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THE EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON THE REVERSE REACTION KINETICS OF FRUCTOSE 1,6-BISPHOSPHATASE FROM BOVINE LIVER*

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SUMMARY. The fructose 1,6-bisphosphatase reaction was investigated in the reverse direction by using fructose 2,6-bisphosphate. The effector was found to be a potent inhibitor of the reverse reaction substrates. Inhibition of fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate was competitive, and slope replots were linear. In the context of other accumulated kinetic data, our results serve to support a Random Bi Uni mechanism as the most likely mechanism for the reverse reaction. In addition, two models consistent with the data are presented for the interaction of fructose 2,6-bisphosphate with fructose 1,6-bisphosphatase.

INTRODUCTION. FBPase[1] (EC 3.1.3.11) catalyzes the hydrolysis of FBP to form F6P and P_i in the presence of a required divalent cation. The enzyme is a crucial control site in gluconeogenesis and, as such, is highly regulated by several effectors, including AMP and F2,6BP. The FBPase reaction has been studied extensively in the forward (physiological) direction (1), but these investigations have been ineffective in elucidating the manner of product release because technical problems for some time precluded the use of product inhibition studies, and in addition, the amount of information obtainable in a Uni system is limited inherently. The exergonic hydrolysis of FBP until recently was considered irreversible (2). However, it was demonstrated that the

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[1]Abbreviations used: FBPase, fructose 1,6-bisphosphatase; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; F2,6BP, fructose 2,6-bisphosphate; phosphate, P_i.

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reaction of FBPase is kinetically reversible (3), and studies of the reverse reaction have substantially narrowed the possible kinetic mechanisms (4).

Using results of product inhibition studies in the forward reaction and initial-velocity studies of the reverse reaction of FBPase, it was possible initially to eliminate from consideration the conventional rapid-equilibrium Ordered Uni Bi mechanism. Further studies on the reverse reaction utilizing substrate analogs indicated that the steady-state Ordered and Ping Pong Uni Bi mechanisms were not consistent with the data. In addition, isotope partitioning experiments indicated that the reverse reaction approximates the rapid-equilibrium assumption.

The results obtained thus far were in accord with a rapid-equilibrium Random Bi Uni mechanism, as well as a unique rapid-equilibrium Ordered Bi Uni mechanism of the type proposed by Frieden (5), with F6P binding to a site other than the active site (4). Several methods have been suggested whereby these two mechanisms may be differentiated, including the use of multisubstrate analogs (6).

Recent reports have indicated that F2,6BP is a potent competitive inhibitor for FBP in the forward reaction (7,8). Pilkis et al. (9) have presented additional evidence suggesting that F2,6BP may be an allosteric effector of the enzyme and also may act at the active site. These results and the obvious similarity of F2,6BP to FBP suggested the use of the former compound as a multisubstrate analog for studying the reverse reaction. This communication presents the results of these studies and the conclusion that the most probable mechanism for the reverse reaction is rapid-equilibrium Random Bi Uni. In addition, our data serves to shed additional light on the interaction of F2,6BP with FBPase.

MATERIALS AND METHODS

Distilled, deionized water was used in the preparation of all reagents. Chemicals and coupling enzymes were of the highest purity available commercially.

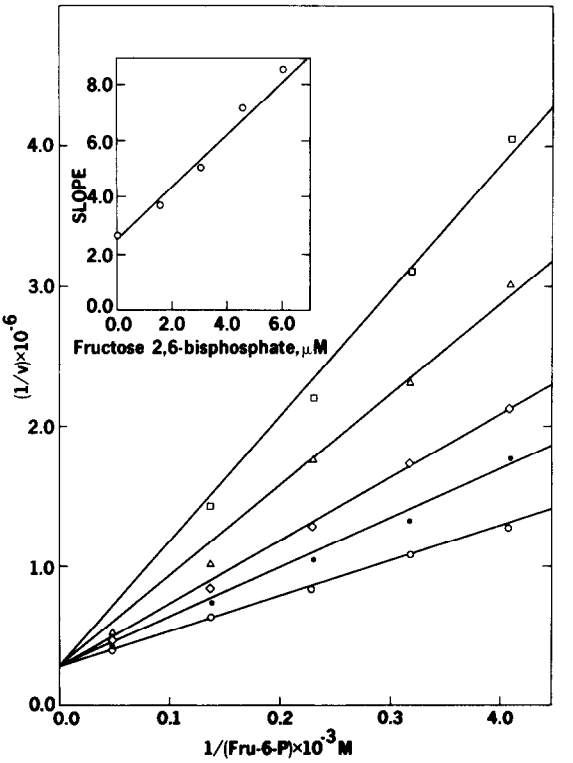


Figure 1. Plot of the reciprocal of initial velocity versus the reciprocal of the molar concentration of F6P in the absence (o) and presence of 1.5 μM (●), 3.0 μM (◇), 4.5 (Δ), and 6.0 μM (□) F2,6BP. Assays were performed as outlined in "Materials and Methods" with a fixed concentration of 25 mM P_i and 0.60 I.U. of FBPase.

