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THE EFFECT OF ARABINOSE 1,5-BISPHOSPHATE ON RAT HEPATIC 6-PHOSPHOFRUCTO-1-KINASE AND FRUCTOSE-1,6-BISPHOSPHATASE

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Summary. The α- and β-anomers of arabinose 1,5-bisphosphate and ribose 1,5-bisphosphate were tested as effectors of rat liver 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase. Both anomers of arabinose 1,5-bisphosphate activated the kinase and inhibited the bisphosphatase. The α-anomer was the more effective kinase activator while the β-anomer was the more potent inhibitor of the bisphosphatase. Inhibition of the bisphosphatase by both anomers was competitive, and both potentiated allosteric inhibition by AMP. β-Arabinose 1,5-bisphosphate was also more effective in decreasing fructose 2,6-bisphosphate binding to the enzyme. Neither anomer of ribose 1,5-bisphosphate affected 6-phosphofructo-1-kinase or fructose-1,6-bisphosphatase, indicating that the configuration of the C-2 (C-3 in Fru 2,6-P_2) hydroxyl group is important for biological activity. These results are also consistent with arabinose 1,5-bisphosphate binding to the active site and thereby enhancing the interaction of AMP with the allosteric site.

Fructose 2,6-bisphosphate (Fru 2,6-P_2)[1] is an important regulator of hepatic carbohydrate metabolism. It activates 6-PF-1-K and inhibits Fru-1,6-P_2ase (1-3). The mechanism whereby Fru 2,6-P_2 inhibits rat liver Fru-1,6-P_2ase has been the subject of some controversy. Since Fru 2,6-P_2 potentiates allosteric inhibition by AMP and induces a sigmoidal substrate concentration dependence, François et al. (4) postulated that Fru 2,6-P_2 must bind to an allosteric site rather than interact with the catalytic site. However, based on kinetic (5-9),

[1]Abbreviations used: arabinose 1,5-P_2, arabinose 1,5-bisphosphate; Fru 1,6-P_2, fructose 1,6-bisphosphate; Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; Fru 2,6-P_2, fructose 2,6-bisphosphate; Fru 6-P, fructose 6-phosphate; 6-PF-1-K, 6-phosphofructo-1-kinase; P_i, inorganic phosphate; ribose 1,5-P_2, ribose 1,5-bisphosphate.

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protein modification (6,10), ligand binding (11), and spectroscopic studies (12,13), others have proposed that Fru 2,6-P_2 binds to the catalytic site. Also, it has been suggested that the sugar bisphosphate interacts with both the catalytic and allosteric sites (14). The Fru 2,6-P_2-induced sigmoidal substrate concentration dependence may also be explained by a "ligand exclusion" phenomenon (9) at the active site. Recently, Maryanoff et al. (15) reported the synthesis of α- and β-arabinose 1,5-P_2 and briefly mentioned their activation of rat liver 6-PF-1-K and inhibition of rat liver Fru-1,6-P_2ase. The object of this work is to characterize further the effects of the arabinose 1,5-P_2 compounds in an attempt to elucidate the site of Fru 2,6-P_2 interaction with Fru-1,6-P_2ase.

MATERIALS AND METHODS

Materials. ATP, Fru 6-P, and Fru 2,6-P_2 were obtained from Sigma Chemical Company. [2-^{32}P]Fru 2,6-P_2 was prepared by a modification (16) of the method of El-Maghrabi et al. (17). [1-^{32}P]Fru 1,6-P_2 was prepared as in Pilkis et al. (5). α- and β-arabinose 1,5-P_2 were synthesized as described by Maryanoff et al. (15)[2]. α- and β-ribose 1,5-P_2 were prepared by related methods, which will be described elsewhere (18)[3].

Enzyme Purifications and Assays. Rat liver 6-PF-1-K was purified to homogeneity by the method of Pilkis et al. (19) and was assayed spectrophotometrically as previously described (19). Homogeneous rat liver Fru-1,6-P_2ase was prepared as described by Riou et al. (20). It was assayed either spectrophotometrically as previously described (20) or by measuring $^{32}P_i$ release from [1-^{32}P]Fru 1,6-P_2 as described by Pilkis et al. (5).

