Substrate Form of D-Fructose 1,6-Bisphosphate Utilized by Fructose 1,6-Bisphosphatase†

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ABSTRACT: Rapid quench kinetic experiments on fructose 1,6-bisphosphatase demonstrate a stereospecificity for the α anomer of fructose 1,6-bisphosphate relative to the β configuration. The β anomer is only utilized after mutarotation to the α form in a process that is not enzyme catalyzed. Studies employing analogues of the acyclic keto configuration indicate that the keto form is utilized at a rate less than 5% that of the α anomer, a finding also confirmed by computer simulation of the rapid quench data. Chemical trapping experiments of the keto analogue, xylulose 1,5-bisphosphate, and the normal substrate suggest that interconversion of the acyclic and anomeric configurations is retarded by their binding to the enzyme. A hypothesis is advanced attributing substrate inhibition of fructose 1,6-bisphosphatase to possible binding of the keto species.

D-Fructose 1,6-bisphosphate (fru-1,6-P_2) exists in solution as an equilibrium mixture of α- and β-cyclic furanose anomers, an acyclic keto species, and a hydrated keto (*gem*-diol) form. Literature values based on natural abundance ^{13}C NMR[1] measurements indicated an α to β distribution of approximately 20:80 (Benkovic et al., 1972; Koerner et al., 1973). More recently, values for the various forms were determined by Rose and co-workers (Midelfort et al., 1976) again using ^{13}C NMR with ^{13}C-enriched fru-1,6-P_2. The reported distribution of fru-1,6-P_2 is 15% α anomer, 81% β anomer, 2.0% acyclic keto, and 1.3% *gem*-diol at pH 7.2 and 25 °C.

A knowledge of the configuration of fru-1,6-P_2 utilized by the gluconeogenic enzyme fructose 1,6-bisphosphatase (FBPase) is necessary for an understanding of the mechanism of enzymatic catalysis. Rapid quench kinetic experiments have shown that, for the alkaline form of rabbit liver FBPase, the α anomer of fru-1,6-P_2 is utilized with a rate coefficient 5- to 10-fold greater than for the β anomer (Benkovic et al., 1974a). A series of comparable rapid quench experiments is reported here for the neutral rabbit liver enzyme. The possible involvement of the acyclic keto form in substrate hydrolysis has also been examined by employing various acyclic substrate analogues, including D-xylulose 1,5-bisphosphate (xylu-1,5-P_2), 5-deoxyfructose 1,6-bisphosphate (5-d-fru-1,6-P_2), and dihydroxyacetone phosphate (DHAP) in the presence and absence of glycerol phosphate. Experiments designed to demonstrate the presence of a FBPase–keto sugar complex by reductive trapping with borohydride are also reported.

Experimental Procedures

Materials

Fru-1,6-P_2, fru-6-P, α-GP, β-GP, DHAP, NAD^+, NADH, $NADP^+$, aldolase, glucose-6-P isomerase, glucose-6-P dehydrogenase, α-glycerophosphate dehydrogenase, and triose phosphate isomerase were purchased from Sigma Chemical Co. Glycolaldehyde-P diethyl acetal, as the dicyclohexylammonium salt, was obtained from Calbiochem, La Jolla, Calif. 5-d-Fru-1,6-P_2 was kindly furnished by I. A. Rose. Chelex-100 from Bio-Rad Co. was treated according to the supplier's procedure prior to use. Instabray scintillation cocktail was obtained from Yorktown Research Co. Buffers and inorganic salts were reagent grade. $NaBH_4$ (Alfa Inorganic) was weighed and dissolved at 0 °C in distilled water or in triethanolamine–diethanolamine buffer (pH 9.2) to give a 1 or 0.1 M solution. Aliquots were added to reaction mixtures within 5 min of dissolution of the solid.

Carrier-free [^{32}P]H_3PO_4 (5 mCi) obtained from New England Nuclear was used with commercially available enzymes and substrates to prepare [γ-^{32}P]ATP by a technique adapted from the method of Keenan et al. (1972). Purified [γ-^{32}P]ATP and fru-6-P were reacted with phosphofructokinase to yield [1-^{32}P]fru-1,6-P_2 which was purified on Dowex 1-X4 chloride (Bartlett, 1959).

