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Energy coupling to ATP synthesis and pyridine nucleotide transhydrogenase in chromatophores from photosynthetic bacteria

A 'dual-consumer' test for localised interactions with electron transport components

N.P.J. Cotton and J.B. Jackson

Department of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, England

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The rate of ATP synthesis and the rate of pyridine nucleotide transhydrogenase were recorded in parallel experiments in steady-state conditions following the onset of illumination in chromatophores of *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata*). The transhydrogenase rate was more resistant than the ATP synthesis rate to inhibition by antimycin A and myxothiazol, the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and the combination of valinomycin with nigericin in the presence of K^+. The relationship between the transhydrogenase rate and the ATP synthesis rate was not influenced by the nature of the inhibitor. These data suggest that the ATP synthase and the transhydrogenase are differentially regulated by Δp but not by local interactions with components of the electron transport chain.

ATP synthesis; Transhydrogenase; Energy coupling; Photosynthetic bacteria; Protonmotive force; (*Rhodobacter capsulatus*)

1. INTRODUCTION

ATP synthesis and pyridine nucleotide transhydrogenase are both driven by free enthalpy released during photosynthetic electron transport in chromatophores. Both reactions are inhibited by uncoupling agents and by agents which block the photosynthetic electron transport chain. Within the framework of the chemiosmotic hypothesis, the ATP synthase and the pyridine nucleotide transhydrogenase are viewed as consumers of the electrochemical proton gradient (Δp) across the chromatophore membrane [1]. In general the chemiosmotic hypothesis has provided a satisfactory working description for energy coupling processes in chromatophores and in other membrane systems like thylakoids and mitochondria. However, there is a body of evidence (see [2,3]) to suggest that the description is incomplete. One proposed modification, not a radical one, is that the activities of the Δp consumers are regulated by chemical signals transmitted from components in the electron transport chain [4–6]. Another suggestion, originally from Padan and Rottenberg [7], is that there are other pathways of energy coupling such as intra-membrane proton transfers in parallel with the bulk phase chemiosmotic proton circuits [3,8–11].

The methods used for the estimation of Δp continue to excite controversy: inaccuracies in these methods have been blamed for some disagreements in interpretation. In this report we have employed an approach (see [12]) to test for local interactions

Correspondence address: J.B. Jackson, Department of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, England

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Δp, proton electrochemical gradient; $\Delta\psi$, membrane potential; J_t, rate of pyridine nucleotide transhydrogenase; J_p, rate of ATP synthesis

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in the energy transduction process which relies only on the uncontentious measurement of the rate of ATP synthesis (J_p) and the rate of pyridine nucleotide transhydrogenase (J_t) in illuminated chromatophore suspensions. Regardless of whether titrations were performed with uncoupler or ionophores, with myxothiazol or antimycin A the relationship between J_p and J_t was similar. The results do not reveal any differential regulatory influence of electron transport components on the ATP synthase and the transhydrogenase. They show that if intra-membrane proton transfer does play a significant role in energy coupling then ATP synthase- and transhydrogenase-coupling units are degenerate in their sensitivity to uncoupler and electron transport inhibitors. The results are consistent with, but do not prove, the simple form of the chemiosmotic hypothesis.

2. MATERIALS AND METHODS

Phototrophic growth of *Rhodobacter capsulatus* and the preparation of chromatophores were as described [13,14]. The rates of ATP synthesis and pyridine nucleotide transhydrogenase were assayed aerobically in a medium containing 10% sucrose, 100 mM KCl, 10 mM $MgCl_2$, 1 mM K^+-phosphate, 0.5 mM Na^+-succinate, 100 μM cresol red, 1 mM ADP, 66 μM thio-NADP, 133 μM NADH, 1 μM nigericin, 1 μg/ml rotenone and chromatophores to give a bacteriochlorophyll concentration of 10 μM. The samples for the transhydrogenase assay contained, in addition, 50 mM Tricine, pH 7.6: the rate of transhydrogenase was estimated from the absorbance change due to the formation of thio-NADPH at 395 nm using an extinction coefficient of 11.3 mM^{-1} [15,16]. The samples for the ATP synthesis assay were adjusted to pH 7.6 with dilute KOH: the rate of ATP synthesis was estimated from the absorbance change of cresol red at 572 nm resulting from the scalar uptake of H^+ accompanying the phosphorylation reaction [17]. All samples were pre-incubated in the dark at 30°C for 10 min before assay. The uncoupler, ionophores and inhibitors were added as methanolic solutions at the start of the pre-incubation period. The rates were recorded with a laboratory-built single-beam spectrophotometer after 10 s of saturating illumination from a collimated 250 W quartz-halogen lamp passed through 10 cm water and one layer of Wratten 88A gelatin filter. Separate samples were used for each measurement.