Rate of Dialysis Measurements of Sugar Bisphosphate Binding to Fructose-1,6-bisphosphatase. A modification of the procedure of McGrane et al. (11) was used. Fru-1,6-P_2ase and [2-^{32}P]Fru 2,6-P_2 were placed in the upper chamber of a microdialysis cell where the contents were stirred continuously with a small stirring bar. Buffer was pumped continuously through the lower chamber, and after 2 min a steady state was achieved such that the rate of entrance of [2-^{32}P]Fru 2,6-P_2 into the lower chamber by dialysis and exit by flow were equal. Thus, the concentration in the effluent may be assumed from Fick's law to be proportional to the free [2-^{32}P]Fru 2,6-P_2 concentrations. Aliquots of increasing concentrations of α- or β-arabinose 1,5-P_2 were added to the enzyme solution in the upper chamber and [2-^{32}P]Fru 2,6-P_2 was counted in fractions of the effluent from the lower chamber. Each experiment resulted in a series of values of free and bound [2-^{32}P]Fru 2,6-P_2 as estimated according to Colowick and Womack (21).

RESULTS

Effect of Arabinose 1,5-P_2 and Ribose 1,5-P_2 on Rat Liver 6-PF-1-K and Fru-1,6-P_2ase Activities. The ability of the α- and β-anomers of

[2]Please note that these substances were not 100% isomerically pure: the α isomer contained 85-90% of that isomer, and the β isomer contained nearly 95% of that isomer.

[3]The β-ribose 1,5-P_2 was >95% isomerically pure; the sample of α-ribose 1,5-P_2 contained 75% α isomer and 25% β isomer. Complete characterization (elemental analysis; ^{1}H and ^{13}C NMR) has been performed.

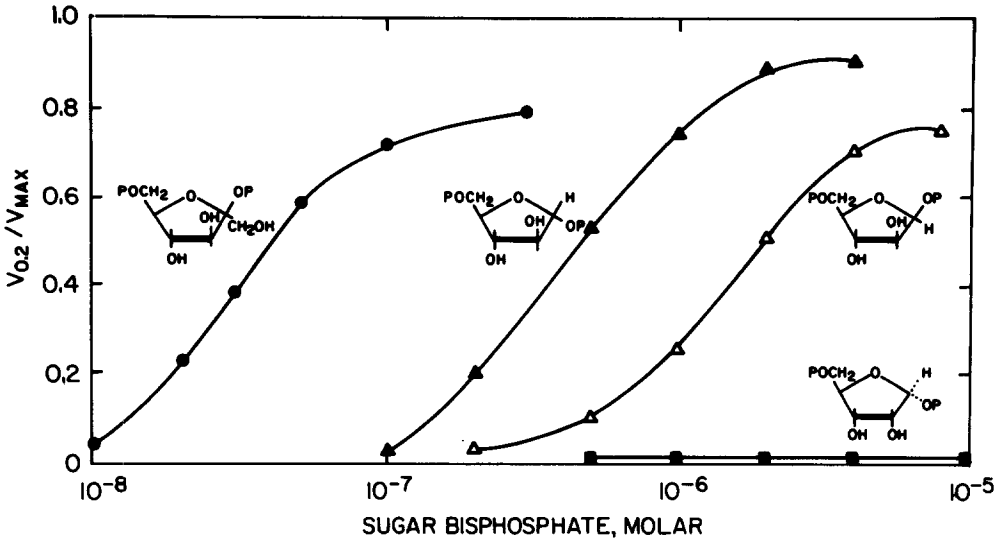

Figure 1. Activation of rat liver 6-PF-1-K by α- and β-arabinose-1,5-P_2 and ribose 1,5-P_2. Enzyme activity was measured spectrophotometrically as described in Methods. Activity is expressed as the ratio of the rate measured with 0.2 mM Fru 6-P to that measured with 4 mM Fru 6-P. The structures of each compound are shown as inserts: β-Fru 2,6-P_2 (●), α-arabinose 1,5-P_2 (▲), β-arabinose 1,5-P_2 (Δ), and α- or β-ribose 1,5-P_2 (■).

arabinose 1,5-P_2 to activate 6-PF-1-K is shown in Fig. 1. The α-anomer of arabinose 1,5-P_2 (K_A = 0.5 μM) was slightly more effective than the β-anomer (K_A = 1 μM). Both compounds, however, had substantially higher K_A's for activation than Fru 2,6-P_2 (K_A = 0.02 μM). Ribose 1,5-P_2 is structurally identical to arabinose 1,5-P_2 except for the configuration of the C-2 hydroxyl group. However, neither the α- nor β-anomer of ribose 1,5-P_2 activated 6-PF-1-K when tested at concentrations up to 100 μM (Fig. 1, and data not shown).

