Effect of Fructose 2,6-Bisphosphate on the Kinetic Properties of Cytoplasmic Fructose 1,6-Bisphosphatase from Germinating Castor Bean Endosperm[1]

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ABSTRACT

The cytoplasmic form of fructose 1,6-bisphosphatase (FBPase) was purified over 60-fold from germinating castor bean endosperm (*Ricinus communis*). The kinetic properties of the purified enzyme were studied. The preparation was specific for fructose 1,6-bisphosphate and exhibited optimum activity at pH 7.5. The affinity of the enzyme for fructose 1,6-bisphosphate was reduced by AMP, which was a mixed linear inhibitor. Fructose 2,6-bisphosphate also inhibited FBPase and induced a sigmoid response to fructose 1,6-bisphosphate. The effects of fructose 2,6-bisphosphate were enhanced by low levels of AMP. The latter two compounds interacted synergistically in inhibiting FBPase, and their interaction was enhanced by phosphate which, by itself, had little effect. The enzyme was also inhibited by ADP, ATP, UDP and, to a lesser extent, phosphoenolpyruvate. There was no apparent synergism between UDP, a mixed inhibitor, and fructose 2,6-bisphosphate. Similarly ADP, a predominantly competitive inhibitor, did not interact with fructose 2,6-bisphosphate. Possible roles for fructose 2,6-bisphosphate and the other effectors in regulating FBPase are discussed.

During germination of castor bean the large lipid reserve in the endosperm is rapidly converted to sugar. The final stage of this conversion, the synthesis of sucrose from oxaloacetate, is probably confined to the cytoplasm (12). Although sucrose synthesis is a major metabolic process in the endosperm our understanding of its control is limited. Present evidence suggests that the production of Fru-6-P[3] from Fru-1,6-P_2 is a regulated step in this pathway (5).

In castor bean, the above reaction could be catalyzed by either FBPase or PFP. About 80% of the FBPase and all of the PFP activity is located in the cytoplasm, and each is sufficient to account for the flux from Fru-1,6-P_2 to Fru-6-P *in vivo* (8). In addition, the development of both enzyme activities during germination closely follows sucrose production (8). Recently, we have shown that the PFP, like that from other sources, is markedly stimulated by low levels of Fru-2,6-P_2 (6). This activation is inhibited by a wide range of metabolic intermediates (7) and

these compounds, together with Fru-2,6-P_2, may regulate PFP activity *in vivo*.

In contrast, apart from its inhibition by AMP, little is known about the properties of FBPase from castor bean endosperm (14, 15, 18). Similar inhibition by AMP has been reported for FBPase from the cytoplasm of spinach leaves (3, 19). The spinach enzyme is also inhibited by Fru-2,6-P_2 (2), which acts synergistically with AMP and Pi (16).

In view of the likely importance of Fru-2,6-P_2 in regulation of carbohydrate metabolism (4), we have studied the properties of FBPase specifically from the site of gluconeogenesis (cytoplasm) in castor bean endosperm and, in particular, investigated the effect of Fru-2,6-P_2 on the response of FBPase to a range of metabolites.

MATERIALS AND METHODS

Materials. Castor bean seeds (*Ricinus communis* cv 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. All biochemicals and auxillary enzymes were purchased from Sigma except for GTP (trilithium salt) and ribulose 1,5-bisphosphate which were from Calbiochem and fructose 1-phosphate from Boehringer Mannheim. DEAE-cellulose (DE 52) and Bio-Gel A-1.5m were from Whatman and Bio-Rad, respectively.

Purification of Cytoplasmic FBPase. 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, for 2 to 3 min, together with 2 volumes of 200 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, 2% (w/v) PVP. This procedure releases essentially all of the FBPase known to be present in this tissue (8). The homogenate was centrifuged at 20,000g for 20 min and the resulting supernatant was filtered through Miracloth (Calbiochem) to remove the fat layer. $(NH_4)_2SO_4$ was added to the filtrate. The material that precipitated between 45 and 62.5% saturation was collected by centrifugation (20,000g, 20 min), redissolved in about 30 ml 20 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol and dialyzed against 2 changes, each 1 L, of the same buffer. The dialyzed extract was adjusted to 8% (w/v) glycerol and applied, at 35 ml/h, to a DEAE-cellulose column (2.5 × 20 cm) equilibrated with 20 mM triethanolamine-HCl (pH 7.7), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 8% (w/v) glycerol. The column was washed with 120 ml of equilibrating buffer and then FBPase was eluted by a linear gradient (500 ml) of 0 to 0.4 M KCl in the same buffer. Two separate peaks of FBPase were observed. The initial peak, eluting at about 0.1 M KCl and containing most of

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[3] Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; FBPase, fructose 1,6-bisphosphatase (EC 3.1.3.11); PFP, pyrophosphate:fructose 6-phosphate phosphotransferase (EC 2.7.1.90).

the FBPase activity, was used in the subsequent steps. The fractions which contained this 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 the same buffer used in the previous column except that KCl was 0.1 M. The column was then washed with equilibrating buffer. The fractions containing most of the FBPase activity were combined to yield the purified FBPase preparation.

