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# Chloroplast Phosphofructokinase

## I. PROOF OF PHOSPHOFRUCTOKINASE ACTIVITY IN CHLOROPLASTS[1]

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### ABSTRACT

**Ammonium sulfate fractionation of an extract from the leaves of spinach (*Spinacia oleracea* L.) produced two fractions of phosphofructokinase activity, the first stimulated by inorganic phosphate and the second inhibited by inorganic phosphate. Only the second fraction was obtained from similar treatment of an extract of isolated spinach chloroplasts. The two fractions differed distinctly with respect to kinetics for the substrate fructose 6-phosphate. Evidence for these two types of phosphofructokinase was also obtained with extracts from the leaves of wheat (*Triticum aestivum* L.), pea (*Pisum sativum* L.), and maize (*Zea mays* L.), and the glumes of oat (*Avena sativa* L.), but not from chive (*Allium schoenoprasum* L.) leaves, pea cotyledons, or pea roots. It was concluded that most leaves contain phosphofructokinase activity in chloroplasts as well as in the cytoplasm. Spinach chloroplast phosphofructokinase activity, which was at least 2.5 μmoles fructose 1,6-bisphosphate formed per mg chlorophyll per hour, did not result from contamination by cytoplasm or by other cellular organelles, and was not detected until after chloroplasts were broken.**

Photosynthetic tissues have been shown to contain the glycolytic enzyme PFK[3] (ATP: D-fructose-6-P 1-phosphotransferase, EC 2.7.1.11) (1, 2, 16, 17, 22), but until recently none of this enzyme was detected in chloroplast preparations (7, 13, 21). This posed a problem since a portion of the glycolytic sequence, including the PFK reaction, seemed essential for the net conversion of chloroplast starch to triose-P which, unlike hexose-P, can be easily exported to the cytoplasm (5, 6). The search for chloroplast PFK was therefore renewed in this laboratory and evidence was obtained that substantial PFK activity could be detected in isolated chloroplasts when care was taken with the assay conditions (10).

The PFK in whole spinach leaves has now been separated into two fractions, and evidence obtained that one of these fractions contains PFK largely of chloroplast origin, while the other contains cytoplasmic PFK. In an accompanying paper (11) the kinetic and regulatory properties of the chloroplast-type PFK are presented.

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[3] Abbreviations: PFK: phosphofructokinase; DTE: dithioerythritol.

### MATERIALS AND METHODS

**Materials.** Green leaves from spinach (*Spinacia oleracea* L.), wheat (*Triticum aestivum* L.), pea (*Pisum sativum* L.), and chive (*Allium schoenoprasum* L.) were obtained from plants grown under glasshouse conditions. Maize leaves (*Zea mays* L.) and oat glumes (*Avena sativa* L.) were obtained from the field. Biochemicals and auxiliary enzymes were purchased from Boehringer, Mannheim, and DTE from Merck, Darmstadt.

**Ammonium Sulfate Fractionations.** In the standard procedure, 15 g plant tissue was ground, using mortar and pestle, with 30 ml extraction solution (5 mM $MgCl_2$, 5 mM DTE, and 1 mM EDTA, adjusted to pH 7.7 with $NaHCO_3$) containing 3 g PVP. The homogenate was squeezed through cheesecloth, centrifuged, and the supernatant fractionated with ammonium sulfate by dropwise addition of a saturated solution of pH 8. The salt concentration was first brought to 20% of saturation and the resultant precipitate removed by centrifugation. Further ammonium sulfate additions were designed to increase the concentration in the preparation by small (5% or 7% of saturation) increments; after each addition precipitated protein was collected by centrifugation and dissolved in 1 to 2 ml of extraction solution. When all samples were available, they were added to separate lengths of dialysis tubing (flat width 1 cm) and dialyzed together for 3 hr in 1 liter of a solution consisting of 5 mM imidazole-HCl, 1 mM $MgCl_2$, 1 mM DTE, and 0.5 mM EDTA (pH 7.7). All centrifugations were at 22,000*g* for 20 min. The temperature was 2 to 4 C.

