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Carbohydrate Substrate Specificity of Bacterial and Plant Pyrophosphate-Dependent Phosphofructokinases†

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Received October 30, 1985; Revised Manuscript Received January 29, 1986

ABSTRACT: Pyrophosphate-dependent phosphofructokinase from the facultative anaerobic bacterium *Propionibacterium freudenreichii* and from the mung bean *Phaseolus aureus* has been purified to homogeneity. Potential utilization of carbohydrate substrate analogues for each enzyme was initially screened by using Fourier transform ^{31}P NMR at pH 8 and 25 °C and monitoring the appearance of the phosphate resonance in the direction of D-fructose 6-phosphate phosphorylation (forward reaction direction) and, with the bisphosphate analogues, the appearance of the pyrophosphate resonance in the direction of phosphate phosphorylation (reverse reaction direction). Both enzymes are strict in their requirements for the sugar phosphate substrate, with only D-fructose 6-phosphate, D-sedoheptulose 7-phosphate, and 2,5-anhydro-D-mannitol 6-phosphate, or their respective bisphosphates in the reverse reaction direction, utilized as substrates at detectable levels. The dissociation constants for D-psicose 6-phosphate, D-tagatose 6-phosphate, and L-sorbose 6-phosphate are an order of magnitude larger than that for D-fructose 6-phosphate, indicating a stringent steric requirement for the D-threo (trans) configuration at the two nonanomeric furan ring hydroxyl groups. These results strongly suggest that the anomeric, epimeric, and tautomeric form of the sugar phosphate substrates favored by both enzymes is the β-D-fructofuranose form. Dissociation constants for nonsubstrate analogues were used to provide information on the nature of the active site. Competitive inhibition patterns vs. fructose 1,6-bisphosphate were obtained for a series of 1,*n*-alkanediol bisphosphates (where $n = 2$–9). The bacterial enzyme binds compounds with $n = 6$, 7, and 8 more tightly ($K_i \cong 200$ μM) than any of the others tested. The plant pyrophosphate-dependent phosphofructokinase, however, binds all analogues less tightly than the bacterial enzyme with 1,8-octanediol bisphosphate ($K_i \cong 650$ μM) binding tighter than any of the other analogues in the alkanediol bisphosphate series. Thus, although the active sites for the two enzymes are similar, there are distinct differences.

Pyrophosphate-dependent phosphofructokinase (pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) catalyzes the reversible reaction:[1]

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

The enzyme has a very strict requirement for pyrophosphate as the phosphoryl donor with as yet no detectable rates reported for analogues of pyrophosphate or for tri- and tetrapolyphosphates (Reeves et al., 1974; O'Brien et al., 1975; Carnal & Black, 1979; Sabularse & Anderson, 1981; Bertagnolli & Cook, 1984; Wood & Goss, 1985). However, the carbohydrate substrate (phosphoryl acceptor) for this enzyme

† This work was supported by NIH Grants GM31686 (to P.F.C.) and AM31676 (to E.S.Y.) and Grant B-1031 from The Robert A. Welch Foundation (to P.F.C.). P.F.C. is the recipient of NIH Research Career Development Award AM 01155.
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[1] Abbreviations: ATP-PFK, ATP-dependent phosphofructokinase; PP_i-PFK, pyrophosphate-dependent phosphofructokinase; ATP, adenosine 5′-triphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; F6P, fructose 6-phosphate; T6P, tagatose 6-phosphate; FBP, fructose 1,6-bisphosphate; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]-1-propanesulfonic acid; F2,6P, fructose 2,6-bisphosphate; G6P, glucose 6-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; Pipes, 1,4-piperazinediethanesulfonic acid; C_nP_2, *n*-alkanediol 1,*n*-bisphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, NAD phosphate.

0006-2960/86/0425-4674$01.50/0

has not been defined as to its anomeric, epimeric, and tautomeric specificity.

Chemically synthesized nonisomerizing locked analogues of the α- and β-furanose configurations have been widely used to study carbohydrate anomeric specificity of ATP-PFKs. Although there are many enzymatic reactions where the free aldehydo or keto forms are the actual substrates (Gracy & Noltmann, 1968), utilization of 2,5-anhydro-D-mannitol-6-P (locked β analogue of F6P) but not 2,5-anhydro-D-glucitol-6-P (locked α analogue of F6P) by both rat liver and rabbit muscle ATP-PFKs clearly demonstrates that these enzymes use the β anomer of the furanose ring with phosphorylation of the C-1 hydroxyl without prior ring opening (Koerner et al., 1973a,b, 1974; Bar-Tana & Cleland, 1974; Benkovic, 1979; Riquelme et al., 1984). These findings are also supported by results obtained from rapid-quench kinetics (Fishbein et al., 1974) and from stopped-flow techniques (Wurster & Hess, 1974). Both ^{13}C and ^{31}P NMR analyses at neutral pH have indicated that 80–85% of F6P and FBP exist in solution as the β-furanose, 15–20% as the α-furanose, and <2% as the keto or hydrated keto forms (Gray, 1971; Koerner et al., 1973a,b; Van Den Berg & Heerschap, 1982).

The epimeric specificity of the rabbit muscle ATP-PFK has also been extensively studied. D-Tagatose-6-P (C-4 epimer) is utilized as a phosphoryl acceptor (Totten & Lardy, 1949; Lardy, 1962; Koerner et al., 1976). This finding has also been obtained by using rapid-quench kinetic techniques (Fishbein et al., 1974). The rabbit muscle ATP-PFK will also apparently utilize D-tagatose 6-sulfate and L-sorbose-6-P (C-5 epimer) as well as D-psicose-6-P (C-3 epimer) (Koerner et al., 1976). With regard to tautomeric specificity, the ATP-PFK favors the furanose ring form over the pyranose ring form. However, Eyer et al. (1971) found that this enzyme will use α-D-glucopyranose-1-P but at 0.67% of the V_{max} obtained for F6P.

