

Short Communication

Fructose 2,6-Bisphosphate and the Regulation of Pyrophosphate-Dependent Phosphofructokinase Activity in Germinating Pea Seeds¹

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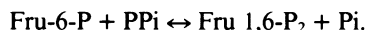
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ABSTRACT

The activity of pyrophosphate:D-fructose-6-phosphate-1-phosphotransferase (EC 2.7.1.90, PPI-PFK) in cotyledons and sprouts of germinating pea seeds (*Pisum sativum* cv Alaska or Green Arrow) increases rapidly during the first 2 to 3 days after imbibition and then declines to a lower activity. The reaction toward fructose 1,6-bisphosphate formation is activated greatly by fructose 2,6-bisphosphate (fru 2,6-P₂); however, the sensitivity of the enzyme's activity to fru 2,6-P₂ activation changes during germination.

The cotyledon enzyme was partially purified and exists in two forms apparently with different molecular weights. The large form shows little sensitivity to fru 2,6-P₂, while the small form shows a high sensitivity to this effector ($K_a = 15$ nanomolar). Gel filtration experiments indicate that fru 2,6-P₂ is involved in converting the small form into the large form. We propose that the interconversion of two forms of the PPI-dependent PFK by fru 2,6-P₂ is one mechanism for regulating glycolysis during seed germination.

A plant PPI-PFK⁴ was discovered in pineapple leaves in 1979 (1) and now has been found in other plant tissues including many leaves (2, 4, 12), potato tubers (14), mung beans (11), and castor bean seedlings (9). The plant PPI-PFK catalyzes the following reversible reaction:



Recent work shows that fru 2,6-P₂, a potent effector of ATP-PFK discovered in animal research on the regulation of glycolysis and gluconeogenesis in liver (3, 13, 14), also is a potent effector of plant PPI-PFK activity (11). But fru 2,6-P₂ has no effect on plant ATP-dependent PFK activity (2, 4, 9, 11, 12, 16) with the exception of the unconfirmed report of stimulating a plastid ATP-PFK (10). The role of PPI-PFK in glycolysis in plants is not certain, but classically the formation of fru 1,6-P₂ from fru-

6-P is a control point in the regulation of sugar breakdown. This led us to postulate that PPI-PFK plays an important role in glycolysis and energy metabolism in plants (1) which is strongly supported by the widespread distribution in many plants and algae of the PPI-PFK often several-fold in excess of the ATP-dependent PFK activity (2).

In this paper, we report that PPI-PFK in germinating pea seeds is activated by fru 2,6-P₂ and that the mechanism of this activation involves the interconversion of two forms of PPI-PFK. Apparently, these two forms have different mol wt, and this interconversion is a new mechanism for regulating plant glycolysis.

MATERIALS AND METHODS

Materials. Pea seeds (*Pisum sativum* cv Alaska or Green Arrow) were soaked overnight in running tap water, and germinated at room temperature in darkness. Zero time on the figures is after the overnight imbibition of water. Sprouts include shoots and roots. Fru 6-P, Fru 1,6-P₂, Fru 2,6-P₂, and the assay enzymes were purchased from Sigma Chemical Co. Sephadex G-200, fine (40–120 μM) was purchased from Pharmacia.

Enzyme Assays. *Assay A.* The forward direction of PPI-PFK was assayed by the production of fru 1,6-P₂ as previously described (1).

Assay B. The reverse direction reaction of PPI-PFK was assayed spectrophotometrically. The assay mixture contained 2.5 mM MgSO₄, 0.2 mM NADP, 100 mM Hepes-NaOH (pH 8.0), 2.5 μg glu 6-P dehydrogenase, and 5 μg phosphoglucose isomerase, 10 mM fru 1,6-P₂ and 5 mM K-phosphate in a 0.5-ml volume. The assay was dependent upon and started by the addition of K-phosphate. All the assays were carried at 30°C in Gilford spectrophotometer model 240. The sensitivity of PPI-PFK to fru 2,6-P₂ was estimated by measuring enzyme activity (in the forward direction) with fru 2,6-P₂ at a saturated concentration (1 μM) (V_a) in comparison with enzyme activity without fru 2,6-P₂ (V_o).