Protein was measured according to Lowry (11) after precipitation by 5% (w/v) TCA. BSA was used as a standard.

Enzyme Assays. FBPase activity was measured as described previously (8). The standard assay contained 100 mM Hepes-NaOH (pH 7.5), 5 mM MgCl₂, 0.5 mM NADP⁺, 0.5 mM Fru-1,6-P₂, 2 IU hexose phosphate isomerase, 1 IU glucose 6-phosphate dehydrogenase. The reaction was started by the addition of Fru-1,6-P₂. During the purification of FBPase the assay also contained 0.25 IU gluconate 6-P dehydrogenase.

Assay conditions for fructose 2,6-bisphosphatase were identical to those for FBPase, except that the buffer was Mes-NaOH (pH 6.5) and 0.5 mM Fru-2,6-P₂ replaced Fru-1,6-P₂. Other enzymes were measured as follows: PFP (8), phosphofructokinase (8), aldolase (12), gluconate 6-P dehydrogenase (12). These assays gave maximum activities in crude homogenates of endosperm.

To check the substrate specificity of the purified FBPase preparation, the extent of Pi release from a range of sugar phosphates was measured. The assay contained 100 mM Hepes-NaOH (pH 7.5), 5 mM MgCl₂, 0.5 mM sugar phosphate, in a total volume of 0.5 ml. The reaction was incubated at 25°C for up to 30 min and then stopped by adding 0.5 ml 10% (w/v) ice-cold TCA. The extract was immediately adjusted to pH 4.0 to 4.2 by adding 4 ml 1 M sodium acetate and Pi was determined as described by Lowry and Lopez (9).

Determination of Inhibition Constants. The competitive and uncompetitive inhibition constants, K_i and K'_i, respectively, were obtained from Dixon and Cornish-Bowden plots as described previously (6).

RESULTS

Purification of Cytoplasmic FBPase. A summary of the purification procedure is presented in Table I. Chromatography on DEAE-cellulose yielded two peaks of FBPase activity. This pattern is identical to that reported by Nishimura and Beevers (13), who demonstrated that the initial peak, which contained most of the activity, was cytosolic FBPase, and the second peak corresponded to FBPase from plastids. Hence, only the initial peak was purified further. Typically, FBPase was purified over 60-fold with a yield of 30 to 40%.

In the final preparation the activities of PFP, phosphofructokinase, aldolase, fructose 2,6-bisphosphatase, and gluconate 6-P dehydrogenase were less than 2% of the activity of FBPase. In addition, activity was specific for Fru-1,6-P₂. No release of Pi was detected when the extract was incubated with either Fru-6-

P, fructose 1-phosphate, glucose 1,6-bisphosphate, ribulose 1,5-bisphosphate, or sedoheptulose 1,7-bisphosphate, each at 0.5 mM. Under the same conditions the rate of Pi release from Fru-1,6-P₂ was 92 to 105% of the activity measured in the standard spectrophotometric assay. The pH optimum of FBPase activity was pH 7.5 to 7.7, and activity declined sharply at lower pH values (Fig. 1). All subsequent assays were performed at pH 7.5.

Effects of AMP and Fru-2,6-P₂ on FBPase. We confirmed that cytoplasmic FBPase was inhibited by AMP. The pattern obtained from Dixon and Cornish-Bowden plots indicate that the inhibition was mixed with respect to Fru-1,6-P₂ (Fig. 2). By mixed inhibition we mean inhibition that is not due solely to increased K_m (competitive inhibition), nor to decreasing V and K_m in constant ratio (uncompetitive inhibition) but to a combination of these effects (1). Values for the competitive (K_i) and uncompetitive (K'_i) inhibition constants, calculated as described in "Materials and Methods," were 0.07 and 0.86 mM, respectively. The correlation coefficient of linear regression for each of the lines was greater than 0.994 demonstrating that AMP did not induce sigmoid kinetics.

