On the extent of localization of the energized membrane state in chromatophores from *Rhodopseudomonas capsulata* N22

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It is now widely accepted that bacterial chromatophores provide an excellent system for studies aimed at furthering our understanding of the processes involved in electron transport phosphorylation (Crofts & Jackson, 1970; Crofts, 1974; Gromet-Elhanan, 1977; Crofts & Wood, 1978; Clayton & Sistrom, 1978; Baccarini-Melandri et al., 1981). In particular, they provide an excellent experimental opportunity to attack the important current problem of whether the energized intermediate between electron transport and ADP phosphorylation is delocalized over the entire chromatophore membrane, as in the chemiosmotic model of membrane energy coupling processes (e.g. Mitchell, 1966, 1979a,b), or whether there exist more localized and direct free-energy-transferring interactions between electron transport complexes and ATP synthase complexes in these energy-coupling membranes (e.g. Del Valle-Tascon et al., 1978; Williams, 1978; Kell, 1979; Petty & Jackson, 1979b; Melandri et al., 1980, 1981; Baccarini-Melandri et al., 1981; Conover & Azzone, 1981).

Since a great deal of uncertainty remains concerning the quantitative reliability of methods for measuring the bulk-phase protonmotive force in bacterial chromatophores (e.g. Elema et al., 1978; Kell et al., 1978; Michels & Konings, 1978; Ferguson et al., 1979; Kell, 1979; Ostroumov et al., 1979; Armitage & Evans, 1981; Baccarini-Melandri et al., 1981; Casadio et al., 1981; Cirillo & Gromet-Elhanan, 1981; Clark & Jackson, 1981; Jackson & Clark, 1981), we have chosen to examine the functional interactions between electron transport and ATP synthase complexes by means of the use of double-inhibitor titrations. The principle of this method (Fig. 1) (cf., e.g., Baum et al., 1971; Kell et al., 1979; Melandri et al., 1981) is that, in the case of electron transport phosphorylation, a titration curve of the rate of phosphorylation versus the concentration of a specific electron transport inhibitor may be established. Then an aliquot of the energy-coupling-membrane preparation may be taken and reacted with a concentration of a covalent ATPase inhibitor sufficient to reduce the rate of phosphorylation, in the absence of added electron transport inhibitor, by, say, 50% compared with that of the control membranes. Under these conditions, the rate-limiting reaction for the phosphorylation may be taken and reacted with a concentration of a covalent ATPase inhibitor sufficient to reduce the rate of phosphorylation, in the absence of added electron transport inhibitor, by, say, 50% compared with that of the control membranes. Under these conditions, the rate-limiting reaction for the phosphorylation may be taken and reacted with a concentration of a covalent ATPase inhibitor sufficient to reduce the rate of phosphorylation, in the absence of added electron transport inhibitor, by, say, 50% compared with that of the control membranes.

1. The principle of the double-inhibitor titration method for assessing competing models of electron transport phosphorylation is expounded. 2. This principle is applied to photophosphorylation by chromatophores from *Rhodopseudomonas capsulata* N22. 3. It is found that, in contrast to the predictions of the chemiosmotic coupling model, free energy transfer is confined to individual electron transport chain and ATP synthase complexes. 4. This conclusion is not weakened by arguments concerning the degree of uncoupling in the native chromatophore preparation or the relative number of electron transport chain and ATP synthase complexes present. 5. Photophosphorylation is completely inhibited by the uncoupler SF 6847 at a concentration corresponding to 0.3 molecules per electron transport chain. 6. The apparent paradox is solved by the proposal, consistent with the available evidence on the mode of action of uncouplers, that uncoupler binding causes a co-operative conformational transition in the chromatophore membrane, which leads to uncoupling and which is not present in the absence of uncoupler.

Abbreviations used: SF 6847, 3,5-di-t-butyl-4-hydroxybenzylidene malononitrile; Tricine, N-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

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electron transport chains or the provision of energy to the functioning ATPases. If the effect of the same electron transport inhibitor upon phosphorylation by the latter preparation, some, say one-half, of whose ATPases have been inhibited with the covalent ATPase inhibitor, is tested, two possible ATPase complexes are obtained, which free energy transfer is restricted to particular ATPases; the electron transport inhibitor is just as effective as in the control experiment (Fig. 1c). Thus a simple distinction is possible between ‘localized’ and ‘delocalized’ models of electron transport phosphorylation in chromatophores.

The purpose of the present article is to report an experimental analysis of electron transport phosphorylation in bacterial chromatophores by using the double-inhibitor titration technique described above. It is found, using this technique, that the localization of free-energy transfer during electron transport phosphorylation by bacterial chromatophores is apparently complete. However, it is also found that full uncoupling of electron transport from phosphorylation may be observed with the uncoupler SF 6847 at a concentration of uncoupler equivalent to 0.31 molecules per electron transport chain. The apparent paradox between this result and that from the double-inhibitor titrations is simply resolved by proposing that the presence of the uncoupler itself can sub-stoichiometrically cause a co-operative uncoupling transition in the energy-coupling proteins of the chromatophore membrane.

