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Demonstration of ΔpH- and $\Delta\psi$-induced synthesis of inorganic pyrophosphate in chromatophores from *Rhodospirillum rubrum*

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It is possible to obtain synthesis of PP_i by artifical ion potentials in *Rhodospirillum rubrum* chromatophores. PP_i can be formed by K^+-diffusion gradients ($\Delta\psi$), H^+ gradients (ΔpH) or a combination of both. In contrast, ATP can only be synthesized by imposed $\Delta\psi$ or $\Delta\psi + \Delta$pH. For ATP formation there is also a threshold value of K^+ concentration below which synthesis of ATP is not possible. Such a threshold is not found for PP_i formation. Both PP_i and ATP syntheses are abolished by addition of FCCP or nigericin and only marginally affected by electron transport inhibitors. The synthesis of PP_i can be monitored for several minutes before it ceases, while ATP production stops within 30 s. As a result the maximal yield of PP_i is 200 nmol PP_i/μmol BChl, while that of ATP is no more than 25 nmol ATP/μmol BChl. The initial rates of syntheses were 0.50 μmol PP_i/μmol BChl per min and 2.0 μmol ATP/μmol per min, respectively. These rates are approx. 50 and 20% of the respective photophosphorylation rates under saturating illumination.

Acid-base jump; Bioluminescence; H^+-ATPase; H^+-Pyrophosphatase; K^+-diffusion potential; (*Rhodospirillum rubrum*)

1. INTRODUCTION

pH jumps and K^+-diffusion potentials have been used extensively as driving forces for ATP synthesis in order to confirm the validity of the chemiosmotic theory (proposed by Mitchell [1]) in a variety of organisms and organelles [2–7].

Leiser and Gromet-Elhanan [8] have reported that ATP can be formed in *Rhodospirillum rubrum* chromatophores under energization by an artificially imposed K^+-diffusion potential alone or together with a ΔpH. A pH jump alone only gave rise to very little formation of ATP.

Membrane-bound pyrophosphatase activity in *R. rubrum* chromatophores was first shown in 1964 [9] and has since been found in a number of organisms [10–14]. A method developed recently [15] for continuous monitoring of inorganic pyrophosphate (PP_i) facilitates the very close study of PP_i synthesis. This novel technique, developed in our laboratory, is based on the luciferin/luciferase enzyme system and is very rapid in response to increases in PP_i concentrations [15]. PP_i at 0.1 nM can be assayed, which makes this procedure by far the most sensitive continuous method for PP_i determination.

Using this method, we demonstrate here for the first time the formation of PP_i in *R. rubrum* chromatophores as a result of artificial ion gradients.

A preliminary account of this work has recently been presented as a short communication [16].

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2. MATERIALS AND METHODS

R. rubrum was grown anaerobically in light. The

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cells were grown, harvested and chromatophores prepared as in [17], with the exception that 0.2 M glycylglycine-NaOH (pH 7.4) was used to wash twice and resuspend the chromatophores. The bacteriochlorophyll (BChl) concentration was determined by using an in vivo extinction coefficient of 140 $(mM \cdot cm)^{-1}$ at 880 nm [18].

The continuous monitoring of PP_i was performed by the new and sensitive method introduced by Nyrén and Lundin [15]. The assays were carried out at 23°C and the emitted luminescence was monitored and recorded using an LKB-Wallac 1250 luminometer. Oligomycin was present to inhibit any possible formation of ATP. The chemicals needed were from the same sources as described in [15]. Standard additions of ATP were used for calibration to correlate PP_i formation with ATP synthesis.

ATP synthesis was followed by using the commercial, luciferin/luciferase based, ATP-monitoring kit from LKB-Wallac (Turku, Finland). The assay was calibrated by means of standard additions of ATP.

To generate a pH gradient, chromatophores (10 μl) were first equilibrated at pH 5.3 or 8.3 for 2 min, except where stated otherwise, in 90 μl medium containing 0.2 M glycylglycine. The pH was checked before and after each experiment. The lowest pH value used was 5.3, since the chromatophores aggregated and precipitated at more acidic pH values. After incubation, chromatophores, corresponding to 1–3 μM BChl were transferred to a more alkaline assay medium situated in a test tube in the luminometer. The assay medium (0.5 ml) contained 0.2 M glycylglycine (pH 8.3), 10 mM Na-P_i, 1 μM P^1,P^5-di(adenosine-5′)-pentaphosphate (an inhibitor of the competing adenylate kinase reaction). When ATP synthesis was monitored, 50 μl ATP-monitoring reagent and 50 μM ADP were included. If instead PP_i formation was studied, the following reagents were present: 5 μg oligomycin, 1 mM 1,4-dithioerythritol, 0.15 U ATP-sulphurylase (EC 2.7.7.4), 5 μM adenosine 5-phosphosulphate, 10 mM Mg acetate, 0.4 mg/ml polyvinylpyrrolidone (M_r 360000), 0.1% BSA, 50 μg D-luciferin, 4 μg L-luciferin and purified luciferase (EC 1.13.12.7) (for the amount of enzyme used, see [15]). The P_i used in PP_i synthesis experiments was subjected to previous incubation with yeast PPase to eliminate PP_i present.

