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The Anomeric Specificity of Yeast Pyruvate Kinase toward Activation by D-Fructose 1,6-Bisphosphate†

Richard Fishbein, Patricia A. Benkovic, and Stephen J. Benkovic*

ABSTRACT: The anomeric specificity of D-fructose 1,6-bisphosphate activation of yeast pyruvate kinase has been investigated utilizing stopped-flow kinetics and synthetic analogs of D-fructose 1,6-bisphosphate. It has been demonstrated that the immediate product, β-D-fructose 1,6-bisphosphate, of the phosphofructokinase-catalyzed reaction increases the catalytic activity of the enzyme. Although exclusive activation by the α anomer is ruled out by these experiments the possibility that the allosteric site is anomerically nonspecific cannot be excluded owing to experimental limits. 2,5-Anhydromannitol 1,6-bisphosphate, 2,5-anhydroglucitol 1,6-bisphosphate, 1,6-diphosphohexitol, and methyl (α + β)-D-fructofuranoside 1,6-bisphosphate were tested as activators or inhibitors of the D-fructose 1,6-bisphosphate activation of pyruvate kinase. No activation was observed but inhibition of D-fructose 1,6-bisphosphate activation by 2,5-anhydromannitol 1,6-bisphosphate and 2,5-anhydroglucitol 1,6-bisphosphate was noted. Methyl (α + β)-D-fructofuranoside 1,6-bisphosphate and 1,6-diphosphohexitol also proved to inhibit weakly. The collective data suggest that the allosteric site on yeast pyruvate kinase may be nonspecific with respect to anomeric configuration, but that a C-2 hydroxyl is necessary for activation by D-fructose 1,6-bisphosphate.

Pyruvate kinase catalyzes one of the rate-limiting steps in glycolysis (Scrutton and Utter, 1968). The kinases found in liver and yeast are subject to feed forward activation by D-fructose-1,6-P_2[1] (Kane, 1973). Since D-fructose-1,6-P_2 exists in solution in three rapidly equilibrating configurations, it became of interest to investigate whether pyruvate kinase from yeast is subject to stereospecific activation by only one of the three possible forms. Several other glycolytic and gluconeogenic enzymes have been observed to show a dependence on the anomeric nature of their substrates; these include phosphoglucose isomerase (Schray et al., 1973), D-fructose-6-P kinase (Fishbein et al., 1974; Wurster and Hess, 1974a; Bar-Tana and Cleland, 1974; Koerner et al., 1974), fructose bisphosphatase (Benkovic *et al.,* 1974), and aldolase (Schray et al., 1975; Wurster and Hess, 1974b). This manuscript presents the results of experiments utilizing stopped-flow kinetic techniques and D-fructose-1,6-P_2 analogs relevant to the question of anomeric specificity.

Experimental Section

Materials. Phosphoenolpyruvate, ADP, ATP, NADH, D-fructose-1,6-P_2, D-fructose-6-P, and $NADP^+$ were all obtained from the Sigma Chemical Co. Lactic dehydrogenase, specific activity 815 units/mg, and D-fructose-6-P kinase, specific activity 170 units/mg, were also obtained from Sigma.

Pyruvate kinase was isolated from fresh Budweiser bakers yeast obtained from Anheuser Busch Inc., Baltimore, Md., according to the procedure of Hunsley and Suelter (1969) resulting in a preparation with a specific activity of 375 units/mg. Pyruvate kinase activity was measured at pH 6.2 utilizing the lactic dehydrogenase enzyme couple.

2,5-Anhydromannitol-1,6-P_2, 2,5-anhydroglucitol-1,6-P_2, and 1,6-diphosphohexitol were previously prepared according to published procedures (Benkovic et al., 1971).

