The Role of Fructose 2,6-Bisphosphate in Regulation of Fructose-1,6-bisphosphatase*

Simon J. Pilkis, M. Raafat El-Maghrabi, Molly M. McGrane, Jo Pilkis, and Thomas H. Claus

From the Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

(Received for publication, April 17, 1981)

The effect of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate on the inhibition of rat liver fructose-1,6-bisphosphatase by AMP was investigated. When the concentration of fructose 1,6-bisphosphate increased from 1 μM to 50 μM, the concentration of AMP that gave 50% inhibition (IC50) decreased from 50 μM to 16 μM. Fructose 2,6-bisphosphate was also found to potentiate allosteric inhibition of the enzyme by AMP but at much lower concentrations than those required for fructose 1,6-bisphosphate. Thus, both the substrate, fructose 1,6-bisphosphate, and an inhibitor, fructose 2,6-bisphosphate, appear to interact with the allosteric site for AMP.

The basis for this interaction was investigated by studying the effect of fructose bisphosphatases on modification of the enzyme by acetylation with acetylimidazole. Inclusion of fructose 2,6-bisphosphate during the acetylation reaction protected catalytic activity, but the AMP inhibition was abolished. Inclusion of AMP led to a large loss of catalytic activity but the sensitivity of the residual enzyme activity to AMP inhibition was not altered. However, inclusion of fructose 2,6-bisphosphate protected both the catalytic activity and the sensitivity of the enzyme to AMP inhibition. Ultraviolet difference spectroscopy showed that acetylation occurred at tyrosine residues and that fructose 2,6-bisphosphate, and an inhibitor, fructose 2,6-bisphosphate, interact with the allosteric site.

The interaction between the two sites was only revealed after treatment of the enzyme with protein-modifying agents (9–11) or in the presence of high concentrations of substrate (12), and its significance in regulation of enzyme activity in intact cells is uncertain. Recently, it has been reported that fructose-2,6-P2, an allosteric activator of 6-phosphofructo-1-kinase (13–21), is a potent inhibitor of fructose-1,6-bisphosphatase with respect to its substrate and that it potentiated the inhibition of the enzyme by AMP (8, 22). In this report, we have analyzed the interaction between the catalytic site and allosteric site of fructose-1,6-bisphosphatase by studying the effect of fructose-2,6-P2 on the AMP inhibition.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glucose-6-P dehydrogenase, rabbit muscle phosphofructokinase, and phosphoglucone isomerase were from Boehringer-Mannheim. Acetylimidazole, unlabeled tetrasodium d-fructose-1,6-P2, adenosine 5'-monophosphate, and disodium fructose-6-P were obtained from Sigma. All other reagents were of the highest purity commercially available. d-Fructose-1,6-[1-32P]P2, (specific activity 3000 to 6000 C/mole) was prepared starting with carrier-free H3[32P]PO4 as described by Pilkis et al. (8). The concentration of stock solutions of fructose-1,6-P2 and fructose-6-P were determined using the spectrophotometric assay described below for fructose-1,6-bisphosphatase.

**Preparation of Fructose-1,6-bisphosphatase**—Rat liver fructose-1,6-bisphosphatase was prepared by the method of Riou et al. (23). It had a specific activity of 40 units/mg of protein at 30 °C and was homogeneous by the criteria of sodium dodecyl sulfate-slab gel electrophoresis with an apparent subunit molecular weight of 41,000 (24). The ratio of activity at pH 7.5 to that at pH 9.2 at either low (2.5 μM) or high (100 μM) concentrations of fructose-1,6-P2 was 3.4, indicating that little or no proteolysis occurred during the preparation (23). The enzyme was precipitated with (NH4)2SO4 (0 to 50%), dissolved in buffer that contained 20 mM TES, pH 7.5, 0.1 mM EDTA, and 1 mM dithiothreitol, and dialyzed extensively in this buffer in order to remove all fructose-1,6-P2. The enzyme was stored at −20 °C at a concentration greater than 2 mg/ml. For rate measurements the enzyme was diluted appropriately in 50 mM Tris-HCl buffer, pH 7.5, that contained 0.2 mM EDTA, 200 mM KCl, and 1 mM KHPO4. Fresh dilutions of enzyme were prepared daily.