Methods

[1-^{32}P]Xylu-1,5-P_2 Synthesis. The reaction mixture (16 mL) contained 25 mM triethanolamine (pH 7.0), 24 mM glycolaldehyde-P, 0.0185 mM [1-^{32}P]fru-1,6-P_2, 2400 units of triosephosphate isomerase, and 100 units of aldolase. The reaction enzymes were dialyzed prior to use against 25 mM triethanolamine (pH 7.0). The mixture was incubated for 2 h at room temperature (25 °C) and applied directly to a 1.2 × 12 cm column of Dowex 1-X4 formate (100–200 mesh). Elution of unreacted monophosphates and [1-^{32}P]xylu-1,5-P_2 was accomplished using 0.2 and 0.6 M ammonium formate (pH 6.45), respectively. The purified [1-^{32}P]xylu-1,5-P_2 was lyophilized to remove the ammonium formate, dissolved in doubly distilled, deionized water, and adjusted to pH 7.0. The analogue concentration was determined spectrophotometrically at 340 nm by aldolase cleavage to glycolaldehyde-P and DHAP (Mehler and Cusic, 1967) and subsequent decrease of NADH in the presence of α-glycerophosphate dehydrogenase. The assay (1 mL) contained 50 mM Tris (pH 7.5), 0.1 mM NADH, 0.2 unit of aldolase, 8 units of α-glycerophosphate dehydrogenase, and [1-^{32}P]xylu-1,5-P_2. The lack of fru-1,6-P_2 contamination in the synthesized substrate analogue was

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[1] Abbreviations used: fru-1,6-P_2, D-fructose 1,6-bisphosphate; fru-6-P, D-fructose 6-phosphate; xylu-1,5-P_2, D-xylulose 1,5-bisphosphate; 5-d-fru-1,6-P_2, 5-deoxyfructose 1,6-bisphosphate; α-GP, α-glycerol phosphate; β-GP, β-glycerol phosphate; DHAP, dihydroxyacetone phosphate; P_i, inorganic phosphate; FBPase, fructose 1,6-bisphosphatase; NMR, nuclear magnetic resonance.

shown by the assay described below for fructose 1,6-bisphosphatase activity. Xylu-1,5-P_2 was added in place of the natural substrate and the change in absorbance observed indicated <1% fru-1,6-P_2.

FBPase. Neutral rabbit liver fructose 1,6-bisphosphatase was purified from the frozen livers (Pel-Freez Biologicals Inc.) of young, 24-h-fasted rabbits by the procedure of Ulm et al. (1975) as modified by Benkovic et al. (1974b). One sharp protein band was observed by both disc and sodium dodecyl sulfate gel electrophoresis performed as previously described (Benkovic et al., 1974b). The purified enzyme was routinely concentrated to 8–15 mg/mL for storage in liquid N_2. All enzyme preparations were passed through a column of Chelex-100 resin to remove contaminating metal ions. Protein concentrations were determined on the basis of the standard absorbance of a 1.0 mg/mL solution of 0.71. The enzyme was assayed spectrophotometrically by following the rate of NADPH accumulation at 340 nm in the presence of excess glucose 6-P isomerase and glucose 6-P dehydrogenase. The routine assay mixture (1 mL) for neutral activity at 25 °C contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM $NADP^+$, FBPase, 7.6 units of glucose 6-P isomerase, 1.7 units of glucose 6-P dehydrogenase, and 0.1 mM fru-1,6-P_2. Purified FBPase has a specific activity of 11 units/mg in the above assay (4 units/mg using 0.1 mM $MnCl_2$ and no EDTA).

Xylu-1,5-P_2 as a Substrate. The hydrolysis of xylu-1,5-P_2 by FBPase was measured by following the release of [^{32}P]-H_3PO_4 from the radioactively labeled substrate. The assay mixture (0.5 mL) for activity at 25 °C contained 50 mM Tris-HCl (pH 7.5), 0.1 mM $MnCl_2$, 0.22 μM FBPase, and 0.5-66 μM [1-^{32}P]xylu-1,5-P_2. Aliquots (0.1 mL) were removed at various times and quenched by the addition of 0.3 mL of 10% $HClO_4$. The extent of reaction at each time was determined by addition of a portion of the acid-quenched reaction mixture to a 0.6 mL total volume two phase extraction mixture prepared as described below. Aliquots from each phase were removed and counted in 10 mL of Instabray scintillation cocktail.

Xylu-1,5-P_2 as an Inhibitor. The effect of xylu-1,5-P_2 on the ability of FBPase to catalyze the hydrolysis of its natural substrate was studied in the spectrophotometric assay used for enzyme activity. The assay mixture (1 mL) for measurement of activity at 25 °C contained 50 mM Tris-HCl (pH 7.5), 0.1 mM $MnCl_2$, 0.01–0.5 unit of FBPase, 0.2 mM $NADP^+$, 15 units of glucose-6-P isomerase, 3 units of glucose 6-P dehydrogenase, 0.08–33 μM xylu-1,5-P_2, and 0.005–2.0 mM fru-1,6-P_2.

