Transport of inorganic pyrophosphate across the spinach chloroplast envelope

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Spinach-leaf chloroplasts take up PP_i at a rate of $1.9 \mu\text{mol/h}$ per mg of chlorophyll (Chl) in the dark and $1.6 \mu\text{mol/h}$ per mg of Chl in the light. The K_m for PP_i transport is $32 \mu\text{M}$ in the dark and $6 \mu\text{M}$ in the light. Uptake is inhibited by pyridoxal phosphate, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid and imidodiphosphate, but not by NaF or EDTA. PP_i does not appear to cross the chloroplast envelope in exchange for P_i , suggesting that it is not transported by the phosphate translocator. Exchange of

PP_i and adenine nucleotides across the chloroplast envelope is very slow and PP_i does not competitively inhibit ATP uptake, suggesting that little, if any, PP_i is transported by the adenine-nucleotide translocator. These results are consistent with the presence of a specific, high-affinity PP_i translocator in the spinach chloroplast envelope. It is proposed that *in vivo* PP_i is taken up into the chloroplast from the cytosol to replenish the P_i pool in the stroma.

INTRODUCTION

The rate of photosynthesis in isolated chloroplasts is greatly influenced by the concentration of P_i in the external medium. P_i enters chloroplasts in exchange for triose phosphates or 3-phosphoglycerate (3PGA) via the phosphate translocator (Heber and Heldt, 1981). If the external P_i concentration is too low the rate of P_i import is insufficient to support the maximal rate of photophosphorylation; however, if the external P_i concentration is too high the rapid export of Calvin-cycle intermediates (triose phosphates and 3PGA) from the chloroplasts limits the rate of CO_2 fixation. PP_i stimulates the rate of photosynthesis in isolated spinach-leaf chloroplasts. Lilley et al. (1973) proposed that PP_i is hydrolysed by alkaline pyrophosphatase released from broken chloroplasts, providing a steady supply of P_i . Although it is probable that PP_i does act to some extent as a source of P_i , it seems rather fortuitous that the resulting P_i concentration should always appear to be near optimal; therefore it is likely that other factors are involved in the stimulation of photosynthesis by PP_i .

Mouriaux (1981) and Dumas et al. (1989) found that the P_i content of P_i -depleted chloroplasts increased with time when the chloroplasts were incubated with PP_i . This net accumulation of P_i in the chloroplasts cannot be accounted for by the normal activity of the phosphate translocator, which carries out a one-for-one exchange of P_i , triose phosphate or 3PGA, and suggests that PP_i may be imported into the chloroplasts where it is hydrolysed to P_i by alkaline pyrophosphatase. Fliege et al. (1978) found that $^{32}\text{P}_i$ was released from pre-loaded chloroplasts when PP_i was added to the external medium, suggesting that the phosphate translocator does in fact also transport PP_i , albeit at a rate (V_{max} of $2.5 \mu\text{mol/h}$ per mg of Chl at 20°C) 100 times lower than for the preferred substrates. However, the possibility that the $^{32}\text{P}_i$ was released from the chloroplasts in exchange for P_i arising from enzymic hydrolysis of the added PP_i cannot be excluded.

In contrast with spinach, the rate of photosynthesis in chloroplasts from young pea and wheat leaves is inhibited by PP_i

(Robinson and Wiskich, 1976; Stankovic and Walker, 1977; Edwards et al., 1978). This inhibition can be relieved by addition of ATP or ADP. Robinson and Wiskich (1977a) and Woldegiorgis et al. (1985) found that addition of PP_i led to release of ^{14}C from young-pea-leaf chloroplasts preloaded with $[^{14}\text{C}]\text{ATP}$. From these results it was suggested that PP_i is transported into chloroplasts in exchange for ATP or ADP via the adenine-nucleotide translocator, and that loss of adenine nucleotides from the chloroplasts was responsible for the inhibition of photosynthesis by PP_i . Interestingly, the rate of photosynthesis in chloroplasts from older pea or wheat leaves is much less sensitive to inhibition by PP_i (Robinson and Wiskich, 1976; Edwards et al., 1978).

