Kinetic Studies on the Activation of Pyrophosphate-Dependent Phosphofructokinase from Mung Bean by Fructose 2,6-Bisphosphate and Related Compounds†

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ABSTRACT: Pyrophosphate-dependent phosphofructokinase (PP_i-PFK) was purified from the mung bean *Phaseolus aureus*. The enzyme is activated by fructose 2,6-bisphosphate at nanomolar concentrations. The enzyme exhibits Michaelis–Menten kinetics, and the reaction mechanism, deduced from initial velocity studies in the absence of inhibitors as well as product and dead-end inhibition studies, is rapid equilibrium random in the presence and absence of fructose 2,6-bisphosphate. In the direction of fructose 6-phosphate phosphorylation, saturating fructose 2,6-bisphosphate (1 μM) increases $V \simeq 9$-fold and increases V/K_{MgPP_i} and V/K_{F6P} about 30-fold. In the reverse direction (phosphate phosphorylation), the same concentration of activator has little if any effect on V or the K_m for inorganic phosphate (P_i) and Mg^{2+} but does increase V/K_{FBP} about 42-fold. No changes were observed in any of the other rate constants. The binding affinity of fructose 2,6-bisphosphate to all enzyme forms is identical. The activator site of the mung bean PP_i-PFK binds fructose 2,6-bisphosphate with a K_{act} of 30 nM with the 2,5-anhydro-D-glucitol 1,6-bisphosphate (the most effective analogue) 33-fold less tightly. Of the alkanediol bisphosphate series, 1,4-butanediol bisphosphate exhibited the tightest binding ($K_{act} \simeq 3$ μM). These and a series of other activating analogues are discussed in relation to the activator site.

The most potent activator of phosphofructokinases was first extracted from rat liver (Furuya & Uyeda, 1980; Van Schaftingen et al., 1980; Claus et al., 1981) and later chemically synthesized and identified as fructose 2,6-bisphosphate (Pilkis et al., 1981; Uyeda et al., 1981b; Van Schaftingen & Hers, 1981). Both ^{13}C and ^{31}P NMR analyses showed F2,6P[1] to exist as the β anomer (Hesbain-Frisque et al., 1981; Uyeda et al., 1981b). The activator was later found to exist in yeast and plant cells (Lederer et al., 1981; Cseke et al., 1982; Stitt et al., 1983; Kruger & Beevers, 1985) but not in procaryotic cells (Cseke et al., 1982).

The ATP-PFK from rat liver and rabbit muscle is activated by F2,6P in the nanomolar-concentration range (Van Schaftingen et al., 1980, 1981; Pilkis et al., 1981; Uyeda et al., 1981a). It is the general consensus that F2,6P binds to a specific activator (allosteric) site on the enzyme that is not equivalent to the FBP active site (Bartrons et al., 1982; Bosca et al., 1982; Kitajima & Uyeda, 1983). The ATP-PFK of plant cells is, however, not activated by micromolar concentrations of F2,6P at basic pH (Kelly & Latzko, 1975; Sabularse & Anderson, 1981b; Cseke et al., 1982; Kombrink & Wober, 1982; Miernyk & Dennis, 1982; Van Schaftingen et al., 1982; Ashihara & Stupavska, 1984). This is also the case for PP_i-PFK from the faculatative anaerobic bacterium *Propionibacterium shermanii* (Bertagnolli & Cook, 1984; Wood & Goss, 1985).

In addition to an ATP-PFK, plants also contain a pyrophosphate-dependent phosphofructokinase (pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90), which catalyzes the readily reversible reaction:

$$F6P + MgPP_i \rightleftharpoons FBP + P_i + Mg^{2+} \quad (1)$$

The enzyme was discovered originally in pineapple leaves (Carnal & Black, 1979) and later in a wide variety of plants (Cseke et al., 1982; Kruger et al., 1983; Kombrink & Kruger, 1984; Kowalczyk et al., 1984; Miyatake et al., 1984; Smyth et al., 1984). It was first reported by Sabularse and Anderson (1981b) that F2,6P activates ($K_{act} \simeq 50$ nM) PP_i-PFK from the mung bean *Phaseolus aureus* primarily by decreasing the K_m for F6P and increasing V.

Although much detailed information exists in the literature on the F2,6P activation of ATP-PFK from mammalian sources, little is known about the activation of PP_i-PFK from plants by F2,6P. In addition, little information exists for either ATP-PFK or PP_i-PFK on the mechanism of activation. We chose to study PP_i-PFK from the mung bean *P. aureus* because of its ease of isolation, stability, hyperbolic saturation curves with respect to reactants in both reaction directions, and the lack of substrate inhibition. In this study we report the overall kinetic mechanism of this PP_i-PFK in the absence and presence of F2,6P, the mechanism of activation by F2,6P, and the specificity of the F2,6P activator site.

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[1] Abbreviations: F2,6P, fructose 2,6-bisphosphate; ATP-PFK, ATP-dependent phosphofructokinase; PP_i-PFK, inorganic pyrophosphate dependent phosphofructokinase; ATP, adenosine 5′-triphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTT, DL-dithiothreitol; PCP, methylenediphosphonate; C_nP_2, 1,*n*-alkanediol bisphosphate; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

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Materials and Methods

Chemicals. Sodium pyrophosphate was obtained from Mallinckrodt Chemical Co. Fructose 6-phosphate (sodium salt), FBP (sodium salt), F2,6P (sodium salt), methylenediphosphonate (sodium salt), sulfate (potassium salt), Taps, glycerol (Sigma grade), Pipes, DTT, and EDTA (sodium salt) were from Sigma Chemical Co. Cellulose phosphate (Cellex P) was obtained from Bio-Rad. NADH (100%, disodium salt) and NADP (98%, disodium salt) were from Boehringer Mannheim.

