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Product Inhibition of Potato Tuber Pyrophosphate:Fructose-6-Phosphate Phosphotransferase by Phosphate and Pyrophosphate[1]

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ABSTRACT

The product inhibition of potato (*Solanum tuberosum*) tuber pyrophosphate:fructose-6-phosphate phosphotransferase by inorganic pyrophosphate and inorganic phosphate has been studied. The binding of substrates for the forward (glycolytic) and the reverse (gluconeogenic) reaction is random order, and occurs with only weak competition between the substrate pair fructose-6-phosphate and pyrophosphate, and between the substrate pair fructose-1,6-bisphosphate and phosphate. Pyrophosphate is a powerful inhibitor of the reverse reaction, acting competitively to fructose-1,6-biphosphate and noncompetitively to phosphate. At the concentrations needed for catalysis of the reverse reaction, phosphate inhibits the forward reaction in a largely noncompetitive mode with respect to both fructose-6-phosphate and pyrophosphate. At higher concentrations, phosphate inhibits both the forward and the reverse reaction by decreasing the affinity for fructose-2,6-bisphosphate and thus, for the other three substrates. These results allow a model to be proposed, which describes the interactions between the substrates at the catalytic site. They also suggest the enzyme may be regulated *in vivo* by changes of the relation between metabolites and phosphate and could act as a means of controlling the cytosolic pyrophosphate concentration.

Control of phosphofructokinase and fructose-1,6-bisphosphatase by $Fru2,6P_2$,[2] adenylates, and respiratory intermediates provides a framework to understand how glycolysis and gluconeogenesis are controlled in animals and fungi. However, plants possess an enzyme, called PFP, which is capable of substituting for both of these enzymes (1, 6, 21). PFP was found originally in *Entamoeba* (19) and *Proprionibacteria* (18) and catalyses the freely reversible reaction Fru6P + PPi⇌$Fru1,6P_2$ + Pi. The significance of PFP for plant metabolism is not yet understood (1, 8, 27).

$Fru2,6P_2$ activates PFP but does not activate phosphofructokinase from higher plants (7, 21, 32). Accordingly, it has been suggested that increasing concentrations of $Fru2,6P_2$ stimulate glycolysis by activating PFP, in analogy to the action of $Fru2,6P_2$ on phosphofructokinase in other tissues (2–4, 8, 11, 16, 23, 25, 32, 33). However, measurements of metabolite levels have revealed that the reaction catalyzed by PFP is close to equilibrium *in vivo* (9, 34). In this case, PFP might equally well operate in the gluconeogenic direction. Indeed, several studies of $Fru2,6P_2$ levels and PFP activities in contrasting tissues have been interpreted as evidence that the reverse reaction provides carbon for synthesis of sugars, or PPi for sucrose mobilization via sucrose synthase (1, 4, 5, 9, 12, 17).

Studies of the kinetic properties of PFP should provide information about the conditions in which the forward or the reverse reaction might operate. Previous studies have concentrated on the activation of PFP by $Fru2,6P_2$. There is general agreement that $Fru2,6P_2$ activates PFP by increasing the V_{max} of the forward reaction and by increasing the affinity for Fru6P and $Fru1,6P_2$ (4, 13, 14, 32). It is also known that Fru6P increases the affinity for $Fru2,6P_2$, while Pi acts in the opposite manner as do a range of other anions (13, 14, 32). These properties have been interpreted as evidence that the physiological role of PFP is in glycolysis (32), or as consistent with it operating in either direction (4), or as not providing any clear evidence about its role (13, 14).

The following article reapproaches this problem by investigating the interactions between the various potential substrates and products of PFP. The products of a reaction are formed at the catalytic site, and are substrates for the reverse reaction. Consequently a product may act as an inhibitor by occupying the same site on the enzyme as the substrate from which it is derived. Since PFP catalyses a freely reversible reaction which lies close to equilibration *in vivo*, study of these interactions could be crucial for understanding how it functions in physiological conditions.