PP_i relieves inhibition of photosynthesis by ADP in pea chloroplasts (Stankovic and Walker, 1977). When pea chloroplasts were treated with *p*-chloromercuriphenylsulphonic acid, which inhibits the phosphate translocator but not the adenine-nucleotide translocator, PP_i was unable to relieve inhibition of photosynthesis by ADP (Robinson and Wiskich, 1977b). This result led Robinson and Wiskich (1977b) to modify their earlier proposal by suggesting that PP_i is transported by both the phosphate translocator and the adenine-nucleotide translocator. However, this assumes that *p*-chloromercuriphenylsulphonic acid is a specific inhibitor of the phosphate translocator, but there is no evidence for this. In fact, *p*-chloromercuriphenylsulphonic acid is a rather general inhibitor of enzymes and proteins containing available thiol groups. Therefore, transport of PP_i by another carrier distinct from the phosphate and adenine-nucleotide translocators cannot be ruled out.

Much of the work on the effect of PP_i on photosynthesis in isolated chloroplasts was carried out before it was known that the cytosol of leaves contains micromolar concentrations of PP_i (Weiner et al., 1987). Given our incomplete understanding of PP_i transport across the chloroplast envelope, and the implication from the results of Weiner et al. (1987) that such transport may occur *in vivo*, we decided to re-examine the uptake of PP_i into chloroplasts. Our aims were to find out if PP_i is transported

Abbreviations used: Chl, chlorophyll; PLP, pyridoxal phosphate; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; 3PGA, 3-phosphoglycerate; ImidoDP, imidodiphosphate.

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across the chloroplast envelope, and if so, whether the transport is mediated by the phosphate translocator, by the adenine-nucleotide translocator or by some other carrier.

EXPERIMENTAL

Plant material

Freshly harvested spinach (*Spinacea oleracea* L.) leaves were obtained from a local grower and stored at 4 °C until required for use.

Chemicals

Radiochemicals were obtained from Amersham, $\text{Na}_4^{32}\text{P}_2\text{O}_7$ was dissolved in water to give a 10 mM solution, diluted 50-fold with 10 mM $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7/\text{NaOH}$, pH 7.6, and stored at -20 °C to reduce the rate of hydrolysis. $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and imidodiphosphate (ImidoDP) were obtained from Sigma; pyridoxal phosphate (PLP) and adenine nucleotides were from Boehringer. All other chemicals were analytical grade. All solutions were made in Milli Q water.

Preparation of chloroplasts

Chloroplasts were prepared from spinach leaves as described by Walker et al. (1987). The leaves were floated on water at 4 °C in the light for 30 min before use. Approx. 50 g of deribbed leaves were ground for 5 s in 250 ml of ice-cold medium A [330 mM sorbitol, 10 mM $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7/\text{KOH}$, 5 mM MgCl_2 , 2 mM sodium ascorbate, 0.2 % (w/v) BSA, pH 6.5] using a Polytron. The brei was squeezed through two layers of miracloth (Calbiochem) enclosing a layer of cotton wool, and the filtrate was centrifuged at 1700 g for 1 min. The supernatants were discarded and the pelleted chloroplasts were resuspended in 5 ml of medium B [330 mM sorbitol, 50 mM Hepes/KOH, 2 mM EDTA, 1 mM MgCl_2 , 1 mM MnCl_2 , 0.2 % (w/v) BSA, pH 7.6]. The chloroplasts were purified by layering 4 ml of chloroplast suspension on to 4 ml of medium B containing 40 % (v/v) Percoll (Pharmacia, Uppsala, Sweden) and centrifuging at 1700 g for 1 min (swinging-bucket rotor, no brake). The supernatant was discarded and the pelleted intact chloroplasts were gently resuspended in medium C (330 mM sorbitol, 50 mM Hepes/KOH, 2 mM EDTA, 1 mM MgCl_2 , 1 mM MnCl_2 , pH 7.6), at a Chl concentration of 2 mg/ml. All procedures were carried out at 4 °C. Chloroplasts prepared as described above were virtually free of broken chloroplasts as judged by phase-contrast light microscopy, and typically showed rates of photosynthetic CO_2 -dependent oxygen evolution of 120–135 $\mu\text{mol/h}$ per mg of Chl at 25 °C.

Preparation of P_i -depleted chloroplasts

P_i -depleted chloroplasts were obtained as described by Mouriaux and Douce (1981), by incubating chloroplasts (20 μg of Chl/ml) at 20 °C for 1 h, followed by centrifugation at 1700 g (swinging-bucket rotor) for 1 min and resuspension in fresh medium C (2 mg of Chl/ml).

Measurement of Chl

Chl concentration was measured by the method of Arnon (1949) as modified by Bruinsma (1961).

