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INORGANIC PYROPHOSPHATASE AND PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

I. CHARACTERISATION OF CHLOROPLAST PYROPHOSPHATASE AND ITS RELATION TO THE RESPONSE TO EXOGENOUS PYROPHOSPHATE

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SUMMARY

1. A soluble, alkaline, Mg^{2+}-dependent inorganic pyrophosphatase (EC 3.6.1.1) has been isolated from the stroma of intact spinach and pea chloroplasts and purified some 100-fold. The enzyme has a high specificity for inorganic pyrophosphate and Mg^{2+}, and exhibits maximal activity at pH 8.2–8.6. The enzyme shows allosteric characteristics with Mg^{2+} as activator and optimal rates are obtained with a ratio of Mg^{2+} to PP_i of approximately 4 to 1. The enzyme is inhibited by anionic PP_i and by its own reaction product, orthophosphate.

2. If Mg^{2+} is excluded from the medium in which isolated chloroplasts are assayed, active photosynthetic oxygen evolution can still be observed. The addition of P_i, but not PP_i, will then offset a phosphate deficiency. If external Mg^{2+} is present PP_i will also offset a phosphate deficiency and in these circumstances the rapidity and nature of the response is related to the external pyrophosphatase activity.

3. Evidence is presented that the chloroplast envelope is relatively impermeable to PP_i and that the response to added PP_i is due to external hydrolysis followed by entry of P_i to the chloroplast. These results have significance concerning proposed mechanisms for control of photosynthesis.

INTRODUCTION

In studies on photosynthesis by isolated chloroplasts we have been impressed by the controlling influence of orthophosphate[1,2], and by the ability of inorganic pyrophosphate and pyrophosphatase to act as an "orthophosphate buffer", maintaining the external P_i at or near its optimal concentration[3]. Originally it was found that both

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, MES, 2-(*N*-morpholino)-ethanesulphonic acid.

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P_i and PP_i could reinitiate photosynthetic O_2 evolution in P_i-deficient chloroplasts with stoichiometric ratios of approximately 3 O_2/P_i and 6 O_2/PP_i[1] Except for an initial delay of about 15 s with PP_i, the kinetics of response were almost identical when the effect of adding PP_i was compared with that of adding P_i at twice the concentration[4]. Subsequently, with very carefully prepared chloroplasts, a longer delay was observed with PP_i and very occasionally this was so prolonged that no response was observed during the 2–3-min period of observation, although the response to P_i was still more or less immediate In these circumstances the original rapidity of response to PP_i could be restored if a small proportion of ruptured chloroplasts were included in the reaction mixture (Ludwig, L. J. and Walker, D. A., unpublished).

These results suggested that PP_i was unable to cross the chloroplast envelope and that the normal response to PP_i followed external hydrolysis by a pyrophosphatase released from ruptured chloroplasts. Previous studies with maize[5], sugarcane[6] and spinach chloroplasts[7,8] have established the presence of an inorganic pyrophosphatase (EC 3.6.1.1.) in green plants[6,9] which has similar properties to the more thoroughly investigated pyrophosphatases from *Escherichia coli*[10] and yeast[11].

It therefore seemed desirable to reinvestigate the pyrophosphatase from spinach and pea chloroplasts, its regulation and localisation, and its relation to the effect of P_i and PP_i on photosynthesis by isolated chloroplasts

