Fructose 2,6-bisphosphate in relation with the resumption of metabolic activity in slices of Jerusalem artichoke tubers

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When slices of Jerusalem artichoke tubers were incubated at 25°C, their concentration in fructose 2,6-bisphosphate increased up to 250-fold within 2 h. Fructose 2,6-bisphosphate was also formed, although at a slower rate, in slices incubated at 0°C. Its formation could not be explained by an increase in the concentration of fructose 6-phosphate or of ATP either by an activation of phosphofructo-2-kinase. Pyrophosphate–fructose-6-phosphate 1-phosphotransferase was the only enzyme present in a tuber extract which was found to be sensitive to fructose 2,6-bisphosphate. An improved procedure for the assay of fructose 2,6-bisphosphate is also reported.

Fructose 2,6-bisphosphate *Phosphofructokinase* *Jerusalem artichoke* *Tuber*

1. INTRODUCTION

We have recently reported a dramatic but transient formation of Fru-2,6-P_2 in fungal spores that were incubated at 25°C in a culture medium immediately after the breaking of dormancy by a heat-shock or by other means [1]. We here explore the possibility that a similar increase in Fru-2,6-P_2 concentration would characterize the resumption of metabolic activity in another biological system. Our attention has been drawn to Jerusalem artichoke tubers whose metabolic activity, like that of other tubers and plant storage organs, is resumed upon incubation of slices at 25°C. This activation consists of a progressive rise in the respiration rate as well as of a delayed increase in the rate of protein synthesis (review [2]). It was reported to be preceded by a drop in cyclic AMP concentration [3]. By analogy with the ability of cyclic AMP to cause the destruction of Fru-2,6-P_2 in the liver (review [4]), this situation may be considered favorable to the formation of Fru-2,6-P_2.

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Abbreviations: PFK 1,6-phosphofructo-1-kinase; PFK 2,6-phosphofructo-2-kinase; PP_i-PFK, pyrophosphate–fructose-6-phosphate 1-phosphotransferase

2. MATERIALS AND METHODS

2.1. *Materials*

Jerusalem artichoke tubers were purchased in December from a local producer and stored at 4°C. Most experiments were performed in March. Indole 3-acetic acid, gibberellic acid and beef heart cyclic AMP-dependent protein kinase were from Sigma (St Louis); cyclic AMP, dibutyryl cyclic AMP and other biochemicals were from Boehringer (Mannheim). Fru-2,6-P_2 was prepared as in [5].

2.2. *Preparation and incubation of slices and of extracts*

Slices of 2 cm^2 section and weighing about 0.3 g were prepared from a cylindrical piece cut along the grand axis of the tubers. Unless otherwise stated, they were rinsed with water, blotted on paper and incubated with vigorous shaking at a concentration of 1 slice per 2 ml water. At the

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times indicated, the slices were removed from the vials, blotted on paper and frozen by immersion in liquid nitrogen. They were kept at −20°C until further processing.

The frozen slices were homogenized in the cold with a Potter Elvehjem device in 10 vols of either 0.05 M NaOH (for measurement of Fru-2,6-P_2), or 5% (w/v) perchloric acid (for measurement of Glc-6-P and ATP). For the assay of enzymes, tubers were peeled and immediately homogenized in a Waring blendor with 3 vols of an ice-cold buffer containing 1 mM dithiothreitol, 20 mM K–acetate and 25 mM Hepes (pH 7.1). Extracts were obtained by centrifuging the homogenates for 10 min at 10000 × *g*.

2.3. *Assay of Fru-2,6-P_2 and of other phosphoric esters*

Fru-2,6-P_2 was assayed by a modification of the method of Van Schaftingen et al. [6] based on the stimulation of potato tuber PP_i-PFK. We have recently discovered that commercial Fru-6-P was contaminated by about 1 ppm Fru-2,6-P_2 identified by its property to stimulate PP_i-PFK, its acid-lability and its behavior upon chromatography on Dowex AG-1X8 performed as in [7]. This contamination was responsible for the greatest part of the blank of the method. To remove it, a 0.1 M solution of Fru-6-P was mixed with an equivalent amount of HCl to reach pH 2, maintained at 30°C for 30 min and then neutralized with NaOH. A second modification, which became possible once the blank had been lowered, was to substitute 50 mM Tris–acetate (pH 7.8) for 50 mM Tris–HCl (pH 8) and 2 mM Mg–acetate for 5 mM $MgCl_2$ in the assay mixture. This modification increased the sensitivity of the test; half-maximal activity was then obtained with about 1.5 nM Fru-2,6-P_2 with a blank value amounting to only 2% of the rate reached with a saturating amount of Fru-2,6-P_2. Fig.1 shows a standard curve obtained under such conditions. Glc-6-P [8] and ATP [9] were measured as described. Concentrations are expressed per g wet weight.

