implied that the protons in the local domains were not in equilibrium with those in the bulk aqueous phase. By performing calculations on such a model on the basis of an analogous electrical circuit for protons

in which the barrier was represented by a resistance between bulk phase and local domain, many of the anomalies observed in studies on energy coupling could be explained.

Both the experiments directed against delocalized chemiosmosis and their interpretations have been criticized (Ferguson & Sorgato, 1982; Azzone *et al.*, 1984). Indeed, some of the original observations may have been subject to systematic experimental errors, as was shown for instance for the measurements of ΔG_p and $\Delta \tilde{\mu}_H$ (e.g. Lolkema *et al.*, 1982; Woelders *et al.*, 1985). In order to avoid the problems concerning the measurement of $\Delta \tilde{\mu}_H$ and still to be able to discriminate between delocalized and localized chemiosmotic behaviour, Baum *et al.* (1971) developed and Hitchens & Kell (1982*a,b*, 1983) extended inhibitor–uncoupler and double-inhibitor titrations. In the inhibitor–uncoupler titration experiment, the dependence of the overall activity of the system (e.g. phosphorylation driven by the respiratory chain or by photosystems) on uncoupling is determined with or without partial inhibition of one of the two proton-pumping systems. In the double-inhibitor titration experiment, the dependence of the overall activity of the system on inhibition of the ‘primary’ proton pump (e.g. that linked to electron transfer) or the ‘secondary’ one (e.g. ATP synthase) is followed with and without partial inhibition of the other proton pump. In such experiments Baum *et al.* (1971), Hitchens & Kell (1982*a,b*, 1983), Wester-

Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

hoff *et al.* (1983a,b) and Berden *et al.* (1984) found evidence that energy transduction was not consistent with delocalized chemiosmotic behaviour.

Other suggestions for the failure of delocalized chemiosmosis to explain free-energy transduction were obtained from kinetic studies in chloroplasts (Vinkler, 1981). It was found that the effect of addition of protonophore on the dependence of photophosphorylation on the ADP concentration is different from the effect of varying the activity of the primary proton pump (i.e. photosystems). This difference was thought to be understandable in terms of a localized chemiosmotic model.

Interpretations of the experiments described above have been criticized, however, because it is not clear how a delocalized system may be expected to behave (Clark *et al.*, 1983; Westerhoff *et al.*, 1983b; O'Shea & Thelen, 1984). Further elaboration of the theory is needed. An alternative approach to investigate the problem of localization is to use a system in which coupling between the proton pumps is expected to be purely delocalized.

A system which may be considered to function in a purely delocalized manner is the co-reconstituted system of bacteriorhodopsin and ATP synthase. It was demonstrated first by Racker & Stoeckenius (1974) and afterwards by others (e.g. Sone *et al.*, 1977; Ryrle *et al.*, 1979; Alfonzo *et al.*, 1981; Takabe & Hammes, 1981) that in liposomes the two proteins could be coupled in a functional way: light-driven ATP synthesis could be observed after co-reconstitution. Besides the fact that this liposomal system offers the possibility to study delocalized chemiosmotic behaviour, also the role of certain factors which may be involved in the coupling of the proton-pumping enzymes may be studied, e.g. factors concerning the so-called proton neural network (Kell & Morris, 1981).

In the experiments reported here, we subjected liposomes containing bacteriorhodopsin and ATP synthase from yeast mitochondria (Van der Bend *et al.*, 1984) to the inhibitor-uncoupler and double-inhibitor experiments and to the experiments in which the dependence of the kinetic parameters of the ATP synthesis reaction on protonophore and partial inhibition of the primary proton pump (bacteriorhodopsin) were studied.

Experimental

Biological material

Preparation and reconstitution of soybean phospholipid liposomes with bacteriorhodopsin and ATP synthase were performed by the sonication-gel filtration method, as described by Van der Bend *et al.* (1984), in 25 mM-K₂SO₄/85 mM-KCl/0.2 mM-EDTA/10 mM-Tricine, pH 7.5 ('standard medium'). ATP synthase was purified from

yeast mitochondria according to Rott & Nelson (1981).

