ATP SYNTHESIS DRIVEN BY INORGANIC PYROPHOSPHATE IN RHODOSPIRILLUM RUBRUM CHROMATOPHORES*.

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Summary

Inorganic pyrophosphate was demonstrated to drive the synthesis of ATP from ADP and P_i in Rhodospirillum rubrum chromatophores. The reaction was inhibited by uncouplers and inhibitors of phosphorylation such as Cl-CCP, S-13, and oligomycin, thus illustrating the energy-linked characteristics of the reaction. Exchange reactions involving the direct transfer of phosphate from PP_i to ADP or ATP were very low in chromatophores and did not appear to contribute to the ATP synthesis. Fluoride inhibited the inorganic pyrophosphatase and $PP_i \rightleftharpoons P_i$ exchange of chromatophores but had no effect on ATP-linked reactions. The PP_i driven synthesis of ATP was inhibited by fluoride also, indicating that the membrane bound inorganic pyrophosphatase may be involved in the energy metabolism in the cell.

Introduction

Inorganic pyrophosphate is a product of photophosphorylation in R. rubrum chromatophores (1) and is an energy donor for several energy-linked reactions in R. rubrum chromatophores including cytochrome reduction (2,3) transhydrogenation (4,5), NAD^+ reduction (6) and the carotenoid shift (7). These observations indicate that pyrophosphate may serve an important role in energy conservation in the cell. The utilization of both ATP and PP_i apparently involves the formation of an energized-state or compound. The utilization

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of the energy of PP_i to form ATP is the subject of this report. In addition, further evidence is presented which indicates that the membrane bound inorganic pyrophosphatase or chromatophores (2) is involved in the energy conservation reactions involving PP_i.

Methods

Cultures of R. rubrum, S1, were grown in the synthetic medium of Cohen-Bazire et al. (8) and chromatophores were prepared as previously described (9). The methods for photophosphorylation and ATPase have been described (10). The reaction mixture for PP_iase contained 50 mM Tris, pH 8, 0.67 mM sodium pyrophosphate, 1 mM $MgCl_2$ and Bchl[1]. The liberation of P_i was determined as for ATPase. The reaction mixture for ATP$\rightleftharpoons{}^{32}P_i$ exchange contained 50 mM Tris, pH 8, 1 mM $MgCl_2$, 0.67 mM ATP, 3.3 mM $^{32}P_i$ and Bchl. The reaction mixture for $PP_i\rightleftharpoons{}^{32}P_i$ exchange contained 50 mM Tris, pH 8, 2.5 mM $MgCl_2$, 6.7 mM $^{32}P_i$, 0.67 mM PP_i and Bchl. Esterified $^{32}P_i$ was determined as for photophosphorylation. S-13 and Cl-CCP were gifts from Dr. P. C. Hamm of the Monsanto Company and Dr. P. G. Heytler of E. I. du Pont de Nemours Company.

Results

Time Course of PP_i-driven ATP Formation. The time course of ATP formation is illustrated in Figure 1. With 1 µmole PP_i the reaction essentially was complete in four minutes while with two µmoles PP_i this time was doubled. These chromatophores contain a very active pyrophosphatase and in separate experiments it was demonstrated that these times corresponded approximately to the exhaustion of

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[1]Abbreviations used are: Bchl, bacteriochlorophyll; PP_iase, pyrophosphatase, PP_i, sodium pyrophosphate, Cl-CCP, m-chlorocarbonyl cyanide phenylhydrazone; S-13, 5-chloro-3,t-butyl-2'-nitrosalicylanilide.

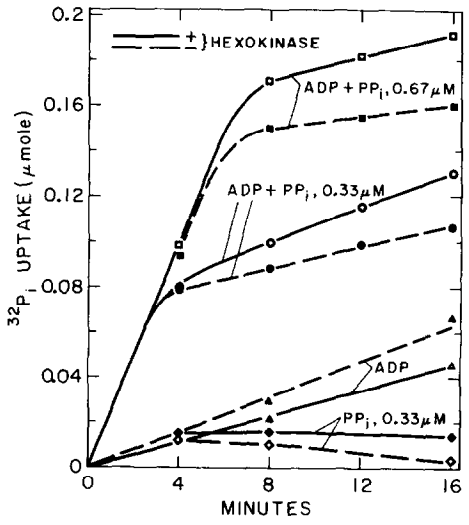

Fig. 1. Time course of the PP_i-driven ATP synthesis. The reaction mixture contained 50 mM Tris, pH 8, 1 mM $MgCl_2$, 3.3 mM $^{32}P_i$, 18 μg/ml Bchl and where indicated 0.33 mM ADP, 0.02 M glucose, 1 unit hexokinase and PP_i in 3 ml. The reaction was terminated by the addition of trichloroacetic acid to 5% and $^{32}P_i$ uptake was assayed as previously described (11).

the pyrophosphate. Addition of hexokinase to trap the ATP in the form of glucose-6-phosphate enhanced the reaction slightly. With no ADP added a very slow labeling of PP_i was observed. This reaction as described below, was sensitive to uncouplers and to fluoride but not to oligomycin and thus has the characteristics of a $PP_i \rightleftharpoons {}^{32}P_i$ exchange reaction. With no PP_i added, a slow labeling of ADP occurred which was not sensitive to uncouplers or oligomycin. These reactions will be documented further in a later publication.

