Kinetic Studies on the Mechanism and Regulation of Rabbit Liver Fructose-1,6-bisphosphatase*

Feng Liu and Herbert J. Fromm
From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

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The interaction of Mg²⁺, AMP, and fructose 2,6-bisphosphate with respect to rabbit liver fructose-1,6-bisphosphatase was investigated by studying initial-rate kinetics of the system at pH 9.5. A rapid-equilibrium Random Bi Bi mechanism is suggested for the rabbit liver enzyme from the kinetic data. Our kinetic findings indicate that Mg²⁺ and the inhibitor AMP are mutually exclusive in their binding to fructose-1,6-bisphosphatase. This probably is the mechanism for AMP regulation of fructose-1,6-bisphosphatase and thus, to some extent, gluconeogenesis. A kinetic model for the interaction of these ligands with respect to rabbit liver fructose-1,6-bisphosphatase is presented.

Fructose 1,6-bisphosphatase occurs at a crucial control point in carbohydrate metabolism. In the presence of divalent metal ions, the enzyme catalyzes the hydrolysis of d-fructose 1,6-bisphosphate (d-Fru-1,6-P₂) to d-Fru-6-P and inorganic phosphate. Fructose-1,6-bisphosphatase from rabbit liver is a homotetramer with a subunit molecular weight of 35,000 (1, 2). It has been shown from binding investigations that there are two metal-binding sites/enzyme subunit: a high affinity "structural" site and a low affinity "catalytic" site (3-5). Binding of metal ions to the catalytic site occurs only in the presence of substrates or substrate analogs (3-6). Although the terms structural and catalytic denote specific functions for the two sites, there is no direct evidence as to how either metal ion participates in the catalytic mechanism.

It is now well established that the coordinated regulation of fructose-1,6-bisphosphatase and phosphofructokinase is controlled by AMP and Fru-2,6-P₂ (7-9). Kinetic (10-12) and binding investigations (13, 14) have shown that Fru-2,6-P₂ is a competitive inhibitor of the substrate, Fru-1,6-P₂. Although the model for competitive inhibition predicts that two ligands are mutually exclusive in their binding to the enzyme, it is possible for an allosteric inhibitor to exhibit competitive kinetics; however, recent results from nuclear magnetic resonance (NMR) spectroscopy (15) and x-ray diffraction studies (16) strongly support the hypothesis that Fru-2,6-P₂ binds to the enzyme at the active site.

AMP is clearly recognized as an allosteric inhibitor of fructose-1,6-bisphosphatase. The inhibition of the enzyme by AMP is nonlinear noncompetitive with respect to Fru-1,6-P₂ from the forward reaction direction (17-19) and nonlinear noncompetitive with respect to both Fru-6-P and Pᵢ in the reverse reaction (19). Recent fluorescence investigations have shown that the binding of FMP, a fluorescent analog of AMP, is enhanced in the presence of Fru-2,6-P₂ (20). This observation is further supported by results from NMR studies that indicate that the rate constant for dissociation of AMP from the enzyme-AMP-Fru-2,6-P₂ complex is 17-fold lower than that of AMP in the AMP-enzyme binary complex (21).

The effect of metal ions on AMP inhibition is unclear. It was reported that Mg²⁺ can reduce both AMP binding and inhibition (22-24). Binding of metal ions at the low affinity metal-binding site is observed in the absence of substrate, and the binding of metal ions is competitive with the binding of FMP (20). In the presence of substrate analogs, however, the concentration of each metal ion required to displace FMP is reduced by approximately one order of magnitude (20). Unpublished NMR studies by Ganson and Fromm suggest that when the stoichiometry of Mn⁺ to enzyme exceeds one/subunit, metal, which according to the known dissociation constant for the enzyme-metal complex should be bound, appears free in solution both in the presence and absence of products. In addition, recent x-ray diffraction studies indicate that there is only one Mg²⁺ bound to each pig kidney enzyme subunit although there seems to be two metal-binding sites.