The effects of α- and β-arabinose 1,5-P_2 on rat liver Fru-1,6-P_2ase were also tested (Fig. 2). The β-anomer was a potent competitive inhibitor of Fru-1,6-P_2ase with a K_i of 3.5 μM. The α-anomer was also a competitive inhibitor, but with a K_i of 20-30 μM. For comparison, the K_i for Fru 2,6-P_2 is about 0.2 μM (5, and data not shown). Thus, while both arabinose 1,5-P_2 anomers are reasonable inhibitors, they are still only 1/10-1/20th as potent as Fru 2,6-P_2. Unlike Fru 2,6-P_2, however, neither anomer of arabinose 1,5-P_2 induced sigmoidicity in the substrate dependence of Fru-1,6-P_2ase, even at high concentrations (Fig. 2 and data not shown). These findings suggest that the specificity of Fru-1,6-P_2ase includes preference for the two phosphate moieties

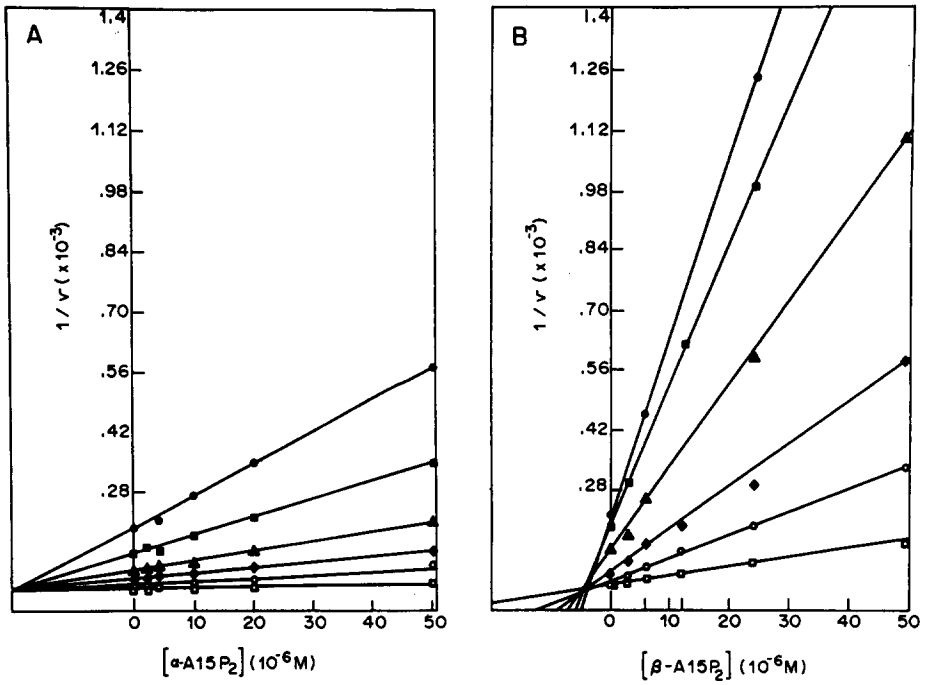

Figure 2. Inhibition of rat liver Fru-1,6-P_2ase by α- and β-arabinose-1,5-P_2. Enzyme activity was measured by the $^{32}P_i$ release assay as described in Methods. Dixon plots of (A) α- (α-A15P_2) and (B) β-arabinose 1,5-P_2 (β-A15P_2) inhibition of Fru-1,6-P_2ase. The enzyme concentration was 1.2 nM and Fru 1,6-P_2 concentrations were (●) 2 μM, (■) 3 μM, (▲) 5 μM, (◆) 10 μM, (○) 20 μM, and (□) 50 μM.

in the *cis* configuration (c.f., β-Fru 2,6-P_2 and β-arabinose 1,5-P_2), in contrast to 6-PF-1-K activation which shows little stereochemical preference. Neither α- nor β-ribose 1,5-P_2 had any inhibitory effect on Fru-1,6-P_2ase (data not shown), just as they had no effect on 6-PF-1-K.

Effect of Arabinose 1,5-P_2 on AMP Inhibition of Fru-1,6-P_2ase.

It has been argued that, since Fru 2,6-P_2 potentiates AMP inhibition of Fru-1,6-P_2ase, the sugar bisphosphate must bind to a separate and distinct allosteric site (4). A corollary to this hypothesis is that a purely competitive inhibitor of the enzyme, which binds only to the active site, would not alter AMP inhibition. Thus, the presence of such an effect on AMP inhibition by a competitive inhibitor would discount the argument that Fru 2,6-P_2 must necessarily bind to an allosteric site. Fig. 3 shows that, in the absence of any effectors, AMP inhibited Fru-1,6-P_2ase with an $I_{0.5}$ of about 42 μM, while in the presence of 3 μM β-arabinose 1,5-P_2 the $I_{0.5}$ was decreased to 14 μM. Thus,