MATERIALS AND METHODS

Plant material, isolation of chloroplasts

Peas (*Pisum sativum*, var. "Feltham First") were grown in vermiculite in a greenhouse for 18–24 days at 15–20 °C. Spinach (*Spinacea oleracea*, var. Elsoms 24) was grown in the field. Freshly harvested leaf and stem material was used from peas, while the midribs were removed from the spinach leaves used. Approximately 100 g of material was homogenised for 2–4 s, using a Polytron blender, in 250 ml of isolation medium, containing 0 33 M sucrose, 25 mM 2-(*N*-morpholino)-ethanesulphonic acid (MES), 8.6 mM NaCl, 9.3 mM sodium D-isoascorbate, adjusted to pH 6.5 with KOH, and chilled to a consistency resembling melting snow. After blending for 3–5 s the suspension was filtered through first 2 layers of muslin and then 8 layers of muslin containing a layer of cotton wool. All subsequent operations were at 0 °C. The filtrate was immediately centrifuged in a swing-out head at $5000 \times g$ for 50 s including acceleration and then braked to a stop in approximately 7 s using a Christ model 17 centrifuge. The supernatant was poured off and the surface of the pellets gently rinsed with a solution containing 0.33 M sucrose and 1/25 dilution of resuspending medium. After draining, the chloroplasts were resuspended by gently mixing the pellets with about 2 ml of resuspending medium, using a small pad of cotton wool on the end of a glas rod. The resuspending medium contained 0.33 M sorbitol, 2 mM EDTA, 1 mM $MnCl_2$ and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES) adjusted to pH 7.6 with KOH. The chlorophyll content of chloroplast suspensions was determined by the method of Arnon[12].

Measurement of photosynthetic oxygen evolution

Photosynthetic oxygen evolution by twin chloroplast reaction mixtures was measured by oxygen electrodes and recorded simultaneously in the two-channel

apparatus described by Delieu and Walker[13]. Each reaction mixture contained 200 μg chlorophyll in a total volume of 2.0 ml, was maintained at 20 °C and illuminated with red light[13]

Isolation of inorganic alkaline pyrophosphatase

Chloroplasts from pea or spinach were isolated as described above, except that the homogenising medium contained in addition 5 mM $MgCl_2$ and, after centrifugation, the pellet was resuspended in homogenising medium (10 ml per 100 g plant material) and centrifuged again. The pellet of intact chloroplasts was resuspended for 30 min at 4 °C in a solution containing 50 mM Tris–HCl, 2 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.8) which ruptured the envelopes entirely. The crude enzyme extract was separated from the chloroplasts by centrifugation at 15 000 $\times g$ for 30 min and stored frozen if not used immediately. The clear supernatant was fractionated with solid ammonium sulphate. The fraction 45–78 % was collected after 30 min precipitation and redissolved in a solution containing 50 mM Tris–HCl and 5 mM $MgCl_2$ (pH 8.0). A 55–75 % fraction was then obtained by the addition of an ice-cold solution of saturated $(NH_4)_2SO_4$ (pH 8.8). This precipitate was redissolved in 50 mM Tris–HCl, 2 mM $MgCl_2$ (pH 8.2) and dialysed against two further changes of this solution for 2 h each.

The dialysed crude enzyme was clarified by centrifugation and applied to a DEAE column (7 5 cm $\times$ 3 8 cm) pre-equilibrated with 25 mM Tris–HCl and 2 mM $MgCl_2$ (pH 8.0). The column was then washed with 100 ml 25 mM Tris–HCl, 2 mM $MgCl_2$ and 100 mM NaCl (pH 8.2). The pyrophosphatase activity was eluted with 25 mM Tris–HCl, 2 mM $MgCl_2$ and 280 mM NaCl (pH 8.8) The active fractions were bulked together and diluted 5 times with redistilled water and applied to a second DEAE column of the same dimensions as the first, pre-equilibrated with 25 mM Tris–HCl (pH 8.2) A gradient of 25 mM Tris–HCl at pH 8.2 (without NaCl) to 100 mM Tris–HCl at pH 8.8 with 300 mM NaCl, of a total volume of 1000 ml was used for elution. Protein that eluted after approximately 450 ml was pooled, diluted as before and collected on a third DEAE column (2.4 cm $\times$ 5 cm) pre-equilibrated with 25 mM Tris–HCl (pH 8 2) This column was eluted in a single step with 200 ml of 50 mM Tris–HCl, 2 mM $MgCl_2$ and 300 mM NaCl (pH 8.2). The eluate between 60 and 110 ml was collected and the protein precipitated with solid $(NH_4)_2SO_4$. The white precipitate was redissolved in a minimum amount of 25 mM Tris–HCl (pH 8.2) and passed through a Sephadex G-200 F column (1.8 cm $\times$ 40 cm), which was equilibrated with the same buffer. The fractions containing maximum activity were pooled for use in subsequent work.

