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Studies on photosynthetic inorganic pyrophosphate formation in *Rhodospirillum rubrum* chromatophores

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Photosynthetic formation of inorganic pyrophosphate (PP_i) in *Rhodospirillum rubrum* chromatophores has been studied utilizing a new and sensitive method for continuous monitoring of PP_i synthesis. Studies of the reaction kinetics under a variety of conditions, e.g., at different substrate concentrations and different electron-transport rates, have been performed. At very low light intensities the rate of PP_i synthesis is twice the rate of ATP synthesis. Antimycin A, at a concentration which strongly inhibited the photosynthetic ATP formation, inhibited the PP_i synthesis much less. Even at low rates of electron transport a significant rate of PP_i synthesis is obtained. The rate of photosynthetic ATP formation is stimulated up to 20% when PP_i synthesis is inhibited. It is shown that PP_i synthesis and ATP synthesis compete with each other. No inhibition of pyrophosphatase activity is observed at high carbonyl cyanide p-trifluoromethoxyhydrazone concentration while ATPase activity is strongly inhibited under the same conditions.

Introduction

Inorganic pyrophosphate is the product of photophosphorylation in *R. rubrum* in the absence of added nucleotides [1]. The proton translocating membrane-bound inorganic H^+-pyrophosphatase is the only known alternative to the well known coupling factor H^+-ATPase. The enzyme has been implicated in a number of different organisms (for a recent review, see Ref. 2). The proton pyrophosphatase, which catalyzes light induced synthesis of PP_i, has been solubilized and extensively purified from *R. rubrum* chromatophores [3]. The purified enzyme is very specific for PP_i and the activity of the purified enzyme is stimulated more than twice by the presence of cardiolipin [4]. Reconstitution studies have been performed on the purified enzyme and show that the proton pyrophosphatase retains both its catalytic and proton translocating functions after purification [5–7]. The synthesis of PP_i is inhibited by electron-transport inhibitors and uncouplers, but is insensitive to oligomycin [8]. Light-dependent synthesis of PP_i was studied by Guillory and Fisher [9] in order to investigate the rates of ATP and PP_i synthesis, respectively. They found that maximal rate of PP_i synthesis was obtained at a lower light intensity than that required for maximal ATP synthesis.

In the detailed study of electron-transport coupled phosphorylation of P_i to PP_i a sensitive and

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Abbreviations: P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; BChl, bacteriochlorophyll; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethyoxyhydrazone; DBMIB, dibromomethylisopropyl-*p*-benzoquinone (dibromothymoquinone); ATP-sulphurylase (ATP: sulphate adenylyltransferase; EC 2.7.7.4); firefly luciferase (EC 1.13.12.7) and yeast inorganic pyrophosphatase (EC 3.6.1.1).

continuous measurement of PP_i is of importance. (^{32}P)P_i has previously been used as a substrate in the study of photophosphorylation of P_i to PP_i. One problem with this technique is the difficulty of separating PP_i from P_i at low ratios of PP_i to P_i [10]. We have recently developed an extremely sensitive and rapid method for the monitoring of PP_i synthesis [11]. The assay, which utilizes the enzyme ATP sulphurylase and purified firefly luciferase is, in contrast to the radiometric method, continuous. Initial velocity kinetics of PP_i synthesis can thus be studied. In the present paper, the use of this new method for monitoring steady-state phosphorylation of P_i to PP_i and the results obtained are illustrated.

Materials and Methods

Trizma base, glycylglycine, 1,4-dithioerythritol, FCCP, oligomycin, valinomycin, nigericin, antimycin A, magnesium acetate, bovine serum albumin, adenosine 5′-phosphosulphate, P^1,P^5-di(adenosine-5′) pentaphosphate, yeast inorganic pyrophosphatase (EC 3.6.1.1), and ATP-sulphurylase (ATP:sulphate adenylyltransferase; EC 2.7.7.4) were purchased from Sigma (St. Louis, USA). Purified luciferase (EC 1.13.12.7), D-luciferin and L-luciferin were obtained from LKB Wallac (Turku, Finland). DCCD was obtained from Fluka AG (Bucks, Switzerland). The DBMIB was a gift from Dr. A. Trebst, which is gratefully acknowledged. Other materials were reagent grade and obtained from commercial sources.

Preparation of chromatophores. R. rubrum (strain S1) was grown anaerobically and prepared as in Ref. 6. BChl was determined using the in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm as reported by Clayton [12].

