Chloroplast Phosphofructokinase

II. PARTIAL PURIFICATION, KINETIC AND REGULATORY PROPERTIES

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ABSTRACT

Chloroplast phosphofructokinase from spinach (Spinacia oleracea L.) was purified approximately 40-fold by a combination of fractionations with ammonium sulfate and acetone followed by chromatography on DEAE-Sephadex A-50. Positive cooperative kinetics was observed for the interaction between the enzyme and the substrate fructose-6-phosphate. The optimum pH shifted from 7.7 toward 7.0 as the fructose-6-phosphate concentration was taken below 0.5 mm. The second substrate was MgATP (Michaelis constant 30 μM). Free ATP inhibited the enzyme. Chloroplast phosphofructokinase was sensitive to inhibition by low concentrations of phosphoenolpyruvate and glycolate 2-phosphate (especially at higher pH); these compounds inhibited in a positively cooperative fashion. Inhibitions by glyceraldehyde-2-phosphate (and probably glyceraldehyde-3-phosphate), citrate, and inorganic phosphate were also recorded; however, inorganic phosphate effectively relieved the inhibitions by phosphoenolpyruvate and glycolate 2-phosphate. These regulatory properties are considered to complement those of ADP-glucose pyrophosphorylase and fructosebiphosphatase in the regulation of chloroplast starch metabolism.

One of the early examples of an enzyme with allostERIC regulatory properties is the glycolytic enzyme PFK3 (ATP: d-fructose-6-P 1-phosphotransferase, EC 2.7.1.11) (3,16,18), and investigations of this enzyme from a variety of plant tissues have established that the higher plant PFK is no exception: commonly reported properties include positive and negative cooperativity toward the substrate fructose-6-P, inhibition by ATP when the concentration of this substrate exceeds that of Mg2+, and allosteric regulatory influences by at least 10 metabolites, most notably P-enolpyruvate (1,5,13,14,16,20,22,23). It seemed probable that the PFK activity in chloroplasts, recently detected in this laboratory (10,12), might be essential not only for the net conversion of chloroplast starch to triose-P, but also for the regulation of this process. A study of the kinetic and regulatory properties of chloroplast PFK was undertaken accordingly and the results, outlined in this paper, indicate that starch degradation could be controlled at the reaction catalyzed by PFK.

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3 Abbreviations: PFK: phosphofructokinase; DTE: dithioerythritol.

MATERIALS AND METHODS

Materials. Italian grown spinach (Spinacia oleracea L.) was purchased from E. Lösch, Freising. Biochemicals and auxiliary enzymes were supplied from Boehringer, Mannheim, and DTE by Merck, Darmstadt. The concentrations of biochemicals in solutions were standardized by enzymic assays.

Partial Purification of Spinach Chloroplast Phosphofructokinase. Deribbed and washed spinach leaves (100 g) were homogenized in 200 ml extraction solution (12) containing 5 g PVP. The homogenate was squeezed through cheesecloth, centrifuged at 18,000g for 25 min and the supernatant fractionated by addition of a cold, saturated solution of ammonium sulfate (pH 8). Protein precipitated between 49 and 61% salt saturation was collected by centrifugation (18,000g, 25 min), dissolved in 8 ml extraction solution and dialyzed against 1 liter of dialysis solution (12). This preparation was diluted with 3 volumes of extraction solution and then fractionated with acetone; protein precipitated between 22 and 30% acetone was collected by centrifugation at 22,000g for 15 min, dissolved in 5 ml extraction solution, dialyzed as above, and finally added to a column of DEAE-Sephadex A-50 (11 cm × 2.3 cm3) equilibrated with eluting buffer (5 mm imidazole-HCl, 2 mm MgCl2, 2 mm DTE, and 0.5 mm EDTA, [pH 7.7]). The column was washed successively with 50-ml portions of eluting buffer containing 0.17 M NaCl, then 0.25 M NaCl, and lastly 0.30 M NaCl. PFK activity was eluted by 0.25 M NaCl (some further activity could be obtained with 0.30 M); this 50 ml of solution was concentrated over a Diaflo XM-50 ultrafiltration membrane, using 2.5 atm N2, to 7 ml and dialyzed as above. All operations were at 2 to 4°C.