**Isolation of Chloroplasts.** Chloroplasts were isolated by homogenizing 8 g leaves for 5 sec in 40 ml of the isolation medium described by Lilley *et al.* (15). The homogenate was filtered through Miracloth, centrifuged at 2,500*g* for 80 sec, and the pellet of chloroplasts collected. The procedure was repeated where necessary. Spinach chloroplasts isolated in this manner evolved $O_2$ in a light-dependent reaction at a rate of 75 μmol/mg Chl·hr. Where chloroplast extracts were required, each pellet was resuspended and chloroplasts broken in 10 ml extraction solution (see above) containing 0.2 g PVP and the suspensions combined and centrifuged at 22,000*g* for 20 min; the supernatant was taken as the extract of soluble chloroplast components.

**Determination of Enzyme Activities.** PFK was assayed (12) in 1-ml reaction mixtures containing 50 μmol imidazole-HCl buffer (pH 7.7), 2.5 μmol $MgCl_2$, 2.5 μmol DTE, 0.08 μmol NADH, 1 unit aldolase, 12 units triose-P isomerase, 1 unit α-glycero-P dehydrogenase, enzyme preparation, and an equilibrium mixture of D-glucose-6-P and D-fructose-6-P generated from 1.5 μmol D-fructose-6-P by 1 unit hexose-P isomerase. The reaction was initiated by addition of 0.5 μmol ATP and the activity calculated from the change in extinction at 340 nm

measured with a UNICAM SP1800 spectrophotometer. The formation of 1 μmol fructose-1,6-$P_2$/min was taken as 1 unit of activity. Hexose-P isomerase was included so that all assay mixtures contained a high activity of this enzyme, rather than variable quantities dependent on the level in each ammonium sulfate fraction. The effect of Pi was routinely tested by including 25 μmol Na-phosphate buffer (pH 7.7) in the reaction mixture. It was essential to dialyze the auxiliary enzymes before use to remove ammonium sulfate which otherwise inhibited enzyme activities, especially that of chloroplast PFK: 10 mM ammonium sulfate reduced the PFK activity in a chloroplast extract by 47%, although there was still a 20-fold excess activity of the auxiliary enzyme system.

Nonreversible glyceraldehyde-3-P dehydrogenase was assayed as described previously for cell-free plant extracts (9). Protein was estimated by the biuret procedure after precipitation with trichloroacetic acid and washing with acetone.

## RESULTS

Two distinct fractions of PFK activity were obtained from ammonium sulfate fractionation of a spinach leaf extract (Fig. 1a). The activity measured in the first fraction (collected between 30 and 35% salt saturation) was doubled when 25 mM Pi was included in assay mixtures, but that in the second (obtained between 50 and 60%) was inhibited more than 50% by the same quantity of Pi (Fig. 1b). When the same experiment was performed with an extract from isolated spinach chloroplasts, only the second fraction of PFK activity which was strongly inhibited by Pi was observed (Fig. 2, a and b); the first fraction, seen with the whole leaf extract, was entirely absent from chloroplasts. These results confirm that spinach leaves contain at least two PFK enzymes, including one in the cytoplasm with activity stimulated by Pi, and a second in chloroplasts with activity inhibited by Pi.

The distribution of whole leaf PFK (Fig. 1a) did not simply follow the distribution of total protein. In fact, most protein

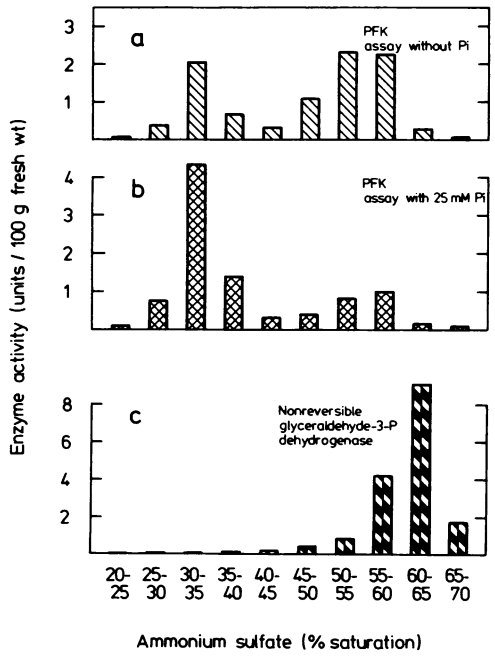


FIG. 1. Distribution of PFK and nonreversible glyceraldehyde-3-P dehydrogenase among 5% ammonium sulfate fractions from an extract of the leaves of spinach (*Spinacia oleracea* L., var. Früremona).