Enzyme Partial Purification. Separated cotyledons or sprouts from developing pea seeds were homogenized in a Waring Blender in a ratio of 1 g fresh weight to 3 ml of 100 mM Bicine-NaOH (pH 8.4, isolation medium), containing 7 mM β -mercaptoethanol, 1 mM disodium EDTA, and 5% (v/v) glycerol. All steps were performed at 4°C. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 25,000g for 20 min. The supernatant fraction was referred to as the crude extract. Polyethylene glycol 6000 (Sigma Chemical Co.) was added slowly to concentration 20% (w/v) and stirred for 10 min. After centrifugation at 25,000g for 15 min, the precipitate was discarded and the supernatant fraction was made up to a concentration of 35% (w/v) polyethylene glycol 6000 with stirring. The 20 to 35% (w/v) polyethylene glycol-insoluble fraction was

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⁴ Abbreviations: PPI-PFK, pyrophosphate-dependent phosphofructokinase; fru 2,6-P₂, fructose 2,6-bisphosphate; fru 1,6-P₂ fructose 1,6-bisphosphate; PPI, inorganic pyrophosphate.

obtained after centrifugation at 25,000g for 20 min and resuspended in the isolation medium in a small volume. Gel filtration chromatography was done as described by Ibsen *et al.* (8). The enzyme was purified over 50-fold by this procedure.

RESULTS

Changes in the Activity of Pyrophosphate-Dependent Phosphofructokinase in Pea Seeds during Germination. PPI-PFK was found in crude extracts from both cotyledons and sprouts at each assay stage during pea seed germination (Fig. 1A). PPI-PFK activity increased over 3-fold and 7-fold in cotyledons and sprouts, respectively, after 2 to 3 d of germination and then declined to a lower activity. The enzyme activity in sprouts was 3 times higher than the activity in cotyledons based on tissue fresh weight. PPI-dependent PFK activity was readily detected in the absence of the activator, fru 2,6-P₂, at all stages of development.

Changes in Fructose 2,6-Bisphosphate Sensitivity of PPI-PFK in Pea Seeds during Germination. Fru 2,6-P₂ was a potent stimulator of pea PPI-PFK in the direction of fru 1,6-P₂ formation throughout germination. However, we found that during germination the sensitivity of PPI-PFK to exogenous fru 2,6-P₂ (at 1 μ M in the assay), changed depending on the seedling age in both cotyledons and sprouts (Fig. 1B). Initially, pea PPI-PFK was sensitive to fru 2,6-P₂ in the assay, followed by a period of relative insensitivity, near day 2 to 7, prior to a later resumption, near day 8 or 9, of fru 2,6-P₂ sensitivity. Total PPI-PFK activity (forward direction, Fig. 1A) was greatest during the period when fru 2,6-P₂ sensitivity was least (Fig. 1B). Subsequent work revealed that similar differences in fru 2,6-P₂ sensitivity were maintained when partially purified enzyme preparations (polyethylene glycol precipitates) were obtained from pea seedlings of different ages (data not shown).

Evidence for Two Forms of PPI-PFK in Pea Cotyledons. In a detailed study of the changing sensitivity to fru 2,6-P₂, using an enzyme preparation partially purified from 2-d-old cotyledons by polyethylene glycol 6000 precipitation, two distinct activity peaks of PPI-PFK activity were eluted via gel filtration chromatography (Sephadex G-200) (Fig. 2). The large form, which was eluted first, has activity without added fru 2,6-P₂ and shows little sensitivity to fru 2,6-P₂, whereas the small form shows activity only when assayed in the presence of fru 2,6-P₂ (1 μ M). We assume elution volume on our gel filtration columns correlates with mol wt, such that large proteins eluted more rapidly than small proteins. Currently, research is in progress on the mol wt determinations and other properties of these proteins.

Further differences between the large and small forms of PPI-PFK appeared when fru 2,6-P₂ was added at varying concentrations. The large form (peak I) was little affected by added fru 2,6-P₂. On the contrary, the activity of the small form (peak II) was activated dramatically, 15- to 20-fold, by fru 2,6-P₂. The K_a for fru 2,6-P₂ was 15 nM for the small form (peak II) of the cotyledon enzyme; a value close to the 12 nM reported for spinach leaves (4) and the 5 nM for the potato tuber enzyme (16).

