The Interaction of Fructose 2,6-Bisphosphate and AMP with Rat Hepatic Fructose 1,6-Bisphosphatase*

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The binding of the inhibitory ligands fructose 2,6-bisphosphate and AMP to rat liver fructose 1,6-bisphosphatase has been investigated. 4 mol of fructose-2,6-P₂ and 4 mol of AMP bind per mol of tetrameric enzyme at pH 7.4. Fructose 2,6-bisphosphate exhibits negative cooperativity as indicated by $K'_1 > K'_2 > K'_3 \geq K'_4$ and a Hill plot of the curvatures of which indicates $K'_a/K'_b < 1$, $K'_a/K'_c < 1$, and $K'_a/K'_d = 1$. AMP binding, on the other hand, exhibits positive cooperativity as indicated by $K'_1 < K'_2 < K'_3 < K'_4$ and an $n_H$ of 2.05. Fructose 2,6- and fructose 1,6-bisphosphates enhance the binding of AMP by an increase in the intrinsic association constants. At pH 9.2, where fructose 2,6-bisphosphate and AMP inhibition of the enzyme are diminished, fructose 2,6-bisphosphate binds with a lower affinity but in a positively cooperative manner, whereas AMP exhibits half-sites reactivity with only 2 mol of AMP bound per mol of tetramer. Ultraviolet difference spectroscopy confirmed the results of these binding studies.

The site at which fructose 2,6-bisphosphate binds to fructose 1,6-bisphosphatase has been identified as the catalytic site on the basis of the following. 1) Fructose 2,6-bisphosphate binds with a stoichiometry of 1 mol/mol of monomer; 2) covalent modification of the active site with acetylimidazole inhibits fructose 2,6-bisphosphate binding; and 3) α-methyl D-fructofuranoside-1,6-P₂ and β-methyl D-fructofuranoside-1,6-P₂, substrate analogs, block fructose 2,6-bisphosphate binding. We propose that fructose 2,6-bisphosphate enhances AMP affinity by binding to the active site of the enzyme and bringing about a conformational change which may be similar to that induced by AMP interaction at the allosteric site.

Hepatic fructose 1,6-bisphosphatase is a key regulatory enzyme in the gluconeogenic pathway which has been shown to be regulated by allosteric effectors (1, 2) and to be subject to AMP-dependent protein kinase-catalyzed phosphorylation both in vitro and in vivo (3). Recent work from this laboratory (4, 5), confirmed by others (6), has shown that fructose-2,6-P₂ is a potent inhibitor of fructose 1,6-bisphosphatase and that it potentiates AMP inhibition of the enzyme. Glucagon (7), insulin (8), epinephrine (9), phenylephrine (10), and vasopressin (10) have all been shown to modulate the hepatic concentration of fructose-2,6-P₂, raising the possibility that the sugar diphosphate is an important regulator of flux through the enzyme in vivo. It has been the purpose of this study to elucidate the physical interaction of fructose-2,6-P₂ with fructose 1,6-bisphosphatase by directly measuring the stoichiometry of binding of this ligand and delineating the independent or cooperative nature of this interaction. In order to investigate further the synergism between fructose 2,6-P₂ and AMP inhibition, the binding of AMP to the enzyme has been measured both in the absence and presence of fructose-2,6-P₂.

Evidence from protein modification experiments and ligand-induced UV difference spectroscopy suggests that fructose-2,6-P₂ interacts with both the catalytic (fructose-1,6-P₂) site and the allosteric (AMP) site. This could be brought about by fructose-2,6-P₂ binding to both sites or by fructose-2,6-P₂ binding to the catalytic site with a concomitant conformational change which results in an enhanced affinity for AMP (4, 5). The binding studies have allowed us to identify the site, i.e. catalytic and/or allosteric, at which fructose-2,6-P₂ binds to the enzyme, thereby enabling us to distinguish between these two possibilities. In order to confirm our conclusions derived from binding analysis, we have employed UV difference spectroscopy to measure ligand-induced conformational alterations involving the active and allosteric sites.