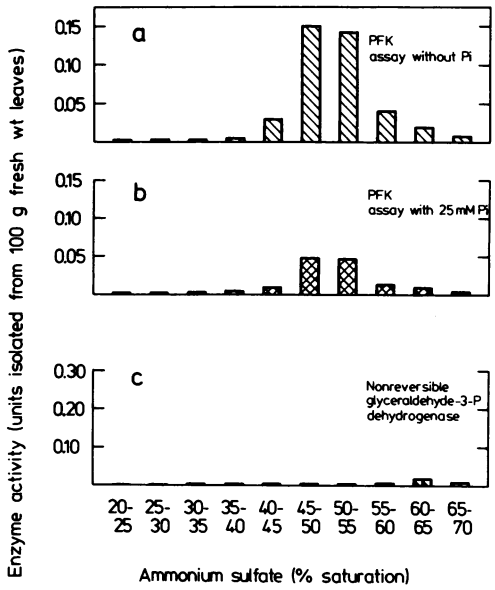


FIG. 2. Distribution of PFK and nonreversible glyceraldehyde-3-P dehydrogenase among 5% ammonium sulfate fractions from an extract of spinach chloroplasts. Chloroplasts isolated from 104 g leaves were broken and the extract obtained after centrifugation was concentrated over a Diaflo XM-50 filter to 25 ml. This preparation was fractionated with ammonium sulfate and enzyme activities were determined as described under "Materials and Methods."

was collected between 40 and 50% salt saturation (not shown) and separation of the two PFK fractions is even more pronounced if expressed as specific activity in each sample. Also, the absence of the first PFK fraction in chloroplasts (Fig. 2a) was not a consequence of this enzyme being unstable in the more dilute preparation obtained after chloroplast breakage since, in a control experiment, a similarly diluted whole leaf extract subsequently yielded both PFK fractions as in Figure 1a. A comparison between the activities of the cytoplasmic nonreversible glyceraldehyde-3-P dehydrogenase (9) and PFK showed that the PFK measured in the chloroplast extract could not have originated from cytoplasmic contamination (Figs. 1c and 2c). Other control experiments, which were an extension of those described previously (10), utilized marker enzymes to establish that the PFK activity found associated with chloroplasts could not have originated from either cytoplasm, mitochondria, or microbodies.

A demonstration that the PFK associated with chloroplasts is located inside the organelle is given in Table I. Detectable PFK activity was at least seven times greater with broken chloroplasts than with chloroplasts kept intact by addition of 0.33 M sorbitol to reaction mixtures. The value of 2.92 μmol fructose-1,6-$P_2$ formed/mg Chl·hr is probably an underestimation of the true activity since control experiments indicated that chloroplast fructosebisphosphatase may have hydrolyzed half of the formed fructose-1,6-$P_2$. Additional experiments (Table I) established that the activity being studied was indeed PFK: both fructose-6-P and ATP were necessary, and inhibition by P-enolpyruvate and by high concentrations of ATP, known inhibitors of plant PFK (12, 16), were observed.

