Plant Physiol. (1985) 77, 999–1003
0032-0889/85/77/0999/05/$01.00/0

Enzyme Regulation in C_4 Photosynthesis[1]

IDENTIFICATION AND LOCALIZATION OF ACTIVITIES CATALYZING THE SYNTHESIS AND HYDROLYSIS OF FRUCTOSE-2,6-BISPHOSPHATE IN CORN LEAVES

Received for publication September 4, 1984 and in revised form November 29, 1984

JÜRGEN SOLL[2], CHRISTIANE WÖTZEL[3], AND BOB B. BUCHANAN*
Division of Molecular Plant Biology, Hilgard Hall, University of California, Berkeley, California 94720

ABSTRACT

Activities catalyzing the synthesis of fructose-2,6-bisphosphate (fructose-6-phosphate,2-kinase or Fru-6-P,2K) and its breakdown (fructose-2,6-bisphosphatase or Fru-2,6-P_2ase) were identified in leaves of corn (*Zea mays*), a C_4 plant. Fru-6-P,2K and Fru-2,6-P_2ase were both localized mainly, if not entirely, in the leaf mesophyll cells. A partially purified preparation containing the two activities revealed that the kinase and phosphatase were regulated by metabolite effectors in a manner generally similar to their counterparts in C_3 species. Thus, corn Fru-6-P,2K was activated by inorganic phosphate (Pi) and fructose-6-phosphate, and was inhibited by 3-phosphoglycerate and dihydroxyacetone phosphate. Fru-2,6-P_2ase was inhibited by its products, fructose-6-phosphate and Pi. However, unlike its spinach equivalent, corn Fru-2,6-P_2ase was also inhibited by 3-phosphoglycerate and, less effectively, by dihydroxyacetone phosphate. The C_4 Fru-6-P,2K and Fru-2,6-P_2ase were also quite sensitive to inhibition by phosphoenolpyruvate, and each enzyme was also selectively inhibited by certain other metabolites.

Fructose-2,6-bisphosphate (Fru-2,6-P_2)[4] is a regulatory metabolite that functions in determining the route of cytosolic carbon processing in plant cells—*i.e.* whether sucrose, the most important plant sugar, is synthesized or broken down (2, 5, 14). Our laboratory has recently described an enzyme preparation that catalyzes both the synthesis via Fru-6-P,2K (Eq. 1) and the breakdown via Fru-2,6-P_2ase (Eq. 2) of Fru-2,6-P_2 in leaves of spinach, a C_3 plant (3, 4, 12). Both the synthetic and degradatory activities in the preparation were regulated allosterically by leaf metabolites in a manner consistent with a role of chloroplasts in determining the fate of cytosolic carbohydrate flux in leaf cells.

$$\text{Fru-6-P} + \text{ATP} \xrightarrow{\text{Fru-6-P,2K}} \text{Fru-2,6-P}_2 + \text{ADP} \quad (1)$$

$$\text{Fru-2,6-P}_2 + \text{H}_2\text{O} \xrightarrow{\text{Fru-2,6-P}_2\text{ase}} \text{Fru-6-P} + \text{Pi} \quad (2)$$

Unlike C_3 plants, leaves of C_4 plants have two types of photosynthetic (parenchyma) cells—mesophyll and bundle sheath—in which there is a division of biochemical labor (8, 9). Despite comprehensive investigations on chloroplast enzymes, relatively little is known about the production and utilization of sucrose in C_4 plants (17) and even less is known about the role of Fru-2,6-P_2 in these species. We have, therefore, undertaken a study to localize the synthesis and breakdown of Fru-2,6-P_2 in *Zea mays* (corn), a classical C_4 plant. In this paper, we summarize results showing that Fru-6-P,2K and Fru-2,6-P_2ase activities are present in corn leaves. The activities, which were localized in mesophyll cells, were regulated by effector metabolites.

MATERIALS AND METHODS

Plant Material. Corn plant (*Z. mays* var Golden Cross Bantam T51; Burpee Seed Co., Warminister, PA) were grown in a greenhouse under normal day/night conditions. Plants were germinated and grown in U.C. mix and watered as needed with half-strength Hoagland solution. Leaves were generally harvested 8 to 12 weeks after planting.