In contrast to the wealth of information available for the ATP-PFK, very little is known concerning the tolerance of PP_i-PFKs toward the carbohydrate substrate. The pyrophosphate-dependent phosphofructokinase (PP_i-PFK) from the parasitic protozoan *Entamoeba histolytica* also appears to have a β-anomeric specificity as deduced from techniques similar to those mentioned above (Koerner et al., 1977). The PP_i-PFK from yeast will also utilize fructose-1-P (Sols & Salas, 1966) while the PP_i-PFK from *Propionibacterium shermanii* (O'Brien et al., 1975), *E. histolytica* (Reeves et al., 1974), and wheat seedlings (Yan & Tao, 1984) did not use fructose-1-P as a phosphoryl acceptor. Finally, the PP_i-PFK from *E. histolytica* (Reeves et al., 1974) and wheat seedlings (Yan & Tao, 1984) will not phosphorylate G6P in the forward reaction, and PP_i-PFK from *P. shermanii* will not use glucose 1,6-bisphosphate as the phosphoryl donor to phosphate in the reverse reaction (O'Brien et al., 1975).

The aim of this study is to explore in detail the anomeric, epimeric, and tautomeric specificity of the bacterial and plant PP_i-PFKs by means of ^{31}P NMR and kinetic analyses. Both enzymes are specific for the β anomer of the sugar phosphate and are much more stringent in their substrate specificity compared to the ATP-dependent enzymes.

Materials and Methods

Chemicals. Fructose 6-phosphate (sodium salt), fructose 1,6-bisphosphate (sodium salt), fructose 2,6-bisphosphate (sodium salt), D-glyceraldehyde 3-phosphate (diethyl acetal, dicyclohexylammonium salt converted to D-glyceraldehyde 3-phosphate as suggested by Sigma), Taps, activated charcoal (acid washed), glycerol (Sigma grade), Pipes, DL-dithiothreitol, ADP (sodium salt, vanadium free), EDTA, D-sedoheptulose 7-phosphate (85%, barium salt), D-sedoheptulose 1,7-bisphosphate (95%, sodium salt), D-arabinose 5-phosphate (>98%, sodium salt), D-glucose 6-phosphate (>98%, sodium salt), and D-glucitol 6-phosphate (labeled commercially as D-sorbitol 6-phosphate) (>98%, barium salt) were obtained from Sigma. Sodium pyrophosphate was obtained from Mallinckrodt. Deuterium oxide (100 atom % deuterium) was purchased from Aldrich Chemical Co. Magnesium chloride, potassium chloride, sodium sulfate, and potassium phosphate (dibasic) were from Fisher Chemical Co. Cellulose phosphate (Cellex P) was purchased from Bio-Rad. Boehringer Mannheim was the source of NAD (100%, free acid), NADH (100%, disodium salt), and NADP (98%, disodium salt). All other chemicals and reagents were obtained from commercial sources and were of the highest quality available. The 5-ketofructose was the generous gift of Dr. John S. Blanchard (Department of Biochemistry and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10401).

The monophosphates of D-psicose, D-tagatose, and L-sorbose and the derivatives of 2,5-anhydro-D-glucitol and 2,5-anhydro-D-mannitol were prepared according to procedures as reported earlier (Koerner et al., 1976, 1980). The bisphosphates of D-psicose and D-tagatose were prepared by the action of ATP-PFK from rabbit muscle on their respective monophosphates. The following compounds were prepared by adaptations of published procedures: D-hexitol bisphosphate (Ginsburg & Mehler, 1966), the mono- and bisphosphates of alkanediols and glycol (Hartman & Barker, 1965), and the isopropylidene derivatives of L-sorbose (Mann & Lardy, 1950).

The 5-ketofructose was converted to the monophosphate at pH 8, in 100 mM Taps, by using 20 mM MgATP and 20 mM 5-ketofructose incubated for 90 min at 25 °C with 20 units of yeast hexokinase. After the reaction was deemed complete (no change in ^{31}P NMR signal intensity beyond 90 min), the reaction mixture was filtered through a PM-10 (Amicon) ultrafiltration membrane to remove hexokinase and was then subjected to activated charcoal filtration to remove MgADP (no ADP signal in ^{31}P NMR). The solution was then adjusted to pH 8 and stored at −20 °C. Barium salts were converted to sodium salts by weighing a small amount (typically 25–50 mg) into a small test tube and then adding a 2-fold weight excess of sodium sulfate. One to two milliliters of 100 mM Taps, pH 8, was added, and the suspension was allowed to sit for 1 h at room temperature with occasional mixing followed by centrifugation to remove the barium sulfate precipitate. The clear supernatant was then adjusted to pH 8 and to volume and was used immediately.

The analogues were pure by ^1H and/or ^{13}C NMR analyses. In addition, proton-decoupled ^{31}P NMR spectra were run on 20 mM solutions at pH 8 to check for extraneous sugar phosphate resonances and the inorganic phosphate resonance. No significant amounts were detected in any of the synthesized analogues mentioned above. The D-arabinose 5-phosphate, D-glucose 6-phosphate, and D-glucitol-6-P also gave only one major resonance in the phosphate ester region with one minor resonance of less than 2% of the intensity of the major resonance. The D-sedoheptulose 7-phosphate gave one major resonance in the phosphate ester region of the spectrum and two resonances of <10% of the intensity of the major peak, while D-sedoheptulose 1,7-bisphosphate gave two major resonances in the phosphate ester region of the spectrum and two resonances of ~17% of the intensity of the major resonances. The 5-ketofructose phosphate gave one major resonance in the phosphate ester region of the spectrum and a peak of ~16% of the intensity of the major resonance. It is probable that

part, if not all, of the minor resonances discussed above could represent the α anomers of the furanose ring or, in some cases, the pyranose form of the sugar phosphate.