EXPERIMENTAL PROCEDURES

Materials

[U,14C]Adenosine 5′-monophosphate (538 mcg/mmol) was obtained from Amersham Corp. Dialysis tubing utilized for rate of dialysis studies was obtained from Schleicher and Schuell. Binding membranes were of the Millipore type GS. The microdialysis cell, on loan from Dr. S. Colowick (Department of Microbiology, Vanderbilt University), was constructed according to the modifications of Feldman (11). Acetylimidazole was obtained from Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were from Boehringer-Mannheim. The fructose 1,6-bisphosphate analogs α-methyl D-fructofuranoside-1,6-P₂ and β-methyl D-fructofuranoside-1,6-P₂ were a generous gift from Dr. Steven Benkovic (Department of Chemistry, Pennsylvania State University).

Methods

Purification and Assay of Hepatic Fructose 1,6-Bisphosphatase—

Rat Liver fructose-1,6-bisphosphatase was purified by a modification of the method of Riu et al. (3). A 6–12% polyethylene glycol fractionation was included prior to column chromatography on DEAE-Sephadex A-50. For all of the concentration steps, a collodion bag apparatus was utilized, as this method resulted in better recovery than did ultrafiltration. Enzyme which was eluted from the CM (carboxymethyl)-Sephadex C-50 column by 2 mM fructose-1,6-P₂ was incubated with 5 mM MgCl₂ for 30 min in order to hydrolyze fructose-1,6-P₂ and then dialyzed extensively versus 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonfyl fluoride, and 0.5 μM/ml of leupeptin. The enzyme was homogeneous as indicated by sodium dodecyl sulfate-slab and -disc gel electrophoresis.

1 The abbreviations used are: DTT, dithiothreitol; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
with an apparent subunit molecular weight of 41,000. The specific activity of the enzyme was 30 units/mg at 32 °C. The enzyme was stored at 0 °C at a concentration of 2 mg/ml. Fructose 1,6-bisphosphatase was assayed either spectrophotometrically as previously described (3) or by following the release of \[^{32}P \]Pi from fructose-1,6-(1-\[^{32}P\])IP, as previously described (4).

Preparation of Fructose-2,6-(2-\[^{32}P\])IP—Fructose-2,6-(2-\[^{32}P\])IP was prepared by incubating fructose-6-P with [\(^{32}P\)]ATP and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase under conditions where the phosphotransferase reaction predominates. The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase was purified to homogeneity by the method of El-Maghrabi et al. (12). The reaction mixture (0.25 ml) contained 20 mM MES, 5 mM fructose-6-P, 0.2 mM [\(^{32}P\)]ATP (1000 cpm/mmol), 10 mM MgCl\(_2\), 100 mM KCl, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 0.2 milliunit of enzyme at pH 7.5. After 5 h, the reaction mixture was made 0.25 N in NaOH, heated for 20 min at 90 °C to hydrolyze fructose-6-P and to destroy 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, and then treated with charcoal (0.5 ml of a 100-mg/ml suspension) to remove nucleotides. The reaction mixture was diluted with 10 ml of 20 mM triethylamine HCO\(_3\) and chromatographed on DEAE-Sephadex A-25. A gradient from 20 to 500 mM triethylamine-HCO\(_3\) was employed to separate and elute [\(^{32}P\)]IP and fructose-6-P. Fractions with radioactivity were freed of 6-phosphofructo-2-kinase were pooled, lyophilized, and resuspended in the appropriate binding buffer. The fructose-2,6-(2-\[^{32}P\])IP concentration was determined by measuring fructose-6-P after acid hydrolysis or by the 6-phosphofructo-1-kinase activation assay as previously described (13).

Association of Fructose 1,6-Bisphosphatase-The reaction mixture was 120-ml Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA with a protein concentration of 3 mg/ml. Acetylmydilazole was added at a concentration which corresponded to a 1200-fold molar excess of enzyme protein. The acetylated protein was precipitated with ammonium sulfate (70% saturation), dissolved in 50 mM Tris buffer, and dialyzed for 90 min at 4 °C before analysis.

Rate of Dialysis Measurements of \[^{32}P\]AMP and Fructose-2,6-(2-\[^{32}P\])IP—Binding to Fructose 1,6-Bisphosphatase—The enzyme solution and radiolabeled ligand were placed in the upper chamber of the microdialysis cell where the contents were stirred continuously with a small stirring bar. The buffer for both fructose-2,6-P, and AMP binding studies was composed of 50 mM Tris-HCl, 100 mM KCl, 1 mM MgCl\(_2\), 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml of leupeptin, pH 7.4. The final volume was 100 μl. Buffer was pumped continuously through the lower chamber with a Buchler Polystaltic pump at a rate of 0.5 ml/min, and 0.5 ml fractions were collected. A steady state, in which rate of ligand was made to the enzyme solution of the sample cell and the buffer solution of the reference cell, and an equal volume of buffer was added to the other respective cell compartments. Additions were made in microliter increments with a Hamilton microsyringe.

