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Kinetic Properties of Pyrophosphate:Fructose-6-Phosphate Phosphotransferase from Germinating Castor Bean Endosperm[1]

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ABSTRACT

Pyrophosphate:fructose-6-phosphate phosphotransferase (PFP) was purified over 500-cold from endosperm of germinating castor bean (*Ricinus commiunis* L. var. Hale). The kinetic properties of the purified enzyme were studied. PFP was specific for pyrophosphate and had a requirement for a divalent metal ion. The pH optimum for activity was 7.3 to 7.7. The enzyme had similar activities in the forward and reverse directions and exhibited hyperbolic kinetics with all substrates. Kinetic constants were determined in the presence of fructose 2,6-bisphosphate, which stimulated activity about 20-fold and increased the affinity of the enzyme for fructose 6-phosphate, fructose 1,6-bisphosphate, and pyrophosphate up to 10-fold. Half-maximum activation of PFP by fructose 2,6-bisphosphate was obtained at 10 nanomolar. The affinity of PFP for this activator was reduced by decreasing the concentration of fructose 6-phosphate or increasing that of phosphate. Phosphate inhibited PFP when the reaction was measured in the reverse direction, *i.e.* fructose 6-phosphate production. In the presence of fructose 2,6-bisphosphate, phosphate was a mixed inhibitor with respect to both fructose 6-phosphate and pyrophosphate when the reaction was measured in the forward direction, *i.e.* fructose 1,6-bisphosphate production. The possible roles of fructose 2,6-bisphosphate, fructose 6-phosphate, and phosphate in the control of PFP are discussed.

During germination of castor bean, there is a massive conversion of fat to sucrose in the endosperm. The final stage of this conversion, the synthesis of sucrose from oxaloacetate, probably occurs exclusively in the cytoplasm (13, 14). Our knowledge of the control of this sequence is limited. The available evidence suggests that the conversion of Fru-1,6-P_2[3] to Fru-6-P is regulated in both castor bean endosperm (8) and the cotyledons of *Cucurbita pepo* (10).

Although castor bean endosperm contains fructose-1,6-biphosphatase and phosphofructokinase, the two enzymes of Fru-6-P/Fru-1,6-P_2 metabolism generally considered to be important in gluconeogenesis and glycolysis, respectively, we have recently demonstrated that this tissue also contains considerable PFP activity (9). At present the physiological role of this enzyme is unclear. The reaction catalyzed by PFP is easily reversible *in vitro*; therefore, it could operate in either direction *in vivo*. In castor bean endosperm, PFP activity is confined to the cytoplasm and is sufficient to account for the flux from Fru-1,6-P_2 to Fru-6-P *in vivo*. Thus, PFP has the capacity to contribute significantly to Fru-6-P production and may be important in the regulation of sucrose synthesis.

Little is known about the properties of PFP from gluconeogenic tissues. We have previously reported that, in crude extracts of castor bean endosperm, PFP is stimulated up to 10-fold by Fru-2,6-P_2 (9). Similar activation by Fru-2,6-P_2 has been reported for PFP purified from mung bean hypocotyl (19), spinach leaf (3), and potato tuber (20). Here we describe the purification of PFP from castor bean endosperm and report on its kinetic and regulatory properties.

MATERIALS AND METHODS

Materials. Castor bean seeds (*Ricinus communis* L. var. Hale) were soaked for 24 h in running tap water, then placed in moist vermiculite and grown in the dark at 30°C in a humidified growth chamber. The plants were harvested 4 d after sowing. Fru-2,6-P_2, substrates, cofactors, and auxiliary enzymes were purchased from Sigma. DEAE-cellulose (DE52) and phosphocellulose (P11) were from Whatman, and Bio-Gel A-1.5m was obtained from Bio-Rad.

Enzyme Assays. All assays were carried out at 25°C in a total volume of 1 ml. Auxiliary enzymes were dialyzed against 5 mM Hepes-NaOH (pH 7.5) before use. NADH oxidation and $NADP^+$ reduction were recorded at 340 nm with a Gilford 250 spectrophotometer. PFP activity in the forward direction was measured by the production of Fru-1,6-P_2. The standard assay contained 100 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 5 mM Fru-6-P, 0.1 mM NADH, 0.2 mM PPi, 1 μM Fru-2,6-P_2, 1 IU aldolase, 10 IU triose-P isomerase, 1 IU glycerol-3-P dehydrogenase. The reaction was started with PPi. This assay was used throughout the purification of PFP. PFP activity in the reverse direction was measured by the production of Fru-6-P in a mixture that contained 100 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM Fru-1,6-P_2, 1 mM $NADP^+$, 5 mM NaH_2PO_4, 2 IU hexose-P isomerase, 1 IU glucose-6-P dehydrogenase. The assay was started by the addition of Pi. For the kinetic analyses, the concentrations of substrates and effectors were varied as indicated in "Results."