Enzymes. Fructose-1,6-bisphosphate aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, ATP-dependent phosphofructokinase, and 3-phosphoglycerate kinase were all from rabbit muscle and were supplied by Sigma as sulfate-free powders. Phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase were all from bakers' yeast and were also supplied by Sigma as sulfate-free powders. Hexokinase from yeast was supplied by Boehringer Mannheim as a lyophilized powder. All enzymes were dissolved in cold 100 mM Taps, pH 7.6, prior to use. To test whether or not the coupling enzymes used in the forward and reverse reaction direction assays (see below) were devoid of interfering activities, all enzyme stocks were checked for phosphoglucose isomerase, inorganic pyrophosphatase, fructose-1,6-bisphosphatase, and FBP aldolase activities as described previously (Bertagnolli & Cook, 1984).

Crude pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* was obtained from Sigma as a lyophilized powder and was purified by methods outlined in Bertagnolli and Cook (1984) based on a procedure developed by O'Brien et al. (1975). Partially purified mung bean (*Phaseolus aureus*) PP_i-PFK (EC 2.7.1.90) was also obtained from Sigma (product no. F8757) as a lyophilized powder and was purified by using the phosphocellulose column step as developed by Sabularse and Anderson (1981). The specific activity of several preparations of the plant enzyme was routinely in the range of 74–82 μmol min^{-1} (mg of protein)$^{-1}$ in the presence of 1 μM F2,6P at pH 8 and 25 °C. Protein concentration was determined by the Bio-Rad reagent microassay based on the method of Bradford (1976). Both PP_i-PFKs were found to be free of interfering enzymatic activities by ^{31}P NMR analyses similar to those described above.

^{31}P NMR Spectra. Potential carbohydrate substrate analogues were initially screened by using Fourier transform ^{31}P NMR and monitoring the appearance of the phosphate resonance in the direction of F6P phosphorylation or the pyrophosphate resonance in the reverse reaction (Figure 1). The proton-decoupled ^{31}P NMR spectra were recorded at 36.3 MHz on a JEOL FX-90Q spectrometer with 10% v/v deuterium oxide as the locking signal. Spectra were obtained with a 90° pulse of 25-μs duration, a pulse delay of 3 s, and a pulse acquisition of 3.4 s. Typically, 100 scans were accumulated and the sweep width was 1200 Hz. Chemical shifts were referenced to 85% H_3PO_4 as the external standard. All measurements were made in 1 cm × 21 cm NMR tubes (Wilmad) at a probe temperature of 28 °C.

A typical sample for the forward reaction direction contained in a 2-mL total volume 100 mM Taps, pH 8, 10% D_2O (v/v), 20 mM $NaPP_i$, 10 mM $MgCl_2$, 20 mM F6P (or analogue), and 1 μM F2,6P (for plant PP_i-PFK only). The solution was then incubated with 0.2 unit of PP_i-PFK for 2 h at 25 °C before the spectra were taken. The pH was routinely measured before and after the spectra were taken. In parallel, a control sample was run, containing all of the above components minus PP_i-PFK. In the reverse reaction direction a typical sample contained in a 2-mL total volume 100 mM Taps, pH 8, 10% D_2O (v/v), 12.5 mM $MgCl_2$, 20 mM inorganic phosphate, 20 mM FBP (or analogue), and 1 μM F2,6P (for plant PP_i-PFK only). The remainder of the conditions and the control sample were in keeping with that stated above for the forward reaction direction.

A lower limit of sensitivity of this method is ~5% conversion in 2 h of reaction time. That is, if less than 5% of the analogue was utilized by PP_i-PFK in 2 h, the signal for the phosphate or pyrophosphate resonance used to monitor the extent of the reaction could not be integrated above base line and, therefore, would not be detected.

Spectrophotometric Enzyme Assays. All assays were carried out at pH 8 and 25 °C with 0.1 full-scale absorbance monitoring changes in absorbance at 340 nm on a Cary/Varian 210 spectrophotometer with a chart speed of 12 cm/min. Reaction mixtures were 1-mL total volume. All assays were carried out as stated previously (Bertagnolli & Cook, 1984) except that 1 μM F2,6P was included in all assays of the mung bean enzyme. For carbohydrate analogues utilized as substrates as shown by ^{31}P NMR screening as described above, the glyceraldehyde-phosphate dehydrogenase/phosphoglycerate kinase couple was used (Bertagnolli & Cook, 1984). For analogues *not* utilized as substrates, K_i values were obtained from inhibition experiments.

Metal Chelate Complex Correction. Correction for metal chelate complexes formed was made according to the method of Bertagnolli and Cook (1984). The dissociation constants for the Mg-(analogue monophosphate) and Mg-(analogue bisphosphate) complexes were assumed to be identical with those for MgF6P and MgFBP, respectively.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and all plots were linear. All data were fit by using the appropriate rate equation and the FORTRAN programs of Cleland (1979). Data for substrate and substrate analogue saturation curves were fit by using eq 2, while data for competitive inhibition were fit by using eq 3. In eq 2 and 3, V is the maximum velocity, K is the K_m for

$$v = VA/(K + A) \quad (2)$$

$$v = VA/[K(1 + I/K_i) + A] \quad (3)$$

the substrate or substrate analogue, and K_i is the dissociation constant for competitive inhibition.

RESULTS

Native PP_i-PFK from *Propionibacterium freudenreichii* (*shermanii*) is a dimer of M_r 96 000 (O'Brien et al., 1975) and has a rapid equilibrium random mechanism as derived from initial velocity studies and product and dead-end inhibition studies in both reaction directions (Bertagnolli & Cook, 1984). At pH 8 and 25 °C there is no effect of micromolar concentrations of F2,6P on any of the kinetic parameters measured for this enzyme in either reaction direction. In fact, only at relatively high concentrations is F2,6P competitive vs. FBP (K_i = 3.9 mM) (Bertagnolli & Cook, 1984). These observations are in agreement with those of Wood and Goss (1985) in that this enzyme is insensitive to F2,6P. Therefore, F2,6P was not included in the ^{31}P NMR screening for potential utilization of analogues or in any of the spectrophotometric assays performed with the bacterial enzyme. The enzyme from mung bean, however, appears to be a homotetramer of M_r 190 000 on the basis of native and sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration. The enzyme is activated by nanomolar concentrations of F2,6P, resulting in changes in several kinetic parameters [see Bertagnolli et al. (1986)]. Saturating F2,6P (1 μM) was therefore included in all the ^{31}P NMR studies and spectrophotometric assays.

