Volume 225, number 1,2, 6–10 FEB 05345 December 1987

Concurrent measurements of the inhibition of ATP synthesis and alanine transport in intact cells of *Rhodobacter capsulatus*

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Received 2 October 1987

Myxothiazol, antimycin A and carboxylcyanide-*m*-chlorophenylhydrazone (CCCP) all had a more pronounced inhibitory effect on the rate of ATP synthesis than on the rate of alanine transport during illumination of intact cells of *Rhodobacter capsulatus*. CCCP had the most potent effect and antimycin A the least. The data suggest that the energy-input requirement for alanine transport is lower than that for ATP synthesis. A new test for direct interactions between the electron transport system and the ATP synthase and alanine transporter is applied to the data. Subject to reservations about the accuracy of rate measurement in intact bacterial cells and about the specificity of inhibition, the test reveals that the proton motive force is not the sole determinant of the rate of ATP synthesis and the rate of alanine transport.

ATP synthesis; Alanine transport; Antimycin A; Myxothiazol; CCCP; (*Rb. capsulatus*)

1. INTRODUCTION

In many bacteria, ATP synthase and some of the solute translocation systems across the cytoplasmic membrane are driven by free enthalpy released during electron transport. The prevalent view (but see [1]) is that the energy coupling reactions of these processes are mediated by a proton motive force (Δp). In its simplest form, the chemiosmotic hypothesis predicts that a reduction in the supply of energy to the bacteria will result in a decrease in the proton motive force and a consequent drop in the rate of solute translocation and ATP synthesis.

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Abbreviations: CCCP, carboxylcyanide-*m*-chlorophenylhydrazone; J_{ala}, initial rate of alanine uptake; J_p, initial rate of ATP synthesis

These two rates need to drop in parallel; in fact differences between the rate versus Δp (the flux/force) relationships might well be expectd and they might be crucial to the survival of the organism. The first objective of the experiments described in this report was to investigate using electron transport inhibitors and uncoupling agents with intact cells of *Rhodobacter capsulatus* how the rates of alanine translocation and ATP synthesis are controlled by the supply of energy.

There have been some indications that the simplest form of the chemiosmotic hypothesis is not adequate to account for the observed relationships between the rate of ATP synthesis or the rate of solute translocation and the value of the proton motive force [1,2]. The matter is controversial and technical difficulties, particularly in the accurate measurement of Δp, have been discussed [3]. To circumvent these difficulties, procedures have been developed, the 'double inhibitor' titrations which do not require measurement of Δp and which are

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

designed to establish whether the energetic intermediate is 'delocalised' as expected by the chemiosmotic hypothesis or whether there is 'direct coupling' between the electron transport chain and the energy consumer. However, the conclusions which can be drawn from these experiments are also equivocal [4]. Here we employ another approach which does not require measurement of Δp and which could lead to minimal but unequivocal information about the existence of 'direct interactions' between energy generators and energy consumers. The thesis is that if the rate of a Δp-consuming process (e.g. ATP synthesis) is reduced to the same extent by a particular concentration of an electron transport inhibitor and a particular concentration of uncoupling agent, then those concentrations should have identical (albeit undefined) effects on the rates of another consumer of Δp (e.g. solute translocation). If, however, there are direct (but different) interactions between the Δp generator and the two Δp consumers then the correlation is not expected.

2. MATERIALS AND METHODS

Rb. capsulatus strain N22 was grown phototrophically in RCV medium [5] as described [6]. Cells were harvested and washed once at 4°C in 10 mM $KHPO_4$, pH 7.0, and used within several hours. Bacteriochlorophyll was extracted in 7:2 acetone/methanol and assayed as in [7].

The rate of light-induced ATP synthesis in anaerobic intact cell suspensions in fresh RCV growth medium was estimated from the initial rate of increase of the ATP content at the onset of illumination. Samples were quenched in perchloric acid/EDTA at 0, 2, 4, 6, 8 and 10 s as described [8] except that the bacteriochlorophyll concentration was 20 μM. ATP was assayed with firefly extract [9]. The rate of increase of the ATP content was approximately linear from 0 to 4 s of illumination. Assuming that the rate of consumption of ATP in this period was negligible, the rate of increase in the ATP level was taken as a direct measure of the rate of synthesis.