Enzyme Preparation - FBPase was purified from bovine liver as previously described (3) and had a specific activity of 22.0 I.U./mg. All enzyme used had a pH 6.5:9.0 activity ratio of at least 2.0.

Enzyme Assays - The coupled spectrophotometric assay used to measure the reverse FBPase reaction has been described previously (3). The final volume of the assay was 1.0 ml, and each assay was incubated 5 min at 28°C before initiation by the addition of FBPase. F2,6BP was added in a small volume of 0.01 N NaOH; uninhibited assays contained the same volume of 0.01 N NaOH alone.

Preparation of F2,6BP - F2,6BP was prepared as described (10) and analyzed by three methods: 1) ^{31}P NMR spectroscopy with and without proton decoupling (11), 2) inhibition of the forward FBPase reaction (8), 3) the assay of acid-revealed F6P (10). Our preparations contained up to 5% FBP; however, this was easily removed by incubation with coupling system as described.

Treatment of Kinetic Data - The kinetic data were analyzed by using a computer program written in the OMNITAB II language (12), with an α value of 0.0.

RESULTS AND DISCUSSION. The results of F2,6BP inhibition of the FBPase reaction are shown in Figures 1 and 2. In both cases, one substrate was held at a constant concentration near its K_m value while the other substrate was varied in the presence of 0-6 μM inhibitor. Figure 1 shows

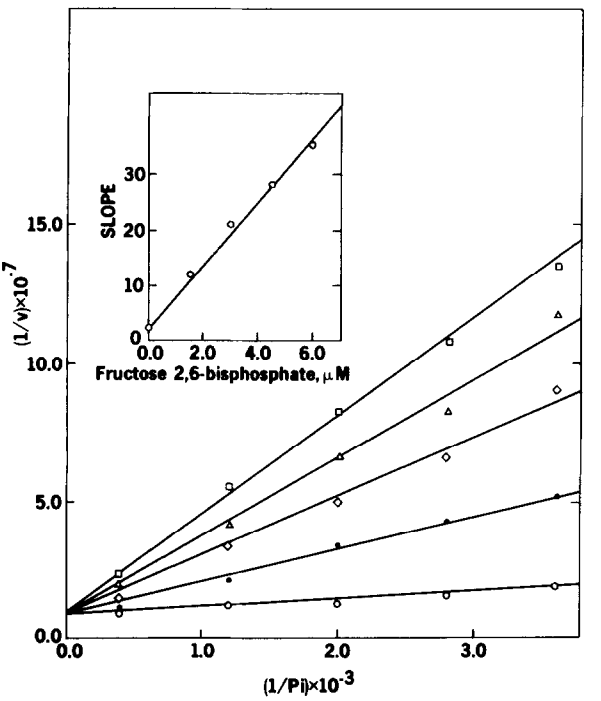


Figure 2. Plot of the reciprocal of initial velocity versus the reciprocal of the molar concentration of P_i in the absence (o) and presence of 1.5 μM (●), 3.0 μM (◇), 4.5 μM (Δ) and 6.0 μM (□) F2,6BP. Assays were performed as outlined in "Materials and Methods" with a fixed concentration of 0.61 mM F6P and 0.33 I.U. of FBPase.

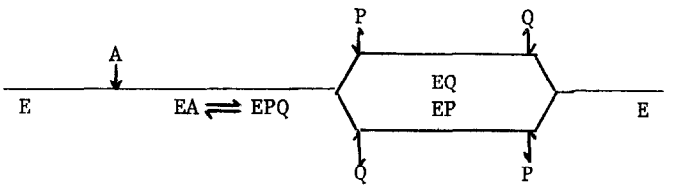
that F2,6BP gives a linear competitive plot with respect to F6P. The slope replot shown in the inset is also linear. Similarly, inhibition with respect to P_i is linearly competitive with a linear secondary plot, as shown. The values for the inhibition constants were determined to be 1.16 ± 0.16 μM for F6P and 0.35 ± 0.06 for P_i from Figures 1 and 2, respectively. Though theoretically the inhibition constants should be equivalent, some error may be introduced because they are calculated by using a combination of other previously determined kinetic constants (4) in addition to the experimental data.

