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Fructose-1,6-Bisphosphate Is an Allosteric Activator of Pyrophosphate:Fructose-6-Phosphate 1-Phosphotransferase[1]

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The activity of highly purified pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP) from barley (*Hordeum vulgare*) leaves was studied under conditions where the catalyzed reaction was allowed to approach equilibrium. The activity of PFP was monitored by determining the changes in the levels of fructose-6-phosphate, orthophosphate, and fructose-1,6-bisphosphate (Fru-1,6-bisP). Under these conditions PFP activity was not dependent on activation by fructose-2,6-bisphosphate (Fru-2,6-bisP). Inclusion of aldolase in the reaction mixture temporarily restored the dependence of PFP on Fru-2,6-bisP. Alternatively, PFP was activated by Fru-1,6-bisP in the presence of aldolase. It is concluded that Fru-1,6-bisP is an allosteric activator of barley PFP, which can substitute for Fru-2,6-bisP as an activator. A significant activation was observed at a concentration of 5 to 25 μM Fru-1,6-bisP, which demonstrates that the allosteric site of barley PFP has a very high affinity for Fru-1,6-bisP. The high affinity for Fru-1,6-bisP at the allosteric site suggests that the observed activation of PFP by Fru-1,6-bisP constitutes a previously unrecognized in vivo regulation mechanism.

PFP (EC 2.7.1.90) catalyzes one of the key reactions of hexosephosphate metabolism; the interconversion of Fru-6-P and Fru-1,6-bisP. PFP uses PPi to phosphorylate the carbon-1 of Fru-6-P concomitantly with the liberation of Pi:

$$\text{Fru-6-P} + \text{PPi} \xleftrightarrow[\text{Mg}^{2+}]{\text{Fru-2,6-bisP}} \text{Fru-1,6-bisP} + \text{Pi}$$

The reaction is fully reversible. PFP from plants is potently activated by Fru-2,6-bisP. This activation is readily observed in enzyme-linked assays measuring the activity in the forward reaction (use of PPi as substrate). It has been reported frequently that Fru-2,6-bisP preferentially enhances the activity of PFP in the forward direction when compared to the reverse reaction (van Schaftingen et al., 1982; for refs., see Stitt, 1990). From a theoretical point of view, Fru-2,6-bisP must activate both the forward and the reverse reaction equally. When this is not observed experimentally, it must be due to the different assay conditions used to determine activity in the two directions. Stitt and Vasella (1988) have shown that in the presence of high concentrations of Pi, potato PFP is indeed activated in both directions by Fru-2,6-bisP. They also suggested that Fru-1,6-bisP may function as an analog to Fru-2,6-bisP in the activation of PFP. This would explain the lack of a requirement for activation by Fru-2,6-bisP when PFP is assayed in the reverse direction, since this assay contains a high concentration of Fru-1,6-bisP. However, the data obtained using analogs to hexosephosphates suggested the affinity for Fru-1,6-bisP as an activator to be quite low (Stitt and Vasella, 1988), and conclusive data to support the activation of PFP by Fru-1,6-bisP are still lacking.

In a previous paper the purification and initial kinetic characterization of barley leaf PFP was reported (Nielsen, 1994). Barley PFP shares kinetic properties with potato PFP: it has an extremely high affinity for the activator Fru-2,6-bisP when assayed in the forward direction. When assayed in the reverse reaction, Fru-2,6-bisP has only a limited effect on the PFP activity. In this paper purified PFP from barley leaves was further characterized with respect to activation by Fru-1,6-bisP and Fru-2,6-bisP. In previous studies of PFP from different plant species the enzyme activity was determined by the continuous assays based on conversion of NAD/NADH by auxiliary enzymes, and this technique was also used for the characterization of the barley PFP (Nielsen, 1994). A new assay procedure was developed in this study. The auxiliary enzymes were excluded and the reaction catalyzed by PFP was allowed to approach equilibrium conditions. The changes in levels of substrates and products was monitored by determination of metabolite concentrations in aliquots retrieved at different time intervals. This methodology allowed us to study the PFP activity in the presence of all four substrates and products of the reaction.