Our purpose in undertaking the studies in the present report was to establish the kinetic mechanism for fructose-1,6-bisphosphatase and to define the role of Mg²⁺ and the regulatory ligands AMP and Fru-2,6-P₂. The results of this study are consistent with a rapid-equilibrium Random Bi Bi mechanism. A kinetic model for the interaction of the substrate, metal, and the regulatory ligands with respect to rabbit liver fructose-1,6-bisphosphatase is presented.

EXPERIMENTAL PROCEDURES

Materials—Rabbit liver fructose-1,6-bisphosphatase was purified from frozen liver (Pel-Freez Biologicals, Rogers, AR) according to the method of Ulm et al. (25). 1 mM phenylmethylsulfonyl fluoride was included in the homogenate buffer. Fructose-1,6-bisphosphatase activity was measured as described elsewhere (25). The specific activity of the enzyme was 20, and the pH 7.5/0.9 activity ratio of the purified enzyme was above 2.0 and remained constant throughout purification. Glucose-6-phosphate dehydrogenase and phosphoglucomutase were purchased from Boehringer Mannheim; cadmium chloride was purchased from Mallinckrodt Chemical Works; 8-hydroxyquinoline was bought from Fisher; and NADP, Fru-6-P, Fru-1,6-P₃, Fru-2,6-P₃.

1 The abbreviations used are: Fru-1,6-P₂, fructose-1,6-bisphosphate; Fru-2,6-P₂, fructose-2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; FMP, formycin 5'-monophosphate.

2 W. Lipscomb, personal communication.
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MgCl₂ and diethanolamine were obtained from Sigma.

Fructose-1,6-P₂ concentration was assayed enzymatically (26), and the acid hydrolysis product of Fru-2,6-P₂ (namely, Fru-6-P) was assayed in the same way after neutralization to determine the concentration of Fru-2,6-P₂ in the stock solution. Atomic absorption spectroscopy was employed to measure the concentrations of the stock Mg₂⁺ solution. The AMP concentration in the stock solution was determined by measuring the absorbance at 259 nm by using an extinction coefficient of ε₉₀₉ = 15,400 M⁻¹ cm⁻¹, at pH 7.0.

Distilled deionized water was used in all experiments, and all the reagents were of the highest purity available commercially. All solutions used in the experiments, including water, were Chelex-treated. Plastic tubes were acid washed and thoroughly rinsed before use.

Methods—Preceding kinetic experiments, fructose-1,6-bisphosphatase was exhaustively dialyzed against buffer containing 5 mM MgCl₂ to assure removal of the Fru-1,6-P₂ used to elute the enzyme from carboxymethylcellulose during purification (26). Subsequently, the enzyme was dialyzed against 5 mM mops, pH 6.5, overnight by using a rocking dialyzer. After dialysis, the enzyme sample was concentrated in an Amicon Centriprep 30 microconcentrator to a final concentration of about 0.1 mM (enzyme monomer), assuming a subunit Mr = 35,000 (7, 8). Protein concentrations were determined by measuring the absorbance at 280 nm by using an extinction coefficient of 0.89 for 0.1% (27). Ammonium sulfate was removed from solutions of auxiliary enzymes by exhaustive dialysis at pH 8.0 against 5 mM diethanolamine.

The stability constants of the complexes of Mg·NADP and Mg·Fruct-2,6-P₂ were determined at pH 8.0 in the diethanolamine/HC1 buffer by using 8-hydroxyquinoline (28). The values are 450 M⁻¹ for Mg·NADP and 180 M⁻¹ for Mg·Fruct-2,6-P₂. The stability constants for Mg·Fruct-1,6-P₂·Mg·AMP, and Mg·Cd are 350 M⁻¹, 40 M⁻¹ (17), and 0.01 M⁻¹ (29), respectively. The free concentrations of Mg₂⁺ in the enzyme assay solutions were calculated on the basis of these values, and they were not adjusted for Mg₂⁺ bound to KCl, which was assumed constant in all assays.

