An enzyme synthesizing fructose 2,6-bisphosphate occurs in leaves and is regulated by metabolite effectors

Csaba Cséke[+] and Bob B. Buchanan*

Division of Molecular Plant Biology, Hilgard Hall, University of California, Berkeley, CA 94720, USA

Received 22 March 1983

An enzyme catalyzing the ATP and fructose 6-phosphate-dependent synthesis of fructose 2,6-bisphosphate, a regulator of glycolysis and gluconeogenesis, has been identified and partially purified from plants, specifically the cytoplasmic fraction of spinach leaf parenchyma cells. The enzyme, designated fructose 6-phosphate,2-kinase, showed no response to a protein phosphorylation system known to inhibit the corresponding enzyme in mammalian cells, but it responded strikingly to metabolite effectors (P_i, an activator/PGA, an inhibitor) through changes in substrate affinity and maximal velocity. The observed pattern of regulation suggests a role for chloroplasts in controlling cytoplasmic carbon processing.

Fructose 2,6-bisphosphate *Fructose 6-phosphate,2-kinase* *Sucrose synthesis* *Gluconeogenesis* *Glycolysis* *Enzyme* *Regulation*

1. INTRODUCTION

One of the recent advances in biochemistry concerns the identification of fructose 2,6-bisphosphate (Fru-2,6-P_2) as a regulatory metabolite first in animals [1,2] and then in plants [3–5]. In [4] we reported that Fru-2,6-P_2 functions in the cytoplasm of leaf parenchyma cells in regulating the synthesis and breakdown of sucrose, an important plant sugar. Until now, nothing was known about the enzyme synthesizing Fru-2,6-P_2 in plants; i.e., either its cellular location or its regulation.

We now report evidence that this enzyme, fructose 6-phosphate,2-kinase (Fru-6-P,2K), which synthesizes Fru-2,6-P_2 from fructose 6-phosphate and ATP (eq.1) [6–8] also occurs in leaves and that, like Fru-2,6-P_2, it is localized in the cytoplasm:

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

Evidence is also presented that leaf Fru-6-P,2K is a regulatory enzyme under the control of metabolite effectors that are transported by chloroplasts [9]. P_i, a chloroplast import, serves as an activator, and 3-phosphoglycerate (PGA), a chloroplast export, serves as an inhibitor. The results are consistent with the conclusion that chloroplasts function in regulating the synthesis of Fru-2,6-P_2 via these transported metabolites and thereby direct the flow of carbon in the cytoplasm.

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) was purchased from a local market. Fructose 6-phosphate, Fru-2,6-P_2,

[+] On leave from: Institute of Plant Physiology, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

* To whom correspondence should be addressed

Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P,2K, fructose 6-phosphatase,2-kinase; PEG, polyethyleneglycol; PGA, 3-phosphoglycerate; PFP, pyrophosphate-linked phosphofructokinase (EC 2.7.1.90)

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

ATP, beef heart protein kinase catalytic subunit and polyethylene glycol (PEG) (M_r ~8000) was obtained from Sigma. Other reagents were obtained from commercial sources and were of the highest purity available.

Fru-6-P,2K was purified at 4°C from spinach leaf homogenates prepared as in [4] except that 1 kg leaf material was blended in 1.5 l of a different buffer (50 mM Tris–HCl buffer (pH 7.8) containing 10% glycerol and 0.1% β-mercaptoethanol; buffer A). To the 27000 × g supernatant fraction an appropriate volume of 50% PEG solution was added to bring the final concentration to 8%. After 1 h incubation, the precipitate that formed was removed by centrifugation (20 min, 13000 × g) and discarded. [PEG] of the supernatant fraction was increased to 12%, the precipitate was collected as above and dissolved in 100 ml buffer A. Further purification of the enzyme was achieved by chromatography on a hydroxyapatite column (2.6 × 20 cm) using a linear 0–0.3 M sodium phosphate gradient, followed by DEAE-cellulose (DE52) chromatography (1.6 × 15 cm column) using a linear 0–0.25 M NaCl gradient, all in buffer A. The preparation was essentially free of phosphofructokinase and fructose 1,6-bisphosphatase and contained <5% of pyrophosphate-linked phosphofructokinase (PFP) activity relative to Fru-6-P,2K. After concentration against 99% glycerol, the enzyme could be stored at −20°C for 1 week without detectable loss of activity.