Other enzymes were measured as follows: P-fructokinase (9), fructose-1,6-bisphosphatase (9), aldolase (13), and hexose P isomerase (13). These assays gave maximum activities in crude homogenates of endosperm. Inorganic pyrophosphatase was as-

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[3] Abbreviations: Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; K_a, activation constant, *i.e.* concentration of effector that gives half-maximum activation; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase (EC 2.7.1.90)

sayed in a mixture containing 100 mM Hepes-NaOH (pH 7.5) or Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 5 mM PPi. The reaction was stopped after 20 min by adding 1.3 volumes of 7.15 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 5% (w/v) TCA and the Pi released was determined according to Leigh and Walker (11).

Purification of PFP. All procedures were carried out at 0 to 4°C. Endosperm (100–150 g fresh weight) from 120 to 200 4-d-old castor bean seedlings was homogenized in a Waring Blendor together with 2 volumes of 50 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2% (w/v) PVP. The homogenate was centrifuged at 20,000*g* for 20 min, and the resulting supernatant was filtered through Miracloth (Calbiochem) to remove the fat layer. Ammonium sulfate was added to the filtrate. The material that precipitated between 35 and 50% saturation was collected by centrifugation (20,000*g*, 20 min), redissolved in about 20 ml 10 mM sodium citrate (PH 6.6), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol and dialyzed against 2 L of the same buffer. The dialyzed extract was adjusted to 20% (w/v) glycerol and applied, at 37 ml/h, to a phosphocellulose column (2.5 × 20 cm) equilibrated with 10 mM sodium citrate (pH 6.6), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 20% (w/v) glycerol. The column was washed with 80 ml of the equilibrating buffer, and then PFP was eluted by a linear gradient (500 ml) of 0 to 0.4 M KCl in the same buffer. The fractions that contained most of the PFP activity were combined and concentrated to 10 ml with an Amicon ultrafiltration system fitted with a Diaflo PM-10 membrane. The concentrated extract was applied, at 20 ml/h, to a Bio-Gel A-1.5m column (2.5 × 86 cm) equilibrated with 50 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 100 mM KCl, 20% (w/v) glycerol, and the column was washed with the same buffer. The peak of PEP activity eluted from the column was dialyzed against 2 L of the same buffer, except that KCl was omitted. The dialyzed extract was applied, at 37 ml/h, to a DEAE-cellulose column (2.5 × 4 cm) equilibrated with dialysis buffer, and the column was washed with 40 ml of the equilibrating buffer followed by a linear gradient (400 ml) of 0 to 0.4 M KCl in the same buffer. The fractions containing most of the PFP activity were combined to yield the purified PFP preparation.

Protein was measured according to Lowry *et al.* (12) after precipitation by 5% (w/v) TCA. BSA was used as a standard.

Determination of Kinetic Constants. Values for K_m^{app} and V^{app} at fixed concentrations of the second substrate were obtained by linear regression of v against v/s (Eadie-Hofstee plot). Similar values were obtained from Hanes plots (s/v against s). Each plot contained at least six points. The absolute kinetic parameters K_m, K_i, and V were obtained from secondary plots of b/V^{app} and $b\cdot K_m^{app}/V^{app}$ against b as described by Cornish-Bowden (5), where b is the concentration of the second substrate. Each secondary plot contained at least five points.

Two different methods were used to calculate the apparent values for the competitive and uncompetitive inhibition constants ${}^{app}K_i$ and ${}^{app}K_i'$, respectively. In Method 1, the constants were obtained by linear regression of $1/v$ and s/v against i at fixed substrate concentrations as described by Dixon (6) and Cornish-Bowden (4), where i is the inhibitor concentration. In these two plots, the lines at various substrate concentrations intersect around a point for which $i = -{}^{app}K_i$ or $-{}^{app}K_i'$, respectively. Individual points of intersection were calculated directly from the regression equations, and the median value of these estimates was taken as the inhibition constant. In Method 2, K_m^{app} and V^{app} were determined from Eadie-Hofstee plots at fixed inhibitor concentrations. The inhibition constants were obtained from secondary plots of K_m^{app}/V_{app} and $1/V^{app}$ against i. These plots yield intercepts on the x axis of ${}^{app}K_i$ and ${}^{app}K_i'$, respectively.

RESULTS

Purification of PFP. A summary of the purification procedure for PFP is presented in Table I. Typically, the enzyme was purified over 500-fold with a yield of 10 to 20%. It was essential to include glycerol during the purification. Attempts to purify PFP in the absence of glycerol resulted in almost complete loss of activity before the final step. After purification, PFP retained about 80% of its activity for 1 week when stored at 4°C in the presence of 20% (w/v) glycerol.

In the final preparation, the activities of P-fructokinase, hexose-P isomerase, aldolase, and inorganic pyrophosphatase were less than 1% of the activity of PFP. The apparent fructose-1,6-bisphosphatase activity was about 2% of that of PFP. However, this is likely to be an over-estimate of the contamination by fructose-1,6-bisphosphatase since any Pi in the assay would allow PFP to function in the reverse direction.

