Inorganic pyrophosphate-driven ATP-synthesis in liposomes containing membrane-bound inorganic pyrophosphatase and F_0–F_1 complex from *Rhodospirillum rubrum*

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PP_i driven ATP synthesis has been reconstituted in a liposomal system containing the membrane-bound energy-linked PP_iase and coupling factor complex, both highly purified from *Rhodospirillum rubrum*. This energy converting model system was made by mixing both enzyme preparations with an aqueous suspension of sonicated soybean phospholipids and subjecting to a freeze–thaw procedure. In the presence of ADP, Mg^{2+}, P_i and PP_i the system catalyzed phosphorylation by up to 25 nmol ATP formed × mg protein^{-1} × min^{-1}, at 20°C, which was sensitive to uncouplers and inhibitors of phosphorylation such as oligomycin, efrapeptin and *N,N′*-dicyclohexylcarbodiimide.

Pyrophosphatase *Liposome* *Reconstitution* *ATPase* *ATP synthesis* *Proton pump*

1. INTRODUCTION

Inorganic pyrophosphatase is a product of photophosphorylation in *Rhodospirillum 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]. The utilization of PP_i apparently involves the formation of an energized state, also indicated by the fact: that PP_i-energized chromatophores of *R. rubrum* generate a membrane potential ($\Delta\psi$) and transmembrane pH difference (ΔpH) [8]; and that a partly purified membrane-bound PPase from *R. rubrum*, incorporated in a phospholipid membrane, acts as a PP_i-dependent electric generator [9].

Abbreviations: DCCD, *N,N′*-dicyclohexylcarbodiimide; DTE, dithioerythritol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IDP, imidodiphosphate; MDP, methylenediphosphonate

Enzymes: Membrane-bound inorganic pyrophosphatase (EC 3.6.1.1) and adenosine 5′-triphosphatase (EC 3.6.1.4)

We have developed a method for obtaining a highly purified membrane-bound PPase from *R. rubrum* chromatophore [10]. This pure membrane-bound PPase, incorporated into phospholipid vesicles in the presence of PP_i and Mg^{2+}, can function as a H^+-pump. PP_i can drive the synthesis of ATP from ADP and P_i in *R. rubrum* chromatophores [11].

Here, we have obtained phosphorylation of ADP to ATP at reasonable rates, by incorporation of the membrane-bound inorganic PPase (functioning as a proton pump) with the DCCD-sensitive ATPase complex from *R. rubrum* into pre-formed liposomes.

2. MATERIALS AND METHODS

Triton X-100, DTE, FCCP, IDP, MDP, valinomycin, oligomycin, nigericin, Trizma-base, cholic acid and asolectin (crude soybean phospholipids used after an acetone wash [12]) were purchased from Sigma. DCCD from Huka AG (Bucks). Luciferin/luciferase assay kit was from LKB. Efrapeptin D was a kind gift of Professor B. Beechey (Shell Research, Sittingbourne, Kent).

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2.1. *Preparation of chromatophores*

Rhodospirillum rubrum strain S1 was grown anaerobically in the medium described in [13]. After 40 h of growth, cells were harvested, washed and chromatophores were prepared as in [14].

2.2. *Preparation of the DCCD-sensitive ATPase complex*

The ATPase complex was prepared from *R. rubrum* chromatophores as in [25]. Isolated chromatophores were solubilized with final concentrations of 0.25% (w/v) sodium cholate plus 15 mM octylglucoside at ~8 mg protein/ml for 20 min at 4°C. The purification on the sucrose gradient was performed in the presence of 0.1% soybean phospholipids and 0.2% Triton X-100 [15]. The most active fraction for ATPase activity was quickly frozen and was stored at −70°C. Protein was 0.5 mg/ml.

2.3. *Preparation of the membrane-bound PPase*

The preparation of the membrane-bound PPase from *R. rubrum* chromatophores was performed as in [10]. The purified PPase was concentrated by ultrafiltration through an Amincon XM100A membrane and was kept frozen at −70°C. Protein was 0.1–0.2 mg/ml.

2.4. *Preparation of ATPase and PPase liposomes*

The freeze–thaw technique of [16] was used to reconstitute the PPase and ATPase into liposomes; 40 mg soybean phospholipids were supplemented with 1 ml medium, containing 10 mM Tris–HCl (pH 7.5), 0.5 mM DTE, 0.5 mM EDTA and 0.05% Na-cholate. The suspension was flushed with nitrogen and sonicated in a covered test tube for 20–25 min at 20°C in a bath-type sonicator model G 112 SP 1T, Lab. Supplies (Hicksville NY). The PPase (0.1 ml desalted preparation [10], containing 10–20 μg protein) and ATPase (0.1 ml, containing 50 μg protein) preparations were then added to 0.5 ml liposomes, sonicated for a few seconds and frozen at −70°C. After thawing at room temperature, the preparation was stored on ice with only minor losses in activity for at least 3 h.

