FRUCTOSE-1-PHOSPHATE-6-SULFATE AS AN ALTERNATIVE SUBSTRATE FOR ALDOLASE AND FRUCTOSE-1,6-DIPHOSPHATASE

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SUMMARY. Fructose-1-phosphate-6-sulfate was prepared by direct sulfurylation of fructose, and selective phosphorylation of the 6-sulfuryl isomer by phosphofructokinase. The ketose derivative was used as a substrate for aldolase and fructose-1,6-diphosphatase. Kinetic studies with aldolase showed that the alternative substrate binds one third as well as fructose-1,6-P_2 yet 900 fold greater than fructose-1-P. The V_m was intermediate between the two ketose phosphates. From kinetic studies with skeletal muscle fructose-1,6-diphosphatase at pH 7.5 a K_m of 8 μM and a V_m approximately 6% that for fructose-1,6-P_2 was obtained.

Recent studies showed that fructose-6-sulfate is an alternative substrate for phosphofructokinase (1). The product of this reaction was identified to be fructose-1-phosphate-6-sulfate (FPS)*. It became of interest to find whether substitution of the phosphoryl moiety by a sulfuryl group on carbon-6 of the ketose diester affects its acceptability as a substrate for aldolase and fructose-1,6-diphosphatase. Since aldolase can utilize fructose-1-P as a substrate (2), it was possible to compare different kinetic parameters obtained with three substrates containing on carbon-6: a monoanion, a dianion or no charged group. Studies on the effect of pH on aldolase activity with both fructose-1,6-P_2 and FPS suggest that a decrease in enzyme activity at low pH values involve primarily substrate binding and not catalysis. Some kinetic parameters for FPS with fructose-1,6-diphosphatase are also reported.

METHODS

Fructose was sulfurylated with pyridine-sulfur trioxide in anhydrous dimethylformamide at 0° (3). This method sulfurylates predominantly primary hydroxyl groups. Free sulfate ion was removed by $BaCl_2$ and centrifugation at 4°. Dimethylformamide was removed *in vacuo*. An aqueous solution of the

*FPS refers to fructose-1-phosphate-6-sulfate.

syrup was adjusted on ice to pH 7.0 and applied to a column of AG2-X8 anion exchange resin (formate form). Gradient elution with pyridine-formic acid (1.2M:2.1M) separated the sulfurylated fructose from unreacted fructose. Pyridine was removed in vacuo and the potassium salt of sulfurylated fructose was precipitated in cold absolute ethanol (Yield 35%). Elemental analysis showed fair agreement with the calculated amount of C/H/S for $C_6H_{11}O_9SK$ and established the absence of phosphorus. Infrared analysis of the material showed absorption bands consistent with modification at primary hydroxyl positions (4). The sulfurylated fructose (30 mMole) was incubated with ATP (10 mMole) $MgCl_2$ (11 mMole) in 100 ml of 0.1M Tris-Cl, pH 8.6 containing 10 mM dithiothreitol, 10 mM EDTA, and 10 mg of heart phosphofructokinase. The pH was maintained at 8.2-8.6 by the addition of powdered Tris in the free base form. Aliquots of the reaction mixture were removed at various times to determine ADP production. The reaction reached a plateau at three hours. Unreacted ATP was precipitated by the addition of $BaCl_2$ and centrifugation at 4°. The reaction mixture was diluted twofold and applied to an AG2-X8 (formate form) column. Gradient elution with pyridine-formic acid (2.4M:4.2M) yielded two ketose containing peaks. The first peak corresponded to the monosulfurylated fructose. The second peak, which eluted well after the first peak, was pooled and the pyridine formate was evaporated. The syrup was dissolved in a small volume of water and passed through a AG50-X8 column (hydrogen form) and titrated on ice to pH 7.5 with KOH (1M). The solution was added dropwise to twenty-fold its volume of cold absolute ethanol. The resulting precipitate of the potassium salt of FPS was washed with absolute ethanol, absolute ethanol-absolute ether (1:1) and absolute ether followed by dessication in vacuo. Product yield was 51% of the ADP produced.

Rabbit skeletal muscle aldolase (from Boehringer Mannheim) activity was determined using a Cary 118C recording spectrophotometer by coupling the reaction with triosephosphate isomerase, α-glycerphosphate dehydrogenase and NADH. The assay mixture contained 58 mM 2(N-morpholino) ethane sulfonic acid, 0.91 mM NADH, 0.73 mM EDTA, 3 units of α-glycerophosphate dehydrogenase, and 36 units of triosephosphate isomerase. Aldolase concentration was 0.74 μg/ml and 4.9 μg/ml in the assay with fructose-1,6-P_2 and FPS, respectively.