Properties of PFP. Enzyme activity was specifically dependent on PPi. Neither ATP, ADP, GTP, CTP, UTP, ITP, phosphoenolpyruvate, nor tripoly-P, each at 1 mM, could replace PPi in the reaction. Low rates were initially observed with several of these compounds, but were attributed to contamination by PPi, since these rates declined to zero after a few minutes and could be totally abolished by incubating the compound, at 20 mM, with inorganic pyrophosphatase (1 unit/ml) for 1 to 5 min before use.

In the forward direction, PFP had a broad pH optimum with maximum activity at pH 7.3 to 7.7, whereas in the reverse direction, the enzyme displayed a sharper optimum at pH 7.75 (Fig. 1). Stimulation by Fru-2,6-P_2 had no effect on the pH optimum in the forward direction. In the reverse direction, Fru-2,6-P_2 did not stimulate PFP activity, since the assay already contained sufficient Fru-1,6-P_2 to full activate the enzyme (18).

To investigate the cation requirement of PFP, we removed from the enzyme preparation Mg^{2+} which had been added during purification to stabilize PFP activity. Purified enzyme (1 unit) was absorbed onto a DEAE-cellulose column (1.5 × 3 cm) equilibrated with 100 mM triethanolamine-HCl (pH 7.7) containing 20% (w/v) glycerol. The column was washed with 10 bed volumes of the above buffer, and then protein was eluted with 0.15 M KCl in the same buffer. About 50% of the PFP activity applied to the column was recovered. In the forward direction, low activity could be measured in the absence of added $MgCl_2$. This residual activity was abolished by including 1 mM EDTA in the assay and maximum activity could be restored by adding an excess of Mg^{2+} (2 mM). Atomic absorption spectrophotometry revealed that, despite ion exchange chromatography, the enzyme preparation still contained sufficient Mg^{2+} to produce a final concentration of approximately 12 μM in the assay. Attempts to reduce this contamination were unsuccessful. Figure 2 shows the effect of Mg^{2+} on PFP activity. The enzyme displayed hyperbolic kinetics under all conditions tested. When PFP was measured in the forward direction, the K_m^{app} for Mg^{2+} was 185 μM, by adding 1 μM Fru-2,6-P_2 the K_m^{app} decreased to 10.3 μM and V^{app} increased 15-fold (Fig. 2A). However, at concentrations exceeding 1 mM, Mg^{2+} inhibited PFP activity. In the reverse direction, when fully activated by Fru-1,6-P_2, the enzyme had a K_m^{app} for Mg^{2+} of 176 μM (Fig. 2B) and was not inhibited by Mg^{2+} concentrations up to 15 mM. Both Mn^{2+} and Co^{2+} could replace Mg^{2+}, but were less effective stimulators, whereas Ca^{2+}, Ni^{2+}, and Zn^{2+} were ineffective and even inhibited the stimulation of PFP by Mg^{2+}. The inhibitory effect of Ca^{2+} could be relieved by increasing concentrations of Mg^{2+} (results not shown). With respect to the above cations, the properties of castor bean PFP are similar to those reported for the enzyme from *Entamoeba histolytica* (17) and mung bean hypocotyl (1), and demonstrate that PFP had a requirement for a divalent metal ion.

Since PFP from other sources has been shown to be affected by several anions (1, 20), we studied the effect of various salts on

Table I. *Purification of PFP from Castor Bean Endosperm*

Purification Step	Volume	Total Protein	Total Activity	Specific Activity	Purification	Yield
	ml	*mg*	*μmol·min⁻¹*	*μmol·min⁻¹·mg protein⁻¹*	*-fold*	*%*
Crude extract	276	3257	47	0.0144		(100)
$(NH_4)_2SO_4$ fraction	37	1332	50	0.038	2.6	104
P-cellulose	74	62	27	0.44	30	56
Bio-Gel A-1.5m	37	14	15	1.07	74	31
DEAE-Cellulose	15	0.57	4.7	8.25	573	10

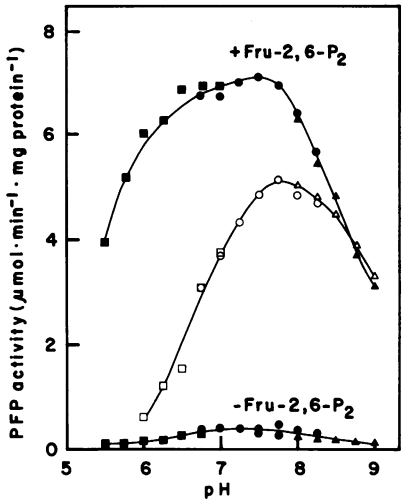

FIG. 1. Effect of pH on PFP activity. PFP was measured in the forward direction (■,●,▲) and the reverse direction (□,○,△) using Mes-NaOH (■,□), Hepes-NaOH (●,○), and Tris-HCl (▲,△) buffers, each at 100 mM. Activity was measured in the presence of 1 μM Fru-2,6-P_2 and in its absence. In the reverse direction PFP activity measured in the absence of Fru-2,6-P_2 was the same as that measured in its presence.