Effect of Fru 2,6-P₂ on the Interconversion of Two Forms of Pea Cotyledon PPI-PFK. The small form (peak II) of pea cotyledon PPI-PFK was obtained from a partially purified enzyme preparation by passing the preparation through a Sephadex G-200 column. The fractions containing the small form were concentrated with 55% saturated (NH₄)₂SO₄ precipitation, resuspended in a small volume of isolation medium, and then applied to the second Sephadex G-200 column (2.0 \times 35 cm) (Fig. 3A). The column was preequilibrated and eluted with the same buffer, and the fractions (each 3 ml) were pooled.

The elution activity profile showed that only a fru 2,6-P₂-dependent PPI-PFK was eluted (V_e = 84 ml), and that finding implied that only the small form of this enzyme was isolated

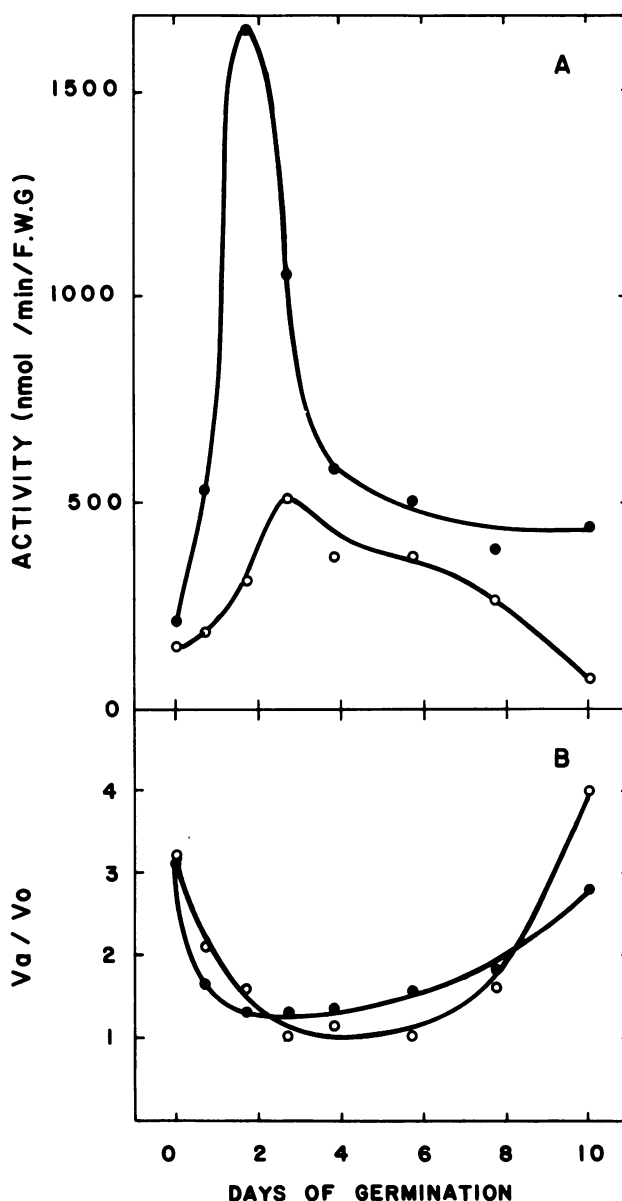


FIG. 1. A, Total activity changes in the PPI-dependent PFK in pea seed tissues during germination. All activities in these assays were without exogenous additions of fru 2,6-P₂. B, Changes in the sensitivity of PPI-dependent PFK activity to fru 2,6-P₂ activation during pea seed germination. V_a and V_o represent the enzyme activities measured with and without 1 μ M fru 2,6-P₂, respectively. Cotyledons (○) and sprouts (●).

(Fig. 3A). This small form of the pea cotyledon PPI-PFK was concentrated by the same procedure given above, and then was treated by incubation with fru 2,6-P₂ (final concentration, 125 μ M) at 4°C for 24 h. This treated preparation was applied to the same column (Sephadex G-200).

The results (Fig. 3C) show that PPI-PFK activity reappeared in the chromatographic position of the large form or peak I (V_e = 63 ml) after fru 2,6-P₂ incubation. Although the small form or peak II of PPI-PFK was observed as well after this treatment, the activity in the position of the small form was less than 40% of the activity in the large form. We interpret this result as a direct demonstration of the interconversion of the small form to the large form after incubation with fru 2,6-P₂.