**Experimental**

**Biological material**

*Rhodopseudomonas capsulata* N22, generously given by Dr. J. B. Jackson, was grown photo-thermally under anaerobic conditions at 30 ± 1°C in filled 500 ml flasks, in a medium containing, per litre: (NH₄)₂SO₄, 1 g; sodium DL-malate, 4 g; KH₂PO₄, 0.6 g; K₂HPO₄, 0.48 g; sodium EDTA, 20 mg; MgSO₄.7H₂O, 0.2 g; CaCl₂, 75 mg; FeSO₄.7H₂O, 12 mg; thiamine HCl, 1 mg; MnSO₄, 4 mg; H₂BO₃, 7 mg; Cu(NO₃)₂.2H₂O, 0.1 mg; ZnSO₄.7H₂O, 0.6 mg; Na₂MoO₄.2H₂O, 1.9 mg; pH 6.8. For the preparation of chromatophores, all procedures were carried out at 4°C. Mid-exponential phase cultures were washed once and resuspended to a volume of 50 ml in a medium containing 50 mm-Tricine/NaOH/50 mm-NaCl/8 mm-MgCl₂, pH 7.4 (Packham et al., 1978). Chromatophores were prepared by sonication (3 x 30s) at the full power of an MSE 150 watt ultrasonic disintegrator, operating at 20 µm peak-to-peak amplitude. Cell debris was removed by centrifugation at 20000 g for 15 min, and chromatophores were sedimented at 115000 g for 90 min. The chromatophores were resuspended by using a glass homogenizer in approx. 10 ml of a medium containing 3 mm-KH₂PO₄/10 mm magnesium acetate/30 mm-potassium acetate, pH 7.8, and were stored in the dark at 0–4°C until required. Bacteriochlorophyll concentrations were estimated after extraction with acetone/methanol (7:2, v/v) using the extinction coefficients given by Clayton (1963). Bacteriochlorophyll concentrations in typical stock

![Fig. 1. Principle of the double-inhibitor titration method for assessing the extent of localization of free energy transfer in bioenergetic systems](image-url)
chromatophore preparations were in the range 0.2–0.4 m~

Photophosphorylation

Photosynthetic phosphorylation was measured by a modification of the method of Nishimura et al. (1962), in a black-painted glass reaction vessel maintained at 25°C by a thermostat. Illumination was effected by means previously described (Ferguson et al., 1979), and control experiments in which the chromatophore concentration was varied were performed to establish that the light intensity under these conditions was saturating for photophosphorylation. The reaction medium for photophosphorylation assays contained, in a final volume of 6 ml, 3 m~KH₂PO₄, 10 m~magnesium acetate, 30 m~potassium acetate, 0.2 m~sodium succinate, 1.5 m~sodium ADP, pH 7.8, chromatophores corresponding to a bacteriochlorophyll concentration of 20 μm and 800 μg of carbonic anhydrase. In addition, P₁,P₅-bis-(5'-adenosyl)pentaphosphate an inhibitor of adenylate kinase (Lienhard & Secemski, 1973), was included at a final concentration of 5 p~

Reaction mixtures were vigorously stirred with a magnetic follower and were maintained under a stream of CO₂-free oxygen. No effect of adenosine 5'-[γ,γ-imidoltriphosphate (0.2 m~) upon the rate of phosphorylation was observed, indicating that negligible uncoupled ATP hydrolase activity was present in these preparations. The scalar photophosphorylation reaction is accompanied, at pH 7.8, by the uptake of approximately 0.95 H⁺ (Nishimura et al., 1962), and the decrease in pH caused by this reaction was used to determine the rate of photophosphorylation, measured as described in the Experimental section. Fig. 2(a) (---), no further additions were made, whilst in trace (b) (----), antimycin A was added at the points, and to the micromolar concentrations, indicated.

Chemicals

Antimycin A, P₁,P₅-bis-(5'-adenosyl)pentaphosphate, adenosine 5'-[β,γ-imido]triphosphate and dicyclohexylcarbodi-imide were purchased from Sigma. Compound SF 6847 was generously given by Dr. Y. Nishizawa, Sumitomo Chemical Company, Osaka, Japan. Other reagents were obtained from Sigma or BDH, and were of the highest grade available. Water was doubly distilled in an all-glass apparatus.