The noninvasive ^{31}P NMR technique was used to screen potential utilization of analogues of F6P/FBP. This is the method of choice for this enzyme system as a result of the

Table I: Sugar Specificity for Bacterial PP_i-PFK in the Forward Reaction Direction[a]

compound	^{31}P NMR[b]	K_m (mM)[c]	K_{is} (mM)[c]	V (μmol min^{-1})[c]	V/K
D-fructose-6-P	100	0.24 ± 0.02		0.0217 ± 0.0009	0.09
D-sedoheptulose-7-P	20	0.38 ± 0.06		0.0036 ± 0.0002	0.0095
2,5-anhydro-D-mannitol-6-P	12	0.97 ± 0.24		0.0027 ± 0.0003	0.0028
2,5-anhydro-D-glucitol-6-P	0		0.32 ± 0.03		
D-psicose-6-P	0		3.78 ± 0.14		
D-tagatose-6-P	0		2.56 ± 0.08		
L-sorbose-6-P	0		2.05 ± 0.21		
2,3-*O*-isopropylidene-α-L-sorbofuranose-1-P	0		2.37 ± 0.12		
D-arabinose-5-P	0		2.36 ± 0.14		
D-glucose-6-P	0		12.14 ± 0.66		
5-ketofructose-P	0		3.29 ± 0.43		
diethylene glycol-mono-P	0		1.49 ± 0.13		
1,6-hexanediol-mono-P	0		2.22 ± 0.18		
D-glucitol-6-P	0		2.16 ± 0.04		

[a] At pH 8 and 25 °C. [b] Peak heights at the phosphate resonance position relative to that obtained with F6P as reactant (set at 100%) after 2-h reaction time. [c] From spectrophotometric assays (see Materials and Methods).

Table II: Sugar Specificity for Plant PP_i-PFK in the Forward Reaction Direction[a]

compound	^{31}P NMR[b]	K_m (mM)[c]	K_{is} (mM)[c]	V (μmol min^{-1})[c]	V/K
D-fructose-6-P	100	0.23 ± 0.05		0.0700 ± 0.0100	0.3
D-sedoheptulose-7-P	7	0.49 ± 0.10		0.0056 ± 0.0005	0.0114
2,5-anhydro-D-mannitol-6-P	4	0.92 ± 0.26		0.0025 ± 0.0003	0.0027
2,5-anhydro-D-glucitol-6-P	0		1.52 ± 0.04		
D-psicose-6-P	0		7.97 ± 0.58		
D-tagatose-6-P	0		2.41 ± 0.12		
L-sorbose-6-P	0		2.39 ± 0.24		
2,3-*O*-isopropylidene-α-L-sorbofuranose-1-P	0		6.86 ± 0.49		
D-arabinose-5-P	0		10.71 ± 1.51		
D-glucose-6-P	0		14.15 ± 0.57		
5-ketofructose-P	0		6.09 ± 0.18		
diethylene glycol-mono-P	0		10.87 ± 0.48		
1,6-hexanediol-mono-P	0		3.62 ± 0.23		
D-glucitol-6-P	0		5.07 ± 0.76		

[a] At pH 8 and 25 °C plus 1 μM F2,6P. [b] Peak heights at the phosphate resonance position relative to that obtained with F6P as reactant (set at 100%) after 2-h reaction time. [c] From spectrophotometric assays (see Materials and Methods).

separation of all phosphate resonances and the ease of interpretation of results. Figure 1 shows typical spectra accumulated in the direction of phosphorylation of inorganic phosphate with FBP as the phosphoryl donor. Both control and experimental samples are depicted 2 h after addition of 0.2 unit of the bacterial enzyme to the experimental sample. Appearance of the PP_i resonance is indicative of reaction. The reaction mixture had not reached equilibrium after 2 h at 25 °C as judged by spectrotometric assays performed on aliquots at various times. The intensities of the pyrophosphate peaks with various analogues of FBP are reported relative to a value of 100 with FBP as reactant, and these values appear in the left-hand column of Tables III and IV. For the forward reaction direction with F6P as reactant, the intensity of the inorganic phosphate resonance after 2 h was set at a relative value of 100 with F6P as the carbohydrate substrate and the phosphate peak intensities appear in the left-hand column of Tables I and II.

The only two analogues of the natural substrates that were found to be utilized at detectable levels in the forward reaction direction were D-sedoheptulose-7-P and 2,5-anhydro-D-mannitol-6-P[2] and their respective bisphosphates in the reverse reaction direction. Other analogues could have been utilized at very low levels, but the ^{31}P NMR technique does serve as a rapid and accurate means of indicating which compounds are favored by both enzymes utilized in this study.

