

Titration of ATP synthesis with uncoupling agents do not provide evidence of localised high energy intermediates in electron transport phosphorylation in bacterial chromatophores

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The dependence of light-driven ATP synthesis by chromatophores upon uncoupling agent concentration in the presence and absence of F_0 inhibitors was investigated. When experiments were carried out using the procedure described by Hitchens and Kell [Biochem. J. (1983) 212, 25–30] it appeared as though uncouplers were more potent in the presence of F_0 inhibitors. This however was the result of uncorrected, time-dependent changes in the rate of ATP synthesis. When the experimental procedure was modified to minimise the effect of time-dependent changes in rate, it was found that uncoupler potency was not significantly changed by the presence of F_0 -inhibitors. These results do not discriminate between localised and delocalised models of energy coupling and are entirely consistent with the chemiosmotic hypothesis.

ATP synthesis

Chemiosmosis

Chromatophore

Photosynthesis

Uncoupling agent

1. INTRODUCTION

The chemiosmotic interpretations for the action of uncoupling agents and energy transfer inhibitors [1] have become almost axiomatic: uncoupling agents are supposed to increase the passive proton conductance of biological membranes and thus, in mitochondria, thylakoids and bacteria, they collapse the proton motive force that is responsible for the coupling of electron transport to ATP synthesis. Energy transfer inhibitors, such as oligomycin, are believed to block proton transport through the F_0 -component of the ATP synthase. Numerous experimental tests have provided direct support for these mechanisms (for reviews see [2,3]) although some observations have led to modified or alternative suggestions (see [4–6] and references therein). Recently authors in

[4–6] described the results of some experiments with chromatophores from photosynthetic bacteria which they believed to be inconsistent with the chemiosmotic view of uncoupling and energy transfer inhibition. Their finding was that less uncoupling agent was required to decrease the rate of ATP synthesis in chromatophores which were partially blocked with energy transfer inhibitors than in control chromatophores; i.e., the uncoupler appeared to be more potent when the number of active ATP synthases was reduced. Authors in [4–6] concluded that a delocalised proton motive force could not be the obligatory high-energy-intermediate between electron transport and ATP synthesis. They argued that the high-energy-intermediate must be strictly localised in the sense that free energy released by an individual electron transport chain may only be used by a particular ATP synthase. To explain the titration result and the fact that very low concentrations of uncoupler are required to inhibit ATP synthesis, these agents were envisaged as shuttling between coupling sites, discharging only those which are energised.

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Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile

We thought that in view of these results, it would be of interest to measure the value of the proton motive force in the presence of limiting quantities of both uncoupler and energy transfer inhibitor. However, in attempting to reproduce the experiments of the above authors we found that their results could only be obtained in special circumstances. When phosphorylation rates were measured after a fixed time of illumination and suitable precautions were taken to ensure that phosphorylation rates were linear in time, then we found that uncoupler potency was similar in the presence and absence of energy transfer inhibitor.

2. METHODS

Bacteria (*Rhodospseudomonas capsulata* strain N22) were grown and chromatophores were prepared by French Press treatment as in [7,8]. ATP synthesis rates were measured with a glass electrode by following the scalar H^+ uptake [9,10]. Unless otherwise noted the assays were carried out in a medium containing 10% sucrose, 100 mM K^+ -acetate, 10 mM Mg^{2+} -acetate, 1 mM ADP, 5 mM K^+ -phosphate, 0.5 mM Na^+ -succinate, 6 μM P^1, P^5 -bis(5'-adenosyl) pentaphosphate, and bacteriochlorophyll concentrations in the range 10–20 μM at a final pH of 7.4 and at 30°C. The samples were illuminated with saturating intensities from a 150 W quartz halogen lamp filtered through one layer Wratten 88A filter and 8 cm of water. The sample of SF6847 was a gift from Dr S.J. Ferguson.

3. RESULTS

A titration of the photophosphorylation rate of chromatophores with the uncoupler FCCP in the presence and in the absence of 0.05 $\mu g/ml$ oligomycin is shown in fig.1A. This experiment was carried out as in [4–6,10] and gave essentially the same result – less uncoupler was required to inhibit ATP synthesis in the presence of oligomycin than in the absence of the F_0 inhibitor. Each FCCP titration (in either the presence or absence of oligomycin) was carried out sequentially on the same chromatophore sample; i.e., the chromatophore suspension was illuminated for 2 min and then an aliquot of FCCP was added. One minute later a further FCCP addition was

