Studies on the Entry of Fructose-2,6-Bisphosphate into Chloroplasts

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ABSTRACT

The regulatory metabolite fructose-2,6-bisphosphate (Fru-2,6-P$_2$) has an important function in controlling the intermediary carbon metabolism of leaves. Fru-2,6-P$_2$ controls two cytosolic enzymes involved in the interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate (fructose-1,6-bisphosphatase and pyrophosphate, fructose-6-phosphate 1-phosphotransferase) and thereby controls the partitioning of photosynthetic carbon and starch. It has been demonstrated that Fru-2,6-P$_2$ is present mainly in the cytosol. Here we present evidence that Fru-2,6-P$_2$ can be taken up by isolated intact chloroplasts but at a very slow rate (about 0.01 micromoles per milligram of chlorophyll per hour). This uptake is time and concentration dependent and is inhibited by PPI. When provided a physiological concentration of Fru-2,6-P$_2$ (10 micromolar), chloroplasts accumulated up to 0.6 micromolar Fru-2,6-P$_2$ in the stroma. Elevated plastid Fru-2,6-P$_2$ levels had no effect on overall photosynthetic rates of isolated chloroplasts. The results indicate that, while Fru-2,6-P$_2$ enters isolated chloroplasts at a sluggish rate, caution should be exercised in ascribing physiological importance to effects of Fru-2,6-P$_2$ on chloroplast enzymes.

Fru-2,6-P$_2$ plays a key regulatory role in the intermediary carbon metabolism of leaves (4, 10, 16). It does so by controlling two cytosolic enzymes involved in the interconversion of Fru-1,6-P$_2$ and Fru-6-P: cytosolic FBPase, which is strongly inhibited by Fru-2,6-P$_2$ (5, 9), and PFP, which is activated by Fru-2,6-P$_2$ (10, 16). PFP activation is mainly in the glycolytic or forward direction (production of Fru-1,6-P$_2$). High levels of Fru-2,6-P$_2$ thus seem to favor glycolytic carbon flow, whereas low levels of Fru-2,6-P$_2$ result in the production of hexose-monophosphates and hence sucrose synthesis (9, 17, 18).

The relative activities that synthesize and degrade Fru-2,6-P$_2$ (Fru-6-P, 2K and Fru-2,6-P$_2$-ase) determine the concentration of Fru-2,6-P$_2$ and thus the flow of carbon in the cell. In plants the major forms of these activities are found on different cytosolic proteins (13, 14). Typical concentrations of cytosolic Fru-2,6-P$_2$ are in the micromolar range (5, 17), although for CAM plants higher concentrations (about 100 $\mu$M) have been reported (6).

Although Fru-2,6-P$_2$ is found mainly in the cytosolic fraction of leaves, the question arises as to whether Fru-2,6-P$_2$ can enter chloroplasts and, if so, whether it affects photosynthesis. We have, therefore, studied Fru-2,6-P$_2$ uptake by isolated intact chloroplasts and now report that, while Fru-2,6-P$_2$ entry is observed, uptake is limited and quite slow. Furthermore, as elevated levels of Fru-2,6-P$_2$ were found to have no effect on photosynthesis, the physiological significance of Fru-2,6-P$_2$ uptake and function in chloroplasts is questionable.

MATERIALS AND METHODS

Materials

DEAE cellulose (DE-52) was obtained from Whatman and alkaline phosphatase from Boehringer (ammonium sulphate precipitate). Percoll, coupling enzymes, and all other chemicals were from Sigma. Spinach (Spinacia oleracea var Hipack) was grown in the greenhouse in hydroponic culture or in a growth chamber in vermiculite under a 16 h light/8 h dark cycle at 25°C.

Chloroplast Isolation

Spinach leaves (50–100 g) harvested from approximately 4-week-old plants were homogenized in a grinding buffer (50–100 mL) containing 50 mM Hepes-KOH (pH 8), 330 mM sorbitol (henceforth called Hepes/sorbitol), 2 mM MgCl$_2$, 2 mM EDTA, and 0.1 mg/mL bovine serum albumin. The homogenate was filtered through 8 layers of cloth and centrifuged for 90 s at 3000 rpm (1000g) in an SS34 rotor (Sorvall). The pellet was washed twice with Hepes/sorbitol and resuspended at a Chl concentration of 3 mg/mL. Alternatively, the first pellet was resuspended in a small volume of Hepes/sorbitol, loaded on a Percoll step gradient in grinding buffer (80-60-40-20% [v/v] Percoll, 5-10-5-0.5 mL, respectively) and centrifuged for 5 min at 5000 rpm (4000g) in a HB4 swing-out rotor (Sorvall). The intact chloroplasts (lower band) were removed, washed twice, and resuspended at a Chl concentration of 3 mg/mL. Chl was determined as in Arnon (1).