Other Assays—A Perkin-Elmer Model 603 atomic absorption spectrophotometer was used to measure Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) at wavelengths of 285, 280, and 214 nm, respectively. Protein concentrations were determined by the method of Lowry et al. (16) using crystalline bovine serum albumin as standard.

Analysis of Binding Data—The microscopic intrinsic association constants were evaluated utilizing the Adair equation (17). The general formula (18) for this equation is as follows:

\[
\frac{\text{binding}}{\text{free}} = 
\frac{r}{q} 
\]  
where \(r\) denotes the fractional saturation of the protein, \(q\) is the number of binding sites, and \(i\) is the number of molecules of ligand bound (\(i = 0, 1, 2, q\)). As each molecule of ligand is bound, an equilibrium is reached characterized by an association constant \(K_i\). This theoretical expression has been used to determine the intrinsic association constants from experimentally derived \(r\) values where \(r\) is equivalent to the number of molecules of ligand bound per mol of enzyme. The form of this equation which we have employed takes into account the statistical factors defining "intrinsic" association constants, i.e.

\[
K' = \frac{[S]}{[S]_0} 
\]

The reaction mixture was then dialyzed against a 1200-fold molar excess of enzyme protein. The acetylated protein was precipitated with ammonium sulfate (70% saturation), dissolved in 50 mM Tris buffer, and dialyzed for 90 min at 4 °C before analysis.

**RESULTS**

The Binding of Fructose 2,6-Bisphosphatase to Fructose 1,6-Bisphosphatase—Fructose-2,6-P\(_2\) is an activator of 6-phosphofructo-1-kinase and an inhibitor of fructose 1,6-bisphosphatase and plays a prominent role in regulation of both glycolysis and gluconeogenesis. The concentration of fructose-2,6-P\(_2\) in livers from fed rats has been estimated to be about 8 mmol/g or about 16 μM (8). This high concentration of the sugar diphosphate would be expected to activate 6-phosphofructo-1-kinase completely in vivo unless some of the fructose-2,6-P\(_2\) were bound to other proteins or sequestered. Since fructose 1,6-bisphosphatase is present at much higher concentrations than 6-phosphofructo-1-kinase or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in rat liver (21), it is reasonable to suggest that it is a major site of bound fructose-2,6-P\(_2\). In order to determine if this is indeed the case, it was necessary to characterize the interaction of fructose-2,6-P\(_2\) with fructose 1,6-bisphosphatase. Fig. 1 illustrates
the binding of fructose-2,6-P$_2$ at pH 7.4 to purified rat liver fructose 1,6-bisphosphatase. When the amount of fructose-2,6-P$_2$ bound to the enzyme (r) was plotted as a function of the free fructose-2,6-P$_2$ concentration, a curve that closely approximated a shallow hyperbola was obtained. Extrapolation of the best fit logitographic curve of Fig. 1 to high concentrations of free fructose-2,6-P$_2$ indicated that 4 mol of fructose-2,6-P$_2$ would bind at a free fructose-2,6-P$_2$ concentration of 400-500 μM. When binding studies were conducted at these high ligand concentrations, 4 mol of fructose-2,6-P$_2$ were found to bind per mol of holoenzyme at a free fructose-2,6-P$_2$ concentration of 380 μM (data not shown). Therefore, the indicated stoichiometry is 4 mol of fructose-2,6-P$_2$/mol of tetrameric enzyme or 1 mol/mol of subunit. This suggests that fructose-2,6-P$_2$ does not bind at both the catalytic and allosteric sites. Scatchard analysis of the data (Fig. 1, inset) indicated that the interaction between sites exhibited negative cooperativity. A Hill plot of the data was curved at the half-saturation point, and the shape of the curve indicated negative cooperativity between K$_i$ and K$_2$ and K$_3$ and no cooperativity between K$_4$ and K$_5$ when compared to the theoretical Hill plots of Cornish-Bowden and Koshland (19). Analysis of the intrinsic binding constants confirmed the negative cooperative binding at pH 7.4 and gave values of K$_i$ > K$_2$ > K$_3$ > K$_4$ (Table I). The subunit interaction free energy has been calculated from the equation ΔG = RT ln K$_i$/K$_4$ and is estimated to be +4.19 kcal/mol.