2.4. *Assay of enzymes*

PP_i-PFK was assayed by the production of Fru-1,6-P_2 in the presence of 50 mM Tris–acetate (pH 7.8), 2 mM Mg–acetate, 0.15 mM NADH, 1 mM PP_i, as well as auxiliary enzymes at the concentrations given previously [6]; except when indicated, 5 μM Fru-2,6-P_2 and 5 mM Fru-6-P were also present. PFK 1 was assayed under the same conditions except that 1 mM PP_i was replaced by 1 mM ATP and that Fru-2,6-P_2 was omitted. PFK 2 was assayed by the production of Fru-2,6-P_2 in the presence of 50 mM K–acetate, 25 mM Hepes (pH 7.1), 2 mM Mg–acetate, 0.5 mM dithiothreitol, 5 mM P_i, 1 mM Fru-6-P and 2 mM ATP. For this assay as well as for that of PP_i-PFK, Glc-6-P was included at 3.5-fold the concentration of Fru-6-P. Fructose-1,6-bisphosphatase was assayed spectrophotometrically as in [10]. All assays were performed at 25°C. One unit of enzymes catalyzes the conversion of 1 μmol of substrate per min under these conditions.

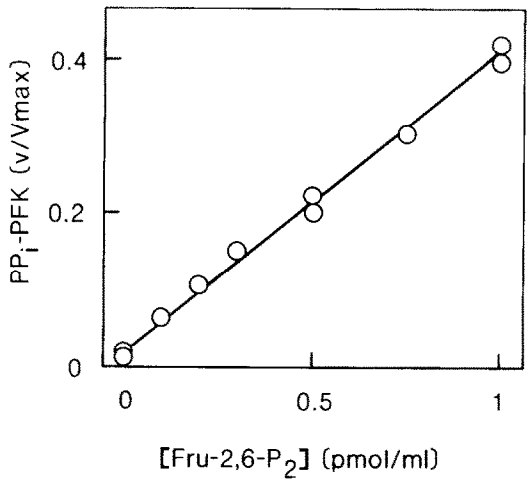

Fig.1. Standard curve for the assay of Fru-2,6-P_2.

3. RESULTS

3.1. *Concentration of Fru-2,6-P_2, Glc-6-P and ATP in slices of Jerusalem artichoke tubers*

The concentration of Fru-2,6-P_2 in dormant tubers kept at 4°C was consistently low (0.01–0.22 nmol/g) from January to April. In half of the tubers kept until June, this concentration reached 0.5–1 nmol/g. This concentration was the same in freshly cut tissue but increased upon incubation of the slices even at 0°C, and in a temperature-dependent manner (fig.2). After 2 h at 25°C, the concentration of Fru-2,6-P_2 reached 2.6 (fig.2,4) to 6 (fig.3) nmol per g, i.e., up to 250-fold the initial value. There was then a slow

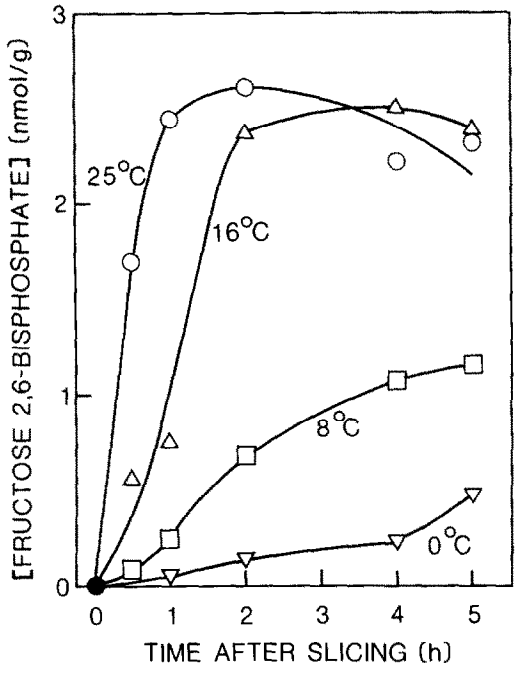

Fig.2. Concentration of Fru-2,6-P_2 in slices of Jerusalem artichoke tuber incubated at different temperatures. For practical convenience, the slices were not shaken. The initial concentration of Fru-2,6-P_2 was 0.01 nmol/g.