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3 Abbreviations: Fru-2,6-P$_2$, fructose-2,6-bisphosphate; Fru-1,6-P$_2$, fructose-1,6-bisphosphate; Fru-6-P, fructose-6-phosphate; FBPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); PFP, pyrophosphate, fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); Fru-6-P, 2K, fructose-6-phosphate, 2-kinase (EC 2.7.1); Fru-2,6-P$_2$-ase, fructose-2,6-bisphosphatase (EC 3.1.3); Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).
Fru-2,6-P₂ Uptake by Chloroplasts

Experiments to measure Fru-2,6-P₂ uptake by chloroplasts were performed in 1.5 mL plastic reaction tubes containing in 0.2 mL final volume: Hepes/sorbitol buffer with chloroplasts corresponding to 0.2 to 0.3 mg Chl, Fru-2,6-P₂ as indicated and other reagents also as indicated. Import reactions were performed as described below at room temperature or on ice for 5 to 10 min. The reactions were terminated by adding 1 mL of ice-cold Hepes/sorbitol buffer and centrifuging for 1 min at 3000 rpm in an Eppendorf variable speed centrifuge. The pellet was resuspended in 0.4 mL buffer and layered on 0.9 mL of 40% (v/v) Percoll in Hepes/sorbitol. Centrifugation for 2 min at 3500 rpm separated the intact chloroplasts (pellet) from the broken ones. The supernatant solution plus half of the Percoll layer was removed and the tube rinsed with buffer to ensure complete substrate removal. Next, buffer and Percoll layers were completely removed, the pellet was resuspended in 1 mL buffer, centrifuged again as before, and finally suspended in 0.1 mL of 0.15 m KOH. The tubes were boiled for 3 min and debris pelleted. The Fru-2,6-P₂ concentration in the supernatant solution was measured using the PFP bioassay (5).

The effectiveness of the 40% Percoll cushion used above for separating intact chloroplasts from broken ones was tested by measuring the rate of ferricyanide dependent-O₂ evolution in intact and lysed chloroplast preparations using an oxygen electrode as described (11, 12). The percentage of intact chloroplasts increased from about 50 to about 95% after the Percoll step.

Fru-2,6-P₂ Analysis

FPP was prepared from potato tubers as described (19). The Fru-2,6-P₂ stimulation of PPI-dependent Fru-1,6-P₂ production by FPP was measured in a coupled assay. The FPP reaction was calibrated using known amounts of Fru-2,6-P₂ and care was taken to stay within the linear response range (up to 10 pmol/mL). Chloroplast Fru-2,6-P₂ concentrations were calculated as pmol/mg protein and converted to micromolar using values of 40 μL/mg Chl (21) and 30 mg protein/mg Chl (our observation) for the chloroplast volume and protein content, respectively.

Photosynthetic Measurements

Oxygen evolution measurements were performed using an oxygen electrode as described (11, 12) except that PPI was omitted. Intact chloroplasts (Percoll prepurified, 0.3 mg Chl) were combined with 10 mM NaHCO₃, 1 mM MgCl₂, 2 mM EDTA, and 0.5 mM Pi in Hepes/sorbitol buffer. To overcome the lag phase, 1 mM phosphoglycerate was added. 

¹⁴CO₂ incorporation by intact chloroplasts was performed in Hepes/sorbitol buffer containing 0.2 mM Pi, 5 mM PPI, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 1 mM [¹⁴C]NaHCO₃ (100 cpm/nmol), 0.5 mM ATP, and 2 mM DTT. Following incubation in the light (30 min, 20°C) chloroplasts were pelleted and the supernatant solution was treated with 0.1 M HCl, dried on filter paper, and counted (soluble metabolites). The chloroplast pellet was extracted with 80% ethanol and the radioactivity in the residue determined (starch). The ethanol supernatant fraction, representing stromal metabolites, contained less than 5% of the radioactivity fixed.

Other Methods

Alkaline phosphatase was dialyzed against Hepes/sorbitol buffer and used at a concentration of 200 to 250 units/mL. Incubations were for 10 min at room temperature. Protein concentrations were determined with the Bradford method (2) using human γ-globulin as a protein standard. Rubisco was partially purified from stromal proteins by PEG/MgCl₂ precipitation and assayed as described (7). The enzyme was activated for 10 min at 30°C in the presence of 2 mM DTT, 10 mM NaHCO₃, 20 mM MgCl₂ immediately before use.