The 2,5-anhydro-D-glucitol-6-P is not phosphorylated by either enzyme but does have a reasonable affinity compared to F6P (Tables I and II). The 3-, 4- and 5-epimers of F6P are not utilized as substrates and have very poor affinity when

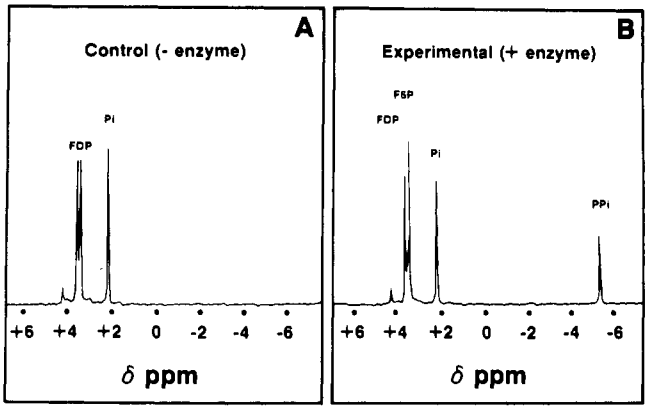

FIGURE 1: Examples of Fourier transform ^{31}P NMR spectra of the bacterial PP_i-PFK reaction in the reverse reaction direction with FBP as the carbohydrate substrate. Initial concentrations of reactants and conditions for running the spectra are listed under Materials and Methods. (A) The reaction mixture minus enzyme after 2 h at pH 8, 25 °C. The two large peaks for β-FBP are due to the phosphorus at C-1 (left) and at C-6 (right). The small resonance at +4.2 ppm is due to the phosphorus at C-1 of α-FBP, and the small broad peak at +3.2 ppm is due to the phosphorus at C-6 of α-FBP. All of the substrate and nonsubstrate analogues listed in Tables I–IV of this paper had resonances falling in the range of +3.2–+4.5 ppm. The large resonance at +2.2 ppm is inorganic phosphate. (B) Duplicate reaction mixture 2 h after addition of 0.2 unit of enzyme. The resonance at the C-6 of FBP has increased due to the appearance of F6P (phosphorus at C-6 of F6P has the same chemical shift as that at the C-6 of FBP), the inorganic phosphate resonance at +2.2 ppm has decreased, and as expected, the pyrophosphate resonance appears at −5.6 ppm.

[2] Nomenclature: Both 2,5-anhydro-D-mannitol and 5-keto-D-fructose have a C-2 axis of symmetry; hence, their 1-P is identical with their 6-P. The 6-P nomenclature is adopted here.

Table III: Sugar Specificity for Bacterial PP_i-PFK in the Reverse Reaction Direction[a]

compound	^{31}P NMR[b]	K_m (mM)[c]	K_{is} (mM)[c]	V (μmol min^{-1})[c]	V/K
D-fructose-1,6-bis-P	100	0.024 ± 0.007		0.0220 ± 0.0002	0.92
D-sedoheptulose-1,7-bis-P	54	0.121 ± 0.004		0.0118 ± 0.0001	0.098
2,5-anhydro-D-mannitol-1,6-bis-P	45	0.136 ± 0.005		0.0098 ± 0.0001	0.072
2,5-anhydro-D-glucitol-1,6-bis-P	0		0.59 ± 0.02		
D-psicose-1,6-bis-P	0		0.88 ± 0.11		
D-tagatose-1,6-bis-P	0		0.82 ± 0.05		
L-sorbose-1,6-bis-P	0		0.46 ± 0.19		
2,3-*O*-isopropylidene-α-L-sorbofuranose-1,6-bis-P	0		1.98 ± 0.13		
1,2-ethanediol-bis-P	0		1.41 ± 0.06		
1,3-propanediol-bis-P	0		1.62 ± 0.08		
1,4-butanediol-bis-P	0		0.84 ± 0.03		
1,5-pentanediol-bis-P	0		0.62 ± 0.02		
1,6-hexanediol-bis-P	0		0.21 ± 0.01		
1,7-heptanediol-bis-P	0		0.25 ± 0.01		
1,8-octanediol-bis-P	0		0.26 ± 0.01		
1,9-nonanediol-bis-P	0		0.45 ± 0.02		
diethylene glycol-bis-P	0		0.59 ± 0.02		
triethylene glycol-bis-P	0		1.28 ± 0.06		
D-hexitol-bis-P	0		3.67 ± 0.53		

[a] At pH 8 and 25 °C. [b] Peak heights at the pyrophosphate resonance position relative to that obtained with FBP as reactant (set at 100%) after 2-h reaction time. [c] From spectrophotometric assays (see Materials and Methods).

Table IV: Sugar Specificity for Plant PP_i-PFK in the Reverse Reaction Direction[a]

compound	^{31}P NMR[b]	K_m (mM)[c]	K_{is} (mM)[c]	V (μmol min^{-1})[c]	V/K
D-fructose-1,6-bis-P	100	0.010 ± 0.002		0.0110 ± 0.0010	1.1
D-sedoheptulose-1,7-bis-P	10	0.197 ± 0.008		0.0014 ± 0.0005	0.0071
2,5-anhydro-D-mannitol-1,6-bis-P	7	0.128 ± 0.004		0.0007 ± 0.0020	0.0055
2,5-anhydro-D-glucitol-1,6-bis-P	0		1.21 ± 0.10		
D-psicose-1,6-bis-P	0		1.58 ± 0.04		
D-tagatose-1,6-bis-P	0		1.31 ± 0.10		
L-sorbose-1,6-bis-P	0		0.52 ± 0.01		
2,3-*O*-isopropylidene-α-L-sorbofuranose-1,6-bis-P	0		2.65 ± 0.11		
1,2-ethanediol-bis-P	0		1.75 ± 0.57		
1,3-propanediol-bis-P	0		3.51 ± 0.72		
1,4-butanediol-bis-P	0		4.37 ± 0.42		
1,5-pentanediol-bis-P	0		3.43 ± 0.34		
1,6-hexanediol-bis-P	0		1.36 ± 0.09		
1,7-heptanediol-bis-P	0		1.03 ± 0.04		
1,8-octanediol-bis-P	0		0.65 ± 0.04		
1,9-nonanediol-bis-P	0		0.96 ± 0.04		
diethylene glycol-bis-P	0		1.83 ± 0.04		
triethylene glycol-bis-P	0		2.77 ± 0.12		
D-hexitol-bis-P	0		6.41 ± 0.28		