The specific activity of the enzyme from pea was increased 620-fold (with respect to the initial chloroplast extract) by this procedure. Preparations hydrolysed $MgPP_i^{2-}$ at rates in the range of 280–496 μmoles P_i released per mg protein per min.

Pyrophosphatase assay

Pyrophosphatase was assayed in a final volume of 1 ml containing 100 mM Tris–HCl, $MgCl_2$ and tetrasodium pyrophosphate in a molar ratio of four to one and at a concentration usually not exceeding 5 mM at pH 8.6 and 25 °C. The reaction was stopped 10 min after addition of protein by $HClO_4$ and liberated P_i measured as its molybdenum complex according to the method of Allen[14]. No dithiothreitol was

used, since it was found to hydrolyse PP_i non enzymically, nor EDTA, in order to avoid chelation of Mg^{2+}.

Purified inorganic pyrophosphatase from yeast was supplied by Boehringer. The enzyme preparation was dialysed against 25 mM Tris–HCl (pH 8 2) for 12 h

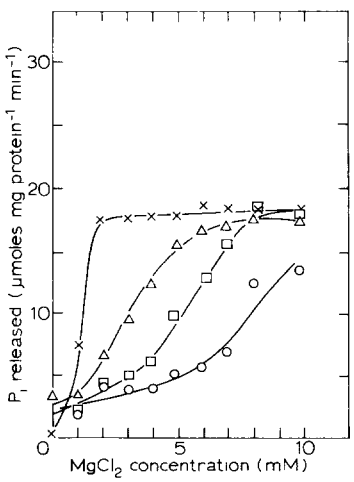
Preparation of fructose-1,6-diphosphatase

Preparations of fructose-1,6-diphosphatase were preparared and assayed by the procedure of Springer-Lederer *et al.*[15]. The fractions obtained by this procedure were also assayed for pyrophosphatase activity.

RESULTS

Properties of chloroplast inorganic pyrophosphatase

Substrate saturation was investigated by increasing Mg^{2+} concentration either at 4 constant PP_i concentrations or while maintaining 3 constant Mg^{2+}/PP_i ratios according to Bloch-Frankenthal[16]. Fig. 1 shows the saturation curves of spinach pyrophosphatase at constant PP_i and increasing Mg^{2+} concentration. The reciprocal plots of these curves in Fig. 3 shows the activation of the pyrophosphatase by Mg^{2+} and in Fig 4 the inhibition of the enzyme by PP_i. These results were generally confirmed by using constant Mg^{2+}/PP_i ratios in the assay, as in Fig 2 The plot of V against the substrate $MgPP_i^{2-}$ concentration reveals sigmoidal kinetics suggesting that the enzyme has allosteric properties, with a variable K_m (in the sense of substrate concentration required for half maximal rate).



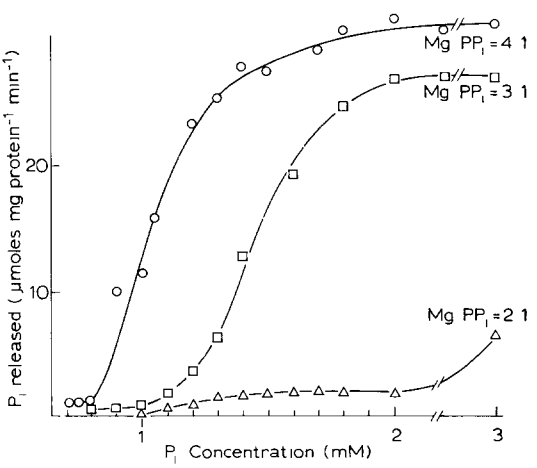


Fig 1 Substrate saturation curve of inorganic pyrophosphatase (spinach) Assay conditions as described in Materials and Methods, using 4 μg of protein 1 mM PP_i, ( ), 4 mM PP_i, (△); 6 mM PP_i, (□), 8 mM PP_i, (○).