MATERIALS AND METHODS

PFP was partially purified (160-fold) from potato (*Solanum tuberosum*) tubers (32) and was stored in 50% (v/v) glycerol at 20°C. The preparation was free of ATP phosphofructokinase, aldolase, fructose-1,6-bisphosphatase, and aldolase. Phosphoglucose isomerase was less than 10% of the PFP activity.

Assays were carried out in 50 mM imidazol-HCl (pH 7.3), 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA. For the forward reaction, 0.12 mM NADH, 5 units/mL glycerol-3-phosphate dehydrogenase, 15 units/mL triose phosphate isomerase, 10 units/mL triose phosphate isomerase, and 10 units/mL al-

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[2] Abbreviations: $Fru1,6P_2$, fructose-1,6-bisphosphate; $Fru2,6P_2$, fructose-2,6-bisphosphate; Fru6P, fructose-6-phosphate; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase; Pi, inorganic phosphate.

dolase were included. For the reverse reaction, 0.4 mM NADP, 10 units/mL glucose-6-phosphate dehydrogenase, and 10 units/mL phosphoglucose isomerase were included. Unless stated, $Fru2,6P_2$ was included at 0.5 μM. For the concentrations of Fru6P, PPi, $Fru1,6P_2$, and Pi, see figure legends. Biochemicals and coupling enzymes were purchased from Boehringer (Mannheim) except for Fru6P and $Fru2,6P_2$, which were from Sigma (St. Louis, MO). $Fru1,6P_2$ and Fru6P were pretreated with acid to ensure they did not contain $Fru2,6P_2$. All assays were carried out using a ZFP 22 dual wavelength photometer (Sigma, Berlin) at an expansion of 0.02E or 0.05E.

RESULTS

Substrate-Substrate Interactions

Before carrying out product inhibition studies, it was necessary to establish whether substrate binding was random or fixed order (Figs. 1, and 2). For each substrate, saturation curves were measured at several fixed concentrations of the reaction partner. Double reciprocal plots of the resulting data intersected on the left hand side of a Lineweaver-Burke plot close to, but just below, the x axis. Such plots are consistent with random order binding, with one ligand slightly decreasing the affinity for the second ligand (22). Figure 1 (forward reaction) and Figure 2 (reverse reaction) show how the K_m^{app} of each substrate varies, depending on the concentration of the reaction partner. The range where the second substrate is

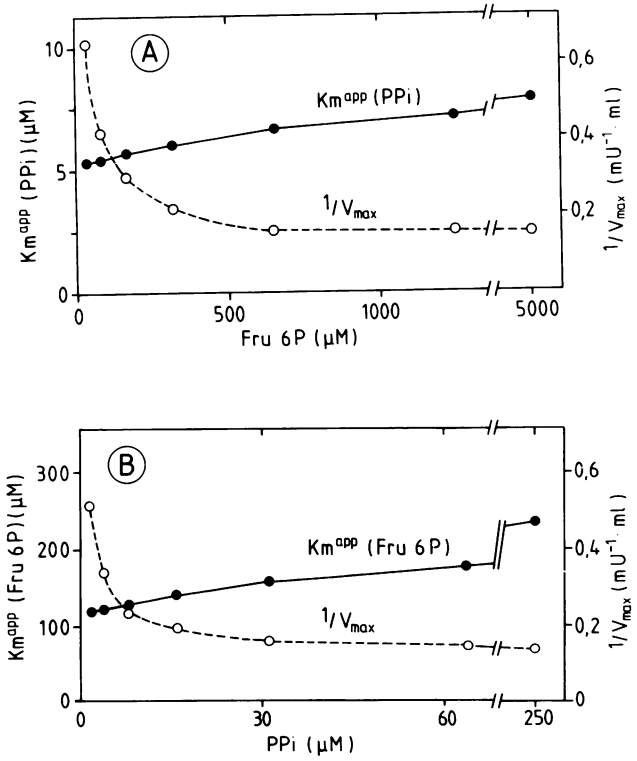

Figure 1. Interactions between substrates for the forward reaction. A, Influence of the Fru6P concentration on the affinity for PPi. B, Influence of the PPi concentration on the affinity for Fru6P. The alteration of the K_s^{app} (●——●) and of the V_{max} (O- - - -O) are shown. All assays included 0.5 μM $Fru2,6P_2$.