2.5. *ATPase activity*

This was assayed in a reaction mixture containing 10 mM $MgCl_2$, 1 mM ATP, 1 ml 0.1 M Tris–HCl (pH 7.5) and H_2O in 2 ml total vol. The assay mixture was incubated at 30°C and the reaction was terminated after 10 min by addition of 1 ml 10% trichloroacetic acid. Blanks were run where trichloroacetic acid was added before the sample. Supernatant (1.6 ml, after centrifugation) was used for P_i determination. P_i was assayed colorimetrically as in [17]. The PPase activity was assayed as the ATPase activity with the exception that 0.5 mM PP_i was used instead of ATP.

2.6. *ATP-synthesis*

The amount of ATP synthesized was measured with the luciferin/luciferase technique, 15–50 μl of reconstituted ATPase, PPase liposomes were added directly to a test solution containing 200 μl luciferin/luciferase assay (LKB kit), 1 ml 0.2 M glycylglycine (pH 7.8), 20 μl 100 mM sodium phosphate, 10 μl 10 mM ADP and 50 μl 10 mM sodium pyrophosphate. The final concentration of $MgCl_2$ was 10 mM as the luciferin/luciferase assay contains a high [$MgCl_2$]. Addition of inhibitors is as indicated in the text and figures. The reaction was carried out at room temperature. The resulting luminescence was measured in an LKB luminometer 1250. The light output was calibrated by addition of a known amount of ATP.

Protein was determined according to the modified Lowry method [18].

3. RESULTS AND DISCUSSION

3.1. *PP_i-driven ATP formation*

The time course of ATP formation in liposomes is illustrated in fig.1. With 0.1 μmol PP_i the reaction was complete in 15 min. At least 50 nmol PP_i must be added to detect phosphorylation. This amount of PP_i might give the approx. K_m for PPase and, thereby, a low rate of PP_i hydrolysis.

No reaction was observed if only PP_i without liposomes or liposomes without PP_i was added to the ATP synthesis assay. ATP hydrolysis was observed with no added PP_i (fig.2). The hydrolyzed ATP originated from the ATP contamination of the ADP in the sample (0.5–1%). ATP hydrolysis could be abolished with oligomycin (fig.2). With ADP, PP_i and liposomes present, ATP was synthesized at 25 nmol.mg protein^{-1}.min^{-1}. This value can be compared with 23 μmol.mg Bchl^{-1}.h^{-1} for the chromatophore

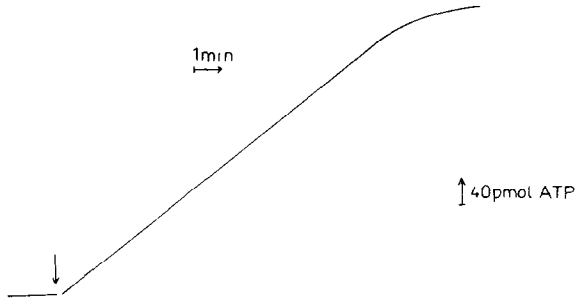

Fig.1. Time course of ATP synthesis in a suspension of PPase,ATPase liposomes; 25 μl liposomes (~0.07 mg protein/ml) were suspended in a medium containing 1 ml 0.2 M glycylglycine (pH 7.8), 0.2 ml luciferin/luciferase assay, 20 μl 100 mM sodium phosphate, 10 μl 10 mM ADP and 50 μl 10 mM sodium pyrophosphate; final [$MgCl_2$] was 10 mM; (⟶) 25 μl liposomes added. The resulting luminescence was measured in an LKB luminometer 1250. The light output was calibrated by addition of a known amount of ATP.

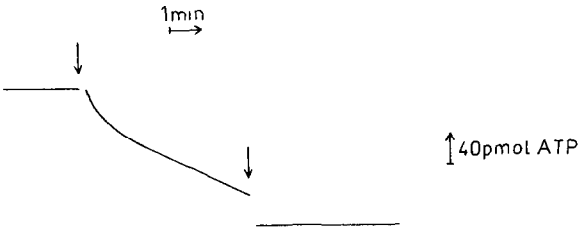

Fig.2. Time course of ATP hydrolysis in a suspension of PPase,ATPase liposomes; 50 μl liposomes were suspended in the same medium as in fig.1 except that no sodium pyrophosphate was added. At the first arrow 50 μl liposomes was added. At the second arrow 50 μl oligomycin (2 mg/ml) was added.

reaction [11]. If we assume that 1 mg Bchl corresponds to ~40 mg protein, the rate becomes 9.6 nmol.mg $protein^{-1}.min^{-1}$. As yet, no attempts have been made to optimize the conditions for the phosphorylation reaction; the reaction with chromatophores was performed at a higher temperature. Over the range of concentrations tested the phosphorylation rate was proportional to the amount of liposomes added (fig.3). The stoichiometry of ATP formation to PP_i hydrolysis was determined by two different methods.