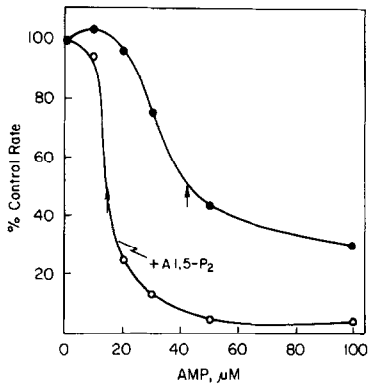

Figure 3. Potentiation of AMP inhibition of Fru-1,6-P_2ase by β-arabinose 1,5-P_2. Enzyme activity was measured by the $^{32}P_i$ release assay as described in Methods. Activity is expressed as the percent of control. The concentration of Fru 1,6-P_2 was 2 μM.

the β-anomer of arabinose 1,5-P_2 which interacts with the active site can also potentiate AMP inhibition. The α-anomer also potentiated AMP inhibition, but 20 to 30-fold higher concentrations were necessary. In the presence of 1 μM Fru 2,6-P_2 the $I_{0.5}$ for AMP inhibition was decreased to 12 μM (data not shown).

Effect of Temperature on K_m for Substrate and Inhibition by Arabinose 1,5-P_2 of Fru-1,6-P_2ase. François et al. found that the effects of Fru 2,6-P_2 and AMP on Fru-1,6-P_2ase vary with temperature in a similar manner, while the K_m for Fru 1,6-P_2 and the $I_{0.5}$ for a substrate analog ([α+β]methyl-Fru 1,6-P_2) did not change with temperature (4). They argued that these findings suggest that Fru 2,6-P_2 does not interact at the active site. We re-examined the effect of temperature on the K_m of Fru-1,6-P_2ase and on inhibition by arabinose 1,5-P_2. As shown in Table I, when the temperature is increased from 15 to 45°C, not only does the K_m increase from 1.6 to 4.4 μM but the $I_{0.5}$ for arabinose 1,5-P_2 also increases from 4.5 to 16 μM. These results are in sharp contrast to those of François et al. (4) and provide more evidence that arabinose 1,5-P_2 interacts at the active site of Fru-1,6-P_2ase.

Effect of Arabinose 1,5-P_2 on Fru 2,6-P_2 Binding to Fru-1,6-P_2ase. It has been shown that Fru 2,6-P_2 binds to Fru-1,6-P_2ase to the extent of 1 mole per mole of enzyme subunit (11). If Fru 2,6-P_2 binds to the active site, it would be expected that a competitive inhibitor would compete for this binding. Fig. 4 illustrates the effect of arabinose 1,5-P_2 upon [2-^{32}P]Fru 2,6-P_2 binding. A

TABLE I

EFFECT OF TEMPERATURE ON K_m FOR SUBSTRATE AND INHIBITION BY β-ARABINOSE 1,5-BISPHOSPHATE OF FRUCTOSE-1,6-BISPHOSPHATASE

Temperature	K_m Fru 1,6-P_2	$I_{0.5}$ β-Arabinose 1,5-P_2
15°	1.6 μM	4.5 μM
30°	3.0 μM	6.8 μM
45°	4.4 μM	16.0 μM

NOTE: Fru-1,6-P_2ase activity was assayed spectrophotometrically as described in Methods. $I_{0.5}$ is defined as the concentration of β-arabinose 1,5-P_2 which gives 50% inhibition at 5 μM Fru 1,6-P_2.

half-maximal decrease in binding was exhibited at 10 μM β-arabinose 1,5-P_2, and 100 μM β-arabinose 1,5-P_2 completely abolished Fru 2,6-P_2 binding. Furthermore, α-arabinose-1,5-P_2 was a less potent inhibitor of Fru 2,6-P_2 binding than the β-anomer; as indicated in Fig. 4, a half-maximal decrease in binding required 80 μM α-arabinose 1,5-P_2. Thus, inhibition of Fru 2,6-P_2 binding exhibited the same anomeric specificity as the inhibition of catalytic activity, providing