Extraction Analysis for [^{32}P]H_3PO_4. Inorganic phosphate was separated from sugar phosphates by an adaptation of the Martin and Doty procedure (1949) as modified by Jencks and Gilchrist (1964). Assay tubes contained six parts of a 1:1 by volume isobutyl alcohol–benzene solution saturated with water, five parts of water, and one part of a solution made from 2 g of ammonium molybdate, 5.55 mL of concentrated H_2SO_4, and water to make 100 mL. A measured aliquot of the quenched reaction mixture was added to the assay tube whose contents were agitated vigorously for two separate intervals of 30 s. Centrifugation at low speed of the assay tube ensured clean separation of the two layers. Aliquots from each layer were removed and ^{32}P activity was measured with a Packard TriCarb liquid scintillation spectrometer. Radioactivity in the organic phase compared with that in the aqueous phase yielded the relative distribution of ^{32}P as H_3PO_4 and organic phosphate which is acid stable at room temperature. Corrections were made for variation in the volume of the aqueous reaction mixture added to the two layer assay solution.

β-GP, DHAP, and 5-d-Fru-1,6-P_2 Assays. The hydrolysis of β-GP was determined by measuring P_i release (Fiske and Subbarow, 1925). The hydrolysis of DHAP was followed by determining the amount of unreacted DHAP in the presence of α-glycerophosphate dehydrogenase. Unreacted 5-d-fru-1,6-P_2 was determined in a coupled enzymatic assay using aldolase, α-glycerophosphate dehydrogenase, and triosephosphate isomerase, all in excess. Aliquots were removed at given times from assay mixtures containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 5–50 mM β-GP or 1 mM DHAP or 74 μM 5-d-fru-1,6-P_2, and FBPase (0.01–0.6 unit).

$NaBH_4$ Trapping. [1-^{32}P]Fru-1,6-P_2 and [1-^{32}P]xylu-1,5-P_2 were reduced by $NaBH_4$ in the presence and absence of FBPase. In the fru-1,6-P_2 reduction, the reaction mixture contained 10 μM fru-1,6-P_2, 5.5 μM FBPase (mol wt 143 000), and 20 mM $NaBH_4$ in 80 mM triethanolamine–diethanolamine (pH 9.2) and a drop of octanol to minimize bubbling. Aliquots (0.06 mL) were removed from two reaction mixtures, one with and one without enzyme, at various intervals up to 8 min after $NaBH_4$ addition. The aliquots were added directly to ampoules containing 0.015 mL of 12 N HCl, sealed, and heated 30 min in a steam bath. The ampoules were cooled, centrifuged at low speed, and opened. The entire sample was removed and added to 0.6 mL of the two phase extraction mixture described above. An analogous procedure was used for xylu-1,5-P_2 at a concentration of 4.5 μM with FBPase at 13, 26, and 85 μM. With this amount of FBPase, it was essential that *all* precipitated protein be removed from the acidified aliquot before the extraction analysis for $^{32}P_i$. Control experiments had indicated that P_i was obtained quantitatively from the 1-phosphate of both keto sugar derivatives under the above conditions whereas the reduced hexitol- and pentitol-P_2 were unaffected. A parallel incubation of 7–28 nM FBPase under identical conditions in the presence of $NaBH_4$ followed by assay for activity demonstrated no loss in activity.

Rapid Quench Experiments. The rapid quench kinetic experiments utilized a Durrum Multi-Mixer apparatus as modified in this laboratory and previously published techniques (Benkovic et al., 1974a). Runs were performed at 25 °C by mixing equal volumes of enzyme–$MnCl_2$ and fru-1,6-P_2–$MnCl_2$ solutions followed by rapid quenching with a double volume of 10% $HClO_4$. The reaction time points were varied randomly for each enzyme concentration to guard against system-generated errors. The reaction mixture for each time point contained 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 0.4–8 μM FBPase, and 9–85 μM fru-1,6-P_2. The quench reaction mixture, maintained at 0 °C, was centrifuged to remove the precipitated protein. The extent of reaction for each point was then determined by measuring the ratio of [^{32}P]H_3PO_4 to [1-^{32}P]fru-1,6-P_2 via the extraction assay already described.