(1) Adding 0.1 mol PP_i to the reaction mixture and detecting the amount of ATP synthesis: This method gave a value of 260 PP_i/ATP. However, the reaction rate leveled off at ~30 μM PP_i and a corrected value of 130 PP_i/ATP is obtained.

(2) Determining PP_i hydrolysis colorimetrically, and ATP synthesis by the luciferase technique: This was done under similar conditions, except that with the colorimetric assay no phosphate was added; the two rates were compared. This method gave 100 PP_i/ATP. About 10 PP_i/ATP was obtained for the chromatophore reaction in [10]. The difference can be explained by assuming that not all liposomes contain both enzymes, so some of the energy will be wasted.

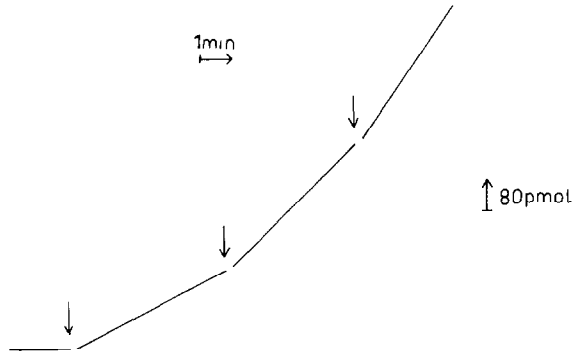

Fig.3. Time course of ATP synthesis as a function of the amount of liposomes added; (⟶) 25 μl liposomes added to the same medium as in fig.1 except that 50 μl 10 mM sodium pyrophosphate was present.

3.2. *Effects of energy-transfer inhibitors*

The energy-transfer inhibitor DCCD inhibits the membrane-bound PPase of chromatophores to ~75% [19]. The titration curves with added DCCP were similar for both PPase and ATPase activities of chromatophores. However, after purification and reconstitution the PPase, but not the ATPase, lost the DCCD-sensitivity [14]. So the 50% inhibition of phosphorylation in fig.4 might be due to the inhibition of the ATPase complex.

Oligomycin inhibits energy transfer in phosphorylation at a site closely connected with the final ATP-forming reaction. Oligomycin is an inhibitor of photophosphorylation to ATP [20] and of the membrane-bound ATPase in chromatophores [2]. However, it does not inhibit photosynthetic PP_i formation [1], nor does oligomycin inhibit membrane-bound PPase activity [2]. Fig.4 shows that oligomycin inhibits phosphorylation in the liposome system to 80%.

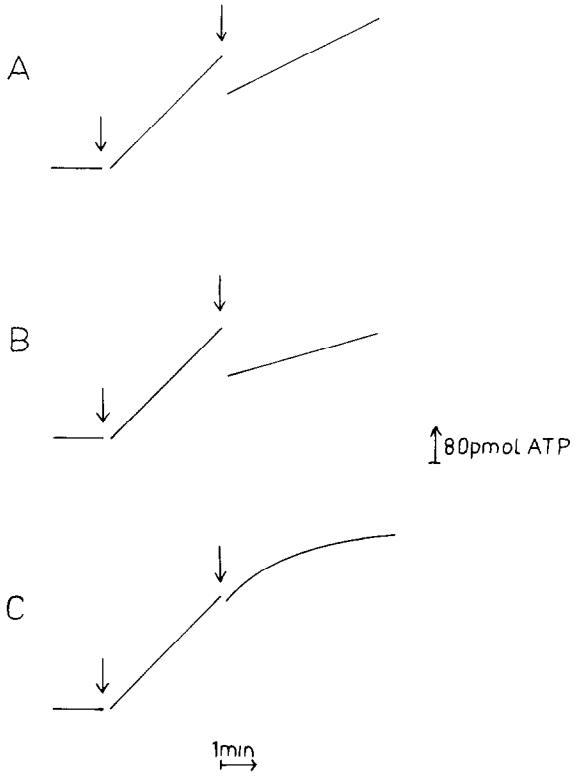

Fig.4. Time course of ATP synthesis in a suspension of PPase,ATPase liposomes; 50 μl liposomes were suspended in a medium described in fig.3. At the first arrow in A, the liposomes were added; at the second arrow 10 μl 10 mM DCCD was added. At the first arrow in B, liposomes were added, at the second arrow 50 μl oligomycin (2 mg/ml) was added. At the first arrow in C, liposomes were added; at the second arrow 4 μl efrapeptin (1 mg/ml) was added.