Previous studies have indicated that fructose-2,6-P$_2$ inhibits fructose 1,6-bisphosphatase activity in both a competitive (4, 6) and noncompetitive (6) manner. The noncompetitive inhibition suggests that fructose-2,6-P$_2$ interacts with the enzyme at a site other than the catalytic site. In order to determine at which site fructose-2,6-P$_2$ interacts with the enzyme, the binding of fructose-2,6-P$_2$ to fructose 1,6-bisphosphatase that had been chemically modified with acetyl-Imidazole was studied. This agent has been shown to acetylate tyrosine residues at both the catalytic site and the AMP site of fructose 1,6-bisphosphatase (22, 23). When the enzyme was incubated in the presence of a 1200-fold molar excess of acetylimaldazole, the specific activity of the enzyme was decreased by 90% and fructose-2,6-P$_2$ did not bind to the enzyme. When the enzyme was incubated with acetylimaldazole in the presence of 1 mM fructose-1,6-P$_2$ to protect the active site, the specific activity was reduced by 50% and fructose-2,6-P$_2$ bound to the enzyme but with an altered stoichiometry (Fig. 2). A Scatchard plot of the data indicated negative cooperativity with a decreased fractional saturation of the enzyme at a given free fructose-2,6-P$_2$ concentration and a stoichiometry of 2 mol of fructose-2,6-P$_2$ bound per mol of enzyme. The fact that half of the sites bind fructose-2,6-P$_2$ when the active site is partially protected, as evidenced by retention of approximately 50% of the specific activity, is consistent with the idea that it is modification of the active site that causes the decreased specific activity.

Table I

| FDPase | pH | S$_{50}$ | Intrinsic binding constants |
|--------|----|---------|-----------------------------|
| 1.8    | 7.4| 2.15    | $3.5 \times 10^4$ | $3.5 \times 10^5$ | $2.0 \times 10^4$ | $2.0 \times 10^4$ |
| 3.5    | 7.4| 5       | $2.96 \times 10^5$ | $1.65 \times 10^5$ | $2.04 \times 10^4$ | $2.0 \times 10^4$ |
| 7.2    | 7.4| 6       | $1.0 \times 10^6$  | $1.0 \times 10^5$  | $1.5 \times 10^4$  | $1.5 \times 10^4$  |
| 10.8   | 7.4| 8       | $1.0 \times 10^6$  | $1.0 \times 10^5$  | $5.0 \times 10^4$  | $2.5 \times 10^4$  |
| 3.5    | 9.2| 12.5    | $2.0 \times 10^4$  | $5.1 \times 10^4$  | $1.18 \times 10^4$ | $3.0 \times 10^4$  |

Fig. 2. Effect of acetylation upon fructose 2,6-P$_2$ binding to rat liver fructose 1,6-bisphosphatase. Fructose 1,6-bisphosphatase (10 μM) was incubated for 90 min at room temperature in one of the following ways: 1) with a 1200-fold molar excess of acetylimaldazole, 2) with a 1200-fold molar excess of acetylimaldazole, and 3) in the absence of acetylimaldazole. Specific activities for each enzyme sample were determined as indicated in the text. Enzyme treated with acetylimaldazole in the absence of fructose-1,6-P$_2$ did not bind fructose-2,6-P$_2$. Enzyme treated with acetylimaldazole in the absence of fructose-1,6-P$_2$ did not bind fructose-2,6-P$_2$. Enzyme treated with fructose-1,6-P$_2$ and acetylimaldazole and that of the control enzyme incubated in the absence of the modifying reagent.
site, and not the allosteric site, that prevents fructose-2,6-P₂ binding.