The separation of chloroplast and cytoplasmic PFKs from spinach leaves encouraged similar experiments with other leaf tissues. Ammonium sulfate fractionation of sugar-beet leaf extracts produced a pattern of PFK activity quite similar to that for spinach leaves, indicating separation of two sugar beet PFKs. However, the patterns obtained with five other examined plant species (Figs. 3 and 4) were somewhat different

Table I. Release of PFK Activity Following Lysis of Isolated Intact Spinach Chloroplasts

Chloroplasts were isolated as described under Methods. After centrifugation, 2 ml resuspension solution (15) was added gently and swirled over the chloroplast pellet to resuspend the upper layer which was discarded. The lower chloroplasts were resuspended in a further 2 ml of solution and 0.1 ml of this chloroplast suspension, containing 66 µg Chl, was used to start reactions. The standard reaction mixture contained, in a final volume of 1 ml, 50 µmoles imidazole-HCl buffer (pH 7.7), 2.5 µmoles $MgCl_2$, 2.5 µmoles DTE, 1.5 µmoles fructose-6-P, 0.5 µmole ATP, and chloroplasts. After 10 min incubation, mixtures were rapidly centrifuged (2 min) and 0.5 ml supernatant immediately added to a spectrophotometer cuvette containing 0.45 ml of 0.35 mM NADH. The absorbance at 340 nm was determined before and after addition, in 0.05 ml, of the three auxiliary enzymes used in the determination of PFK activity (see Methods) and the amount of fructose-1,6-$P_2$ plus triose-P formed was calculated.

| Reaction Mixture | Fructose-1,6-$P_2$ + Triose-P Formed |
|---|---|
| | µmoles fructose-1,6-$P_2$/mg Chl/hr |
| Standard | 2.92 |
| Plus 0.33 M sorbitol | 0.41 |
| Plus 0.33 M sorbitol added 1 min after chloroplasts | 2.22 |
| Minus fructose-6-P | 0.39 |
| Minus ATP | 0.42 |
| Plus 0.1 mM P-enolpyruvate | 0.68 |
| ATP increased to 8 mM | 0.76 |

from spinach and sugar beet in that a clear separation of PFK activity into two fractions was absent. Nevertheless, enzyme precipitated with lower ammonium sulfate concentrations was consistently stimulated by Pi whereas that obtained with higher salt concentrations was generally inhibited. Thus, both types of PFK appear to be present, and the amount of activity inhibited by Pi might represent a rough estimate of the level of chloroplast PFK. For example, about half of the PFK activity in wheat leaves (Fig. 3a) and oat glumes (Fig. 3b) was of the chloroplast type, but there was little, if any, of this PFK in chive leaves (Fig. 3d).

The difference between spinach leaves (Fig. 1) and the leaves of other plants (Fig. 3) prompted a comparison with nonphotosynthetic plant tissues. For this purpose the distribution of PFK in the pea plant was studied (Fig. 4). Pea cotyledon PFK (Fig. 4a) was precipitated by relatively lower concentrations of ammonium sulfate compared to the PFKs from roots and shoots; the distribution of enzyme activity among ammonium sulfate fractions from roots, etiolated shoots, and green leaves was similar for each tissue (Fig. 4, b, c, and d), but Pi inhibition was seen only with etiolated shoots and, more noticeably, with green leaves, suggesting that some chloroplast-type PFK is already present in etioplasts. Like spinach chloroplasts, isolated pea chloroplasts contained a PFK precipitated by relatively high ammonium sulfate concentrations and strongly inhibited by Pi (Fig. 4e).