Efrapeptin, a small peptide (16 amino acids long) isolated from the fungus *Folypocladium inflatum* [21] inhibits phosphorylation and ATP hydrolysis by the coupling factor ATPases from mitochondria [22], chloroplasts [23] and *Escherichia coli* [21]. Efrapeptin also inhibits ATP hydrolysis and phosphorylation in *R. rubrum* [24], but has no effect on the adenylate kinase or energy-linked PPase [25]. Efrapeptin is a time-dependent inhibitor. Efrapeptin at 4 μg/ml inhibits the phosphorylation in the liposomal system to nearly 100% after 5 min (fig.4). Unlike oligomycin, efrapeptin does not inhibit luciferase at concentrations that inhibit the *R. rubrum* ATPase [24].

We infer from the above results that inhibition by DCCD, oligomycin and efrapeptin of liposomal phosphorylation is due to the effect on the ATPase complex and not on membrane-bound PPase.

3.3. *Effects of PPase inhibitors*

Fluoride is a selective inhibitor of the inorganic pyrophosphatase that has no effect on ATPase or photophosphorylation in *R. rubrum* chromatophores [26]. NaF at 10 mM inhibited phosphorylation in liposomes to 100% (fig.5). Methylene diphosphonate is an effective inhibitor of inorganic PPase while not affecting the ATP-linked reactions [11]. MDP at 0.5 mM inhibited phosphorylation to 50% and 0.5 mM IDP, a com-

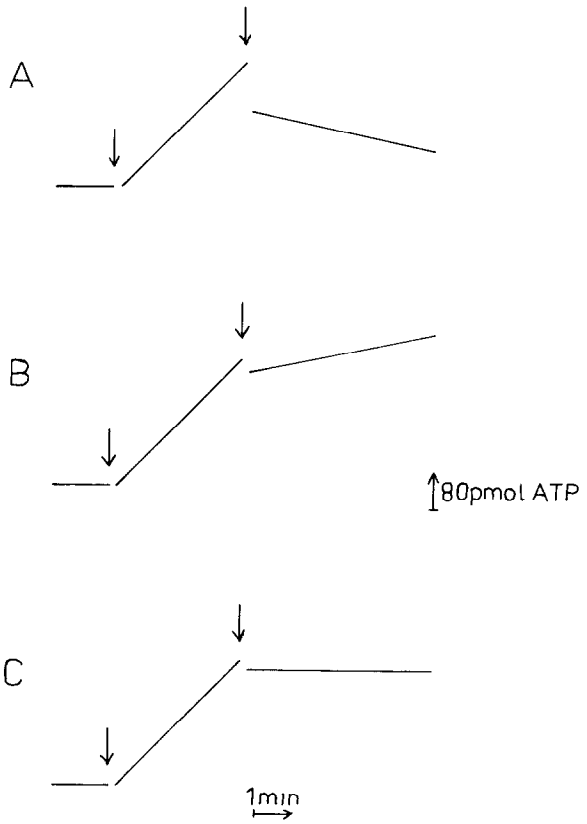

Fig.5. Time course of ATP synthesis in a suspension of PPase,ATPase liposomes; 50 μl liposomes were suspended in a medium described in fig.3. At the first arrow in A, the liposomes were added; at the second arrow 30 μl 0.4 NaF was added. At the first arrow in B, liposomes were added; at the second arrow 30 μl 20 mM MDP was added. At the first arrow in C, liposomes were added; at the second arrow 30 μl 20 mM IDP was added.

petitive inhibitor of PPase, decreased phosphorylation by 5-fold (fig.5).

3.4. *Effects of uncouplers and ionophores*

Valinomycin, which is a cyclic depsipeptide antibiotic, creates a specific K^+ permeability in membranes. The PP_i-driven ATP synthesis in chromatophores was inhibited to nearly 100% by a combination of valinomycin and NH_4Cl [11]. A combination of valinomycin and KCl inhibits the liposomal PP_i-driven ATP synthesis to 50%, whereas nigericin, an ionophore of the type that forms an uncharged complex with K^+ and transports this ion in exchange for H^+, inhibits completely (fig.6). The uncoupler FCCP inhibits phosphorylation to 100% (fig.6). This effect is expected if the ATP synthesis is driven by the proton gradient created by the PPase, since FCCP would prevent the formation of a pH gradient across the liposomal membrane.