[a] At pH 8 and 25 °C. [b] Peak heights at the pyrophosphate resonance position relative to that obtained with FBP (set at 100%) after 2-h reaction time. [c] From spectrophotometric assays (see Materials and Methods) in the presence of 1 μM F2,6P.

compared to F6P (Tables I and II). In addition, neither enzyme tolerates the pyranose form of sugar phosphates as judged by G6P (Tables I and II), but the bacterial enzyme does, at least, tolerate the open-chain form reasonably well, as indicated by diethylene glycol monophosphate, 1,6-hexanediol monophosphate, and D-glucitol-6-P (Table I). All open-chain forms are bound less tightly by the plant enzyme (Table II). Somewhat surprisingly, arabinose-5-P, which is F6P minus the hydroxymethyl group at C-1, is also bound very poorly, as is 5-ketofructose-P (Tables I and II). None of the analogues tested bind as well as F6P. [In this case $K_{F6P} = K_{i\,F6P}$, since the mechanism is rapid equilibrium as shown by Bertagnolli and Cook (1984).]

In the direction of phosphorylation of inorganic phosphate, the story is qualitatively identical as shown in Tables III and IV. Again, none of the analogues tested bind as well as FBP. A number of 1,*n*-alkanediol bisphosphates were also tested (Tables III and IV). These data are plotted in Figure 2 as K_i vs. carbon chain length. As can be seen, all analogues bind more tightly to the bacterial enzyme than to the plant enzyme. In addition, the bacterial enzyme accommodates the alkanediol bisphosphate with 6, 7, and 8 carbons equally well while the plant enzyme appears to be more selective, binding the octanediol bisphosphate with highest affinity.

Discussion

For the bacterial enzyme, F6P is 10-fold better as a substrate than S7P, which is about 3.5-fold better than 2,5-anhydro-D-mannitol-6-P as suggested by the V/K values listed in Table I. Similarly, FBP is about 10-fold better than SBP, but there is little discrimination between SBP and the anhydromannitol bisphosphate, with SBP being favored by about 40%. Results for the mung bean enzyme are similar to those obtained for the bacterial enzyme, with F6P better by 30-fold than S7P, which is 4-fold better than anhydromannitol-6-P. The discrimination is even more pronounced in the reverse reaction, with FBP better by 150-fold than SBP. Again, little discrimination is observed between SBP and the anhydromannitol bisphosphate. Thus, increasing the chain length and replacing the hydroxyl group at C-1 with a proton have a dramatic effect on activity. This will be discussed in more detail below.

All of the above-mentioned cyclic compounds exist in solution with the furanose ring form as the predominant tautomeric configuration (Figure 3). The D-glucose-6-P that exists almost entirely as the pyranose ring form (38% α and

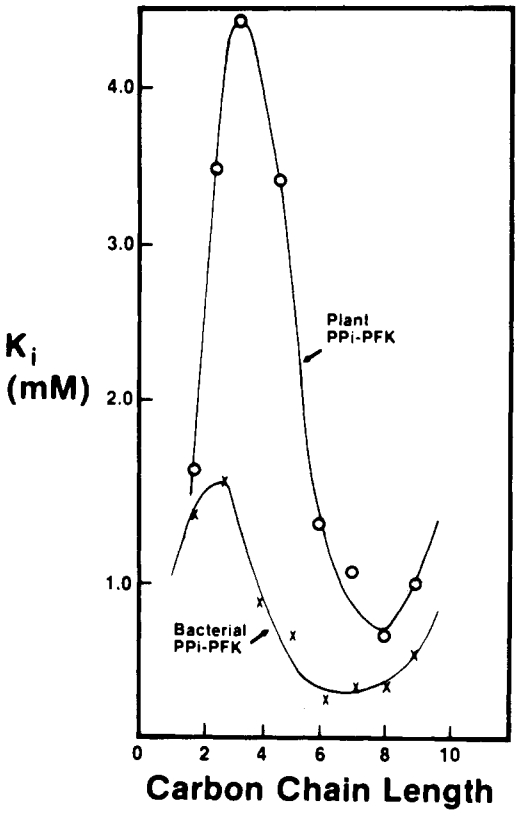

FIGURE 2: Comparative study of the binding of a series of alkanediol bisphosphates (1,2-ethanediol-bis-P through 1,9-nonanediol-bis-P; see Tables III and IV) to the active site of bacterial and plant PP_i-PFKs. The K_i values were calculated from competitive inhibition patterns vs. FBP at saturating P_i and Mg^{2+}, pH 8, and 25 °C.

62% β; Rose, 1975) has the lowest affinity for either PP_i-PFK. Although yeast hexokinase will phosphorylate the α or β anomer of 5-ketofructose (Benkovic, 1979) at the C-6 position (Crane, 1962), Blanchard et al. (1982) proposed that the product is 5-ketofructose-6-P existing in solution as 95% β-pyranose and 2% β-furanose. Neither PP_i-PFK employed in this study appeared to utilize the phosphorylated 5-ketofructose compound prepared by using yeast hexokinase.

Utilization of the locked β analogue, 2,5-anhydro-D-mannitol-6-P, but not the α analogue, 2,5-anhydro-D-glucitol-6-P, by both the bacterial and plant PP_i-PFKs demonstrates an absolute requirement for the β-anomeric configuration at C-2. This is also apparently true for the rabbit muscle ATP-PFK (Koerner et al., 1974) and the PP_i-PFK from *E. histolytica* (Koerner et al., 1977). It is interesting that, in the reverse reaction direction, the maximum distance between the two phosphate groups of 2,5-anhydro-D-mannitol 1,6-bisphosphate and 2,5-anhydro-D-glucitol 1,6-bisphosphate as measured on Dreiding models is 10.2 and 10.0 Å, respectively (Hartman & Barker, 1965). Since the former but not the latter is utilized by both enzymes in this study, the 0.2-Å difference in distance between the phosphorus atoms is not as important for substrate activity as is the interaction of enzyme with substrate and the anomeric configuration at C-2. It should be mentioned that, in addition to the anomeric configuration at C-2, the chemical nature of the C-1 group is also very important for catalysis, as suggested by the failure of D-arabinose-5-P, which has a hydroxyl in place of the CH_2OH at C-1, to serve as a substrate for either PP_i-PFK. These observations are also in agreement with the fact that rat or rabbit muscle ATP-PFK will not utilize either α- or β-D-arabinose-5-P as a substrate (Koerner et al., 1974; Maryanoff et al., 1984). D-Arabinose-5-P does inhibit both PP_i-PFKs, although the K_i values, especially for the plant enzyme, are significantly larger than those reported for ATP-PFK (Koerner et al., 1974), indicating that these enzymes are even more strict in their requirements for the $-CH_2OH$ at C-1.