All other mono- and bisphosphate compounds listed in Table V as well as L-sorbose 6-phosphate and 1,8-octanediol bisphosphate in Table III were prepared as described in the accompanying paper (Bertagnolli et al., 1986). All samples were shown to be pure by ^1H and/or ^{13}C NMR analyses. In addition, no significant amount of contaminant was detected by proton-decoupled ^{31}P NMR. Compounds obtained as barium salts were converted to sodium salts as discussed in the accompanying paper (Bertagnolli et al., 1986).

All other chemicals and reagents used in this study were obtained from commercial sources and were of the highest quality available.

Enzymes. All enzymes and coupling enzymes were obtained and prepared for coupled enzyme assay as described in the accompanying paper (Bertagnolli et al., 1986).

Impure pyrophosphate-dependent phosphofructokinase from *P. aureus* (mung bean) was obtained from Sigma (product no. F8757) as a lyophilized powder of specific activity varying from 5 to 13 μmol min^{-1} (mg of protein)$^{-1}$. This enzyme was purified by a modification of the procedure originally developed by Sabularse and Anderson (1981b). Typically, 80 units of the lyophilized powder was dialyzed in the cold (4 °C) for 2 h vs. 10 mM Pipes–NaOH, pH 6.6, in the presence of 1 mM DTT and 0.1 mM EDTA. One unit of PP_i-PFK will convert 1 μmol of F6P to FBP per minute at pH 8 and 25 °C. The dialysate (2–3 mL) was then carefully loaded onto a phosphocellulose (Bio-Rad Cellex P) column (1.5 × 10 cm) thoroughly equilibrated with the same buffer mixture. The column was then washed until no A_{280} material was detected in the effluent. The column was finally eluted with 50 mL of the same buffer mixture plus 17 mM sodium pyrophosphate (pH 6.6, 4 °C). The peak fractions were pooled, concentrated, and dialyzed (PM-10 ultrafiltration membrane) with a buffer consisting of 20 mM Pipes–NaOH, pH 7.4 (4 °C), with 100 mM KCl, 1 mM DTT, and 0.1 mM EDTA. The final concentrate (1.0–1.5 mL) was made up to 25% with glycerol (v/v) and stored at −20 °C with a trace of pyrophosphate (5 μM). The specific activity of several preparations ranged from 74 to 82 μmol min^{-1} (mg of protein)$^{-1}$. Protein was determined by the Bio-Rad reagent microassay procedure, which is a modification of the dye-binding procedure developed by Bradford (1976).

The native molecular weight of the plant PP_i-PFK was estimated to be 190 000 by the method of Hedrick and Smith (1968) and by gel filtration on a (1.5 × 50 cm) Bio-Rad A0.5-m column in the presence and absence of 1 μM F2,6P. There was no evidence that F2,6P causes dissociation in agreement with Reinhart (1983) for the ATP-PFK and two additional reports on plant PP_i-PFKs (Wu et al., 1983; Yan & Tao, 1984). The subunit molecular weight was estimated by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to O'Farrell (1975) to be 48 000, indicating that the native plant enzyme is a tetramer as opposed to the enzyme from *Propionibacterium*, which is a dimer (O'Brien et al., 1975). The mung bean PP_i-PFK preparations were free of competing enzymatic activities as judged by ^{31}P NMR analyses similar to those used in the accompanying paper.

Enzyme Assays. All assays were performed at pH 8, 25 °C, by monitoring the change in absorbance at 340 nm with a Cary/Varian 210 spectrophotometer according to the method of Bertagnolli and Cook (1984). In all cases, saturation with coupling enzymes was assured by linear velocity vs. PP_i-PFK plots.

To test whether or not any of the analogues were activators in the direction of F6P phosphorylation, the assay mixture contained 100 mM Taps–KOH, pH 8, 2.3 mM Mg^{2+}, 0.5 mM $MgPP_i$, 20.8 mM F6P, 0.2 mM NADH, and 0.05 unit of PP_i-PFK. After a background rate was established (that is, the rate in the absence of the analogue) 0.2 mM analogue was added to determine if any enhancement of the rate occurred. Since FBP and L-sorbose-1,6-bis-P are substrates for FBP aldolase (Tung et al., 1954), one of the coupling enzymes in the forward reaction, the K_{act} values for these compounds were calculated from velocities determined by assaying P_i production by methods given in Bertagnolli and Cook (1984). The reciprocal of the stimulated velocity (above that in the absence of activator) was plotted against the reciprocal of the activator concentration to obtain the activation constant (K_{act}).