In the absence of inhibitors, the apparent K_m for Fru-1,6-P₂ was less than 2.5 μM and the enzyme was slightly inhibited by excess substrate (Fig. 3). For technical reasons we were unable to reliably assay FBPase in the presence of less than 2.5 μM Fru-1,6-P₂ and, consequently, cannot provide a more accurate value for K_m. FBPase was markedly inhibited by low concentrations of Fru-2,6-P₂ (Fig. 3). This effector decreased the affinity of the enzyme for Fru-1,6-P₂ and at high Fru-2,6-P₂ the saturation curve with respect to Fru-1,6-P₂ became increasingly sigmoid (Fig. 3).

The effects of Fru-2,6-P₂ were enhanced by low concentrations of AMP. The sigmoid kinetics of FBPase were considerably strengthened by levels of AMP which alone caused only slight inhibition, and sigmoid curves were obtained even at 1 μM Fru-2,6-P₂ (Fig. 4). Apparently Fru-2,6-P₂ and AMP act synergistically since the inhibition by a combination of these two compounds was greater than that expected from their individual effects (results not shown).

The interaction between the above effectors was investigated further as illustrated in Figures 5 and 6. At fixed substrate concentration, inhibition of FBPase by Fru-2,6-P₂ was sigmoid. The amount of Fru-2,6-P₂ required to produce 50% inhibition was reduced by a factor of four, from 1.7 to 0.4 μM, in the presence of 50 μM AMP (Fig. 5). Similarly, Fru-2,6-P₂ had a marked effect on inhibition by AMP. Reduction of FBPase activity by 50% was achieved by 7 μM AMP in the presence of 5 μM Fru-2,6-P₂ (Fig. 6), but about 500 μM AMP was required in the absence of Fru-2,6-P₂ (calculated from Fig. 2).

Pi, which inhibits spinach leaf FBPase (3), had only a very weak effect on the enzyme from castor bean endosperm either on its own (Fig. 7) or in combination with Fru-2,6-P₂ (results not shown). However, Pi greatly enhanced the inhibition by a combination of low concentrations of both Fru-2,6-P₂ and AMP,

Table I. *Purification of Cytoplasmic FBPase from Castor Bean Endosperm*

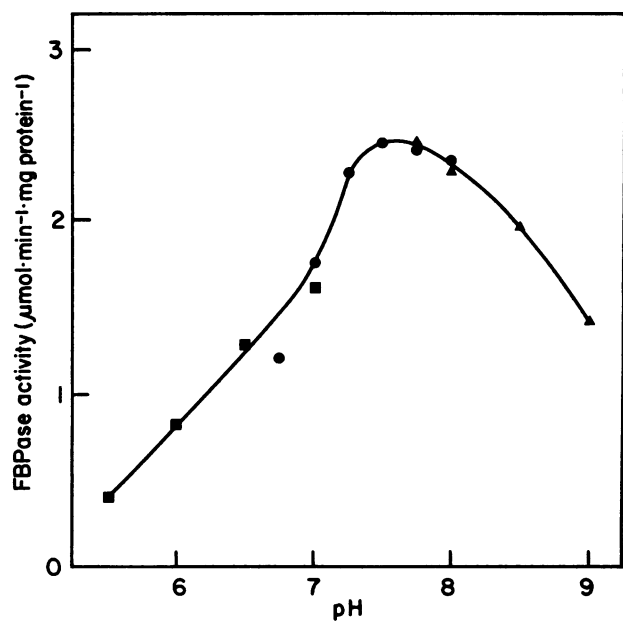| Purification step	Volume	Total Protein	Total Activity	Specific Activity	Purification	Yield
	ml	*mg*	*μmol·min⁻¹*	*μmol·min⁻¹·mg protein⁻¹*	*-fold*	*%*
Crude extract	390	4250	149	0.0351		(100)
(NH₄)₂SO₄ fraction	63	1065	139	0.131	3.7	93
DEAE-cellulose						
Peak I	55	225	75	0.33	9.5	50
(Peak IIᵃ)	(37)	(47.5)	(21)	(0.44)	(12.6)	(14)
Bio-Gel A-1.5m	29	21.2	48	2.26	64	32

ᵃ Not used in subsequent purification step.