Chemicals and Biochemicals. PFP (EC 2.7.1.91) used in the Fru-6-P,2K and Fru-2,6-P_2ase assays was isolated from spinach leaves as in Cséke *et al.* (5). All other biochemicals were purchased from Sigma Chemical Co. Reagents were purchased from commercial sources and were of the highest quality available.

Methods. The buffers used in this investigation were adjusted to the indicated pH at room temperature. Purification steps were carried out at 4°C.

Isolation of Fru-6-P,2K. Corn leaves, 0.5 kg, were harvested, deribbed, and homogenized in a Waring Blendor containing buffer solution made up of 50 mM Tricine-KOH (pH 7.4), 1.1 M glycerol, 14 mM 2-mercaptoethanol, and 0.5 mM PMSF (buffer A). The volume to tissue ratio was 3:1. The homogenate was filtered through a 20-μm aperture net and the filtrate was centrifuged at 45,000g for 30 min. The precipitate was discarded and PEG (mol wt 8000) was added to the supernatant fraction to a final concentration of 15% (w/v). After standing for 1 h, the slurry was centrifuged at 45,000g for 20 min. The pellet was solubilized in a buffer solution containing 50 mM Tricine-KOH (pH 7.4), 1.1 M glycerol, and 14 mM 2-mercaptoethanol (buffer B) and applied to a DEAE (DE52) cellulose column (2.5 × 20 cm) that had been equilibrated with buffer B. The column was eluted with a linear NaCl gradient prepared by mixing 150 ml each of 0 and 300 mM NaCl in buffer B; fractions of 4.5 ml were

[1] Supported by a grant-in-aid from Chevron Chemical Company. Fifth paper of a series. The earlier papers are identified in Jacquot JP, P Gadal, AN Nishizawa, BC Yee, NA Crawford, BB Buchanan 1984 Arch Biochem Biophys 228: 170–178. A preliminary account of this work has been published (Soll J, J Wötzel, BB Buchanan, 1983. Sixth International Congress on Photosynthesis, Abstracts, Brussels, Belgium, 1983, pp 112–128).

[2] Present address: Botanisches Institut der Universität München, Menzinger Strasse 67, 8000 München 19, F.R.G.

[3] Present address: Botanisches Institut der Universität Köln, II. Lehrstuhl, Gryhofstrasse 15, D-5000 Köln 41, F.R.G.

[4] Abbreviations: Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru-6-P, fructose-6-phosphate; PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; Fru-6-P,2K, fructose-6-phosphate,2-kinase; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; PFP, pyrophosphate-D-fructose-6-phosphate-1-phosphotransferase; PMSF, phenymethylsulfonyl fluoride; $I_{0.5}$, concentration of inhibitor giving 50% inhibition; $S_{0.5}$, concentration of substrate supporting half-maximal velocity.

collected. The fractions containing Fru-6-P,2K activity were combined, reprecipitated with PEG, and collected by centrifugation as described above. The precipitate, containing Fru-6-P,2K and Fru-2,6-P_2ase activities, was dissolved in buffer B and applied to a 1.0 × 15 cm hydroxyapatite column that was equilibrated and developed in this same buffer. The column was eluted with a linear Pi gradient prepared by mixing 35 ml each of 0 and 300 mM K-phosphate in buffer B; fractions of 3.0 ml were collected. The active fractions were pooled, collected by PEG precipitation as before, dissolved in 1.5 ml of 50 mM Tricine-KOH (pH 7.4) buffer containing 14 mM 2-mercaptoethanol and 6 M glycerol, and stored at −20°C. Depending on the preparation, 10 to 20 μl and 5 to 10 μl were used for the Fru-6-P,2K and Fru-2,6-P_2ase assays, respectively.

Purification of Fru-2,6-P_2ase. Fru-2,6-P_2ase was found to copurify with Fru-6-P,2K throughout the procedure described above. Following the hydroxyapatite chromatography and PEG precipitation concentration steps, the preparation was subjected to additional purification for studying Fru-2,6-P_2ase. The sample was applied to a second DE52 column (1 × 10 cm) previously equilibrated with buffer B and eluted with a linear gradient prepared by mixing 25 ml each of 0 and 250 mM NaCl in buffer B. The fractions showing Fru-2,6-P_2ase activity were pooled, concentrated, dissolved, and stored as described for the final Fru-6-P,2K step above.