Silicone-oil-filtering centrifugation

Uptake of PP_i and other compounds was measured by silicone-oil-filtering centrifugation (Heldt, 1980) in 0.4 ml polypropylene tubes (length 47 mm, internal diam. 4 mm). The tubes contained 20 μl of 1 M HClO_4 , 70 μl of AR200 silicone oil (Wacker Chemie, Munich, Germany) and 190 μl of medium C containing the radiolabelled substrate. Uptake was started by addition of 10 μl of freshly prepared chloroplast suspension (20 μg of Chl/assay), using a disposable piston from a Gilson M250 Microman positive-displacement pipette to achieve rapid dispersion of the chloroplasts in the medium containing the radiolabelled substrate. After incubation for 0.5–4 min at room temperature under low-light conditions unless stated otherwise, uptake was stopped by centrifuging the tubes in a Beckman B microfuge (10000 g, horizontal rotor) for 20 s. An aliquot (20–100 μl) of the supernatant was taken for determination of radioactivity. The tubes were cut just above the HClO_4 layer and the pellets were resuspended by vigorously shaking the bottom of the tube in 300 μl of water. An aliquot (200 μl) of the resuspended pellet was taken for determination of radioactivity. The sorbitol and water spaces were determined from parallel samples incubated with 37 kBq $^3\text{H}_2\text{O}$ and 7.4 kBq [^{14}C]sorbitol for 1 min. The difference between the sorbitol and water spaces was taken to be the stromal volume; no correction was made for the volume of the thylakoids, which typically represent only 13 % of the total internal chloroplast volume (Heldt, 1980). The mean $^3\text{H}_2\text{O}$ space was $55.7 \pm 3.7 \mu\text{l/mg}$ of Chl and the mean [^{14}C]sorbitol space was $32.4 \pm 2.3 \mu\text{l/mg}$ of Chl, giving a mean stromal volume of $23.3 \pm 1.9 \mu\text{l/mg}$ of Chl (mean $\pm$ S.D., $n = 8$). The amount of substrate taken up was calculated as described by Heldt (1980).

Back-exchange of [^{14}C]adenine nucleotides

Freshly prepared chloroplasts were loaded with [^{14}C]ATP by incubation in the dark with 100 μM [^{14}C]ATP (20.1 TBq/mol) for 2 h at 0 °C. The chloroplasts were washed by dilution with approx. 100 vol. of ice-cold medium C and centrifugation at 1700 g (swinging-bucket rotor). The supernatant was discarded and the pelleted chloroplasts were gently resuspended in fresh medium C at a concentration of 2 mg of Chl/ml. Back-exchange was measured by incubating 10 μl of [^{14}C]ATP-loaded chloroplasts in 190 μl of medium C containing unlabelled substrates for 1–4 min, followed by silicone-oil-filtering centrifugation as described above.

RESULTS

The rate of uptake of $^{32}\text{PP}_i$ by isolated chloroplasts under high- and low-light conditions was measured at various concentrations of PP_i by silicone-oil-filtering centrifugation (Figure 1). The maximum rates of PP_i uptake were observed at 200 μM and 500 μM PP_i in the light and dark respectively, with the rates observed at higher PP_i concentrations being significantly lower. Values for K_m and V_{max} obtained from plots of $[\text{PP}_i]/v$ versus $[\text{PP}_i]$ (Figure 2) were 6 μM and 1.62 $\mu\text{mol/h}$ per mg of Chl respectively in the light and 32 μM and 1.90 $\mu\text{mol/h}$ per mg of Chl respectively in the dark.

In order to test whether the apparent uptake of $^{32}\text{PP}_i$ was in fact due to uptake of $^{32}\text{P}_i$ arising from enzymic hydrolysis of $^{32}\text{PP}_i$ by alkaline pyrophosphatase, uptake of PP_i was measured in the presence of NaF and EDTA which inhibit the chloroplast alkaline pyrophosphatase (El Badry and Bassham, 1970). It can be seen from Table 1 that neither 5 mM NaF nor 5 mM EDTA significantly reduced the rates of PP_i uptake. The dependence of PP_i transport on the presence of P_i in the chloroplasts was tested by

Figure 1 Effect of PP_i concentration on the rate of uptake of PP_i by spinach-leaf chloroplasts in the light and dark

Spinach-leaf chloroplasts (20 μg of Chl) were incubated in 200 μl of 0.02–2 mM Na₂H₂³²P₂O₇/NaOH, pH 7.6 (991 GBq/mol) at room temperature for up to 2 min. At intervals of 30 s uptake of PP_i was stopped by centrifuging the chloroplasts through a layer of AR200 silicone oil (Wacker Chemie, Munich, Germany). The rate of uptake of PP_i was calculated from measurements of the radioactivity in the pelleted chloroplasts, and measurements of the water and sorbitol spaces from chloroplasts incubated for 1 min with 37 kBq ³H₂O and 7.4 kBq [¹⁴C]sorbitol (Heldt, 1980). Measurements were made in dim light (dark) or under bright white-light illumination (light).