Alanine transport was estimated as described for intact cells of *Rb. sphaeroides* [1]. Anaerobic cell suspensions of *Rb. capsulatus* in fresh RCV medium at 20 μM bacteriochlorophyll were preilluminated for 60 s before addition of

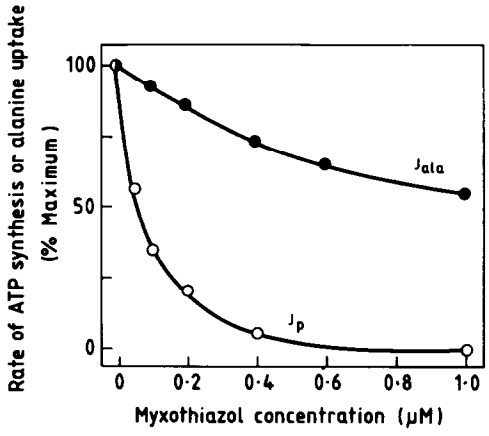

Fig.1. Dependencies of J_{ala} and J_p on myxothiazol concentration in intact cells of *Rb. capsulatus*. The data were collected as described in section 2. The rates of both alanine uptake and ATP synthesis were normalised to 100% as shown in the figure.

[^{14}C]alanine to a final concentration of 50 μM. Alanine uptake, assayed at 30 s intervals, was linear for at least 3 min.

Inhibitors and uncoupling agents were added as methanolic solutions before the dark anaerobic preincubation period (40 min for both the transport and the ATP synthesis assays).

3. RESULTS

The effects on the rates of alanine uptake (J_{ala}) and ATP synthesis (J_p) of myxothiazol, antimycin

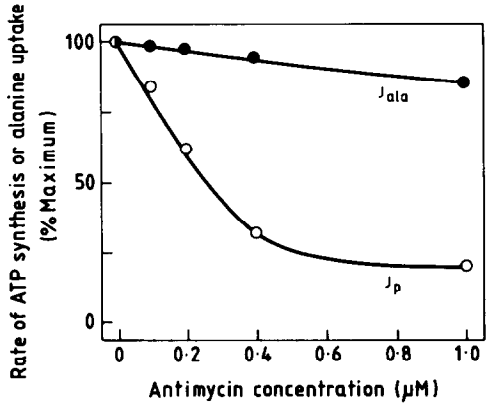

Fig.2. Dependencies of J_{ala} and J_p on antimycin A concentration in intact cells of *Rb. capsulatus*. Other details as for fig.1.

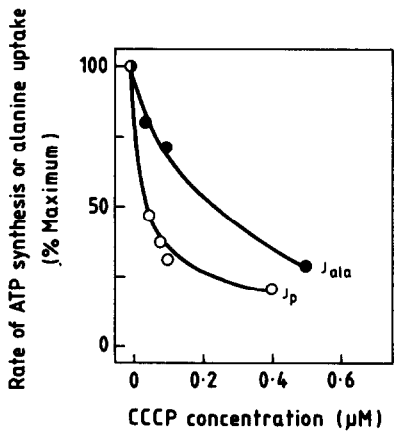

Fig.3. Dependencies of J_{ala} and J_p on CCCP concentration in intact cells of *Rb. capsulatus*. Other details as for fig.1.

A and CCCP are shown in figs 1–3, respectively. The measurements of J_{ala} and J_p were carried out under similar but not identical conditions (see section 2): the assay procedures demand that J_p is measured at the onset of illumination and that J_{ala} is measured after 1 min of preillumination. In each of figs 1–3 it is clear that J_{ala} is less sensitive to inhibition than is J_p. At very low concentrations, CCCP was the most potent inhibitor of both processes. Antimycin A was the least potent inhibitor.

4. DISCUSSION

The first conclusion to be drawn from the above data is that the energy requirement for alanine transport is lower than that for ATP synthesis: each of the three reagents used to interrupt the bioenergetic apparatus of the cytoplasmic membrane had a more pronounced inhibitory effect on ATP synthesis than on alanine transport. Evidently, alanine transport can be driven by lower levels of energy input than can ATP synthesis. This could be for largely thermodynamic or for kinetic reasons. For example it might be that the chemical affinity of the alanine transport is lower (has a less negative ΔG) than that of the ATPase reaction at the beginning of the period of measurement. Perhaps more likely, is the possibility that the alanine transporter and/or the ATP synthase are kinetically regulated, for example by the value of the proton motive force. Irrespective of the controlling parameter there is an important consequence for the physiology of the organism: in these cells of *Rb. capsulatus* there is evidently a priority for solute translocation at low rates of energy input to the cells. It is clear that there are instances in figs 1–3 where the rate of ATP synthesis has been reduced to almost zero but where the alanine transporter is operating at rates in excess of 50% of the maximum.

The question arises as to whether the data in figs 1–3 are consistent with the view that a single delocalised product of electron transport (such as Δp) has a unique effect on the rate of alanine translocation and on the rate of ATP synthesis. If Δp is the sole determinant of (say) ATP synthesis, then in two separate experiments in which the same J_p is recorded in the presence of two different inhibitors (or an inhibitor and an uncoupler), we can predict that the values of Δp must also have been the same in those two experiments. Consequently, those two concentrations of inhibitors should lead to the same value of Δp in experiments to measure alanine transport and therefore (if Δp is the sole determinant of the rate) to identical values of J_{ala}. The results of this test from the data of figs 1–3 are summarised in table 1.