The small form of PPI-PFK in pea cotyledon isolated by gel

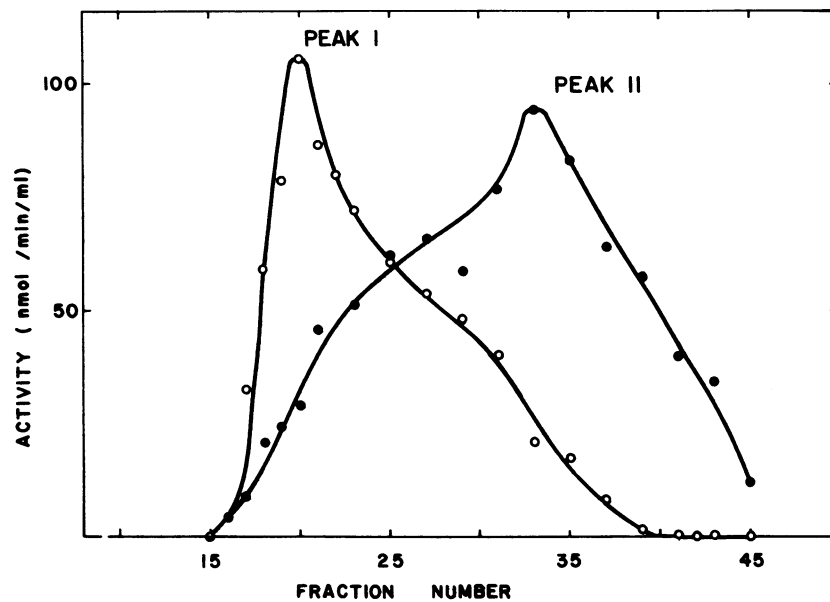


FIG. 2. Elution profile from a Sephadex G-200 column of PPI-PFK activity partially purified from pea cotyledons. Peak I is activity without the activator (V_o) and peak II is the fru 2,6- P_2 -dependent activity measured in the presence of $1 \mu\text{M}$ fru 2,6- P_2 ($V_a - V_o$).

filtration chromatography (Fig. 3A) still showed the function of catalyzing the reverse reaction (Fig. 3B), although it had no significant activity in the forward direction in the absence of fru 2,6- P_2 (Fig. 3A).

Unlike the case in the forward direction, the reverse direction was stimulated only slightly (Fig. 3B) by a fru 2,6- P_2 concentration ($1 \mu\text{M}$) saturating for activation of the forward direction of PPI-PFK. These results suggest that the two forms of PPI-PFK favor two opposing reactions, and that fru 2,6- P_2 probably plays a role to alter the equilibrium of the two reactions (at least in the situation presented in these *in vitro* studies).

These results further indicate that two activity peaks obtained from a Sephadex G-200 column (Fig. 3C) show different ratios of reverse direction activity to forward direction activity (R/F) when measured at the same concentration of substrate, metal ion, and protein. The ratio of R/F from peak II is much larger than that from peak I, and fru 2,6- P_2 diminished this difference in ratio of R/F between these two preparations (data not shown). This phenomenon is interpreted as a result of fru 2,6- P_2 altering the relative proportion of the two forms of PPI-PFK.

DISCUSSION

Pyrophosphate-dependent PFK activity has been found in pineapple leaves (1), mung bean seedlings (11), spinach leaves (4), wheat protoplasts (12), potato tubers (16), castor bean seedlings (9), and many other plant leaves (2). Most work on plant sources of the enzyme shows this enzyme is activated and sometimes completely dependent on fru 2,6- P_2 . However, in some plant tissues, in particular Crassulacean acid metabolism plants, very high activity is present and only a small activation by fru 2,6- P_2 occurs at V_{max} (2). Fru 2,6- P_2 also is a potent stimulator of ATP-PFK in animals and an inhibitor of fru 1,6-bisphospha-