3. RESULTS

Fig.1 shows the inhibitory effects of FCCP, myxothiazol and antimycin and of valinomycin in the presence of nigericin on J_t and J_p measured in parallel experiments. All reagents had a more pronounced effect on J_p than on J_t. Inhibition of both

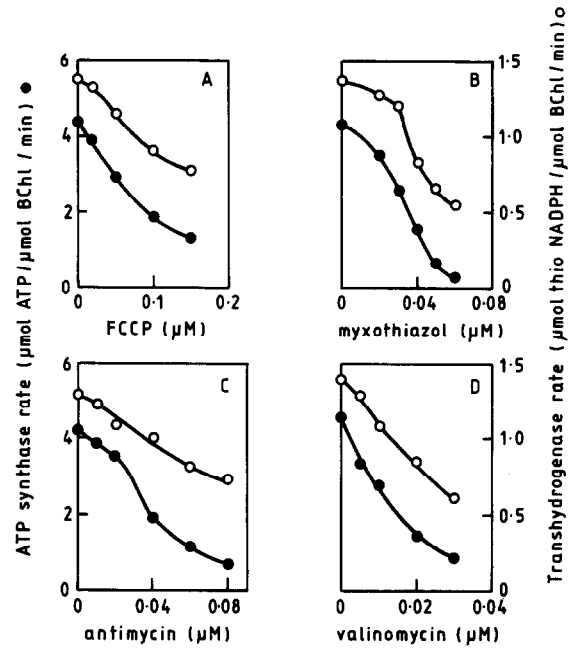

Fig.1. Inhibition of J_p and J_t by FCCP, myxothiazol, antimycin and valinomycin (in the presence of nigericin). See section 2. The experiments in A and B were performed on the same batch of chromatophores on the same day. Experiments in C were performed on the same batch of chromatophores as in A and B but on the following day. Experiments in D were performed on a separate batch of chromatophores.

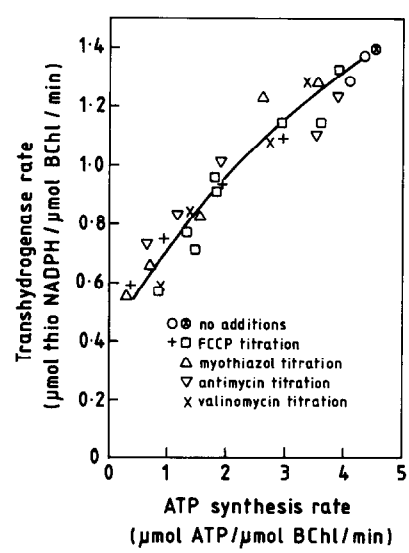

Fig.2. Relationship between J_p and J_t in titrations with FCCP, myxothiazol, antimycin and valinomycin (in the presence of nigericin). Includes data from two batches of chromatophores. (○, □, △, ▽) Batch 1, (⊗, +, ×) batch 2.

J_t and J_p by myxothiazol (fig.1B) and by antimycin (fig.1C) was sigmoidal. Antimycin was the least potent inhibitor although sensitivity to antimycin of energy-consuming reactions is affected by the experimental conditions [16]. Valinomycin in the presence of nigericin and K^+ was extremely potent, only 12.5 nM valinomycin being necessary to reduce J_p by 50% (fig.1D).

Fig.2 illustrates the relationship between J_t and J_p during titrations performed with FCCP, myxothiazol, antimycin and with valinomycin in the presence of nigericin. The figure is compiled from experiments performed on two batches of chromatophores. For each batch, a parallel titration of J_t and J_p was carried out with FCCP as a reference. The reproducibility was good. The crucial point in fig.2 is that irrespective of whether the titrations were performed with FCCP, antimycin, myxothiazol or valinomycin in the presence of nigericin, the relationship between J_t and J_p was similar. In uninhibited samples, J_p was routinely about 4-times the value of J_t. Through the course of each titration the ratio of J_t/J_p increased, reflecting the insensitivity of J_t to inhibition relative to J_p.

4. DISCUSSION

The objective of this work was to reveal information about the energy transduction process without having to rely on measurements of the protonmotive force. However, it is interesting first to compare the results of fig.1 with recent electrochromic measurements of $\Delta\psi$ using the simple predictions of chemiosmotic theory. Note that in the experiments described above the routine presence of nigericin ensured that $\Delta pH = 0$ so that $\Delta p = \Delta\psi$. Two features merit attention. (i) Myxothiazol is a more potent inhibitor (of both J_p and J_t) than antimycin A. This is consistent with the finding that although both are tight-binding inhibitors of the cytochrome b/c_1 complex, myxothiazol, an inhibitor of the quinol oxidase site, is more effective at reducing $\Delta\psi$ in continuous illumination than antimycin which inhibits at the quinone reductase site ([18] and see [19]). This observation was explained by a coincidence of two factors: the limited rate of electron transport through the b/c_1 complex in the presence of antimycin and the diodic dependence of the dissipative membrane ionic current upon $\Delta\psi$ [18]. (ii) The transhydrogenase rate was more resistant to inhibition (by all the reagents tested) than was the ATP synthesis rate (see also fig.2). This is consistent with the finding that J_p has a higher $\Delta\psi$ threshold than has J_t [16,17]. It was established many years ago in *Rhodospirillum rubrum* chromatophores that ATP synthesis has a higher light-intensity requirement than transhydrogenation [20] and that in submitochondrial particles ATP synthesis is more sensitive to inhibition by FCCP than transhydrogenase [21].