Measurement of photophosphorylation of P_i to PP_i. Continuous monitoring of PP_i synthesis was measured as described earlier [11]. Continuous illumination for 10 s to 1 min was obtained from a 20 W halogen lamp, with light passing through double layers of Wratten 88A gelatin filter which only transmits light above 720 nm. Light intensity was adjusted with a rheostat and measured with an YSI-Kettering Model 65A Radiometer (Yellow Springs Instrument Co., OH). All reactions were carried out at room temperature. The standard assay volume was 1 ml and contained the following components: 0.1 M glycylglycine (pH 7.75), 2 mM EDTA, 10 mM $Mg(Ac)_2$, 0.1% bovine serum albumin, 1 mM 1,4-dithioerythritol, 0.1 mg D-luciferin, 8 μg L-luciferin, 5 μM adenosine 5′-phosphosulphate, 0.3 Units ATP-sulphurylase, 10 mM NaPi, 1 μM P^1,P^5-di(adenosine-5′) pentaphosphate, 10 μg oligomycin, purified luciferase (for the amount, see Ref. 11). Variation of BChl and phosphate concentrations are given in the appropriate tables and figures. Phosphate solutions were incubated with yeast inorganic pyrophosphatase in order to remove endogenous PP_i (for details see, Ref. 11). Internal calibration was obtained by the addition of a PP_i or ATP standard to each assay.

Measurement of photophosphorylation of ADP to ATP. The assay was performed as for PP_i determination with the exception that the standard assay volume contained 50 μM ADP. Oligomycin, adenosine-5′-phosphosulphate and ATP-sulphurylase were omitted.

Assay of pyrophosphatase activity. The inorganic pyrophosphatase activity was assayed in a reaction mixture containing 1.0 mM $Mg(AC)_2$, 0.5 mM PP_i, 1 ml 0.1 M glycylglycine (pH 7.75), 0.1 mM sodium succinate, chromatophores corresponding to 0.38 μM BChl, and H_2O, in a total volume of 2 ml. The assay mixture was incubated at room temperature and the reaction was terminated after 10 min by addition of 0.4 ml 30% trichloroacetic acid and cooled in an ice bath. In the blanks, trichloroacetic acid was added before the sample. P_i was assayed colorimetrically, using a modification of the method of Rathbun and Betlach [13]. The cooled reaction mixture was quickly mixed with 0.5 ml of an acetate-formaldehyde mixture (1 part of a 1 : 1 mixture of 7 M NaAc and 7 M HAc mixed with 0.24 parts of 37% formaldehyde). The molybdenum blue color was developed by addition of 0.1 ml 2% ammonium molybdate and, within 15–20 s, of 0.2 ml 6.75 mM stannous chloride to the test tube down the opposite wall. The tube contents were rapidly mixed and allowed to stand at room temperature. The absorbance was measured at 735 nm, 25 min after addition of the molybdate.

Assay of ATPase activity. The assay was performed as for inorganic pyrophosphatase activity

determination with the exception that 0.5 mM ATP was used instead of 0.5 mM PP_i and that the BChl concentration was 1.5 μM.

Results and Discussion

Fig. 1 illustrates the rate of light driven PP_i synthesis in chromatophores as a function of the P_i concentration. The substrate saturation curve is S-shaped (see insert in Fig. 1), which is to be expected if a two-substrate reaction is proceeding with two identical molecules:

$$v = \frac{V_{max}[P_i]^2}{[P_i]^2 + K_1[P_i] + K_2} \quad (1)$$

The S-shaped curve may also simply be a consequence of the linked assay used here. Half maximal rate of PP_i synthesis was obtained with 0.4 mM P_i. The effect of free magnesium and MgP_i on the rate of PP_i synthesis was not considered in this experiment. The optimal Mg^{2+} concentration and pH have been determined by Guillory and Fisher [9]. Although the conditions used by us were not optimal for PP_i synthesis as compared to the results of those workers, the decrease of PP_i synthesis was less than 10%. However, the assay system was optimal with respect to the luciferase system [11]. In subsequent experiments 10 mM P_i was used. In order to eliminate ATP synthesis or hydrolysis by the H^+-ATPase, oligomycin was included in the assay system. Similarly, P^1,P^5-di-(adenosine-5′) pentaphosphate was included to avoid ATP synthesis by the adenylate kinase activity. Variations in luciferase and ATP-sulphurylase activity due to sample composition or reagent inactivation can easily be detected and compensated for by internal calibration with a ATP or PP_i standard in each single assay.