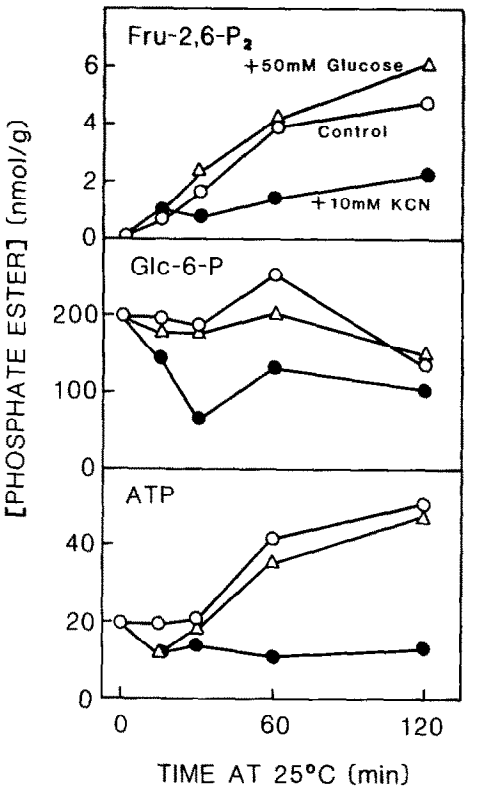

Fig.3. Concentration of Fru-2,6-P_2, Glc-6-P and ATP in slices of Jerusalem artichoke tuber incubated at 25°C in the presence of 50 mM glucose or 10 mM KCN or without addition. The initial concentration of Fru-2,6-P_2 was 0.15 nmol/g.

and continuous decline (fig.4). A similar increase in Fru-2,6-P_2 concentration was obtained without shaking the slices (fig.2) and when freshly cut unwashed slices were maintained in a wet atmosphere without incubation medium.

Assuming that Fru-2,6-P_2 is formed in Jerusalem artichoke tubers like in the liver [4], from Fru-6-P and ATP, it was of interest to verify if the biosynthesis of Fru-2,6-P_2 could be related to the appearance of one of its two precursors. Experiments in which hexose 6-phosphates and ATP were measured together with Fru-2,6-P_2 are illustrated in fig.3,4. In 2 out of 6 experiments of this type, Glc-6-P concentration did not change significantly (see fig.3); in other experiments, illustrated by fig.4, this concentration increased transiently up to 2-fold. It has been verified that the concentration of Fru-6-P was about 25% of that of Glc-6-P, as expected from the equilibrium of phosphohexoisomerase. In the long-scale experiment, shown in fig.4, there was no parallelism between the late changes in concentration of Fru-2,6-P_2 and of hexose 6-phosphates. The concentration of ATP remained stable during the first 30 min and increased 2–3-fold during the following 60 min. The presence of glucose (fig.3), fructose or sucrose (not shown) had no marked effect on the biosynthesis of Fru-2,6-P_2 in the slices nor on the concentration of Glc-6-P or ATP. The presence of 10 μM 2,4-dinitrophenol or the replacement of air by nitrogen in the gas phase also had little effect (not shown). The addition of KCN decreased the concentration of ATP as well as the rate of synthesis of Fru-2,6-P_2 by about one half (fig.3). The addition of 10 μM indole 3-acetic acid, gibberellic acid, cyclic AMP, or dibutyryl cyclic AMP to the incubation medium had no detectable effect on the parameters studied (not shown).

Preliminary experiments have indicated that there was also an increase in Fru-2,6-P_2 concentration in potato tuber slices incubated at 25°C. The