Enzyme Assays. Fru-6-P,2K was assayed in a two-stage procedure by following Fru-2,6-P_2 formation enzymically with PFP (3). The Fru-6-P,2K sample was preincubated in a mixture containing 100 mM Hepes-NaOH buffer (pH 7.6), 5 mM Pi, 2 mM ATP, 5 mM $MgCl_2$, and 4 mM Fru-6-P (final volume, 0.1 ml). Aliquots were removed at different times and assayed for newly synthesized Fru-2,6-P_2 by measuring the ability to activate spinach PFP in the second part of the assay. Fru-2,6-P_2ase was assayed in a manner similar to Fru-6-P,2K by following the decrease of Fru-2,6-P_2 enzymically with PFP (4). The Fru-2,6-P_2ase sample was preincubated in a mixture containing 100 mM Hepes buffer (pH 7.0) and 150 nM Fru-2,6-P_2 in a final volume of 0.1 ml. Aliquots were removed at different times and assayed for the ability to activate spinach PFP. For both Fru-6-P,2K and Fru-2,6-P_2ase, PFP was assayed by measuring change at A_{340nm} in a reaction mixture containing, in 0.5 ml final volume, coupling enzymes: 50 μg of aldolase (EC 4.1.2.13) (0.5 unit); 4.5 μg of α-glycerophosphate dehydrogenase (EC 1.1.1.94) (0.5 unit); and 3 μg of triose-P isomerase (EC 5.3.1.1) (5 units), and the following (mM): Hepes buffer (pH 7.3), 50; $MgCl_2$, 5; tetrasodium EDTA, 1: NADH, 0.1; Fru-6-P, 1; sodium pyrophosphate, 0.2. Activities of both Fru-6-P,2K and Fru-2,6-P_2ase are expressed in either nmol Fru-2,6-P_2 hydrolyzed or nmol NADP reduced 'per min · ml of partially purified enzyme.'

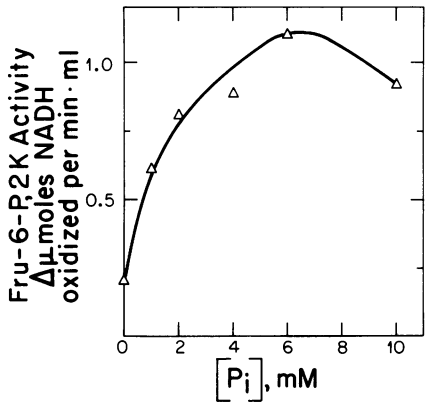

FIG. 1. Activation of corn leaf Fru-6-P,2K by Pi. Fru-6-P,2K was assayed in a two-stage procedure by following Fru-2,6-P_2 formation enzymically with PFP.

Table I. *Inhibition of Corn Leaf Fru-6-P,2K by Metabolites*

Metabolite[a]	Fru-6-P,2K Inhibition ($I_{0.5}$)	Maximal Inhibition Observed	Highest Concn. Tested
	mM	%	*mM*
3-P-glycerate	0.5	96	8.0
Oxalacetate	0.6	70	6.0
Dihydroxyacetone-P	1.0	75	5.0
P-enolpyruvate	0.05	92	2.0

[a] No effect was seen with pyruvate, malate, UDP-glucose, or sucrose. The initial Fru-6-P,2K activities observed were (Δ μmol NADH oxidized/min · ml): 3-P-glycerate (0.57); oxalacetate (0.58); dihydroxyacetone-P (0.68); and P-enolypyruvate (0.58).

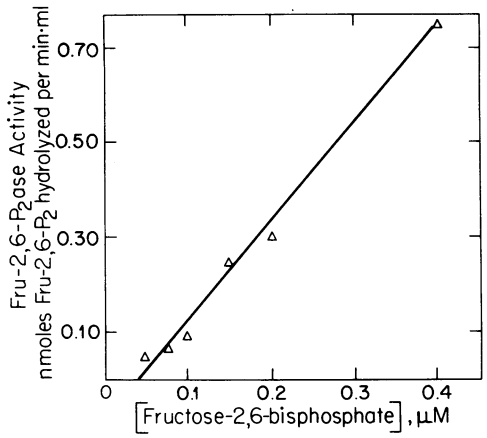

FIG. 2. Effect of Fru-2,6-P_2 on Fru-2,6-P_2ase from corn leaves. Fru-2,6-P_2ase was assayed by following Fru-2,6-P_2 disappearance enzymically with PFP.

