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Fructose 2,6-bisphosphate and the control of the energy charge in higher plants

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The mechanism by which cutting Jerusalem artichoke tubers into slices causes a large increase in their content in fructose 2,6-bisphosphate was analysed by investigating the changes in metabolite concentrations occurring within the first minutes after slicing. The first event was a rapid consumption of ATP, and also of glycerate-3-P and phospho*enol*pyruvate, two potent inhibitors of phosphofructokinase 2. The synthesis of fructose 2,6-bisphosphate started after a lag of 5 min, corresponding to the time required for the decrease in concentration of the two inhibitors. This sequence of events is proposed as a new mechanism for the preservation of ATP, which also operates in intact tubers maintained in anoxia. The role of fructose 2,6-bisphosphate in the Pasteur effect in higher plants is discussed.

Fructose 2,6-bisphosphate; Glycolysis; Pasteur effect; (Jerusalem artichoke)

1. INTRODUCTION

A dramatic increase (up to 250-fold) in the concentration of fructose 2,6-bisphosphate (fructose-2,6-P_2) is known to occur in Jerusalem artichoke tubers (*Helianthus tuberosus* L.) after slicing. This occurs without a significant increase in the activity of PFK 2, the enzyme that forms fructose 2,6-P_2 from ATP and Fru-6-P, or in the concentration of these two substrates [1]. Until now, the mechanism that initiates this synthesis has remained unexplained.

More recently, spinach leaf PFK 2 has been purified and its kinetic properties have been defined [2]. This activity belongs to a bifunctional protein which acts both as a 6-phosphofructo-2-kinase and as a fructose-2,6-bisphosphatase, being in this respect similar to the liver and muscle PFK 2/FBPase 2 [3]. Contrary to the liver and yeast enzymes (review [4]), its activity does not appear to be controlled by phosphorylation and dephosphorylation, but only by the concentration of substrates and inhibitors; among these, a series of C-3 glycolytic intermediates (glycerate-3-P, glycerate-2-P and P-*enol*pyruvate) decreases its affinity for Fru-6-P and ATP, with a K_i close to 0.15 mM.

Here, we have investigated the changes in concentration of these C-3 derivatives and of other metabolites during the first few minutes after slicing, using for this purpose a quick-freezing procedure. We reached the conclusion that the rise in fructose 2,6-P_2 concentration is part of a mechanism by which a decrease in ATP concentration automatically increases the rate of glycolysis and allows the initial ATP value to be restored. This

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Abbreviations: PFK, phosphofructokinase; FBPase, fructose bisphosphatase

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mechanism appears to apply also to the stimulation of glycolysis by anoxia and could be of general application in higher plants.

2. MATERIALS AND METHODS

Jerusalem artichoke tubers were obtained from a local producer and stored at 4°C in moist sand. Experiments were performed before germination of the tubers. Bovine serum albumin and antipain were from Sigma (St. Louis, USA); phenylmethylsulfonyl fluoride and dithiothreitol from Janssen Chimica (Beerse, Belgium); enzymes and other biochemicals from Boehringer (Mannheim). Other chemicals were from Merck (Darmstadt) or Fluka (Buchs, Switzerland) and were of analytical grade. PP_i-PFK [5] and fructose 2,6-P_2 [6] were prepared as described.

Pieces of intact tubers (about 0.5 cm thick) were rapidly frozen in liquid nitrogen and broken therein; pieces without peel were selected. Slices of about 4 cm^2 section and 1 mm thickness were prepared from a cylindrical piece cut along the tuber. The two first prepared slices were maintained at room temperature in air for 1 and 2 min respectively, and then frozen in liquid nitrogen. Other slices were incubated with vigorous shaking in distilled water at 25°C for the times indicated, blotted on paper and frozen in liquid nitrogen. Samples were stored at −80°C until further processing. Frozen slices were homogenized at 0°C with a Potter-Elvejhem device in 10 vols of either 0.05 M NaOH, for the measurement of fructose 2,6-P_2, or 5% perchloric acid, for the measurement of other metabolites. Both mixtures were centrifuged for 5 min at 5000 × *g* and the pellets were discarded. Anoxia was initiated by introducing a nitrogen stream at a rate of approx. 10 l/min in a 5 l jar. For the assay of enzymes, tubers were peeled and immediately homogenized in a Potter-Elvejhem device with 4 vols ice-cold buffer containing 5 mM dithiotreitol, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, 0.5% bovine serum albumin and 50 mM Tris-acetic acid, pH 7.5; the homogenates were centrifuged for 10 min at 10000 × *g* and the pellets were discarded. Fructose 2,6-P_2 was measured by the stimulation of PP_i-PFK [1,5]. Glc-6-P [7], ATP [8], glycerate-3-P [9] and P-*enol*pyruvate [10] were measured using an Aminco DW2 spectrophotometer in the dual-wavelength mode. PFK 2 was assayed and ^{32}P labelling of FBPase 2 was performed as in [2]. Pyruvate kinase was assayed by monitoring ATP production: the assay mixture contained 5 mM magnesium acetate, 0.15 mM NADP, 2 mM glucose, 1.7 U Glc-6-P dehydrogenase, 1.4 U hexokinase and 50 mM Hepes, pH 7.5; P-*enol*pyruvate and ADP were at variable concentrations. Enzyme measurements were made at 30°C.