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal reactant concentrations, and all plots were linear. All reactant and inhibitor concentrations were corrected for chelate complex formation as suggested in the accompanying paper. Data were fitted by using the appropriate rate equation and the FORTRAN programs of Cleland (1979). Data adhering to a sequential initial velocity pattern were fitted by using eq 2 while saturation curves were fitted by using eq 3. Data for

$$V = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$V = VA/(K_a + A) \quad (3)$$

$$V = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (4)$$

$$V = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (5)$$

$$y = a(1 + x/K_{IN})/(1 + x/K_{ID}) \quad (6)$$

competitive and noncompetitive inhibition were fitted by using eq 4 and 5. Hyperbolic replots of kinetic parameters vs. F2,6P concentration were fitted by using eq 6. In eq 2–5, V is the maximum velocity, K_a and K_b are Michaelis constants for A and B, respectively, K_{ia} is the dissociation constant for A, K_{is} and K_{ii} are inhibition constants obtained from the slope and intercept, respectively, while A, B, and I are reactant and inhibitor concentrations, respectively. In eq 6, y is the kinetic parameter, e.g., V and V/K, a is the value of y at $x = 0$, $a(K_{ID}/K_{IN})$ is the value of y at $x = \infty$, x is F2,6P concentration, K_{ID} is the dissociation constant[2] for F2,6P, and K_{IN} is a ratio of rate constants that causes y to level off at $x = \infty$.

Results

Initial Velocity Studies in the Absence of Products. The PP_i-PFK originally isolated from the amoebic parasite *Entamoeba histolytica* appears to have a rapid equilibrium random mechanism (Reeves et al., 1976). On the basis of a more complete kinetic study in both reaction directions including initial velocity as well as product and dead-end inhibition patterns, the PP_i-PFK from *Propionibacterium freudenreichii (shermanii)* also has a mechanism that approximates rapid

[2] For the theoretical treatment of the mechanism of modulation by allosteric effectors see Cook (1982).

Table I: Kinetic Parameters for Plant PP_i-PFK at pH 8 and 25 °C

forward reaction direction			reverse reaction direction		
parameter[a]	−F2,6P	+F2,6P[b]	parameter	−F2,6P	+F2,6P[b]
V	0.012 ± 0.01	0.070 ± 0.005	V	0.011 ± 0.002	0.011 ± 0.002
K_{MgPP_i}	0.035 ± 0.003	0.007 ± 0.001	K_{P_i}	0.467 ± 0.039	0.546 ± 0.043
K_{F6P}	1.025 ± 0.127	0.213 ± 0.035	K_{FBP}	0.065 ± 0.008	0.009 ± 0.002
$K_{i\,MgPP_i}$	0.012 ± 0.001	0.006 ± 0.001	K_{Mg}[c]	0.028 ± 0.001	0.021 ± 0.001
$K_{i\,F6P}$	0.815 ± 0.062	0.214 ± 0.015	$K_{i\,P_i}$	0.392 ± 0.047	0.316 ± 0.173
			$K_{i\,FBP}$	0.055 ± 0.006	0.006 ± 0.001
			$K_{i\,Mg}$	nd[e]	nd
V/E_T[d] (s^{-1})	9.1	53	V/E_T (s^{-1})	nd	8.3
$V/K_{MgPP_i}E_T$ ($M^{-1}\,s^{-1}$)	2.6×10^5	7.6×10^6	$V/K_{P_i}E_T$ ($M^{-1}\,s^{-1}$)	nd	1.5×10^4
$V/K_{F6P}E_T$ ($M^{-1}\,s^{-1}$)	8.9×10^3	2.5×10^5	$V/K_{FBP}E_T$ ($M^{-1}\,s^{-1}$)	nd	9.2×10^5

[a] Units for V are micromoles per minute and for K and K_i are millimolar. [b] 1 μM F2,6P (saturating). [c] Saturation curve for Mg^{2+} obtained at saturating concentrations of FBP and P_i. [d] Calculations based on monomer M_r of 46 000. Values for the unregulated bacterial PP_i-PFK (monomer 48 000) were miscalculated in the report by Bertagnolli and Cook (1984). The actual values are as follows: in the forward reaction direction, V/E_T = 890 s^{-1}, $V/K_{MgPP_i}E_T = 1.9 \times 10^8$ $M^{-1}\,s^{-1}$, and $V/K_{F6P}E_T = 7 \times 10^6$ $M^{-1}\,s^{-1}$; in the reverse reaction, V/E_T = 1035 s^{-1}, $V/K_{P_i}E_T = 1.8 \times 10^6$ $M^{-1}\,s^{-1}$, and $V/K_{FBP}E_T = 3.5 \times 10^7$ $M^{-1}\,s^{-1}$. [e] nd, not determined.

Table II: Product Inhibition Patterns[a]

variable substrate[b]	fixed substrate[c]	product	pattern[d]	K_{is} + SE (mM) −F2,6P	K_{is} + SE (mM) +F2,6P
			Forward Reaction		
F6P	$MgPP_i$	P_i	C	0.032 ± 0.02	2.20 ± 0.13
$MgPP_i$	F6P	P_i	C	1.65 ± 0.08	4.03 ± 0.16
			Reverse Reaction		
P_i	Mg, FBP	$MgPP_i$	C	0.035 ± 0.001	0.017 ± 0.001
FBP	Mg, P_i	$MgPP_i$	C	0.158 ± 0.004	0.121 ± 0.005

[a] At pH 8 (100 mM Taps–KOH), 25 °C. [b] Concentration of variable substrate varied around its K_m value. [c] Concentration of fixed substrate(s) maintained at saturating levels. [d] Data adhering to competitive inhibition were fitted by using eq 4.