A coupled spectrofluorometric assay was employed to measure the rate of NADPH formation at 25 °C.Velocity is expressed in arbitrary fluorescence units/min. The excitation and emission wavelengths of a Spex spectrofluorometer were 340 and 476 nm, respectively. Slits were fixed at 1.25 mm. The assay mixture contained 50 mM diethanolamine, pH 9.5, 0.1 mM NADP, 2 units of glucose-6-phosphate dehydrogenase, and 2 units of phosphoglucomutase, in a final volume of 2.5 ml. Fructose-1,6-bisphosphatase employed in the experiments, which varied in concentration from 0.3 to 0.5 mM, had been preincubated with MgCl₂ for at least 20 min, and the reaction was started by the addition of Fru-1,6-P₂. Initial rate data were analyzed by using a computer program written in OMNITAB II language, with an α value of 2.0 (30). The kinetic data were fit to a variety of models and tested for goodness of fit using the F test (30).

RESULTS

Fructose-1,6-bisphosphatases isolated from different sources exhibit many common properties. The enzyme has an absolute requirement for divalent metal ions for its activity, and its activity is synergistically inhibited by AMP and Fru-1,6-P₂ in many species (9).

It has been proposed that fructose-1,6-bisphosphatase has two sets of divalent metal binding sites, a structural and a catalytic metal-binding site (3-5). Results from binding experiments showed that metal ions can bind to the second site only in the presence of substrate analogs (3-5). Recent studies from our laboratory have shown that binding of metal ions is competitive with respect to FMP, a fluorescence analog of AMP (20). However, we did not in any way infer from this finding that the metal and AMP sites overlap. In addition, we also found that metal binding occurred in the absence of substrates (20). Kinetic experiments were undertaken in an attempt to further understand the function of the metal ions involved in the enzyme kinetic mechanism and to understand the interactions of the substrates, metal ions, and inhibitors (AMP or Fru-2,6-P₂). It had been reported that when data from primary plots are replotted, the slope and intercept replots against free [Mg²⁺] are parabolic at neutral pH, whereas they are linear at alkaline pH (17). It was for this reason that all our kinetic experiments were done at pH 9.5.

Kinetic Studies in the Absence of Inhibitors—Fig. 1 shows the double-reciprocal plots of initial velocity against [Fru-1,6-P₂] (Fig. 1A) or against free [Mg²⁺] (Fig. 1B). When Fru-1,6-P₂ concentration was varied at different fixed concentrations of Mg²⁺, a family of lines intersecting below the abscissa was obtained (Fig. 1A). The family of curves for various concentrations of free Mg²⁺ at different fixed concentrations of Fru-1,6-P₂ is similar to those observed when Fru-1,6-P₂ was varied (Fig. 1B). When the data presented in Fig. 1 were computer fitted to a variety of kinetic equations, which included the Ping Pong Bi Bi case, the sequential Bi Bi mechanism, and steady-state and rapid-equilibrium mechanisms in which the sequence of ligand addition is metal, substrate, metal, in that order, leading to a ternary complex with enzyme, the best fit was obtained with the rate equation:

$$U = \frac{V_{max}A·B}{A·B + K_a·B + K_a·A + K_{a}·K_b}$$  

(1)

where \(V_{max}\), \(A\), \(B\), \(K_a\), \(K_b\), and \(K_{a}\) represent the maximum velocity of the reaction, the concentration of the free Mg²⁺, the concentration of the free Fru-1,6-P₂, the Michaelis constant for Mg²⁺, the Michaelis constant for Fru-1,6-P₂, and the dissociation constant for Mg²⁺, respectively. The values of the constants were determined by using the computer program of Siano et al. (30) with an α = 2.0 and are summarized in Table