Results

Rapid Quench Kinetic Experiments. Figure 1 is a representative plot of mole fraction fru-6-P (f_t) produced vs. time. Over a 20-fold range of enzyme activities at fru-1,6-P_2/FBPase ratios of 10–55, a biphasic plot is observed with an initial rapid formation of product followed by a second slower rate. Extrapolation of the second phase to the ordinate for the ten experiments yielded an average intercept of 0.18 ± 0.05. The calculated rate coefficient for the second phase is independent

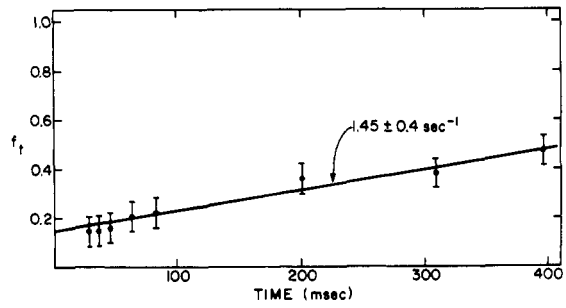


FIGURE 1: Representative plot of fru-6-P formation from fru-1,6-P_2 in the rapid quench experiments ([FBPase] = 0.72 μM; [fru-1,6-P_2] = 9.65 μM). The ordinate, f_t, is the mole fraction of fru-6-P at each experimental time point. The linear portion of the plot is based on a least-squares fit of the data.

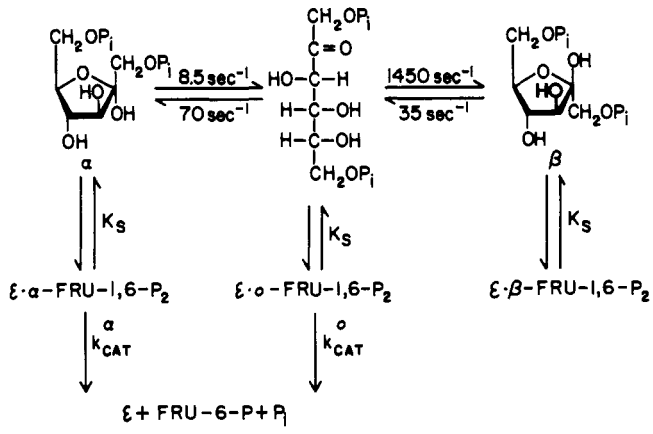


FIGURE 2: The general model for utilization by fructose 1,6-bisphosphatase of the α and keto isomeric forms of fru-1,6-P_2 where k_{cat}^{α} and k_{cat}^{o} represent simulated catalytic rate constants for dephosphorylation of the α and open chain (keto) forms. A single value for $K_S \simeq 0.050$ μM was employed for the dissociation of the enzyme-substrate complexes.

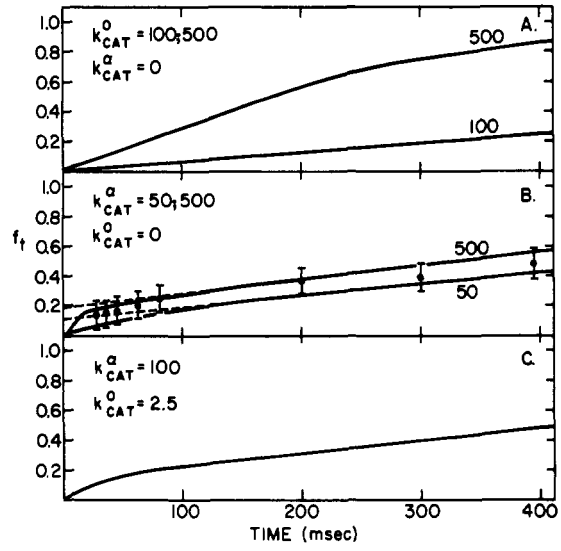


FIGURE 3: Plots of f_t vs. time calculated using the scheme of Figure 2 and assuming keto, α, and both keto and α utilization by fructose 1,6-bisphosphatase (A, B, and C, respectively) at various values for k_{cat}. The solid lines shown are computer generated at the indicated values for k_{cat}. The experimental points from the representative plot of Figure 1 are shown in B.

of enzyme concentration with an average value of 1.45 ± 0.4 s^{-1}.