Table III: Dead-End Inhibition Patterns[a]

variable substrate	fixed substrate[b]	inhibitor	pattern[c]	K_{is} + SE (mM) −F2,6P	K_{is} + SE (mM) +F2,6P	K_{ii} + SE (mM) −F2,6P	K_{ii} + SE (mM) +F2,6P
			Forward Reaction				
F6P	$MgPP_i$	L-sorbose-6-P	C	2.4 ± 0.1	1.8 ± 0.1		
$MgPP_i$	F6P	L-sorbose-6-P	NC	3.2 ± 0.6	0.8 ± 0.3	3.7 ± 0.3	2.0 ± 0.3
$MgPP_i$	F6P	PCP	C	2.6 ± 0.1	1.1 ± 0.1		
F6P	$MgPP_i$	PCP	NC	2.8 ± 0.3	2.1 ± 0.2	2.9 ± 0.2	1.8 ± 0.2
			Reverse Reaction				
FBP	Mg, P_i	1,8-octanediol-bis-P	C	0.10 ± 0.01	0.09 ± 0.01		
P_i	Mg, FBP	1,8-octanediol-bis-P	NC	0.32 ± 0.10	0.37 ± 0.07	0.12 ± 0.01	0.14 ± 0.01
P_i	Mg, FBP	SO_4^{2-}	C	9.79 ± 0.47	7.32 ± 0.24		
FBP	Mg, P_i	SO_4^{2-}	NC	5.53 ± 0.11	6.63 ± 0.37	21.04 ± 0.78	14.49 ± 0.56

[a] At pH 8 (100 mM Taps–KOH), 25 °C. [b] Concentration of fixed substrate(s) maintained at K_m concentrations. [c] Data for competitive and noncompetitive inhibition were fitted by using eq 4 and 5.

equilibrium random (Bertagnolli & Cook, 1984). However, to our knowledge, there is no report of the kinetic mechanism for PP_i-PFK from any plant source. It was important, therefore, to first determine the kinetic mechanism of the plant enzyme in the absence and presence of F2,6P to aid in the interpretation of the mechanism of its action on this enzyme.

In both reaction directions initial velocity patterns that intersected to the left of the ordinate were obtained. In the direction of phosphorylation of P_i, the uncomplexed Mg^{2+} concentration was maintained at 1 mM. These experiments were then repeated in the presence of 1 μM F2,6P. Kinetic parameters are listed in Table I.

Product Inhibition. Inhibition by P_i was obtained vs. either F6P or $MgPP_i$ in the absence or presence of 1 μM F2,6P. Inhibition by $MgPP_i$ was obtained vs. either FBP or P_i in the absence or presence of 1 μM F2,6P. In all cases, inhibition is competitive and the results are summarized in Table II.

Dead-End Inhibition. In the forward reaction, L-sorbose-6-P was used as an analogue of F6P and methylenediphosphonate (PCP) with a carbon atom in place of the bridge oxygen as an analogue of pyrophosphate. L-Sorbose-6-P is competitive vs. F6P and noncompetitive vs. $MgPP_i$, while MgPCP is competitive vs. $MgPP_i$ and noncompetitive vs. F6P. These data are summarized in Table III.

For the reverse direction, 1,8-octanediol-bis-P was used as an analogue of FBP and inorganic sulfate as an analogue of phosphate. The 1,8-octanediol bisphosphate is competitive vs. FBP and noncompetitive vs. P_i, while sulfate is competitive vs. P_i and noncompetitive vs. FBP. These data are summarized in Table III.

None of the dead-end analogues were utilized as substrates as judged by coupled spectrophotometric or ^{31}P NMR analyses. In addition, as indicated in Table III, the dead-end inhibition patterns in both reaction directions were carried out in the absence and presence of a saturating concentration of F2,6P.

Effect of F2,6P on the Kinetic Parameters of Plant PP_i-PFK. The mechanism of activation of the plant PP_i-PFK by F2,6P was determined by using the procedure outlined by Cook (1982). Using this method, one obtains an initial velocity pattern by varying both reactants, e.g., F6P and $MgPP_i$, in the absence of F2,6P and then repeating this experiment at

Table IV: Effect of F2,6P on Kinetic Parameters of Plant PP_i-PFK[a]

enzyme form	kinetic paramater	x-fold stimulation	K_{act} for F2,6P[b] (nM)
	Forward Reaction		
$E{\cdot}MgPP_i{\cdot}F6P \rightarrow E{\cdot}FBP{\cdot}P_i$	V	9	54 ± 27
$E{\cdot}MgPP_i$	V/K_{F6P}	30	31 ± 3
E·F6P	V/K_{MgPP_i}	30	30 ± 1
E	$V/(K_{i\,MgPP_i})(K_{F6P})$	90	31 ± 15
	Reverse Reaction		
$E{\cdot}P_i$	V/K_{FBP}	42	nd[c]

[a] At pH 8, 25 °C. [b] Activation constants calculated from a fit using eq 6. [c] nd, not determined.