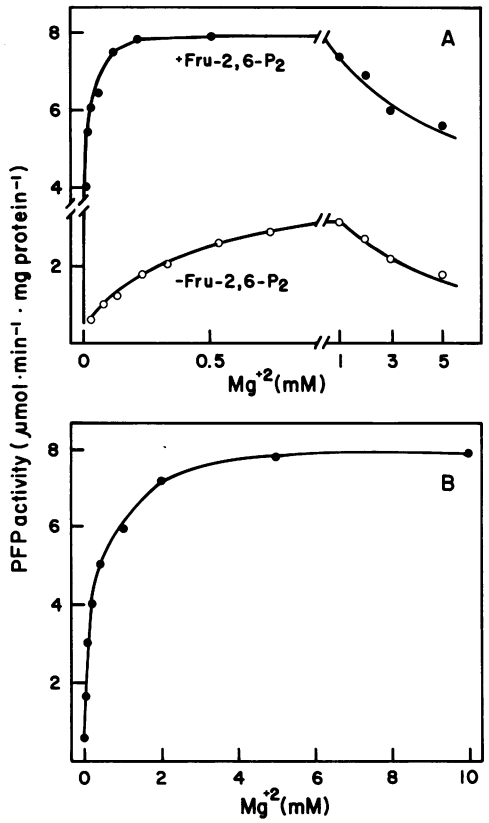

FIG. 2. Effect of Mg^{2+} on PFP activity. PFP was measured in the forward direction (A) in the presence of 1 μM Fru-2,6-P_2 (●) and in its absence (○). In the reverse direction (B), PFP activity measured in the absence of Fru-2,6-P_2 was the same as that measured in its presence. $MgCl_2$ was used to vary Mg^{2+} as shown.

PFP in both the presence and absence of Fru-2-6-P_2 (Table II). There was no difference between the effects of sodium, potassium, and ammonium salts. Phosphate caused substantial inhibition and was investigated in detail (see below). Sulfate caused marked inhibition at high concentrations; this inhibition was greater in the presence of Fru-2,6-P_2. Nitrate and chloride both caused some inhibition at high concentrations, while acetate was the least inhibitory anion tested.

Kinetics Constants of PFP. PFP exhibited hyperbolic kinetics with Fru-6-P, PPi, Fru-1,6-P_2, and Pi in both the presence and absence of Fru-2,6-P_2 (Figs. 3–6). At high concentrations, both Pi and PPi inhibited the enzyme. This inhibition was partially relieved by Fru-2,6-P_2. We found no evidence of sigmoid kinetics in the absence of Fru-2,6-P_2 as reported by others (3, 20). Under none of the conditions examined was the Hill coefficient significantly greater than one (Fisher's probability value > 0.1).

In a separate series of experiments, we determined for each substrate the Michaelis constant at saturating levels of the second substrate (K_m), the dissociation constant in the absence of the second substrate (K_i), and the maximum velocity at saturating concentrations of both substrates (V) in the presence of 1 μM Fru-2,6-P_2 as described in "Materials and Methods." The substrate concentrations used in this analysis were as follows. In the forward direction, Fru-6-P was 10, 5, 2, 1, 0.5, 0.2, and 0.1 mM, and PPi was 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, and 0.005 mM; in the reverse direction Fru-1,6-P_2 was 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 mM, and Pi was 5, 2, 1, 0.5, 0.2, and 0.1 mM. The correlation coefficient of linear regression for each of the plotted lines was greater than 0.975. The values for the constants are shown in Table III. For each substrate, Eadie-Hofstee plots of our data yielded a series of lines intersecting in the fourth quadrant. This pattern (equivalent to intersections to the left of the y axis in a Lineweaver-Burk plot) is consistent with a ternary complex mechanism which has previously been reported for PFP from both *Entamoeba histolytica* (17) and *Propionibacterium shermanii* (15). Recently a substituted enzyme mechanism (ping-pong mechanism) has been proposed for the enzyme from pineapple leaf (2). The initial velocity patterns we observe are incompatible with PFP from castor bean endosperm operating via this later mechanism.

Activation of PFP by Fru-2,6-P_2. In the forward reaction, the major effect of Fru-2,6-P_2 was to increase V^{app} about 20-fold and to decrease K_m^{app} for Fru-6-P by a factor of 10, from 2.5 to 0.25 mM (Fig. 3). K_m^{app} for PPi decreased by about 50%, from 0.035 to 0.017 mM, on addition of Fru-2,6-P_2 (Fig. 4). Inhibition of PFP by PPi was observed at concentrations greater than 1 mM and could be relieved by Fru-2,6-P_2. Activation of PFP by Fru-

Table II. *Effect of Various Salts on the Activity of PEP*

PFP activity was measured in the forward direction as described in "Materials and Methods" at two concentrations of each salt in the absence and presence of 1 μM Fru-2,6-P_2. The results are expressed as the percentage of the activity measured in the absence of salts.