Computer Simulations. In order to simulate the expected rapid quench results based on enzymatic utilization of various isomeric forms, a series of rapid equilibrium models was generated using the Chemical Reaction Analysis and Modeling System (CRAMS) program previously described (Fishbein et al., 1974). The general scheme shown in Figure 2 was employed. The rate constants for mutarotation are those reported by Rose and co-workers (Midelfort et al., 1976) for the ring-opening and ring-closing rates of the α, β, and keto forms. It is assumed in these simulations that the distribution of the enzyme-substrate complexes of the α, β, and keto forms is identical with the anomeric composition of the free sugar phosphate. The concentrations employed for FBPase and fru-1,6-P_2 were identical with those used in one of the rapid quench experiments. The values used for k_{cat}^{o} and k_{cat}^{α} were varied and the resulting data were plotted as mole fraction of fru-6-P (f_t) vs. time. The results of these simulations are presented in Figure 3. Figure 3A shows that, setting $k_{cat}^{\alpha} = 0$ and varying k_{cat}^{o}, the observed f_t data do not yield a biphasic plot at low values of f_t. With $k_{cat}^{o} = 0$ and different assumed values for k_{cat}^{α} as illustrated in Figure 3B, the f_t plot is biphasic and, depending on the chosen value for k_{cat}^{α}, intercepts at an f_t value of 0.12–0.2. The upper plot in Figure 3B represents a limiting case because further increases in k_{cat}^{α} do not significantly alter the results. Doubling the enzyme concentrations for these conditions does not change the observed rate constant for the second phase. Thus, calculation of the rate constant for a line drawn through the second phase of Figure 3B yields a value of 1.5 s^{-1} that is independent of enzyme concentration. A third set of conditions was also simulated where the enzyme utilized both the α and keto forms. When $k_{cat}^{o} \gg k_{cat}^{\alpha}$, a behavior similar to Figure 3A is observed. With $k_{cat}^{o} = k_{cat}^{\alpha}$, the results indicated that the f_t plot is not biphasic. With $k_{cat}^{o} \ll k_{cat}^{\alpha}$, however, Figure 3C shows the desired biphasic behavior with a value for the rate constant of the second phase of 1.5 s^{-1} that is independent of enzyme concentration. This result, as expected, is almost identical with Figure 3B where the α anomer was considered the only reactive anomer.

Substrate Analogues. Assays for xylu-1,5-P_2 activity indicated that the purified neutral enzyme catalyzed the cleavage of xylu-1,5-P_2 to xylu-5-P and P_i. Figure 4 shows a linear region (based on a least-squares fit) in the double-reciprocal plot of velocity vs. xylu-1,5-P_2 concentration and self-inhibition at concentrations >5 μM. Extrapolation of the linear portion yields a value of 0.018 mM for K_M and 0.074 units/mg for V_M. Essentially identical values were obtained by the alternate linearization plotting technique described by Cleland (1970).

The inhibition of FBPase activity toward fru-1,6-P_2 by xylu-1,5-P_2 was examined in two concentration regions, i.e., at concentrations greater than 10 μM where the latter substrate is self-inhibiting, and at less than 1 μM where it functions as a poor substrate. In the former range the percent inhibition is dependent only on the concentration of xylu-1,5-P_2 and independent of fru-1,6-P_2 concentration typical of noncompetitive inhibition; in the latter range, the percent inhibition at a given concentration of xylu-1,5-P_2 can be decreased by increasing concentrations of fru-1,6-P_2 in accord with competitive inhibition.[2]

Additional analogues of the acyclic keto configuration were

[2] The degree of inhibition is dependent on the order of addition of inhibitor and substrate precluding evaluation of K. Addition of xylu-1,5-P_2 prior to fru-1,6-P_2 leads to maximal inhibition; simultaneous addition of both materials reduces the degree of inhibition observed.

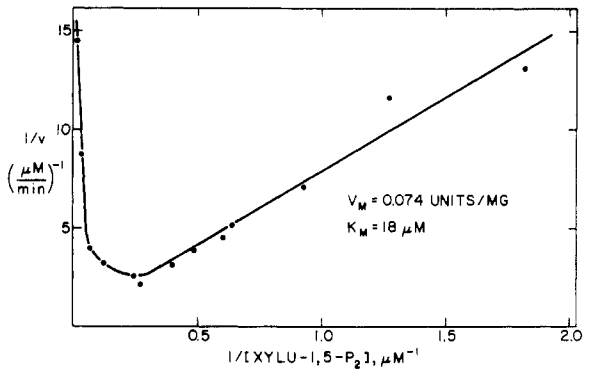


FIGURE 4: Plot of $1/v$ vs. $1/[\text{xylu-1,5-P}_2]$. The values for K_M and V_M were determined by extrapolation of the linear portion of the data as calculated from a least-squares fit.

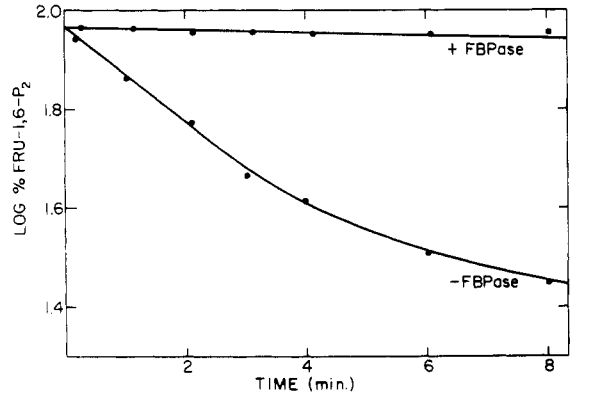


FIGURE 5: Reduction of fru-1,6-P_2 in the presence of FBPase. Fru-1,6-P_2 (10 μM) was mixed with 20 mM $NaBH_4$ at pH 9.2 with or without 5.5 μM FBPase.

tested for activity toward FBPase relative to the natural substrate at pH 7.5 (Table I). None proved to be effective substrates including β-glycerol phosphate which is hydrolyzed at a rate 23% that of fru-1,6-P_2 by the FBPase from bovine liver at pH 9.5 (Cohen et al., 1971). The combination of DHAP and α-GP was no more active than DHAP alone. The 5-d-fru-1,5-P_2 analogue is a competitive inhibitor of FBPase activity at 10–78 μM (plot not shown) with $K_i \simeq K_M$.