**Assay of Fructose-1,6-bisphosphatase**—Fructose-1,6-bisphosphatase activity was assayed spectrophotometrically (23) or by determining the rate of formation of [1-32P]P2 fructose-1,6-1,2-P2 at 25 °C as previously described (8).

**Preparation and Assay of Fructose 2,6-Bisphosphatase**—Fructose 2,6-P2 was synthesized by base-catalyzed ring opening of fructose 1,2-cyclic 6-bisphosphate and purified by ion exchange chromatography as described by Pilkis et al. (21). The synthetic compound contained no detectable fructose-1,6-P2. Fructose-2,6-P2 is not hydrolyzed by fructose-1,6-bisphosphatase (21).

**Stability Constants of Metal Complexes**—The stability constants of the complexes MgAMP and Mg-Fructose-1,6-P2 at pH 7.5 and 9.2 were taken from Nimmo and Tipton (6). Values for the free concentrations of divalent metal ions and ligands in assays of fructose-1,6-bisphosphatase were calculated on the basis of these values, assuming that no other component of the assay mixture except EDTA chelated the metal ions significantly and that EDTA bound the metal ions quantitatively.

**Acetylation of Fructose 1,6-bisphosphatase**—The reaction with acetylimidazole was carried out at 22 °C in 20 mM TES, pH 7.5, 0.1
mm EDTA, 1 mm dithiothreitol with a protein concentration of 1 mg/ml. The reagent was added at a concentration of 1 mg/ml at the beginning of the incubation. The amount of acetylimidazole added corresponded to a 1000-fold molar excess over the molar quantity of enzyme protein. The acetylated protein was precipitated with ammonium sulfate (60% saturation), dissolved in 10 mM Tris-HCl, pH 7.5, plus 0.9% NaCl, and dialyzed against this buffer for 90 min at 4 °C before analysis. Fructose-1,6-bisphosphatase treated with acetylimidazole was acetylated to the extent of 10 to 12 tyrosine residues/mol of enzyme as determined by changes in absorbance at 278 nm (3) (data not shown). Associated with the acetylation was an 85% loss of enzyme activity. The enzyme acetylation could be reversed by treatment of the enzyme with hydroxylamine with restoration of 85% of the enzyme activity (data not shown).

Determination of Ultraviolet Difference Spectra of Fructose-1,6-bisphosphatase—The ultraviolet difference spectra were recorded on a Cary 219 spectrophotometer using a 0- to 0.05-A scale expansion. Fructose-1,6-bisphosphatase was equilibrated in 20 mM TES, pH 7.5, 0.1 mM EDTA, and 1 mM dithiothreitol. For titration of the enzyme with substrate, fructose-2,6-P₂, or AMP, the tandem-cell method of Henskens and Laskowski (25) was used with a pair of Helma cuvettes (2 x 0.437 cm pathlength). Additions of AMP were made with a Hamilton syringe as described by O'Brien and Kapoor (30). The magnitude of the difference spectrum was a linear function of the protein concentration up to 4 mg/ml. Most measurements were performed on solutions containing 1 to 2 mg/ml. Protein was determined by the method of Lowry et al. (27).

RESULTS

Effect of Fructose-1,6-P₂ on Inhibition by AMP—The inhibition of fructose-1,6-bisphosphatase by AMP as a function of the fructose-1,6-P₂-concentration was investigated at pH 7.5 and at pH 9.2 where the nucleotide has been reported to have a much smaller effect on the enzyme (4, 6, 28, 29). Fig. 1A shows that at pH 7.5 AMP inhibition of enzyme activity was enhanced as the concentration of substrate increased from 1 to 50 μM. The concentration of AMP that gave 50% inhibition (S₀.₅) decreased from 49 μM with 1 μM fructose-1,6-P₂ to 16 μM with 50 μM fructose-1,6-P₂. Increasing the fructose-1,6-P₂ concentration also resulted in a small decrease in the cooperative interaction of AMP as judged by a decrease in the slope of a Hill-type plot from 1.98 with 1 μM fructose-1,6-P₂ to 1.65 with 50 μM fructose-1,6-P₂ (Fig. 1A, inset).