In the following experiments we studied the effect of electron transport rate on the rates of PP_i and ATP synthesis, respectively. Photosynthetic electron flow was reduced either by reducing the intensity of the exciting light thereby reducing the rate of the electron flow, or by inhibition of the ubiquinol/cytochrome b-c_1 complex with antimycin A and DBMIB. At saturating light intensity the maximal rate of PP_i synthesis is 10–15% of the maximal rate of ATP synthesis (results not shown). The light saturation curve for PP_i synthesis as compared with the ATP synthesis was studied (not shown) and the results confirmed those of Guillory and Fisher [9]; K_m values for light being 1 and 12 W/m^2 for the PP_i synthesis and the ATP synthesis, respectively. The influence of light intensity on the synthesis of PP_i is similar to that on the transhydrogenase activity [14]. When the actual rates of the reactions are compared on a molar basis (see Fig. 2) at low light intensities (10 mM NaF was present when ATP synthesis was

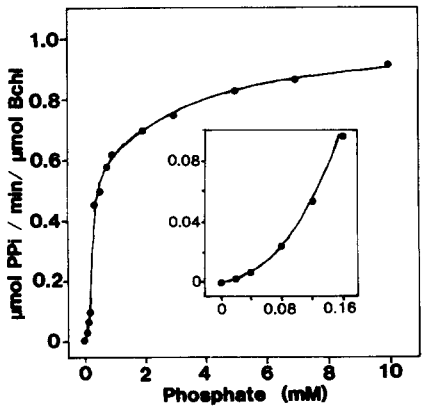

Fig. 1. The effect of phosphate on the absolute rate of photosynthetic PP_i formation. BChl concentration 0.4 μM. The light intensity was 625 W/m^2.

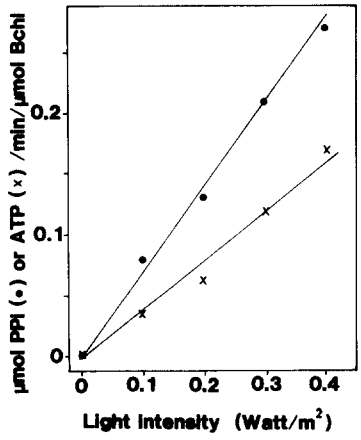

Fig. 2. The light intensity dependence of the absolute rate of photosynthetic PP_i (●) and ATP (×) synthesis at low light intensities. ATP synthesis was measured in the presence of 10 mM NaF to inhibit all PP_i synthesis activity. BChl concentration, 0.4 μM (●, ×).

studied to inhibit all PP_i synthesis activity) the rate of PP_i synthesis is twice the rate of ATP synthesis.

At high light intensity a much higher amount of antimycin A was required for inhibition of PP_i synthesis than was required for inhibition of ATP synthesis (Fig. 3). Furthermore, it was found that antimycin A failed to inhibit PP_i synthesis completely, while it almost completely inhibited ATP synthesis. Even at 0.1 μM or higher concentrations of antimycin A, the light-induced PP_i synthesis was not inhibited beyond 75%, and the residual activity was 0.4 μmol PP_i formed/min per μmol BChl. However, at high antimycin concentration the rate of PP_i and ATP synthesis are similar if they are compared on a molar basis. This residual activity was totally abolished if, in addition to antimycin, a low concentration of DBMIB (15 μM) was added. One of us has shown [15] that both antimycin A and DBMIB are necessary for complete inhibition of electron transport in the cytochrome region of *R. rubrum* chromatophores. Antimycin at high concentrations only partially inhibits electron transport [15,16], and the transport through the 'leak' apparently is sufficient to drive the residual ATP and PP_i synthesis.

As was expected, a lower concentration of FCCP was required to inhibit PP_i synthesis at low light intensity than at high light intensity (Fig. 4). Both light intensities were maximal to give almost identical control activities. The maximal rates of PP_i synthesis were 1 μmol PP_i formed/min per μmol BChl at 625 W/m^2 and 0.95 μmol PP_i formed/min per μmol BChl at 5.7 W/m^2.