Skeletal muscle fructose diphosphatase (from Boehringer Mannheim) activity was assayed in 50 mM Tris-Cl, pH 7.5 containing 5 mM $MgCl_2$ and the indicated amount of [^{32}P]FPS. Enzyme concentration was 1.0 μg/ml. Maximal substrate utilization was 17%. Reactions were terminated by the addition of 10 mM EDTA and 10 mM fructose-1,6-P_2. Samples were applied to small columns containing approximately 0.3 g AG1-X4 anion exchange resin (chloride form). The column was washed with 3.0 ml of 150 mM H_3PO_4 adjusted to pH 2.0 which quantitatively eluted all $^{32}P_i$. The eluate was neutralized with 0.8 meq of Tris and the phosphate precipitated by the addition of 0.5 meq $BaCl_2$. After centrifugation the precipitate was dissolved by the addition of 0.5 meq HCl and quantitatively transferred to a scintillation vial. Samples were counted in 5.0 ml of Insta-Gel.

RESULTS & DISCUSSION

FPS was shown to be distinct from fructose-1-P, fructose-6-P and fructose-1,6-P_2 on thin layer cellulose chromatography and high voltage electrophoresis. The position of attachment of the phosphoryl moiety at carbon-1 was confirmed by: a) The selective hydrolysis of phosphoryl groups at carbon-1 of hexoses by phenylhydrazine (5) which removed 98 ± 1% of the total phosphate b) A linear

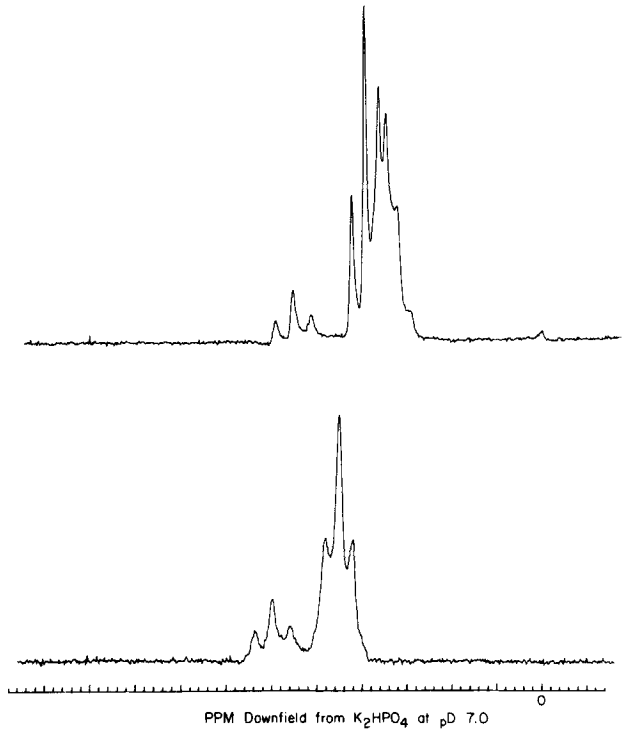

Fig. 1. ^{31}P NMR spectra of the potassium salts of fructose-1,6-P_2 (Top) and FPS (bottom) in D_2O (pD 7.0) at concentrations of 50 mM. Spectra were obtained in a Varian XL-100 spectrometer operating at 23.5 KG for ^{31}P acquisition at 40.5 MHz in the Fourier transform mode.

first order hydrolytic constant in 1N HCl at 95° which was nearly identical to that of fructose-1-phosphate c) The position of attachment was investigated further by ^{31}P NMR. Fig. 1 shows the spectra of FPS and fructose-1,6-P_2. The two triplets observed for FPS are consistent with phosphorylation at carbon-1 of the α and β anomers of fructose-6-sulfate. Assignment of coupling constants and chemical shifts were based on the work of Grey (6) and are shown in Table 1 Coupling constants were measured from a computer listing of line positions. Integration of peak areas were used to determine anomeric compositions. Values for the coupling constants of fructose-1,6-P_2 were approximately 0.8 Hz greater than was previously reported yet the assignments for FPS can be made on the

TABLE I

^{31}P Chemical Shifts and ^{31}P-^{1}H Coupling constants of the Anomeric Forms of D-Fructose-1,6-diphosphate and D-Fructose-1-phosphate-6-sulfate.