At pH 9.2, the S₀.₅ for AMP was 158 μM in the presence of 1 μM fructose-1,6-P₂, and increasing the substrate concentration to 50 μM did not reduce the S₀.₅ (Fig. 1B). However, the cooperative interaction of AMP was decreased at the higher pH. The slope of a Hill-type plot was 1.33 in the presence of 1 μM fructose-1,6-P₂ and increasing the substrate concentration decreased the slope so that at high substrate concentrations it approached 1.0 and no cooperativity was evident (Fig. 1B, inset).

Effect of Fructose-2,6-P₂ on Fructose-1,6-bisphosphatase as a Function of pH—Fructose-2,6-P₂ is a potent inhibitor of rat liver fructose-1,6-bisphosphatase (8, 22). Fig. 2 shows the pH dependence of enzyme activity in the absence and presence of fructose-2,6-P₂. The enzyme exhibited a pH optimum at pH 7.45. Addition of increasing concentrations of fructose-2,6-P₂ resulted in increasing inhibition of activity at pH 7.5 until the curve became almost flat in the presence of 5 μM fructose-2,6-P₂. Little or no inhibition was observed at pH 9.2. This pH dependency is similar to that observed for AMP; both effectors had optimal effects at pH 7.5 and had little effect at pH 9.2 (Fig. 1) (4, 6, 28, 29).

Effect of Mg²⁺ Ions on Fructose-1,6-bisphosphatase—Since fructose bisphosphatase from ox (6), rabbit (30), and beef liver (31) have all been shown to be allosterically activated by divalent cations, we investigated the role of Mg²⁺ ions on the activity of the rat liver enzyme. Double-reciprocal plots of initial velocity versus fructose-1,6-P₂ concentration at fixed levels of free Mg²⁺ ions were linear at pH 7.5 (Fig. 3A) and at pH 9.2 (data not shown). When the slopes were replotted against 1/[free Mg²⁺], the lines were parabolic at pH 7.5 (inset, Fig. 3A) but linear at pH 9.2 (data not shown). Furthermore, the double-reciprocal plots of initial velocity versus free Mg²⁺ concentration at several fructose-1,6-P₂ concentrations were parabolic at pH 7.5 (Fig. 3B) but linear at pH 9.2 (data not shown). Thus, rat liver fructose-1,6-bisphosphatase appears to...
respond to Mg$^{2+}$ ions sigmoidally at pH 7.5 but hyperbolically at pH 9.2.

We also investigated the effects of fructose-2,6-P$_2$ on the response of fructose-1,6-bisphosphatase to Mg$^{2+}$ ions. Double-reciprocal plots of initial velocity versus substrate concentration at fixed levels of free Mg$^{2+}$ ions and in the presence of 0.5 mM fructose-2,6-P$_2$ were linear at pH 7.5 (Fig. 4) and at pH 9.2 (data not shown). A replot of the slopes obtained at pH 7.5 versus 1/[free Mg$^{2+}$] (inset, Fig. 4) gave a curve that was less parabolic than that observed in the absence of fructose-2,6-P$_2$ (inset, Fig. 3A). The slope replot was linear at pH 9.2 (data not shown). These results suggest that fructose-2,6-P$_2$ can influence the sigmoidal response of the enzyme to activating cation at pH 7.5.

Effect of Fructose-2,6-P$_2$ on Inhibition by AMP—Fructose-2,6-P$_2$ also influences the allosteric inhibition of the enzyme by AMP (8, 22). Fig. 5 shows that this effect is dependent upon the concentration of fructose-2,6-P$_2$. At pH 7.5, the $S_{0.5}$ for AMP was estimated to be 40 μM in the absence of fructose-2,6-P$_2$ and 14 μM, 10 μM, and 6 μM in the presence of 0.1, 0.2, and 1 μM fructose-2,6-P$_2$, respectively (Fig. 5A). The effect of fructose-2,6-P$_2$ on AMP inhibition was decreased at pH 9.2 (Fig. 5B). The $S_{0.5}$ for AMP was estimated to be 158 μM in the absence of fructose-2,6-P$_2$ and 100 μM and 27 μM in the presence of 0.2 and 1 μM fructose-2,6-P$_2$, respectively.