The specific activity of chloroplast PFK in this preparation was about 40 times that in the crude extract. The cytoplasmic PFK which is stimulated by Pi (12) was largely separated from the chloroplast enzyme at the ammonium sulfate fractionation (12), and any remaining activity was removed during the ion exchange chromatography (not shown). P-hexose isomerase, glyceraldehyde-3-P dehydrogenase, enolase, adenylate kinase, and phosphatases acting on fructose-6-P, fructose-1,6-P2, P-enolpyruvate, and ATP were not detected in the preparation, but P-glyceromutase and glyceraldehyde-3-P kinase were present at activities about equal to that of the PFK.

Assay of Phosphofructokinase Activity. Standard reaction mixtures contained, in a final volume of 1 ml, 50 μmol glycylglycine-NaOH buffer (pH 7.7), 2.5 μmol MgCl2, 2.5 μmol DTE, 1 μmol fructose-6-P, 0.08 μmol NADH, 1 unit aldolase, 12 units triose-P isomerase, 1 unit α-glycerophosphate dehydrogenase, and 20 μl (containing 12 μg protein) partially purified chloroplast PFK preparation. Addition of 0.5 μmol ATP initiated the reaction, and enzyme activity was calculated from the change in absorbance at 340 nm followed with a UNICAM SP1800
RESULTS

Effect of Concentration of Substrates. Spinach chloroplast PFK responded to increased levels of fructose-6-P as shown in Figure 1. The enzyme activity with 6 mm fructose-6-P (not shown) was only slightly greater than that with 1 mm. The sigmoid shape of the curve in Figure 1 (Hill coefficient of 1.7) indicates that a moderate degree of positive cooperativity existed for the interaction between fructose-6-P and the enzyme. Such positive cooperativity has been observed for other plant PFKs (1, 5) and contrasts with the distinctive negative cooperativity first reported for pea seed PFK (14) and subsequently observed with the enzymes from banana fruit (20), Atriplex spongiosa leaves (22), and spinach leaf cytoplasm (12).

Varying the levels of ATP and Mg\textsuperscript{2+} (Fig. 2) showed that PFK activity became greater with increasing concentrations of ATP until the concentration of this substrate was similar to that of Mg\textsuperscript{2+}; further ATP inhibited the enzyme. This observation is consistent with utilization of MgATP\textsuperscript{2-} as the phosphate donor substrate, and inhibition of enzyme activity by free (i.e. uncomplexed) ATP. Quite similar relationships between ATP and Mg\textsuperscript{2+} concentrations and enzyme activity have been documented for PFK from other plant tissues (1, 5, 13). Chloroplast PFK had a relatively high affinity for MgATP\textsuperscript{2-} (Km 30 \mu M, Fig. 2). CTP, UTP, and ITP, but not pyrophosphate, could replace ATP, and Mn\textsuperscript{2+} was a substitute for Mg\textsuperscript{2+}.

Influence of pH. The optimum pH for chloroplast PFK activity was 7.7 when assayed with 0.5 mm fructose-6-P (Fig. 3). However, this optimum was shifted toward pH 7.0 by lowering the fructose-6-P concentration (Fig. 3A) or, more noticeably, by the presence of the inhibitor P-enolpyruvate (Fig. 3B). The effect of lowering the fructose-6-P concentration is more clearly depicted in Figure 4. With 0.5 mm fructose-6-P, enzyme activity at pH 8 was 92% of that at pH 7, but with 0.05 mm fructose-6-P the activity at pH 8 was only 38% of that at pH 7.

Regulation by Inorganic Phosphate, Glycolate 2-Phosphate, and Phosphorlated Intermediates of Glycolysis. A characteristic feature of chloroplast PFK is the inhibition caused by Pi. At a concentration of 21 mm this anion reduced enzyme activity by 50% (Fig. 5). This Pi inhibition contrasts with the generally observed stimulation by Pi of other plant PFKs (23) and indeed the spinach leaf cytoplasmic enzyme belongs in the latter category (12). The effect of Pi on a mixture of chloroplast and cytoplasmic PFKs, such as in a crude leaf extract, therefore depends on the relative amounts of each enzyme present. The previously reported effects of Pi on PFK from plant leaves (5, 16) might consequently be evalu-

Figure 1. Effect of fructose-6-P concentration on spinach chloroplast PFK activity. Reaction mixtures were as described under "Materials and Methods," except that concentration of fructose-6-P was varied as shown. Inset: Hill plot; the slope of the line is 1.7.