To obtain a K^+-diffusion potential, 10 μM valinomycin and different concentrations of KCl were added to the assay medium within the luminometer. This concentration of valinomycin gave the highest yield of synthesized ATP.

In the experiments where inhibitors were tested, these were included in the assay medium. Controls of the assay system were performed upon addition of incubation buffer and inhibitors (no significant effects).

3. RESULTS AND DISCUSSION

When chromatophores, incubated at low pH, were transferred to the alkaline solution containing valinomycin and K^+, synthesis of PP_i or ATP commenced immediately. Fig.1 shows typical traces from these recordings. A clear difference in the respective time courses between ATP and PP_i syntheses can be seen. Whereas the ATP synthesis ceased within 30 s of incubation, PP_i synthesis continued for several minutes.

The yields of ATP and PP_i in these experiments were dependent on the time of incubation of the chromatophores in the acidic medium. 2 min incubation gave 10% lower yield than 5 min incubation (not shown). Despite this fact a 2 min incubation period was chosen in the following experiments.

In fig.2 the rate of ATP synthesis is shown. In one set of experiments, only a K^+ gradient was used ($\Delta pH = 0$), whereas in another set a pH gradient was superimposed ($\Delta pH = 3.0$). Clearly, a pH gradient of this size alone is insufficient to accomplish phosphorylation of ADP. A concentration of 4 mM K^+ in the assay medium failed to induce ATP synthesis. This has previously been found in chloroplasts [3,19–22] and photosynthetic bacteria [23–25]. The synthesis of ATP had to be monitored at high sensitivity, which gave noisy recordings and was the reason for the scattering of the points in fig.2. The time in which the ATP synthesis was completed was fairly constant in all experiments whereas the rate and amount of ATP production were a function of K^+ concentration.

The corresponding experiments for PP_i synthesis are shown in fig.3. No threshold was found

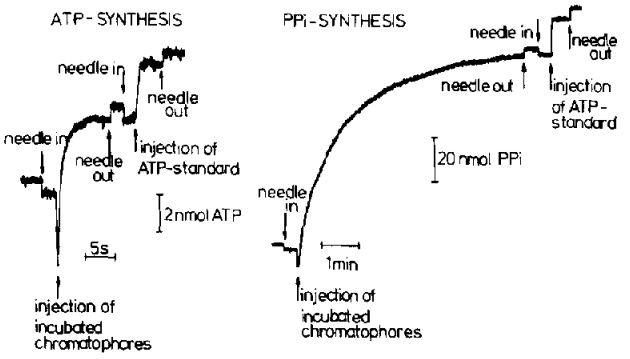

Fig.1. Typical traces from monitoring of ATP and PP_i synthesis. Chromatophores were incubated at pH 5.3 for 2 min. 2 μM BChl of equilibrated chromatophores was transferred to the assay medium (pH 8.3) containing valinomycin and K^+. The medium is described in section 2. Each experiment was calibrated with standard additions of ATP.

for the formation of PP_i by the H^+-PPase, in accordance with [26]. This may also be the reason for the higher yield of PP_i compared to ATP as the H^+-PPase might utilize the $\Delta\tilde{\mu}H^+$ to a greater extent, especially at low levels of the protonmotive force. It probably also reflects the lack of a $\Delta\psi$-dependent activation step for this enzyme [26].

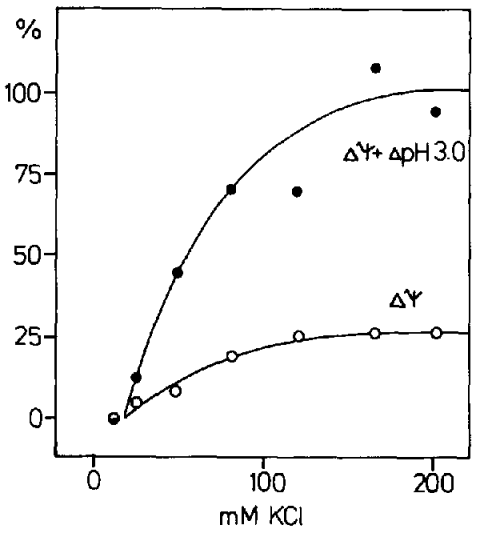

Fig.2. Rate of ATP synthesis as a function of K^+ concentration at two different ΔpH values. The pH in the assay medium was 8.3. 100% corresponds to 2 μmol ATP/μmol BChl per min. Experimental conditions were as described in section 2.