Light-driven ATP synthesis

Light-driven ATP synthesis was assayed as described by Van der Bend *et al.* (1984). During incubation in the dark the formation of ATP was less than 2 nmol/min per mg of ATP synthase. Light-driven ATP synthesis activities were corrected for this. The light intensity was varied with neutral density filters (Oriel Corp., Stanford, CT, U.S.A.). Inhibition with 8-azido-ATP (Wagenvoort *et al.*, 1977) was achieved by illuminating liposomes containing bacteriorhodopsin and ATP synthase on ice for various periods of time in the presence of 200 μ M-8-azido-ATP at 350 nm. Samples were kept in the dark and on ice. Residual 8-azido-ATP was removed by the quick gel filtration method (Van der Bend *et al.*, 1984). In control experiments it was found that illumination at 350 nm without 8-azido-ATP did not affect the light-driven ATP synthesis activity. The initial and steady-state proton uptake in liposomes containing bacteriorhodopsin only was not affected by illumination in the presence of 8-azido-ATP.

Chemicals

FCCP, gramicidin D and hexokinase were obtained from Sigma. Glucose 6-phosphate was obtained from Boehringer Mannheim. All other chemicals were of analytical grade and obtained from commercial sources. 8-Azido-ATP was synthesized by Mr. A. F. Hartog according to the method described by Wagenvoort *et al.* (1977).

Results

Effect of protonophore and light intensity on kinetic parameters of the ATP-synthesis reaction

Analogous to studies in chloroplasts and submitochondrial particles, the effect of protonophore and partial inhibition of the primary proton pump (i.e. bacteriorhodopsin) on the kinetics of the light-driven ATP synthesis was studied in the co-reconstituted system. In Fig. 1(a) it is shown that different light intensities gave parallel lines in a double reciprocal plot of rate and ATP concentration. In Fig. 1(b) it can be seen that at different FCCP concentrations lines were obtained that intersect in the second quadrant. Thus, we observe the same kinetic picture as Vinkler (1981) and Aflalo & Shavit (1983) found for chloroplasts. For submitochondrial particles parallel lines were found when the respiratory chain was inhibited with malonate, but intersecting lines upon addition of protonophore (Hatefi *et al.*, 1982; Yagi *et al.*, 1984).

Fig. 1. Effect of protonophore and variation in light intensity on Lineweaver-Burk plots for the light-driven ATP synthesis in liposomes containing bacteriorhodopsin and ATP synthase

(a) Light intensity was varied; (b) FCCP concentration was varied (c) gramicidin concentration was varied.

Instead of FCCP we also used gramicidin as protonophore which acts by forming pores. Although the uncoupling effect of gramicidin was not large (the light-driven ATP synthesis could be inhibited by maximally 40%), a similar effect as with FCCP was observed (Fig. 1c).

Analogous to the conclusion of Vinkler (1981), the findings in Fig. 1 do not seem to agree with delocalized behaviour.

Inhibitor-uncoupler titrations

Hitchens & Kell (1982a, 1983) observed that, compared with the uninhibited case, less protonophore was needed to uncouple photophosphorylation completely in chromatophores when the photosystems or the ATP synthase were partly inhibited. They also observed that the relative

Fig. 2. Titration with protonophore of the light-driven ATP synthesis activity with the ATP synthase inhibited to different extents

Inhibition was obtained by preincubating for different periods at 350 nm in the presence of 8-azido-ATP. (■), 0 min; (□), 10 min; (●), 20 min of illumination at 350 nm. In (a) absolute activities are plotted; in (b) relative activities are plotted.

Fig. 3. Titration with protonophore of the light-driven ATP synthesis activity at different light intensities

(■), 100%; (□), 24%; (●), 10% light intensity. In (a) absolute activities are plotted; in (b) relative activities are plotted. The FCCP concentration used during the illumination assay is given.

change in activity upon addition of protonophore became larger when one of the proton pumps was inhibited. In Figs. 2 and 3 results of similar experiments are shown for the co-reconstituted system. In the experiment of Fig. 2 the ATP synthase was partly inhibited by 8-azido-ATP; in

the experiment of Fig. 3 the bacteriorhodopsin activity was lowered by using lower light intensities. The insets in Figs. 2 and 3 (Figs. 2*b* and 3*b*) present the normalized data. When the ATP synthase was partly inhibited, we found that the amount of protonophore needed for completely abolishing ATP synthesis was the same as that needed for the preparation that was not inhibited (Fig. 2). From the normalized curves (Fig. 2*b*) it can be concluded that the relative change in activity upon addition of protonophore remained constant. This differs from the findings of Hitchens & Kell (1982*a*, 1983) and also from the data of Westerhoff *et al.* (1983) and Berden *et al.* (1984), who studied reversed electron flow in submitochondrial particles.