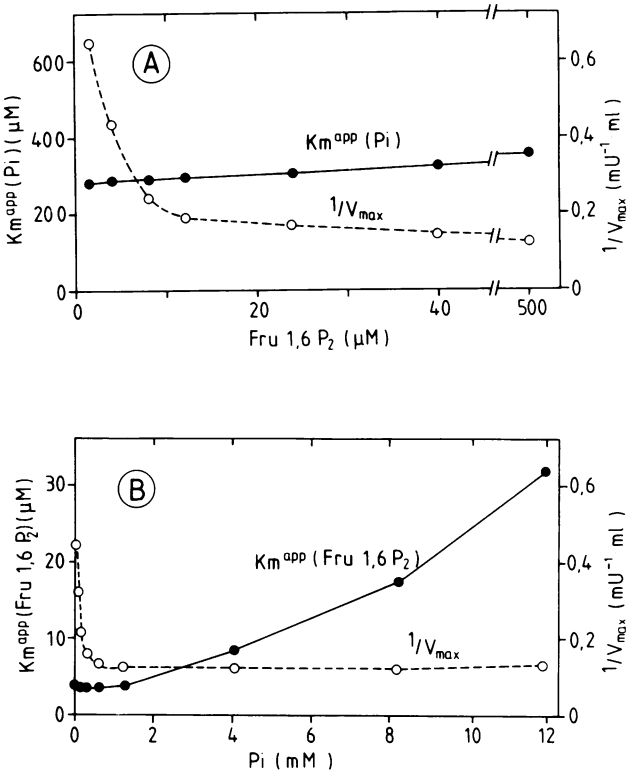

Figure 2. Interactions between substrates for the reverse reaction. A, Influence of $Fru1,6P_2$ on the affinity for Pi. B, Influence of Pi on the affinity for $Fru1,6P_2$. The alterations of K_s^{app} (●——●) and of the V_{max} (O- - - -O) are shown. All assays included 0.5 μM $Fru2,6P_2$.

required for catalysis is revealed by a rising V_{max} value. The K_m^{app} (Fru6P) and K_m^{app} (PPi) only increased by about 30% as the concentration of their reaction partner was increased (Fig. 1), and the K_m^{app} (Pi) only rose by 14% as the $Fru1,6P_2$ concentration was increased (Fig. 2A).

The response of K_m^{app} ($Fru1,6P_2$) to rising Pi was more complicated (Fig. 2B). When Pi was increased up to about 1.5 mM, which is the range where it is needed for catalysis, the K_m^{app} ($Fru1,6P_2$) was unaffected or even declined slightly. When the Pi concentration was increased over the range from 2 to 12 mM, the K_s^{app} ($Fru1,6P_2$) increased 10-fold. This decreased affinity for $Fru1,6P_2$ was not accompanied by any change in V_{max}.

PPi and the Reverse Reaction

PPi was a very effective inhibitor of the reverse reaction (Fig. 3), inhibiting competitively to $Fru1,6P_2$ (Fig. 3A). Derivative plots of K_m^{app} ($Fru1,6P_2$) against the PPi concentration yielded a K_i value of about 9 μM in the presence of 0.25 mM Pi, or 16 μM in the presence of 8 mM Pi (Fig. 3B). The inhibition by PPi could not be relieved by increasing the $Fru2,6P_2$ concentration (not shown), and PPi did not significantly alter the K_a^{app} ($Fru2,6P_2$) (Fig. 3C). The inhibition could not be overcome by increasing Pi (Fig. 3D); conversely PPi did not prevent the inhibition found when high Pi was present (Fig. 3D, compare also Fig. 3B).