Methods. Stopped-flow studies were performed on a Durrum Gibson stopped-flow spectrophotometer at 25° and 340 nm. The spectrophotometer is designed to have a mixing ratio of 1:1 in a minimum volume of approximately 0.15 ml; a total dead time of 10 msec was estimated by mixing 0.01 *M* $Fe(NO_3)_3$ in 0.1 *N* H_2SO_4 with 0.01 *M* KCNS and following the appearance of product at 650 nm. Reactions were started by mixing an equal volume of 0.1 *M* Tris (pH 7.5), 0.025 *M* $MgCl_2$, 0.1 *M* KCl, 2 m*M* ADP, 2 m*M* P-

† Contribution from the Department of Chemistry, 152 Davey Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received April 28, 1975.* This work was supported by National Institutes of Health Grant AM 16464.

[1] The abbreviations P_2 and P will designate bisphosphate and monophosphate, respectively.

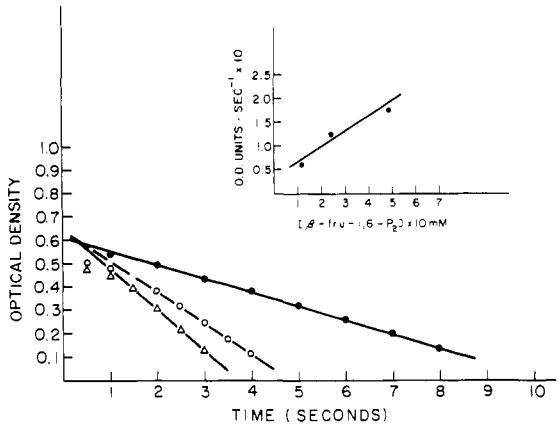

FIGURE 1: Plot of the time course of the pyruvate kinase catalyzed reaction utilizing D-fructose-6-P kinase to generate β-D-fructose-1,6-P_2. Initial concentrations of reagents and enzymes were: 1 m*M* P-enolpyruvate; 1 m*M* ADP; 0.8 m*M* ATP; 0.7 m*M* NADH; 0.15 m*M* (●), 0.30 m*M* (○), 0.60 m*M* (△) D-fructose-6-P; 3.5 units/ml of pyruvate kinase; 21.5 units/ml of lactic dehydrogenase; and 93 units/ml of D-fructose-6-P kinase. The insert is a replot of the slopes as a function of β-D-fructose-1,6-P_2 concentration.

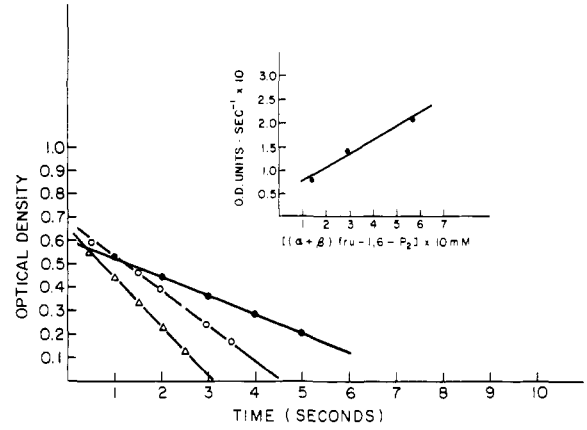

FIGURE 2: Plot of the time course of the pyruvate kinase catalyzed reaction utilizing (α + β)-D-fructose-1,6-P_2. Initial concentrations of reagents and enzymes were: 1 m*M* P-enolpyruvate; 1 m*M* ADP; 0.8 m*M* ATP; 0.7 m*M* NADH; 0.14 m*M* (●), 0.29 m*M* (○), 0.57 m*M* (△) D-fructose-1,6-P_2; 3.8 units/ml of pyruvate kinase; 21.5 units/ml of lactic dehydrogenase; and 93 units/ml of D-fructose-6-P kinase. The insert is a replot of the slopes as a function of (α + β)-D-fructose-1,6-P_2 concentration.