One inherent danger in the present experiments was that

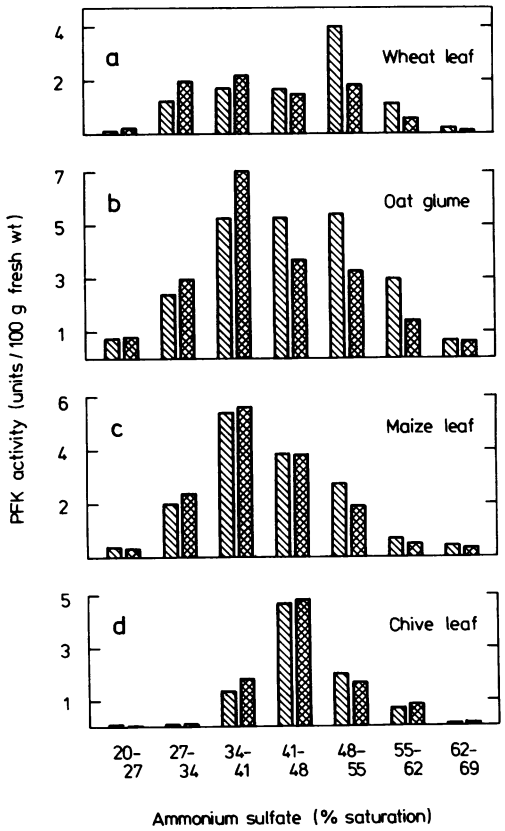


FIG. 3. Distribution of PFK among 7% ammonium sulfate fractions from extracts of the leaves of wheat, maize, and chive, and the glumes of oat. Fractions were prepared as described under "Materials and Methods" except that for the initial extractions only 4.4 g wheat leaves, maize leaves, and oat glumes, and 10 g chive leaves were used; the wheat and maize leaves and oat glumes were ground under a nitrogen stream in extraction solution containing 5 g PVP and, in the case of maize, DTE increased to 20 mM. PFK was assayed in the absence (single-hatched columns) and presence (double-hatched columns) of 25 mM Pi.

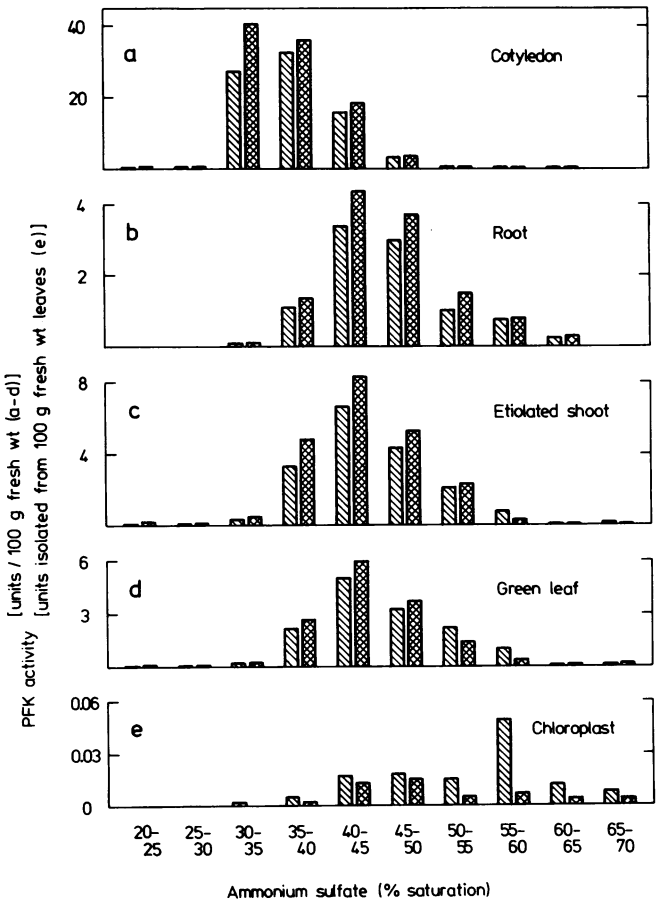


FIG. 4. Distribution of PFK among 5% ammonium sulfate fractions from extracts of various tissues of the pea (*Pisum sativum* L., var. Kleine Rheinländerin). The experiment with pea chloroplasts, isolated from 54 g leaves, was basically as for spinach chloroplasts (Fig. 2). PFK was assayed in the absence (single-hatched columns) and presence (double-hatched columns) of 25 mM Pi.

the restriction of the Pi inhibition of PFK activity to the higher ammonium sulfate fractions may have been connected with the possibly higher residual ammonium sulfate concentrations in these fractions after dialysis; Pi and sulfate anions have similar effects on plant PFK (12). However, two sets of results indicate that this was not the case. Firstly, no Pi inhibition was detected in any of the pea cotyledon and pea root fractions (Fig. 4, a and b); and secondly, mixing two pea leaf fractions with different responses to Pi (Table II) gave values quantitatively consistent with Pi stimulating PFK activity in the first and inhibiting that in the second, whether or not they were assayed together.