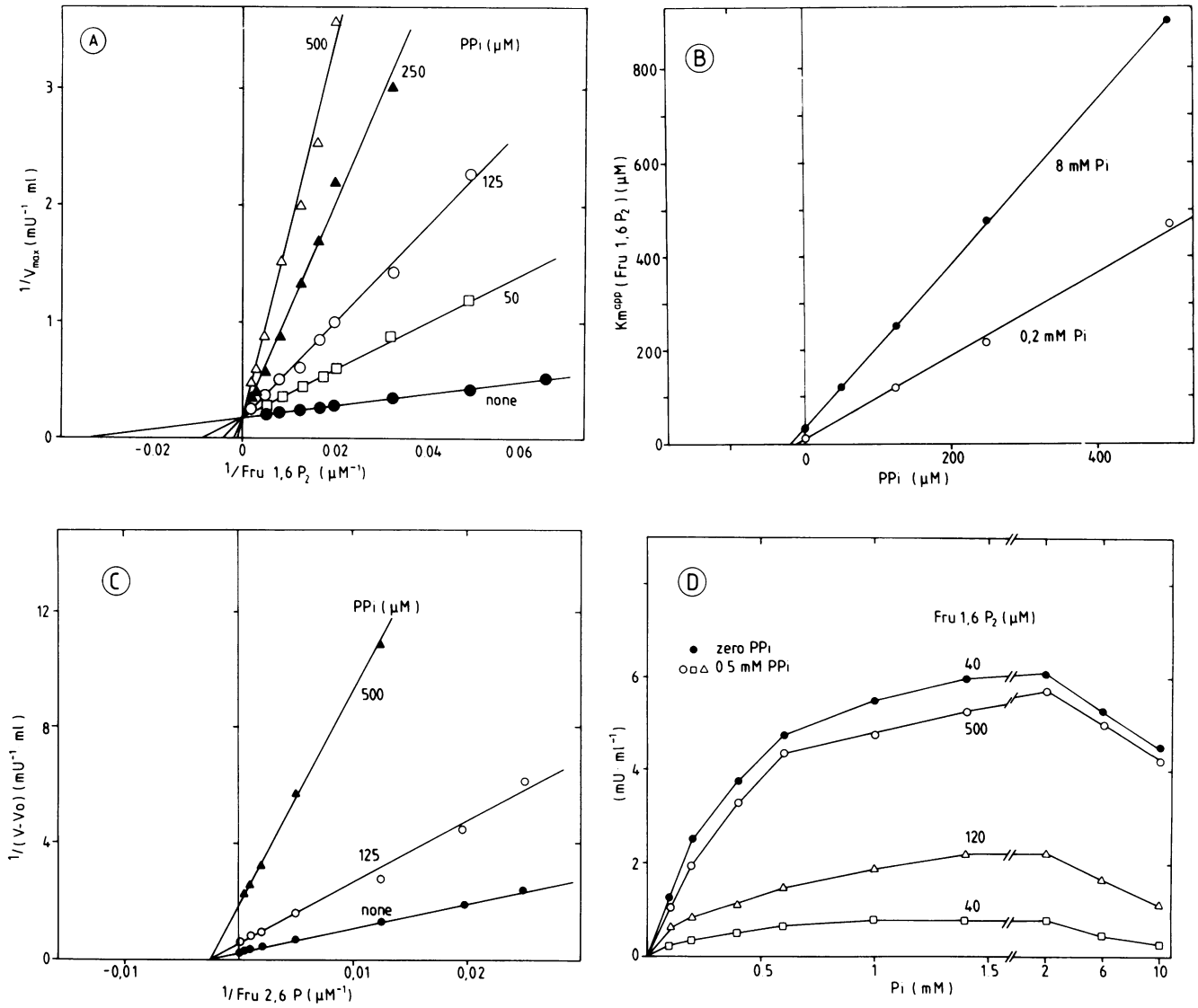

Figure 3. Influence of PPi on the reverse reaction. A, Noncompetitive inhibition with respect to Fru1,6P_2. Assayed with 8 mM Pi, 0,5 μM Fru2,6P_2. B, Derivative plot of K_s^{app} (Fru1,6P_2) versus PPi concentration. The data are taken from A, and a similar experiment carried out with 0.2 mM Pi. C, Influence of PPi on the K_a^{app} (Fru2,6P_2). The term ($V - V_0$) was obtained by subtracting the activity in the absence of Fru2,6P_2 from the activity with Fru2,6P_2 present. All assays were carried out with 8 mM Pi and 40 μM Fru1,6P_2. D, Response to rising concentrations of Pi in the absence of PPi (●) and with 0.5 mM PPi (○, □, △).

Pi and the Forward Reaction

As previously reported (13, 14, 32), Pi inhibits the forward reaction, affecting both the K_m and the V_{max}. However, Pi does not act like a normal mixed-type inhibitor. When 1/Fru6P or 1/PPi were plotted against $1/V$ for different Pi concentrations, the lines did not intersect at one point (data not shown). Instead, the point of intersection shifted to the right as the Pi concentration was increased. The resulting values for the K_m^{app} (Fru6P), K_m^{app} (PPi) and $1/V_{max}$ are given in Figure 4. These do not change in a linear manner as Pi is increased. At low Pi, the K_m values are less strongly affected than the V_{max}. Above 2 mM Pi, the K_m values are more strongly affected than the V_{max}.