PFP was assayed as in [4]; protein was determined as in [10].

3. RESULTS AND DISCUSSION

As found for Fru-2,6-P_2 [4], Fru-6-P,2K was localized in the cytoplasmic fraction of leaf parenchyma cells. Like its liver counterpart, leaf Fru-6-P,2K was substrate-specific and showed no activity when fructose-6-phosphate was replaced by glucose 6-phosphate, glucose 1-phosphate, fructose 1,6-bisphosphate or when ATP was replaced by ADP, UDP or UTP.

We quickly observed that Fru-6-P,2K rapidly lost activity during purification. While partly due to enzyme instability, much of this loss resulted from removal of an activator, identified as P_i, that was present in crude preparations. Thus, when tested under our standard assay conditions, purified Fru-6-P,2K showed little or no activity in the absence of P_i (fig.1). P_i was found to act by increasing enzyme affinity (lowering the $S_{0.5}$) for ATP and fructose 6-phosphate substrates and by increasing V_{max} (table 1). In the case of fructose 6-phosphate, P_i also changed the kinetics from sigmoidal to hyperbolic (Michaelian) type. Significantly, P_i was effective at concentrations generally believed to be physiological in plant systems [5,11].

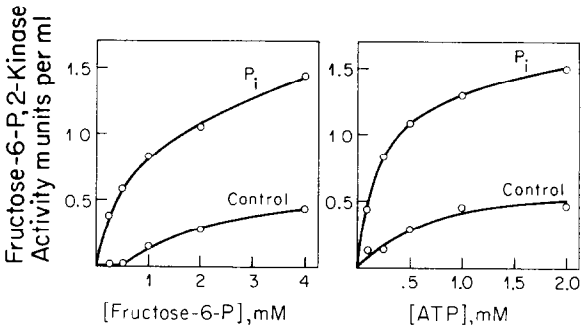

Fig.1. Effect of P_i on the activity of cytoplasmic Fru-6-P,2K from spinach leaves. The enzyme was assayed at 20°C in a 1.0 × 7.5 cm test tube containing (mM): Tris–HCl buffer (pH 7.4), 50; fructose 6-phosphate, 2 (unless indicated otherwise); ATP, 1 (unless indicated otherwise); $MgCl_2$, 5; and, as indicated, P_i, 5; final vol., 0.1 ml. The reaction was started by adding 10 μl (12 μg) purified Fru-6-P,2K. At zero time, 3 and 6 min, an aliquot of the Fru-6-P,2K reaction mixture was analyzed for Fru-2,6-P_2 by the PFP method in [4]. The aliquot of the newly synthesized Fru-2,6-P_2 was added to a cuvette (1 cm, 1 ml capacity) containing 15 munits spinach leaf PFP and the following (mM): HEPES–NaOH buffer (pH 7.3), 50; $MgCl_2$, 5; tetrasodium ethylene diamine tetracetic acid (EDTA), 1; NADH, 0.1; fructose 6-phosphate, 1; inorganic pyrophosphate, 1; and coupling enzymes: 10 μg aldolase (1 unit); 8 μg α-glycerophosphate dehydrogenase (1 unit); and 6 μg triose phosphate isomerase (10 units). The amount of Fru-2,6-P_2 synthesized was calculated by comparing the extent of PFP activation in this assay to that effected by known amounts of Fru-2,6-P_2. One unit of Fru-6-P,2K activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol Fru-2,6-P_2/min. Fru-2,6-P_2 synthesized by spinach Fru-6-P,2K was identified by its capability to activate PFP, by its acid lability (15 min, 0.1 N HCl, 30°C), and by its stability in the presence of aldolase.