FIG. 1. Effect of pH on FBPase activity. FBPase was measured in the presence of 50 μM Fru-1,6-P₂ in Mes-NaOH (■), Hepes-NaOH (●), and Tris-HCl (▲) buffers, each at 100 mM.
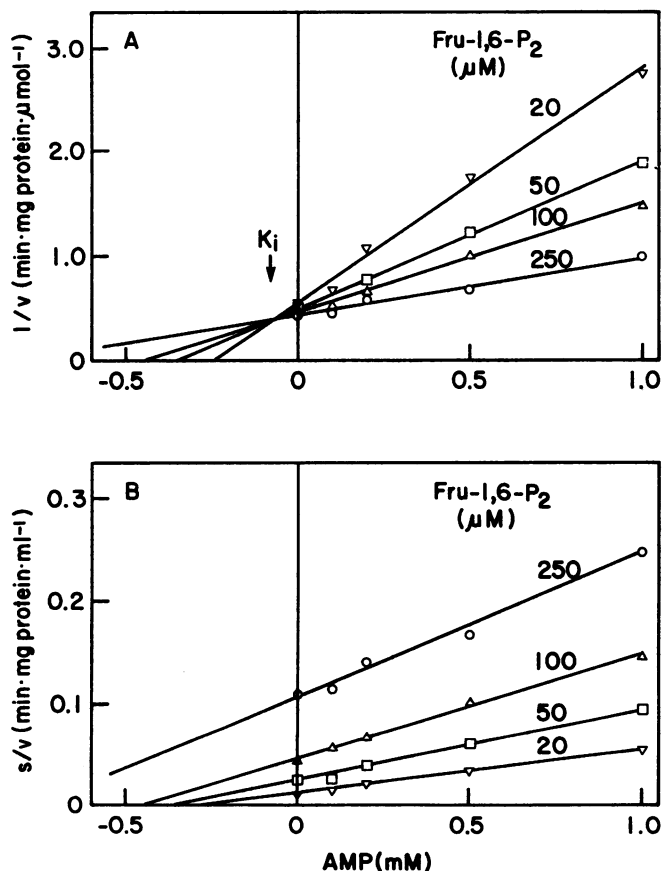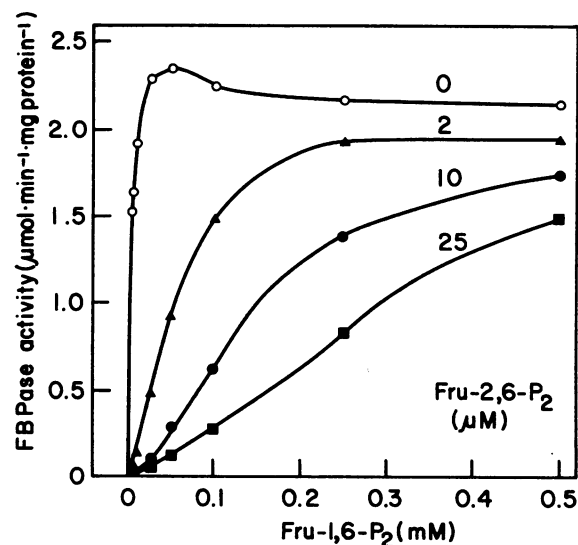
FIG. 3. Effect of Fru-2,6-P₂ on the affinity of FBPase for Fru-1,6-P₂. FBPase was measured in the presence of no (O), 2 μM (▲), 10 μM (●), and 25 μM (■) Fru-2,6-P₂. Fru-1,6-P₂ was varied as shown.

FIG. 2. Effects of AMP on the affinity of FBPase for Fru-1,6-P₂. FBPase was measured in the presence of 20 μM (▽), 50 μM (□), 100 μM (△), and 250 μM (O) Fru-1,6-P₂. AMP was varied as shown. A. Dixon plot. B. Cornish-Bowden plot.
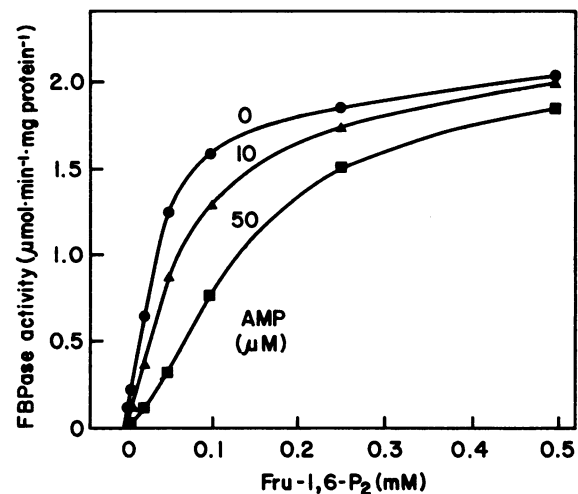
FIG. 4. Effect of a combination of AMP and Fru-2,6-P₂ on the affinity of FBPase for Fru-1,6-P₂. FBPase was measured in the presence of 1 μM Fru-2,6-P₂ and no (●), 10 μM (▲), and 50 μM (■) AMP. Fru-1,6-P₂ was varied as shown.

and under certain conditions FBPase activity was almost completely abolished (Fig. 7).