Pi and Fru2,6P_2

Previous studies have shown Pi alters the affinity for Fru2,6P_2, and the V_{max} in a complex manner (13). This interaction was reinvestigated, paying attention to the possibility that the effect of Pi might vary, depending on the concentration used. The influence of Pi on the Fru2,6P_2 affinity was tested when PFP was assayed in the forward (Fig. 5A) and the reverse (Fig. 5B) direction. At concentrations below 1.7 mM, Pi decreased the V_{max} of the forward reaction and increased the V_{max} of the reverse reaction, without substantially altering the K_a^{app} (Fru2,6P_2) for either reaction. At higher concentrations, Pi led to a very large increase of the K_a^{app} (Fru2,6P_2) for both the forward and the reverse reactions. The V_{max} of the reverse reaction was hardly affected. The V_{max}

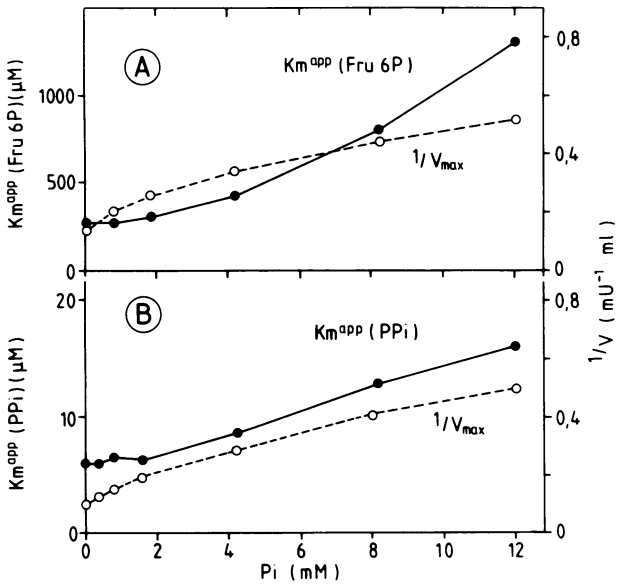

Figure 4. Influence of Pi on the forward reaction. A, Change of K_s^{app} (Fru6P) (●——●) and V_{max} (O- - - -O), assayed with 0,5 μM Fru2,6P_2 and 0.1 mM PPi. B, Changes of K_s^{app} (PPi) (●——●) and V_{max} (O- - - -O), assayed with 0.5 μM Fru2,6P_2 and 0.6 mM Fru6P.