The experiments with antimycin A and myxothiazol are in good agreement with the simple prediction. Antimycin A is an inhibitor of the quinol reductase site [10] in the cytochrome b/c_1 complex and myxothiazol of the quinol oxidase site [11]. Although the potency of the two inhibitors was different they had equivalent effects on J_p and J_{ala} (compare the last two columns of the top two rows in table 1). The difference in the potency is consistent with the relative effects of these inhibitors on membrane potential as measured by electrochromism [12].

The comparison of data with myxothiazol and CCCP are not consistent with the simple model. The bottom two rows of table 1 show that myxothiazol concentrations and CCCP concentrations which had similar effects on J_p had quite different effects on J_{ala}. CCCP (relative to myxothiazol) was more effective in reducing J_{ala} than it was (relative to myxothiazol) in reducing J_p. If the experimental precision can be accepted (see below) then this suggests that the inhibitory effects of these reagents are not a sole consequence of their effect on Δp. It suggests that J_p and J_{ala} are influenced by other

Table 1

A unique dependence of J_p and J_{ala} on Δp? Predictions and measurements

Inhibition of J_p (%)	Concentration of modifier I required for that inhibition of J_p	Concentration of modifier II required for that inhibition of J_p	Inhibition of J_{ala} by that concentration of modifier I (%)	Predicted inhibition of J_{ala} by that concentration of modifier II (%)	Recorded inhibition of J_{ala} by that concentration of modifier II (%)
66	0.1 μM myxothiazol	0.39 μM antimycin	9	9	7
81	0.2 μM myxothiazol	1.0 μM antimycin	15	15	17
66	0.1 μM myxothiazol	0.088 μM CCCP	9	9	30
81	0.2 μM myxothiazol	0.3 μM CCCP	15	15	58

Data were collected from the experiments described in figs 1–3

parameters, such as chemical interactions between the electron transport chain and either the ATP synthase or the alanine transporter [13]. It is evident of course that uncouplers and electron transport inhibitors will probably have differential effects on the redox state of electron transport components and that this might be the source of the differential effect on J_{ala} and J_p.

It should be emphasised that the conclusions based on this method do not rely on the measurement of Δp across the membrane and, subject to the experimental limitations described below, the experiments provide independent evidence in support of the existence of complex regulatory interactions between the generators and the consumers of the proton motive force.

There are major difficulties in making measurements of J_{ala} and J_p under comparable conditions in intact bacterial cells and the above conclusions cannot be accepted without qualification. In the first place, ATP synthesis is measured at the onset of illumination whereas alanine transport is measured after a 1 min preillumination period: when alanine transport is recorded at the onset of illumination, the rate is not linear with time [14]. Moreover, there are technical difficulties in the measurement procedures, particularly those associated with J_p. (i) There is doubt as to whether we are measuring a true initial rate of increase in cellular ATP levels, undistorted by ATP consumption by metabolism; (ii) the approximately linear increase in ATP levels persists for only three sample collections (0, 2 and 4 s of illumination). The manual sampling technique that we employed, coupled with uncertainties about the speed of the cold perchloric acid quench, compound this problem. Finally there is concern about the specificity of the inhibitors used in the investigation. The protocol required prolonged incubation (40 min) with the inhibitors prior to the assays. Whereas myxothiazol and antimycin A were used at concentrations only slightly in excess of the photosynthetic reaction centre concentration (approx. 0.2 μM) and are likely to be fairly specific, there may be some problems with the uncoupling agent: it has been shown that concentrations of FCCP and S-13 as low as 0.5 μM (at comparable bacteriochlorophyll concentrations to those used above) do appear to have secondary effects on the respiratory activity of intact cells of *Rb. capsulatus*. In *Rb. sphaeroides* analogous experiments have been performed and reveal some differences between myxothiazol and antimycin A (unpublished and [13]). Differences in the potency of the two bc_1-complex-inhibitors in *Rb. sphaeroides* have also been reported by other authors [2].

In general this test for differential localised interactions in energy coupling may be of value in other bioenergetic membranes. Indeed there are systems (like chromatophores) in which rate measurements can be made with more confidence than in intact bacterial cells and where the test would take on more significance.

ACKNOWLEDGEMENTS

This work was supported by a NATO grant to J.B.J. and W.N.K. J.M.F. was the recipient of an SERC postgraduate studentship. W.C. was supported by The Netherlands Foundation for Chemical Research (SON) which is subsidized by

the Netherlands Organization for the Advancement of Pure Research (ZWO).

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