Identification of Products by Thin-Layer Chromatography. Figure 2 is a radioautogram of a PEI-cellulose thin-layer sheet on which the products of the PP_i-driven ATP synthesis have been chromatographed. The products were adsorbed on activated charcoal, eluted with 40% acetone-0.1% NH_4OH in water, concentrated by lyophilization and applied to the thin-layer. With ADP alone, a small amount of both $AD^{32}P$ and $AT^{32}P$ was present. With PP_i alone, no product is visible since any

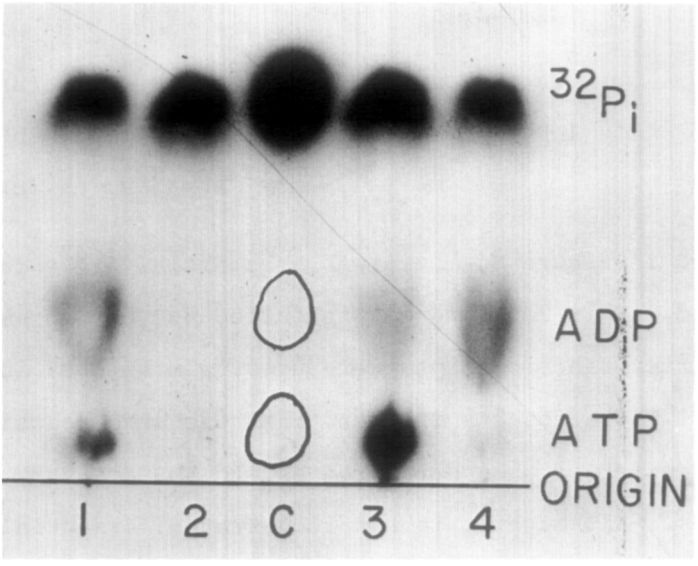

Fig. 2. Identification of the $^{32}P_i$-labeled products by radioautography of a thin-layer chromatogram. The products of the reaction were adsorbed on activated charcoal following trichloroacetic precipitation of the protein. The products were eluted from the charcoal with 40% acetone-0.1% NH_4OH in water, concentrated 10-fold and 20 µl was applied to a cellulose-PEI thin layer sheet in 2.5 µl aliquots. The chromatogram washed with anhydrous methanol, dried, developed for 5 min. in 0.25 M LiCl then transferred to 1.0 M LiCl and developed for 13 cm. The control (c) ADP and ATP spots which are outlined were visualized under UV light. The reaction mixture was the same as for Figure 1. (1) ADP alone; (2) PP_i alone; (3) ADP plus PP_i; (4) ADP, PP_i, glucose and hexokinase.

$^{32}PP_i$ formed would not be adsorbed on the charcoal. A small amount of $^{32}PP_i$ is formed however (see Figure 1) and this was identified by its sensitivity to yeast inorganic pyrophosphatase and thin-layer chromatography using a different solvent system to separate PP_i and ATP. When both PP_i and ADP were present the principle product was $AT^{32}P$ and this has disappeared when hexokinase was added. The glucose-6-phosphate was not adsorbed on the charcoal.

Origin of the $^{32}P_i$ in ATP. In order to determine the contribution of exchange reactions involving $^{32}PP_i$ and ADP following a $PP_i \rightleftharpoons {}^{32}P_i$ exchange, we measured ATP formation using $^{32}PP_i$. Some possible exchange reactions giving rise to labeled ADP and ATP are:

$$ADP + {}^{32}P\underline{{}^{32}P}_i \rightleftharpoons \begin{cases} AMP{}^{32}P + P\underline{{}^{32}P}_i & (a) \\ AMP{}^{32}P{}^{32}P + P_i & (b) \\ ADP{}^{32}P + {}^{32}P_i & (c) \end{cases}$$

As illustrated in Figure 3, there was substantial $AT^{32}P$ formed when $^{32}PP_i$ was used. This labeling was inhibited 89% by 3.3 mM "cold" P_i and if shorter reaction times were used, almost 100% inhibition was observed. These results indicate that exchange reactions involving the direct transfer of phosphate from PP_i to ADP are very low in chromatophores. Although it is not illustrated, essentially similar results were found when we measured exchange reactions between ATP and $^{32}PP_i$. Thus the labeling of ATP by $^{32}PP_i$ can be accounted for by $ATP \rightleftharpoons {}^{32}P_i$ exchange following hydrolysis of the $^{32}PP_i$ by the very active chromatophore inorganic pyrophosphatase.