enolpyruvate, 1.6 m*M* ATP, 0.1–0.15 m*M* NADH, and either 0.07–0.7 m*M* D-fructose-1,6-P_2 or 0.25–1.2 m*M* D-fructose-6-P with 0.1 *M* Tris (pH 7.5), 25 m*M* $MgCl_2$, 0.1 *M* KCl, 0.2 m*M* EDTA, 0.95 m*M* ATP, 1.4–8.3 units/ml of pyruvate kinase, 43 units/ml of lactic dehydrogenase, and 142–186 units/ml of D-fructose-6-P kinase. Data from the oscilloscope were obtained with percent transmission as the ordinate and time as the abscissa, which were then replotted as optical density vs. time. The main source of error in obtaining the data was in measuring the percent transmission from the photographs taken of the oscilloscope traces. Data points at the beginning of a reaction could be estimated to ±0.010 OD units and points toward the end of a reaction to ±0.004. Thus, the slope of any line is accurate to within 4–6%.

D-Fructose-6-P was assayed by coupling with phosphoglucose isomerase and glucose-6-P dehydrogenase. D-Fructose-1,6-P_2 was assayed by coupling with hexose diphosphatase, phosphoglucose isomerase, and glucose-6-P dehydrogenase.

Studies utilizing 2,5-anhydromannitol-1,6-P_2, 2,5-anhydroglucitol-1,6-P_2, 1,6-diphosphohexitol, and methyl (α + β)-D-fructofuranoside-1,6-P_2 as inhibitors were performed at 25° utilizing the lactic dehydrogenase enzyme couple. The final reaction mixtures contained 5 μ*M* D-fructose-1,6-P_2, 1 m*M* ADP, 1 m*M* P-enolpyruvate, 10 m*M* $MgCl_2$, 0.2 *M* KCl, 50 m*M* Tris (pH 7.5), 0.2–0.3 m*M* NADH, and ~0.02 unit of pyruvate kinase. The reactions were followed at 340 nm utilizing a Gilford Model 2000 spectrophotometer. Periodically 1-μl aliquots of 0.02 *M* analog were added and the effect on the rate was observed.

Results

In order to determine the anomeric activator specificity, a method of rapidly synthesizing pure β-D-fructose-1,6-P_2 and observing the effect of this species on the pyruvate kinase catalyzed reaction velocity was developed. It has been observed that the β anomer of fructose-6-P is phosphorylated during the reaction catalyzed by D-fructose-6-P kinase (Fishbein et al., 1974; Wurster and Hess; 1974a). Since ring opening of D-fructose-6-P is not a requisite for phosphorylation, it is reasonable to assume that the β anomer is the product (Fishbein et al., 1974; Bar-Tana and Cleland, 1974; Koerner et al., 1974). Thus if β-D-fructose-1,6-P_2 were generated at a rate which is fast with respect to its expected mutarotation rate and the rate of the pyruvate kinase catalyzed reaction, it should be possible to observe the effect of β-D-fructose-1,6-P_2 on the velocity of the pyruvate kinase catalyzed process. In addition it might be possible to observe any change in the rate of pyruvate production due to mutarotation of β- to α-D-fructose-1,6-P_2.

The results of the stopped-flow experiments at pH 7.5 are displayed in Figures 1 and 2. Figure 1 presents the time course of the pyruvate kinase catalyzed reaction over approximately 10 sec for that case where β-D-fructose-1,6-P_2 is generated by D-fructose-6-P kinase. The plots are linear after exclusion of an ca. 1 sec lag phase. Sufficient D-fructose-6-P kinase was present to convert all of the D-fructose-6-P to β-D-fructose-1,6-P_2 in less than 0.4 sec. The insert to Figure 1 is a replot of the slopes as a function of β-D-fructose-1,6-P_2 concentration. Figure 2 shows the time course of the pyruvate kinase catalyzed reaction at pH 7.5 over approximately 10 sec for the case where (α + β)-D-fructose-1,6-P_2 is initially present. Although the slope for the insert in Figure 1 should be 20% greater than that in Figure 2, if the β anomer solely were the activator, a deduction regarding anomeric specificity on this basis is tenuous owing to the possibility of curvature imposed by the approach to saturating concentrations of D-fructose-1,6-P_2 (Kuczenski and Suelter, 1971).