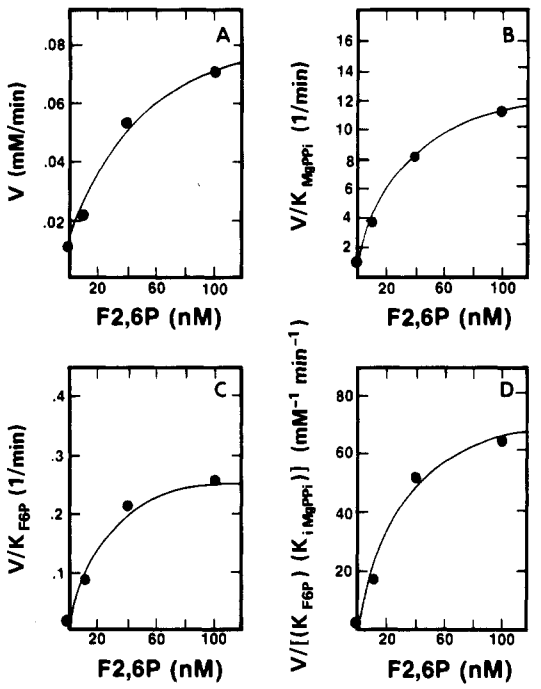


FIGURE 1: Stimulation of kinetic parameters of mung bean *P. aureus* PP_i-PFK in the forward reaction direction with 0, 10, 40, and 100 nM F2,6P: (A) V_{max} (mM/min), (B) V/K_{MgPP_i} (1/min), (C) V/K_{F6P} (1/min), and (D) $V/K_{F6P}(K_{i\,MgPP_i})$ (mM^{-1} min^{-1}). The solid curve is from a computer fit using eq 6.

several concentrations of F2,6P. The resulting values of V, V/K_{MgPP_i}, V/K_{F6P}, and $V/K_{i\,MgPP_i}$ (K_{F6P}) are then plotted vs. F2,6P. Results are shown in Table IV and Figure 1. Note that, under all conditions, a stimulation in rate is obtained. This experiment was also carried out for the reverse direction with only the V/K_{FBP} affected (Table IV).

Activators in the Forward Reaction Direction. In the presence of saturating $MgPP_i$ and F6P at pH 8 and 25 °C, the rate of the forward reaction is stimulated by a wide variety of mono- and bisphosphate compounds. Since the background rate in the absence of activator is subtracted, one can obtain a first-order rate (V/K_{act}) for activation. All activators are listed in Table V in order of decreasing effectiveness (lower V/K_{act}). The K_{act} values for even the best activators are 1–2 orders of magnitude weaker than F2,6P. For the alkanediol bisphosphate series, a plot of K_{act} values vs. carbon chain length for C_nP_2 (n = 2–6) suggests, as shown in Figure 2, that the four-carbon compound binds tightest.

None of the compounds listed in Table V, as well as many similar compounds not listed, would stimulate the velocity of the reverse reaction in the presence of saturating Mg^{2+}, FBP, and phosphate. In agreement with this, Sabularse and Anderson (1981a) reported no effect of D-glucose 1,6-bisphosphate, a poor activator of the forward reaction, on the reverse reaction direction of the mung bean PP_i-PFK.

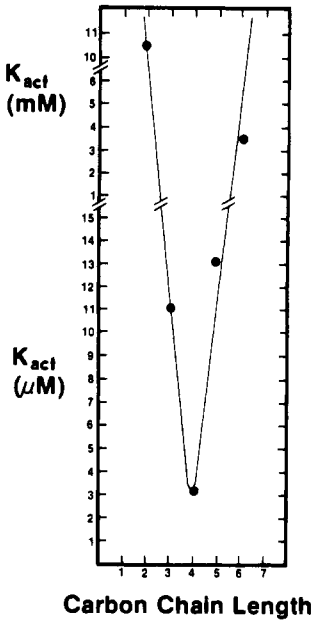


FIGURE 2: Activation of the forward reaction rate of mung bean *P. aureus* PP_i-PFK using a series of alkanediol bisphosphate compounds in the presence of saturating $MgPP_i$ and F6P. The carbon chain length indicates the number of carbons between the phosphates.

Table V: Activators of the Forward Reaction[a]

compound	K_{act}[b]	V (mM min^{-1})	V_{rel}[c]	V/K_{act} (min^{-1})
D-fructose-2,6-bis-P	30 nM	0.0150	1.00	500
2,5-anhydro-D-glucitol-1,6-bis-P	0.9 μM	0.0112	0.75	12.5
diethylene glycol-bis-P	1.7 μM	0.0125	0.83	7.4
1,4-butanediol-bis-P	3.1 μM	0.0090	0.60	2.9
1,3-propanediol-bis-P	11.2 μM	0.0110	0.73	0.98
1,5-pentanediol-bis-P	13.2 μM	0.0065	0.43	0.48
D-fructose-1,6-bis-P	40 μM	0.0148	0.98	0.37
diethylene glycol-mono-P	77 μM	0.0100	0.67	0.13
2,5-anhydro-D-mannitol-6-P	132 μM	0.0098	0.65	0.074
2,5-anhydro-D-mannitol-1,6-bis-P	398 μM	0.0231	1.53	0.058
L-sorbose-1,6-bis-P	82 μM	0.0028	0.19	0.034
2,3-*O*-isopropylidene-α-L-sorbofuranose-1-P	80 μM	0.0017	0.11	0.021
D-tagatose-6-P	320 μM	0.0062	0.41	0.019
D-glucitol-6-P	6.6 mM	0.0180	1.20	0.003
D-arabinose-5-P	1.0 mM	0.0028	0.19	0.0028
D-hexitol-1,6-bis-P	2.5 mM	0.0060	0.40	0.0024
D-psicose-6-P	2.3 mM	0.0026	0.17	0.0011
2,3-*O*-isopropylidene-α-L-sorbofuranose-1,6-bis-P	8.3 mM	0.0064	0.42	0.00076
triethylene glycol-bis-P	9.3 mM	0.0063	0.42	0.00068

[a] At pH 8 (100 mM Taps-KOH), 25 °C, saturating $MgPP_i$ and F6P. [b] Activation constant. [c] V_{max} relative to that with F2,6P.