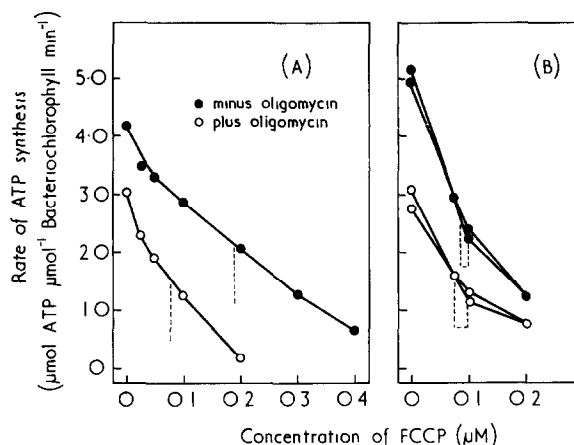


Fig.1. The combined effect of FCCP and oligomycin on the rate of ATP synthesis in chromatophores. Experiments were carried out in air in a clear perspex vessel with a final vol. of 4 ml. The light-induced pH change was kept within 0.1 unit. (A) Sequential titration of FCCP on the same chromatophore sample. Two experiments were set up, one in the absence (●) and one in the presence (○) of 0.05 $\mu g/ml$ oligomycin. The chromatophores (10.1 μM bacteriochlorophyll) were pre-incubated in the dark for 20 min in phosphorylation medium (see section 2) in the presence or absence of oligomycin. The samples were illuminated for 2 min and then the FCCP titration was begun. One minute was allowed between each FCCP addition. The rate was measured immediately before the subsequent FCCP addition and is shown as a function of the final FCCP concentration at that time. (B) FCCP titration on separate samples (rate measured after a constant time of illumination). Each data point was taken from a separate experiment. Preincubation conditions were as described in (A). Each sample, in either the presence (○) or absence (●) of 0.05 $\mu g/ml$ oligomycin was illuminated for 1.5 min. A single addition of FCCP to the concentration shown, was then added. The rate was measured at 3.5 min after the onset of illumination. The dotted lines show the FCCP concentration giving half-inhibition of the maximum rate of ATP synthesis. In (B) the pair of dotted lines arise from the small discrepancies in the duplicate samples. The solid lines between the points are drawn to the extremity of the duplicates.

made, and so on. The entire titration was performed on a single sample with a total illumination time of 6–8 min. Clearly in this procedure, the time of illumination as well as the FCCP concentration was varied during the course of the experiment.

In fig.1B a different experimental protocol was employed in an attempt to eliminate any possible

deleterious effects of prolonged illumination. In this case a separate chromatophore sample was used for each experimental point. Each sample was pre-illuminated for 1.5 min under phosphorylating conditions and then treated with a single dose of FCCP. The rate of ATP synthesis was measured 2 min after the addition of uncoupler. This figure shows a different result from that in fig.1A – the potency of the uncoupler in inhibiting ATP synthesis was similar in the presence or absence of the limiting concentration of oligomycin. This result is entirely compatible with the chemiosmotic interpretation of the action of uncoupling agents and F_0 inhibitors. The aim of the remainder of this report is to demonstrate that the results in [4–6] arise from their adopting an experimental protocol in which the uncoupler titration is performed sequentially on a single sample of chromatophores. From a study of the kinetics of ATP synthesis during prolonged illumination (5–10 min) we shall argue that in order to avoid or compensate for slow changes in the rate, it is necessary to carry out the experiments, one at a time, with a single concentration of uncoupler for each chromatophore sample.

Fig.2 shows a glass electrode recording of chromatophore photophosphorylation in the presence of a limiting concentration of oligomycin. The rate of ATP synthesis was not constant but declined progressively during the course of the illumination period. Authors in [11] described a similar effect in chromatophores from *Rhodospirillum rubrum*. These workers found that the development of the inhibition was independent of the 'contact time' between the chromatophores and the oligomycin and our findings were completely in agreement with this – the development of inhibition during illumination was similar whether the chromatophores were pre-incubated with the oligomycin for 5 min or for 20 min before switching on the light.