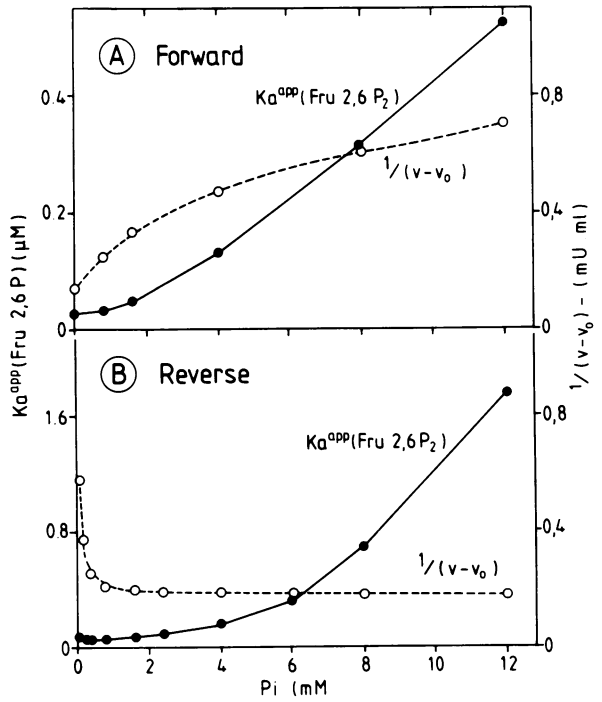

Figure 5. Influence of different concentrations of Pi on the Fru2,6P_2 affinity. A, Forward reaction, assayed with 0.1 mM PPi and 0.6 mM Fru6P. B, Reverse reaction, assayed with 16 μM Fru1,6P_2. For each reaction the V_{max} (O- - - -O) and the K_a^{app} (Fru2,6P_2 (●——●) was estimated by plotting $(V - V_0)^{-1}$ *versus* $(Fru2,6P_2)^{-1}$, where V = activity with Fru2,6P_2 and V_0 = activity in the absence of Fru2,6P_2.

of the forward reaction was decreased but this was less marked than at lower Pi concentrations.

The influence of other anions was also investigated, for comparison with Pi. Table I summarizes the influence of NaCl, and similar results were obtained with malate and nitrate (not shown). These anions led to a large decrease of the affinity for Fru2,6P_2, decreased the affinity for Fru6P and Fru1,6P_2, but had only a small effect on the affinity for Pi and PPi. These results are very similar to these obtained with 3-phosphoglycerate on PFP from castor bean endosperm (13).

DISCUSSION

Substrate-Product Interactions

There is little interaction between the binding of Fru6P and PPi for the forward reaction. There is also little interaction between the binding of the substrates for the reverse reaction, provided Pi is kept in the range of 0 to 1.7 mM. Since the K_m^{app} (Pi) was in the range of 0.25 to 0.4 mM, these results suggest that binding of Pi at the catalytic site does not strongly interfere with binding of Fru1,6P_2 (see below for further discussion). These results confirm the conclusion from studies of the castor bean endosperm enzyme (13) that PFP higher plants has random order binding, rather than the ping-pong mechanism proposed for the enzyme from pineapple (5).

PPi is a powerful inhibitor of the reverse reaction. The *Ki* values for inhibition are comparable to the K_m (PPi) for the forward reaction. Fru2,6P_2 does not relieve the inhibition by PPi (Fig. 3D), nor does it strongly alter the K_m (PPi) for the forward reaction (13, 14). Both are also only weakly modified by Pi (Figs. 1B and 3C). Taken together, these results suggest PPi is acting as a true product inhibitor of the reverse reaction. Significantly, PPi inhibits competitively to Fru1,6P_2 and non-competitively to Pi (see below for further discussion).