When bacteriorhodopsin was inactivated by using lower light intensities, the relative change in activity upon addition of protonophore became larger (Fig. 3*b*). This is analogous to the finding of Hitchens & Kell (1983) when they inhibited the photosystems with antimycin A. From the findings in Fig. 3 it can also be concluded that, within the accuracy of the determination of the ATP synthesis activity, less protonophore is needed to abolish ATP synthesis completely at lower activities of bacteriorhodopsin. This is also analogous to the findings of Hitchens & Kell (1983) and Berden *et al.* (1984) when they inhibited the primary pump.

Thus, according to the predictions made by Hitchens & Kell (1983) the findings in Fig. 2 are in agreement with delocalized chemiosmosis, but those in Fig. 3 are not.

Double-inhibitor titrations

Using two inhibitors, one would, on the basis of the classic interpretation of chemiosmotic behaviour, expect that the dependence of the overall activity on inhibition of one of the proton pumps becomes less when the other proton pump is partly inhibited (Baum *et al.*, 1971; Hitchens & Kell, 1982*b*). In contradiction to this, it was found that the relative change in activity in double-inhibitor titrations remained constant in the case of submitochondrial particles (Baum *et al.*, 1971; Westerhoff *et al.*, 1983*b*; Berden *et al.*, 1984) and bacterial chromatophores (Hitchens & Kell, 1982*a,b*). This was taken as evidence for localized chemiosmosis. In Fig. 4 we present an experiment with the co-reconstituted system in which the ATP synthase was inhibited with 8-azido-ATP and the bacteriorhodopsin activity was varied by changing the light intensity. When the ATP synthase became inhibited, we found that the relative change in activity upon lowering light intensity remained constant (Fig. 4*b*). In Fig. 5 the data from Fig. 4 were plotted in a different way: we now obtain titration curves at different light intensities, the ATP synthase

Fig. 4. Titration by varying light intensity of the light-driven ATP synthesis activity, the ATP synthase being inhibited to different extents

Inhibition of ATP synthase was obtained by incubating for different periods at 350 nm in the presence of 8-azido-ATP. (□), 0 min; (○), 10 min; (×), 20 min of illumination at 350 nm. In (a) absolute activities are plotted; in (b) relative activities are plotted.

Fig. 5. Titration, by inhibiting the ATP synthase to different extents, of the light-driven ATP synthesis activity at different light intensities

(□), 100%; (○), 63%; (×), 51%; (▽), 17%; (●), 10% light intensity. Data are the same as used in the experiment of Fig. 4. In (a) absolute activities are plotted; in (b) relative activities are plotted.

being 'titrated' by inhibition with 8-azido-ATP. When the light intensity was lowered from 100% to 17% (but perhaps not down to 10%), the relative change in activity upon inhibition of the ATP

synthase remained constant, as can be seen in Fig. 5(b).

According to predictions made by Baum *et al.* (1971) and Hitchens & Kell (1982*b*) the findings in Fig. 4 and Fig. 5 (at the higher light intensities) are not compatible with delocalized chemiosmosis.

Discussion

Effect of protonophore and varying light intensity on kinetic parameters of the ATP synthesis reaction

In Fig. 1 it is shown that the dependence of the light-driven ATP synthesis on the ADP concentration was differently affected by adding protonophore from lowering the light intensity. The findings for the co-reconstituted system are similar to those found for chloroplasts (Vinkler, 1981; Aflalo & Shavit, 1983) and for submitochondrial particles (Hatefi *et al.*, 1982; Yagi *et al.*, 1984). Vinkler (1981) suggested from her results obtained with chloroplasts that a direct regulatory effect of the electron-transfer system on the ATP synthase may be involved. The fact that for the co-reconstituted system, which should be considered as a purely delocalized system, the same kinetic behaviour is found as for the chloroplast and mitochondrial preparations leads us to look, however, whether the results still may be consistent with delocalized chemiosmotic behaviour. A direct regulatory effect of the primary on the secondary proton pump as suggested by Vinkler (1981), which most likely would occur by protein-specific interactions, would not be expected in the case of the co-reconstituted system, since the two proteins in this system (bacteriorhodopsin and ATP synthase) are from completely different organisms.