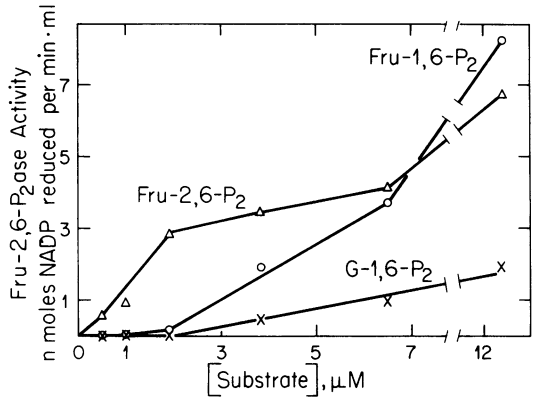

FIG. 3. Substrate specificity of corn leaf Fru-2,6-P_2ase was assayed by following the appearance of hexose-6-P from the indicated hexose bisP spectrophotometrically with a DW-2 spectrophotometer. The assay was carried out for 10 min in a 1.5-ml cuvette (light path, 1 cm) in 100 mM Hepes buffer (pH 7.0), 1 mM NADP, 1 unit phosphohexoseisomerase, 1 unit glucose-6-P dehydrogenase, 10 μl of partially pruified enzyme, and the indicated amounts of the different sugar bisphosphates. *A* at 340 nm was measured in a range of 0 to 0.02. One nmol of hexose bisP hydrolyzed corresponds to 1 nmol of NADP reduced.

Isolation of Mesophyll and Bundle Sheath Extracts from Corn Leaves. Mesophyll and bundle sheath cells were separated from corn leaves by differential homogenization steps (7). In the procedure below, 'filtration' refers to filtration through a 20-μm aperture net.

After removing midribs, 60 g leaves were cut into small pieces,

Table II. *Inhibition of Fru-2,6-P_2ase from Corn Leaves by Various Metabolites*

Compound C_4 Intermediates[a]	Fru-2,6-P_2ase Inhibition ($I_{0.5}$)	Maximal Inhibition Observed	Highest Concn. Tested
	mM	%	mM
P-enolpyruvate	0.04	100	0.25
Pyruvate	1.5	60	5.0
Malate	7.0	75	10.0
Chloroplast transport metabolites			
3-P-glycerate	0.1	100	0.5
Pi	0.25	86	5.0
Dihydroxyacetone-P	0.6	100	2.0
Other metabolites			
UDP-glucose	2.0	50	2.0
Fru-6-P	3.0	63	5.0
Mn^{2+}	0.15	80	3.0
Mg^{2+}	3.0	50	10.0

[a] No effect was observed with oxalacetate. [b] Figures correspond to concentrations required to give 50% inhibition. The initial Fru-2,6-P_2ase activity observed was (nmol Fru-2,6-P_2 hydrolyzed/min·ml): P-enolpyruvate (0.36); pyruvate (0.31); malate (0.31); 3-P-glycerate (0.33); Pi (0.28); dihydroxyacetone-P (0.40); UDP-glucose (0.31); Fru-6-P (0.68); Mn^{2+} (0.44); and Mg^{2+} (0.44).

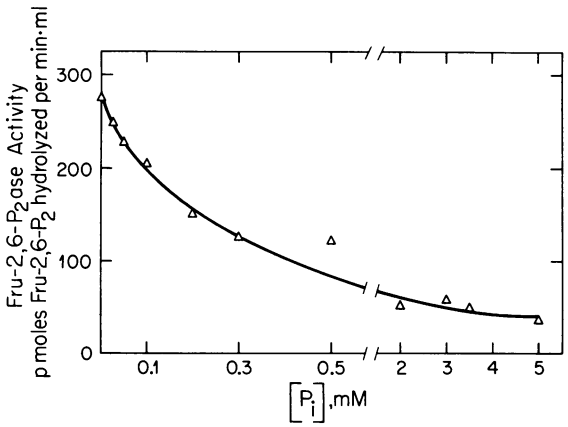

FIG. 4. Inhibition of corn leaf Fru-2,6-P_2ase by Pi. Fru-2,6-P_2ase was assayed by following Fru-2,6-P_2 disappearance enzymically with PFP.