Discussion

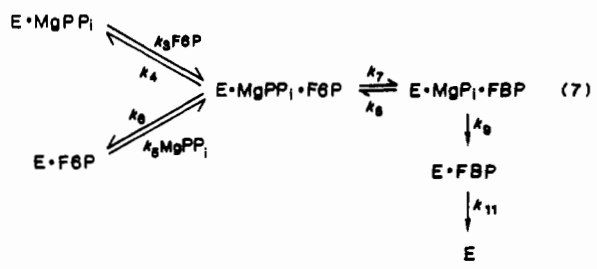
Kinetic Mechanism. Intersecting initial velocity patterns in both reaction directions indicate a sequential kinetic mechanism for the plant enzyme. Product inhibition (Table II) by P_i and $MgPP_i$ is competitive vs. either reactant with the other reactant saturating, suggesting a rapid equilibrium random mechanism in which P_i can bind to E·F6P and E·$MgPP_i$ and in which $MgPP_i$ can bind to E·P_i and E·FBP. This is qualitatively identical with the mechanism obtained for PP_i-PFK from *Propionibacterium* (Bertagnolli & Cook, 1984). The competitive patterns for P_i and $MgPP_i$ indicate that these reactants bind only as dead-end inhibitors. The randomness

is confirmed by the dead-end inhibition patterns (Table III). For this type of mechanism, the inhibitory analogue of a reactant (e.g., L-sorbose-6-P) is competitive vs. the reactant it resembles (e.g., F6P) while it is noncompetitive vs. the other reactant (e.g., $MgPP_i$). This is true in all cases.

When all of these experiments were repeated in the presence of 1 μM F2,6P, qualitatively identical results are obtained. The only definite quantitative changes observed are in the values of the kinetic parameters in the forward reaction direction, the parameters for FBP in the reverse direction (Table I), and the K_i for P_i as a product inhibitor (Table II). Thus, the mechanism appears to be rapid equilibrium random in the absence or presence of F2,6P. The ATP-PFK from rabbit muscle also appears to have a random mechanism at basic pH (Bar-Tana & Cleland, 1974; Merry & Britton, 1985). However, the nonregulated ATP-PFK from *Lactobacillus plantarum* has been reported to have an ordered mechanism in which the sugar substrate binds first (Simon & Hofer, 1978).

Mechanism of Activation by F2,6P. Cook (1982) has shown that, by carrying out a systematic variation of reactants and allosteric effectors, one can begin to understand the specific rate processes affected by the allosteric modulator. Initial velocity patterns were carried out at several different levels of F2,6P including zero. From the results shown in Tables I and IV and Figure 1, it can be seen that all rate constants in the forward reaction direction are affected as well as the K_i for F6P from E·F6P, with little effect on the K_i for $MgPP_i$. In the reverse reaction, the only parameters apparently affected are K_{FBP} and $K_{i\,FBP}$, with a possibility that V_r is also increased by F2,6P. The latter phenomenon is masked however, since FBP is also an activator with a K_{act} of 40 μM (Table V), similar to the K_m of 65 μM for FBP in the absence of F2,6P (Table I). Therefore, the actual unactivated V in this direction will certainly be less than in the presence of F2,6P. One can at least calculate the V/K for FBP in the absence of F2,6P but in the presence of saturating free Mg^{2+} using the Haldane relationship $K_{eq} = [(V_f/K_{F6P})(K_{i\,P_i})]/[(V_r/K_{FBP})(K_{i\,MgPP_i})]$. In the absence of 1 μM F2,6P, the $K_{eq} = 14.2$ by using the data in Table I. Since $K_{i\,P_i}$ and K_{eq} are insensitive to F2,6P, the only unknown is V_r/K_{FBP}, giving a calculated value of 2.2×10^4 M^{-1} s^{-1}. Thus, a 42-fold increase is obtained in V/K_{FBP} upon stimulation with saturating F2,6P.

The data in Tables I–IV and Figure 1 suggest that F2,6P activates largely by affecting a single rate process, that is, the interconversion of the central complexes. There is relatively little effect on the binding constants of dead-end inhibitors. There may be some increase in affinity for sugar phosphates as indicated by the decrease in K_i for L-sorbose-6-P in the presence of F2,6P, but these changes, if real, are relatively small. Thus, most of the 30–40-fold changes in the V/K values for F6P, $MgPP_i$, and FBP (Table IV) are likely a result of an increase in the rate of interconversion of the central complexes. Since the catalytic rate is increased 30–40-fold, it is necessary to explain why only a 9-fold stimulation of V is observed. This can best be illustrated by the mechanism



(7)

where k_7 and k_8 represent the catalytic steps and k_9 and k_{11} represent the off-rates for Mg^{2+} and P_i and for FBP, respectively. The expressions for V/K_{F6P}, V/K_{MgPP_i}, and V are

$$V/K_{F6P} = \frac{k_7}{K_{I\,F6P}(1 + k_7/k_4 + k_8/k_9)} \quad (8)$$

$$V/K_{MgPP_i} = \frac{k_7}{K_{I\,MgPP_i}(1 + k_7/k_6 + k_8/k_9)} \quad (9)$$

$$V = \frac{k_7}{1 + k_7(1/k_9 + 1/k_{11}) + k_8/k_9} \quad (10)$$

The $K_{I\,F6P}$ and $K_{I\,MgPP_i}$ represent k_4/k_3 and k_6/k_5, respectively. Since the mechanism approximates rapid equilibrium random, k_4, k_6, k_9, and k_{11} must be greater than k_7 and k_8. Thus, V/K_{F6P} and V/K_{MgPP_i} reduce to $k_7/K_{I\,F6P}$ and $k_7/K_{i\,MgPP_i}$, respectively. As a result, when k_7 is increased 30-fold, the entire 30-fold stimulation is observed for both V/K values. In the case of V, however, either k_9 or k_{11} must become rate limiting when k_7 is increased 9-fold; i.e., either k_9 or k_{11} is 9-fold less than k_7 in the absence of F2,6P or one of these rate constants decreases in the presence of F2,6P. Since the off-rate for P_i increases, if anything, in the presence of F2,6P (the K_i values for product inhibition increase; see Table II), k_{11} is most likely the rate-limiting step in the presence of F2,6P.