Figure 2. Effect of ATP concentration on spinach chloroplast PFK activity at two concentrations of MgCl\textsubscript{2}. Enzyme activity was determined as described under "Materials and Methods," except that the concentration of ATP was varied as shown and the MgCl\textsubscript{2} concentration was either 2.5 mm (○) or 0.25 mm (●). Inset: double reciprocal plot of values obtained with noninhibitory levels of ATP.

Figure 3. Influence of pH on spinach chloroplast PFK activity. Reaction mixtures were as described for the standard assay, except that the fructose-6-P concentration was 0.2 mm or 0.5 mm (A) and 0.5 mm (B). P-enolpyruvate was added as shown (V, ●, ●, □, △, ▲), and the pH varied as shown. Symbols distinguish the buffer compounds used. For this experiment, PFK eluted from DEAE-Sephadex A-50 by 0.30 M NaCl was used.
CHLOROPLAST PHOSPHOFRACTOKINASE. II


Effect of fructose-6-P concentration on spinach chloroplast PFK activity at four pH values. Reaction mixtures were as described under "Materials and Methods," except that the concentration of fructose-6-P and the pH were varied as shown. Buffering compounds were imidazole (O), glycylglycine (• and () and triethanolamine (■).

Influence of Pi on spinach chloroplast PFK activity in the absence and presence of P-enolpyruvate. Enzyme activity was determined as described under "Materials and Methods" and Pi and P-enolpyruvate were added as shown.

Figure 6. Inhibition of spinach chloroplast PFK activity by P-enolpyruvate and relief of the inhibition by Pi. Reaction mixtures were as described for the standard assay, except that the concentration of fructose-6-P was varied and P-enolpyruvate and Pi were added as shown.

Figure 7. Evidence for inhibition of spinach chloroplast PFK by glycerate-2-P and glycerate-3-P. Reaction mixtures were as described under "Materials and Methods," except that the fructose-6-P concentration was 0.5 mM and either glycerate-2-P (A) or glycerate-3-P (B) was added as shown.

Figure 8. Influence of pH on inhibition of spinach chloroplast PFK activity by P-enolpyruvate. Reaction mixtures were as described for the standard assay, except that the fructose-6-P concentration was 0.5 mM and either glycerate-2-P (A) or glycerate-3-P (B) was added as shown.

cause of the somewhat different influence of Pi on the chloroplast enzyme.

Glycerate-3-P and glycerate-2-P are also strong inhibitors of pea seed PFK (23), and in the present experiments indications that these metabolites inhibit chloroplast PFK were obtained (Fig. 7). These results are complicated by the presence of P-glyceromutase and glycerate-3-P kinase in the PFK preparation. Nevertheless, it seems most likely that the inhibition seen with glycerate-2-P (Fig. 7A) was authentic due to this compound, and calculation showed that the inhibition observed with glycerate-3-P (Fig. 7B) could not be attributed to the formation of glycerate-2-P, although it is not known what contribution glycerate-1,3-P2, formed from the glycerate-3-P, might have made to the observed inhibition.

Glycolate-2-P can also inhibit PFK. This inhibition was first reported for the enzyme from chloroplasts (11). Further studies (Fig. 8) have shown that the glycolate-2-P inhibition, like the P-enolpyruvate inhibition (Fig. 3), is more pronounced at higher pH; glycolate-2-P at 40 μM was sufficient to reduce
chloroplast PFK activity by 50% at pH 8, but at pH 7 more than twice this concentration was required for a similar inhibition. The glycolate-2-P and P-enolpyruvate inhibitions were also similar with respect to their extent of cooperativity (Fig. 9). In other experiments (not shown), the inhibition of chloroplast PFK (prepared as described previously [11]) by 80 µM glycolate-2-P was reversed by 10 mM Pi; the relationships between glycolate-2-P, Pi, and enzyme activity were qualitatively similar to those (Figs. 5 and 6) between P-enolpyruvate, Pi, and enzyme activity.