Fig. 5 shows the results of titrating the light driven PP_i and ATP synthesis with valinomycin (which specifically collapses the electrical potential component of the proton-motive force). 40%

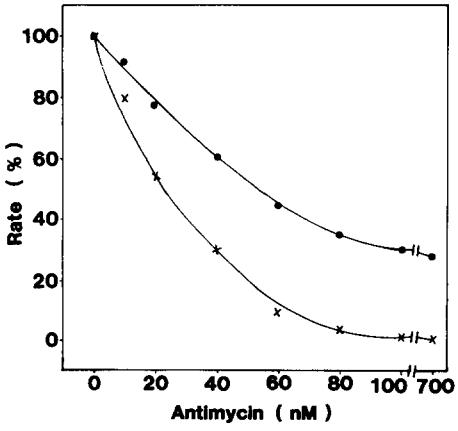

Fig. 3. The effect of antimycin on the relative rate of photosynthetic PP_i (●) and ATP (×) synthesis. BChl concentrations 0.4 μM (●) and 0.04 μM (×). The light intensity was 625 W/m^2. Control activities were 1.3 μmol PP_i formed/min per μmol BChl and 13.8 μmol ATP formed/min per μmol BChl.

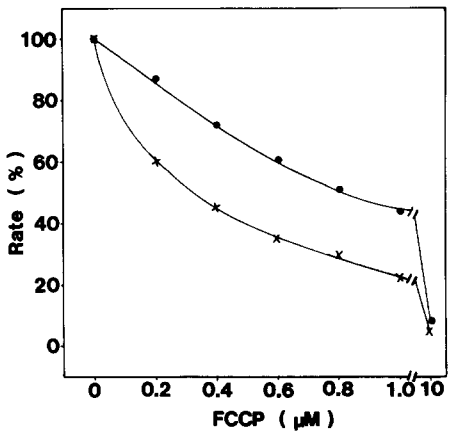

Fig. 4. The effect of different concentrations of FCCP on the relative rate of photosynthetic PP_i formation at two different light intensities. ● 625 W/m^2; ×, 5.7 W/m^2; BChl concentration 0.4 μM. Control activities were 1 μmol PP_i formed/min per μmol BChl (●) and 0.95 μmol/PP_i formed/min per μmol BChl (×).

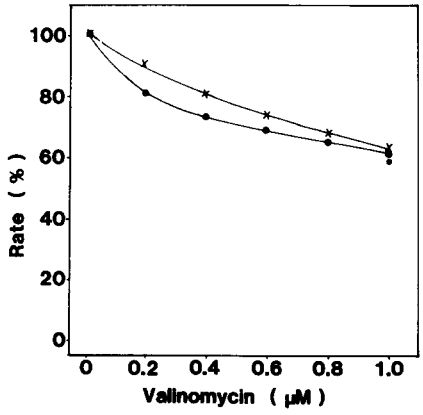

Fig. 5. The effect of valinomycin on the photosynthetic PP_i (●, *) and ATP (×) formation (relative rates). Chromatophores corresponding to a BChl concentration of 0.4 μM (●) and 0.04 μM (×, *) were preincubated for 5 min with the valinomycin concentration indicated in the presence of 50 mM KCl. The light intensity was 625 W/m^2. Control activities were 1.2 mol PP_i formed/min per μmol BChl and 9.0 μmol ATP formed/min per μmol BChl.

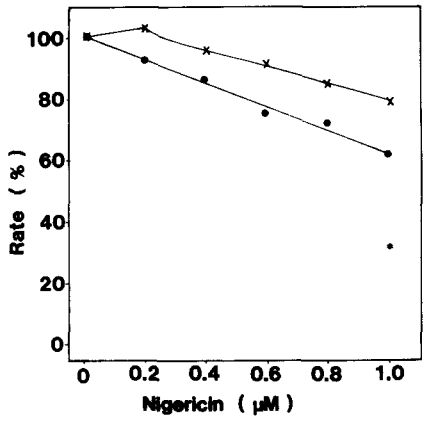

Fig. 6. The effect of nigericin on the photosynthetic PP_i (○, *) and ATP (×) formation (relative rates). Chromatophores corresponding to a BChl concentration of 0.4 μM (○) and 0.04 μM (×, *) were preincubated for 5 min with the nigericin concentration indicated in the presence of 50 mM KCl. The light intensity was 625 W/m^2. Control activities were 1.1 μmol PP_i formed/min per μmol BChl and 9.0 μmol ATP formed/min per μmol BChl.

inhibition was obtained using 1.0 μM valinomycin for both PP_i and ATP synthesis. The effect of nigericin (which specifically collapses the proton gradient component of a proton-motive force) on photosynthetic PP_i and ATP formation, is shown in Fig. 6. Nigericin has a greater effect on the rate of PP_i synthesis compared with that on the rate of ATP synthesis. We have earlier shown that nigericin has no inhibitory effect directly on the proton-pumping inorganic pyrophosphatase [6].