Salt	Activity					
	[Salt] − Fru-2,6-P_2			[Salt] + 1 μM Fru-2,6-P_2		
	5 mM	10 mM	50 mM	5 mM	10 mM	50 mM
	% of control					
NaCl		93	54		101	74
$Na(CH_3COO)$		101	90		109	105
$NaNO_3$		80	42		88	59
Na_2SO_4		87	42		60	22
Na_2HPO_4	56	26		61	21	
KCl		89	65		102	82
$K(CH_3COO)$		108	96		111	104
KNO_3		79	47		86	52
K_2SO_4		93	43		69	22
K_2HPO_4	58	25		58	20	
NH_4Cl		104	64		89	72
$NH_4(CH_3COO)$		98	81		105	96
NH_4NO_3		103	51		89	52
$(NH_4)_2SO_4$		104	51		62	24
$(NH_4)_2HPO_4$	71	32		63	27	

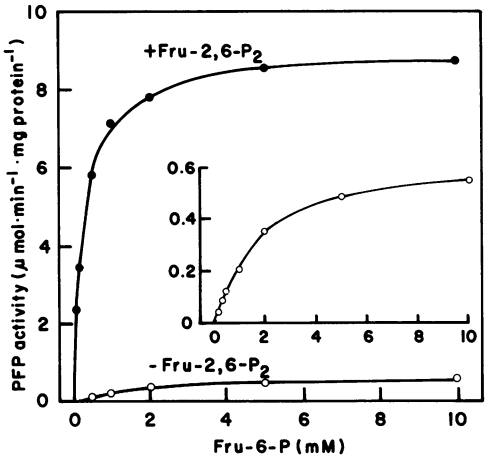

FIG. 3. Effect of Fru-6-P on PFP activity. PFP was measured in the forward direction in the presence of 1 μM Fru-2,6-P_2 (●) and in its absence (○). PPi was 0.2 mM and Fru-6-P was varied as shown. Inset is PFP activity in the absence of Fru-2,6-P_2.

2,6-P_2 was hyperbolic and dependent on the Fru-6-P concentration (Fig. 7). Decreasing the concentration of Fru-6-P from 5 to 0.5 mM increased the K_a for Fru-2,6-P_2 2-fold, from 10 to 22 nM. Pi also had a marked effect on the stimulation of PFP by Fru-2,6-P_2. K_a for Fru-2,6-P_2 at 5 mM Fru-6-P was increased more than 10-fold, to 123 nM, by the addition of 5 mM Pi. PFP could also be activated by glucose-1,6-P_2 but at much higher concentrations (K_a = 0.19 mM at 5 mM Fru-6-P and 0.2 mM PPi). Moreover, the maximum stimulation was only about half that obtained with Fru-2,6-P_2 (results not shown).

When PFP was measured in the reverse direction, addition of Fru-2,6-P_2 decreased the K_m^{app} for Fru-1,6-P_2 from 0.09 to 0.015 mM, but did not affect V^{app} significantly (Fig. 5). There was a marked inhibition of PFP at Pi concentrations greater than 2 mM. This inhibition was partially relieved by Fru-2,6-P_2 which caused a 2- to 4-fold stimulation of activity (Fig. 6). However, Fru-2,6-P_2 actually increased K_m^{app} for Pi from 0.14 to 0.36 mM.

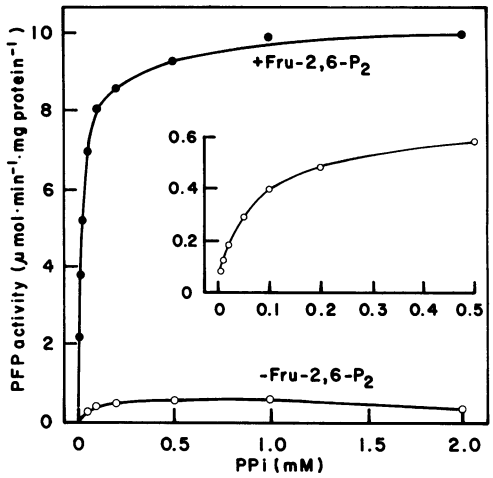

FIG. 4. Effect of PPi on PFP activity. PFP was measured in the forward direction in the presence of 1 μM Fru-2,6-P_2 (●) and in its absence (○). Fru-6-P was 5 mM and PPi was varied as shown. Inset is PFP activity in the absence of Fru-2,6-P_2.