Effect of Substrate Analog on Binding of Fructose 2,6-Bisphosphate to Fructose 1,6-Biphosphatase- In order to obtain additional evidence that fructose-2,6-P₂ interacts with the catalytic site, binding of the ligand was measured in the presence of two substrate analogs, α-methyl D-fructofuranoside-1,6-P₂ and β-methyl D-fructofuranoside-1,6-P₂, which are known to bind to the catalytic site. Substitution of the methyl group at the 2-position in the d-fructofuranoside-1,6-P₂ and p-methyl D-fructofuranoside-1,6-P₂, binds to the active site, it would be expected that was required to inhibit binding by 70%. P-Methyl D-fructofuranoside, under identical conditions, inhibited fructose-2,6-P₂, 3.5 mM α-methyl fructofuranoside, under identical conditions, inhibited fructose-2,6-P₂ binding completely; in the presence of 10 PM group at the 2-position in the tose-2,6-P, binds to the active site, it would be expected that substitution of the methyl side-1,6-P₂ and p-methyl D-fructofuranoside-1,6-P₂ are known to bind to the catalytic site. The addition of α-methyl D-fructofuranoside-1,6-P₂ during rate of dialysis measurements indicated that this α analog must be present in molar excess in order to inhibit fructose-2,6-P₂ binding completely; in the presence of 10 μM fructose-2,6-P₂, 3.5 mM α-methyl D-fructofuranoside-1,6-P₂ was required to inhibit binding by 70%. β-Methyl D-fructofuranoside, under identical conditions, inhibited fructose-2,6-

P₂ binding by 50% at a concentration of 3.5 mM (Table II). When higher concentrations of the analogs (8.5 mM) were employed, binding of fructose-2,6-P₂ was abolished (Fig. 3). Millimolar concentrations of the α or the β analog are also required to induce 50% inhibition of the enzyme, as indicated by steady state kinetic measurements (24). The α and β substrate analogs appear to inhibit fructose-2,6-P₂ binding at similar concentrations, although the β analog appears less effective at high concentrations than does the α analog, a purely competitive inhibitor.

Effect of Metal Ions upon Binding of Fructose-2,6-P₂ to Fructose 1,6-Biphosphatase- When purified fructose 1,6-biphosphatase was analyzed by atomic absorption spectrophotometry for the presence of Mg²⁺, Mn²⁺, and Zn²⁺, it was found that 4 mol of Zn²⁺ and 4 mol of Mg²⁺ were present per mol of tetramer (data not shown). Mn²⁺ was not detected. Treatment of the enzyme with Chelex 100 did not remove this endogenous metal as indicated by atomic absorption measurements. This was an unexpected finding and indicated that the 4 mol of Zn²⁺ and Mg²⁺ are very tightly bound. In

![FIG. 3. Fru-1,6-P₂ analog competition for fructose-2,6-P₂ binding to rat liver fructose 1,6-biphosphatase. Fructose-2,6-P₂ binding to rat liver fructose 1,6-biphosphatase was measured by rate of dialysis method in the absence (●) and in the presence (O) of the fructose-1,6-P₂ analog α-methyl D-fructofuranoside-1,6-P₂. The specific activity of the fructose-2,6-[2-³²P]P₂ was 46 mCi/μmol. After radioactivity in the effluent reached a steady state, increments of unlabeled fructose-2,6-P₂ were added to give the final concentrations indicated under the arrows. The fructose 1,6-biphosphatase concentration was 4 μM, and the α-methyl D-fructofuranoside-1,6-P₂ concentration was 8.5 mM.](http://www.jbc.org/)

![FIG. 4. Concentration dependence, Hill plot, and Scatchard plot of AMP binding to rat liver fructose 1,6-biphosphatase. A, the number of moles of AMP bound per mol of fructose 1,6-biphosphatase (r) are plotted versus the free AMP concentration. Enzyme concentration was 30 μM; AMP concentrations were micromolar. The symbols represent experimental points, and the curve has been shown to fit that theoretically determined from the Adair equation (17). Inset, Hill plot of data in A. B, Scatchard plot of data in A.](http://www.jbc.org/)
Regulation of Fructose 1,6-Bisphosphatase

The binding of AMP to Fructose 1,6-Bisphosphatase—Fructose-2,6-P₂ not only inhibits fructose 1,6-bisphosphatase activity directly but it also enhances the inhibition by AMP (5). In order to investigate the interactions of these two ligands, it was first necessary to characterize the binding of AMP at pH 7.4 to purified rat liver fructose 1,6-bisphosphatase. When the amount of AMP bound to the enzyme (r) was plotted as a function of the free AMP concentration, a sigmoidal curve was obtained (Fig. 4A). This suggested that the binding was cooperative in nature. This was confirmed by the bell-shaped curve obtained from a Scatchard plot of the data (Fig. 4B). The data also indicated that 4 mol of AMP were bound per mol of tetrameric enzyme and that half-saturation (S₀₅) was obtained with a concentration of free AMP of 37 μM. A Hill plot of the data (Fig. 4A, inset) gave an nₜₜ value of 2.05. Analysis of the intrinsic binding constants confirmed the positive cooperative binding of AMP to the enzyme and gave values of Kᵣ'< Kₜ'< Kₜ'< K₁'.