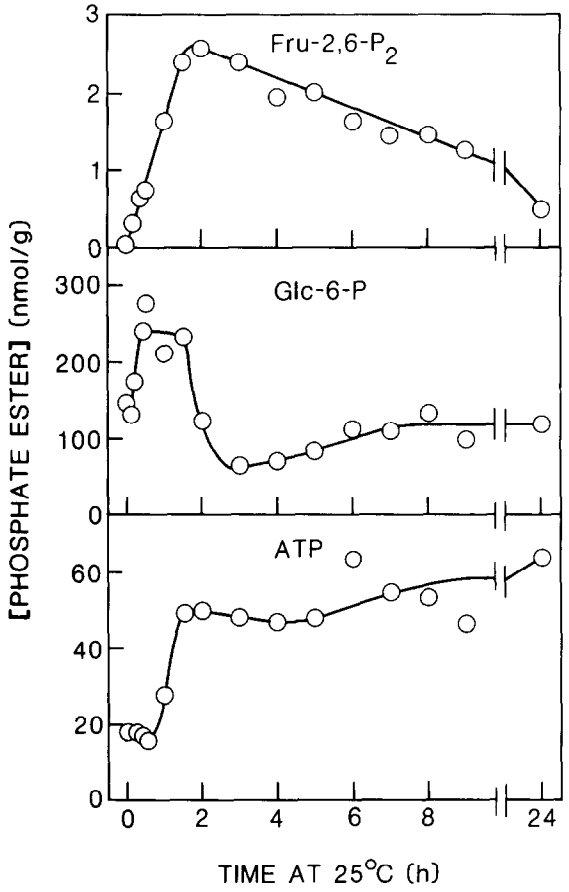

Fig.4. Concentration of Fru-2,6-P_2, Glc-6-P and ATP in slices of Jerusalem artichoke tuber incubated at 25°C. The initial concentration of Fru-2,6-P_2 was 0.05 nmol/g.

concentration reached, however, did not exceed 0.5 nmol per g.

3.2. *Effect of Fru-2,6-P_2 on Jerusalem artichoke enzymes*

Jerusalem artichokes contain about 0.15 unit of PFK 1 per g and the activity of that enzyme, even when measured at subsaturating concentrations of Fru-6-P and at pH 7, was not affected by the presence of Fru-2,6-P_2 (not shown). The tissue contains about 0.08 units/g of PP_i-PFK. As previously reported for the same enzyme obtained from other plants [6,11] this activity was greatly dependent upon the presence of Fru-2,6-P_2. However, the K_a for that effector was about 20 nM (fig.5), i.e., 8-fold greater than for the potato

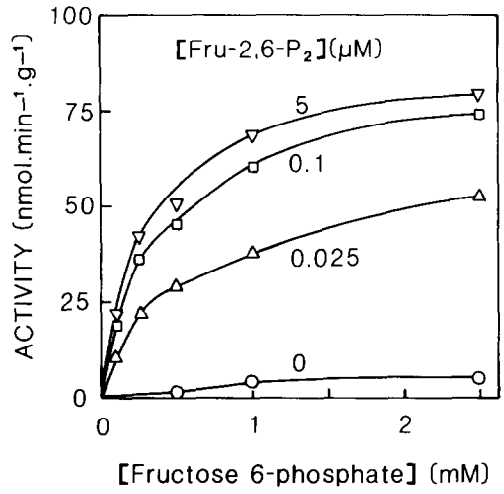

Fig.5. Effect of Fru-2,6-P_2 on the saturation curve of Jerusalem artichoke tuber PP_i-PFK for Fru-6-P.

tuber enzyme measured under the same conditions (not shown). Another striking difference with the properties previously reported for the potato enzyme (see fig.1 in [6]) was that the artichoke enzyme was almost inactive in the absence of Fru-2,6-P_2 (fig.5). This is explained by the fact that the batch of Fru-6-P used in [6] was contaminated by 1 ppm Fru-2,6-P_2 (see section 2). In the absence of this contaminant, the potato enzyme was also very poorly active (results not shown) with a V_{max} amounting to about 10% of that measured in the presence of a saturating concentration of Fru-2,6-P_2.

No indication was obtained in favor of the presence of a magnesium-dependent specific fructose-1,6-bisphosphatase in extracts of Jerusalem artichoke tubers.

3.3. *Properties of PFK 2 from Jerusalem artichoke tubers*

Jerusalem artichoke tubers contained about 0.15 mU PFK 2 per g. The K_m values for Fru-6-P and for ATP were close to 0.25 mM when measured in the presence of 5 mM P_i. The K_m for Fru-6-P was increased 5–10-fold when no P_i was added. Incubation of extracts in the presence of ATP-Mg, cyclic AMP and cyclic AMP-dependent protein kinase did not change the properties of the enzyme.

4. DISCUSSION

4.1. *Mechanism of Fru-2,6-P_2 formation*

Since Fru-2,6-P_2 did not accumulate in the tubers kept for several weeks at 4°C but was formed in the slices incubated at 0°C or at higher temperatures, the latter phenomenon obviously cannot be attributed only to a rise in temperature. Shaking the slices in oxygen as well as washing out potential inhibitors do not appear to be important factors. One is therefore drawn to believe that cutting the slices may be the trigger that initiates Fru-2,6-P_2 formation. The potential effect of light has not been investigated.