![Fig. 1. Plot of the reciprocal initial velocity against the reciprocal molar concentration of Fru-1,6-P₂ at 0.33 mM (A), 0.25 mM (B), 0.2 mM (C), 0.167 mM (D), and 0.14 mM Mg²⁺ (E) and the reciprocal molar concentration of Mg²⁺ at 5 mM (F), 1.67 mM (G), 1.67 µM (H), and 0.714 µM (J), and 0.556 µM Fruc-1,6-P₂ (O). The coupled spectrofluorometric assay was performed at 25 °C in 50 mM diethanolamine buffer, pH 9.5, containing 0.1 mM KCl.](http://www.jbc.org)
The findings illustrated in Fig. 1, A and B, serve to exclude the participation of more than 1 mol of metal binding to 1 mol of fructose-1,6-bisphosphatase. Had the binding stoichiometry been two metal ions/ enzyme subunit, [metal]² terms would have been generated in the rate expression analogous to Equation 1. On the basis of these results, it is reasonable to conclude that the suggestion that the sequence of ligand binding to enzyme: metal, substrate, metal, is untenable at pH 9.5. The requirement for structural and catalytic metal-binding sites as an integral part of the fructose-1,6-bisphosphatase mechanism in the physiological pH range (3-5) is clearly not excluded by these kinetic results.

**Inhibition Studies with Fru-2,6-P₃ and Cd²⁺**—The initial rate experiments shown in Fig. 1, A and B, eliminated a number of possible kinetic mechanisms from consideration for fructose-1,6-bisphosphatase. However, they did not permit a choice to be made between the steady-state Ordered Bi Bi mechanism and the rapid-equilibrium Random Bi Bi case. The use of competitive dead-end inhibitors is a valuable tool for distinguishing between these two possibilities (31). Studies were therefore carried out with a competitive inhibitor of the substrate, Fru-2,6-P₃, and a competitive inhibitor of the metal activator, Cd²⁺.

A number of investigators have shown from kinetic studies that Fru-2,6-P₃ is a competitive inhibitor of the substrates in both the forward (10, 11) and the reverse (12) fructose-1,6-bisphosphatase reactions. Similar findings were also obtained in our experiments for rabbit liver fructose-1,6-bisphosphatase using 0.1 M KCl, 5 μM Fru-1,6-P₂.

The coupled spectrofluoroscence assay was performed at 25 °C in 50 mM diethanolamine buffer, pH 9.5, containing 0.1 M KCl, 5 μM Fru-1,6-P₂.

**TABLE I**

<table>
<thead>
<tr>
<th>Kinetic constants for the fructose-1,6-bisphosphatase reaction</th>
<th>Enzyme interaction</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$</td>
<td>$E + Mg^{2+} = E-Mg^{2+}$</td>
<td>0.3 ± 0.18 mM</td>
</tr>
<tr>
<td>$K_i$</td>
<td>$E + Fru-1,6-P_2 = E.Fru-1,6-P_2$</td>
<td>1.3 ± 0.14 μM</td>
</tr>
<tr>
<td>$K'_i$</td>
<td>$E.Fru-1,6-P_2 + Mg^{2+} = E.Fru-1,6-P_2.Mg^{2+}$</td>
<td>1.1 ± 0.12 mM</td>
</tr>
<tr>
<td>$K_{in}$</td>
<td>$E.Mg^{2+} + Fru-1,6-P_2 = E.Fru-1,6-P_2.Mg^{2+}$</td>
<td>4.9 ± 0.76 μM</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Kinetic constants for the inhibition by AMP, Fru-2,6-P₃, and Cd²⁺ in the fructose-1,6-bisphosphatase reaction</th>
<th>Enzyme interaction</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{i}$</td>
<td>$E + Fru-2,6-P_3 = E.Fru-2,6-P_3$</td>
<td>1.3 μM</td>
</tr>
<tr>
<td>$K_{ai}$</td>
<td>$E.Mg^{2+} + Fru-2,6-P_3 = E.Mg^{2+}.Fru-2,6-P_3$</td>
<td>1.5 μM</td>
</tr>
<tr>
<td>$K_{ain}$</td>
<td>$E.Fru-2,6-P_3 + Mg^{2+} = E.Mg^{2+}.Fru-2,6-P_3$</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>$K_{o}$</td>
<td>$E + Cd^{2+} = E.Cd^{2+}$</td>
<td>0.7 μM</td>
</tr>
<tr>
<td>$K_{oi}$</td>
<td>$E.Fru-1,6-P_2 + Cd^{2+} = E.Fru-1,6-P_2.Cd^{2+}$</td>
<td>4.1 μM</td>
</tr>
<tr>
<td>$K_{oin}$</td>
<td>$E.Cd^{2+} + Fru-1,6-P_2 = E.Fru-1,6-P_2.Cd^{2+}$</td>
<td>0.3 μM</td>
</tr>
<tr>
<td>$K_{ai}$</td>
<td>$E + 2AMP = E.2AMP$</td>
<td>26.0 (μM)²</td>
</tr>
<tr>
<td>$K_{ai}$</td>
<td>$E.Fru-1,6-P_2 + 2AMP = E.Fru-1,6-P_2.2AMP$</td>
<td>18.6 (μM)²</td>
</tr>
<tr>
<td>$K_{ain}$</td>
<td>$E.2AMP + Fru-1,6-P_2 = E.Fru-1,6-P_2.2AMP$</td>
<td>0.9 μM</td>
</tr>
</tbody>
</table>