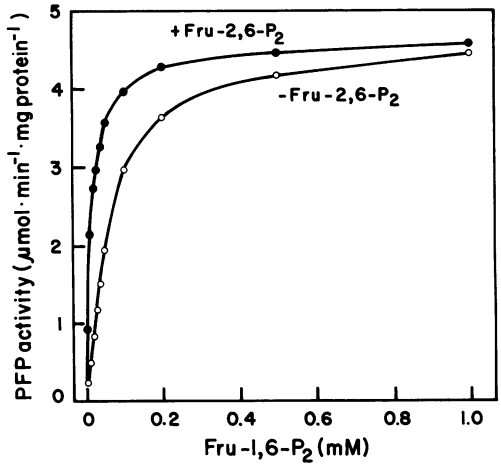

FIG. 5. Effect of Fru-1,6-P_2 on PFP activity. PFP was measured in the reverse direction in the presence of 1 μM Fru-2,6-P_2 (●) and in its absence (○). Pi was 2 mM and Fru-1,6-P_2 was varied as shown.

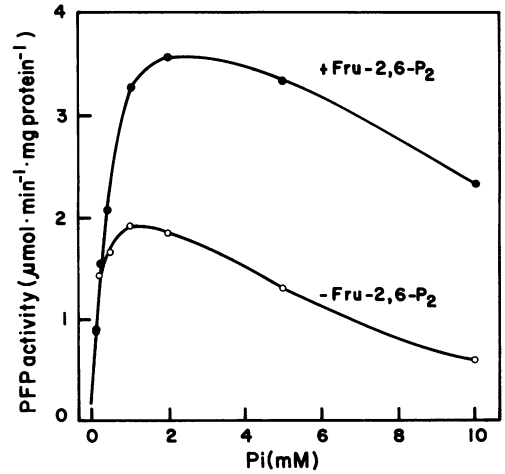

FIG. 6. Effect of Pi on PFP activity. PFP was measured in the reverse direction in the presence of 1 μM Fru-2,6-P_2 (●) and in its absence (○). Fru-1,6-P_2 was 50 μM and Pi was varied as shown.

Table III. *Kinetic Constants of PFP*

Enzyme activity was measured in the forward and reverse direction in the presence of 1 μM Fru-2,6-P_2, and the kinetic constants were determined as described in "Materials and Methods."

Assay	Substrate	K_m	K_i	V
		mM		*μmol·min⁻¹·mg⁻¹ protein*
Forward				9.55
	Fru-6-P	0.300	0.113	
	Ppi	0.015	0.0025	
Reverse				9.49
	Fru-1,6-P_2	0.023	0.013	
	Pi	0.630	0.390	

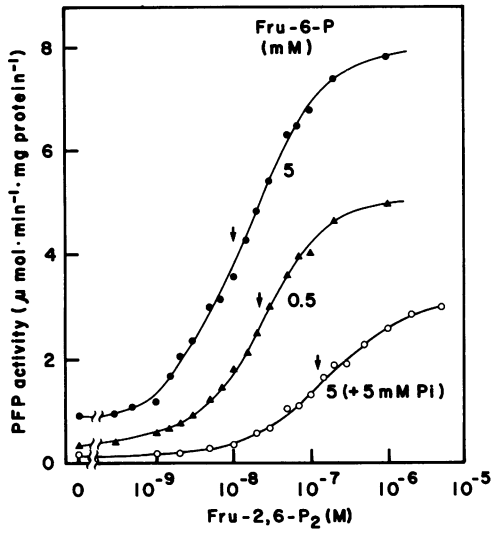

FIG. 7. Effect of Fru-6-P and Pi on the activation of PFP by Fru-2,6-P_2. PFP was measured in the forward direction. PPi was 0.2 mM. Calculated K_a values (arrows) were 10 nM at 5 mM Fru-6-P (●), 22 nM at 0.5 mM Fru-6-P (▲), and 123 nM at 5 mM Fru-6-P with 5 mM Pi (○).

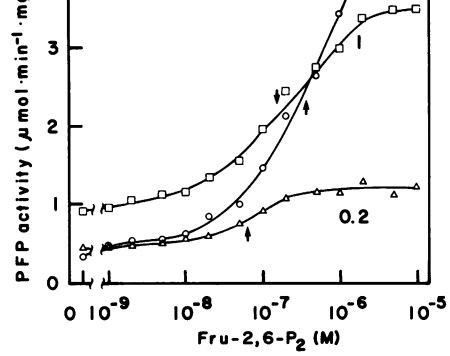

FIG. 8. Effect of Pi on the activation of PFP by Fru-2,6-P_2. PFP was measured in the reverse direction. Fru-1,6-P_2 was 20 μM. Calculated K_a values (arrows) were 63, 150, and 370 nM at 0.2 mM (△), 1.0 mM (□), and 5.0 nM (○) Pi, respectively.