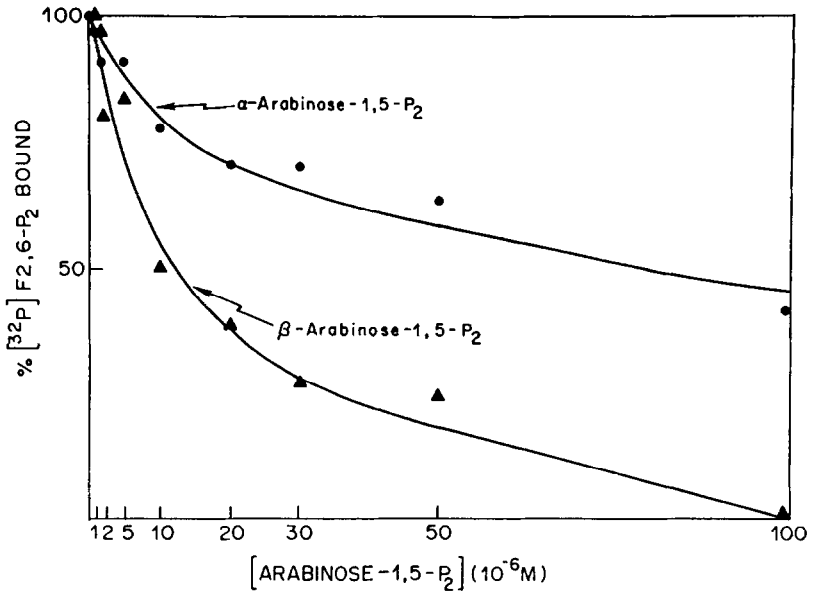

Figure 4. Effect of α- and β-arabinose 1,5-P_2 upon Fru 2,6-P_2 binding to rat liver Fru-1,6-P_2ase. Binding of [2-^{32}P]Fru 2,6-P_2 (F2,6-P_2) to Fru-1,6-P_2ase was measured by the rate of dialysis technique (11,21). The enzyme concentration was 1.5 μM, the Fru 2,6-P_2 concentration was 10 μM, and the increasing arabinose 1,5-P_2 concentrations were as indicated in the figure.

persuasive evidence that Fru 2,6-P_2 is indeed binding at the active site of the Fru-1,6-P_2ase.

DISCUSSION

Both anomers of arabinose 1,5-P_2 are less potent effectors than Fru 2,6-P_2, indicating that the 1-hydroxymethyl group of β-Fru 2,6-P_2, though important, is not essential for activation of 6-PF-1-K or inhibition of Fru-1,6-P_2ase. β-Arabinose 1,5-P_2 was a much more potent inhibitor of Fru-1,6-P_2ase than the α-anomer. Consistent with this finding is evidence indicating that Fru-1,6-P_2ase acts only on the α-anomer of Fru 1,6-P_2 (22) (i.e., the anomer in which the phosphate moieties are in the *cis* configuration). The lack of any effect of α- or β-ribose 1,5-P_2 on either 6-PF-1-K or Fru-1,6-P_2ase activity suggests that the configuration of the C-2 (C-3 in Fru 2,6-P_2) hydroxyl group is important. Although Rose and Warms (23) reported that ribose 1,5-P_2 was a potent activator of erythrocyte 6-PF-1-K, no definite evidence for the identity, composition, or purity of this putative ribose 1,5-P_2 was provided, and it is uncertain that the activating agent was actually ribose 1,5-P_2.

A number of groups have studied the mechanism of Fru 2,6-P_2 potentiation of the inhibition of Fru-1,6-P_2ase by AMP (8,10,11). Binding studies demonstrated that Fru 2,6-P_2 enhances the affinity of the enzyme for AMP (11). The sensitivity of Fru-1,6-P_2ase to AMP inhibition is also increased by the substrate, Fru 1,6-P_2, which enhances AMP binding (11). These results suggest that Fru 2,6-P_2 and Fru 1,6-P_2 both bind to the active site and bring about a conformational change that facilitates AMP binding. Moreover, Fru 2,6-P_2 binding at the active site might induce a conformational change in the enzyme similar to that induced by AMP interaction with the allosteric site. Compatible with this hypothesis is the finding that Fru 2,6-P_2 and AMP both induce UV-difference spectra with saturable absorbance maxima at the same wavelengths (6,11). NMR and EPR studies are consistent with this hypothesis since they indicate that the catalytic site and the AMP binding sites are in close proximity to one another (24). Recently, ^1H and ^{31}P NMR studies also suggested that Fru 2,6-P_2 affects the interaction of AMP with Fru-1,6-P_2ase by interacting with the active site (12,13). In this vein, the present study shows that arabinose 1,5-P_2 is a purely competitive inhibitor of the bisphosphatase and

competitively decreases Fru 2,6-P_2 binding to the enzyme, while potentiating AMP inhibition. The observation that interaction of substate and substrate analogs with the active site can potentiate allosteric behavior is intriguing and, in the case of Fru-1,6-P_2ase, is more consistent with the "ligand exclusion" hypothesis (9,25) than with a classic active versus allosteric site model.

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