Using the newly devised assay procedure we demonstrated that Fru-1,6-bisP is a potent allosteric activator of barley leaf PFP. A significant activation is observed at concentrations of 5 to 25 μM. The high affinity for Fru-1,6-bisP at the allosteric site suggests that this metabolite contributes to the regulation of PFP activity in vivo.

MATERIALS AND METHODS

Enzyme Preparation

PFP from etiolated barley seedling leaves was purified more than 250-fold as described by Nielsen (1994). The

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Abbreviations: DHAP, dihydroxyacetonephosphate; PFP, pyrophosphate:fructose-6-phosphate 1-phosphotransferase.

enzyme preparation had a specific activity of 20 units mg^{-1}. The purified preparation was essentially free of pyrophosphatase, aldolase, phosphoglucose isomerase, and hexosephosphate phosphatases (Nielsen, 1994).

Assay Conditions

PFP activity was monitored by adding purified PFP (10–40 milliunits) into 2 mL of a reaction mixture containing 50 mM Mops-KOH (pH 7.3), 10 mM $MgCl_2$, 1 mM EDTA, and variable amounts of Fru-6-P, Fru-1,6-bisP, Fru-2,6-bisP, Pi, and PPi as indicated in "Results." The reaction mixture was incubated at 20°C for up to 140 min. At intervals 200-μL aliquots from the reaction mixture were mixed with 50 μL of 4 N HCl. After 3 min the sample was neutralized with 50 μL of 4 N KOH and placed on ice. The concentrations in these samples of Fru-6-P, Fru-1,6-bisP, and free Pi was then determined.

The preparations of Fru-1,6-bisP and Fru-6-P used for the PFP reaction were acid treated before use to remove any impurities of Fru-2,6-bisP; 50 μmol of substrate were added to 50 μL of 4 N hydrochloric acid (200 μmol) and left at 20°C for 3 min. Assay buffer (900 μL) was added and the mixture was neutralized with 50 μL of 4 N KOH and stored on ice. It was further checked to determine that there were no impurities of Fru-2,6-bisP present in any of the assay components by using the same stock solutions of buffer and substrates and the same PFP preparation in a conventional enzyme-coupled assay, in which barley PFP activity shows dependence on activation by Fru-2,6-bisP (Nielsen, 1994).

Determination of Metabolites

Fru-6-P and Fru-1,6-bisP were determined by enzyme-linked reduction/oxidation of NAD/NADH. The assay for Fru-6-P contained 50 μL of sample, 50 mM Mops-KOH (pH 7.3), 5 mM $MgCl_2$, 1 unit of phosphoglucoisomerase, 1 unit of Glc-6-P dehydrogenase (Leuconostoc), and 0.5 mM NAD in a final volume of 350 μL. The assay for Fru-1,6-bisP contained 50 μL of sample, 50 mM Mops-KOH (pH 7.3), 5 mM $MgCl_2$, 1 unit of aldolase, 8 units of triosephosphate isomerase, 1 unit of α-glycerophosphate dehydrogenase, and 0.2 mM NADH in a final volume of 350 μL. The A_{340} was detected using a Ceres-UV-9000 microplate reader (Bio-Tek, Winooski, VT). Pi was determined by the method of Lanzetta et al. (1979).

RESULTS

In the present study a new assay procedure for PFP activity was used (here called "noncoupled assays") in which the reaction will approach equilibrium during the experimental period. Figure 1 shows typical reactions when the assays are started in the forward direction, in the reverse direction, or near equilibrium. Initial concentrations were 1 mM Fru-6-P and 1 mM PPi (forward reaction), 1 mM Fru-1,6-bisP and 1 mM Pi (reverse reaction), or 0.5 mM of each of the four substrates. Thus, the sums of the phosphate and the hexose equivalents, respectively, were unaltered. In all tested combinations of substrates, PFP catalyzed interconversion of the substrates and within a reaction period of 140 min the same equilibrium was approached irrespective of the start condition. Under all conditions PFP appeared to be fully active, and the inclusion of 1 μM Fru-2,6-bisP in the reaction mixture had little effect on the reaction rate and no effect on the obtained equilibrium (Fig. 1).