This suggests that, at low Pi concentrations, Fru-2,6-P_2 is a less effective activator. By comparison with the forward reaction, the stimulation of PFP in the reverse direction by Fru-2,6-P_2 was weak. This is probably because the enzyme was already partially

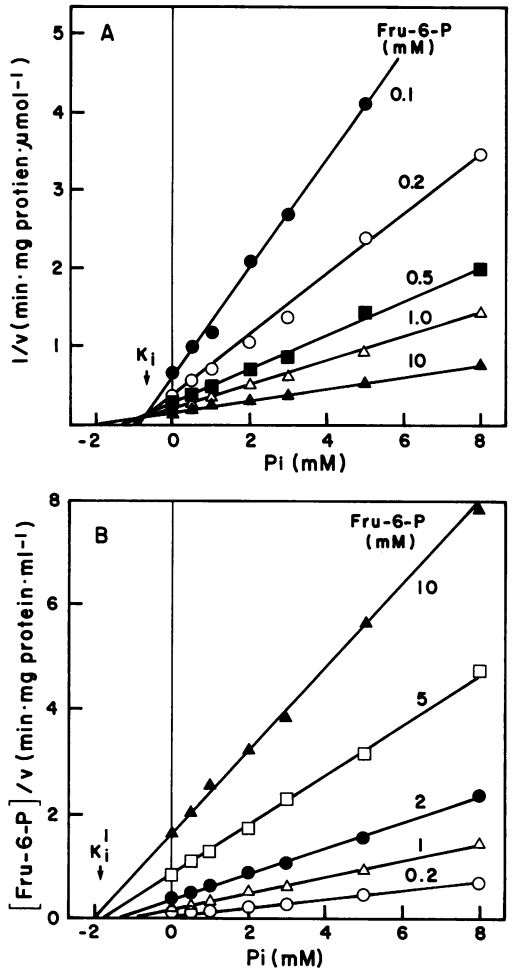

FIG. 9. Effect of Pi on the affinity of PFP for Fru-6-P. PFP was measured in the forward direction in the presence of 1 μM Fru-2,6-P_2. PPi was 0.2 mM. Fru-6-P and Pi were varied as shown. A. Dixon plot. B. Cornish-Bowden plot.

activated by Fru-1,6-P_2, a substrate of the reaction, as described by others (18). The observation that the stimulation by Fru-2,6-P_2 increased as the concentration of Fru-1,6-P_2 was decreased (Fig. 5) is consistent with this idea. In the reverse reactions, the activation by Fru-2,6-P_2 was hyperbolic at low Fru-1,6-P_2 concentrations. At 20 μM Fru-1,6-P_2 and 0.2 mM Pi, the K_a for Fru-2,6-P_2 was 60 nM. This value was increased up to 5-fold at higher Pi concentrations (Fig. 8).

Inhibition of PFP by Pi. Pi increased the K_a for Fru-2,6-P_2 in both the forward and reverse directions (Figs. 7 and 8). However, even at saturating concentrations of Fru-2,6-P_2, Pi strongly inhibited PFP. The pattern obtained from Dixon and Cornish-Bowden plots (Figs. 9 and 10) indicates that the inhibition was mixed with respect to both Fru-6-P and PPi. We determined the apparent competitive ($^{app}K_i$) and uncompetitive ($^{app}K'_i$) inhibition constants as described in "Materials and Methods." The substrate concentrations were varied as follows: Fru-6-P was 10, 5, 2, 1, 0.5, 0.2, and 0.1 mM at constant PPi (0.2 mM); PPi was 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 mM at constant Fru-6-P (5 mM). Pi was included at the concentrations indicated in Figures 9 and 10. To avoid complications due to the effect of Pi on the K_a for Fru-2,6-P_2, the assays contained 1 μM Fru-2,6-P_2 to fully activate PFP. For each plotted line, the correlation coefficient was greater than 0.954. Apparent K_i values calculated from Figures 9 and 10 were 0.66 mM for Fru-6-P and 1.19 mM for PPi. From the same data, values of $^{app}K'_i$ were 1.78 and 1.99 mM, respectively. Similar