In the presence of saturating Mg^{2+}, the reverse reaction of FBPase is Bi Uni. The kinetics of the reverse reaction have been investigated by initial-velocity studies and the use of substrate and product analogs. Fromm and Stone showed the product analog 2,5-anhydromannitol 1,6-bisphosphate is a competitive inhibitor for both P_i and F6P (4), as well

as a competitive inhibitor of FBP (13). These data served to eliminate both steady-state Ordered Bi Uni and Ping Pong Bi Uni mechanisms because the rate equation for both predicted that the analog would be a non-competitive inhibitor with respect to F6P. However, the data still were consistent with two mechanisms described by the rate equation:

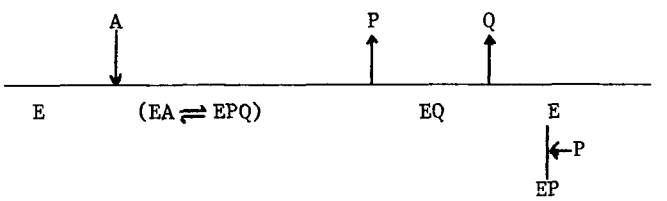
$$\frac{1}{v} = \frac{1}{V_2}\left[1 + \frac{K_q}{Q} + \frac{K_p}{P} + \frac{K_{iq}}{(P)}\frac{K_p}{(Q)}\left[1 + \frac{A'}{K_{ia'}}\right]\right] .$$

One possible mechanism is a rapid-equilibrium Random Bi Uni of the type



where P, Q, A, and A' represent F6P, P_i, FBP and product analog, respectively; K_q, K_p, and $K_{ia'}$ are the Michaelis constants for P_i and F6P, and the dissociation constant for the product analog. In previous work on forward reaction, F6P was shown to be a noncompetitive inhibitor for FBP. Because product inhibition should be competitive for a rapid-equilibrium random Uni Bi mechanism, it is necessary to propose the formation of an inactive enzyme·FBP·F6P complex for this mechanism to be in harmony with all the data.

An equally likely mechanism of the type proposed by Frieden (5) is



This rapid-equilibrium Ordered mechanism involves the formation of an inactive enzyme·F6P complex, and is consistent with all the data obtained to this point for the reverse reaction.

The two aforementioned mechanisms are not distinguishable by classical kinetic techniques. It has been pointed out, however, that

three criteria will permit a differentiation to be made between the rapid-equilibrium Random mechanism and the unique Ordered mechanism (6). One such procedure involves the use of a multisubstrate analog. For a Random mechanism, the multisubstrate analog would be a competitive inhibitor for both substrates. Because the analog would bind both substrate sites on the enzyme simultaneously, only the enzyme term of the rate equation would be effected. Primary plots for this mechanism would be linearly competitive, and secondary plots would be slope linear.

Alternatively, in the case of Frieden's mechanism, the analog would be expected to bind to a subsite for P, as well as to the active site. The binding of more than one molecule of the analog per substrate site will result in several squared terms in the rate equation (6). Consequently, the primary plots for this mechanism would be competitive for Q and noncompetitive for P, and secondary plots would be parabolic.

It is well documented that F2,6BP is a potent competitive inhibitor of FBPase in the forward reaction, and the compound seemed an ideal choice as a multisubstrate analog for F6P and P_i. The results of our study on the effect of F2,6BP indicate that it is a powerful inhibitor of the reverse reaction as well. In light of the previous discussion, it also is possible to select the steady-state Random Uni Bi mechanism in which the rapid-equilibrium assumption is approximated (4) as the best possibility for FBPase in the forward direction. In the reverse direction FBPase seems to be rapid-equilibrium Bi Uni.

The nature of the actual interaction of F2,6BP with FBPase is far from clear. At low concentrations (<10μM), F2,6BP seems to be strictly a competitive inhibitor of the forward reaction; however, higher concentrations of F2,6BP cause the hyperbolic substrate concentration curve to become sigmoidal (9). In addition, even at low concentration, the effector potentiates AMP inhibition. Hence, it has been proposed that the action of F2,6BP on FBPase involves at least some interaction with an allosteric site (9).

It is possible to conceive of two mechanisms for F2,6BP interaction with FBPase that are in harmony with the data presented here. If the effector were to interact only with the active site, we would expect competitive plots for both substrates and linear replots as seen in Figures 1 and 2. This would, however, be kinetically indistinguishable from a situation in which F2,6BP binds exclusively to an allosteric site, causing simultaneous release of product or substrates at the active site.

On the basis of our previous study of the effect of the product analog 2,5-anhydromannitol-1,6-bisphosphate on the reverse reaction, interaction with the active site seems most likely. The product analog, thought to act at the FBP active site, gave kinetic plots of the same pattern seen with F2,6BP. It also is possible that F2,6BP binds an allosteric site only at concentrations higher than were used in this study. In any case, physical studies on the binding of F2,6BP to FBPase should serve to clarify the nature of the interaction.

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