Figure 2 Effect of PP_i concentration on the rate of uptake of PP_i by spinach-leaf chloroplasts in the light and dark

The results from Figure 1 are presented as a Hanes plot.

measuring the uptake of PP_i by P_i-depleted chloroplasts incubated with 100 μM ³²PP_i. The rates of PP_i uptake by untreated and P_i-depleted chloroplasts were 1.18 μmol/h per mg of Chl and 1.16 μmol/h per mg of Chl respectively. In contrast, the rates of uptake of ³²P_i (100 μM) by untreated and P_i-depleted chloroplasts were 35.9 μmol/h per mg of Chl and 23.7 μmol/h per mg of Chl respectively.

The identity of the PP_i transporter was investigated by looking at the effects of PLP and DIDS, inhibitors of the phosphate translocator (Rumpho et al., 1988), on PP_i transport. From the results in Table 1 it can be seen that uptake of PP_i was strongly inhibited by both PLP and DIDS, with DIDS being a particularly potent inhibitor. DIDS also strongly inhibited uptake of [¹⁴C]-ATP by chloroplasts; however, PLP had no effect on ATP transport (Table 1). ImidoDP, an analogue of PP_i, strongly inhibited the uptake of PP_i by chloroplasts, but neither ImidoDP nor PP_i had any great effect on the transport of ATP. However,

Table 1 Effect of ATP, PP_i and various inhibitors on uptake of PP_i and ATP by spinach-leaf chloroplasts

Spinach-leaf chloroplasts (20 μg of Chl) were incubated with 200 μl of 1 mM Na₂H₂³²P₂O₇/NaOH, pH 7.6 (465 GBq/mol) or 1 mM [¹⁴C]ATP (5.5 GBq/mol) under low-light conditions at room temperature for 1–4 min. Uptake of the labelled substrate was stopped by centrifuging the chloroplasts through a layer of AR200 silicone oil (Wacker Chemie, Munich, Germany). The rates of uptake of PP_i and ATP were calculated from measurements of the radioactivity in the pelleted chloroplasts, and measurements of the water and sorbitol spaces from chloroplasts incubated for 1 min with 37 kBq ³H₂O and 7.4 kBq [¹⁴C]sorbitol (Heldt, 1980). Uptake of PP_i and ATP was also measured in the presence of various inhibitors and the rates of uptake are expressed as percentages of the rates in the absence of inhibitors. For uptake of PP_i the superscript indicates the appropriate control rate ('None'). Abbreviation: N.D., not determined.

Addition	Rate of uptake (μmol/h per mg of Chl)		Proportion of control rate (%)	
	PP _i	ATP	PP _i	ATP
None	2.36*, 2.12†	1.00		
NaF (5 mM)	2.22†	0.86	105	87
EDTA (5 mM)	2.15*	N.D.	91	—
DIDS (10 μM)	1.10*	N.D.	47	—
DIDS (100 μM)	0.13*	N.D.	6	—
DIDS (500 μM)	0.00†	0.07	0	7
PLP (500 μM)	0.91*	1.11	39	111
ImidoDP (5 mM)	0.12†	0.89	6	90
ATP (1 mM)	1.61*	N.D.	68	—
PP _i (1 mM)	N.D.	1.09	—	110

Figure 3 Back-exchange of [¹⁴C]adenine nucleotides across the spinach chloroplast envelope

Spinach-leaf chloroplasts were loaded with [¹⁴C]ATP by incubation in the dark with 100 μM [¹⁴C]ATP (20.1 TBq/mol) for 2 h at 0 °C. The chloroplasts were washed to remove excess [¹⁴C]ATP, and contained 347 650 d.p.m./ml. Back-exchange was measured by incubating 10 μl of [¹⁴C]ATP-loaded chloroplasts with 190 μl of 330 mM sorbitol, 50 mM Hepes/KOH, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, pH 7.6, containing unlabelled substrates (0.5 mM) at room temperature for 1–4 min. Exchange was stopped at 1 min intervals by centrifuging the chloroplasts through a layer of AR200 silicone oil (Wacker Chemie, Munich, Germany). The amount of ¹⁴C released, in exchange for the unlabelled substrate, was found by measuring the radioactivity in the supernatant.

the rate of PP_i transport was reduced by 32 % in the presence of 1 mM ATP.