3. RESULTS

3.1. *The early changes in metabolite concentration after slicing*

As shown in fig.1, the quick-freezing technique allowed us to detect several remarkable changes occurring during the first few minutes after slicing. The concentrations of ATP, P-*enol*pyruvate and glycerate-3-P (not shown) decreased immediately to reach 30–40% of their initial value after 10 min. There was simultaneously a transient rise in ADP. The concentration of fructose 2,6-P_2 started to increase only after a latency of several minutes, which corresponded to the time required for the C-3 derivatives to reach a low value, and, in agreement with [1], rose to about 3 nmol/g after 60 min. During this time, the concentration of P-*enol*pyruvate remained low, whereas that of ATP increased slowly to reach its initial level after more than 1 h. As reported in [1], the concentration of Glc-6-P did not vary significantly and Fru-6-P concentration was continuously one-quarter of that of Glc-6-P (not shown). Glycerate-3-P concentration was 3-fold that of P-*enol*pyruvate (fig.2).

3.2. *The effect of fluoride*

Because the results shown above supported the hypothesis that the activity of PFK 2 is controlled by the concentration of the C-3 derivatives, we attempted to monitor the latter parameter by inhibiting enolase with fluoride. We show in fig.2 (closed symbols) that when 10 mM NaF was present in the incubation medium, the concentration of metabolites in the slices started to be affected after a delay of 20 min, indicating a slow penetration of the inhibitor. As expected if glycolysis was in operation, we found an accumulation of glycer-

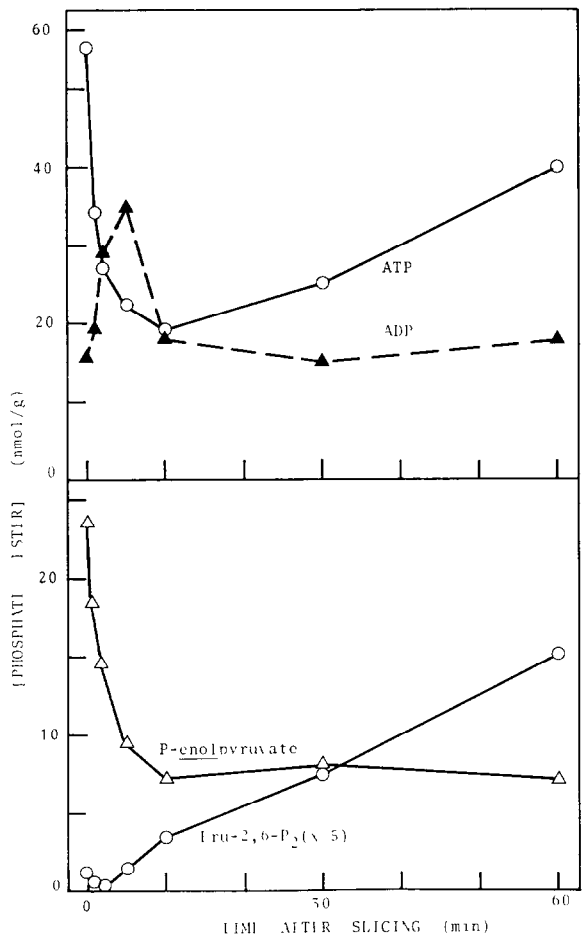

Fig.1. Concentration of phosphate esters in slices of Jerusalem artichoke tuber. Values shown are expressed per g fresh wt.