Results

Fig. 2 shows a trace of the scalar light-induced pH increase accompanying the phosphorylation of ADP, measured as described in the Experimental section. Fig. 2(a) shows that this rate is constant, despite the build-up of ATP, throughout the time, phosphorylation potential and pH change considered, whilst Fig. 2(b) shows that the rate of phosphorylation is decreased by the addition of the electron transport inhibitor antimycin A. Similar data for Rps. sphaeroides chromatophores, together with strong evidence that this inhibition by antimycin A is not due to any uncoupling (cf. Wikström & Krab, 1979), have been provided by Melandri et al. (1981). A separate aliquot of chromatophores from the same preparation was preincubated with dicyclohexylcarbodi-imide (50 μm) in the reaction vessel for 25 min in the dark, and a similar titration of photophosphorylation to that of the control chromatophores shown in Fig. 2(b) was performed with antimycin A. The results of the two titrations are shown in Fig. 3. It is clear that the relative titration curves correspond quite closely to the idealized plots given in Fig. 1(c), indicating that the energy coupling process between individual electron transport chains and ATPase enzymes is completely
Fig. 3. Effect of dicyclohexylcarbodi-imide and antimycin A on phosphorylation by Rps. capsulata chromatophores

The rate of photophosphorylation, performed as described in the Experimental section, is plotted against the concentration of the electron transport inhibitor antimycin A for two cases. In trace (a), the data from Fig. 2 (b) are plotted. An aliquot of chromatophores from the same preparation was reacted with 50 μM-dicyclohexylcarbodi-imide as described in the text, and the titration was repeated. It may be observed that these titration curves correspond rather closely to the idealized titration curves for fully localized energy transfer given in Figs. 1(a) and 1(c).

localized, without even a hint of delocalization. Similar data (not shown) were obtained when the amount of dicyclohexylcarbodi-imide added was varied so as to inhibit phosphorylation by between 30 and 70% of the rate observed in its absence. A similar conclusion concerning the lack of energetic interaction between the different electron transport and ATPase complexes, together with evidence presented to show that, as expected, dicyclohexylcarbodi-imide does not act as an electron transport inhibitor, was reached by Melandrì et al. (1981) on the basis of double-inhibitor titrations under single-turnover-flash conditions (and cf. Baccarini-Melandrì et al., 1977; Casadio et al., 1978).

However, earlier work with chromatophores from Rps. sphaeroides using the ionophore valinomycin (Saphon et al., 1975), and, in particular, experiments with rat-liver mitochondria using the uncoupler SF 6847 (Terada & van Dam, 1975) have shown that uncoupling can occur at concentrations of uncoupler of less than 1 molecule per electron transport chain. We have therefore carried out a titration of the rate of photophosphorylation by Rps. capsulata chromatophores with the uncoupler SF 6847, with the resulting data plotted in Fig. 4. It is seen that full uncoupling of photophosphorylation is caused by 80nM-SF 6847 when chromatophores corresponding to 20 μM-bulk bacteriochlorophyll are present. Since there is 1 reaction centre for approx. every 77 molecules of bulk bacteriochlorophyll (Packham et al., 1978), the maximum number of functioning electron transport chains under the conditions of Fig. 4 is equivalent to 260nM. Thus, even assuming that every added molecule of SF 6847 is active, full uncoupling is achieved by 0.31 molecules of SF 6847 per electron transport chain. Thus although the energy-coupling process appears to be completely localized, uncoupling seems to exhibit co-operative or delocalized features.

Discussion

As indicated in Fig. 1, the use of double-inhibitor titrations offers, in principle, a simple and clear-cut tool for making an experimental distinction between competing models of electron transport phosphorylation. The data in Fig. 3 indicate that in bacterial chromatophores, and in contrast to the predictions of the chemiosmotic coupling hypothesis, the energetic coupling between the reactions of the electron transport chains and the ATP synthases is quite localized in nature, and does not extend over the entire chromatophore membrane vesicle. We now consider alternative interpre-
tations of these experiments that might make such a conclusion unwarranted.

One possibility, discussed by Baum et al. (1971), is that there is a significant native 'energy leak' in the system. Thus, if electron transport-derived free energy is partitioned between ATP synthesis and a significant competing energy 'leak', inhibition of either electron transport or the ATP synthase enzymes would, even in the chemiosmotic coupling model, lead to a decrease in photophosphorylation, since a greater proportion of the electron transport-derived energy would go to the energy 'leak'. Amongst the abundant evidence that bacterial chromatophores do not in general have a significant energy leak of this type are the following: (i) the phosphorylation rates of our chromatophores (1.5 μmol of ATP·min⁻¹·μmol of bacteriochlorophyll⁻¹) compare favourably with those obtained by others (e.g. Petty & Jackson, 1979a); (ii) under multiple-flash conditions the P/2e⁻ ratio of bacterial chromatophores is close to the maximal theoretical efficiency of a system with the calculated degree of coupling (Jackson et al., 1981) and, more importantly, is essentially independent of the flash frequency when this is varied between 3.3 and 12.5 Hz (Melandi et al., 1981); (iii) no rapid decay phase of the electrochromic carotenoid response ascribable to significant uncoupling is observed in bacterial chromatophores (Jackson et al., 1978). We therefore conclude that the present results are not attributable to the presence in chromatophores of a significant energy 'leak'.