Table 1

Effect of P_i on substrate affinity and maximal velocity of cytoplasmic Fru-6-P,2K from spinach leaves

Treatment	Substrate required for half-maximal activity ($S_{0.5}$)		Maximal velocity (V_{max})
	Fructose-6-P (mM)	ATP (mM)	(munits/ml)
Control	5.8	1.25	1.0
P_i (5 mM)	0.64	0.26	1.5

Constants were calculated from the data in fig.1 by Lineweaver-Burk analysis

Because of its importance in cellular reactions, the finding that P_i plays a role in regulating cytoplasmic Fru-6-P,2K is of considerable interest. The chloroplast envelope contains a translocator that promotes the transport of P_i into chloroplasts and of certain C_3 metabolites in the reverse direction; e.g., PGA or glyceraldehyde-3-phosphate [9]. It appears that translocation of these C_3 compounds is the major route by which chloroplasts provide energy-rich products of photosynthesis to the cytoplasm and, at the same time, retrieve P_i for future use in carbon assimilation. Accordingly, any enzyme of the cytoplasm that is strongly influenced by P_i or one of these C_3 metabolites would be under control of the chloroplast.

With this perspective, it became interesting to know whether a transported C_3 metabolite functions in a manner opposite to that of P_i; i.e., to inhibit Fru-6-P,2K. The results in fig.2 show this to be the case: PGA, a metabolite transported counter to P_i by the translocator, inhibited the P_i-activated enzyme. Concomitant with the inhibition by PGA was a change in kinetic constants of Fru-6-P,2K back to the original (control) values (not shown). In a separate experiment (table 2) we observed that the PGA inhibition of Fru-6-P,2K was reversed by P_i. Interestingly, the other C_3 metabolites transported by the translocator (glyceraldehyde-3-phosphate and dihydroxyacetone phopshate) had no effect on Fru-6-P,2K in the concentration range effective for PGA. Also without effect were covalent modification agents of the redox type functional for the regulation of key chloroplast enzymes [12] (reduced thioredoxins/oxidized disulfides) and of the phosphorylation type functional in the regulation of mammalian Fru-6-P,2K [13] (beef heart protein kinase catalytic subunit). Thus, although P_i seems to be an effector of mammalian Fru-6-P,2K [8], capacity for regulation via phosphorylation is an important distinction between the Fru-6-P,2K enzymes of plant and animal sources. It is also worth noting that Fru-6-P,2K, an enzyme important in controlling sucrose processing in the cytoplasm, is regulated in a manner antipodal to ADP-glucose pyrophosphorylase, an enzyme functional in starch synthesis in chloroplasts that is activated by PGA and inhibited by P_i [14].

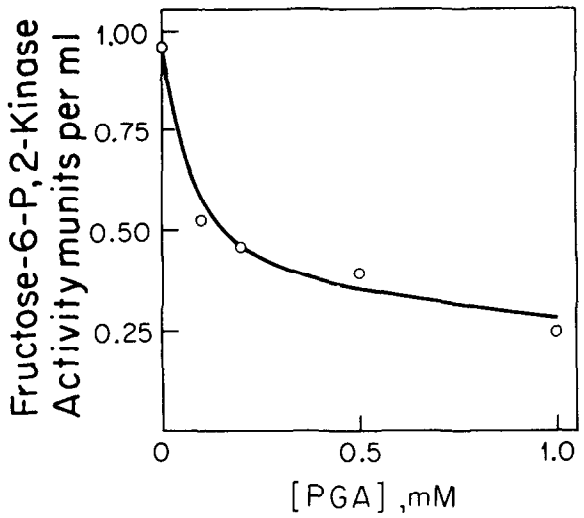

Fig.2. Inhibition of P_i-activated cytoplasmic Fru-6-P,2K from spinach leaves by PGA. Except for adding PGA as indicated, Fru-6-P,2K was assayed in the presence of 5 mM P_i as in fig.1.

