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Localized energy coupling during photophosphorylation by chromatophores of *Rhodopseudomonas capsulata* N22

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The principle of the dual inhibitor titration method for testing models of electron-transport phosphorylation is outlined, and the method is applied to the study of photophosphorylation in bacterial chromatophores. It is concluded that energy coupling is strictly localized in nature in this system, in the sense that free energy released by a particular electron-transport chain may be used only by a particular H^+-ATP synthase. Dual inhibitor titrations using the uncoupler SF 6847 and the H^+-ATP synthase inhibitor oligomycin indicate that uncouplers act by shuttling rapidly between the localized energy-coupling sites.

An important current problem in bioenergetics may be summarized by the question "is the 'energized intermediate' coupling electron transport and ATP synthesis delocalized at the level of the intact vesicle, as in macroscopic versions of the chemiosmotic coupling hypothesis (see e.g. Mitchell, 1979; Nichols, 1982), or do there exist more localized and direct free-energy-transferring interactions between the redox and ATP synthase proton-pumping complexes in energy-coupling membranes (e.g. Ernster, 1977; Williams, 1978; Kell, 1979; Baccarini-Melandri et al., 1981; Conover & Azzone, 1981)?"

We (Hitchens & Kell, 1982), and others (Melandri et al., 1981; Venturoli & Melandri, 1982) have recently addressed ourselves to this question by using the double inhibitor titration method (Baum et al., 1971; Kell et al., 1979) with bacterial chromatophores, and have concluded that the free energy transfer between electron transport and ATP synthase complexes in chromatophores is fully localized in nature, in the sense that the 'high-energy intermediate' generated by a particular electron-transport chain may be used only by a particular H^+-ATP synthase. This conclusion was arrived at from the results of experiments in which the effect on the photophosphorylation rate of the covalent H^+-ATP synthase inhibitor dicyclohexyl carbodiimide (DCCD) was studied at different rates of electron transport (Melandri et al., 1981; Hitchens & Kell, 1982; Venturoli & Melandri, 1982). However, to strengthen this conclusion further, it would be desirable to test other H^+-ATP synthase inhibitors, and it has been suggested (Westerhoff et al., 1982a,b) that the double inhibitor titration method

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is only completely reliable when the effect of an electron-transport inhibitor on the titration curve of an ATP synthase inhibitor for inhibiting phosphorylation is tested in this type of experiment. It is the purpose of the present article to report the results of such experiments in bacterial chromatophores, using the electron-transport inhibitor Antimycin A and the H^+-ATP synthase inhibitor oligomycin, and it is concluded that the energy coupling in electron-transport phosphorylation is indeed 'localized' in nature in those membranes. Further, it is shown that the potent uncoupler 3,5-di-t-butyl-4-hydroxybenzylidene malononitrile (SF 6847) acts 'substoichiometrically' by shuttling rapidly between the localized energy-coupling sites in these membranes.

Materials and Methods

The growth and maintenance of *Rhodopseudomonas capsulata* N22, the preparation of chromatophores therefrom, the assay of bacteriochlorophyll content and photophosphorylation rate, and the sources of chemicals and biochemicals have been described elsewhere (Hitchens & Kell, 1982), except that oligomycin was obtained from Sigma (Cat. No. O 4876).

Results

The principle of the double inhibitor titration method (see Baum et al., 1971; Kell et al., 1979; Hitchens & Kell, 1982) may briefly be summarized as follows. If a reaction sequence $A \xrightarrow{E_1} B \xrightarrow{E_2} C$ is envisaged, in which, implicitly, A represents electron transport, B stands for a 'high-energy' intermediate, and C constitutes phosphorylation, inhibition of the net rate of phosphorylation by the addition of an H^+-ATP synthase (E_2) inhibitor will, if the 'high-energy' intermediate B is fully delocalized, make the electron-transport-driven formation of B less rate-limiting, i.e. the addition of an electron-transport (E_1) inhibitor such as antimycin A will have relatively less effect on the rate of phosphorylation in the presence of an ATPase inhibitor than in its absence. If, however, B is fully localized, in the sense that the 'high-energy' intermediate generated by a particular electron-transport chain may be used only by a particular H^+-ATP synthase, then the electron-transport inhibitor will be equally effective in each case.