Fig 2. Substrate saturation curve of inorganic pyrophosphatase (peas) employing constant Mg^{2+}/PP_i ratios Assay conditions as described in Materials and Methods using 18 μg of protein Mg^{2+}/PP_i ratio 4/1, (○), Mg^{2+}/PP_i ratio 3/1, (□); Mg^{2+}/PP_i ratio 2/1, (△)

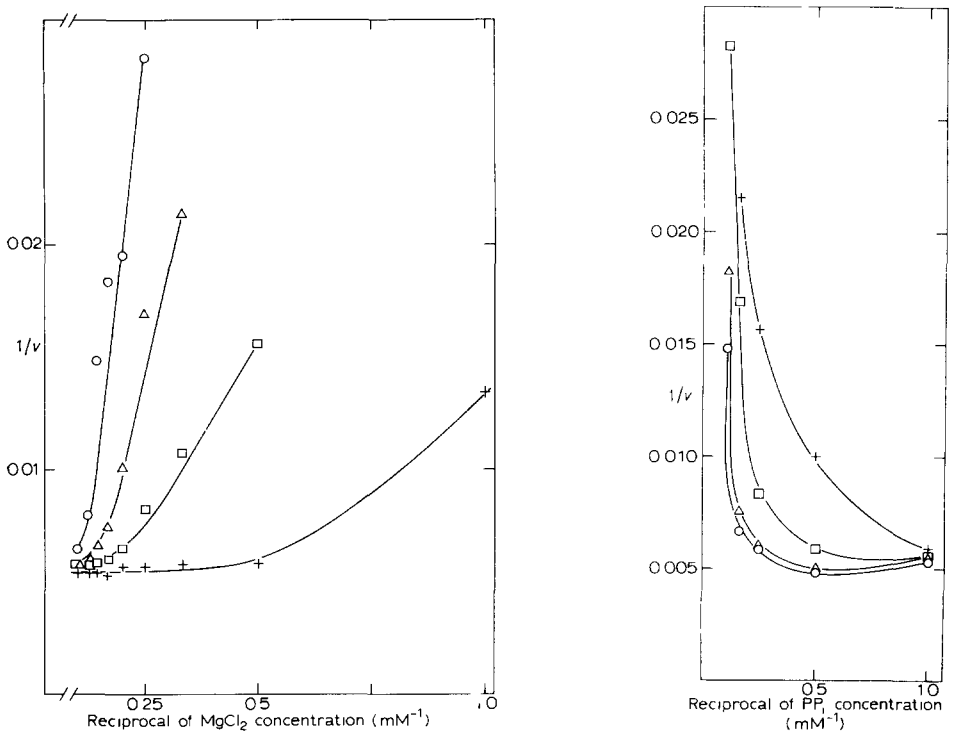


Fig 3 Activation of pyrophosphatase by Mg^{2+} (spinach). Reciprocal plot of pyrophosphatase activity against Mg^{2+} concentration at four different PP_i concentrations showing the activation by increasing Mg^{2+} (conditions and symbols as in Fig. 1).

Fig. 4. Inhibition of pyrophosphatase (spinach) by free PP_i^{4-}. Reciprocal plot of pyrophosphatase activity against PP_i concentration at four different Mg^{2+} concentrations showing the inhibition by increasing PP_i^{4-} (assay conditions as in Fig. 1). 2 mM $MgCl_2$, (+); 4 mM $MgCl_2$, (□); 6 mM $MgCl_2$, (△), 8 mM $MgCl_2$, (○).

The activity of the pea pyrophosphatase was measured over a range of pH (Fig. 5). No P_i could be detected in the absence of Mg^{2+} at acidic or neutral pH, indicating that no acid phosphatase was present. Activity was optimal at pH 8.4, but decreased to 30 % at pH 7, showing that a relatively small shift in the stromal pH could regulate the activity of the enzyme.

Table I shows the activity of pea pyrophosphatase with various substrates in the presence of excess Mg^{2+}. The pyrophosphatase showed only negligible fructose-1,6-diphosphatase activity. Mg^{2+} could not be replaced by manganese, copper or iron salts confirming earlier findings for pyrophosphatase from different sources [5-8,10].