These observations help to explain the discrepancy between fig.1A and B. We suggest that in the presence of oligomycin, the increased potency of the FCCP in the sequential titration (fig.1A) was not real but arose partly from the time-dependent effect of the F_0 -inhibitor. When the experiments were carried out one at a time, with the rate of ATP synthesis measured a fixed time after the onset of illumination, this problem was eliminated and the FCCP potency was unaffected

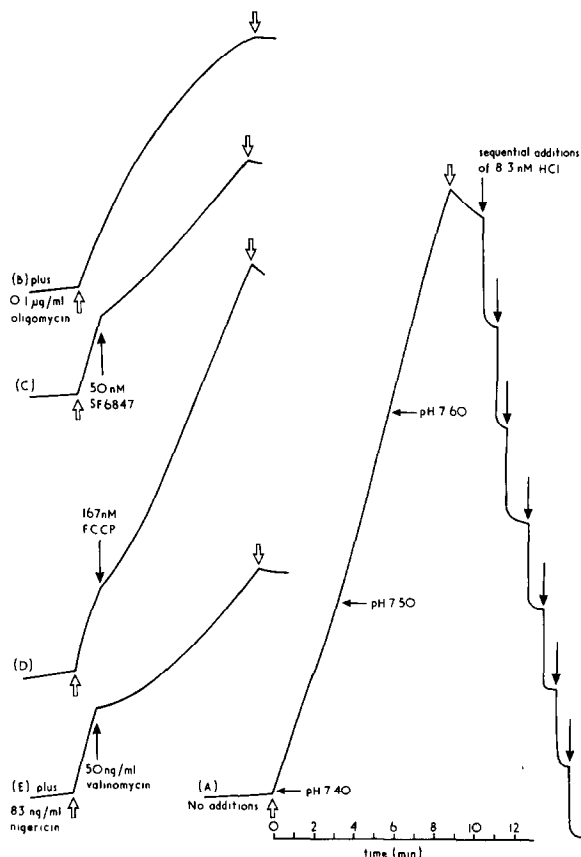


Fig.2. Kinetics of ATP synthesis during prolonged illumination. Experimental conditions as in fig.1 except that the vessel was made of glass, the final vol. was 6 ml, the medium was supplemented with 875 μ g of carbonic anhydrase, a stream of O_2 was directed over the surface of the medium and the bacteriochlorophyll concentration was 21.3 μ M. In each case the chromatophores were pre-incubated under these conditions for 20 min in complete darkness. The deviation from linearity in the buffering capacity is shown by the HCl additions at the end of trace (A). The time-scale marks and absolute pH values shown in (A) also apply to the other traces. In (B) the oligomycin pre-incubation was for 20 min. In (E) the nigericin preincubation was for 1 min. Other additions as indicated. The unlabelled, broad arrows show light on (up) and off (down).

by the presence of oligomycin.

Another problem that we encountered in following the procedure in [10] was that the addition of uncoupling agent also introduced non-linearity into the ATP synthesis rate. Fig.2D shows that the photophosphorylation rate was maximally in-

hibited by FCCP within a few seconds of the addition, but thereafter there was a partial recovery. A steady-state rate was not reached until at least 4 min after the addition. This effect was not peculiar to FCCP — it was also observed with the potent uncoupling agent SF6847 (fig.2C) and with a combination of the ionophores, valinomycin and nigericin in the presence of K^+ (fig.2E). The same effect was observed in a glass or in a perspex vessel, arguing against a slow binding to the walls of the chamber. It was similar when the carbonic anhydrase and the oxygen stream were omitted (cf. fig.2) or when the P^1, P^5 -bis(5'-adenosyl) pentaphosphate was omitted. Similar results were observed when Cl^- replaced acetate in the medium, at P_i concentrations between 1–10 mM, at medium pH values between 7.4 and 7.8, at diminished actinic light intensities, and at bacteriochlorophyll concentrations in the range 9–21 μM .

It may be noted that when small, incremental changes in uncoupler concentration are made in sequence with only 1–2 min between additions [4–6] then the non-linearity, which is seen clearly in fig.2C,D and E, may be easily obscured. However, the impact of this response on the uncoupler/ F_0 inhibitor titration is shown in fig.3. In this experiment venturicidin was used instead of oligomycin. This reagent did not lead to the pronounced time-dependent inhibition of ATP synthesis characteristic of oligomycin (see above). The data of fig.3A and B were taken from the same experiments. Two sets of experiments were carried out: in the presence and in the absence of venturicidin. In each set, a separate experiment was performed for each concentration of FCCP. The rates of ATP synthesis 1 min after and 4 min after the FCCP addition are shown in fig.3A and 3B, respectively. The apparently increased potency of the uncoupler in the presence of venturicidin was only observed when the rates were measured a short time after the uncoupler addition (fig.3A). When the assay time was sufficient to approach linear rates of photophosphorylation, this result was not obtained. Instead, it was found that the inhibitory effect of FCCP was similar in the presence and absence of venturicidin (fig.3B).