Fig. 1 shows the data from such an experiment with chromatophores from *Rhodopseudomonas capsulata* N22, where it may be seen that, far from making the electron-transport-driven formation of the 'high-energy' intermediate less rate-limiting, partial restriction of photophosphorylation with oligomycin actually decreases the concentration of antimycin required to inhibit photophosphorylation completely. The symmetrical experiment, in which the photophosphorylation rate is titrated with oligomycin in the presence and absence of a partially inhibitory concentration of Antimycin A, is displayed in Fig. 2, wherein it may be observed that data entirely comparable to those of Fig. 1 are obtained, as predicted by the 'thumb rule' (see Westerhoff et al., 1982a,b) analysis of this type of experiment when energy coupling is fully localized in nature.

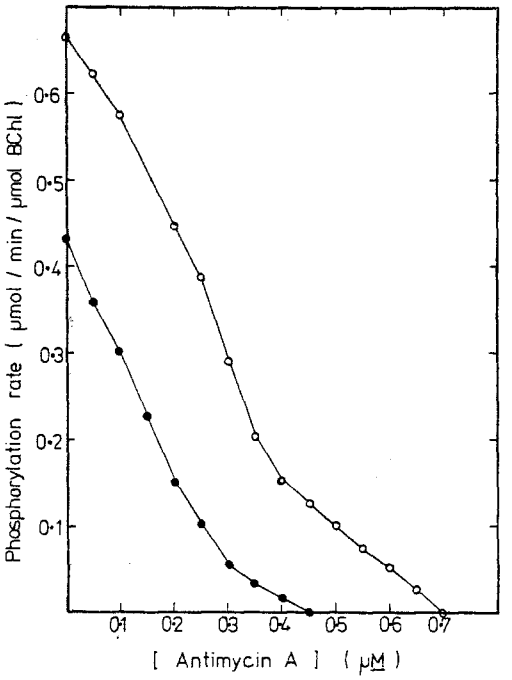

Fig. 1. Effect of antimycin A and oligomycin on photophosphorylation by chromatophores of *Rps. capsulata* N22. Photophosphorylation was measured as described in 'Materials and Methods' in a 6-ml reaction mixture containing, at 25°C and pH 7.8, 3 mM KH_2PO_4, 10 mM magnesium acetate, 30 mM potassium acetate, 0.2 mM sodium succinate, 1.5 mM sodium ADP, 5 μM P^1,P^5-bis-(5'-adenosyl) pentaphosphate, 800 μg of carbonic anhydrase, and chromatophores corresponding to a bacteriochlorophyll concentration of 20 μM. Antimycin A was added to the concentrations indicated (○, ●), and where indicated (●) the chromatophores were preincubated for 20 min with oligomycin (0.1 μg/ml).

The uncoupler SF 6847 is apparently the most potent uncoupler known (Terada, 1981), and causes complete uncoupling of photophosphorylation in our chromatophore system at a concentration corresponding to 0.3 molecules per electron-transport chain (Hitchens & Kell, 1982). The question arose (Hitchens & Kell, 1982) as to how one might reconcile the localized energy coupling observed (Hitchens & Kell, 1982; and see above) with the 'substoichiometric' uncoupling catalysed by SF 6847. We countenanced two possibilities (Hitchens & Kell, 1982): either (i) SF 6847 shuttled between the localized energy-coupling sites at a rate sufficiently rapid to uncouple at least 3 sites during one turnover of the photophosphorylation apparatus, or (ii) the simple *binding* of a given SF 6847 molecule could lead to the cooperative uncoupling of (here) 3 or more 'coupling sites'. The