Some final evidence that chloroplasts contain a PFK different from that in the cytoplasm is given in Figures 5 and 6. Spinach leaf cytoplasmic PFK was stimulated by Pi at concentrations up to 10 mM but little affected by the addition of further Pi (Fig. 5). This response was almost identical to that observed earlier for pea seed PFK (8). In contrast, spinach chloroplast PFK was progressively inhibited by Pi as the anion concentration was increased to 35 mM (Fig. 5). The two enzymes also reacted differently to increases in the concentration of fructose-6-P (Fig. 6). The activity of the cytoplasmic enzyme increased rapidly between zero and 0.05 mM fructose-6-P but thereafter responded sluggishly to higher concentrations of this substrate; kinetic analysis indicated pronounced negative cooperativity with a Hill coefficient of 0.4 (Fig. 6, inset). On the other hand, the interaction between chloroplast PFK and fructose-6-P concentration was not far removed from hyperbolic kinetics (Fig. 6) with the result that the chloroplast PFK, in comparison to the cytoplasmic enzyme, was proportionately much less active with fructose-6-P concentrations below 0.05 mM.

## DISCUSSION

From the enzymological point of view, the existence in plant leaves of two enzymes having PFK activity but differing with respect to kinetic properties and intracellular location is of particular interest, especially since PFK is an enzyme important to the regulation of glycolysis. It will be interesting to see whether the chloroplast and cytoplasmic PFKs are true isoenzymes or whether one is a modified form of the other, and to what extent the development of chloroplast PFK activity is light-dependent. There seemed to be some activity in etiolated pea shoots (Fig. 4c) and it is interesting that Dennis and Green (3) recently detected PFK activity in proplastids isolated from castor bean endosperm.

Table II. Evidence that the Response of Pea-leaf PFK Activity to Pi was not Influenced by Residual Sulfate in Ammonium Sulfate Fractions

The 40% to 45% and 50% to 55% ammonium sulfate fractions shown for green pea leaves in Fig. 4d were assayed for PFK activity as described under Methods, in the presence and absence of Pi.

| Ammonium Sulfate Fractions | Amount of Protein | Fructose-1,6-$P_2$ Formed | |
|---|---|---|---|
| | | No Pi | Plus 25 mM Pi |
| | mg | nmoles/min | |
| 20 µl 40% to 45% | 0.80 | 7.1 | 7.4 |
| 30 µl 50% to 55% | 0.45 | 5.8 | 2.6 |
| 50 µl 50% to 55% | 0.75 | 8.2 | 5.2 |
| 20 µl 40% to 45% + 30 µl 50% to 55% | 1.25 | 12.6 | 10.4 |
| 20 µl 40% to 45% + 50 µl 50% to 55% | 1.55 | 15.0 | 13.5 |

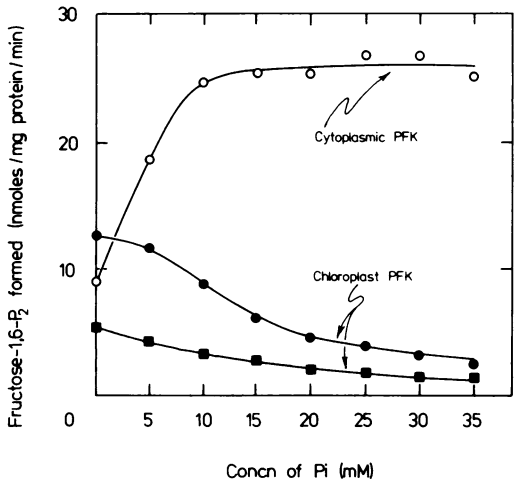


FIG. 5. Effect of Pi on the activities of chloroplast PFK and cytoplasmic PFK from spinach leaves. Partially purified enzymes (O and ●) were obtained from whole leaf extracts by ammonium sulfate fractionation essentially as described under "Materials and Methods," except broader fractions were taken: based on the results in Figures 1 and 2, cytoplasmic PFK was obtained between 25 and 37% saturation and chloroplast PFK between 49 and 61%. In addition, PFK in a soluble extract from isolated chloroplasts (■) was tested. Enzyme activity was determined as described under "Materials and Methods," except the buffer was glycylglycine-NaOH (pH 7.7) and Pi was added as shown.