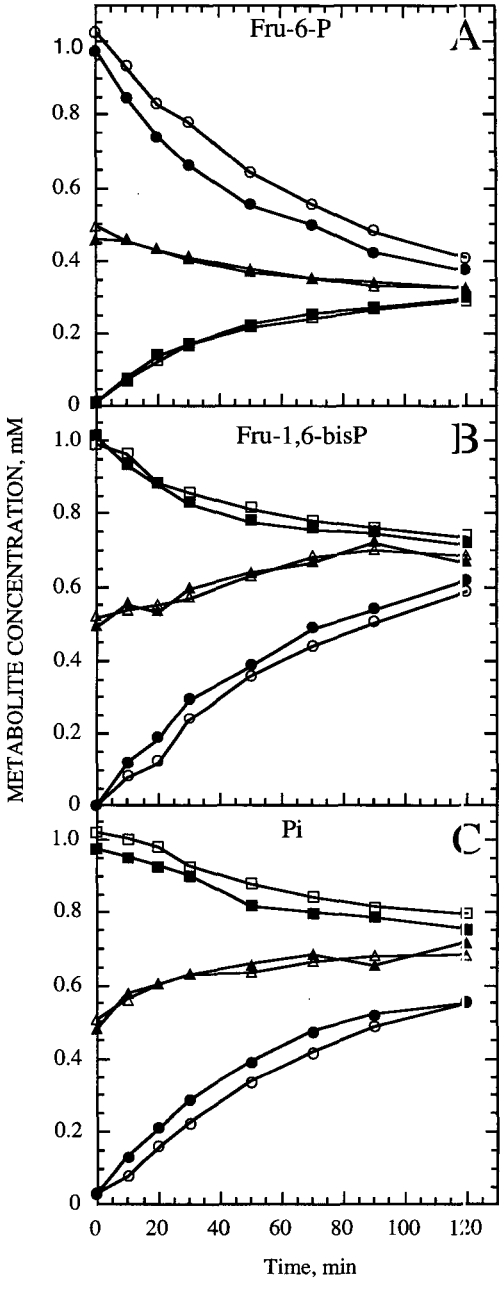

Figure 1. Levels of Fru-6-P (A), Fru-1,6-bisP (B), and Pi (C) in reaction mixtures during the PFP reaction in noncoupled assays. Reactions were initiated by adding PFP. Initial concentrations were 1 mM Fru-6-P and 1 mM PPi, (○, ●); 1 mM Fru-1,6-bisP and 1 mM Pi (□, ■); or 0.5 mM of each of Fru-6-P, Fru-1,6-bisP, PPi, and Pi (△, ▲). Reactions were performed in the absence (open symbols) or the presence (filled symbols) of 1 μM Fru-2,6-bisP. Each assay contained 20 units mL^{-1} PFP.

The following experiments were all focused on investigating the noncoupled assays starting in the forward direction. The noncoupled assays (Fig. 1) and enzyme-coupled assays in the forward direction differ with respect to the presence of auxiliary enzymes (aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase) and the presence of reaction intermediates, substrates, and products (Fru-1,6-bisP, triosephosphates, α-glycerophosphate, NAD, NADH). Potentially any of these could cause the differential effect of Fru-2,6-bisP on PFP activity in the two types of assays. However, it was observed that only the addition of aldolase would reintroduce the dependence of PFP activity on Fru-2,6-bisP in the noncoupled assays (Fig. 2). Aldolase removes Fru-1,6-bisP and forms triosephosphates to reach an equilibrium situation. The effect of aldolase was temporary, and after some time the reaction rate started to increase (Figs. 2–5). This may be explained if the effect of aldolase is due to removal of Fru-1,6-bisP produced by a slow reaction catalyzed by the unactivated PFP. The inclusion of 0.2 mM DHAP has little effect on the reaction rates (Fig. 3), and similar results were obtained for glyceraldehyde-3-P (data not shown). This demonstrates that the effect of aldolase on PFP activation cannot be due to the formation of triosephosphates.