The possibility that transport of PP_i across the chloroplast envelope is mediated by the adenine-nucleotide translocator was tested by measuring the stimulation of [^{14}C]adenine-nucleotide efflux from preloaded chloroplasts by PP_i . Addition of unlabelled ATP to the external medium led to rapid efflux of [^{14}C]adenine nucleotides from the chloroplasts (Figure 3). Efflux of [^{14}C]adenine nucleotides was also stimulated, although to a lesser extent, by ADP and AMP. However, the rate of efflux in the presence of PP_i was only slightly greater than in the absence of any added metabolite or in the presence of P_i .

DISCUSSION

Our first aim was to find out whether PP_i is transported across the chloroplast envelope. We needed to distinguish between true uptake of PP_i and uptake of P_i , arising from hydrolysis of PP_i in the medium, giving the appearance of PP_i uptake. PP_i is unstable at alkaline pH, so all solutions containing PP_i were adjusted to near neutral pH. Any alkaline pyrophosphatase released from broken chloroplasts would also hydrolyse PP_i . However, our chloroplast preparations showed a high degree of intactness; therefore the activity of alkaline pyrophosphatase in the medium would be minimal. In addition, NaF and EDTA which inhibit the chloroplast alkaline pyrophosphatase (El Badry and Bassham, 1970) had little or no effect on the rate of PP_i uptake. If the apparent transport of PP_i were in fact caused by uptake of P_i via the phosphate translocator, depletion of the P_i pool in the chloroplast stroma would be expected to reduce the rate of uptake observed. However, the rates of uptake of PP_i in untreated and P_i -depleted chloroplasts were the same. As expected, depletion of the stromal P_i pool significantly reduced the rate of P_i uptake by the chloroplasts. The final evidence that PP_i is not transported as P_i comes from the K_m values observed for PP_i transport, namely 6 μ M and 32 μ M in the light and dark respectively, which are much lower than the K_m of the phosphate translocator for P_i , which is reported to be 170–300 μ M (Fliege et al., 1978; Gross et al., 1990). Taken together these results lead us to the conclusion that PP_i is transported intact across the chloroplast envelope at a rate of 1.9–2.4 μ mol/h per mg of Chl in the dark, which is very close to that reported by Fliege et al. (1978).

Transport of PP_i across the chloroplast envelope was found to obey Michaelis–Menten kinetics with some substrate inhibition at PP_i concentrations higher than $10 \times K_m$. These results showed that PP_i does not enter the chloroplasts by simple diffusion, but is transported by a saturable carrier. Our second aim was to identify the PP_i transporter.

Two alternative transporters have been proposed to carry PP_i : the phosphate translocator (Fliege et al., 1978) and the adenine-nucleotide translocator (Robinson and Wiskich, 1977a; Woldegiorgis et al., 1985). In support of the first proposal we found that PP_i transport is inhibited by PLP and DIDS which both inhibit the phosphate translocator (Rumpho et al., 1988), although the inhibition of PP_i transport was less marked than the inhibition of P_i transport found by Gross et al. (1990), who reported 85 % and 75 % inhibition of P_i transport by 500 μ M PLP and 1 μ M DIDS respectively. In addition, PLP was found to bind to at least 10 polypeptides in the chloroplast envelope (Rumpho et al., 1988). The greater specificity claimed for DIDS is true to the extent that it binds covalently only to the phosphate translocator, but this does not preclude non-covalent binding of DIDS to other polypeptides in the chloroplast envelope including a possible PP_i translocator with consequent inhibition of activity. Indeed we

found that DIDS is a very effective inhibitor of the adenine-nucleotide translocator in spinach chloroplasts. Thus the observed effects of PLP and DIDS are not conclusive because neither inhibitor is specific for the phosphate translocator. If PP_i were transported by the phosphate translocator it would be expected to enter the chloroplast in exchange for P_i . However, we found that reducing the amount of P_i available in the chloroplasts for exchange with PP_i had no effect on the rate of PP_i transport, whereas the rate of P_i transport was significantly reduced. This result suggests that PP_i is not transported by the phosphate translocator.