The binding constants for AMP interaction with fructose 1,6-bisphosphatase indicate that the reaction is a highly exergonic one. Taketa and Pogell (1) reported that the molar enthalpy change (ΔH) was -42.6 kcal/mol, as calculated from the effect of temperature on the association constants for the rat liver enzyme. In accordance with this characterization, we have found that AMP binding to rat liver fructose 1,6-bisphosphatase was also enhanced at lower temperatures. At 4 °C the S₀₅ values for AMP was decreased to 13 μM compared to 37 μM at 24 °C and the fractional saturation of the enzyme at a given AMP concentration was correspondingly increased (data not shown).

The binding of AMP to the enzyme was observed in the absence of substrate, fructose-1,6-P₂ (Fig. 4). Pilkis et al. (5) reported that fructose-1,6-P₂ potentiated AMP inhibition of fructose 1,6-bisphosphatase; at pH 7.5, fructose-1,6-P₂ decreased the concentration of AMP which gave half-maximal...
inhibition \( I_{50} \) from 49 \( \mu M \) in the presence of 1 \( \mu M \) substrate to 16 \( \mu M \) in the presence of 60 \( \mu M \) substrate. This suggests that fructose-1,6-P\(_2\) may enhance the enzyme’s affinity for AMP. As seen in Fig. 5, fructose-1,6-P\(_2\) did enhance AMP binding. Increasing concentrations of fructose-1,6-P\(_2\) from 0.1 to 50 \( \mu M \) elicited a steady increase in the fractional saturation of the enzyme at a given AMP concentration. At a total AMP concentration of 160 \( \mu M \), 500 \( \mu M \) fructose-1,6-P\(_2\) gave a maximal increase in the \( r/\{\text{AMP}\}_{\text{tot}} \) value, increasing it from 0.034 \( \mu M^{-1} \) (in the absence of fructose-1,6-P\(_2\)) to 0.078 \( \mu M^{-1} \). Further increasing the concentrations of fructose-1,6-P\(_2\) decreased the affinity of the enzyme for AMP and decreased the \( r/\{\text{AMP}\}_{\text{tot}} \) value to 0.050 \( \mu M^{-1} \) in the presence of 4.3 mM fructose-1,6-P\(_2\). This may indicate that at high concentrations, fructose-1,6-P\(_2\) alters enzyme conformation or interacts with an inhibitory allosteric site. It is well known that high concentrations of fructose-1,6-P\(_2\) alter enzyme conformation or interacts with an inhibitory allosteric site. It is well known that high concentrations of fructose-1,6-P\(_2\) inhibit the enzyme (25). Interestingly, fructose-2,6-P\(_2\) reversed the inhibition of AMP binding by millimolar concentrations of fructose-1,6-P\(_2\). The addition of 0.2 \( mM \) fructose-2,6-P\(_2\) to an enzyme solution which contained 4.3 \( mM \) fructose-1,6-P\(_2\) increased the \( r/\{\text{AMP}\}_{\text{tot}} \) value to 0.087 \( \mu M^{-1} \) (Fig. 5). Thus, fructose-2,6-P\(_2\) could compete with fructose-1,6-P\(_2\) such that AMP affinity was restored to its maximum value.

The Effect of Fructose 2,6-Bisphosphate on AMP Binding to Fructose 1,6-Bisphosphatase—Fig. 6 illustrates the effects of fructose-2,6-P\(_2\) on AMP binding to purified rat liver fructose 1,6-bisphosphatase at pH 7.4. When the amount of AMP bound to the enzyme was plotted as a function of the free AMP concentration, the \( S_{0.5} \) value was reduced from 37 to 20 \( \mu M \) in the presence of 0.2 \( mM \) fructose-2,6-P\(_2\) (Fig. 6A). Increasing the concentration of fructose-2,6-P\(_2\) from 0.1 to 200 \( \mu M \) elicited corresponding increases in the affinity of the enzyme for AMP as indicated by increases in the moles of AMP bound per mol of enzyme at given free AMP concentrations (Fig. 6B). The intrinsic association constants for AMP increased in the presence of increasing concentrations of fructose-2,6-P\(_2\) (Table III).