Table III. *Cellular Localization of Fru-6-P,2K and Fru-2,6-P_2ase in Corn Leaves*

The numbers indicate the ratio of the activities of each activity in isolated mesophyll and bundle sheath cells.

	Mesophyll	Bundle Sheath
Fru-6-P,2K	11.3	1[a]
Fru-2,6-P_2ase	20	1[b]
P-enolpyruvate carboxylase	300	1[c]
NADP-malate dehydrogenase	8	1[c]
Ribulose-1,5-bisp carboxylase/oxygenase	1[c]	4.3

[a] Fru-6-P,2K activity was 1.36 pmol/min·mg protein. [b] Fru-2,6-P_2ase activity was 1.23 pmol/min·mg protein. [c] Determined immunologically by the ELISA assay.

blended in 20 g lots in 250 ml of buffer A, and filtered. Blending was accomplished by a 40-s homogenization in a Waring Blendor set at 40% voltage output. The residue was set aside for isolation of bundle sheath cells, the filtrate was adjusted to 250 ml with buffer A, and the second 20-g lot of leaves was blended in the adjusted filtrate solution. The process was repeated using the same filtrate adjusted to 250 ml with fresh buffer A until all of the leaves were blended. By reusing the filtrate solution repeatedly, dilution problems were minimized during protein precipitation. The final filtrate was centrifuged for 20 min at 48,000*g*, the precipitate was discarded, and the supernatant fraction was used as a source of mesophyll extract.

The combined residue fractions containing the bundle sheath cells from above were resuspended in 400 ml of buffer A, blended for 40 s in the Waring Blendor, 100% voltage output, and filtered. The filtrate, containing a mixture of broken mesophyll and bundle sheath cells, was discarded. The residue from this step was resuspended in 600 ml of buffer A, and 150-ml lots were subjected to homogenization for 2 × 20 s at 6 units output with a polytron, type PT 10136 (Brinkmann Instruments) regulated by a PCU1 (units 0-11) Kinematica GMBH. The homogenate was filtered and, once again, the filtrate was discarded. The residue containing the bundle sheath strands was suspended in 35 ml buffer A and broken by grinding in a mortar with sand until the mixture began to foam. The slurry was then filtered, the residue discarded, and the filtrate, greatly enriched in broken bundle sheath cells, was centrifuged for 20 min at 48,000*g*. The mesophyll and bundle sheath crude extracts were checked for cross-contamination by the ELISA (enzyme-linked immunosorbent assay) described previously (10). NADP-malate dehydrogenase (EC 1.1.1.82 and P-enolpyruvate carboxylase (EC 4.1.1.31) were used as mesophyll markers and ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) served as a marker for the bundle sheath. Generally, both extracts showed a 5 to 15% contamination with proteins of the other cell type. Prior to measuring Fru-6-P,2K and Fru-2,6-P_2ase, each of the extracts was purified by DE52 chromatography (1 × 7 cm) carried out as described above for Fru-6-P,2K. Elution was accomplished with a linear gradient prepared by mixing 10 ml each of 0 and 300 mM NaCl in buffer A. Fractions of 1 ml were collected.

Other Procedures. All antibodies were raised in rabbits. Antibody against NADP-malate dehydrogenase was prepared in earlier work (10). Ribulose-1,5-bisphosphate carboxylase/oxygenase (spinach) and P-enolpyruvate carboxylase (corn) antibodies were a gift of Prof. W. C. Taylor, Department of Genetics, University of California, Berkeley. Protein was determined by the method of Bradford (1). $I_{0.5}$ and $S_{0.5}$ values were determined from concentration curves each containing at least five points.