The difference in the effect of adding protonophore and varying light intensity on the kinetics of the ATP synthesis may also be explained by the fact that protonophore (FCCP) has a specific interaction with the ATP synthase (see for example Hanstein, 1976). However, when we performed the experiment with gramicidin, which acts in a different way from the protonophore FCCP, similar results were obtained (Fig. 1*c*).

An explanation for the observed kinetic behaviour may be that $\Delta\tilde{\mu}_H$ is affected differently by addition of protonophore from variation in the light intensity at equal ADP concentrations. This is supported by the results of Yagi *et al.* (1984). Next to the kinetic analysis performed in mitochondrial particles, they also measured in separated experiments the oxonol VI response in order to monitor the membrane potential under the different conditions. Upon addition of malonate in concentrations at which parallel Lineweaver–Burk plots for oxidative phosphorylation were found, the membrane potential was unaffected. However,

upon addition of protonophore in concentrations at which intersecting Lineweaver–Burk plots were obtained, the membrane potential decreased. Yagi *et al.* (1984) measured the oxonol VI response only at one ADP concentration. At the different ADP concentrations in Lineweaver–Burk plots, however, $\Delta\tilde{\mu}_H$ may also vary due to the difference in proton pump activity of the ATP synthase. For this reason, apparent K_m and V_{max} values calculated from the Lineweaver–Burk plots (Vinkler, 1981; Hatefi *et al.*, 1982; Yagi *et al.*, 1984) may not be considered as real apparent K_m and V_{max} values according to Michaelis–Menten kinetics, as the ADP concentration is not the only parameter that varies.

For a better understanding $\Delta\tilde{\mu}_H$ should be measured under the different conditions. As mentioned before, no reliable methods exist to measure $\Delta\tilde{\mu}_H$, at least for the co-reconstituted system. Besides this, in the case of the co-reconstituted system, $\Delta\tilde{\mu}_H$ measurements will also be hampered by the heterogeneity of the liposome preparations.

Inhibitor–uncoupler titrations

The findings in Fig. 2 are compatible with delocalized behaviour of free-energy transduction (Hitchens & Kell, 1983). As a fraction of the ATP synthesis became inactivated, the relative change in activity upon addition of protonophore remained constant (Fig. 2*b*). In contrast to the findings in Fig. 2, the findings in Fig. 3 resemble the findings of Hitchens & Kell (1982*a*, 1983) with chromatophores and in terms of their interpretations would seem to be incompatible with delocalized behaviour. At lower light intensities, the relative change in activity upon addition of protonophore became larger (Fig. 3*b*). The findings in Fig. 3 extend, however, the observation made by Van der Bend *et al.* (1984), namely that the light-driven ATP synthesis activity became less sensitive to protonophore at higher light intensities. For the proton gradient measured in bacteriorhodopsin liposomes, a similar dependence on light intensity was observed (Arents *et al.*, 1981). Thus the bulk-bulk proton gradient was affected in the same way as the light-driven ATP synthesis. It will be clear from this that the findings in Fig. 3 may still be in agreement with delocalized behaviour. The fact that the proton gradient created by bacteriorhodopsin became less dependent upon addition of protonophore at higher light intensities can be explained through the inhibitory effect of $\Delta\tilde{\mu}_H$ on the pumping rate of bacteriorhodopsin. At higher light intensities the pumping rate is low due to the fact that $\Delta\tilde{\mu}_H$ will be high; upon addition of protonophore $\Delta\tilde{\mu}_H$ will decline, which will be compensated, however, by a higher

pumping rate of bacteriorhodopsin. Taking this into account, the findings on the proton gradient can be completely understood from a delocalized chemiosmotic model, as Westerhoff *et al.* (1979) showed using mosaic non-equilibrium thermodynamics.