Although fructose-2,6-P\(_2\) dramatically enhanced AMP binding, the reverse effect was not as pronounced. AMP did not affect the stoichiometry of fructose-2,6-P\(_2\) binding, nor did it alter the negative cooperativity observed in the absence of AMP (data not shown). AMP did enhance the binding of the first mole of fructose-2,6-P\(_2\) to the enzyme. This is indicated by Scatchard plots of the data which displayed an enhanced fractional saturation of fructose 1,6-bisphosphatase at concentrations of free fructose-2,6-P\(_2\) which induced the binding of 1 mol/mol of enzyme (data not shown). The first

### Table III

| Fructose-2,6-P\(_2\) | pH | \( S_{0.5} \) | Intrinsic binding constants |
|-------------------|---|----------|--------------------------|
| \( \mu M \) | \( \mu M \) | \( K'_{1} \) | \( K'_{2} \) | \( K'_{3} \) | \( K'_{4} \) |
| 50 | 7.4 | 2.05 | 37.5 | \( 1.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) | \( 3.5 \times 10^{4} \) | \( 1.0 \times 10^{5} \) |
| 100 | 7.4 | 1.52 | 31 | \( 2.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) |
| 200 | 7.4 | 1.50 | 20 | \( 2.0 \times 10^{4} \) | \( 2.25 \times 10^{4} \) | \( 2.25 \times 10^{4} \) | \( 3.0 \times 10^{4} \) |
| 400 | 9.2 | 2.38 | 27 | \( 2.5 \times 10^{4} \) | \( 2.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) | \( 2.0 \times 10^{4} \) |
| 700 | 9.2 | 1.15 | 5 | \( 4.0 \times 10^{4} \) | \( 4.0 \times 10^{4} \) | \( 4.0 \times 10^{4} \) | \( 4.0 \times 10^{4} \) |
mole of fructose-2,6-P$_2$ was bound at a free fructose-2,6-P$_2$ concentration of 2.5 $\mu$M in the absence of AMP, but at only 1 $\mu$M in the presence of AMP. This is approximately a 50% decrease in the fructose-2,6-P$_2$ concentration at which 1 mol of fructose-2,6-P$_2$ is bound; this is similar to the 50% decrease in the $S_{0.5}$ for AMP which was induced by fructose-2,6-P$_2$. These effects on fructose-2,6-P$_2$ binding were observed at physiological concentrations (~30$^{-}$mol of fructose-2,6-P$_2$ is not as effective an inhibitor of fructose 1,6-bisphosphatase at pH 9.2 as it is at neutral pH (5). Binding studies at pH 9.2 indicated that the stoichiometry of fructose-2,6-P$_2$ binding was not altered since 4 mol were bound per mol of enzyme (Fig. 7A). However, the affinity of the enzyme for this ligand was significantly decreased as indicated by the lower $r/[\text{fructose-2,6-P}_2]_{\text{int}}$ values. In contrast to the binding at pH 7.4 which was negatively cooperative (Fig. 1), the interaction between binding sites at pH 9.2 was positively cooperative as indicated by the bell-shaped curve obtained from a Scatchard plot of the data (Fig. 7B) and by an $n_H$ value of 2.07 obtained from a Hill plot of the data (Fig. 7A, inset). Calculation of the intrinsic association constants indicated that the first and second mole of fructose-2,6-P$_2$ bound with a lower affinity than at neutral pH, but the positive interaction between the subunits led to an increased affinity for the third and fourth mole at pH 9.2 (Table I).