We also studied the effect of the above compounds on peak II of FBPase from the DEAE-cellulose column. This we consider to be the plastid form of the enzyme (13). In contrast to cytoplasmic FBPase, this enzyme was not affected by up to 5 mM AMP and was only inhibited about 20% by 10 μM Fru-2,6-P₂ in the presence of 0.5 mM Fru-1,6-P₂. This weak inhibition was not enhanced by AMP or Pi, either separately or in combination (results not shown).

Effects of Other Metabolic Intermediates on FBPase. We investigated the effect of a wide range of compounds (both singly and in combination with Fru-2,6-P₂) on cytoplasmic FBPase. The following compounds, each at 1 mM, had no significant effect on FBPase activity measured with 50 μM Fru-1,6-P₂ either in the presence of 1 μM Fru-2,6-P₂ or in its absence: sucrose, glucose, fructose, UDPglucose, glycerate 3-P, glycerate 2-P, PPi, pyruvate, malate, succinate, and citrate. Sucrose, glucose, and fructose were also tested at 10 mM, under the same conditions, and were without effect. In contrast, UDP, ADP, ATP and, to a
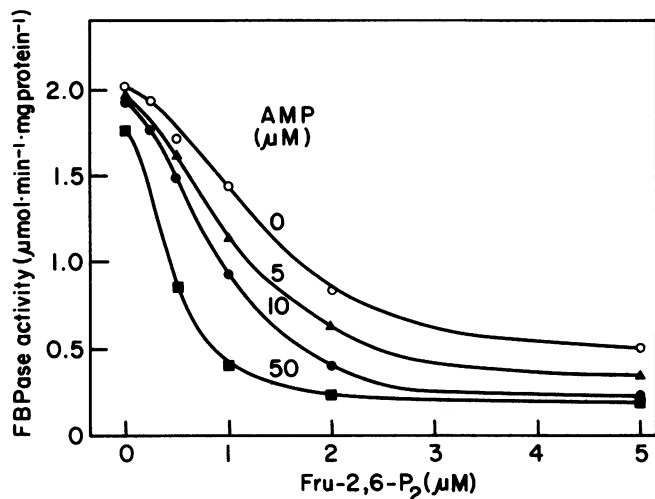
FIG. 5. Effect on AMP on the inhibition of FBPase by Fru-2,6-P₂. FBPase was measured in the presence of 50 μM Fru-1,6-P₂ and no (O) 5 μM (▲), 10 μM (●), and 50 μM (■) AMP. Fru-2,6-P₂ was varied as shown.
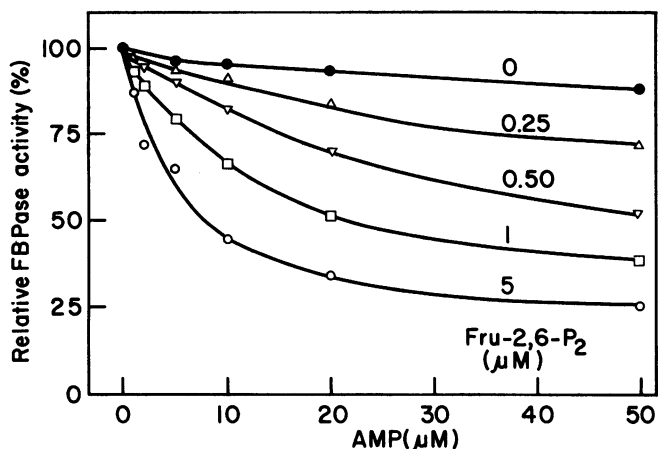

FIG. 6. Effect of Fru-2,6-P₂ on the inhibition of FBPase by AMP. FBPase was measured in the presence of 50 μM Fru-1,6-P₂ and AMP as shown. The results at each concentration of Fru-2,6-P₂ are expressed as a percentage of the activity in the absence of AMP. These activities were 2.01, 1.93, 1.71, 1.44, and 0.50 μmol·min⁻¹·mg protein⁻¹ in the presence of no (●), 0.25 μM (△), 0.5 μM (▽), 1 μM (□), and 5 μM (O) Fru-2,6-P₂, respectively.

lesser extent, phosphoenolpyruvate each inhibited FBPase in the absence of Fru-2,6-P₂ (Fig. 8). GTP, CTP, and UTP, each at 1 mM, also inhibited enzyme activity with 50 μM Fru-1,6-P₂ by 54, 71, and 45%, respectively. We checked that these inhibitions were not due to contamination by AMP. None of the above nucleotides contained significant amounts of AMP, which was measured enzymically as described (10).