If the Fru-1,6-bisP formed by PFP activity in the forward assay activated PFP allosterically and hereby created an autocatalytic forward reaction, this could be tested by varying the PFP activity. Increasing the amount of PFP in the assay resulted in earlier activation of PFP (Fig. 4). This kinetics behavior suggests that an autocatalytic reaction is taking place.

Direct addition of Fru-1,6-bisP activated PFP in the presence of aldolase (Fig. 5). In these assays the PFP and aldolase were added simultaneously to start the reaction and PFP will, therefore, be exposed to an initial concentration of Fru-1,6-bisP as indicated in the figure. However, aldolase will remove Fru-1,6-bisP, and PFP will form Fru-1,6-bisP. Therefore, only the initial concentration of Fru-1,6-bisP is known precisely. This concentration influences the onset of the autocatalytic reaction, and from Figure 5 it can be seen that only 5 μM Fru-1,6-bisP was enough to partially activate PFP as compared to assays with no added Fru-1,6-bisP. With increasing concentrations of Fru-1,6-bisP the activation was more pronounced, and in presence of 100 to 200 μM Fru-1,6-bisP, the PFP reaction appeared to be activated to reach the same rate as in the absence of aldolase (omitting Fru-1,6-bisP in the starting mixture). The reaction was slightly faster in presence of 1 μM Fru-2,6-bisP, indicating that Fru-2,6-bisP was activating PFP more efficiently than Fru-1,6-bisP (see also Figs. 1–3).

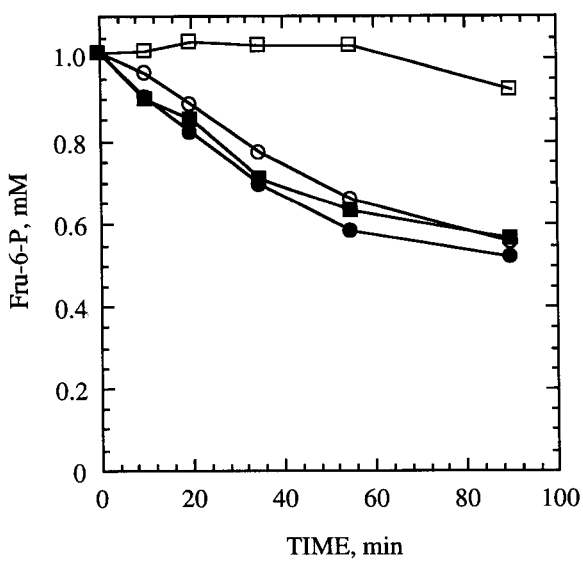

Figure 2. The effect of including aldolase in the PFP assays. Assays were performed as in Figure 1A measuring the concentration of Fru-6-P as used by PFP (forward assay: initial concentrations were 1 mM Fru-6-P and 1 mM PPi). Reactions were performed in the absence (open symbols) or the presence (filled symbols) of 1 μM Fru-2,6-bisP and in the absence (○, ●) or presence (□, ■) of 1 unit mL^{-1} aldolase.