The inhibition by Pi is more complex, but can be explained if Pi is acting in two different ways. One involves a largely noncompetitive inhibition of the forward reaction. This response is found when Pi is varied in the range up to 1.5 mM, which compares well with the concentration range where Pi is needed for catalysis in the reverse direction (K_m = 0.29–0.45 mM). These effects may therefore reflect binding of Pi at the catalytic site. The second effect of Pi becomes apparent at concentrations above 2 mM, and results in a parallel inhibition of *both* the forward and the reverse reaction. This inhibition is associated with a very large decrease of the affinity for

Table I. *Influence of NaCl on the Kinetic Properties of PFP*

PFP was assayed in the forward direction with 0.5 mM PPi, 2 μM Fru2,6P_2, and 3.3 mM Fru6P, and in the reverse direction with 0.8 mM Pi, 40 μM Fru1,6P_2 and 0.5 μM Fru2,6P_2. For determination of K_s or K_a value, one of the substrates on the Fru2,6P were varied, while holding the others constant.

Reaction Direction	Parameter	NaCl Added	
		None	100 mM
Forward	V_{max} (mU/mL)	6.4	6.0
	K_a^{app} (Fru2,6P_2) (μM)	0.005	0.095
	K_s^{app} (Fru6P) (mM)	0.30	0.81
	K_s^{app} (PPi) (mM)	0.008	0.016
Reverse	V_{max} (mU mL^{-1})	5.4	5.0
	K_a^{app} (Fru2,6P_2)	0.083	0.56
	K_s^{app} (Fru1,6P_2) mM	0.005	0.12
	K_s^{app} (Pi) mM	0.20	0.48

Fru2,6P_2. The smaller fall in the affinity for Fru6P and Fru1,6P_2 would then be the result of a lowered affinity for Fru2,6P_2. A similar response is found with many other anions. The effects of Pi at high concentrations may therefore be due to it interfering with binding of Fru2,6P_2, as has previously been suggested for many other anions (13, 14).

Based on these results, a model can be proposed which describes the interactions between the substrates at the active site of PFP. Pi appears to bind at a site which is separated from the other three substrates, because it does not compete with PPi, Fru6P or Fru1,6P_2, when added at the concentrations which are needed for catalysis. PPi binds competitively to Fru1,6P_2, but does not compete strongly with Fru6P, suggesting PPi binds at a site shared by the phosphate esterified to the first carbon of Fru1,6P_2. In this case, PPi is bound in position for one of its constituent phosphates to be transfered onto the first carbon of Fru6P. The other Pi molecule will be extruded into the spatially separated binding site. This model predicts that a enzyme-Fru6P-PPi-Pi complex can be formed, and that this complex will be catalytically inactive because Pi is occupying the site into which the unutilized phosphate from PPi would normally be moved. The noncompetitive inhibition of the forward reaction by low Pi (see above) is consistent with the formation of this dead end complex.

PFP from higher plants has many similarities to PFP from Entamoeba (20). This enzyme also has random order substrate binding, PPi inhibits competitively to Fru1,6P_2, and Pi inhibits noncompetitively to Fru6P and Pi. The higher plant PFP differs, of course, in being activated by Fru2,6P_2. It is therefore interesting that no secondary effects of Pi on the substrate affinities of the *Entamoeba* enzyme were found, provided Mg^{2+} was not limiting. This comparison suggests the protozoan and plant enzyme have a basically similar mechanism at the active site, but that additional properties associated with the Fru2,6P_2 activation have been added in the higher plant enzyme.

Physiological Consequences

It has been shown elsewhere that Fru2,6P_2 binds at one kind of activator site and activates the forward and reverse reactions in parallel (29), by increasing the affinity for Fru6P and Fru1,6P_2. The resulting flux will depend upon the concentrations of the four potential substrates for two different reasons. First, the direction of the net flux will be determined by the relation between the mass action ratio [Fru6P]·[PPi]/[Fru1,6P_2]·[Pi] and the equilibrium constant of this reaction. Secondly, changes in the concentration of one metabolite will alter the affinity of PFP to the other metabolites, as well as the sensitivity to activation by Fru2,6P_2. These thermodynamic and kinetic effects may interact, to allow marked alterations in the flux rate and direction as metabolic conditions alter.