TABLE I

EFFECT OF DCCD ON PHOTOSYNTHETIC PP_i AND ATP FORMATION

Chromatophores corresponding to 0.4 μM BChl were preincubated with indicated concentration of DCCD for 10 min. The light intensity was 625 W/m^2. Control activities were 0.9 μmol PP_i formed/min per μmol BChl and 7 μmol ATP formed/min per μmol BChl.

DCCD (μM)	Rate of PP_i form (% of control)	Rate of ATP form (% of control)
Control	100	100
5	90	50
20	70	5
40	50	1
100	8	0

This may indicate that PP_i-synthesis utilizes mainly the ΔpH component of the electrochemical gradient $\Delta\mu_{H^+}$. Preliminary results in our laboratory show that a ΔpH jump is alone sufficient to drive PP_i synthesis, but not ATP synthesis which requires a supplementary membrane diffusion potential ($\Delta\psi$) (unpublished data; cf. Ref. 17).

DCCD at concentrations which have been reported not to inhibit the light-induced generation of membrane potential [18] inhibited the photosynthetic PP_i formation to a high degree (Table I). Half maximal inhibition was obtained with 40 μM DCCD, and more than 90% with 100 μM. The photosynthetic ATP formation was inhibited by lower concentrations of DCCD than the photosynthetic PP_i formation, half maximal inhibition was obtained with 5 μM DCCD and over 90% inhibition was obtained with 20 μM DCCD. We have earlier shown that DCCD inhibits not only the hydrolytic activity of the H^+-ATPase, but also the hydrolytic activity of the proton-pumping inorganic pyrophosphatase in *R. rubrum* chromatophores [19].

Fig. 7 shows a typical trace obtained in the study of photosynthetic ATP and PP_i formation, using the new method. The dark synthesis of PP_i seen is due to the high $\Delta G^{0\prime}$ for the ATP sulphurylate catalyzed reaction ($\Delta G^{0\prime}$ is −48 kJ/mol [20]), which will drive the H^+-PP_i synthase catalyzed reaction ($\Delta G^{0\prime} = -16.7$ kJ/mol [21]) toward PP_i synthesis. The dark synthesis of ATP may be due to the residual activity of adenylate kinase in the assay which cannot be totally inhibited by P^1,P^5-di(adenosine-5′) pentaphosphatase. One point that has always been overlooked and which has to be considered in the study of ATP synthesis in organisms containing energy-linked PP_i synthetic activity, is the 'energy leak' due to simultaneous PP_i synthesis. From Table II it is evident that the rate of ATP synthesis (measured with the ATP-monitoring technique without addition of ATP sulphurylase) is increased by 20% in the presence of 10 mM NaF, which completely inhibits the simultaneous PP_i synthesis. It should be mentioned that 10 mM NaF inhibits the assay system about 20%, but this inhibition is compensated for by internal calibration with an ATP or PP_i standard. The interpretation of these data is that the proton-motive force

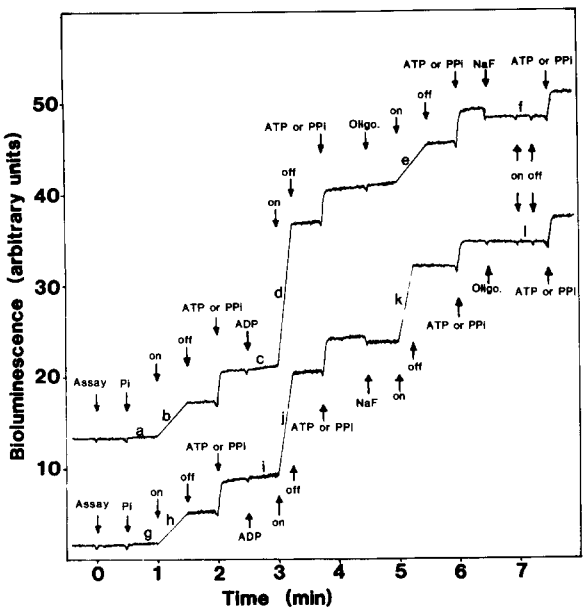

Fig. 7. Simultaneous determination of photosynthetic ATP and PP_i formation (absolute activities and both luciferase and ATP sulphurylase present). BChl concentration, 0.1 μM. The reaction mixture contained both P_i (10 mM) and ADP (50 μM), and oligomycin was omitted. The following additions were made: 100 pmol ATP, 100 pmol PP_i, 10 μg oligomycin and 10 μmol NaF. The illumination intensity was 625 W/m^2 (light on and off as indicated). The rate ATP or PP_i synthesis in μmol/min per μmol BChl in a, b, c, d, e, f, g, h, i, k and l were 0.10, 2.16, 0.22, 18.9, 2.45, 0.00, 0.10, 1.95, 0.16, 13.50, 12.60 and 0.00, respectively.