RESULTS

Fru-6-P,2K. Following its identification, Fru-6-P,2K was partially purified from corn leaves to relate its properties to those recently described for the C_3 (spinach) enzyme (3). As was the case for spinach (4), the Fru-6-P,2K preparation also contained Fru-2,6-P_2ase activity (see below). The preparation described was free of major interfering activities (phosphofructokinase, nonspecific phosphatase).

Partially purified corn Fru-6-P,2K showed a relatively narrow pH optimum at pH 7.5 (the activity at pH 6.5 and 8.5 was 40% and 65% of that at pH 7.5, respectively). The enzyme showed an absolute requirement for its substrates, with an $S_{0.5}$ of 1.5 mM for Fru-6-P and of 0.5 mM for ATP (Pi was 5 mM in both cases). These values are about 2-fold higher than those for spinach Fru-6-P,2K. The corn Fru-6-P,2K resembled its spinach counterpart in depending on a divalent cation, with Mn^{2+} being more effective than Mg^{2+} on a molar basis. At 1 mM, the Fru-6-P,2K activity (Δ μmol NADH oxidized/min·ml) was 1.2 for $MnCl_2$ and 0.8 for $MgCl_2$. Ca^{2+}, 0.02 to 0.1 mM, did not replace Mn^{2+} or Mg^{2+}.

As found for spinach Fru-6-P,2K (3, 12), Pi increased the total activity of the corn enzyme (Fig. 1). Pi achieved this activation by lowering the $S_{0.5}$ for its Fru-6-P and ATP substrates. The $S_{0.5}$

for Fru-6-P was 2.8, 1.5, and 1.3 mM in the presence of 1, 5, and 15 mM Pi, respectively. Also, as for spinach Fru-6-P,2K, the activation by Pi was reversed by PGA, a metabolite transported counter to Pi by chloroplasts. PGA effectively inhibited Fru-6-P,2K in both the absence and presence of Pi. Furthermore, the $I_{0.5}$ of 0.5 mM is well below PGA concentrations reported to occur in the cytoplasm of higher plants (Table I) (14).

Another metabolite transported counter to Pi by chloroplasts, DHAP, was also an inhibitor of Fru-6-P,2K (Table I). Inhibition in this case, as for spinach, was much stronger in the presence than in the absence of added Pi (12). Thus, while activity decreased up to 75% in the presence of 5 mM Pi, total inhibition was only 35% in the absence of added Pi. In these experiments, the initial Fru-6-P,2K activity (μmol NADH oxidized/min·ml) was 0.68 and 0.39 in the presence and absence of 5 mM Pi, respectively.

Typical intermediates of C_4 photosynthesis were also tested for their capacity to influence activity of corn leaf Fru-6-P,2K. Of this group, P-enolpyruvate was found to be a particularly strong inhibitor ($I_{0.5}$ of 0.05 mM) and oxalacetate a much weaker one ($I_{0.5}$ of 0.6 mM) (Table I). Pyruvate and malate did not influence Fru-6-P,2K at concentrations tested (1–10 mM). An immediate precursor of sucrose, UDP-glucose, also did not influence Fru-6-P,2K in the 0.3 to 2 mM concentration range. The effects of these various inhibitors on Fru-6-P,2K are summarized in Table I.

Fru-2,6-P_2ase. As noted above, the corn Fru-6-P,2K preparation used in these studies also contained the enzyme catalyzing the hydrolysis of Fru-2,6-P_2 (Fru-2,6-P_2ase). During purification, no separation of the Fru-2,6-P_2ase and Fru-6-P,2K was achieved. Nevertheless, as for spinach (4), it remains to be seen whether these two activities reside on a single protein as in mammalian tissues (3, 16).

Fru-2,6-P_2ase from corn was found to have a high affinity for Fru-2,6-P_2. Activity of the preparation used was linear in the 0.05 to 2 μM range (Figs. 2 and 3) but became nonlinear at higher Fru-2,6-P_2 concentrations (Fig. 3). Up to a concentration of 4 μM, Fru-2,6-P_2ase activity was greater with Fru-2,6-P_2 as substrate than with Fru-1,6-P_2 or glucose-1,6-bisP (Fig. 3). Only at concentrations greater than 5 μM did the preparation show significant activity with either of the latter substrates. These results (obtained in the absence of Mg^{2+}) suggested that Fru-2,6-P_2ase is an enzyme different from Fru-1,6-P_2ase, and other results strengthened this conclusion (see below). A similar substrate-selective Fru-2,6-P_2ase was recently isolated from corn seedlings (11).