**Fig. 2.** Double-reciprocal plot of $V/ initial velocity$ against $1/Mg^{2+}$ concentration at 0 μM (○), 0.4 μM (△), 0.8 μM (◇), 1.2 μM (□), and 1.6 μM Fru-2,6-P₃ (○). The coupled spectrofluorescence assay was performed at 25 °C in 50 mM diethanolamine buffer, pH 9.5, containing 0.1 M KCl, 5 μM Fru-1,6-P₂.

**Fig. 3.** Double-reciprocal plot of $V/ initial velocity$ against $1/Mg^{2+}$ concentration at 0 μM (○), 1 μM (△), 2 μM (◇), 3 μM (□), and 4 μM Cd²⁺ (■). The coupled spectrofluorescence assay was performed at 25 °C in 50 mM diethanolamine buffer, pH 9.5, containing 0.1 M KCl, 5 μM Fru-1,6-P₂.
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(Fig. 4). The best fit of the experimental data was to Equation 3:

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} \left[ 1 + \frac{K_0}{A} \left( 1 + \frac{I}{K_{i0}} \right) + \frac{K_0}{B} + \frac{K_u K_i}{AB} \left( 1 + \frac{I}{K_{i0}} \right) \right]$$

where $K_{i0} = [E]/[EI]$ and $K_{i0} = [EB]/[EBI]$. If the mechanism is rapid-equilibrium Random Bi Bi, a third dissociation constant $K_{u0} = [EI]/[EBI]$. Other models tested with the data of Fig. 3 were for linear noncompetitive and linear uncompetitive inhibition.

The data depicted in Fig. 4 were also tested for linear competitive and linear uncompetitive inhibition, which gave inferior fits compared with the linear noncompetitive model. The values of the inhibition constants were calculated from the data presented in Fig. 4 and are summarized in Table II. From these results, and those shown in Fig. 2, it is clear that an Ordered Bi Bi mechanism in which Fruct-1,6-P binds to the enzyme before the Mg$^{2+}$ is unlikely.

Kinetic Studies of AMP Inhibition—It was found from fluorescence experiments that the binding of metal ions to rabbit liver fructose-1,6-bisphosphatase is competitive with the binding of FMP (20). This observation led us to further characterize the role of AMP with respect to the catalytic metal ion involved in the enzyme kinetic mechanism. Figs. 5 and 6 show Lineweaver-Burk plots of kinetic data at different fixed AMP concentrations. A number of mechanisms were eliminated by fitting the data to different kinetic equations involving linear and nonlinear inhibition. The best fits obtained for the data shown in Figs. 5 and 6 are consistent with the rapid-equilibrium Random mechanism along with the interactions:

$$K_i E + 2I = EI_i$$

$$K_u EB + 2I = EBI_i$$

$$K_u E + B = EBI_i$$

where the derived rate equation predicts slope-parabolic competitive inhibition (Fig. 5) and intercept-parabolic, slope-parabolic noncompetitive inhibition (Fig. 6). Equation 7 best describes the data shown in Figs. 5 and 6.