It was concluded from Figs. 2 and 3 that, to abolish ATP synthesis completely, the amount of protonophore needed was independent of the remaining activity of ATP synthase (Fig. 2a), but that less protonophore was needed when the activity of bacteriorhodopsin was decreased (Fig. 3a). Hitchens & Kell (1983) found for chromatophores that less protonophore was needed to obtain a fully uncoupled system when either the photosystem or the ATP synthase was partly inhibited. A similar finding has been reported by Westerhoff *et al.* (1983a) and Berden *et al.* (1984) for ATP-energized reversed electron transfer at site 1 in submitochondrial particles. This was considered to be strong evidence for localized chemiosmotic behaviour. In the case of inhibition of the secondary proton pump (ATP synthase) we would go along with these arguments, but not in the case where the primary pump is inactivated since, because of this fact, the titrations are done at lower values of $\Delta\tilde{\mu}_{\text{H}}$. Indeed, we consider the observation that less protonophore was needed to uncouple completely at lower activities of bacteriorhodopsin (Fig. 3a) as compatible with delocalized chemiosmotic behaviour. Thus, our co-reconstituted system behaves according to delocalized chemiosmosis in the experiment of Fig. 2 and Fig. 3.

Double-inhibitor titrations

According to the predictions of Baum *et al.* (1971) and Hitchens & Kell (1982a,b) the findings in Figs. 4 and 5 cannot be understood from a chemiosmotic viewpoint. Westerhoff *et al.* (1983b) and Berden *et al.* (1984) found similar results with double-inhibitor titrations for ATP-energized reversed electron transfer at site 1 in submitochondrial particles. However, Westerhoff *et al.* (1983b) argued that interpretation of the result may be dubious by not taking into account the possible effects of the existing proton leakage. Using the thermodynamic control theory (Westerhoff *et al.*, 1983b), they examined the results in more detail. They concluded that the results can still be readily explained in terms of delocalized chemiosmotic behaviour, except when partial inhibition of the primary proton pump leads to an increased relative effect of an inhibitor of the secondary proton pump (Westerhoff *et al.*, 1983b). In the case where the dependence of the overall activity on inhibition of one of the proton pumps remains constant when the other proton pump is inhibited, this may still be compatible with delocalized chemiosmosis. In

fact, this is illustrated by the experiments performed by us (Figs. 4 and 5) with the co-reconstituted system, which may be considered to be delocalized.

Can the inhibitor-uncoupler and double-inhibitor titration experiments discriminate between localized and delocalized chemiosmosis in the liposome system?

The question may arise as to whether the inhibitor-uncoupler and the double-inhibitor experiments are able to discriminate between localized and delocalized chemiosmosis in the case of the liposomal system. At least two or more molecules of both proteins should be present per liposome (Hitchens & Kell, 1983). Structural analysis on the liposome preparation by sucrose gradient centrifugation (Van der Bend *et al.*, 1984), size estimation by electron microscopy (negative stain procedure, results not shown) assuming molecular sizes of 470 kDa, 26 kDa and 775 Da for ATP synthase (Tzagoloff & Meagher, 1971), bacteriorhodopsin (Oesterhelt & Stoerkenius, 1971) and phospholipid, respectively, and assuming an average area per phospholipid molecule of 0.70 nm², revealed that at least 2 ATP synthase and at least 140 bacteriorhodopsin molecules are present per liposome (with an average diameter of 50 nm).

Also, protonophore molecules should be able to shuttle between the coupling units in which the proton pumps are coupled in a localized way (cf. Kell & Morris, 1981) within the turnover time, the time needed for formation of ATP on the ATP synthase after absorption of a photon by bacteriorhodopsin. From the values given above, an average distance between the coupling units in case of the liposomal system of 18 nm (centre to centre) can be calculated. For the chromatographs used by Hitchens & Kell (1982a,b, 1983) an average distance of 8 nm can be calculated (Reed *et al.*, 1975). Assuming a diffusion coefficient of 10⁻⁸ cm²/s for a protonophore molecule, similar to the diffusion coefficient for lipids in model lipid bilayers (Smith & McConnell, 1978) and assuming a turnover time of 1 ms for the ATP synthesis reaction after absorption of light, it can be calculated that the distance which can be covered by protonophore in the plane of the membrane within the turnover time is about 60 nm (Saffman & Delbruck, 1975). These considerations indicate that the uncoupler-titration experiments may also be used for our liposome preparations to discriminate between localized and delocalized behaviour.

The experiment shown in Fig. 2 is completely in agreement with delocalized chemiosmosis. The other experiments gave indications for non-delocalized behaviour. As the system used in this study may be expected to behave in a delocalized way, it

is clear that a more careful analysis is needed before further conclusions can be made from the latter experiments. So far, it seems that only experiments of the type of that shown in Fig. 2 can discriminate between delocalized chemiosmotic and direct interactions.

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