The corn Fru-2,6-P_2ase was inhibited by metabolites which also inhibited the corresponding Fru-6-P,2K—*i.e.* P-enolpyruvate, DHAP, and PGA. Substances which decreased Fru-2,6-P_2ase activity but were without effect on Fru-6-P,2K were pyruvate, UDP-glucose, and malate. The effects of the various metabolites on Fru-2,6-P_2ase are summarized in Table II. It may be noted that the corn Fru-2,6-P_2ase is especially sensitive to a metabolite important in the C_4 cycle, *viz.* P-enolpyruvate. It is also significant that corn Fru-2,6-P_2ase was, in contrast to its spinach counterpart, inhibited by PGA and, less effectively, by DHAP (*cf.* 12). Both the corn and spinach enzymes are sensitive to inhibition by Pi (Fig. 4) (4, 12).

Cellular Localization of Fru-6-P,2K and Fru-2,6-P_2ase. The finding of enzymes catalyzing the synthesis and hydrolytic breakdown of Fru-2,6-P_2 in corn leaves raises the question of their cellular location—*i.e.* whether they are present in the mesophyll or bundle sheath. We therefore prepared cell-free extracts from each of these two cell types and assayed them for Fru-6-P,2K and Fru-2,6-P_2ase activities. Fru-6-P,2K and Fru-2,6-P_2ase activities were found mainly in mesophyll cells (Table III). The low activity of the enzymes found in the bundle sheath can be ascribed to contaminating mesophyll proteins as evidenced by the recovery of marker enzymes of that tissue detected immunologically (*i.e.* P-enolpyruvate carboxylase and NADP-malate dehydrogenase). The results suggest that both the synthesis and degradation of Fru-2,6-P_2 in corn leaves take place in mesophyll cells. The recent demonstration that Fru-2,6-P_2 is localized exclusively in the mesophyll supports this view (Stitt and Heldt, see "Note Added in Proof").

CONCLUDING REMARKS

The present findings provide evidence that enzymes catalyzing the synthesis and hydrolysis of Fru-2,6-P_2 are present in leaves of corn, a classical C_4 plant. The synthetic enzyme, Fru-6-P,2K, and the degradative enzyme, Fru-2,6-P_2ase, were regulated by metabolite effectors in a manner generally similar to their C_3 spinach counterparts (2–4). Thus, Pi was a strong activator of Fru-6-P,2K and an inhibitor of Fru-2,6-P_2ase. DHAP and PGA inhibited Fru-6-P,2K but, unlike spinach, they inhibited corn Fru-2,6-P_2ase as well. P-enolpyruvate, a metabolite important in C_4 photosynthesis, strongly inhibited both of the corn enzymes. Corn Fru-2,6-P_2ase was also considerably less sensitive to inhibition by Fru-6-P than was the corresponding spinach enzyme ($I_{0.5}$ of 3.0 mM *versus* $I_{0.5}$ of 0.3 mM for the spinach Fru-2,6-P_2ase). Finally, with corn, certain metabolites selectively inhibited only one enzyme—oxalacetate (Fru-6-P,2K), pyruvate, UDP-glucose, and Fru-6-P (Fru-2,6-P_2ase).

Of particular interest in the current study is the finding that both Fru-6-P,2K and Fru-2,6-P_2ase are localized in mesophyll cells and are apparently absent in the bundle sheath. Such a distribution of the system would align cytosolic functions of C_4 mesophyll cells with processes taking place largely in the cytosol of parenchyma cells of C_3 plants. These processes would include the synthesis of sucrose which, in contrast to current concepts (17), now seems to take place mainly in the mesophyll cells of C_4 plants (Stitt and Heldt, see "Note Added in Proof"). Furthermore, because of the nature of the photosynthetic carbon reactions taking place in the mesophyll, the C_4 Fru-6-P,2K and Fru-2,6-P_2ase may have become adapted to regulation by metabolites that are generally ineffective in regulating these enzymes in C_3 plants.