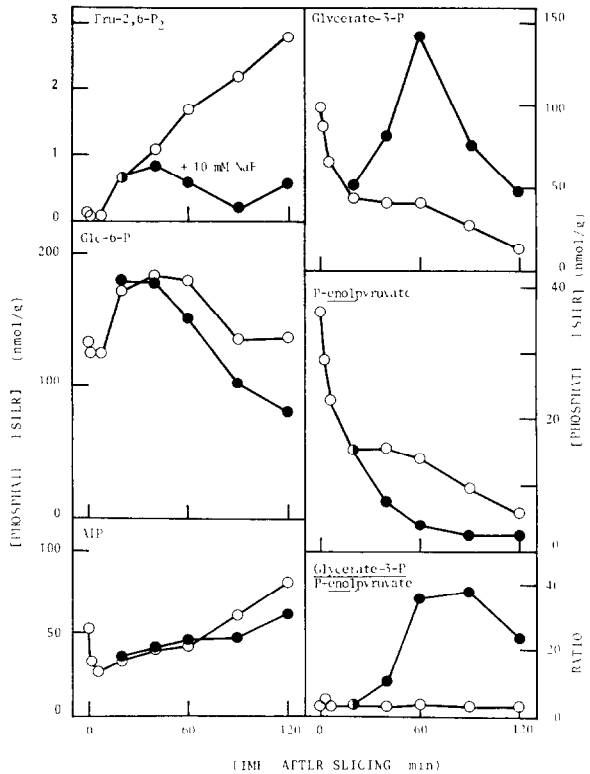

Fig.2. Concentration of phosphate esters in slices of Jerusalem artichoke tuber incubated in water or in 10 mM NaF. Values shown are expressed per g fresh wt.

ate-3-P and a further decrease in the concentration of P-*enol*pyruvate; the ratio of these two metabolites rose from 3 to 40, whereas the sum of their concentrations increased 2–3-fold, thus exceeding the initial value. Simultaneously the rise in fructose 2,6-P_2 concentration was completely arrested. Fluoride had only a minimal effect on the concentration of ATP and Glc-6-P (fig.2). It has been verified that the addition of 10 mM NaCl to the incubation medium had none of the effects observed with 10 mM NaF (not shown).

3.3. *The effect of anoxia*

Because, as indicated in section 4, our data indicated for the first time a role of fructose 2,6-P_2 in the control of the energy charge of the cell, we also investigated the potential role of this phosphoric ester in the stimulation of glycolysis by anoxia. For this purpose, Jerusalem artichoke tubers that had been kept for 4 months at 4°C were maintained at the same temperature, but in nitrogen. As shown in fig.3, the first detectable change was, after 4 h of anoxia, a decrease in concentration of ATP followed by a decrease in that of glycerate-3-P. The concentration of fructose 2,6-P_2, which was very low at the beginning of the experiment, increased abruptly after 4 h to reach about 12-fold its initial value. Glycerol-3-P was undetectable throughout the experiment.

3.4. *Properties of PFK 2 and pyruvate kinase*

As in [1], we obtained no indication of a significant change in PFK 2 activity in extracts of intact or excised tissues under the various experimental conditions used. We also verified that 10 mM NaF had no effect on the PFK 2 assay. In the presence of 2 mM P_i, the K_m of the enzyme for Fru-6-P and

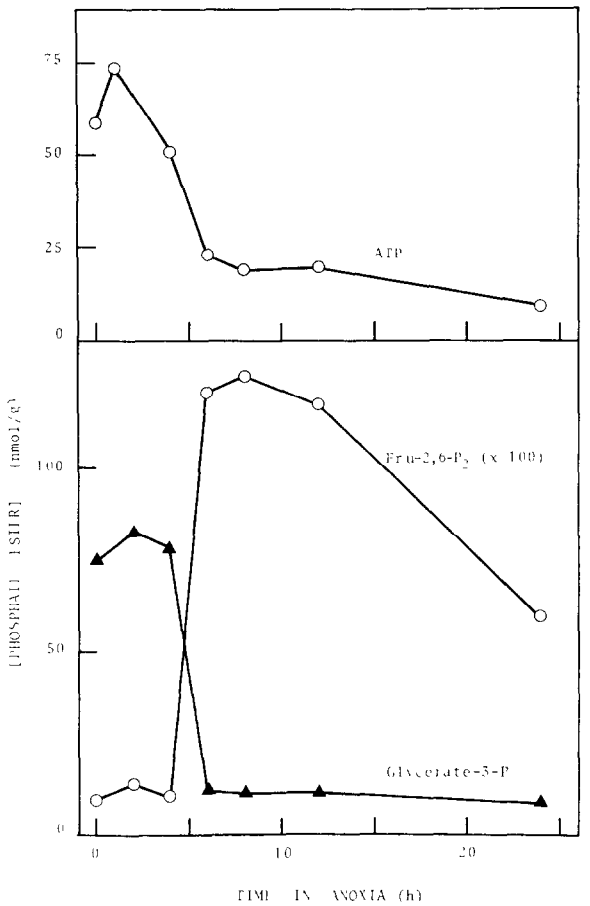

Fig.3. Concentration of phosphate esters in Jerusalem artichoke tuber incubated in anoxic conditions at 4°C. Values shown are expressed per g fresh wt.