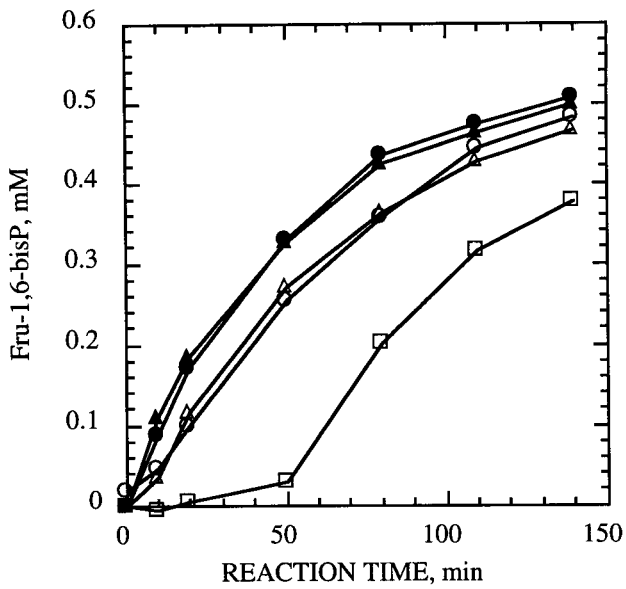

Figure 3. The effect of including DHAP in the PFP assays. Assays were performed as in Figure 1C measuring the concentration of Fru-1,6-bisP formed by PFP (forward assay: initial concentrations were 1 mM Fru-6-P and 1 mM PPi). Reactions were performed in the absence (○, ●, □) or presence (△, ▲) of 0.2 mM DHAP and in the absence (open symbols) or presence (filled symbols) of 1 μM Fru-2,6-bisP. For comparison aldolase was included in one assay (□). The other assays did not contain aldolase.

DISCUSSION

Fru-2,6-bisP is known to be an important regulator of key enzymes in the primary cytosolic carbohydrate metabolism (Stitt, 1990). Two enzymes, cytosolic Fru-1,6-bisphosphatase and PFP are regulated by Fru-2,6-bisP at extremely low concentrations. PFP is potently activated by Fru-2,6-bisP, and typically K_a (Fru-2,6-bisP) values in the range of 3 to 50 nM have been reported, depending on plant species and tissue from which PFP is isolated (van Schaftingen, 1982; Yan and Tao, 1984; Bertagnolli et al., 1986; Stitt, 1989; Nielsen, 1994).

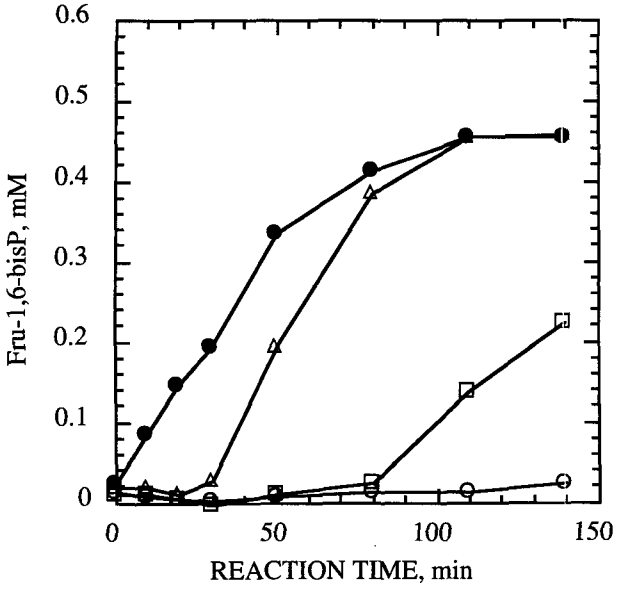

Figure 4. The effect of amount of PFP in the assays. Assays were performed as in Figure 1C measuring the concentration of Fru-1,6-bisP formed by PFP (forward assay: initial concentrations were 1 mM Fru-6-P and 1 mM PPi). Reactions were performed in the absence (open symbols) or the presence (filled symbols) of 1 μM Fru-2,6-bisP. All reactions were performed in the presence of 1 unit mL^{-1} aldolase. Assays included PFP activities of 40 milliunits mL^{-1} (△), 20 milliunits mL^{-1} (●, □), or 10 milliunits mL^{-1} (○).

In a previous study Nielsen (1994) documented that purified barley leaf PFP had a very high affinity for Fru-2,6-bisP (K_a 2.8 nM), and in assays with limited substrates up to 30-fold activation was observed when PFP was assayed in the forward direction. In contrast to this, Fru-2,6-bisP had little effect when PFP was assayed in the reverse direction.

By using the newly devised assay procedure we have shown here that PFP no longer had a requirement for Fru-2,6-bisP as an activator (Fig. 1). However, the inclusion of aldolase in the reaction mixture reintroduced the dependence of PFP on activation by Fru-2,6-bisP (Fig. 2).