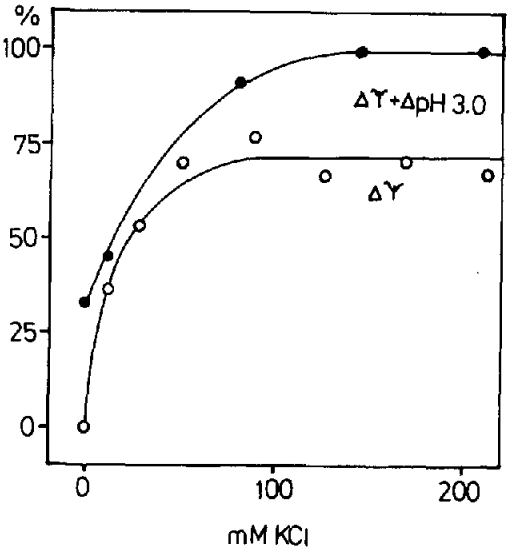

Fig.3. Rate of PP_i synthesis as a function of K^+ concentration at two different ΔpH values. The pH in the assay medium was 8.3. 100% corresponds to 0.5 μmol PP_i/μmol BChl per min. The procedure was as described above. Points are means of several measurements.

The responses of the ATP yield and the rate of PP_i formation to additions of various compounds, such as FCCP, nigericin and electron transport inhibitors, are listed in tables 1 and 2, respectively. Additions of FCCP and nigericin abolished synthesis. This is to be expected if phosphorylation is coupled to an ion gradient. The electron transport inhibitors did not affect the results to any considerable extent. These results support the idea that the formation of both ATP and PP_i is the result of imposed ion gradients implicating a mechanism of the chemiosmotic type.

The samples seem to contain contaminating amounts of P_i, since a slight extent of formation of ATP and PP_i can be seen when no phosphate is added (see tables 1,2).

The amounts of PP_i and ATP synthesized varied among different chromatophore preparations. Typical values were 25 nmol ATP/μmol BChl and 200 nmol PP_i/μmol BChl. The difference in yields of ATP and PP_i is due to the fact that the phosphorylation yielding PP_i continued for a much longer period than that of ATP. Notably, the actual rate of ATP synthesis was approx. 5-fold greater than the rate of formation of PP_i.

Table 1

The effect of omission or addition of different compounds in the assay medium on the amount of ATP synthesized

Additions/ omissions	$\Delta\psi$ (170 mM K^+) (%)	ΔpH 3.0 + $\Delta\psi$ (%)
None	100[a]	100[b]
− ADP	0	0
− P_i	8	18
+ Oligomycin (10 μg/ml)	0	0
+ Fluoride (10 mM)	102	119
+ Antimycin A (4 μM)	97	76
+ Myxothiazol (4 μM)	101	97
+ FCCP (2 μM)	0	0
+ Nigericin (4 μM)	0	0

[a] 100% corresponds to 10 nmol ATP/μmol BChl
[b] 100% corresponds to 25 nmol ATP/μmol BChl

The amounts are given here instead of the initial velocities, since the latter showed larger variances (fig.2). Experimental conditions were as described in section 2. Values are corrected for the effects of inhibitors on the assay system

Table 2

Effect of addition or omission of different compounds in the PP_i formation assays

Additions/ omissions	ΔpH 3.0 (%)	$\Delta\psi$ (170 mM K^+) (%)	ΔpH + $\Delta\psi$ (%)
None	100[a]	100[b]	100[c]
− P_i	10	16	26
− Oligomycin	113	101	95
+ Fluoride (10 mM)	0	0	0
+ Antimycin A (4 μM)	108	82	100
+ Myxothiazol (4 μM)	108	100	94
+ FCCP (2 μM)	0	0	0
+ Nigericin (4 μM)	n.d.	0	0

[a] 100% corresponds to 0.13 μmol PP_i/μmol BChl per min
[b] 100% corresponds to 0.30 μmol PP_i/μmol BChl per min
[c] 100% corresponds to 0.45 μmol PP_i/μmol BChl per min

Assay conditions are described in section 2. Values are corrected for the effects of the different compounds on the assay system

There are a number of possible interpretations for the difference between the two enzyme systems:
(i) The differences in the value of $\Delta G^{0\prime}$ of the two reactions, 4.0 kcal/mol for formation of PP_i at 1 mM free Mg^{2+} [27] and 7.3 kcal/mol for ATP formation [28].
(ii) Differences in the amounts of the two enzymes present in the chromatophore membranes.
(iii) Cessation of ATP synthesis when $\Delta\psi$ drops below a certain threshold value. This idea is supported by the fact that a concentration of 4 mM K^+ in the assay medium was insufficient to accomplish ATP production. Also, ΔpH alone did not give rise to any ATP synthesis. The idea that an activation step induced by a membrane potential is necessary has been put forward previously for the ATPase in different systems [3,19–25].
(iv) Less significant overall control exerted over the H^+-PPase than on the H^+-ATPase by $\Delta\tilde{\mu}H^+$. One possibility is that fewer protons are needed for synthesis of PP_i than of ATP.

Any one of these possibilities, or a combination, might explain our results.

This paper has demonstrated for the first time PP_i synthesis by artificial ion gradients. Work is in progress to establish further the characteristics of ATP and PP_i formation induced by such gradients.

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