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} \left[ 1 + \frac{K_u}{A} \left( 1 + \frac{I}{K_{i0}} \right) + \frac{K_u K_i}{AB} \left( 1 + \frac{I}{K_{i0}} \right) \right]$$

In Equation 7, $K_{i0}$ and $K_{u0}$ represent the dissociation constants for AMP dissociation from the enzyme-AMP$^+_2$ and enzyme-Fruct-1,6-P$_2$-AMP$^+_2$ complexes, respectively. The values of these constants were determined from the data presented in Figs. 5 and 6 and are given in Table II.

It is clear from Equation 7 that a dead-end competitive inhibitor, I, for substrate A of the Random Bi Bi mechanism will be a noncompetitive inhibitor of the other substrate in the reaction. Therefore, when Fruct-1,6-P$_2$ is the variable substrate, the double-reciprocal plot at different levels of AMP will exhibit increases in both slopes and intercepts. Fig. 6 shows the fit of the data from these experiments to the kinetic mechanism described by Equation 7. As expected, when a double-reciprocal plot of 1/velocity against 1/[Fruct-1,6-P$_2$] was made at different concentrations of AMP, a family of intersecting lines was obtained (Fig. 6). This result is also consistent with the findings of others (15, 17, 19, 32). It is well known that AMP is not a simple linear inhibitor of fructose
1,6-bisphosphatase (15, 17, 32). In addition, Stone and Fromm (19) found by means of progress curve analysis that AMP caused slope-parabolic, intercept-parabolic noncompetitive inhibition with respect to Fru-1,6-P$_2$. All these data are consistent with the rapid-equilibrium Random Bi Bi mechanism.

Although the initial rate kinetics are in harmony with the rapid-equilibrium Random Bi Bi mechanism, the mechanism may be steady-state Random Bi Bi (33). A choice between these possibilities can be made by using either isotope exchange or pulse-chase experiments (34). The latter protocol is relatively more feasible in the case of fructose-1,6-bisphosphatase. The results of experiments from Benkovic’s laboratory (35) showing that substrate cannot be trapped using the pulse-chase protocol, along with the results of this study, suggest that the kinetic mechanism for fructose-1,6-bisphosphatase is rapid-equilibrium Random Bi Bi. On the other hand, pulse-chase experiments by Stone and Fromm (36) showed that phosphate could be trapped in the reverse reaction. These findings suggest that the kinetic mechanism may in fact be steady-state Random Bi Bi, rather than equilibrium Random. However, those studies were carried out at saturating levels of Mg$^{2+}$ and are relevant only for the reverse reaction.

**DISCUSSION**

Although the kinetic mechanism of the fructose-1,6-bisphosphatase reaction has received a good deal of attention during the past 15 years, a definitive conclusion has not been reached regarding the sequence of enzyme, metal, and substrate interaction. Kinetic results obtained from studies of the bovine hepatic fructose-1,6-bisphosphatase reaction in the physiological pH range have shown that double-reciprocal plots involving Mg$^{2+}$ are intersecting, indicating a sequential mechanism for Mg$^{2+}$ and Fru-1,6-P$_2$ (37). On the basis of these kinetic results, Marcus et al. (37) suggested that a rapid-equilibrium Ordered Bi Bi mechanism is unlikely. However, they indicated a preference for the Random Bi Bi mechanism although their data did not exclude the steady-state Ordered Bi Bi case. Similar suggestions have also been made from binding experiments (36). Because fructose-1,6-bisphosphatase can also bind Fru-1,6-P$_2$ in the absence of Mg$^{2+}$ (1, 2, 39), a Random mechanism for the fructose-1,6-bisphosphatase reaction was suggested (38). This hypothesis is also supported by kinetic data obtained at pH 9.5 (17). Nimmo and Tipton (17) found that, at this pH, the enzyme obeys either a rapid-equilibrium Random Bi Bi mechanism or a steady-state Ordered Bi Bi mechanism in which Fru-1,6-P$_2$ binds to the enzyme before the cation. None of these earlier studies provided definitive proof of the rapid-equilibrium Random Bi Bi mechanism for fructose-1,6-bisphosphatase from kinetic experiments.