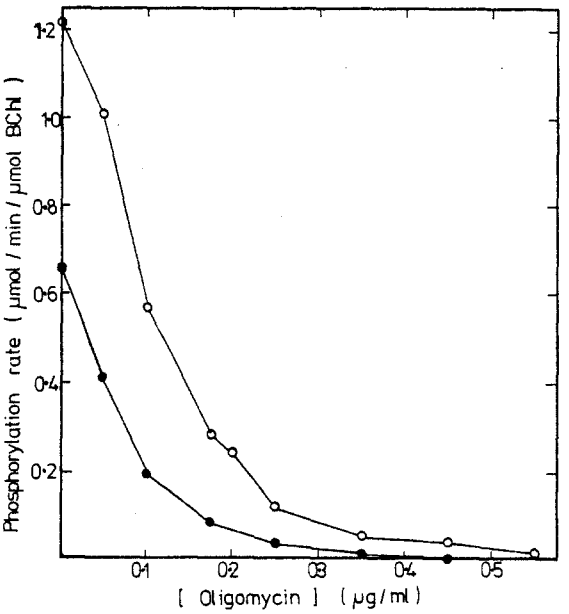

Fig. 2. Effect of oligomycin and antimycin A on photophosphorylation by chromatophores of *Rps. capsulata* N22. Photophosphorylation was assayed as described in the legend to Fig. 1, except that chromatophores were preincubated with the concentrations of oligomycin indicated for periods ranging from 20 to 45 min, prior to initiating photophosphorylation by illuminating the reaction vessel. Where indicated (●) antimycin A (0.2 µM) was also present.

following experiment was performed to distinguish these possibilities. A titration curve for the inhibition of photophosphorylation by SF 6847 was established. Then photophosphorylation was partially inhibited by preincubating the chromatophores with an appropriate titre of oligomycin. For a 'shuttling' model it is to be expected that SF 6847 will uncouple even more effectively when the rate of phosphorylation in the absence of uncoupler is partially decreased by oligomycin, whilst the 'binding' model would predict that a similar titre of the uncoupler would be required for full inhibition of photophosphorylation in both the presence and absence of a partially inhibitory concentration of oligomycin. The experimental data are shown in Fig. 3; it is evident that they strongly support a model in which the uncoupler molecules can shuttle rapidly between the (localized) energy-coupling sites.

Discussion

It is important, in experiments of the type described herein, that the chromatophores do not contain a significant native energy 'leak', and the evidence against this possibility, and also against that of the

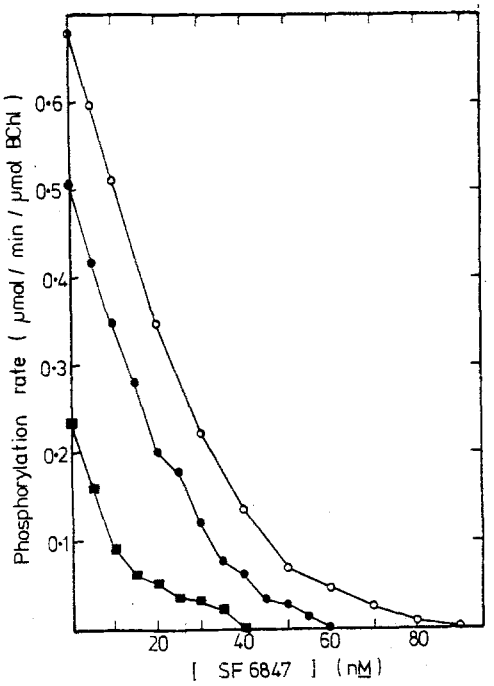

Fig. 3. Effect of SF 6847 and oligomycin on photophosphorylation by chromatophores of *Rps. capsulata* N22. Photophosphorylation was assayed as described in the legend to Fig. 1. SF 6847 was added to the concentrations indicated (○, ●, ■). Where shown, the chromatophores were preincubated with oligomycin at a final concentration of 0.1 µg/ml (●) or 0.2 µg/ml (■).

occurrence of a significant chromatophore heterogeneity (as regards energy-coupling properties), has been discussed at length elsewhere (Hitchens & Kell, 1982). We do not therefore reiterate these arguments here.

That oligomycin enhances the inhibitory effect on photophosphorylation of Antimycin A (Fig. 1), and vice versa (Fig. 2), shows very clearly that the coupling between electron transport and phosphorylation in this system is quite strictly localized in nature, and cannot thus be effected via a delocalized intermediate such as a bulk-to-bulk phase electrochemical protonic potential difference. The slight difference between the data observed with oligomycin in Fig. 1 and that reported earlier in analogous experiments with DCCD (Hitchens & Kell, 1982) is to be expected, and is due to the fact that oligomycin, although a tight-binding inhibitor (see Linnett & Beechey, 1979), is not quite as tightly bound in the steady state as is the covalent modifier DCCD.