	J(Hz)		γ (ppm)[a]	
	P_1	P_6	P_1	P_6
α-D-Fructose-1,6-diphosphate	8.0	b	2.8	b
β-D-Fructose-1,6-diphosphate	6.1	5.0	2.0	1.7
α-D-Fructose-1-phosphate-6-sulfate	7.8	-	3.0	-
β-D-Fructose-1-phosphate-6-sulfate	6.2	-	2.3	-

a) Chemical shifts measured relative to internal phosphate at pH 7.0.
b) Could not be measured - obscured by other resonances.

relative larger values of coupling constants and chemical shifts for the phosphoryl group at carbon-1. The anomeric composition of FPS (α=19%, β=81%) is identical to that reported by Koerner et al. (7) for fructose-1,6-P_2 using ^{13}C NMR.

Using the coupled enzyme containing α-glycerophosphate dehydrogenase and triosephosphate isomerase only one mole of NADH per mole of FPS was oxidized. The same amount of NADH was oxidized when triosephosphate isomerase was excluded from the assay system. Since dihydroxyacetone sulfate could be reduced in the presence of α-glycerophosphate dehydrogenase (8), the stoicheometry obtained suggest that cleavage of FPS by aldolase produces dihydroxyacetone phosphate and glyceraldehyde-3-sulfate. Presumably the triosephosphate isomerase cannot use glyceraldehyde-3-sulfate as a substrate.

Table II summarizes the relative kinetic constants obtained from double reciprocal plots with FPS (Fig. 2), fructose-1,6-P_2 (Fig. 3) and for fructose-1-P as reported by Penhoet et al. (9). The monovalent sulfuryl analogue (FPS) binds 900-fold greater than fructose-1-P but only 3-fold less than fructose-1,6-P_2. The K_m values are representative of the K_d values since Ginsburg and Mehler (10) showed that the binding of substrates corresponded well with the

TABLE II

Relative K_m and V_m values for aldolase with fructose-1,6-P_2, FPS, and fructose-1-P

Values express ratios of K_m or V_m for the enzyme with the indicated substrates to the K_m or V_m with fructose-1,6-P_2. Values for V_m with fructose-1,6-P_2 are expressed as the percentage.

Parameter	Fructose-1,6-P_2	FPS	Fructose-1-P
K_m	1[a]	3.4[b]	3000[c]
V_m	100[a]	14[b]	2[c]

a) Actual K_m = 5.5 μM and V_m = 9.0 μmols/min/mg (Fig. 3)
b) Actual K_m = 16 μM and V_m = 1.24 μmols/min/mg (Fig. 2)
c) Calculated from the data of Penhoet et al. (9), K_m Fructose-1,6-P_2 was 3.3 μM and K_m Fructose-1-P was 10 mM.

K_m values. The effect of valency of the anionic substituent at carbon-6 on V_m is interesting in that the V_m with fructose-1-P is about 2% that for fructose-1,6-P_2 while the V_m with the monovalent sulfuryl analogue (FPS) was 14% that of the hexose diphosphate. Thus the effect of the monovalent sulfuryl group at carbon-6 on V_m is intermediate between no charge and the divalent phosphoryl anion while the effect on binding affinity is far closer to that of the phosphoryl dianion (Table II).

Plots of kinetic data at different pH values can be used to determine the pK's of ionizable groups involved in substrate-enzyme interactions (11). Figs. 2 and 3 summarize kinetic results on the effect of pH on aldolase activity using fructose-1,6-P_2 and FPS. Using FPS a plot of log V_m v pH showed a small change of slope (0.04) (lower inset, Fig. 2) suggesting that there was no essential catalytic groups which were titrated between pH 5.0 to 7.0. However, the plot of log V_m/K_m v showed a large change in the slope with a limiting value of 0.7. The intersection point of asymptotes of the curve at low and high pH values gives the pK value of the ionizing group at 6.0. Decreasing the pH from 7.0 to 5.0 results in the protonization of the phosphoryl group

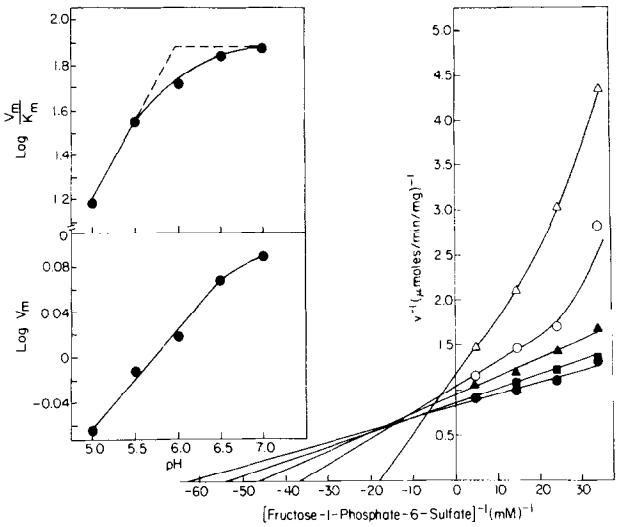

Fig. 2. Lineweaver-Burk plots of aldolase activity with FPS at pH 7.0 (●), 6.5 (■), 6.0 (▲), 5.5 (○), and 5.0 (△). Insets show replots of kinetic data as indicated.