RESULTS

Uptake of Fru-2,6-P₂ into Chloroplasts

To investigate whether Fru-2,6-P₂ can be taken up by chloroplasts we used an in vitro uptake system consisting of isolated intact spinach chloroplasts and Fru-2,6-P₂. Following incubation, the chloroplasts were washed, intact chloroplasts were isolated by a rigorous procedure and, after boiling in KOH, were analyzed for Fru-2,6-P₂ and protein. The intactness of the chloroplasts following isolation was tested using an oxygen electrode and was found to be approximately 95%. All calculations are based on intact chloroplasts only.

To analyze the uptake process, a time-course experiment was performed in which chloroplasts were incubated with 0.25 mM Fru-2,6-P₂ for up to 90 min (Fig. 1). Fru-2,6-P₂ was taken up by chloroplasts and uptake leveled off after 10 min.

![Figure 1. Time-dependent uptake of Fru-2,6-P₂ into isolated spinach chloroplasts at room temperature. The Fru-2,6-P₂ concentration used was 0.25 mM.](image-url)
at an internal Fru-2,6-P₂ concentration of 16 μM (calculated as described in "Materials and Methods"), corresponding to 6.4% of the external Fru-2,6-P₂ concentration. Similar results were obtained with crude chloroplast preparations and with intact chloroplasts prepared by Percoll gradient centrifugation.

If Fru-2,6-P₂ was indeed taken up into chloroplasts and was not simply adhering to the envelope, we reasoned that Fru-2,6-P₂ recovered in the chloroplast fraction would be resistant to alkaline phosphatase treatment. An example of such a phosphatase experiment is shown in Table I. We consistently found that Fru-2,6-P₂ taken up by chloroplasts was completely resistant to external alkaline phosphatase using conditions that degrade Fru-2,6-P₂ present in solution. Hypotonic lysis of chloroplasts followed by alkaline phosphatase treatment efficiently hydrolyzed internal Fru-2,6-P₂ (Table I), showing that the Fru-2,6-P₂ was still present in a phosphatase sensitive form. The plastid Fru-2,6-P₂ was recovered mainly in the stroma. The low levels associated with the thylakoids could be removed by washing with 0.2 mM KCl (results not shown). Fru-2,6-P₂ proved to be remarkably resistant to degradation by stromal proteins; incubation of 50 μM Fru-2,6-P₂ with a stromal extract at room temperature for 1 h did not reveal significant degradation.

To investigate whether Fru-2,6-P₂ uptake is energy dependent, we performed experiments in the light and dark and in the presence of ATP (up to 1 mM) and the ionophore nigericin (400 nM). As none of these treatments had an effect on uptake (results not shown) we concluded that uptake most likely is a passive process. Incubations performed on ice showed a four- to fivefold reduction in import rate when compared to experiments performed at room temperature.

The data presented thus far support the conclusion that Fru-2,6-P₂ can transverse the chloroplast envelope and accumulate in the stroma.

Further Characterization of the Uptake Process

To determine whether Fru-2,6-P₂ uptake into chloroplasts is carrier mediated, we carried out kinetic experiments in which chloroplasts were incubated with increasing concentrations of Fru-2,6-P₂ (up to 12.8 mM) for short incubation times (5 min). As shown in Figure 2, saturation of uptake was not observed at the higher (>3.2 mM) Fru-2,6-P₂ concentration. However, at the lower concentration (up to 3.2 mM) the curve is hyperbolic. This is the behavior one would expect if more than one component were involved in the uptake process, e.g., a component saturable at low Fru-2,6-P₂ concentrations and a diffusional (nonsaturable) component.

Trypsin is a low mol wt protease that can degrade both outer membrane proteins and proteins exposed on the outer face of the inner membrane (3). If a protein were involved in Fru-2,6-P₂ uptake it might be sensitive to externally added trypsin. To test this point, trypsin-treated, washed chloroplasts were combined with 0.25 mM Fru-2,6-P₂ and incubated for 10 min at room temperature in the dark. The results (not shown) revealed that even high concentrations of trypsin (up to 2.5 mg/mL, 10 min at room temperature) had no effect on uptake.

We have also tested the effect of several intermediates of carbohydrate metabolism on Fru-2,6-P₂ uptake. Chloroplasts were incubated with 0.1 mM Fru-2,6-P₂ and increasing concentrations (up to 1 mM) of each metabolite. Though there was some variability between experiments, the following picture emerges: Fru-6-P, glyceraldehyde-3-phosphate, dihydroxyacetone-phosphate, and Pi had no effect on uptake, while Fru-1,6-P₂ and 3-phosphoglycerate gave about 50% inhibition at 1 mM concentration. Interestingly, PPI strongly inhibited Fru-2,6-P₂ uptake (Fig. 3), apparently in a competitive manner (Kᵢ=0.25 mM). This inhibition is independent of magnesium as other experiments showed no effect of magnesium ions on the inhibition of Fru-2,6-P₂ uptake by PPI. Recently, the cytosolic concentration of PPI has been determined to be 0.2 to 0.3 mM for spinach leaves (20). If available as free PPI, this concentration is within the range found to inhibit Fru-2,6-P₂ uptake in isolated chloroplasts.