In order to augment the results obtained utilizing stopped-flow techniques, synthetic analogs of β-, α-, and acyclic D-fructose-1,6-P_2 were tested as models for D-fructose-1,6-P_2 activation. These results are presented in Table I. It can be seen from Table I that both 2,5-anhydroglucitol-1,6-P_2 (an α analog) and 2,5-anhydromannitol-1,6-P_2 (a β analog) do not activate but inhibit activation of pyruvate kinase by fructose-1,6-P_2 to nearly the same extent. In addition, hexitol-1,6-P_2 (acyclic analog) and methyl (α + β)-D-fructofuranoside-1,6-P_2 likewise do not act as activators of pyruvate kinase activity. In the case of both the methyl (α + β)-D-fructofuranoside-1,6-P_2 and hexitol-1,6-P_2 the observed inhibition may be reversed by the addition of fructose-1,6-P_2, suggesting that the analogs compete with fruc-

Table I: Inhibition of Pyruvate Kinase by Substrate Analogs.

Compound	Concn (μM)	% Inhib.
2,5-Anhydromannitol-1,6-P_2[a]	40	42
	80	78
	120	83
2,5-Anhydroglucitol-1,6-P_2	40	27
	80	56
	120	71
Hexitol-1,6-P_2[b]	184	33
Methyl (α + β)-D-fructofuranoside-1,6-P_2	112	33

[a] D-Fructose-1,6-P_2 = 5 μM. [b] D-Fructose-1,6-P_2 = 10 μM.

tose-1,6-P_2 for a common binding site. For example, at 112 μM methyl (α + β)-D-fructofuranoside-1,6-P_2, 50 and 74% of the activation are restored at 9.2 and 18 μM fructose-1,6-P_2, respectively.

Discussion

At pH 7–8 D-fructose-1,6-P_2 exists in a β/α ratio of approximately 4:1 with essentially no acyclic configuration present (Gray, 1971; Benkovic et al., 1974; Koerner et al., 1973). The anomeric distribution of D-fructose-6-P at similar pH has been calculated as 19:76:5 (α/β/keto) from ^{13}C NMR (Gray, 1971; Fishbein et al., 1974) and ir data (Swenson, 1971).

It has been demonstrated that D-fructose-6-P kinase catalyzes the stereospecific phosphorylation of β-D-fructose-6-P, a conversion that is virtually quantitative owing to the high affinity of the enzyme for the substrate and the very favorable equilibrium, 10^3 (Hanson et al., 1973). In the presence of sufficient D-fructose-6-P kinase, the anomerization of the remaining α-D-fructose-6-P will be rate determining. Likewise the initially generated β-D-fructose-1,6-P_2 undergoes subsequent anomerization to the α anomer. This sequence of events is described in

$$\alpha\text{-D-fru-6-P} \xrightarrow{k_1} \beta\text{-D-fru-6-P} \xrightarrow{\text{fast}}$$

$$\beta\text{-D-fru-1,6-P}_2 \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \alpha\text{-D-fru-1,6-P}_2 \quad (1)$$

The concentrations of D-fructose-6-P kinase utilized were sufficient to consume all of the available β-D-fructose-6-P in nearly 0.4 sec—a time span that is short with respect to the conversion of β- to α-D-fructose-6-P, the D-fructose-1,6-P_2 mutarotation process, and the velocity of the pyruvate kinase catalyzed reaction. Based on the anomeric distribution of D-fructose-6-P in neutral aqueous solution it may then be assumed that the concentration of the β-D-fructose-1,6-P_2 initially formed is four times that of the α-D-fructose-6-P remaining. Equations 2 and 3 (Rodiguin and Rodiguina, 1964) may then be used to calculate at any time the concentrations of β- and α-D-fructose-1,6-P_2 respectively.[2] The results of these calculations are displayed graphically in Figure 3.