Note Added in Proof. While this article was at the printer, the above referred to paper of M. Stitt and H.W. Heldt was accepted for publication (Regulation of photosynthetic sucrose synthesis by fructose-2,6-bisphosphate. IV. Intercellular metabolite distribution and properties of cytosolic fructosebisphosphatase in maize leaves. Planta. In press, 1985). Also, it was brought to our attention that H. Usuda and G.E. Edwards earlier concluded that some enzymes of sucrose synthesis in C_4 leaves are enriched in mesophyll tissue (1980 Localization of glycerate kinase and some enzymes for sucrose synthesis in C_3 and C_4 plants. Plant Physiol 65: 1017–1022).

LITERATURE CITED

1. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–256
2. Cséke C, Á Balogh, JH Wong, BB Buchanan, M Stitt, B Herzog, HW Heldt 1984 Fructose-2,6-bisphosphate: a regulator of carbon processing in leaves. Trends Biochem Sci 9: 533–535
3. Cséke C, BB Buchanan 1983 An enzyme synthesizing fructose-2,6-bisphosphate occurs in leaves and is regulated by metabolite effectors. FEBS Lett 155: 139–142
4. Cséke C, M Stitt, A Balogh, BB Buchanan 1983 A product-regulated fructose-2,6-bisphosphatase occurs in green leaves. FEBS Lett 162: 103–106
5. Cséke C, NF Weeden, BB Buchanan, K Uyeda 1982 A special fructose bisphosphate functions as a cytoplasmic regulatory metabolite in green leaves. Proc Natl Acad Sci USA 79: 4322–4326
6. El-Maghrabi MR, TH Claus, J Pilkis, E Fox 1982 Regulation of rat liver fructose-2,6-bisphosphatase. J Biol Chem 257: 7603–7607
7. Ghirardi M, A Melis 1983 Localization of photosynthetic electron transport

components in mesophyll and bundle sheath chloroplasts of *Zea mays*. Arch Biochem Biophys 224: 19–28
8. HATCH MD 1978 Regulation of enzymes in C_4 photosynthesis. *In* BL Horecker, ER Stadtman, eds, Current Topics in Cellular Regulation. Academic Press, New York, p 1
9. KELLY GJ, G ZIMMERMANN, E LATZKO 1976 Regulatory aspects of photosynthetic carbon metabolism. Annu Rev Plant Physiol 27: 181–205
10. PERROT-RECHENMANN C, JP JACQUOT, P GADAL, NF WEEDEN, C CSÉKE, BB BUCHANAN 1983 Localization of NADP-malate dehydrogenase of corn leaves by immunological methods. Plant Sci Lett 30: 219–226
11. SMYTH DA, MX WU, CC BLACK 1984 Phosphofructokinase and fructose-2,6-bisphosphatase activities in developing corn seedlings. Plant Sci Lett 33: 61–70.
12. STITT M, C CSÉKE, BB BUCHANAN 1984 Regulation of fructose-2,6-bisphosphate concentration in spinach leaves. Eur J Biochem 143: 89–93
13. STITT M, R GERHARDT, K KÜRZEL, HW HELDT 1983 A role for fructose-2,6-bisphosphate in the regulation of sucrose synthesis in spinach leaves. Plant Physiol 72: 1139–1141
14. STITT M, G MIESKES, H-D SÖLING, HW HELDT 1982 On a possible role of fructose-2,6-bisphosphate in regulating photosynthetic metabolism in leaves. FEBS Lett 145: 217–222
15. STITT M, W WIRTZ, HW HELDT 1980 Metabolite levels during induction in the chloroplast and extrachloroplast compartments of spinach protoplasts. Biochim Biophys Acta 593: 85–102
16. VAN SCHAFTINGEN E, DR DAVIES, H-G HERS 1982 Fructose-2,6-bisphosphatase from rat liver. Eur J Biochem 124: 143–149
17. WITTINGHAM CP, JJ KEYS, IF BIRD 1979 The enzymology of sucrose synthesis in leaves. *In* M Gibbs, E Latzko, eds, Photosynthesis II. Photosynthetic Carbon and Related Processes. Springer-Verlag, New York, pp 313–316
18. Deleted in proof.