From plots of the kind shown in Figure 1, the dissociation constants for F2,6P from the different enzyme forms along the reaction path can be obtained (Cook, 1982). Note that no matter which reactant is bound to the active site, the affinity for F2,6P is the same ($K_{act} \simeq 30$ nM). Apparently, F2,6P has no effect on the quaternary structure of PP_i-PFK, as suggested by native gel electrophoresis and gel filtration in the absence and presence of F2,6P. Thus, activation most likely occurs as a result of a conformational change of the active site. Moreover, since no change in the affinity of enzyme for F2,6P is observed irrespective of the substrate bound at the active site and the K_i values for substrates are essentially unchanged whether or not F2,6P is bound, this conformational change must only affect the chemical interconversion step.

The K_{act} value reported in this study for F2,6P agrees well with the values in the 5–50 nM range for PP_i-PFKs from several plant sources (Sabularse & Anderson, 1981b; Cseke et al., 1982; Van Schaftingen et al., 1982; Kruger et al., 1983; Yan & Tao, 1984). For mammalian systems a K_{act} of 24 nM has been reported for rat liver ATP-PFK and 10 nM for rabbit muscle ATP-PFK (Uyeda et al., 1981a).

Specificity of the Activator Site. In addition to the most potent activator F2,6P, a number of mono- and bisphosphate compounds have been found to stimulate the rate of the forward reaction (F6P phosphorylation) in the presence of saturating reactants $MgPP_i$ and F6P (Table V). Since all of these related compounds were synthesized chemically under conditions in which F2,6P could not have been produced, the observed stimulation could not be a result of contaminating F2,6P.

Molecular models made of the first four bisphosphate compounds listed in Table V indicate that similar distances exist between the phosphorus atoms. As mentioned in an earlier report on FBP aldolase (Hartman & Barker, 1965), other factors such as positioning of functional groups around the furan ring are very important for tight binding. This can be illustrated by the observation that the locked α analogue 2,5-anhydro-D-glucitol-1,6-bis-P has a V/K_{act} value 2 orders of magnitude larger than the locked β analogue 2,5-anydro-D-mannitol-1,6-bis-P, although the maximal distance between the phosphorus atoms differ by only 0.2 Å for the two compounds. For the alkanediol bisphosphate series C_3P_2, C_4P_2,

and C_5P_2, the maximum distance between phosphorus atoms has been reported to be 8.2, 9.4, and 10.6 Å, respectively (Hartman & Barker, 1965). Although the maximal distance between phosphorus atoms for this series varies by almost 2.5 Å, the V/K_{act} values given in Table V vary by less than a factor of 10. In addition, FBP and L-sorbose 1,6-bisphosphate are relatively weak activators since the phosphate is incorrectly positioned in both. The monophosphate of L-glucitol, D-tagatose, and D-psicose are very poor activators, indicating not only the importance of the second phosphate but also the need for correct orientation of the hydroxyl groups. Replacing the phosphate at C-2 with a proton results in a 4400-fold decrease in affinity (5.3 kcal/mol), which is certainly consistent with ion pairing of this phosphate. The 2,5-anhydro-D-glucitol 1,6-bisphosphate gives a 40-fold decrease in affinity compared to F2,6P, and when this is compared to butanediol bisphosphate, one obtains an additional 4-fold decrease. It thus appears that F2,6P is tightly bound with ion pairing of both phosphates and hydrogen bonds to all hydroxyl groups.

In summary, plant PP_i-PFK in the presence of F2,6P is quantitatively similar to the unregulated bacterial enzyme. The allosteric activator appears to exert its effect on the catalytic interconversion steps. The allosteric site best accommodates a β-D-furanose ring phosphorylated at the C-2 anomeric and C-6 hydroxyls and prefers the D-threo configuration at C-3 and C-4.

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Registry No. PP_i-PFK, 55326-40-4; F2,6P, 77164-51-3; FBP, 488-69-7; F6P, 643-13-0; P_i, 14265-44-2; $MgPP_i$, 13446-24-7; $H_2O_3PO(CH_2)_2O(CH_2)_2OPO_3H_2$, 45163-42-6; $H_2O_3PO(CH_2)_4OPO_3H_2$, 763-26-8; $H_2O_3PO(CH_2)_3OPO_3H_2$, 674-67-9; $H_2O_3PO(CH_2)_5OPO_3H_2$, 763-27-9; $H_2O_3PO(CH_2)_2O(CH_2)_2OH$, 64864-14-8; $H_2O_3PO(CH_2)_2O(CH_2)_2O(CH_2)_2OPO_3H_2$, 52329-58-5; 2,5-anhydro-D-glucitol-1,6-bisphosphate, 4429-47-4; 2,5-anhydro-D-mannitol-6-P, 52011-52-6; 2,5-anhydro-D-mannitol-1,6-bis-P, 671-08-9; L-sorbose-1,6-bis-P, 96480-54-5; 2,3-*O*-isopropylidene-α-L-sorbofuranose-1-phosphate, 103024-66-4; D-tagatose-6-P, 73544-42-0; D-glucitol-6-P, 20479-58-7; D-arabinose-5-P, 13137-52-5; D-hexitol-1,6-bis-P, 4300-29-2; 2,3-*O*-isopropylidene-α-L-sorbofuranose-1,6-bis-P, 103024-68-6.