ATP was 110 and 80 μM, respectively. Half-maximal inhibitions by P-*enol*pyruvate, glycerate-2-P, glycerate-3-P, glycolate-2-P and pyrophosphate were obtained with concentrations in the range 0.2–0.3 mM, as reported previously for the spinach leaf enzyme [2]. We could not detect a glycerol-3-P dehydrogenase activity in the extracts, and 2 mM glycerol-3-P was without effect on PFK 2/FBPase 2 activity.

FBPase 2 could only be detected by its ability to form a (2-^{32}P)-labelled protein upon short incubation in the presence of fructose 2,6-[2-^{32}P]P_2 [2]. As reported earlier [2], analysis by SDS-PAGE and autoradiography revealed a major band of M_r 90 000 and additional ones between M_r 60 000 and 40 000, and this pattern was not modified after slicing (not shown). The tuber contained about 0.4 U pyruvate kinase/g tissue, when measured at 0.5 mM ADP and 0.4 mM P-*enol*pyruvate.

4. DISCUSSION

4.1. *The mechanism by which wounding causes a large increase in fructose 2,6-P_2 concentration*

Because of the presence of a large vacuole in the Jerusalem artichoke tuber cell, the cytosolic concentration of glycerate-3-P and of P-*enol*pyruvate is presumably at least 10-fold greater than those measured in the complete tissue and reported in the figures, therefore reaching 1–1.4 mM in the intact tuber and 0.3–0.6 mM after wounding. Since the K_i for PFK 2 is close to 0.2 mM, one may expect a nearly complete inhibition of this enzyme in the intact tissue and about 70% inhibition 5 min after wounding. This partial inhibition is consistent with the difference between the maximal rate of fructose 2,6-P_2 formation in slices (30 pmol/min per g) and the extractable activity of PFK 2 (150 μU/g). These results allow us to conclude that the dramatic increase in fructose 2,6-P_2 concentration occurring after the preparation of the slices may be explained by a decrease in the concentration of glycerate-3-P and of P-*enol*pyruvate. Remarkably, this decrease is concomitant with a significant decrease in ATP concentration and a transient rise in ADP (fig.1) and also in AMP (not shown), leading to a decrease of the adenylate energy charge from 0.82 to 0.55 10 min after slicing. The following sequence of events can therefore be proposed:

(i) wounding the tissue collapses the ionic gradient, stimulates the membraneous ATPase and initiates the consumption of ATP;

(ii) the decrease in ATP/ADP ratio favors the activity of pyruvate kinase and the consumption of the C-3 phosphate esters;

(iii) PFK 2 ceases to be completely inhibited and fructose 2,6-P_2 is formed;

(iv) PP_i-PFK, which is the only tuber enzyme known to be sensitive to fructose 2,6-P_2, is activated and glycolysis is stimulated, allowing ATP to be regenerated. In this hypothesis, point (iv) is the most controversial, since the respective role of PP_i-PFK and of PFK 1 in glycolysis in higher plants is not clear. However, the fact that PP_i-PFK is submitted to regulation by fructose 2,6-P_2 suggests that it plays an important role in the control

of glycolysis. There are also in the literature several reports [11,12] showing that PFK 1 purified from various plants is inhibited by physiological concentrations of P-*enol*pyruvate and glycerate-3-P. It seems therefore that both PP_i-PFK and PFK 1 activities are dependent on the concentration of the C-3 inhibitors, although by different mechanisms.

The energy charge of intact tuber (0.82) was close to the values reported in intact plant cells (0.8–0.9), and in tuber slices as in other tissues, a decrease of the adenylate energy charge was associated with a decrease of the total adenylates [13] (see section 3).

4.2. *Fructose 2,6-P_2 and the Pasteur effect in plants*

There is now general agreement that, in animals and in yeast, fructose 2,6-P_2 plays an important role in the control of glycolysis by metabolites and by hormones, but that AMP (or the lack of ATP) is the main effector that stimulates PFK 1, and therefore glycolysis, in anoxia.

Indeed it is remarkable that, by contrast with the present data, Hue [14] has observed a decrease in the concentration of fructose 2,6-P_2 in the liver and other rat tissues in anoxia. This difference may be explained by the fact that glycerol-3-P, which accumulates in aminal cells but not in the tuber during anoxia, is an activator of liver FBPase 2 and an inhibitor of liver PFK 2 and has no action on the plant bifunctional enzyme. By contrast, plant PFK 2 is strongly inhibited by glycerate-3-P and P-*enol*pyruvate, both of which decrease in plants during anoxia, as reported here and elsewhere [15,16]. The sequence of events described in the preceding paragraph is therefore a new mechanism by which anoxia, as well as any ATP-consuming process, can stimulate glycolysis and regenerate the energy charge.

Preliminary experiments performed with imbibed oat and soya seeds indicate a 3-fold increase in fructose 2,6-P_2 concentration within a few minutes in anoxia (not shown).

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