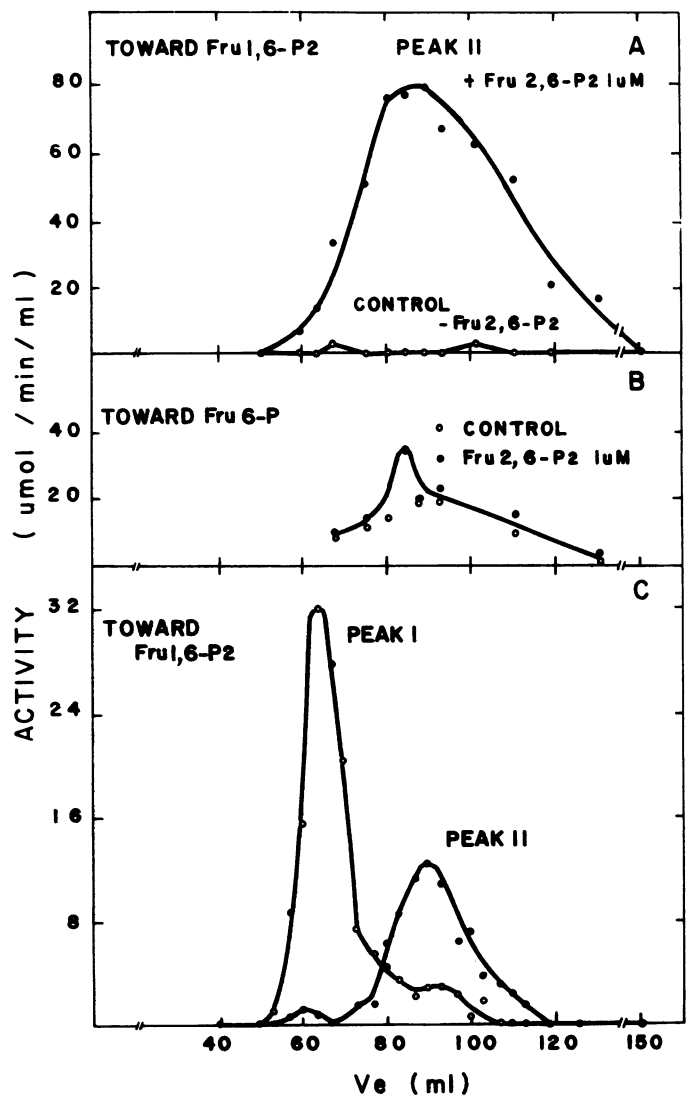


FIG. 3. Separation of the two forms of PPI-PFK using Sephadex G-200 chromatography and interconversion of the two forms by incubation with fru 2,6- P_2 . The small form (or peak II) was measured towards fru 1,6- P_2 formation (A) and towards fru 6-P formation (B). The formation of the large form (peak I) from the small form (peak II) by incubation with $125 \mu\text{M}$ fru 2,6- P_2 was measured towards fru 1,6- P_2 formation (C).

tase in plants and animals (7). The mechanisms of fru 2,6-P₂ activation or inhibition are unknown.

The results presented here indicated that during pea seed germination, PPI-PFK is readily detected (Fig. 1A) and it is further activated by fru 2,6-P₂ (Fig. 1B). PPI-PFK is found in two forms, each showing a difference in sensitivity to fru 2,6-P₂ (Figs. 2 and 3). The two forms apparently have different mol wt (a small and large form) because of their elution patterns off G-200 gel filtration columns (Figs. 2 and 3). The change in sensitivity of PPI-PFK to fru 2,6-P₂ during pea seed germination could be due to the change of relative proportions of these two forms of PPI-PFK in these unpurified tissue preparations. And the level of fru 2,6-P₂ could be modulated during germination by the enzymes found in animal (6, 15), and recently detected in plant leaves (5; Smyth, Wu, and Black, unpublished) which catalyze the synthesis and hydrolysis of fru 2,6-P₂. We propose that fru 2,6-P₂ is involved in the interconversion of the small form of PPI-PFK with the large form.

The experimental observations described in this paper also suggested that the small form of pea cotyledon PPI-PFK probably is the inactive species for the forward reaction in the absence of fru 2,6-P₂ (Fig. 3A), but it still keeps significant activity for the reverse reaction (Fig. 3B). Whereas the large form, presumably a result of association by the small form of PPI-PFK which is induced by fru 2,6-P₂, has a much lower proportion of the reverse to forward reaction (R/F ratio). These results imply that the rate of glycolytic flux in plants can be controlled by the concentration of fru 2,6-P₂ through its regulating role on dissociation-association of PPI-PFK, e.g. during pea seed germination as in this study.

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