The data presented in this report are consistent with a rapid-equilibrium Random Bi Bi mechanism for the rabbit liver fructose-1,6-bisphosphatase reaction involving Fru-1,6-P$_2$ and Mg$^{2+}$ at pH 9.5. If the kinetic mechanism for rabbit liver fructose-1,6-bisphosphatase was, in fact, Ordered Bi Bi, then at least one uncompetitive inhibition plot should have been observed in the double-reciprocal graphs for inhibition by Cd$^{2+}$ and Fru-2,6-P$_2$ (31). Examination of the data shown in Figs. 2 and 4 suggests that this certainly is not the case. In addition, the fact that AMP is a competitive inhibitor of Mg$^{2+}$ and a noncompetitive inhibitor of Fru-1,6-P$_2$ gives further support to the suggestion that the kinetic mechanism is Random Bi Bi.

It has been suggested, from the use of Mn$^{2+}$ or Zn$^{2+}$ as the activating ion, that the metal binds to the structural site, after which substrate bindings occurs (4, 5). Binding to the catalytic metal site, which takes place after the binding of Fru-1,6-P$_2$ to the enzyme, gives rise to the catalytically competent quaternary complex (4, 5). This suggestion is thought to be supported by pulse-chase experiments (35). However, as we pointed out in 1976 (34), if a kinetic mechanism is steady state Random Bi Bi, one can expect to trap either substrate on the enzyme by using the pulse-chase protocol, whereas if the mechanism is rapid-equilibrium Random Bi Bi, neither substrate will be trapped. Studies from Benkovic’s laboratory (35) demonstrated that Fru-1,6-P$_2$ could not be trapped. These findings are fully consistent with the rapid-equilibrium Random Bi Bi mechanism.

The fact that the double-reciprocal plots in which 1/velocity is plotted against 1/Mg$^{2+}$ are linear, mitigates against the suggestion that more than a single metal ion binds to fructose-1,6-bisphosphatase. Thus, the proposal that there are structural and catalytic metal sites associated with the enzyme (3–5, 35) is not consistent with the data of this report nor the findings of Nimmo and Tipton (17), at least at pH 9.5. Unpublished x-ray diffraction studies from Dr. William Lipscomb’s laboratory reveal that only one Mg$^{2+}$ ion binds to enzyme monomer at neutral pH. However, at very high concentrations of Mg$^{2+}$ (10 mM), a second metal ion does bind to the enzyme.

It is possible to calculate the dissociation constants shown in Tables I and II from the initial rate data obtained in this report. The data depicted in Table I reveal that Mg$^{2+}$, when bound to the enzyme, decreases the affinity of the enzyme for the substrate relative to Fru-1,6-P$_2$, binding to the free enzyme. An analogous situation holds for metal binding to the free and substrate-complex form of the enzyme. These effects are a consequence of the intersection of the lines shown in Fig. 1, A and B, below the abscissa axis. In most, but by no means all, instances when a substrate or activator is bound to an enzyme, the affinity of the enzyme for the second ligand is enhanced and intersection of the double-reciprocal plots would be above the x axis. One explanation of the data shown in Fig. 1, A and B, involves the relationship known to exist between binding energy and energy required to induce the required conformation for catalysis or binding by the enzyme of additional ligands. Because Zn$^{2+}$, rather than Mg$^{2+}$, is believed to be the physiological activator of fructose-1,6-bisphosphatase (7, 8), it is possible that more binding energy is utilized with Mg$^{2+}$ than with Zn$^{2+}$ in order to achieve the proper conformation of the active ternary complex of enzyme, metal, and substrate. The net result of this loss of binding energy would be manifested as a decrease in affinity of the enzyme for either the activator or the substrate in the formation of the active ternary complex. It is of course possible that the relationship observed is independent of the nature of metal ion and that ligands are simply held less tightly in the ternary complex than in the binary complex based on the same thermodynamic rationale suggested for Mg$^{2+}$. Finally, it is possible that the kinetic mechanism is not truly rapid-equilibrium Random Bi Bi, but has instead some steady state Random character. In this case $K_a$ and $K_s$ are not true dissociation constants and cannot be compared as such to Michaelis constants (59).