In considerations based on the delocalized-chemiosmotic-coupling theory of the relationship between the apparent or theoretical bulk-phase protonmotive force and the rate of phosphorylation (see

e.g. Jencks, 1980; Jackson, 1982; Schlodder et al., 1982), it is taken that this relationship, although highly non-linear, is monotonic in nature. It is also to be assumed that partial restriction of the rate of phosphorylation by an H^+-ATP synthase inhibitor such as oligomycin will not decrease the supposed protonmotive force in the absence of added uncoupler. On this basis, therefore, a delocalized coupling model can predict only that the presence of a partially inhibitory concentration of oligomycin will either have no effect on, or will cause a decrease in, the efficiency of uncoupling that is displayed by a particular uncoupler in the absence of the H^+-ATP synthase inhibitor, whatever the presumed relationship between the protonmotive force and the rate of ATP synthesis. The observation that the addition of a partially inhibitory concentration of oligomycin caused an *increase* in the uncoupling effectiveness of SF 6847 is explicable only within the framework of a model in which uncoupler molecules shuttle rapidly between the 'energized' and localized coupling sites present, and in which the rate-limiting step in the uncoupler's action lies in the uncoupling step itself, and not in the rate of diffusion of uncoupler molecules between their sites of uncoupling action.

In conclusion, the present results, obtained with the dual inhibitor titration approach in bacterial chromatophores, show clearly that energy coupling in photophosphorylation is strictly localized in nature, and are in harmony with other data obtained using this approach in chromatophores (Melandri et al., 1981; Venturoli & Melandri, 1982; Hitchens & Kell, 1982), in submitochondrial particles (Baum et al., 1971; Westerhoff et al., 1982a,b), and in phosphorylating membrane vesicles from *Paracoccus denitrificans* (Kell et al., 1979).

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References

Baccarini-Melandri A, Casadio R & Melandri BA (1981) Curr. Top. Bioenerg. **12**, 197-258.

Baum H, Hall GS, Nalder J & Beechey RB (1971) in *Energy Transduction in Respiration and Photosynthesis* (Quagliariello E, Papa S & Rossi CS, eds), pp 747-755, Adriatica Editrice, Bari.

Conover TE & Azzone GF (1981) in *Mitochondria and Microsomes* (Lee C-P, Schatz G & Dallner G, eds), pp 481-518, Addison-Wesley, New York.

Ernster L (1977) Ann. Rev. Biochem. **46**, 981-995.

Hitchens GD & Kell DB (1982) Biochem. J. **206**, 351-357.

Jackson JB (1982) FEBS Lett. **139**, 139-143.

Jencks WP (1980) Adv. Enzymol. **51**, 75-106.

Kell DB (1979) Biochim. Biophys. Acta **549**, 55-99.

Kell DB, Ferguson SJ & John P (1979) Biochem. Soc. Trans. **6**, 1292-1295

Linnett PE & Beechey RB (1979) Meth. Enzymol. **55**, 472-518.
Melandri BA, Baccarini-Melandri A & Venturoli G (1981) in *Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria* (Palmieri F, Quagliariello E, Siliprandi N & Slater EC, eds), pp 381-388, Elsevier/North Holland, Amsterdam.
Mitchell P (1979) Science **206**, 1148-1159.
Nicholls DG (1982) *Bioenergetics: An Introduction to the Chemiosmotic Theory*, Academic Press, London.
Schlodder E, Gräber P & Witt HT (1982) in *Electron Transport and Photophosphorylation* (Barber J, ed), pp 105-175, Elsevier Biomedical Press, Amsterdam.
Terada H (1981) Biochim. Biophys. Acta **639**, 225-242.
Venturoli G & Melandri BA (1982) Biochim. Biophys. Acta **680**, 8-16.
Westerhoff HV, de Jonge PC, Colen A, Groen AK, Wanders RJA, van den Berg GB & van Dam K (1982a) EBEC Reports **2**, 267-268.
Westerhoff HV, Colen A & van Dam K (1982b) Biochem. Soc. Trans., in press.
Williams RJP (1978) FEBS Lett. **85**, 9-19.