Effect of Fru-2,6-P₂ on Photosynthesis

The finding that Fru-2,6-P₂ can be taken up by chloroplasts raises the question of its physiological role once inside this organelle. To gain information on this point we examined whether Fru-2,6-P₂ has an effect on the rate of photosynthetic

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**Table I. Effect of Alkaline Phosphatase Treatment on Fru-2,6-P₂ Recovered from Intact and Lysed Chloroplasts**

<table>
<thead>
<tr>
<th>Chloroplast Type</th>
<th>Fru-2,6-P₂ [pmol/mg protein]</th>
</tr>
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<tbody>
<tr>
<td>Intact Control</td>
<td>6.6</td>
</tr>
<tr>
<td>+ Phosphatase</td>
<td>6.7</td>
</tr>
<tr>
<td>Lysed Control</td>
<td>9.0</td>
</tr>
<tr>
<td>+ Phosphatase</td>
<td>0.5</td>
</tr>
</tbody>
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CO₂ assimilation by isolated intact chloroplasts. Chloroplasts purified on a Percoll gradient were incubated for 5 min with increasing concentrations of Fru-2,6-P₂ and put on ice. Next, CO₂-dependent O₂ evolution was measured with an oxygen electrode. This measurement gives a good representation of the overall photosynthetic capability of chloroplasts. As shown in Figure 4, increasing internal Fru-2,6-P₂ concentrations had no effect on oxygen evolution under these conditions. An experiment based on measurement of ¹⁴CO₂ incorporation gave similar results. Here we observed that internal Fru-2,6-P₂ had no effect on the light dependent conversion of CO₂ into soluble (sugar-phosphates and related metabolites) or insoluble (starch) products (not shown). Also in related experiments Fru-2,6-P₂ (added up to 0.5 mM) had no effect on a partially purified Rubisco preparation (10 min preincubation with DTT, 1 min reaction time).

From these experiments we conclude that Fru-2,6-P₂ probably has no effect on the most important function of the chloroplast—i.e. photosynthesis.

**DISCUSSION**

The results presented in this paper show that Fru-2,6-P₂ can be slowly taken up by isolated chloroplasts. At present, we have no conclusive evidence that Fru-2,6-P₂ uptake is carrier mediated. Both the complex nature of the saturation curve (Fig. 2) and the inhibition by PPI (Fig. 3) speak against diffusion as the only uptake component. However, the slow rate of transport (see below) makes it unlikely that a specific carrier is involved. Possibly, transport is effected in part by a carrier whose major function lies in the transport of other metabolites. The initial rate of Fru-2,6-P₂ uptake (0.01 μmol/mg Chl/h, calculated from the data in Fig. 2) is about 1/100th that of e.g. the ADP/ATP translocator (8). Such a slow rate of import indicates that Fru-2,6-P₂ is unlikely to be physiologically important in chloroplasts. This conclusion is supported by the finding that exposure of chloroplasts to elevated levels of Fru-2,6-P₂ (50 times those of the cytosol of C₃ leaves) had no effect on photosynthesis. In view of these results one should be cautious in ascribing physiological significance to effects of Fru-2,6-P₂ on chloroplast enzymes, such as the recently reported stimulatory effect of Fru-2,6-P₂ on chloroplast FBPase (15).

The observation that PPI can inhibit Fru-2,6-P₂ uptake at physiological concentrations warrants comment. This finding suggests that the detection of Fru-2,6-P₂ in plastids is dependent on the cytosolic content of PPI, which could change in a way we do not yet understand. For example, we consistently found significant Fru-2,6-P₂ levels in chloroplasts isolated from leaves of hydroponically grown greenhouse spinach (1–2 μM; harvest time: 10–11 AM) while Fru-2,6-P₂ levels in chloroplasts from spinach grown in vermiculite in a growth chamber were always below detection (harvest time: 2–3 h after start of photoperiod). These results attest to the diversity of results possible when plants are grown under different conditions.

In summary, the present results provide evidence that Fru-2,6-P₂ can enter chloroplasts at a very slow rate. At the same time, these results indicate that Fru-2,6-P₂, even at elevated (unphysiological) levels, has no effect on photosynthesis. Ob-
served effects of Fru-2,6-P2 on chloroplast enzymes should be interpreted and evaluated against this background.

LITERATURE CITED