A number of other metabolites previously reported to influence the activity of PFK from other tissues (3, 23) were tested for effects on the activity of chloroplast PFK and the results are listed in Table I. As is common with the plant enzyme (23),

Table I. Influence of Reported Effectors of PFK Activity on the Enzyme from Chloroplasts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc</th>
<th>Substrate Conc</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>per cent</td>
</tr>
<tr>
<td>5'-ADP</td>
<td>1</td>
<td>0.5</td>
<td>7</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>3',5'-cyclic AMP</td>
<td>1</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>Gluconate-6-P</td>
<td>1</td>
<td>1.0</td>
<td>7</td>
</tr>
<tr>
<td>Glycerate-2,3-P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.5</td>
<td>1.0</td>
<td>55</td>
</tr>
<tr>
<td>Creatine-P</td>
<td>0.9</td>
<td>1.0</td>
<td>7</td>
</tr>
<tr>
<td>Citrate</td>
<td>4</td>
<td>1.0</td>
<td>92</td>
</tr>
</tbody>
</table>

inhibitions by millimolar concentrations of ADP, AMP, and citrate were observed. Enzyme activity was not significantly affected by either 10 mM NH₄Cl, 10 mM KCl, 2 mM glycolate, 2 mM glyoxylate, 2 mM oxalate, 2 mM ADP-glucose, or 0.1% starch. Inhibition by 2.8 mM ATP was not relieved by either 1 mM ADP or 1 mM AMP (Table I), or by 10 mM NH₄Cl, 10 mM KCl, or 3 mM Pi. No decrease in activity followed preincubation of the enzyme at 2°C for 1 hr in reaction mixtures containing either 10 mM or 50 mM DTE.

**DISCUSSION**

There is little doubt that chloroplast PFK could, contribute to the regulation of chloroplast starch degradation: the enzyme possesses allosteric regulatory properties, catalyzes a physiologically irreversible reaction, and exists in leaves with an activity just sufficient to accommodate starch degradation (12). It would seem to be more than coincidence that metabolites and conditions affecting chloroplast PFK activity not only influence the activities of ADP-glucose pyrophosphorylase and chloroplast fructose-1,6-bisphosphatase (two enzymes closely involved in the regulation of chloroplast starch synthesis) (2, 7, 15), but often do so in a reciprocal manner. Thus, ADP-glucose pyrophosphorylase is stimulated by P-enolpyruvate and glycerate-3-P and inhibited by Pi which also counteracts the glycerate-3-P stimulation (7), whereas chloroplast PFK is inhibited by P-enolpyruvate and, quite possibly, glycerate-3-P, and although Pi itself is inhibitory, it may effectively act as a stimulator in vivo by relieving more potent inhibitions, such as that of P-enolpyruvate. In addition, increased pH (such as the change from 7.2 to 8.0 which occurs in the stroma of chloroplasts following illumination [24]) not only reduces PFK activity, especially in the presence of inhibitors and low levels of fructose-6-P (Figs. 3, 4, and 8), but also facilitates the stimulation of ADP-glucose pyrophosphorylase (7) and favors the expression of fructose-1,6-bisphosphatase activity (2).

Chloroplast fructose-1,6-bisphosphatase undergoes a light-induced activation following illumination (15) and a simultaneous decrease in PFK activity would seem appropriate. This might entail a light-mediated inactivation similar to that reported for pea leaf (presumably cytoplasmic) PFK (8). However, although dithiothreitol inactivated PFK in those experiments (8), in this laboratory no inactivation of partially purified spinach chloroplast enzyme by 50 mM DTE was detected. The proclivity of PFK to allosteric regulation may be sufficient
to accommodate any reduction in activity required in the light.

It is conceivable that certain regulatory properties of chloroplast PFK are of little importance in vivo, and simply reflect the relationship of this enzyme to the PFK of other cell types where such regulatory properties are important. For example, it seems unlikely that inhibition by citrate (Table I) would occur in vivo, and from a comparison of chloroplast ATP and Mg2+ levels (4, 17, 19), the possibility of inhibition by free ATP (Fig. 2) might also be questioned. The strong inhibition by P-enolpyruvate, considered feasible and potentially valuable in pea seeds (13) and carrot roots (6), is currently difficult to imagine while the presence of enolase and P-glyceromutase in chloroplasts has yet to be firmly established. On the other hand, strong inhibition by glycolate-2-P, which is produced by chloroplasts during illumination (9) and which inhibits PFK more effectively at the higher pH present in the stroma of chloroplasts in the light (Fig. 8 [24]), and the effectiveness of Pi in relieving this inhibition, possibly carry considerable influence for the in vivo regulation of the enzyme. The importance of Pi was recently emphasized by Steup et al. (21) who found that chloroplast starch breakdown in darkness required a concentration of Pi 10 times greater than the optimum (of about 0.25 mM) for starch synthesis in the light.

LITERATURE CITED