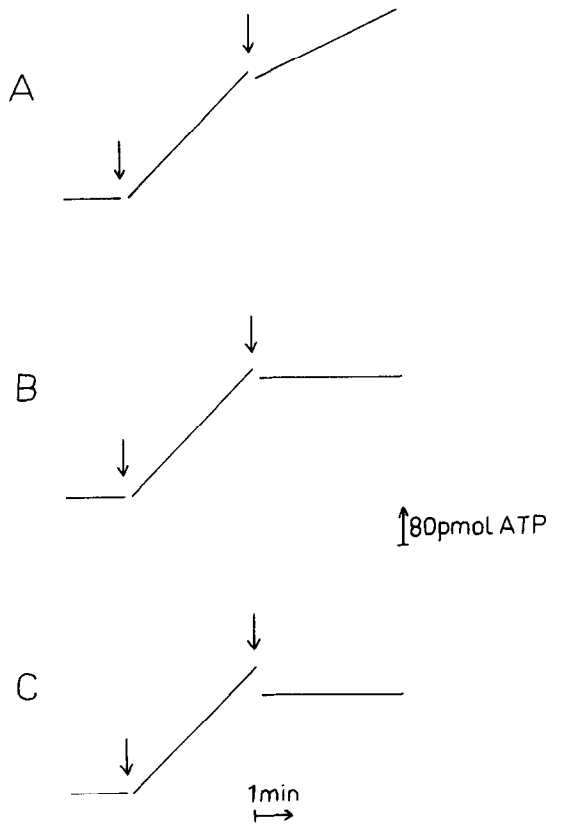

Fig.6. Time course of ATP synthesis in a suspension of ATPase,PPase liposomes; 50 μl liposomes (~0.07 mg protein/ml) were suspended in a medium containing 1 ml 0.2 M glycylglycine (pH 7.8), 0.2 ml luciferin/luciferase assay, 20 μl 100 mM sodium phosphate, 10 μl 10 mM ADP, 50 μl 10 mM sodium pyrophosphate and 20 μl 1 M KCl; final [$MgCl_2$] was 10 mM. At the first arrow in A, liposomes were added; at the second arrow 5 μl 1 mM valinomycin was added. At the first arrow in B, liposomes were added; at the second arrow 2 μl 10 mM nigericin was added. At the first arrow in C, liposomes were added; at the second arrow 3 μl 1 mM FCCP was added.

Table 1 gives a summary of the effects of different compounds tested on the liposomal phosphorylation. The ATPase and PPase activity in the liposomes, measured by the colorimetric method, under similar conditions as the experiments with the luciferase technique, are shown in table 2.

Taken in total, these inhibitor-studies illustrate the dependence of the PPase, the coupling factor ATPase and coupled liposomes for the phosphorylation reaction in the model liposomal system. Liposomes with only the PPase and the ATPase were also examined (not shown) and the result clearly showed the necessity of both enzymes for the phosphorylation reaction.

Purified energy-generating membrane components like bacteriorhodopsin [27] and photosystem I [28] have been reconstituted

Table 1

Effect of different compounds tested on the liposomal phosphorylation

Additions	Concentration	Phosphorylation rate in liposomes (% control)
DCCD	80 μM	50
Oligomycin	75 μg/ml	20
Efrapeptin	3 μg/ml	0
NaF	10 mM	0
MDP	0.5 mM	50
IDP	0.5 mM	20
Valinomycin (15 mM KCl)	4 μM	50
Nigericin (15 mM KCl)	15 μM	0
FCCP	3.0 μM	0

50 μl liposomes were assayed for ATP-synthesis as in section 2, with different inhibitors present

Table 2

The PPase and ATPase activity in liposomes

	ATPase and PPase activity (μmol PP_i or ATP hydrolyzed.mg^{-1}.min^{-1})
PPase activity	0.590
+ 3 μM FCCP	0.800
ATPase activity	0.150
+ 3 μM FCCP	0.690

20 μl and 140 μl liposomes were used for PPase and ATPase activity measurements, respectively. The measurements were done under the same conditions as ATP-synthesis measurements and the amount of P_i realized was measured colorimetrically (see section 2 for details)

together with purified CF_0–F_1 into liposomes. In these cases the membrane was energized by light-driven proton transport, and coupled ATP synthesis was observed. In a model system we have used a chemically-driven (PP_i) proton-pump for energization of the membrane and coupled the energy liberated by PP_i hydrolysis to ATP synthesis.

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