The effects of UDP and ADP were studied in more detail. UDP was a mixed inhibitor with K_i and K'_i values of 0.16 and 0.79 mM, respectively (Fig. 9). The correlation coefficient of linear regression for each of the lines was greater than 0.985, indicating that this compound is a linear inhibitor (1). No synergism was observed between UDP and Fru-2,6-P₂. Up to 50 μM UDP had no effect on FBPase activity in the presence of 1 μM Fru-2,6-P₂ at seven concentrations of Fru-1,6-P₂ over the range 5 to 500 μM. Moreover, under a variety of conditions (varying Fru-2,6-P₂ from 0.25 to 5 μM and UDP from 5 to 50 μM at 50 μM Fru-1,6-P₂), the inhibition did not significantly decrease FBPase activity below that expected from the effect of the two compounds separately.

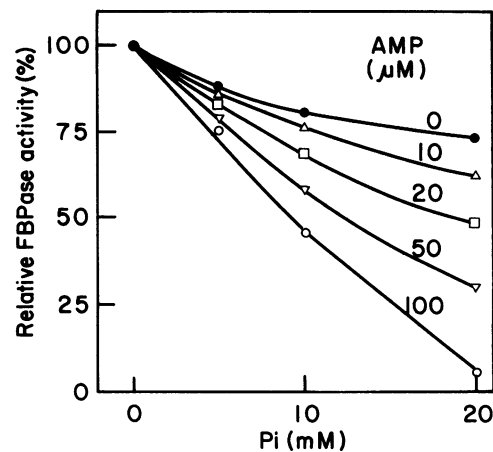FIG. 7. Effect of Pi on the inhibition of FBPase by Fru-2,6-P₂ and AMP. FBPase was measured in the presence of 50 μM Fru-1,6-P₂, 1 μM Fru-2,6-P₂ and Pi as shown. The results at each concentration of AMP are expressed as a percentage of the activity in the absence of Pi. These activities were 1.45, 0.95, 0.74, 0.41, and 0.29 μmol·min⁻¹·mg protein⁻¹ in the presence of no (●), 10 μM (△), 20 μM (□), 50 μM (▽), and 100 μM (O) AMP, respectively.
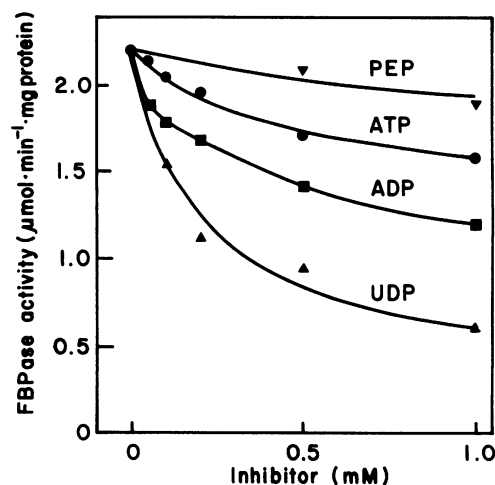

FIG. 8. Effect of various compounds on FBPase activity. FBPase was measured in the presence of 50 μM Fru-1,6-P₂. The concentration of phosphoenolpyruvate (▼), ATP (●), ADP (■), and UDP (▲) was varied as shown.

ADP was also a mixed inhibitor, although with a comparatively strong competitive component (Fig. 10). Calculated values for K_i and K'_i were 0.094 and 1.84 mM, respectively. Again the correlation coefficient of linear regression for each of the lines was greater than 0.997, demonstrating that ADP is a linear inhibitor of FBPase and, by itself, does not induce sigmoid kinetics. Unlike AMP, ADP apparently did not interact with Fru-2,6-P₂. Even at low Fru-1,6-P₂ the inhibition by a combination of Fru-2,6-P₂ and ADP was no greater than expected from the effect of each compound in isolation. Pi, at up to 20 mM, had relatively little effect on the inhibition by a combination of Fru-2,6-P₂ and ADP (results not shown).