Table 1 shows the results of an extensive series of experiments in which the rate of ATP synthesis was measured in the presence of combinations of

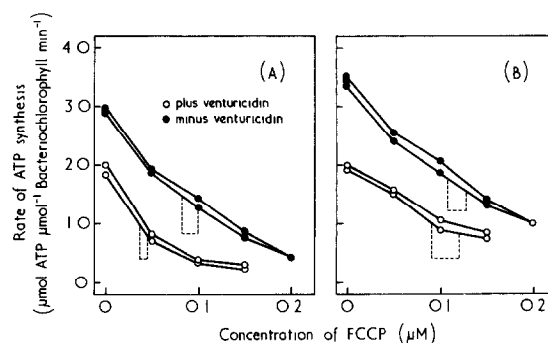


Fig. 3. The combined effect of venturicidin and FCCP on the rate of ATP synthesis. Experimental conditions as in fig.1B (i.e., a separate sample for each FCCP concentration) except the 0.005 $\mu g/ml$ venturicidin replaced the oligomycin and the bacteriochlorophyll concentration was 13.6 μM . In (A) the rate of ATP synthesis was measured 1 min after FCCP addition (2 min after the onset of illumination) and in (B) 4 min after the FCCP addition (5 min after the onset of illumination). The data in (A) and (B) were taken from the same sets of experiments. The light-induced pH change was kept within 0.1 unit.

the F_0 -inhibitor, venturicidin and the uncoupler SF6847. Four sets of experimental conditions (A–D) were chosen in which to examine the potency of SF6847, either in the presence or in the absence of venturicidin. With the experience gained from the results of fig.2, experiments were carried out, one at a time (in duplicate or triplicate) and ATP synthesis was measured from the glass electrode traces when the rate approached a constant value, in each case at a fixed time after the onset of illumination. The experimental conditions in part (A) of table 1 were similar to those described in fig.3B, except that SF6847 was used instead of FCCP. In (B) the uncoupling agent was added much later in the illumination period. In (C) the SF6847 (a smaller quantity on an uncoupler/bacteriochlorophyll basis) was added and in this case before switching on the light. In (D) the experiments were carried out in the presence of carbonic anhydrase under an atmosphere of CO_2 -free oxygen (see fig.2). In each of these cases, within the experimental error, the potency of the SF6847 was similar in the presence and absence of venturicidin. Even with sufficient venturicidin to reduce considerably the rate of ATP synthesis (as in D) the uncoupler potency was not increased.

Table 1

The combined effect of venturicidin and SF6847 on the rate of ATP synthesis

Experimental	Rate ATP synthesis ($\mu\text{mol} \cdot \mu\text{mol}$ bacteriochlorophyll $^{-1} \cdot \text{min}^{-1}$)		
	Minus SF6847	Plus SF6847	% Inhibition by SF6847
(A) Absence of venturicidin	5.93	3.14	50
	6.47	3.02	
	2.46	1.33	47
	2.44	1.26	
(B) Absence of venturicidin	6.84	1.92	72
	6.74	1.87	
	3.00	0.94	70
	2.81	0.80	
(C) Absence of venturicidin	5.85	3.80	31
	5.85	4.10	
	5.80	4.15	
	2.50	2.15	26
	2.85	2.15	
	3.00	1.85	
(D) Absence of venturicidin	4.70	2.85	42
	4.91	2.73	
	1.63	1.00	41
	1.76	1.01	

Experiments A, B and C were carried out essentially as in fig.1B (perspex vessel, in air with no carbonic anhydrase) and experiment D was carried out essentially as in fig.2 (glass vessel, under CO₂-free oxygen, plus carbonic anhydrase). Each rate shown in the table was calculated from separate experiments performed in either duplicate or triplicate. The percentage inhibition by SF6847 is calculated from averaged values. (A) Samples were pre-incubated in the dark for 20 min in either the presence or absence of 0.015 $\mu\text{g}/\text{ml}$ venturicidin. The SF6847 (30 nM), where shown, was added 1 min after the onset of illumination. The rate of ATP synthesis was measured 5 min after the onset of illumination. Bacteriochlorophyll, 18.7 μM . (B) Samples were pre-incubated in the dark for 5 min in either the presence or absence of 0.005 $\mu\text{g}/\text{ml}$ venturicidin. The SF6847 (20 nM), where shown, was added 5 min after the onset of illumination. The rate of ATP synthesis was measured 9 min after the onset of illumination. Bacteriochlorophyll, 10 μM . (C) Samples were pre-incubated in the dark for 5 min in either the presence or absence of 0.005 $\mu\text{g}/\text{ml}$ venturicidin or 10 nM SF6847. The rate of ATP synthesis was measured 6 min after the onset of illumination. Bacteriochlorophyll, 10 μM . (D) Samples were pre-incubated in the dark for 20 min in either the presence or absence of 0.02 $\mu\text{g}/\text{ml}$ venturicidin. The SF6847 (25 nM), where shown, was added 1 min after the onset of illumination. The rate of ATP synthesis was measured 4.5 min after the onset of illumination.