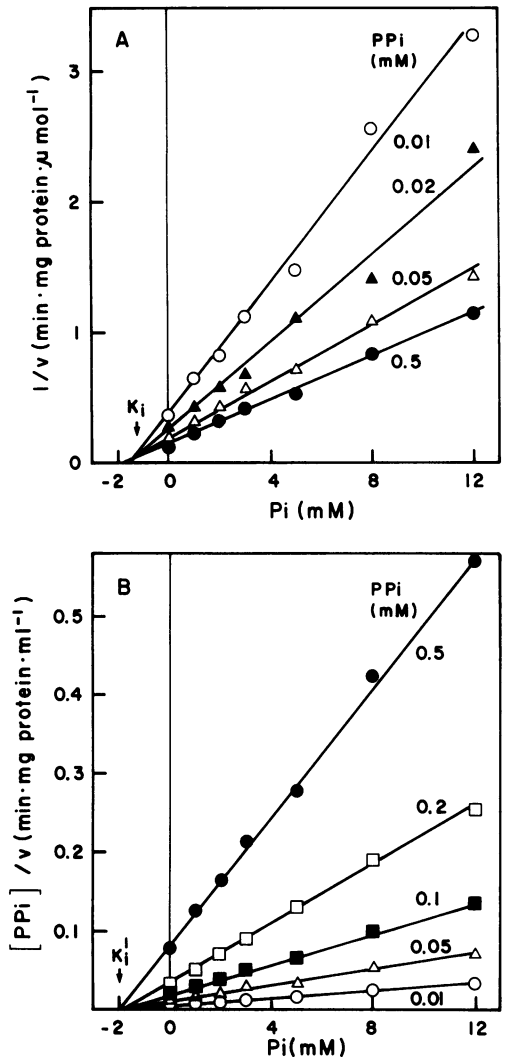

FIG. 10. Effect of Pi on the affinity of PFP for PPi. PFP was measured in the forward direction in the presence of 1 μM Fru-2,6-P_2. Fru-6-P was 5 mM. PPi and Pi were varied as shown. A. Dixon plot. B. Cornish-Bowden plot.

values for the inhibition constants were obtained by an alternative method of computation (Method 2, "Materials and Methods").

DISCUSSION

The purification procedure we have used provides a preparation that is essentially free from other enzymes capable of metabolizing either the substrates or products of PFP. In general, the properties of PFP from castor bean endosperm are similar to those described previously for the enzyme purified from mung bean hypocotyl (1), spinach leaf (3), and potato tuber (20). The most striking feature is the marked stimulation of the enzyme from each source by low concentrations of Fru-2,6-P_2, which was first discovered in mammalian tissues as a potent stimulator of P-fructokinase and an inhibitor of fructose-1,6-bisphosphatase (7, 16). For castor bean PFP, the K_a for Fru-2,6-P_2 in the forward direction was much lower than in the reverse direction, as was previously reported for PFP from potato tuber (20). This can be explained by two complementary properties. Fru-6-P decreased the K_a for Fru-2,6-P_2, whereas Pi had the opposite effect. However, due to these two factors we suggest that *in vivo* the K_a for Fru-2,6-P_2 in the forward direction is likely to be much greater than the minimum value, 10 nM, reported here. Although glucose 1,6-P_2 also stimulated enzyme activity, the K_a of about 0.2 mM makes it unlikely that this compound is an important activator of PFP *in vivo*.

Additionally, castor bean PFP is inhibited by Pi. In the forward direction, this may be expected since product inhibition is characteristic of two-substrate reactions (5). However, this is not the only mechanism of inhibition, because Pi also inhibits PFP in the reverse direction. The inhibition can, in part, be relieved by Fru-2,6-P_2, but even at optimum levels of the activator, Pi causes substantial reduction of PFP activity. The little that has been reported on the effects of Pi on PFP from other tissues suggests that the inhibition described above is not unique to the enzyme from castor bean (20).

Despite the general similarity, the properties of castor bean PFP differ from those reported for the spinach leaf and potato tuber enzyme in two ways. First, in the absence of Fru-2,6-P_2, the castor bean enzyme has very low activity. Therefore, we observed a greater stimulation by Fru-2,6-P_2 and a much larger effect of this activator on both V and K_m^{app}. Second, in contrast to the sigmoid kinetics for Fru-6-P reported for spinach leaf and potato tuber PFP in the absence of Fru-2,6-P_2 (3, 20), the castor bean enzyme exhibited hyperbolic kinetics with all substrates. In these two respects, castor bean PFP is far more similar to that from mung bean hypocotyl (1, 19). The above differences might merely reflect differences in the source of PFP. Alternatively, they may be explained by contaminants in the commercial substrates. We have observed that Fru-6-P from some sources contains a compound, probably Fru-2,6-P_2, that activates PFP (unpublished results). Obviously, such contaminated Fru-6-P could considerably distort the apparent kinetic properties of PFP. Fru-6-P used in the experiments described in the present paper did not contain a significant amount of this activator.

The kinetic properties of PFP that we report do not suggest an obvious physiological role for the enzyme. Castor bean PFP is quite capable of catalyzing the forward and reverse reactions at almost equal maximum rates (Table III). Before we can decide which direction PFP operates *in vivo*, it is necessary to know the concentrations of the reactants. In particular, if PFP is to function in the forward direction, the cytoplasm must contain an adequate supply of PPi. However, the properties described in the present paper suggest that PFP is capable of being tightly regulated in castor bean endosperm. The enzyme is almost completely dependent on the presence of Fru-2,6-P_2. Therefore, effective control could be obtained by variations in the level of Fru-2,6-P_2. In addition, the effect of Fru-2,6-P_2 could be modulated by changes in the concentrations of both Fru-6-P and Pi. An increase in the ratio [Fru-6-P]/[Pi] would increase the activation of PFP at a fixed, limiting concentration of Fru-2,6-P_2. Pi could also function by directly inhibiting the enzyme. Thus, changes in the levels of Fru-2,6-P_2, Fru-6-P, and Pi in the cytoplasm could combine to dramatically alter PFP activity *in vivo*. At present, there is insufficient data on the levels of these metabolites to assess their importance in the regulation of PFP in castor bean endosperm.

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