The K_m (also K_i for these enzymes as mentioned above) values for the locked β analogue 2,5-anhydro-D-mannitol-6-P (and its respective bisphosphate in the reverse reaction) as well as the K_i values for the locked α analogue 2,5-anhydro-D-glucitol-6-P (and its bisphosphate) agree fairly well (within a factor of ~2–5) with those published for ATP-PFK (Koerner et al., 1974; Riquelme et al., 1984) and for PP_i-PFK from *E. histolytica* (Koerner et al., 1977). In all cases, however, the K_m values of the analogues and their bis-

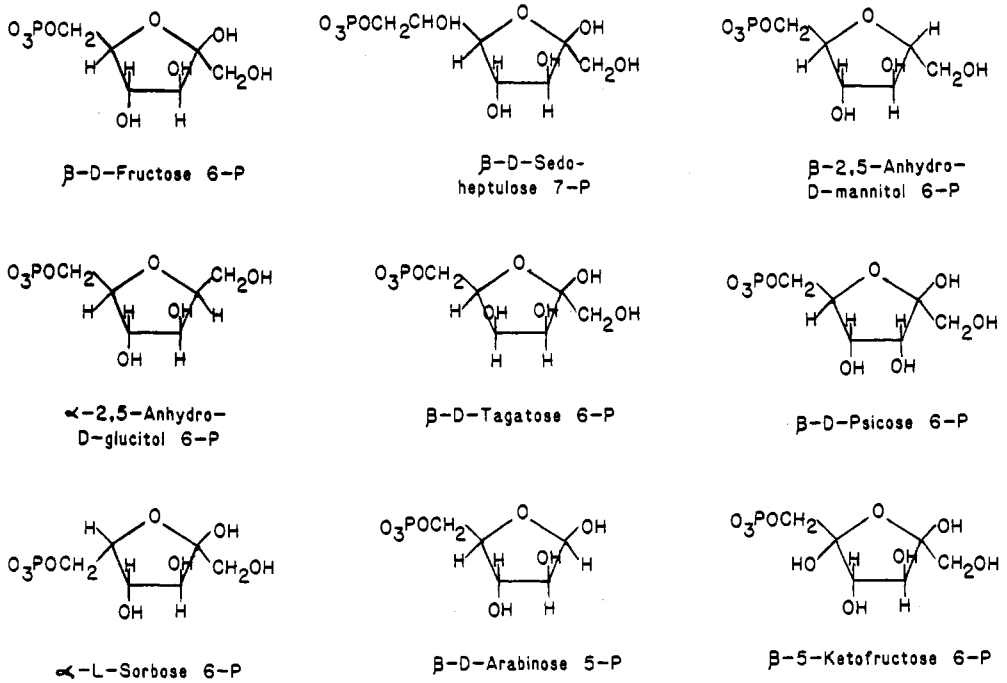

FIGURE 3: Structure of the furanose form of the analogues of F6P.

phosphates are significantly larger than those for F6P and FBP, respectively. Since the only difference between 2,5-anhydro-D-mannitol-6-P and F6P is that the hydroxyl group at C-2 is replaced by a hydrogen in the locked β analogue, the OH at C-2 is not essential for either binding or catalysis with this analogue. However, replacing the OH with H has a dramatic effect on catalysis (8–30-fold) in the direction of F6P phosphorylation with less effect on binding (4-fold). The effect on binding becomes more apparent in the reverse reaction (6–12-fold). The decreased effect on catalysis observed in the reverse reaction is most likely a result of the additional phosphate binding site serving to help align the molecule for phosphoryl transfer, but the effect on catalysis is not completely eliminated, with 2- and 16-fold decreases observed for bacterial and plant enzymes. Thus, PP_i-PFK requires an OH at C-2 for tightest binding and optimum catalysis. This observation is quite distinct from published reports for the ATP-PFK where V_{max} is little affected by replacement of the OH at C-2 by a H (Koerner et al., 1974; Benkovic, 1979). With the 2,5-anhydroglucitol-6-P analogue, very little binding affinity is lost for the bacterial enzyme, suggesting that the hydroxyl at C-1 may be able to form a H-bond with the enzyme group that normally hydrogen bonds the C-2 hydroxyl. The plant enzyme, on the other hand, appears to be much more stringent in its requirement for the hydroxyl groups at C-1 and C-2. Thus, from the above, it would appear that both bacterial and plant enzymes are specific for the β-D-furanose form of the sugar substrates.

At the C-3 position of the D-fructofuranose ring of F6P the hydroxyl group is in the *S* configuration. The C-3 epimer, D-psicose-6-P, in which the hydroxyl group at C-3 is in the *R* configuration, exists in solution as approximately 80% α anomer (Koerner et al., 1980). This analogue and its respective bisphosphate are not utilized at detectable levels by either the bacterial or plant PP_i-PFK in either reaction direction although they are bound loosely as inhibitors by both enzymes. However, rabbit muscle ATP-PFK will utilize this epimer at a V_{max} ~50% that of the F6P with a relatively high K_m of 3 mM (Koerner et al., 1976). Clearly, there is a major difference between PP_i-PFK and ATP-PFK in that the former requires the *S* configuration of the C-3 hydroxyl for proper binding to enzyme while the latter enzyme is less restrictive and will utilize compounds with either the *R* or *S* configurations of the C-3 hydroxyl, although the *S* configuration is preferred.