Trapping by $NaBH_4$. The rate of reduction of [1-^{32}P]fru-1,6-P_2 and [1-^{32}P]xylu-1,5-P_2 in the presence of borohydride was monitored by measuring acid-labile $^{32}P_i$ remaining as a function of time. Control experiments indicated that the 1-phosphate in the unreduced sugar bisphosphate could be quantitatively hydrolyzed in acid media presumably via the reactive acyclic keto species whereas the reduced sugar bisphosphate was stable to these conditions. The rate of reduction of fru-1,6-P_2 is proportional to borohydride concentration. At pH 9.2, the half-life for reduction of fru-1,6-P_2 (10 μM) in the presence of 20 mM $NaBH_4$ is ca. 3–4 min at 23 °C, whereas with xylu-1,5-P_2 (4.5 μM) the reduction is complete within less than 1 min. Typical data for the rate of reduction of fru-1,6-P_2 (10 μM) in the absence and presence of FBPase (5.5 μM) are shown in Figure 5 which illustrate that the enzyme completely protects the sugar bisphosphate from reduction by $NaBH_4$. In contrast the reduction of xylu-1,5-P_2 (4.5 μM) in the presence of FBPase (26.2 μM) is still complete within less than 1 min. Complete reduction after correction for the 33% of xylu-1,5-P_2 dephosphorylated by the enzyme is also observed in a reaction mixture (pH 9.2) containing $MnCl_2$ (0.4 mM) and FBPase (85 μM) preincubated for 10 min at 25 °C followed by simultaneous addition of xylu-1,5-P_2 (4.5 μM) and $NaBH_4$ (20 mM) and quenched with HCl after 0.5 min.

TABLE I: Relative Activities of FBPase toward Substrate Analogues.

Substrate analogue	Concn	% of Fru-1,6-P_2 act.[a]
D-Fructose 1,6-bisphosphate	0.1 mM	100
Dihydroxyacetone phosphate	1 mM	4.4
Dihydroxyacetone phosphate + α-glycerol phosphate	1 mM	3.5
β-Glycerol phosphate	5 mM	1.6
D-Xylulose 1,5-bisphosphate		1.9[b]
5-Deoxyfructose 1,6-bisphosphate	78 μM	0.7, 1.0[c]

[a] See Methods for assay conditions. [b] Based on the calculated V_M using 0.1 mM $MnCl_2$. [c] With 5 mM $MnCl_2$–40 mM glycine (pH 9.4).

Discussion

Previously published rapid quench data for alkaline FBPase indicated that about 20% of the available fru-1,6-P_2 is converted to product at a much faster rate than the remaining substrate (Benkovic et al., 1974a). The observed percentage corresponds to preferred utilization of the α anomer of fru-1,6-P_2. The conversion of the β anomer, however, was also catalyzed by the enzyme with a rate coefficient 5- to 10-fold less than that for the α anomer. Several possibilities to explain these results were proposed: (a) the enzyme catalyzes both the hydrolysis of α-fru-1,6-P_2 and conversion of β to the reactive α anomer, (b) the enzyme is relatively nonspecific and can act on both anomers, or (c) the enzyme catalyzes the ring opening of both α and β to the common keto intermediate prior to dephosphorylation. Alternatively, the apparent hydrolysis of the β anomer might be artifactual due to the presence of a nonspecific phosphatase.

The biphasic plot of Figure 1, with the average intercept of 0.18 ± 0.05, suggests that the neutral enzyme also prefers the α anomer. Unlike the alkaline enzyme, however, the rate coefficient for the second phase of the f_t plot is independent of enzyme concentration. The rate-limiting process during the second phase of the rapid quench experiment, therefore, is nonenzymatic in nature and would most likely involve the mutarotation of the remaining fru-1,6-P_2 to the catalytically reactive isomer(s). Although in principle it is possible that the proteolysis which results in alkaline enzyme also causes a loss in substrate specificity, we favor an explanation based on the presence of a contaminating phosphatase. The neutral FBPase employed is homogeneous by sodium dodecyl sulfate gel electrophoresis criterion (one sharp band), a fact that is more difficult to ascertain in the case of alkaline FBPase since the preparation gives rise to multiple bands. Using the published rate constants for the interconversion of the α, β, and keto forms (Midelfort et al., 1976), the scheme shown in Figure 2 can be used to simulate the expected behavior of the rapid quench results for utilization of the various isomers. The scheme does not consider the β anomer as a reactive isomeric form because the results of Figure 1, with the intercept of 0.18, clearly rule out this possibility.