Mg$^{2+}$ seems to have little effect on Fru-2,6-P$_2$ dissociation from the ternary enzyme-Mg$^{2+}$-Fru-2,6-P$_2$ complex. In fact, Fru-2,6-P$_2$ enhances binding of Mg$^{2+}$ to the binary complex as compared with the substrate. AMP, when bound to fructose-1,6-bisphosphatase, seems to have very little effect on substrate binding. However, as kinetic results of this study
indicate, metal and AMP are mutually exclusive in their binding to enzyme.

One question that arises from studies of the type presented in this report concerns the validity of the extrapolation from experiments at pH 9.5 to those in the physiological pH range. It is clear that the cooperativity observed for the metal ion activator is lost in the alkaline pH region; on the other hand, a number of the kinetic parameters remain unchanged. For example, the Michaelis constant and dissociation constant for Fru-1,6-P₂ shown in Table I approximate the literature reported values at neutral pH (4) as does the dissociation constant for AMP (4, 40). It is of course possible that the kinetic mechanism changes when there is a change in pH, however, this remains an open question, not only in the case of fructose-1,6-bisphosphatase in this report, but also with the large number of enzymes investigated by pH kinetics (41).

Fructose-1,6-bisphosphatase is a key regulatory enzyme in gluconeogenesis. It is now well recognized that the enzyme is regulated by Fru-2,6-P₂ and AMP (9). Results from binding experiments have shown that the synergistic inhibition by AMP and Fru-2,6-P₂ is due to the ability of Fru-2,6-P₂ to enhance the affinity of the enzyme for AMP (14). Fluorescence investigations from our laboratory have shown that the catalytic metal and AMP binding is competitive (20), α-Methyl-α-fructofuranoside 1,6-bisphosphate, an analog of the substrate Fru-1,6-P₂ as well as Fru-6-P and inorganic phosphate, enhances metal-mediated FMP displacement from rabbit liver fructose-1,6-bisphosphatase. Fru-2,6-P₂, on the other hand, has no such effect (20). On the basis of these observations, Scheffler and Froom (20) proposed a model for the molecular regulation of fructose-1,6-bisphosphatase by metal, AMP, and Fru-2,6-P₂. In their hypothesis, AMP displaces the catalytic metal from fructose-1,6-bisphosphatase, and its affinity for the enzyme is enhanced by Fru-2,6-P₂.

The results presented in this paper demonstrate clearly that AMP is a competitive inhibitor of the metal ion, i.e. they are mutually exclusive in their binding to fructose-1,6-bisphosphatase. However, they probably do not occupy overlapping sites (16, 42). This competitive property may explain how the inhibitor regulates the activity of the enzyme. It has been known for some time that the level of Fru-2,6-P₂ in liver is under control of the hormone glucagon (43). Fru-2,6-P₂ regulates fructose-1,6-bisphosphatase in two ways. First, it binds to the catalytic site of the enzyme, competitively inhibiting the enzyme activity. Second, it enhances the affinity of the enzyme for AMP, resulting in synergistic inhibition. The inhibition of the enzyme by AMP may be due to mutually exclusive binding of the ligand and the catalytic metal ion. Scheme I summarizes our hypothesis interpreting the interaction of AMP, Fru-1,6-P₂, Fru-2,6-P₂, metal ions, and fructose-1,6-bisphosphatase. In this model, F and Mg²⁺ represent fructose-1,6-bisphosphatase and divalent metal ion, respectively. This interaction pathway is consistent with data from binding (24), fluorescence (20), and kinetic investigations by others (17, 37, 38) and from our own work presented in this report.

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