Since inhibition by AMP at pH 7.5 was also enhanced by increasing concentrations of fructose-1,6-P$_2$ (Fig. 1A), we investigated the effect of increasing concentrations of substrate on AMP inhibition of the enzyme in the presence of 1 μM fructose-2,6-P$_2$ (Fig. 6). Increasing the concentration of substrate from 2.5 to 50 μM only lowered the $S_{0.5}$ for AMP from about 7 to 4 μM at pH 7.5 (Fig. 6A). This small effect is due to the fact that the high concentration of fructose-2,6-P$_2$ had already decreased the $S_{0.5}$ for AMP from 40 to 7 μM in the presence of 2.5 μM fructose-1,6-P$_2$ (Fig. 5). In contrast to the results of pH 7.5, increasing the substrate concentration from 1 μM to 50 μM at pH 9.2 raised the $S_{0.5}$ for AMP from about 20 μM to 120 μM (Fig. 6B). Thus, at pH 9.2, where fructose-2,6-P$_2$ is not an effective inhibitor of the enzyme (Fig. 2), high concentrations of substrate actually prevented fructose-2,6-P$_2$ from potentiating the AMP inhibition.

Table I summarizes the data on the effect of fructose-1,6-P$_2$ and fructose-2,6-P$_2$ on the $S_{0.5}$ for AMP and on the cooperativity of the enzyme for AMP as measured by the slope (H) of Hill plots. At pH 7.5, both fructose-1,6-P$_2$ and fructose-2,6-P$_2$ lowered the $S_{0.5}$ for AMP. Fructose-2,6-P$_2$ was much more effective than fructose-1,6-P$_2$ in potentiating AMP inhibition. AMP inhibition also exhibited marked cooperativity even in

![Fig. 3](image)

**Fig. 3.** Effect of magnesium concentration on fructose-1,6-bisphosphatase activity. Panel A, double-reciprocal plots of initial velocity against fructose-1,6-[1-32P]P$_2$ concentration at pH 7.5 and at a series of fixed concentrations of free Mg$^{2+}$ ions. The radioactive assay was used and the concentrations of free Mg$^{2+}$ ions are given in the figure. Inset, the variation of the slopes as a function of free Mg$^{2+}$ ion concentration at pH 7.5 and at a series of fixed concentrations of fructose-1,6-[1-32P]P$_2$. The fructose-1,6-P$_2$ concentrations are given beside each curve.
the presence of saturating concentrations of fructose-1,6-P₂ as evidenced by H values of 1.6 to 2.0. The presence of fructose-2,6-P₂ reduced the cooperativity of the enzyme with respect to AMP and the H values approached 1.0 in the presence of 1 μM fructose-2,6-P₂. Very different results were seen at pH 9.2. Increasing the substrate concentration increased rather than decreased the S₀₅₀ for AMP, even though H values decreased as they did at pH 7.5. The addition of fructose-2,6-P₂ had little inhibitory effect on enzyme activity in the absence of AMP. In the presence of AMP, fructose-2,6-P₂ had no effect on H values even though it decreased the S₀₅₀ for AMP at all concentrations of fructose-1,6-P₂.