[2] Estimations of the rate constants of the mutarotation processes used in eq 2 and 3 were obtained from several sources (Fishbein et al., 1974; Wurster and Hess, 1974a,b; and Schray et al., 1975). It should be noted that there is some variation in these numbers. The following values were used: k_1 = 1.45–1.6 sec^{-1}, k_2 = 0.125–0.136 sec^{-1}, k_3 = 0.5–0.548 sec^{-1}.

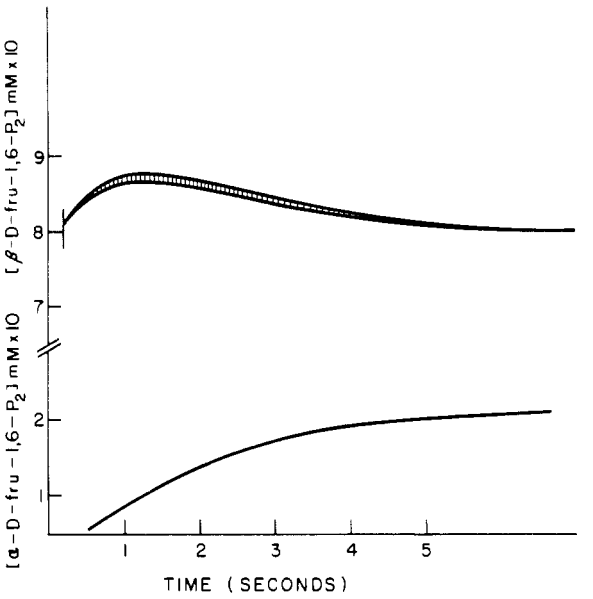

FIGURE 3: Plot of the behavior of β-D-fructose-1,6-P_2 (top) and α-D-fructose-1,6-P_2 (bottom) as a function of time according to eq 2 and 3. Initial conditions were assigned as follows: β-D-fructose-1,6-P_2, α-D-fructose-1,6-P_2, and α-D-fructose-6-P were 0.08, 0, and 0.02 mM, respectively.

$$[\beta\text{-D-fru-1,6-P}_2] = k_1[\alpha\text{-D-fru-6-P}^0] \times \left[\frac{k_3}{k_1(k_3+k_2)} - \frac{k_3-k_1}{k_1(k_3+k_2-k_1)}e^{-k_1t} + \frac{k_2}{(k_3+k_2)(k_1-k_3-k_2)}e^{(-k_3-k_2)t}\right] + [\beta\text{-D-fru-1,6-P}_2^0]\left[\frac{k_3}{k_3+k_2} + \frac{k_2}{k_3+k_2}e^{(-k_3-k_2)t}\right] \quad (2)$$

$$[\alpha\text{-D-fru-1,6-P}_2] = k_2k_1[\alpha\text{-D-fru-6-P}^0] \times \left[\frac{1}{k_1(k_3+k_2)} - \frac{1}{k_1(k_3+k_2-k_1)}e^{-k_1t} - \frac{1}{(k_3+k_2)(k_1-k_3-k_2)}e^{(-k_3-k_2)t}\right] + [\beta\text{-D-fru-1,6-P}^0]k_2\left[\frac{1}{k_3+k_2} - \frac{1}{k_3+k_2}e^{(-k_3-k_2)t}\right] \quad (3)$$