The event which allows the synthesis to start does not appear to be a rise in the concentration of the two substrates of PFK 2 either a change in the kinetics of that enzyme as could result from the drop of cyclic AMP concentration. Indeed, the addition of cyclic AMP to the incubation medium did not affect Fru-2,6-P_2 formation. Furthermore, we did not obtain any indication that a change in the kinetic properties of PFK 2 could occur in the presence of cyclic AMP in a cell-free extract. This is in agreement with the belief that cyclic AMP has little role to play in plant metabolism [12]. We must therefore admit that the nature of the early event that initiates Fru-2,6-P_2 formation is still unknown.

4.2. *Significance of Fru-2,6-P_2 formation in the resumption of metabolic activity*

The dramatic and early increase in Fru-2,6-P_2 concentration that accompanies the resumption of metabolic activity in Jerusalem artichoke tubers may be of interest from the point of view of developmental biochemistry for the following reasons:

(i) It shows that the phenomenon initially observed in germinating fungal spores [1] also extends to the plant kingdom. Similar observations made in various seeds which were allowed to germinate (unpublished) reinforce this concept.

(ii) Our findings in Jerusalem artichoke slices allow one to differentiate clearly the biosynthesis of Fru-2,6-P_2 from that of other hexose phosphates. Indeed, in fungal spores, the biosynthesis of Fru-2,6-P_2 was always accompanied by but slightly subsequent to that of Fru-6-P. This biosynthesis was, however, completely stopped when the concentration of hexose 6-phosphates was still elevated. The situation in Jerusalem artichokes tubers is much clearer since in several experiments, Fru-2,6-P_2 was rapidly synthesized in the absence of any change in the concentration of hexose 6-phosphates. The fact that the concentration of Fru-2,6-P_2 is not controlled by that of its precursors but by another, as yet unknown factor, is in agreement with its role as a metabolic signal (see [4]).

(iii) A potential role for Fru-2,6-P_2 in germinating fungal spores was the stimulation of PFK 1 and inhibition of fructose-1,6-bisphosphatase. This cannot be the case in Jerusalem artichoke tubers whose PFK 1 is insensitive to Fru-2,6-P_2 stimulation and which do not contain fructose-1,6-bisphosphatase. The only artichoke enzyme known to be highly sensitive to Fru-2,6-P_2 stimulation is PP_i-PFK. The maximal activity of that enzyme in the tubers is only half of that of PFK 1 and the respective role of the two PFKs in glycolysis is still unknown. One effect of PP_i-PFK is also to favor various biosynthetic processes thanks to the removal of PP_i formed by several pyrophosphorylases. The fact that potato PP_i-PFK is about 10-times more sensitive to Fru-2,6-P_2 than the artichoke enzyme may be an explanation for the 10-fold lower accumulation of Fru-2,6-P_2 in potato tuber slices.

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REFERENCES

[1] Van Laere, A., Van Schaftingen, E. and Hers, H.G. (1983) Proc. Natl. Acad. Sci. USA, 80, 6601–6605.

[2] Chapman, J.M. and Edelman, J. (1967) Plant Physiol. 42, 1140–1146.

[3] Giannattasio, M., Mandato, E. and Macchia, V. (1974) Biochem. Biophys. Res. Commun. 57, 365–371.

[4] Hers, H.G. and Van Schaftingen, E. (1982) Biochem. J. 206, 1–12.
[5] Van Schaftingen, E. and Hers, H.G. (1981) Eur. J. Biochem. 117, 319–323.
[6] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) Eur. J. Biochem. 129, 191–195.
[7] Van Schaftingen, E., Hue, L. and Hers, H.G. (1980) Biochem. J. 192, 897–901.
[8] Hohorst, H.J. (1963) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) pp.134–138, Academic Press, New York.
[9] Lamprecht, W. and Trautschold, I. (1963) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) pp.543–551, Academic Press, New York.
[10] Van Schaftingen, E. and Hers, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2861–2863.
[11] Sabularse, D.C. and Anderson, R.L. (1981) Biochem. Biophys. Res. Commun. 103, 848–855.
[12] Amrhein, N. (1977) Annu. Rev. Plant Physiol. 28, 123–132.