The reintroduced requirement for Fru-2,6-bisP by addition of aldolase also excludes the possibility that contamination by Fru-2,6-bisP was the reason for lack of requirement for Fru-2,6-bisP activation of PFP in the noncoupled assays.

Aldolase could have an effect by either removing Fru-1,6-bisP or by forming triosephosphates. The latter proved not to be the case, since neither DHAP (Fig. 3) nor glyceraldehyde 3-P (data not shown) had any significant effect on the activation of PFP. When aldolase was included, the noncoupled forward assay behaved as an autocatalytic reaction (Fig. 4), which also suggests that the product of the reaction is activating PFP. In accordance with these data it was demonstrated that in the presence of aldolase Fru-1,6-bisP will activate PFP (Fig. 5). Therefore, it is concluded that Fru-1,6-bisP is an allosteric activator that will substitute for Fru-2,6-bisP. This result explains why PFP activity is typically observed not to be dependent on Fru-2,6-bisP when assayed in the reverse direction. In these assays the concentration of Fru-1,6-bisP is apparently sufficient to substitute for Fru-2,6-bisP as activator of PFP.

The results presented here have implications for the justification of previous estimates of kinetic parameters based on enzyme-coupled forward assays. During the enzyme-coupled forward reactions a steady-state level of Fru-1,6-bisP is built up in the assay. Increasing substrate levels will increase the steady-state level of Fru-1,6-bisP. In the absence of Fru-2,6-bisP this "extra" Fru-1,6-bisP will activate PFP and thereby influence the estimation of both substrate affinity and V_{max}. For example, Nielsen (1994) reported that in the absence of Fru-2,6-bisP the V_{max} (forward assay) was about one-third of the activity seen in the presence Fru-2,6-bisP. This high activity in the absence of Fru-2,6-bisP is in contrast to the results reported here (an almost total dependence in the presence of aldolase). Most likely this is due to the steady-state levels of Fru-1,6-bisP in the enzyme-coupled assays. If PFP from other plant species are activated by Fru-1,6-bisP like the barley enzyme, these considerations will be valid for all kinetics done with enzyme-linked assays. The results obtained in the individual study will depend not only on the substrate and activator affinities of PFP but also on the relative ratio of activities of PFP and auxiliary enzyme included in the assay, as well as the substrate affinities of the auxiliary enzyme.

To understand the observed time-dependent development of PFP activity in the presence of aldolase, it is helpful to recall that the equilibrium reaction catalyzed by aldolase is dependent on the concentration of substrates. Aldolase

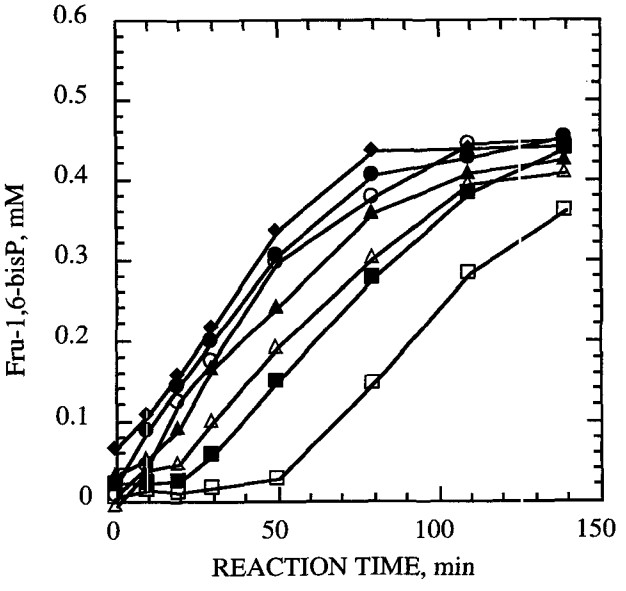

Figure 5. The effect of including Fru-1,6-bisP in the PFP assays. Assays were performed as in Figure 1C measuring the concentration of Fru-1,6-bisP (forward assay: initial concentrations were 1 mM Fru-6-P and 1 mM PPi). Reactions were performed in the presence of either 1 μM Fru-2,6-bisP (●) or Fru-1,6-bisP: 0 μM, ○, □; 5 μM, ■; 10 μM, △; 25 μM, ▲; 100 μM, ◆. One assay (○) did not contain aldolase. All other reactions were performed in the presence 1 unit mL^{-1} aldolase.

