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KINETIC HYSTERESIS FOR FRUCTOSE BISPHOSPHATASE: A CHANGE IN SUBSTRATE CONFIGURATION SPECIFICITY

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SUMMARY: Kinetic hysteresis for rabbit liver fructose bisphosphatase in the presence of Mg^{2+} (pH 7.6) is exhibited by the varied rates at which product formation is reduced on the addition of different inhibitors under cycling conditions. Two different states of the enzyme are detected: the initial resting state which binds α-, β- and keto analogs of fructose 1,6-bisphosphate; and the active cycling state which binds, and is inhibited by, only the α-analog. Both enzyme states, however, bind the allosteric modifier, AMP, and a product analog, (α+β)methyl-D-fructofuranoside 6-phosphate to the same extent so that the resulting inhibition is state independent. A relatively slow first-order transition (0.13 min^{-1}) characterizes the reversion of the active enzyme to its resting state. The implications of this phenomenon for regulating fructose bisphosphatase activity in vivo are discussed.

Stopped-flow kinetic experiments, where phosphate release is monitored by an acid-base indicator method, showed that the reaction sequence for rabbit liver fructose 1,6-bisphosphatase (E.C. 3.1.3.11), FBPase, proceeds through several steps (1). Binding of substrate, D-fructose 1,6-bisphosphate, (Fru-1,6-P_2) is followed by a first-order conformational change of the FBPase·Fru-1,6-P_2 complex prior to hydrolytic cleavage and release of P_i. Once the catalytic cycle begins, turnover continues until Fru-1,6-P_2 levels become so low that relaxation of the enzyme to the initial conformational state can compete. Given this model it appeared that the addition of an inhibitor (i) before initiation of the catalytic process by addition of Fru-1,6-P_2 and (ii) during the steady-state cycle might indicate a different effect of the inhibitor on specific steps. We wish to report experiments employing rabbit liver FBPase in the presence of Mg^{2+} (pH 7.6) and substrate analog inhibitors (for the α- or β-anomers, or the acyclic keto species) that reveal a response

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to a given analog dependent on its chemical configuration. The observation of a kinetic hysteresis (2) for FBPase may play an important role in the regulation of its activity *in vivo*.

MATERIALS AND METHODS

Fru-1,6-P_2, glucose 1,6-P_2, AMP, assay reagents, and auxiliary enzymes were purchased from Sigma Chemical Company. Already published are the methods of preparation for α- (and β-)methyl-D-fructofuranoside 1,6-P_2, 2,5-anhydro-D-glucitol 1,6-P_2, 2,5-anhydro-D-mannitol 1,6-P_2, 1,6-hexanediol 1,6-P_2, *cis*-2,5-bis(hydroxymethyl)tetrahydrofuran 1,6-P_2 (3), xylulose 1,5-P_2 (4), and (α+β)methyl-D-fructofuranoside 6-P (5).

Neutral FBPase was purified from frozen livers (Pel-Freez Biologicals) of young, 24-hour-fasted rabbits by the procedure of Ulm et al. (6), as modified by Benkovic et al. (7).

The enzyme was assayed spectrophotometrically at 340 nm by following the rate of NADPH production in the presence of excess glucose 6-phosphate isomerase and glucose 6-phosphate dehydrogenase. The routine assay solution for activity at 25°C contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM $NADP^+$, 7.6 units of glucose 6-P isomerase, 1.7 units of glucose 6-P dehydrogenase (both freed of $(NH_4)_2SO_4$ by dialysis), FBPase, and 0.2 mM Fru-1,6-P_2. Where noted, the Tris was replaced by glycine (pH 9.4) and the $MgCl_2$-EDTA by 0.5 mM $MnCl_2$ or 10 μM $Zn(NO_3)_2$. Hysteresis effects were demonstrated with assay solutions whose components were added in different sequences: Mode 0 - water, buffer, Mg^{2+}, EDTA, $NADP^+$, auxiliary enzymes, FBPase (12 min. incubation), Fru-1,6-P_2; Mode 1 - identical to Mode 0 but with the assay allowed to progress for 4 minutes before addition of the inhibitor; and Mode 2 - water, buffer, Mg^{2+}, EDTA, inhibitor, $NADP^+$, auxiliary enzymes, FBPase (12 min. incubation), Fru-1,6-P_2.