The results shown in Figure 3B are consistent with a mechanistic picture where the α anomer is the major isomeric form utilized in the catalytic process. Values for k_{cat}^{α} from 50 to 500 s^{-1} were capable of simulating the experimental results

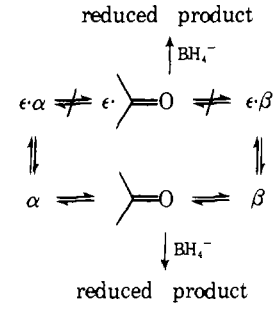
of Figure 1. Under the experimental conditions, the enzyme remains saturated with substrate throughout the course of data collection. Values of $K_S \simeq 0.050$ μM were employed in the simulation for all three substrate configurations based (1) on the kinetically determined K_M at 0.5 mM $MnCl_2$ (pH 7.5) for neutral FBPase;[3] (2) on previous studies with the competitive inhibitors α- and β-methyl D-fructofuranoside-1,6-P_2 which revealed that the calculated K_i's were virtually insensitive to the anomeric configuration and; (3) on the competitive inhibition observed with 5-d-fru-1,6-P_2 where $K_i \simeq K_M$. An increase in K_S as great as tenfold would not alter the simulated result. The k_{cat} value for FBPase determined from the steady-state assay under these conditions is 9.5 s^{-1}. This observed value, however, is only 0.12 times the lower value for k_{cat}^{α} suggesting a higher activity might be associated with the more concentrated enzyme, a possibility which we are currently investigating. Preferred utilization of the α anomer is also confirmed by the enzyme-independent rate constant for the second phase of Figure 1. The rate constant of 1.45 $\pm$ 0.4 s^{-1} is comparable to the reported overall rate constant for β to α mutarotation of 1.6 s^{-1} (Midelfort et al., 1976). The keto form may also be utilized, although at a rate less than 3% of that observed for the α anomer, in order to generate the experimentally determined rapid quench results. A better understanding of keto involvement in the enzymatic process can be obtained by considering the results for the substrate analogues.

Xylu-1,5-P_2 and 5-d-fru-1,6-P_2 are identical with the natural substrate with respect to the first four carbon atoms (refer to the open-chain structure of Figure 2). Because these analogues exist only in the acyclic keto or gemdiol forms, their behavior as potential substrates for FBPase was examined. The observed V_M for xylu-1,5-P_2 is only about 2% of that for fru-1,6-P_2; an equivalent comparison for 5-d-fru-1,6-P_2 indicates about 1% the activity of the normal substrate. Since the latter analogue has an equivalent carbon chain length to fru-1,6-P_2, the lowered activity of the former cannot be due entirely to a chain length factor. Although the shorter acyclic keto analogue, DHAP, exhibits slightly increased activity, its effectiveness as a substrate is not augmented by the addition of α-GP as a substitute for the missing three-carbon segment. The low activity of these analogues is similar to that observed with nonketo analogues, namely, β-GP (1.6%), phosphoenolpyruvate (2–4%, pH 7.2) (Kirtley and Dix, 1969), and p-nitrophenyl phosphate (4%, pH 9.2) (Chou and Kirtley, 1971). An interpretation of the higher activity (10%) of the acyclic analogue 5-keto-D-fru-1,6-P_2 (Avigad and Englard, 1974) is complicated by the possible existence of cyclic forms generated from the hydrate. However, both xylu-1,5-P_2 and 5-d-fru-1,6-P_2 are competitive inhibitors at concentrations <10 μM, suggesting, but not proving, that the keto analogues are bound, albeit nonproductively, at the active site. Their low activity markedly contrasts with their kinetic properties toward muscle aldolase where the V_M/K_M index is more favorable for the acyclic analogues than fru-1,6-P_2 itself (Mehler and Cusic, 1967; Midelfort et al., 1976). Consequently, utilization of the open chain does not appear to be a major contributor to the observed rate. These experiments, however, do not rule out the special situation where the enzyme catalyzes the α anomer and keto interconversion with the latter species then being inaccessible to exchange with the media.

[3] Justification for treating K_M values as a close approximation for the dissociation of the fru-1,6-P_2-FBPase-Mn^{2+} complex is discussed by Libby et al. (1975).