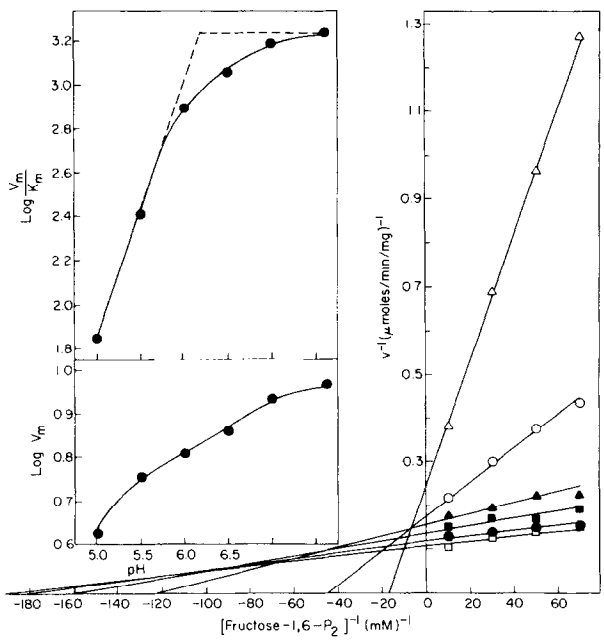

Fig. 3. Lineweaver-Burk plots of aldolase activity with fructose-1,6-P_2 at pH 7.6 (□), 7.0 (●), 6.5 (■), 6.0 (▲), 5.5 (○), and 5.0 (△). Insets show replots of kinetic data as indicated.

at carbon-1. The pK of monophosphorylated fructose is 6.1 (12). Plots with fructose-1,6-P_2 (Fig. 3) as the substrate are qualitatively similar to those obtained with the alternative substrate. A limiting slope of 1.2 for the plot of lot V_m/K_m v pH (Fig. 3, upper inset) is approximately twice that obtained

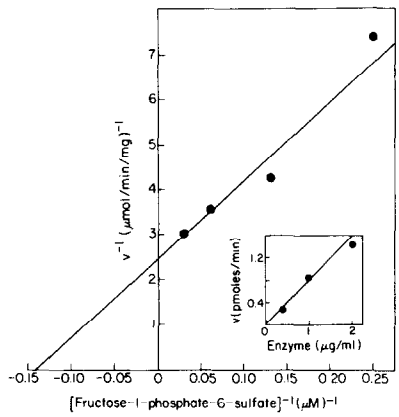

Fig. 4. Lineweaver-Burk plot of fructose-1,6-diphosphatase activity with FPS. Enzyme concentration as 1.0 μg/ml and the pH was 7.5. Inset, plot of product formation versus enzyme concentration.

with FPS and the intersection of the asymptotes gives a pK value of 6.25 which is closely related to the pK_2 (6.32) for fructose-1,6-P_2 (12). If the deprotonation of both phosphoryl groups is essential for binding a limiting slope value of two should be observed. The reason this was not seen may be due to the fact that the range of iructose-1,6-P_2 concentrations used was too high to entirely show the effect of titration of both phosphoryl groups. The highest concentration in the kinetic studies of the alternative substrate was 12-fold the K_m value at pH 7.0 while that with the natural substrate was 22-fold. Our data indicate that the changes in activity at low pH involve binding but not catalysis. This is supported by other studies which showed that the phosphoryl moiety at carbon-1 is involved in the formation of the enzyme substrate complex (c.f. ref. 13,8). Possible participation of a histidyl moiety in binding can be excluded from the studies of Horecker and his associates (13).

A kinetic plot of the activity of fructose-1,6-diphosphatase with FPS at pH 7.5 is shown in Fig. 4. The double reciprocal plot appears to be linear in the concentration range tested. The K_m was determined to be 6 to 8 μM and the V_m was about 0.08 μmoles/min/mg. Maximal specific activity with fructose-1, 6-P_2 was 1.2 μmoles/min/mg. A K_m could not be determined because of substrate inhibition. The low V_m with the sulfurylated substrate when compared to that with fructose diphosphate suggests that a divalent anionic substituent at carbon six of the natural substrate is important for maximal catalysis.

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