In a recent publication we described how inhibitor titrations of twin consumers of the protonmotive force can be used to probe for direct interactions with the protonmotive generators [12]. In that work we used the rate of alanine transport and the rate of ATP synthesis in intact cells of *R. capsulatus* as the two Δp consumers. An element of localised interaction was implied by these results but the interpretations were limited by the reliability of measurements performed with suspensions of intact bacteria.

In the present experiments with chromatophores the reliability of the methods of measuring J_p and J_t is assured. The analysis can be applied to the data in fig.1 as follows. The concentration of FCCP which gives (for example) 50% inhibition of J_p is 0.08 μM by interpolation (fig.1A). This concentration of FCCP depresses J_t by 29% in the parallel experiments (fig.1A). If, under the conditions of these experiments, J_p and J_t are determined by Δp alone, then the concentration of an inhibitor (for example, antimycin or myxothiazol) which leads to a 50% reduction in J_p should also lower J_t by that same proportion of 29%. If, on the other hand, the ATP synthase and the transhydrogenase are differentially regulated by direct interactions with the electron transport chain then, recognising that uncoupler and inhibitor will have different effects on the redox state of the electron transport components [22], we should expect a significant deviation from the 29% reduction in J_t. The actual values for the reduction in J_t were 25% for myxothiazol (fig.1B), 23% for antimycin (fig.1C) and 28% for valinomycin (fig.1D). These values are similar to the 29% for FCCP within the precision of the measurements.

A more extended analysis is presented in fig.2. If J_p and J_t are each uniquely determined by Δp

without the intervention of localised interactions, then the relationship between J_p and J_t in the steady state should not be influenced by the manner in which Δp is varied: the form of the curve relating J_p to J_t although it cannot be explicitly predicted by chemiosmotic theory should only reflect differences between the individual dependences of J_p and of J_t on Δp. The same curve should result from titrations with electron transport inhibitors which block the generation of Δp and with uncouplers and ionophores which accelerate the dissipation of Δp. It should not be affected by the difference in potency of the reagents used to modify Δp nor should it depend on the redox state of components in the electron transport chain. In the compilation of data shown in fig.2 the relationship between J_p and J_t was similar when FCCP, antimycin A, myxothiazol or valinomycin in the presence of nigericin were used as titrants. The predictions of the simple theory are therefore satisfied.

There are indications from experiments with chromatophores [4,5,23] and other membranes (see [2,3]) that the relationship between the rate of ATP synthesis, for example, and the value of Δp is modulated by other parameters: it has been speculated that cooperative, direct interactions between electron transport components and the ATP synthase might modify the enzyme activity. The data described above would only be consistent with such a view if in the conditions of our experiments both the ATP synthase and the pyridine nucleotide transhydrogenase were modified in precisely the same manner by those postulated regulatory interactions. However, it is not easy to understand why these two enzymes, which carry out different functions in bacterial metabolism, should be co-regulated in parallel in this way.

Recent descriptions of energy transduction have included the possibility that protons can be exchanged between the electron transport complexes and the energy consumers without coming into (complete) equilibrium with the bulk phase water on either side of the membrane [3,8–11]. A second mode of action of protonophorous uncouplers in addition to trans-membrane proton conduction is therefore envisaged as an accelerated 'slip' or discharge pathway in the local circuit. Our data do not eliminate this possibility but assuming that, for example, myxothiazol (or antimycin) is equally effective in blocking all cytochrome b/c_1 complexes, it is required that the slip pathway in the transhydrogenase has the same sensitivity to FCCP as that in the ATP synthase. Modified hypotheses usually have provision for limited delocalised coupling through the bulk phase proton circuits: the local circuits connect to the bulk phases via conductance pathways (e.g. [8]) or via proteolytic chemical reactions (e.g. [9]). Since the combination of valinomycin, nigericin and K^+ only affects bulk phase electrochemical potential gradients our data suggest that the proportion of localised/delocalised coupling must be similar for ATP synthesis and for transhydrogenation. Thus, the similar sensitivities of J_t and J_p to myxothiazol, antimycin, FCCP and valinomycin/nigericin do not reveal any differential localised interactions under conditions in which they might reasonably be expected.

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