RESULTS

Table I contains data for the relative inhibition of liver FBPase by various substrate analogs and the allosteric modifier AMP under two sets of conditions. In the case of relative inhibition expressed in terms of (Mode 1/ Mode 0) the inhibitor has been added to FBPase turning-over under steady-state conditions at saturating Fru-1,6-P_2 (Mode 1) relative to turnover in the absence of inhibitor (Mode 0). For (Mode 2/ Mode 0) the inhibitor has been preincubated with FBPase and the reaction initiated by the addition of Fru-1,6-P_2 (Mode 2) relative to turnover in the absence of inhibitor (Mode 0). Several salient observations are: 1) the allosteric modifier AMP as well as product analog, (α+β)methyl-D-fructofuranoside 6-P, act within the time limits of these experiments (less than 2 min) to inhibit FBPase to an extent independent of the mode of addition of the inhibitor; 2) the α- and β-anomer analogs of Fru-1,6-P_2 are more efficient inhibitors when initially incubated in the presence of the

Table I.

Rate of enzymatic cleavage of 0.2 mM Fru-1,6-P_2 (Mg^{2+}-EDTA, pH 7.6) in the presence of the indicated inhibitor (Modes 1 and 2, defined in text) relative to the rate without inhibitor (Mode 0).

Inhibitor[a]	(Mode 1/Mode 0)	(Mode 2/Mode 0)
AMP[b]	0.27	0.33
α-methyl-D-fructofuranoside 1,6-P_2	0.65[c]	0.38
β-methyl-D-fructofuranoside 1,6-P_2	0.83[c]	0.30
xylulose 1,5-P_2	1	0.55
glucose 1,6-P_2	1	0.96
1,6-hexanediol 1,6-P_2	1	0.92
2,5-anhydro-D-glucitol 1,6-P_2	0.27[c]	0.15
2,5-anhydro-D-mannitol 1,6-P_2[d]	0.71[c]	0.39
cis-2,5-bis(hydroxymethyl)-tetrahydrofuran 1,6-P_2	1	0.93
(α+β)-methyl-D-fructofuranoside 6-P	0.81	0.81

[a] At a concentration of 0.5 mM, unless noted.

[b] At 25 μM

[c] Estimated from tangent to progress curve at 2 min following introduction of inhibitor. (The Mode 1/Mode 0) ratio approached that of (Mode 2/Mode 0) at times >2 min in a first-order manner.

[d] At 0.25 mM

enzyme but the β-anomer is ca. 2-fold less inhibitory in the (Mode 1/Mode 0) assay; 3) the acyclic keto analog, xylulose 1,5-P_2, is ineffective as a inhibitor when added to the steady-state assay (4) whereas this compound functions as a competitive inhibitor with a K_i value similar to those for the α- and β-methyl-D-fructofuranoside 1,6-P_2 analogs in terms of (Mode 2/Mode 0); and 4) additional materials such as 1,6-hexanediol 1,6-P_2 and cis-2,5-bis-

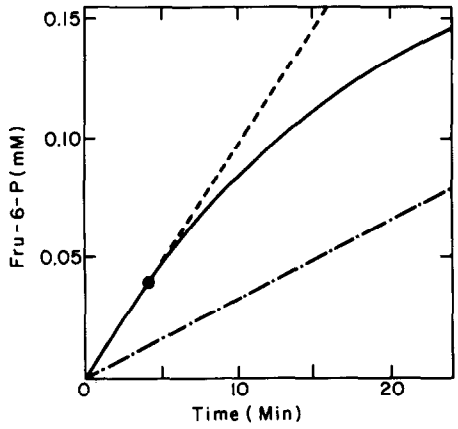


Figure 1. Plots showing Fru-6-P formed at indicated times by FBPase at pH 7.6 with Mg^{2+}-EDTA. The solid line from time zero to "●" and the ---- line denote the system without inhibitor (Mode 0). At "●" 0.5 mM β-methyl-D-fructofuranoside 1,6-P_2 was added quickly and the solid line denotes a decreasing rate of reaction (Mode 1). The -·-·- line denotes the Mode 2 system where enzyme and inhibitor are incubated prior to addition of substrate.

Table II.

First-order rate coefficients for the decreasing rate of Fru-1,6-P_2 cleavage on addition of the indicated concentrations of β-methyl-D-fructofuranoside 1,6-P_2.