Another situation (J. B. Jackson, personal communication) that might artefactually lead to the conclusion that the energy coupling process was localized, rather than chemiosmotic in nature, would arise if bacterial chromatophore preparations contained a significant fraction of chromatophore vesicles that possessed but one or two ATPase molecules. The evidence that this is not the case includes the following: (i) titrations of Rps. capsulata chromatophores with the tight-binding and specific ATPase inhibitors venturicidin, efrapeptin and 4-chloro-7-nitrobenzofurazan all suggest that there is at least 1 ATPase per 100–200 bulk bacteriochlorophyll molecules (Petty & Jackson, 1979a), i.e. 5–10 ATPases per chromatophore; if the ATPases per chromatophore were distributed normally, an insignificant fraction of chromatophores would have only 1 or 2 ATPases; (ii) electron microscopic data demonstrates rather clearly that bacterial chromatophores contain ATPases and reaction centre complexes in approximately equal amounts (Reed & Raveed, 1972); (iii) the presence of any chromatophores containing only 1 or 2 ATPases would have caused an initial shoulder in the antimycin A titration curves (Fig. 3), which was not observed. We thus retain the conclusion that, in contrast to the requirements of the chemiosmotic coupling model, free energy transfer during photophosphorylation by bacterial chromatophores is not delocalized over the entire chromatophore vesicle, but appears, in the absence of protonophores or ionophores, to be localized between individual electron transport chain and ATPase complexes.

However, it was found (Fig. 4) that the potent uncoupler SF 6847 caused complete uncoupling of electron transport phosphorylation at a concentration of 0.31, i.e. <1, molecule per electron transport chain. In fact this is a minimal estimate of its potency, since the actual concentration of active SF 6847 molecules bound to the chromatophore membranes is unknown. In discussing the potency of SF 6847 in uncoupling rat liver mitochondria, in which complete uncoupling occurred at an SF 6847 concentration of 0.2 molecules per respiratory chain (0.06 molecules per 'coupling site'), Terada & van Dam (1975) considered two possibilities. The first was that the uncoupler molecules themselves could move rapidly between sites, with, in this case, only 6% of coupling sites active at any one moment (Margolis et al., 1967). The second possibility was that all coupling sites in a given vesicle released their free energy to a common pool, as in the chemiosmotic coupling theory, and the uncoupler molecules worked at full speed (perhaps as protonophores) to dissipate this supply. Although there is abundant evidence that a variety of uncouplers can act in this way to shuttle H⁺ across the bilayer portions of phospholipid membranes (McLaughlin & Dilger, 1980), such a view would be inconsistent with the data from the double inhibitor titrations (Fig. 3) discussed earlier. Further, the latter model would not be consistent with the following, more recently discovered, features of uncoupling (Kell, 1981): (i) the presence in mitochondria, at a concentration of approx. 1 per respiratory chain, of high-affinity proteinaceous uncoupler-binding sites (Hatefi, 1975; Hanstein, 1976a,b; Katre & Wilson, 1978); (ii) the existence of a number of uncoupler-resistant mutant strains of bacteria (Decker & Lang, 1977, 1978; Guffanti et al., 1981; Ito & Ohnishi, 1981) whose lesions appear to lie in proteins; (iii) the demonstration of recognition sites in the bacterial cytoplasmic membrane for uncoupling molecules (Ordal, 1976; Brummet & Ordal, 1977; Nicholas & Ordal, 1978).

An alternative view of uncoupling that is consistent both with the observed effects of uncouplers generally (see above) and with the uncoupler and double-inhibitor titrations obtained in the present work is that the binding of uncouplers to proteinaceous components in energy-coupling membranes leads in some manner to a co-operative and uncoupling conformational transition in the energy-coupling membrane (Weinbach & Garbus, 1969).
that does not take place in the absence of uncoupler. Such a conclusion is also required to account for the mode of uncoupling by membrane-active bacteri-<ref>2</ref>ins (Kell et al., 1981; Kell & Morris, 1981).

Such considerations lead to the view that the processes of energy coupling and their uncoupling are not related to each other in a simple fashion.

Venturoli & Melandri (1982) have recently extended their work on double-inhibitor titrations of chromatophores from Rps. sphaeroides, and conclude, in harmony with the present work, that the energetic coupling between the redox reactions and phosphorylation is localized in nature.

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References


Energy coupling in bacterial chromatophores