In order to investigate the possible inhibition of pea and spinach chloroplast pyrophosphatase by P_i, the enzyme was preincubated with different amounts of P_i in the presence of Mg^{2+} (20 mM) at 25 °C for 5 min, the reaction started by addition of PP_i (5 mM). Under these conditions we found an inhibition of 50 % at a P_i concentration of 1.1 mM. The enzyme was not inhibited by glucose 6-phosphate, DL-glyceraldehyde, sulphate or *p*-chloromercuribenzoate.

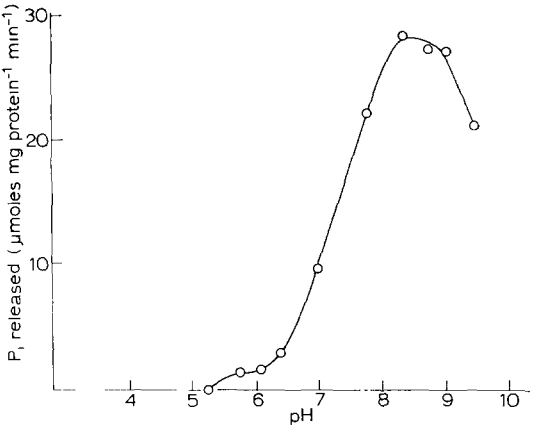


Fig. 5. pH optimum of pyrophosphatase (peas) Assay conditions: 100 mM Tris–MES buffer solutions, 5 mM PP_i, 20 mM $MgCl_2$ and 18 μg of protein in 1 ml. Assay run for 5 min at 25 °C. No orthophosphate detected in the absence of Mg^{2+}.

TABLE I

SPECIFICITY OF INORGANIC PYROPHOSPHATASE

Assay conditions 100 mM Tris–HCl (pH 8 6), either 5 mM of each substrate and 20 mM $MgCl_2$, or 10 mM of each metal ion and 2.5 mM PP_i, and pea pyrophosphatase containing 18 μg protein in a final volume of 1 ml. Assay run for 5 min (PP_i) or 15 min (other substrates) at 25 °C

Substrate	*Relative activity*	*Metal salt*	*Relative activity*
PP_i	1.00	$MgCl_2$	1.00
Fructose 1,6-diphosphate	0.07	$MnCl_2$	0 01
Glucose 6-phosphate	0 02	$CaCl_2$*	0 00
Ribose 5-phosphate	0 01	$CuCl_2$	0.00
3-phosphoglycerate	0 02	$FeSO_4$*	0.00
ATP	0.02	$MgSO_4$	0 96
ADP	0 00		

* Precipitates

Effects of exogenous PP_i and pyrophosphatase activity on photosynthesis by isolated chloroplasts

Fig. 6 shows the time course of CO_2-dependent oxygen evolution by intact chloroplasts prepared and assayed in media from which both P_i and $MgCl_2$ had been omitted. Following the normal induction period[17] there was a detectable acceleration in rate followed by a decline attributed to P_i deficiency[1,4]. Certainly the addition of P_i at this stage produced an immediate and rapid increase in rate (Fig. 6A) However, the addition of PP_i was without effect until the subsequent addition of $MgCl_2$ (Fig. 6C) when acceleration of O_2 evolution followed in about 1 min. Even with added pyrophosphatase in the medium (Fig. 6B) PP_i was without effect until the addition of Mg^{2+} but in these circumstances the attainment of maximal rate occurred within seconds rather than minutes.