β-methyl-D-fructofuranoside 1,6-P_2	k (min^{-1})
0.3 mM	0.10 ± 0.02
0.5 mM	0.17 ± 0.03
1.0 mM	0.12 ± 0.02

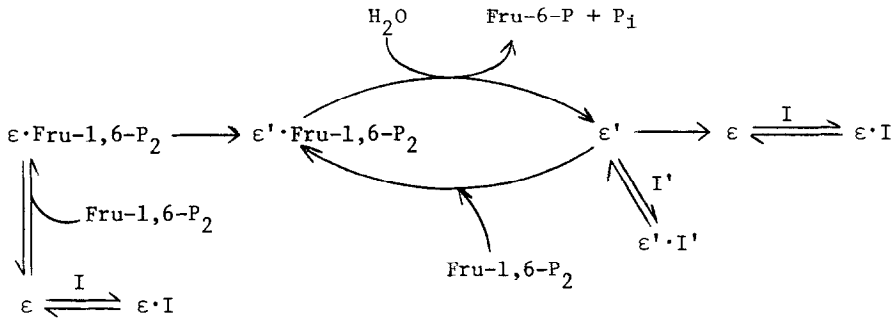
(hydroxymethyl)-tetrahydrofuran 1,6-P_2, which are not potent inhibitors in the normal (Mode 2/Mode 0) assay, exhibit no increase in their inhibitory efficacy in the steady-state assay.

The relative inhibition observed upon addition of either the α- or β-anomer analogs to the steady-state FBPase assay progressively increases to approach that observed upon preincubating FBPase and the inhibitor before initiating turnover through addition of substrate, Fru-1,6-P_2. In Fig. 1 is plotted the exponetial transition observed upon addition of β-methyl-D-fructofuranoside 1,6-P_2 to the steady-state FBPase system. In Table II are listed the calcu-

lated first-order rate coefficients for this transition at varying levels of β-methyl-D-fructofuranoside 1,6-P_2. Within the error limits of the experiments, this transition is independent of the analog concentration.

Similar measurements with α- (or β-)methyl-D-fructofuranoside 1,6-P_2 were made with FBPase activated by Mg^{2+} at pH 9.4, Mn^{2+} (0.5 mM, pH 7.6 or 9.4) and Zn^{2+} (10 μM, pH 7.6). Under these conditions, no difference was observed in the (Mode 1/Mode 0) and (Mode 2/Mode 0) ratios at the arbitrary 2 min. after addition of inhibitor, suggesting that the first-order transition, if present, is more rapid with these metal ions and at higher pH.

A minimal kinetic model consistent with these and previous observations is proposed in Scheme I. It appears that the ε' does not bind the acyclic



where ε and ε' = FBPase

I = α-, and β- and keto analogs of Fru-1,6-P_2; AMP, and fructoside 6-P

I' = α-analogs of Fru-1,6-P_2; AMP, and fructoside 6-P

SCHEME I

keto-analog or the β-anomer analog, and must revert to ε for these compounds to act as potent competitive inhibitors ($K_I \simeq 1$ μM), as previously demonstrated (3,4,8). The more rapid inhibition of cycling FBPase by the α-anomer analogs, particularly 2,5-anhydro-D-glucitol 1,6-P_2, suggests that these compounds can also interact with ε' in accord with the α-anomeric specificity of the enzyme (4). The degree of inhibition by AMP and (α+β)methyl-D-fructo-

furanoside 6-P is independent of whether they are initially present or are added to the steady-state assay thus suggesting they can bind to ε' as well as ε. It is also noteworthy that the rate constant for the ε' → ε (0.13 min^{-1}) is at least an order of magnitude slower than k_{cat} (4-6 sec^{-1}). This coupled with the high affinity of FBPase for Fru-1,6-P_2 ($K_M \simeq 1$ μM) (9) acts to preserve the catalytic loop until nearly all the Fru-1,6-P_2 is consumed.

From a physiological viewpoint, it is of considerable interest, that this phenomenon is only observed with the Mg^{2+} ion cofactor, presumably the more available metal ion in the liver tissue (10). Moreover, if one assumes that Fru-1,6-P_2 is at or near anomeric equilibrium in the liver cell, the enzyme would be insensitive to the β-Fru-1,6-P_2 (ca. 80% of the total Fru-1,6-P_2) when in the active ε' state, but competitively inhibited by this anomeric form in the inactive ε state. Thus there exists a novel means for modulating FBPase activity that would permit it to function efficiently in the presence of β-Fru-1,6-P_2 but reinforce its inactivity in the resting state.

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