REFERENCES

Ashihara, H., & Stupavska, S. (1984) *J. Plant Physiol. 116*, 241.

Bar-Tana, J., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 1271.

Bartrons, R., Van Schaftingen, E., Vissers, S., & Hers, H. G. (1982) *FEBS Lett. 143*, 137.

Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101.

Bertagnolli, B. L., Younathan, E. S., Voll, R. J., Pittman, C. E., & Cook, P. F. (1986) *Biochemistry* (preceding paper in this issue).

Bosca, L., Aragon, J. J., & Sols, A. (1982) *Biochem. Biophys. Res. Commun. 106*, 486.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Carnal, N. W., & Black, C. C. (1979) *Biochem. Biophys. Res. Commun. 86*, 20.

Claus, T. H., Schlump, J., Pilkis, J., Johnson, R. A., & Pilkis, S. J. (1981) *Biochem. Biophys. Res. Commun. 98*, 359.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Cook, P. F. (1982) *Biochemistry 21*, 113.

Cseke, C., Weeden, N. F., Buchanan, B. B., & Uyeda, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4322.

Furuya, E., & Uyeda, K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5861.

Hartman, F. C., & Barker, R. (1965) *Biochemistry 4*, 1068.

Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155.

Hesbain-Frisque, A. M., Van Schaftingen, E., & Hers, H. G. (1981) *Eur. J. Biochem. 117*, 315.

Kelly, G. J., & Latzko, E. (1975) *Nature (London) 256*, 429.

Kitajima, S., & Uyeda, K. (1983) *J. Biol. Chem. 258*, 7352.

Kombrink, E., & Wober, G. (1982) *Arch. Biochem. Biophys. 213*, 602.

Kombrink, E., & Kruger, N. J. (1984) *Z. Pflanzenphysiol. 114*, 443.

Kowalczyk, S., Januszewska, B., Cymerska, E., & Maslowski, P. (1984) *Physiol. Plant. 60*, 31.

Kruger, N. J., & Beevers, H. (1985) *Plant Physiol. 77*, 358.

Kruger, N. J., Kombrink, E., & Beevers, H. (1983) *FEBS Lett. 153*, 409.

Lederer, B., Vissers, S., Van Schaftingen, E., & Hers, H. G. (1981) *Biochem. Biophys. Res. Commun. 103*, 1281.

Merry, S., & Britton, H. G. (1985) *Biochem. J. 226*, 13.

Miernyk, J. A., & Dennis, D. T. (1982) *Biochem. Biophys. Res. Commun. 105*, 793.

Miyatake, K., Enomoto, T., & Kitaoka, S. (1984) *Agric. Biol. Chem. 48*, 2857.

O'Brien, W. E., Bowien, S., & Wood, H. G. (1975) *J. Biol. Chem. 250*, 8690.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.

Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., Claus, T. H., & Cumming, D. A. (1981) *J. Biol. Chem. 258*, 3171.

Reeves, R. E., Serrano, R., & South, D. J. (1976) *J. Biol. Chem. 251*, 2958.

Reinhart, G. D. (1983) *J. Biol. Chem. 258*, 10827.

Sabularse, D. C., & Anderson, R. L. (1981a) *Biochem. Biophys. Res. Commun. 100*, 1423.

Sabularse, D. C., & Anderson, R. L. (1981b) *Biochem. Biophys. Res. Commun. 103*, 848.

Simon, W. A., & Hofer, H. W. (1978) *Eur. J. Biochem. 88*, 175.

Smyth, D. A., Wu, M. X., & Black, C. C. (1984) *Plant Physiol. 76*, 316.

Stitt, M., Gerhardt, R., Kurzel, B., & Heldt, H. W. (1983) *Plant. Physiol. 72*, 1139.

Tung, E. L., Ling, K. H., Byrne, W. L., & Lardy, H. A. (1954) *Biochim. Biophys. Acta 14*, 488.

Uyeda, K., Furuya, E., & Luby, L. J. (1981a) *J. Biol. Chem. 256*, 8394.

Uyeda, K., Furuya, E., & Sherry, A. D. (1981b) *J. Biol. Chem. 256*, 8679.

Van Schaftingen, E., & Hers, H. G. (1981) *Eur. J. Biochem. 117*, 319.

Van Schaftingen, E., Hue, L., & Hers, H. G. (1980) *Biochem. J. 192*, 887.

Van Schaftingen, E., Jett, M. F., Hue, L., & Hers, H. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3483.

Van Schaftingen, E., Lederer, B., Bartrons, R., & Hers, H. G. (1982) *Eur. J. Biochem. 129*, 191.

Wood, H. G., & Goss, N. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 312.

Wu, M. X., Smyth, D. A., & Black, C. C. (1983) *Plant Physiol. 73*, 188.

Yan, T. F. J., & Tao, M. (1984) *J. Biol. Chem. 259*, 5087.