DISCUSSION

Based on previous evidence (13), the procedure described in this paper provides a preparation of cytoplasmic FBPase which is not contaminated by the plastid form of the enzyme, and which is essentially free from other enzymes capable of interfering
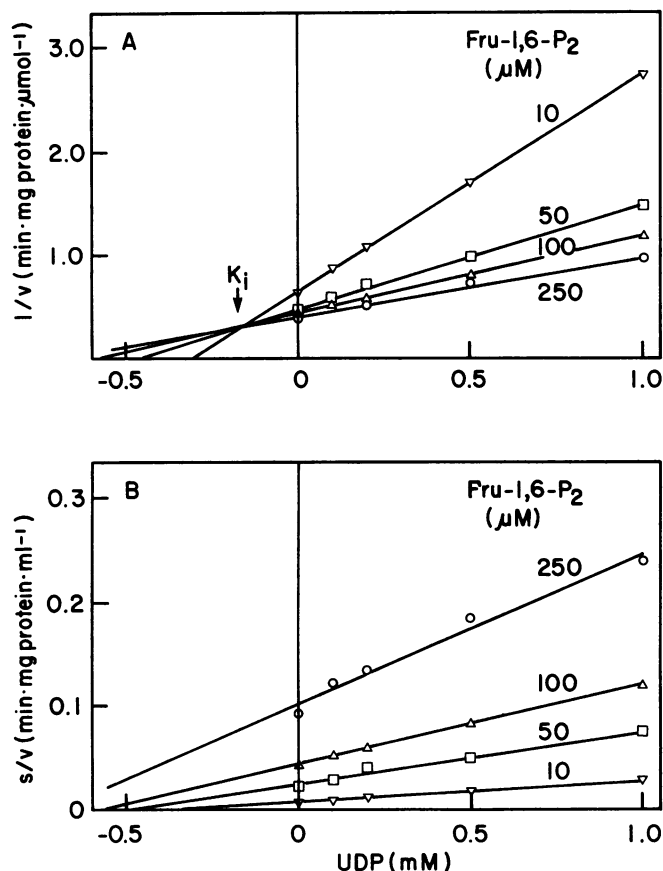
FIG. 9. Effect of UDP on the affinity of FBPase for Fru-1,6-P₂. FBPase was measured in the presence of 10 μM (∇), 50 μM (□), 100 μM (△), and 250 μM (○) Fru-1,6-P₂. UDP was varied as shown. A. Dixon plot. B. Cornish-Bowden plot.

FIG. 10. Effect of ADP on the affinity of FBPase for Fru-1,6-P₂. FBPase was measured in the presence of 10 μM (∇), 25 μM (□), 50 μM (△), and 100 μM (○) Fru-1,6-P₂. ADP was varied as shown. A. Dixon plot. B. Cornish-Bowden plot.

with the assay of FBPase. Previously, Youle and Huang (18) have demonstrated that FBPase from castor bean endosperm can be considerably modified during extraction. Characteristics of this modification are a shift in pH optimum for activity from pH 7.5 to pH 6.7 or below, a decrease in the affinity for Fru-1,6-P₂ and a loss of sensitivity to AMP. None of these changes was observed under the extraction conditions used in the present study. The pH optimum for FBPase in the final preparation was pH 7.5 to 7.7, the enzyme had a very high affinity for Fru-1,6-P₂ and activity was inhibited by AMP. However, if the buffer concentration was decreased, or if phenylmethylsulfonylfluoride was omitted from the extraction buffer, then considerable modification occurred (Kruger, unpublished results).

In general the properties of cytoplasmic FBPase from castor bean endosperm are similar to those reported previously for the enzyme from spinach leaf (2, 3, 16, 19). Perhaps the most striking similarity is the synergism between Fru-2,6-P₂ and AMP. Each of these compounds, at low levels, can enhance the sensitivity of FBPase to the other inhibitor. Additionally, the combined effect of these two compounds is itself enhanced by Pi, which alone has little effect on FBPase. This interaction between Fru-2,6-P₂ and AMP, first observed with liver FBPase (17), is apparently quite specific. We have been unable to demonstrate any synergism between Fru-2,6-P₂ and a range of other nucleotides, even though several of these also inhibit FBPase.