If cytosolic Pi fell below 1 mM, there would be a selective activation of the forward reaction and restriction of the reverse reaction. However, the available estimates for the cytosolic Pi concentration are between 5 and 25 mM (15, 26). At these concentrations, Pi modifies the activity of the forward and reverse reaction in parallel, by altering the sensitivity to Fru2,6P_2. This activation of PFP by falling Pi would be amplified by rising Fru6P or Fru1,6P_2. These properties suggest that PFP will tend to become more active as the general level of metabolites in the cytosol increases, and Pi decreases. This effect would be amplified, if Fru2,6P_2 rises in these conditions (24, 27, 28, 30), due to activation of Fru6P,2-kinase and inhibition of Fru2,6P_2ase by Fru6P (27).

The levels of PPi found in plant tissues suggest the cytosolic concentration is about 200 μM (9, 34). This lies well above the K_m (PPi) for the forward direction, although it might be noted that rising Fru1,6P_2 probably lowers the K_m (PPi) (20). The PPi concentrations found *in vivo* will also lead to a large increase of the Fru1,6P_2 concentration which is needed to support catalysis in the reverse mode.

It has been suggested that PFP could operate in a cycle with the phosphofructokinase or fructose-1,6-bisphosphatase to generate or consume PPi, respectively (1, 5). The properties of PFP would suit it for a role in controlling the PPi concentration. When PPi is high, the reverse reaction would be inhibited, and the forward reaction would provide a way of consuming PPi. Conversely, falling PPi would activate the reverse reaction, so more PPi can be generated. Thus, these properties suggest PFP might be able to operate as a PPi-stat, which is engaged by rising Fru2,6P_2 and/or falling Pi.

An integration of these potential roles of PFP in catalysing a net flow of carbon and in controlling the PPi concentration will only be possible when more is known about other reactions involved in the generation and consumption of PPi in the cytosol of plant cells. However, there are revealing differences between PFP and other enzymes involved in carbohydrate metabolism. During gluconeogenesis, high Fru1,6P_2 and low Pi would stimulate both PFP and the cytosolic fructose-1,6-bisphosphatase (27). These two enzymes, however, will have opposite responses to Fru2,6P_2, and will also differ in their response to the energy status in the cytosol. The Fru1,6Pase will be powerfully inhibited by AMP (27), but PFP will be modulated by PPi. It can be envisaged how rising levels of Fru1,6P_1, and falling Pi signal that surplus carbon is available which cannot be used for respiration or growth (31). Depending on the conditions, either the cytosolic fructose-1,6-bisphosphatase or PFP could catalyse their conversion to hexose P. There are also differences between PFP, and the enzymes which provide the classical control sites for glycolysis. Phosphofructokinase is activated high Pi, and is inhibited by phospho*enol*pyruvate (10, 31). These are the properties expected for an enzyme which is responding to the demand for respiratory substrates. In contrast, PFP is more likely to become active in conditions of low Pi and high sugar phosphates (see above) and is also subject to higher levels of control, via Fru2,6P_2. Such properties suggest PFP does not just respond to changes in the demand for respiratory substrates. Rather, it may provide a way of modifying fluxes in response to external factors, or when large amounts of carbon are being moved between starch, sucrose and organic acids. This resembles the idea that plants may have 'maintenance' and 'adaptive' pathways for carbohydrate metabolism (5). The reversibility of the reaction catalysed by PFP, and its modulation by changes of Fru2,6P_2, PPi, Pi, Fru1,6P_2 and

Fru6P could allow a considerable flexibility in the response of primary metabolism to these contrasting demands.

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