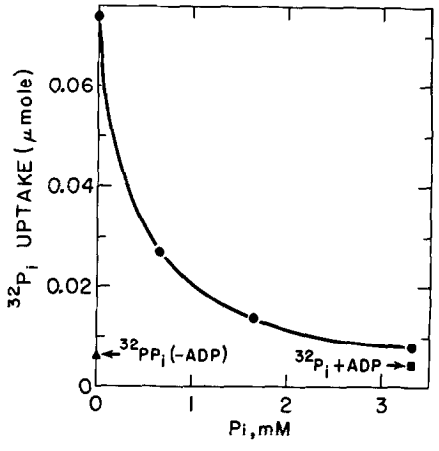

Fig. 3. Incorporation of ^{32}P from $^{32}PP_i$ into ATP and dilution by "cold" P_i. The reaction mixture contained 50 mM Tris, pH 8, 1 mM $MgCl_2$, 0.67 mM $^{32}PP_i$, 0.33 mM ADP, 0.02 M glucose, 1 unit hexokinase, 20 μg/ml Bchl and P_i as indicated. The reaction time was 5 min. at 25°C. The reaction was stopped by TCA to 5%. The unreacted $^{32}PP_i$ was hydrolyzed by boiling for 20 min. in 1 N HCl. Glucose-6-phosphate is not hydrolyzed under these conditions.

Inhibitors. The effect of various inhibitors on the reaction is shown in Table I. The uncouplers Cl-CCP and S-13 completely inhibited

Table I. Effect of Inhibitors

Additions	P_i Uptake (mμmoles)			
	PP_i	ADP	PP_i + ADP	
				% Inhibition[a]
None	14	22	90	
Cl-CCP, 3 μM	0	22	16	100
S-13, 1 μM	1	25	15	100
Antimycin a 0.33 μM	21	22	95	4
None	18	36	115	
Oligomycin, 1 μg/ml	17	29	51	92
NaCl, 10 mM	16	36	128	
NaF, 10 mM	6	38	68	61
No Mg^{++}	0	11	0	100

The reaction mixture contained 50 mM Tris, pH 8, 1 mM $MgCl_2$, 3.3 mM $^{32}P_i$, 0.02 M glucose, 1 unit hexokinase and 20 μg Bchl/ml in 3 ml and 0.33 mM ADP and 0.67 mM PP_i where indicated. Expt. 1 was run for 5 min. and Expt. 2 for 6 min. at 25°C. [a]Calculated using the PP_i alone plus the ADP alone as the endogenous.

Table II. Effect of Fluoride on Various Reactions

Activity	Rate (μmoles/mg Bchl/hr)	% Inhibition by 10 mM F^-
Inorganic Pyrophosphatase	145	84
$PP_i \rightleftharpoons {}^{32}P_i$ Exchange	5	90
ATPase	57	3
$ATP \rightleftharpoons {}^{32}P_i$ Exchange	25	12
Photophosphorylation	448	3

Cl^- at 10 mM had little effect on the reactions.

the PP_i-driven ATP formation, whereas, the electron transport inhibitor antimycin a, had no effect. Oligomycin also inhibited the reaction illustrating that the terminal enzymes involved in oxidative and photophosphorylation are involved in this ATP synthesis. The effect of these inhibitors is consistent with this being an energy-linked reaction.

The involvement of the inorganic pyrophosphatase in the ATP synthesis is indicated by the fluoride inhibition. Thus far, fluoride is the only selective inhibitor that we have observed for this enzyme. As illustrated in Table II, fluoride inhibited the pyrophosphatase and the $PP_i \rightleftharpoons {}^{32}P_i$ exchange activities of chromatophores but had no effect on the ATPase, $ATP \rightleftharpoons {}^{32}P_i$ exchange or photophosphorylation.

No inhibitor that we used had any effect on the slow ${}^{32}P_i$ uptake observed in the presence of ADP alone. The labeled product is $AD{}^{32}P$ as identified by thin-layer chromatography and we do not know the mechanism of this labeling. With PP_i alone, there is a slow labeling of PP_i and this is attributed to a $PP_i \rightleftharpoons {}^{32}P_i$ exchange with characteristics similar to the $ATP \rightleftharpoons {}^{32}P_i$ exchange reaction.

Discussion

We have described a new reaction, the PP_i-driven synthesis of ATP, which may be involved in energy-conservation reactions of the cell. Based on the fluoride inhibition, this reaction apparently involves the very active inorganic pyrophosphatase of the chromatophores. We have found little evidence of any direct exchange reaction between either ADP or ATP and PP_i. Thus we propose the following equation for the reaction where "~" represents an energized compound or state:

$$PP_i \xrightarrow{\text{pyrophosphatase}} \text{"}\sim\text{"} + 2P_i$$

$$ADP + P_i + \text{"}\sim\text{"} \longrightarrow ATP$$

The stoichiometry of the reaction that we have observed at best is about 12 moles PP_i hydrolyzed per mole ATP synthesized and is similar to that which we previously observed for the PP_i-driven transhydrogenase (5). This poor stoichiometry thus questions the importance of PP_i in energy conservation reactions in the cell, but at this stage we have made no attempts to prepare chromatophores specifically to enhance the PP_i-driven reactions. Thus it may be possible to couple PP_i hydrolysis to energy conserving reactions more efficiently in the future.

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