Table 2

Reversal of PGA inhibition of spinach leaf cytoplasmic Fru-6-P,2K by P_i

Treatment	Fru-6-P,2K (munits/ml)	%
Control	0.51	100
0.2 mM PGA	0.15	29
0.2 mM PGA + 2 mM P_i	0.56	110
2 mM P_i	0.78	153

Except for adding PGA and P_i as indicated, Fru-6-P,2K was assayed as in fig.1

The reversible regulation of leaf cytoplasmic Fru-6-P,2K by P_i and PGA indicates that chloroplasts may function in modulating Fru-6-P,2K-linked enzymes and in that way determine the fate of photosynthetically fixed carbon that is transported to the cytoplasm and processed there. Accordingly, extensive export of chloroplast metabolites into the cytoplasmic PGA pool would stimulate sucrose synthesis via inhibition of Fru-6-P,2K, whereas elevated P_i levels, resulting from sucrose synthesis and other P_i-liberating processes in the cytoplasm, would stimulate Fru-6-P,2K and thereby accelerate sucrose breakdown. Thus, Fru-2,6-P_2 seems to be an important factor in regulating the accumulation of sucrose by controlling both its synthesis (via inhibition of cytoplasmic fructose 1,6-bisphosphatase) and its breakdown (via stimulation of PFP):

$$\text{Sucrose} \underset{\text{PFP}}{\overset{\text{Fru-2,6-P}_2\text{ inhibits}}{\overset{\text{fructose bisphosphatase}}{\rightleftharpoons}}} \text{Triose phosphates} \quad (2)$$

Fru-2,6-P_2 stimulates

These results show that an enzyme synthesizing Fru-2,6-P_2 is present in plants. This enzyme, Fru-6-P,2K, is located in the cytoplasm of photosynthetic cells of spinach leaves and is under allosteric regulation by key metabolites formed and transported by chloroplasts and conceivably other leaf organelles as well (viz., vacuoles and mitochondria). In this manner, chloroplasts may 'sense' the overall metabolic needs of the plant and thereby play a rôle in determining the rate of sucrose synthesis. It remains to be seen how the regulation of Fru-2,6-P_2 degradation fits into the overall pattern of carbon flow. A related unanswered question is the extent to which PFP functions in the reverse direction [15,16] and thereby complements fructose bisphosphatase in the synthesis of sucrose.

ACKNOWLEDGEMENTS

We acknowledge the counsel of Professor K. Uyeda during the course of this work. This work was supported by a grant from the National Science Foundation.

REFERENCES

[1] Furuya, E. and Uyeda, K. (1980) Proc. Natl. Acad. Sci. USA 77, 5861–5864.
[2] Van Schaftingen, F., Hue, L. and Hers, H.G. (1980) Biochem. J. 192, 887–895.
[3] Sabularse, D.C. and Anderson, R.L. (1981) Biochem. Biophys. Res. Commun. 103, 848–855.
[4] Cséke, C., Weeden, N.F., Buchanan, B.B. and Uyeda, K. (1982) Proc. Natl. Acad. Sci. USA 79, 4322–4326.
[5] Stitt, M., Mieskes, G., Söling, H.-D. and Heldt, H.W. (1982) FEBS Lett. 145, 217–222.
[6] Furuya, E. and Uyeda, K. (1981) J. Biol. Chem. 256, 7109–7112.
[7] El-Maghrabi, M.R., Claus, T.H., Pilkis, J. and Pilkis, S.J. (1981) Biochem. Biophys. Res. Commun. 101, 1071–1077.
[8] Van-Shaftingen, E. and Hers, M.G. (1981) Biochem. Biophys. Res. Commun. 101, 1078–1084.
[9] Heber, U. and Heldt, H.W. (1981) Annu. Rev. Plant Physiol. 32, 139–168.
[10] Bradford, M.M. (1976) Anal. Biochem. 72, 248–256.
[11] Stitt, M., Wirtz, W. and Heldt, H.W. (1980) Biochim. Biophys. Acta 593, 85–102.
[12] Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[13] Furuya, E., Yokoyama, M. and Uyeda, K. (1982) Proc. Natl. Acad. Sci. USA 79, 325–329.
[14] Preiss, J. (1982) Annu. Rev. Plant Physiol. 33, 431–454.
[15] Reeves, R.E. (1976) Trends Biochem. Sci. 1, 53–55.
[16] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) Eur. J. Biochem. 129, 191–195.