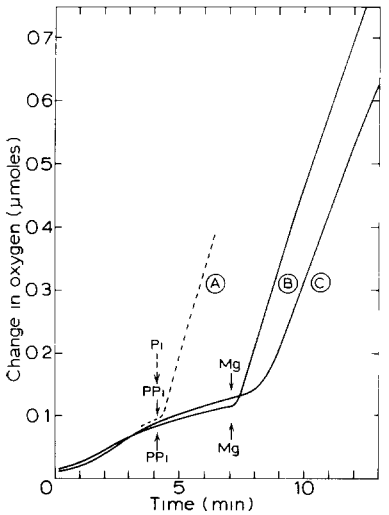


Fig 6 Oxygen evolution by spinach chloroplasts prepared in P_i and Mg^{2+}-deficient media, showing a requirement for Mg^{2+} in PP_i-dependent photosynthesis. Reaction mixtures contained 0 33 M sorbitol, 1 mM EDTA, 1 mM $MnCl_2$, 50 mM HEPES, 10 mM $NaHCO_3$, 1 mM ribose 5-phosphate and 0.1 mM 3-phosphoglycerate, pH 7 6, and chloroplasts equivalent to 200 μg chlorophyll but, initially, no $MgCl_2$ Experiment B also contained 100 μl yeast inorganic pyrophosphatase solution. Additions of P_i to A (0.5 μmole), PP_i to B and C (0 25 μmoles) and $MgCl_2$ to B and C (4 μmoles) were made as indicated

In Table II, the rates of O_2 evolution by a pair of chloroplast suspensions similar to those of Fig. 6, are compared with the pyrophosphatase activity which was

TABLE II

PYROPHOSPHATASE ACTIVITY AND CO_2-DEPENDENT O_2 EVOLUTION BY CHLOROPLAST SUSPENSIONS PREPARED IN P_i AND Mg^{2+}-FREE MEDIA

Conditions as in Fig. 6 except that the reaction mixtures initially contained 0 25 μmole PP_i but no ribose 5-phosphate or 3-phosphoglycerate The pyrophosphatase activity in samples of reaction mixtures were determined as in Materials and Methods

	Consecutive additions	*O_2 evolution (μmoles O_2 mg chlorophyll^{-1} h^{-1})*	*P_i release (P_i · mg chlorophyll^{-1} h^{-1})*
Reaction mixture 1	—	4 8	11
	4 μmoles $MgCl_2$	23.6	130
	2 5 μmoles PP_i	25.7	—
	2 μmoles 3-phosphoglycerate	36.9	—
Reaction mixture 2 + added pyrophosphatase	4 μmoles $MgCl_2$	20 8	840
	2.5 μmoles PP_i	10 8	—
	2 μmoles 3-phosphoglycerate	36 9	—

assayed simultaneously. The increase in the rate of O_2 evolution following the addition of $MgCl_2$ is associated with an increase in pyrophosphatase activity of more than 100-fold in the chloroplast suspension without added enzyme. The further addition of ten times the initial amount of PP_i to the reaction mixture containing added pyrophosphatase, however, resulted in an inhibition of the rate of O_2 evolution. That this inhibition was due to excess P_i released by the increased pyrophosphatase activity is shown by the subsequent reversal of the inhibition by the addition of 3-phosphoglycerate[1,17,18]

Partial purification of fructose-1,6-diphosphatase

The final fractions containing fructose-1,6-diphosphatase activity which were obtained by the preparation procedure used[15] were found to be heavily contaminated with pyrophosphatase activity

Pyrophosphatase activity in washed and shocked chloroplasts

In experiments described above, Mg^{2+}-free media were used to diminish external pyrophosphatase activity because of the difficulties experienced in preparing intact chloroplasts free of this activity. Table III shows that washing of intact chloro-

TABLE III

PYROPHOSPHATASE ACTIVITY IN WASHED AND SHOCKED CHLOROPLASTS

Rates of P_i released in μmoles · mg chlorophyll^{-1} h^{-1} Assay conditions chloroplasts containing 100 μg chlorophyll in 100 mM HEPES (pH 7.6), ±0 33 M sucrose, 5 or 20 mM $MgCl_2$, 5 mM $Na_4P_2O_7$, 0.4 mM EDTA in 1 ml, incubated 5 min at 25 °C.