Despite the overall similarity to cytoplasmic FBPase from spinach leaf, the enzyme from castor bean endosperm differs in several respects. Most notably, Fru-2,6-P₂ clearly induces sigmoid substrate kinetics in the latter enzyme, similar to the sigmoidal substrate dependence with Fru-2,6-P₂ reported for liver FBPase
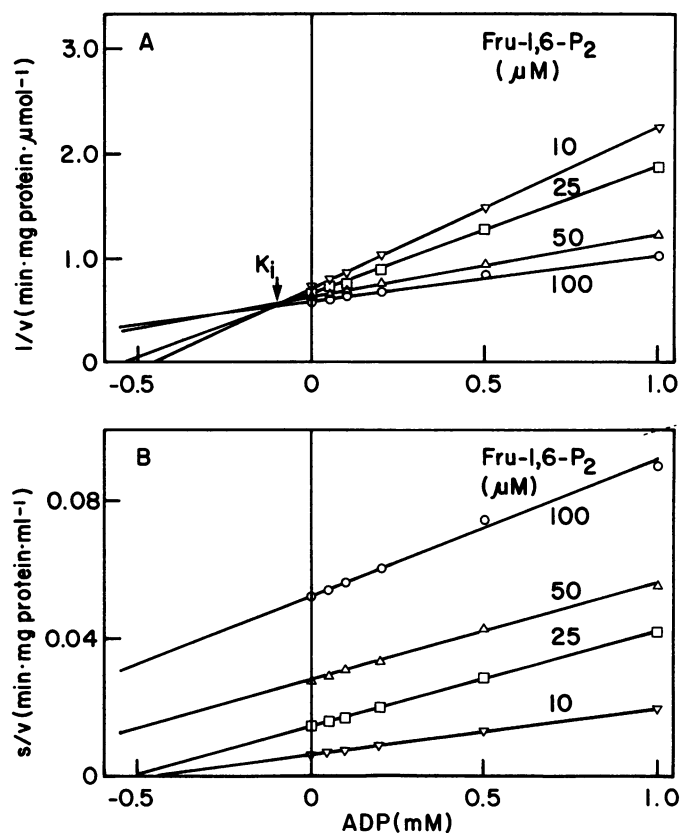
(17). Additionally as with liver FBPase (17), AMP can enhance the sigmoid kinetics of castor bean endosperm FBPase caused by Fru-2,6-P₂. In contrast, the enzyme from spinach leaf is apparently competitively inhibited by Fru-2,6-P₂ (2, 16). However, even with the spinach enzyme there are some indications that Fru-2,6-P₂ induces sigmoid kinetics at low levels of Fru-1,6-P₂ (16). Therefore, this difference may reflect variation in the sensitivity of FBPase from different sources to Fru-2,6-P₂ rather than any qualitative difference in the mechanism of inhibition of the enzyme. The enzyme from castor bean endosperm is less sensitive to Pi than that from spinach leaf (3), but Pi appears to be far more effective in enhancing the inhibition of the former enzyme by a combination of Fru-2,6-P₂ and AMP (16). We have also shown that castor bean endosperm FBPase is inhibited by several nucleotides. UDP has previously been shown to inhibit the spinach leaf enzyme (3), but at present too little is known about the effects of these compounds on FBPase from other sources to decide whether such inhibition is typical for this enzyme in plants.

The properties described above suggest several ways in which FBPase activity could be modulated. The enzyme is almost completely inhibited by a combination of Fru-2,6-P₂, AMP, and Pi. Changes in the level of any one of these compounds could have a profound effect on FBPase. In addition, because of their effect on the substrate kinetics of the enzyme, the levels of these compounds will also determine the response of FBPase to changes in the level of Fru-1,6-P₂. The above controls could be supplemented by the effects of variations in the levels of other inhibitors described in this paper. The concentration of Fru-2,6-P₂ may be particularly important since this compound also markedly activates PFP from the same source (6). If PFP operates

in the glycolytic direction *in vivo*, then the level of Fru-2,6-P_2 could play a pivotal role in determining the direction and net flux between Fru-1,6-P_2 and Fru-6-P_2, through its reciprocal effects on FBPase and PFP.

The extent to which the mechanisms described above contribute to the regulation of FBPase *in vivo* is uncertain. The limited data on changes in the levels of intermediates in endosperm during anoxia (when gluconeogenesis becomes restricted and glycolysis is stimulated) are inconclusive. The large transient increase in Fru-1,6-P_2 at the onset of anoxia (5) indicates that FBPase is not limited solely by substrate concentration. However, despite a similar, temporary increase in the amount of AMP at the beginning of anoxia, the low steady-state level of this compound seems quite inadequate, by itself, to account for the restriction of FBPase activity (5). ATP and ADP both decline during anoxia (5) and, thus, are unlikely to play a major role in the inhibition of FBPase under these conditions. In contrast, results to be published in a subsequent paper demonstrate that variations in the level of Fru-2,6-P_2 in endosperm are consistent with the idea that this compound contributes to the control of gluconeogenesis during germination. However, since the effects of Fru-2,6-P_2 are dependent on the concentration of both AMP and Pi, the levels of these latter two compounds may also be important by determining the response of the enzyme to Fru-2,6-P_2.

Obviously, the above considerations are restricted by our ignorance of the extent to which changes in metabolic levels in endosperm tissue reflect changes in the metabolic concentration in the cytoplasm as opposed to those in plastids or other organelles. A detailed analysis of the subcellular levels of metabolic intermediates and how such levels change is required before the contribution of the various mechanisms to the control of FBPase *in vivo* can be more adequately assessed.

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