Effect of Fructose-2,6-P₂ on Acetylation of Fructose-1,6-bisphosphatase by Acetylimidazole—It has been shown that both the catalytic activity and sensitivity to AMP of “alkaline” fructose-1,6-bisphosphatase from rabbit liver are altered when the enzyme is treated with reagents such as diazobenzene sulfonic acid or acetylimidazole (9-11). Although those results were obtained with a proteolytically modified form of the enzyme (9, 10), they are consistent with the presence of tyrosyl residues in regions of the enzyme that interact with the substrate and with AMP. We chose to investigate the effect of acetylation of the native rat liver enzyme by acetylimidazole in an attempt to determine whether fructose-2,6-P₂ interacts with tyrosine groups at the enzyme’s catalytic or allosteric site. The results are summarized in Table II. Incubation of the enzyme with acetylimidazole in the absence of any additions led to loss of 80 to 90% of catalytic activity and to complete loss of inhibition by 100 μM AMP. Acetylation of the enzyme in the presence of fructose-1,6-P₂ protected catalytic activity but allosteric inhibition by AMP was still lost. AMP protected the allosteric site but 80 to 85% of the catalytic activity was lost. Fructose-2,6-P₂ protected both catalytic activity and allosteric inhibition by 100 μM AMP. The S₀₅₀ for AMP was also unaltered if fructose-2,6-P₂ was present during the acetylation (data not shown). These results suggest that native rat liver fructose-1,6-bisphosphatase, like the alkaline rabbit liver enzyme, contains two groups of essential tyrosine

**Table I**

Summary of the effects of fructose-1,6-P₂ (F-1,6-P₂) and fructose-2,6-P₂ (F-2,6-P₂) on the S₀₅₀ and Hill slope (H) for AMP inhibition of fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase was assayed with the radioactive assay as described under “Experimental Procedures.” Per cent inhibition by fructose-2,6-P₂ refers to the inhibition obtained at a given substrate concentration in the absence of AMP. The S₀₅₀ for AMP and the values of the Hill slope (H) were obtained from Hill-type plots similar to those shown in Fig. 1. The determination of S₀₅₀ and H were repeated 4 to 6 times with a standard error of ±5% for both H and S₀₅₀.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity</th>
<th>AMP inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
<td>90%</td>
</tr>
<tr>
<td>Acetylimidazole</td>
<td>5</td>
<td>0%</td>
</tr>
<tr>
<td>Acetylimidazole + AMP (1 μM)</td>
<td>5</td>
<td>85%</td>
</tr>
<tr>
<td>Acetylimidazole + fructose-1,6-P₂ (1 μM)</td>
<td>38</td>
<td>10%</td>
</tr>
<tr>
<td>Acetylimidazole + fructose-2,6-P₂ (1 μM)</td>
<td>40</td>
<td>88%</td>
</tr>
</tbody>
</table>

**Table II**

Effect of acetylation on the activity of rat liver fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase (1 mg/ml, specific activity 40 units/mg of protein) equilibrated in 20 mM TES buffer, pH 7.5. Acetylimidazole (1 mg/ml) was added at the beginning of the experiments and incubation was continued for 90 min. The protein was precipitated with ammonium sulfate (80% saturation), dissolved in buffer, and dialyzed against buffer for 2 h at 0 °C. Catalytic activity was assayed at pH 7.5 using the spectrophotometric assay with 100 μM fructose-1,6-P₂ as substrate in the presence or absence of 100 μM AMP.
residues which can undergo acetylation; one associated with the catalytic site and the other with the allosteric site.

_Ultraviolet Different Spectra of Fructose-1,6-bisphosphatase Induced by AMP in the Presence and Absence of Fructose-1,6-P_2 and Fructose-2,6-P_2—In order to further define the interaction of AMP, fructose-1,6-P_2, and fructose-2,6-P_2 with the catalytic and allosteric site of fructose-1,6-bisphosphatase, we have used UV difference spectroscopy to examine the consequence of binding of these effectors to the enzyme. Fructose-2,6-P_2 and fructose-1,6-P_2 induced distinctly different difference spectra (Fig. 7). The addition of 125 μM fructose-1,6-P_2 produced distinct minima at 298 nm and 242 nm. Fructose-2,6-P_2 (12.5 μM) produced the same minima as did fructose-1,6-P_2 but the magnitude of the change was greater.