Our results argue strongly against the second proposition that PP_i is transported by the adenine-nucleotide translocator. PP_i transport was inhibited by PLP and ImidoDP, but these had little or no effect on the uptake of ATP. If PP_i and ATP were transported by the same carrier, they would be expected to competitively inhibit the transport of each other. We found that PP_i did not inhibit the uptake of ATP, but ATP did slightly inhibit the uptake of PP_i . It has been reported that addition of ATP to chloroplasts in the dark leads to an increase in the stromal pH (Heber and Heldt, 1981). We found that the maximum rate of PP_i uptake by chloroplasts in the light, where there would also be an increase in the stromal pH, was lower than in the dark. Therefore, it is possible that the inhibition of PP_i transport by ATP was due to an indirect effect of ATP on the pH gradient across the chloroplast envelope, rather than to competition between ATP and PP_i for a common binding site. It is also clear that PP_i is at best a very poor substrate for exchange with adenine nucleotides across the spinach chloroplast envelope, and given the low activity of the adenine-nucleotide translocator in spinach-leaf chloroplasts this leads us to the conclusion that this translocator mediates little if any of the transport of PP_i .

As noted earlier, PP_i is readily transported into chloroplasts from young pea leaves in exchange for ATP (Robinson and Wiskich, 1977a), leading to inhibition of photosynthesis. The inhibitory effect of PP_i on photosynthesis is greatly reduced or even absent in chloroplasts from older pea and wheat leaves (Robinson and Wiskich, 1977a; Edwards et al., 1978), which more closely resemble spinach-leaf chloroplasts where PP_i stimulates photosynthesis. Significantly, the activity of the adenine-nucleotide translocator in young-pea-leaf chloroplasts (19.8 μ mol/h per mg of Chl; Woldegiorgis et al., 1985) is much higher than is found in spinach-leaf chloroplasts (2.0 μ mol/h per mg of Chl; Heldt, 1969). One possible explanation for the conflicting results from pea and spinach is that the adenine-nucleotide translocators from the two species have different substrate specificities. However, an alternative explanation is that in chloroplasts from young leaves, e.g. pea, PP_i is transported predominantly by the very active adenine-nucleotide translocator even though PP_i may not be the preferred substrate; but in older leaves, e.g. spinach, where this translocator is much less active, PP_i is transported by a different carrier independently of adenine-nucleotide transport. Overall our results are consistent with the presence of a specific PP_i translocator in the spinach chloroplast envelope. The concentration of PP_i in the spinach-leaf cytosol is estimated to be 0.2–0.3 mM (Weiner et al., 1987), which is well above the K_m for PP_i transport, therefore the PP_i translocator is likely to be fully active *in vivo*.

One possible role for the transport of PP_i into the chloroplast is to remove the PP_i generated in the cytosol during protein and sucrose synthesis. However, PP_i is generated at a rate of 10–15 μ mol/h per mg of Chl by sucrose synthesis alone (Stitt et al., 1987) which is much higher than the rate of PP_i transport into the chloroplast. Measurements obtained by n.m.r. spectroscopy show that the concentration of P_i in the chloroplast

may be much higher (≥ 12 mM) than in the cytosol (0.6–1.2 mM) (Bligny et al., 1990), thus any passive diffusion of P_i across the chloroplast envelope would drain the P_i pool in the stroma, inhibiting photophosphorylation. Loss of P_i from isolated intact chloroplasts in low-P_i media has been observed by Mourioux and Douce (1981). In addition P_i will also be lost from the stroma, as SO₄²⁻ is imported into the chloroplast in exchange for P_i (Mourioux and Douce, 1979). Assuming a stromal volume of 23.3 μ l/mg of Chl and uptake of PP_i at a rate of 1.9 μ mol/h per mg of Chl, the concentration of P_i in the stroma would increase at a rate of 2.7 mM/min. This increase in the total P_i pool will allow increased accumulation of Calvin-cycle intermediates in the stroma and thus higher rates of CO₂ fixation, hence the observed stimulation of photosynthesis in isolated chloroplasts by PP_i.

In conclusion, from the results presented here and those of Mourioux (1981) and Dumas et al. (1989) we propose that the level of P_i in the chloroplast stroma is maintained *in vivo* by uptake of PP_i from the cytosol, and in spinach this transport is mediated by a specific, high-affinity PP_i translocator.

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