The C-4 epimer, D-tagatose-6-P, in which the hydroxyl at C-4 is in the *R* configuration, exists in solution as ~80% β-anomeric species (Fishbein et al., 1974; Koerner et al., 1980). This epimer and its respective bisphosphate are also not utilized by either PP_i-PFK but inhibit with low affinity. Mammalian ATP-PFKs will, however, utilize this epimer with a V_{max} and a K_m comparable to those for F6P (Totten & Lardy, 1949; Fishbein et al., 1974; Koerner et al., 1976). These bindings suggest that, although the ATP-PFK does not interact with the C-4 hydroxyl, both the bacterial and plant PP_i-PFKs have a strict steric requirement for the D-threo (trans) configuration of the hydroxyls at C-3 and C-4 for proper binding and catalysis. Interestingly, a recent X-ray analysis of the sodium salt of FBP shows the furanose ring in a conformation with the carbon at C-3 displaced 0.58 Å down from the plane of the ring (Narendra et al., 1985). This would cause a different positioning of the hydroxyls at C-3 and C-4.

The C-5 epimer, L-sorbose-6-P, exists in solution as ~80% α-anomeric species (Koerner et al., 1980). The rabbit muscle ATP-PFK utilizes this C-5 epimer, although the V_{max} is only 15% of that with F6P and the K_m is very high (11 mM) compared to that for F6P (Koerner et al., 1976). Again, neither the bacterial nor plant PP_i-PFK appeared to utilize this epimer or its respective bisphosphate, indicating a strict requirement for the D configuration. Both PP_i-PFKs will, however, bind these epimers loosely as competitive inhibitors vs. F6P. In addition to the configuration at C-5, the nature of the substituent groups is also very important. The analogue 5-keto-D-fructose-6-P, which differs from D-fructose-6-P in that it has a hydroxyl in place of the hydrogen at C-5, is a substrate for ATP-PFK with a V_{max} ~25% of that with F6P and a K_m about the same as that for F6P (Avigad & Englard, 1974). However, neither the bacterial nor plant PP_i-PFK uses this analogue at detectable levels. The contention that PP_i-PFKs will not tolerate hydroxyl substitution for the hydrogen at C-5 but ATP-PFKs will is supported by the observations that rabbit muscle ATP-PFK will utilize D-fructose-1-P, although with a low V_{max} compared with D-fructose-6-P (Uyeda, 1972), while PP_i-PFKs from several sources will not utilize D-fructose-1-P (see introduction). Neither PP_i-PFK tolerates the existence of large bulky groups at C-2 and C-3 as evidenced by a 4–5-fold increase in the K_i with 2,3-*O*-isopropylidene-α-L-sorbofuranose-1,6-bis-P compared with L-sorbose-1,6-bis-P.

At the C-6 position it is evident from the data presented in Tables I–IV for D-sedoheptulose-7-P and its respective bisphosphate that extension of the phosphate group by adding a hydroxymethylene group does not limit the ability of either PP_i-PFK to utilize this compound to an appreciable extent in both reaction directions.

Interestingly, it has been reported for rabbit muscle ATP-PFK that acyclic compounds like D-mannitol-1-P, D-glucitol-6-P, and D-xylulose-5-P, which are analogues of the linear keto and hydrated keto forms of F6P, are neither substrates nor inhibitors (Koerner et al., 1974). However, all of the acyclic compounds tested in this study do compete with F6P or FBP for either enzyme. This is illustrated in Figure 2 for the alkanediol bisphosphate series C_nP_2 (n = 2–9) in the reverse reaction direction where analogues compete with FBP. In fact, the tightest binding of these compounds binds as tight if not tighter than any of the cyclic or acyclic analogues tested. A study similar to the one reported here has been carried out with FBP aldolase (Hartman & Barker, 1965). In that study the lowest K_i value for the alkanediol series was obtained for the C_6P_2 compound. Hartman and Barker (1965) reported the maximum distances between the phosphorus atoms as measured on Dreiding models as follows: ethylene glycol bisphosphate, 7 Å; C_3P_2, 8.2 Å; C_4P_2, 9.4 Å; 2,5-anhydro-D-glucitol 1,6-bisphosphate, 10 Å; 2,5-anhydro-D-mannitol 1,6-bisphosphate, 10.2 Å; C_5P_2, 10.6 Å; C_6P_2, 11.8 Å; C_8P_2, 14.2 Å. The maximum distance between the phosphorus atoms for the substrate 2,5-anhydro-D-mannitol 1,6-bisphosphate is 10.2 Å (a value probably very close to that for FBP), and the maximum distances for the tightest binding compounds of the alkanediol bisphosphate series given in Figure 2 for the PP_i-PFKs fall in the 11.8–14.2-Å range. This suggests that although the acyclic compounds could bind to the active site of the PP_i-PFKs in a slightly "puckered" (i.e., bent) shape to achieve an approximately 10-Å separation between the phosphorus atoms for relatively tight binding, the acyclic compounds would still lack the β-D-furanose ring system necessary for proper orientation. From the sequence-data and active-site studies, Hellinga and Evans (1985) report that several ATP-PFKs contain six residues binding the substrate F6P with two arginines and one histidine exclusively binding the 6-phosphate. Although the amino acid sequence and

three-dimensional structures for any PP_i-PFK have not been reported to our knowledge, we would predict from the data gathered in this report a more extensive number of interactions, particularly at the C-2, C-3, C-4, and C-5 positions around the D-furanose ring, than those that have been found for the ATP-PFK.

ACKNOWLEDGMENTS

We thank Rita Huber and Wanda Ford for typing the manuscript and Matt Vrzal for his technical assistance.

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