will remove Fru-1,6-bisP efficiently when this metabolite is present in low concentrations. At higher concentrations of Fru-1,6-bisP, aldolase will convert only a small part to triosephosphates. At the equilibrium concentrations of Fru-1,6-bisP in the PFP assays (about 0.4 mM, Fig. 1), aldolase will cleave a minor fraction of the Fru-1,6-bisP, and aldolase will have only limited effect on the PFP-catalyzed equilibrium.

As another result the aldolase equilibrium will shift toward Fru-1,6-bisP as a low activity of PFP slowly builds up Fru-1,6-bisP. Eventually, sufficient Fru-1,6-bisP is formed to activate the PFP reaction, leading to the formation of even more Fru-1,6-bisP. Therefore, the aldolase-induced requirement for activation by Fru-2,6-bisP is eventually overcome as observed in all experiments (Figs. 2–5). Including less or more PFP will speed up or slow down this activation pattern as seen in Figure 4; the reaction thus behaves as an autocatalytic reaction.

Because of the formation of Fru-1,6-bisP during the forward PFP reaction, it is difficult to obtain precise data on the affinity of PFP for Fru-1,6-bisP at the allosteric site. As soon as the reaction is sufficient to be detected, the concentration of Fru-1,6-bisP has increased dramatically above the concentration required for the allosteric activation. Apparently, the activation of PFP is significant at 5 to 25 μM Fru-1,6-bisP, and PFP is fully activated at 100 to 200 μM Fru-1,6-bisP. The concentration of Fru-1,6-bisP in the cytosolic compartment of plant tissue will depend strongly on the metabolic activity. Thus, photosynthetic activity in leaves will induce a large increase in Fru-1,6-bisP concentration. Gerhardt et al. (1987) estimated the level of Fru-1,6-bisP in spinach leaf cytoplasm to vary between 1 and 4 nmol (mg Chl)$^{-1}$, which is equivalent to approximately 20 μM in the cytosol. On the basis of this estimate it is reasonable to assume that PFP may be activated by Fru-1,6-bisP in photosynthetic leaves. Thus, the concentration of Fru-1,6-bisP may be an important factor for determining in vivo activity of barley PFP. A rather specific activation of PFP by Fru-1,6-bisP would suggest a gluconeogenetic role of the enzyme. The activity of PFP is quite high in barley leaf tissue, especially during the early development (Nielsen, 1992), suggesting that PFP may contribute to Suc synthesis in the young leaves in which the activity of cytosolic Fru-1,6-bisP is typically low. Collis and Pollock (1991) reached a similar conclusion by showing that the activity of cytosolic Fru-1,6-bisP in leaves of *Lolium temulentum* was insufficient to account for the observed Suc synthesis.

Presumably, Pi will modify the affinity of the allosteric site for Fru-1,6-bisP (Stitt and Vasella, 1988; Stitt, 1989). The noncoupled assays included moderate concentrations of Pi, which changed from 1 to about 0.5 mM during the assays. This further complicates the estimation of the affinity for Fru-1,6-bisP at the allosteric site.

The precise role of PFP in metabolism is still not clear (Stitt, 1990; Hajirezai et al., 1993). Most likely, it depends on the specific tissue studied, leaving PFP as an adaptive enzyme as already suggested by Black et al. (1987). The activation of barley PFP by Fru-1,6-bisP should be taken into consideration, since it represents a previously unrecognized possibility for activation of PFP in tissues with gluconeogenetic activity. It still remains to be investigated whether PFP from other plant species are equally sensitive to allosteric activation by Fru-1,6-bisP.

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