TABLE II

EFFECT OF FLUORIDE AND OLIGOMYCIN ON PHOTOSYNTHETIC PP_i AND ATP FORMATION

PP_i and ATP formation in the presence and absence of oligomycin and fluoride, respectively, was measured as described in Materials and Methods. BChl concentration, 0.1 μM. The illumination intensity was 625 W/m^2.

Conditions	μmol PP_i formed min per μmol BChl	μmol ATP formed min per μmol BChl
Dark	0.03	0.04
Light	1.5	10.0
Light + 10 mM NaF	0.0	12.1
Light + 10 μg/ml oligomycin	2.1	0.0

becomes slightly increased if PP_i synthesis is inhibited at high light intensity. On the other hand, PP_i synthesis is stimulated 40% by oligomycin in the absence of ADP. This has been shown by Baltscheffsky et al. [8], to be due to blockage of the passive proton leakage through H^+-ATPases in submitochondrial particles [22] and chromatophores [23]. This would lead to a higher proton-motive force available for the PP_i synthesis machinery. The above results show that both the PP_i synthesis and the ATP synthesis reactions compete with each other.

It has been shown that the H^+-ATPase in *R. rubrum* is regulated by the proton-motive force [24]. At low concentrations of FCCP the activity was increased, but at higher concentrations the activity was completely inhibited [24]. We have also found that the proton pyrophosphatase is regulated by the proton-motive force, but in quite a different way (Fig. 8). Low concentrations of FCCP give rise to an increased rate of PP_i hydrolysis. This is a consequence of the dissipation of the proton-motive force produced by the hydrolysis reaction. The FCCP titration curve becomes steeper in the presence of oligomycin which blocks the passive proton leakage through H^+-ATPase. The proton pyrophosphatase is, in contrast to the H^+-ATPase, not inhibited by high FCCP concentrations (Fig. 8).

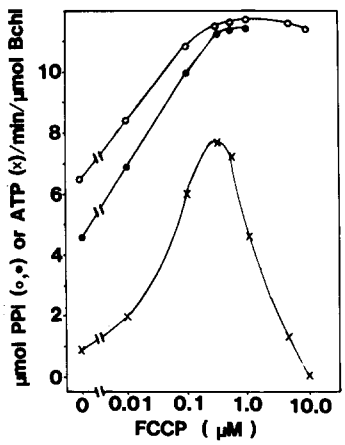

Fig. 8. The effect of FCCP on the absolute rate of ATP (×) and PP_i (●, ○) hydrolysis in darkened *R. rubrum* chromatophores. (●) PP_i hydrolysis in the presence of 10 μg/ml oligomycin. The rates were estimated from the rates of P_i production as described in the Materials and Methods section. BChl concentration 1.5 μM (×) and 0.38 μM (●. ○).

One interpretation is that, in contrast to the H^+-ATPase, the proton pyrophosphatase does not need a proton-motive force for activation. The activation step of the H^+-ATPase by an proton motive force might consist of the release of an inhibitor protein, which may be absent in the proton pyrophosphatase. Johansson and Baltscheffsky [25] have briefly reported the isolation of an inhibitor protein from the *R. rubrum* H^+-ATPase. The results from the FCCP titration experiments (Fig. 8), explain the different effects of PP_i and ATP on the energization of the chromatophore membrane in the absence [26] and presence [14,27] of a coupled reaction. Coupled reactions as to energy-linked transhydrogenase and succinate-linked NAD^+ reduction can be compared with an uncoupler effect on the chromatophore membrane. An uncoupler effect is only to dissipate electrochemical gradient $\Delta\mu_{H^+}$ formed which allows the hydrolysis reactions to proceed with maximal rate.

The main results obtained may be summarized as follows: (1) at low light intensities, i.e., low levels of energization, PP_i synthesis prevails over ATP synthesis. This has also been shown to be the case when the energy source is reverse transhydrogenation [28]. (2) PP_i synthesis and ATP synthesis compete with each other. (3) A ΔpH appears to be the predominant driving force for PP_i synthesis and it cannot be fully compensated for by a $\Delta\psi$. (4) The proton pyrophosphatase does not need a proton-motive force for activation in contrast to the H^+-ATPase.

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