With the premise that the acyclic analogues are bound at the active site of FBPase, the data derived from the borohydride reductions give insight into whether the α,β anomers and keto form are in equilibrium on the enzyme. Consider the following scheme (Scheme I) for the trapping by borohydride

SCHEME I



where the reduction of the keto form takes place both on and off the enzyme. In the absence of enzyme, the pseudo-first-order rate constant for reduction of fru-1,6-P_2 is 3.1 $\pm$ 0.7 $\times$ 10^{-3} s^{-1} where [borohydride] is 20 mM. In the presence of FBPase under conditions where >95% of the substrate is bound,[4] the pseudo-first-order rate constant at 20 mM borohydride is ca. 1.2 $\times$ 10^{-4} s^{-1} or 26-fold slower. As expected the reduction of xylu-1,5-P_2 under identical conditions in the absence of FBPase is considerably faster since complete conversion to the pentitol-1,5-P_2 occurs within 1 min. One calculates that $t_{1/2}$ for the reaction should be about 4 s [(3.7 min $\times$ 2/100 $\times$ 60 s)] since fru-1,6-P_2 contains 2.0% keto isomer at room temperature. In the presence of FBPase under conditions where >90% of the substrate is bound,[5] no protection from reduction is observed even in the presence of $MnCl_2$ at times down to 30 s. If a similar decrease in the rate of xylu-1,5-P_2 reduction had occurred, then about 80% xylu-1,5-P_2 should remain at our first time point. It seems reasonable to conclude that, in so far as xylu-1,5-P_2 approximates the behavior of the keto form of fru-1,5-P_2 at the active site of FBPase, the latter is accessible to borohydride reduction when bound. The protection afforded by FBPase is, thus, interpreted in terms of a lack of interconversion in the absence of metal ions of the three configurations of fru-1,6-P_2 due to immobilization on the enzyme.[6]

The observation of noncompetitive inhibition by xylu-1,5-P_2 at >10 μM (5-d-fru-1,6-P_2 was not varied above 10 μM) offers an additional rationale for the substrate inhibition of FBPase generally observed at concentrations >1 mM. This latter inhibition cannot be caused by competitive binding of the β anomer at the active site since both the α and β anomers vary in a constant ratio as total fru-1,6-P_2 is changed.[7] Consequently the velocity curve will appear normal, although the kinetic constants are only apparent constants. However, if β or keto act noncompetitively, a maximum in the velocity curve will be

[4] Computed on the basis of K_M = 0.315 μM at 0.5 mM $MnCl_2$ (pH 9.2) adopted for the three species in Scheme I at 10 μM fru-1,6-P_2 and 5.5 μM FBPase (subunit concentration = 22 μM).

[5] Computed on the basis of K_M = 18 μM at 4.5 μM xylu-1,5-P_2 and 85 μM FBPase.

[6] Under conditions where [fru-1,6-P_2] $\approx$ [FBPase], the second phase in Figure 1 should be less than 1.45 s^{-1} owing to the presence of the bound noninterconverting β-anomer complex. This inference is presently being tested.

[7] Consequently the argument by Marcus (1976) that the α form of fru-1,6-P_2 is responsible for substrate inhibition of FBPase from bovine liver on the basis of competitive inhibition by α-substrate analogues also is erroneous.

observed (Segel, 1975). If the keto form of fru-1,6-P_2 is responsible for substrate inhibition, then, at total concentrations >1 mM, the concentration of the former would be approaching 20 μM. Its possible involvement thus is suggested by analogy to the kinetic behavior of xylu-1,5-P_2 at concentrations >10 μM, but other mechanisms, such as binding of the β or keto species at an allosteric site, remain plausible.

In summary, the FBPase from rabbit liver is highly stereospecific for the α anomer[8] and the hypothesis is advanced that both anomers and the keto form are bound but may not be rapidly interconverting on the enzyme even in the presence of a metal ion. The acyclic keto form, however, may function as a regulator of FBPase activity.

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[8] The conclusion of β specificity for the bovine enzyme is based on (1) the 20-fold lower K_i for 2,5-anhydro-D-mannitol-1,6-P_2 (an analogue of the β anomer of fru-1,6-P_2) compared with 2,5-anhydro-D-glucitol-1,6-P_2 (an α-analogue) and (2) the ability of this preparation of enzyme to hydrolyze 2,5-anhydro-D-mannitol-1,6-P_2 at 1.1% the rate of fru-1,6-P_2 at pH 9.0 (Marcus, 1976). No activity is observed at pH 6.5, however, nor at either pH for the α analogue. In view of the lower level of nonspecific substrate activity observed with FBPase from several sources, this would be tenuous evidence for such an assignment. Both analogues are competitive inhibitors of the neutral rabbit liver enzyme (Benkovic et al., 1971).