Number of washes	*Mg PP$_i$ = 1 1*		*Mg PP$_i$ = 4 : 1*	
	Intact	*Ruptured*	*Intact*	*Ruptured*
none	19 4	282	80 5	323
1	4 1	276	83 6	327
2	5 2	271	87 4	373
3	2 7	208	81 8	357

plasts could diminish pyrophosphatase activity to a relatively low level if the $MgCl_2$ concentration in the assay medium (containing 5 mM PP_i) was kept at 5 mM. At 20 mM $MgCl_2$ the activity was substantial. At both Mg^{2+} concentrations osmotic shock was followed by a large increase in activity. This is consistent with the proposal that the intact envelope normally constitutes a barrier between stromal pyrophosphatase and its substrate. Residual pyrophosphatase activity in intact chloroplast suspensions could be associated with the envelope or adsorbed onto its surface but the difficulty of obtaining chloroplast preparations which are entirely intact makes it more likely that the washing procedure itself always ruptures a small percentage of envelopes.

Direct evidence that the chloroplast envelope is relatively impermeable to PP_i

Direct measurements of $^{32}PP_i$ uptake were undertaken in this laboratory in collaboration with Dr Hans Heldt. The uptake of metabolites by intact chloroplasts

has been extensively studied by Heldt *et al.*[19-21], who have employed preincubation with labelled intermediates followed by centrifugal filtration. Preliminary experiments carried out in this way showed that uptake of $^{32}P_i$ by spinach chloroplasts was at least 300 times as rapid as the uptake of $^{32}PP_i$. The results of a more extensive investigation using this technique will be published by Dr Heldt elsewhere.

DISCUSSION

The pyrophosphatase isolated from the stroma of pea and spinach chloroplasts requires Mg^{2+} and is only fully active at pH values around 8.4. In these respects its properties closely resemble those previously reported for other green plants[5-8], *E. coli*[10] and yeast[11] pyrophosphatases, with the exception of an enzyme from spinach described by Forti[22]. The substrate saturation curves (Figs 2 and 3) reveal allosteric characteristics of the enzyme with Mg^{2+} as activator. The pyrophosphatases from maize[5], sugar cane[6] and spinach[7,8] have not been described as allosteric although Klemme *et al.*[23] have reported that the soluble pyrophosphatase of *Rhodospirillum rubrum* is an allosteric enzyme. In view of the proposed Mg^{2+} movement between stroma and thylakoids[24] the regulation of this enzyme by Mg^{2+} seems of some importance.

The loss of activity associated with decreasing Mg^{2+}:PP_i ratios indicates first, that Mg^{2+} is needed to form stoichiometric amounts of the active substrate $MgPP_i^{2-}$ (ref. 25), and second, that PP_i^{4-} combines competitively with the enzyme. This substrate inhibition was observed with pyrophosphatases from yeast, *E. coli* and *Rhodospirillum*. Butler[11] and Josse and Wong[10] showed that PP_i^{4-} acts as a strong competitive inhibitor. Josse[25,26] found that the *E. coli* pyrophosphatase binds the free PP_i^{4-} 50 times more strongly than the substrate $MgPP_i^{2-}$ and that Mg_2PP_i, which occurs at high Mg^{2+} concentrations does not act as a substrate.

The results of Fig. 6 and Table II clearly indicate that the extent and rapidity of the response of photosynthesis by intact chloroplasts to added PP_i is determined by the external pyrophosphatase activity Since the permeability of the chloroplast envelope to PP_i is shown to be relatively very low, the responses which normally accompany the addition of PP_i are confirmed to be due to P_i formed in external hydrolysis by pyrophosphatase released from damaged chloroplasts. In practical terms the results again support the view[1,17,18] that external P_i concentration is a major factor in governing the rate of photosynthesis, and that PP_i, Mg^{2+} and pyrophosphatase in the external medium exert their control, (largely, if not entirely), by interacting to produce P_i.

Bassham *et al.*[27] have suggested a regulation of photosynthesis in intact chloroplasts by a protein fraction from spinach containing a fructose-1,6-diphosphatase, in the presence of PP_i and Mg^{2+}. The present results show that protein fractions prepared by their technique[15] may also contain large quantities of pyrophosphatase. Evidence will be presented in a following paper[28] which shows that very similar results to those of Bassham *et al*[27] can be obtained using the highly purified spinach pyrophosphatase described here and that the effects which it produces are ultimately related to the ensuing proportions of P_i, PP_i and Mg^{2+} in the assay medium.

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