In addition, fructose-2,6-P_2 produced two clearly distinguishable maxima at 288 nm and 279 nm which were not seen with fructose-1,6-P_2. Higher concentrations of fructose-2,6-P_2 caused no further increase in magnitude of the difference spectra. The concentration of fructose-2,6-P_2 that was necessary to elicit a half-maximal change in absorbance at 279 nm or 288 nm was about 4 μM but the same concentration of fructose-1,6-P_2 did not elicit any discernible difference spectra (data not shown). The addition of AMP resulted in a difference spectrum which showed the same two maxima at 288 nm and 279 nm as did fructose-2,6-P_2 (Fig. 8). The minimum at 298 nm seen with fructose-1,6-P_2 and fructose-2,6-P_2 was barely visible when AMP was added alone. The major peaks exhibited an increase in amplitude in the presence of increasing concentrations of AMP and this change reached a plateau value at about 125 μM. The concentration of AMP which was necessary to give a half-maximal change in absorption at 279 nm was about 48 μM.

These spectral differences are characteristic of perturbations in the environment of aromatic residues of the enzyme, and several lines of evidence suggest that the changes are due to interaction of the effectors with tyrosine rather than tryptophan residues. First, the changes occur at wavelengths which are characteristic of tyrosine residues in proteins (32). Second, acetylation of the enzyme with acetylimidazole in the absence of any substrate or effector obliterated all of the changes observed with AMP, fructose-1,6-P_2, or fructose-2,6-P_2 (data not shown). Acetylimidazole reacts with tyrosine but not tryptophan residues (33). Third, rat liver fructose-1,6-bisphosphatase has been reported to contain little or no tryptophan (5, 34, 35).²

Spectral titration of the enzyme with AMP was also performed in the presence of fructose-2,6-P_2 and fructose-1,6-P_2. Fig. 9 shows a plot of the increase in absorption difference at 279 nm against the concentration of added AMP. In the presence of 2.5 or 12.5 μM fructose-2,6-P_2, the concentration of AMP necessary to elicit a half-maximal change in extinction at 279 nm decreased from 48 μM in the absence of fructose-

² We have been unable to detect any tryptophan in our preparation of the rat liver fructose-1,6-bisphosphatase. M. McGrane and S. J. Pilks, unpublished results.
2.6-P$_2$ to 38 and 16 μM, respectively. In the presence of 125 μM fructose-1,6-P$_2$, the concentration of AMP necessary to elicit a half-maximal change in extinction at 279 nm was reduced to 22 μM. Thus, fructose-2,6-P$_2$ was more effective in enhancing the perturbation of tyrosine residues in the enzyme by AMP than was fructose-1,6-P$_2$. These results are in excellent agreement with the kinetic experiments where fructose-2,6-P$_2$ was more effective than fructose-1,6-P$_2$ in enhancing the affinity of the enzyme for AMP (Table I).

**DISCUSSION**

Fructose-2,6-P$_2$ has been shown to be a potent competitive inhibitor of fructose-1,6-bisphosphatase (8), but a number of observations suggest that this effector also acts at the allosteric site for AMP. First, fructose-2,6-P$_2$ acts via the sigmoidal-type response of the enzyme to Mg$^{2+}$ ions (Figs. 3 and 4). Second, it potentiates AMP inhibition and decreases the $H$ values from 2.0 to 1.0 (Table I). Third, it protects both the allosteric and catalytic site from acetylation by acetylimidazole while fructose-1,6-P$_2$ only protects the catalytic site (Table II). Fourth, fructose-2,6-P$_2$ enhances AMP interaction with the enzyme as measured by UV difference spectroscopy to a greater extent than fructose-1,6-P$_2$ (Fig. 9). Fructose-2,6-P$_2$ and AMP both induce a maximum at 279 nm while a relatively high concentration of fructose-1,6-P$_2$ does not (Figs. 8 and 9). These results support the notion that binding of fructose-1,6-P$_2$ to the catalytic site induces a conformational change in the enzyme that enhances the effects of AMP. While it seems reasonable to assume that fructose-2,6-P$_2$ behaves in a manner similar to fructose-1,6-P$_2$, further elucidation of the mechanism of its effects may await the results of studies on the binding of fructose-2,6-P$_2$ in the absence and presence of AMP and fructose-1,6-P$_2$. These studies are currently in progress.

**REFERENCES**


3 M. McGrane and S. J. Pilks, unpublished results.