Although an apparent initial fast production of pyruvate is indicated by Figure 1, exclusive β-D-fructose-1,6-P_2 activation of pyruvate kinase cannot be shown under the present experimental conditions. The time spans studied may be divided into short (<2 sec) and long reaction times (between 2 and 5 sec). An inspection of Figure 1 reveals a lag in the velocity of the pyruvate kinase catalyzed reaction, lasting approximately 1.5 sec. In Figure 3 the concentration of α- and β-D-fructose-1,6-P_2 increases during ca. the first 1.5 sec of the reaction. Thus the nonlinear response in rate could be due to continuing synthesis of β-D-fructose-1,6-P_2 by D-fructose-6-P kinase, to the production of α-D-fructose-1,6-P_2 by mutarotation, or to a combination of both processes. In addition, Spivey et al. (1974) have observed a similar lag phenomenon in activation of liver pyruvate kinase by equilibrated D-fructose-1,6-P_2. Thus assignment of anomeric specificity based on the processes occurring in less than 1.5 sec is tenuous.

After approximately 1.5 sec the concentration of the β

anomer begins to decline while the concentration of the α anomer continues to rise (Figure 3). Yet, an examination of Figure 1 after approximately 1.5 sec indicates a linear rate of pyruvate production. The result of complete specificity by the enzyme for the α anomer would have been a doubling of the velocity of the pyruvate kinase catalyzed reaction between 1.5 and 5 sec. Thus this possibility is eliminated. However, exclusive activation by the β anomer remains plausible. The net decrease in β-D-fructose-1,6-P_2 concentration between 1.5 and 5 sec is only 9% so that changes in rate would not readily be detected under our conditions. Likewise an absence of anomeric specificity would also be manifest by a linear production of pyruvate, since there is a compensating change in the concentration of the α and β anomers of D-fructose-1,6-P_2.

In an attempt to distinguish between exclusive activation by the β anomer and a lack of anomeric specificity, analogs of D-fructose-1,6-P_2 were tested as possible modifiers of pyruvate kinase. All of the analogs examined proved to be inhibitors of the D-fructose-1,6-P_2 activation of pyruvate kinase. Both 2,5-anhydromannitol-1,6-P_2 and 2,5-anhydroglucitol-1,6-P_2 have a proton in place of the hydroxyl group of the natural modifier. The methyl (α + β)-D-fructofuranoside-1,6-P_2 mixture possesses a methoxyl function in place of the hydroxyl at C-2. These results indicate the need for a free hydroxyl function at the anomeric carbon for activation of the enzyme. Inhibition of D-fructose-1,6-P_2 activation by both 2,5-anhydromannitol-1,6-P_2 and 2,5-anhydroglucitol-1,6-P_2 to roughly the same extent could be interpreted as indicating a lack of anomeric specificity. However, inhibition albeit more weakly by 1,6-diphosphohexitol implies inhibition of D-fructose-1,6-P_2 activation of the enzyme may be accomplished by compounds that are structurally dissimilar to (α + β)-D-fructose-1,6-P_2.

Yeast pyruvate kinase from *Saccharomyces carlsbergenesis* likewise is activated by fructose-1,6-P_2 and by glucose-1-P; although at concentrations five times that of fructose-1,6-P_2 only ca. 50% of the latter's activation is achieved. Hexitol-1,6-P_2 is a potent inhibitor of fructose-1,6-P_2 activation, decreasing its effect by 82% at equal concentrations (Haeckel et al., 1968). Although glucose-1,6-P_2 does not stimulate this particular yeast enzyme, it acts as an activator for the rat liver enzyme (Van Berkel et al., 1974). These and the above results collectively suggest that the structural specificity of the allosteric site may be species as well as strain dependent. In the present case, failure of 2,5-anhydromannitol-1,6-P_2 to activate the kinase from *S. cerevisiae* indicates that the presence of two properly oriented phosphoryl moieties substituted 1,6 on the furanose ring are insufficient structural features for mimicking the modifier. The presence of a C-2 hydroxyl in an acyclic compound, e.g., hexitol-1,6-P_2, also is inadequate. Thus there are rather specific structural requirements for maximal activation. Although the anomeric specificity of this yeast kinase remains a moot point, it is important to realize that the observed activation by the β anomer alone of fructose-1,6-P_2 accounts for minimally 80% of the total stimulating effect.

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