Bacteriochlorophyll, 21.3 μM

Contrastingly, in similar conditions but using a sequential titration on the same sample of chromatophores, it has been found [6] that the SF6847 concentration which reduced the rate by 56% in the absence of venturicidin was 85% effective in the presence of the inhibitor.

We believe that the apparent effect of F_o -inhibitors on the uncoupler potency in sequential titrations may arise artefactually from slow changes in the ATP synthesis rate similar to those seen in fig.2 but we do not know the origin of these slow changes in rate. They do not result from changes in the buffering capacity of the medium. Three effects may be considered in this context:

- (i) Calibration with aliquots of standard HCl showed that the buffering capacity ($\Delta H^+/\Delta pH$) decreased by about 15% in the range pH 7.4–7.5;
- (ii) Following a 5 min period of ATP synthesis at $4.45 \mu\text{mol} \cdot \mu\text{mol bacteriochlorophyll}^{-1} \cdot \text{min}^{-1}$ with $18.2 \mu\text{M}$ bacteriochlorophyll the buffering capacity measured at pH 7.4 was decreased by about 8%;
- (iii) From the data in [9] the stoichiometry of protons assimilated per ATP synthesized increases by about 2% in the range pH 7.4–7.5.

These three effects are in the wrong direction to account for the progressive decrease in rate seen in the presence of oligomycin and they are too small to explain the changes in rate following addition of uncoupler. They may however, have been responsible (at least partly) for the slight increase in rate that was routinely observed in the control samples (in the absence of uncoupler or inhibitor), after about the second minute of illumination (see fig.2A). This was not pursued in detail. Here, all the calculations of the ATP synthesis rate were based on calibration with standard HCl at the end of the experiment, across the entire pH excursion which had taken place during the illumination period. In fig.1 and 3 and in table 1A, B, and C the light-induced pH change was limited to less than 0.1 units. In fig.2 a high bacteriochlorophyll concentration and a prolonged illumination period were used to demonstrate the change in buffering capacity across a wider pH range. Similar condi-

tions were used in table 1D in order to improve the accuracy on the low rates in the presence of SF6847 at high venturicidin concentrations. Two other features in fig.2A are noted here without further discussion:

- (i) During the first minute of illumination the initially rapid rate of H^+ disappearance may be a direct consequence of light-induced proton translocation;
- (ii) The tailing off of the rate of H^+ disappearance after the eighth minute of illumination was probably the result of the approach to the maximum attainable value of ΔG_p – it can be calculated that about 70% of the ADP is phosphorylated by this time at the high chromatophore concentration used in the experiment.

4. DISCUSSION

The above results show that the conclusion in [4–6] that the effectiveness of uncoupling agents depends on the number of active F_o complexes, may be wrong. We believe that their finding, that the potency of uncoupling agents in chromatophores is increased in the presence of F_o inhibitors, arises from non-linearity in the ATP synthesis rates (fig.2). When measurements were taken in controlled conditions a fixed period from the onset of illumination and the approach to constant rates, the uncoupling agents decreased the phosphorylation rate by the same factor in the presence and absence of F_o inhibitor (fig.1B, 3B and table 1).

The results are consistent with any model of electron transport phosphorylation in which uncouplers act by dissipating the high energy state or intermediate prior to the block produced by F_o inhibitors. They do not discriminate between models of uncoupler action and they are certainly not inconsistent with the existence of a delocalised intermediate such as the proton motive force of the chemiosmotic hypothesis.

The origin of the slow changes in ATP synthesis rate during prolonged illumination is not known. We have taken precautions to check that artefacts of the glass electrode technique (used also in [4–6]) do not produce these effects, but this possibility

cannot be entirely eliminated. If further work on this interesting problem is to be pursued, then another method of measurement of ATP synthesis would be desirable. The existence of the slow rate changes in both KCl-containing and nigericin-containing media suggests that they do not arise from changes in the chromatophore ΔpH . Conceivably they are related to changes in the conformational state of the ATP synthase resulting from shifts in the level of chromatophore energisation (see [11]). These rate changes are however too slow (on a timescale of minutes) to play a role in the mechanism of electron transport phosphorylation.

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