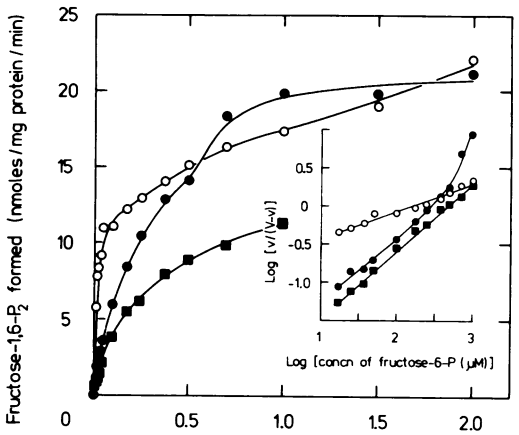


FIG. 6. Effect of fructose-6-P concentration on the activities of chloroplast PFK and cytoplasmic PFK from spinach leaves. The enzymes were obtained as described under Figure 5. Fructose-6-P concentrations shown are those present after equilibration by hexose-P isomerase. Inset: Hill plot. O, ●, and ■: see Figure 5.

Although the present results show that only one of the two PFKs detected is found in the chloroplast, the possibility that this chloroplast-type PFK also exists in the cytoplasm (in addition to the form restricted to the cytoplasm [*i.e.* that stimulated by Pi]) cannot be ruled out until there is irrefutable evidence to the contrary. However, the more straightforward situation, whereby the chloroplast-type PFK is restricted to this organelle, is at present favored since: (*a*) there was no evidence for chloroplast-type PFK in nonphotosynthetic pea tissues (Fig. 4, a and b); and (*b*) with preparation of partially purified chloroplast-type PFK, curves of enzyme activity *versus* concentration of substrate (fructose-6-P) or inhibitor (P-enolpyruvate) were sigmoid between 10 and 90% saturation with these ligands (11), indicating that only one kinetically distinguishable form of enzyme was present; the sensitivity of

this form to Pi was almost identical to that of the PFK in an extract from chloroplasts (Fig. 5).

Of the enzymes necessary for the net conversion of starch to triose-P, PFK is the last to be found in chloroplasts. Association of the other enzyme activities with chloroplasts was reported earlier (13, 21). It is therefore possible that all carbon originating from the degradation of chloroplast starch passes through the chloroplast PFK reaction prior to its exit into the cytoplasm in the form of triose-P or glycerate-3-P; Levi and Gibbs (14) recently observed formation of glycerate-3-P from starch in intact chloroplasts held in darkness. Starch could also be metabolized to triose-P in the chloroplast through the oxidative pentose phosphate pathway, but since this route would entail a loss of half of the carbon as $CO_2$ it might not be considered judicious for the carbon economy of the plant. In addition, in recent experiments (14), neither ribulose-5-P nor gluconate-6-P was detected as a product of starch degradation in intact chloroplasts.

An activity of chloroplast PFK around 2.5 $\mu$mol fructose-1,6-$P_2$ formed/mg Chl·hr (Table I) appears just sufficient to accommodate the degradation of chloroplast starch. Reported levels of starch in plant leaves, as per cent of the leaf dry weight, include up to 4% in sugar beet (19) and temperate grasses, as much as 11.6% in the tropical grass *Paspalum dilatatum* (4, 23), and 23% in soybean (24). Calculation shows a level of 5% is roughly equivalent to 20 $\mu$mol starch glucosyl units/mg Chl, an amount which the above PFK activity could utilize (after conversion to hexose-P) in 8 hr. It is conceivable that the amount of chloroplast PFK activity contained in plant leaves is related to their potential chloroplast starch content; the considerable enzyme activity in wheat leaves (Fig. 3a), which can synthesize starch in their chloroplasts (18), contrasts with the essential absence of the enzyme from *Allium* leaves (Fig. 3d) which contain no starch (25) and are reported to lack ADP-glucose pyrophosphorylase (20).

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