The inhibition of fructose 1,6-bisphosphatase activity by AMP is also not as effective at pH 9.2 as it is at neutral pH, but fructose-2,6-P$_2$ is still able to potentiate the inhibition by AMP (5). Fig. 8 illustrates the binding of AMP to fructose 1,6-bisphosphatase at pH 9.2 in both the absence and presence of 0.2 $\mu$M fructose-2,6-P$_2$. When the amount of AMP bound to the enzyme was plotted as a function of the free AMP concentration, a sigmoidal curve was obtained in the absence of fructose-2,6-P$_2$. In the presence of fructose-2,6-P$_2$, the curve was shifted to the left and closely approximated a hyperbolic curve. The $S_{0.5}$ value for AMP binding was decreased from 27 $\mu$M in the absence of fructose-2,6-P$_2$ to 5 $\mu$M in its presence. In both the absence and presence of fructose-2,6-P$_2$, only 2 mol of AMP were bound per mol of tetrameric enzyme. The $n_H$ value was decreased from 2.58 in the absence of fructose-2,6-P$_2$ to 1.15 in its presence (Fig. 8A, inset). Scatchard analysis gave bell-shaped curves in both the absence and presence of fructose-2,6-P$_2$, but the $r/[\text{AMP}]_{\text{int}}$ values were increased in the presence of the sugar diphosphatase. The intrinsic association constants at pH 9.2, in the absence of fructose-2,6-P$_2$, indicated a low affinity for the first mole of AMP ($K'_1 = 2.5 \times 10^6$) as compared to the second mole of AMP ($K'_2 = 2.0 \times 10^6$). In the presence of fructose-2,6-P$_2$ at pH 9.2, the intrinsic association constants were not as disparate. In this case, the $K'$ values were $K'_1 = 4.0 \times 10^6$ and $K'_2 = 4.0 \times 10^6$ (Table III); this would account for the hyperbolic curve and the lower $n_H$ value.

**Ligand-induced Conformational Changes As Measured by UV Difference Spectroscopy**—It has been reported that fructose-1,6-P$_2$ binds to the enzyme at identical and independent sites (26), in a positively cooperative manner (27), or in a negatively cooperative manner (28). As mentioned earlier, fructose-1,6-P$_2$ binding to rat liver fructose 1,6-bisphosphatase could not be measured directly because the substrate was rapidly hydrolyzed in the presence of Mg$^{2+}$ ions. This being the case, we monitored the fructose-1,6-P$_2$-induced perturbations in UV spectra in an attempt to investigate the nature of the fructose-1,6-P$_2$-enzyme interaction. In confirmation of our earlier study (5), addition of fructose-1,6-P$_2$ induced an absorbance maximum at 288 nm and minima at 298, 283, and 250 nm (Fig. 9). These absorbance changes were dependent on the concentration of fructose-1,6-P$_2$ and were saturable. A plot of the change in molar absorption at 298 nm as a function of the concentration of fructose-1,6-P$_2$ yielded a sigmoidal curve which indicated that the substrate site interaction displayed positive cooperativity (Fig. 9, inset). The half-maximal absorbance change was obtained with 146 $\mu$M fructose-1,6-P$_2$, while maximal changes were observed with 335 $\mu$M fructose-1,6-P$_2$. In order to determine if fractional saturation of the enzyme

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$^3$ Atomic absorbance spectrophotometry measurements indicated that the fructose 1,6-bisphosphatase-bound Mg$^{2+}$ content was 4 mol of Mg$^{2+}$/mol of enzyme; this should have made the Mg$^{2+}$ concentration approximately 35 $\mu$M in the enzyme solution employed for these UV difference spectra studies. When this enzyme solution was measured by atomic absorbance spectrophotometry, the Mg$^{2+}$ concentration was found to be 88 $\mu$M. This indicated that the Mg$^{2+}$ added to the enzyme, after fructose-1,6-P$_2$ elution from the CM-Sephadex column, had not been totally removed by dialysis. Therefore, the free Mg$^{2+}$ concentration was approximately 30 $\mu$M. UV difference spectra measurements were done in the presence of 0.1 mM EDTA (as described under "Experimental Procedures"). This should have significantly reduced the free Mg$^{2+}$ concentration, but may not have chelated endogenous Mg$^{2+}$ ions (as Chelex treatment did not). Therefore, the importance of Mg$^{2+}$ in the fructose-1,6-P$_2$ and fructose-2,6-P$_2$-induced UV difference spectra studies cannot be determined.
with fructose-2,6-bisphosphate, as measured directly in the binding studies (Figs. 1 and 4), was consistent with fractional changes in enzyme conformation, as measured by UV difference spectroscopy, titration curves were run with this ligand. In the presence of fructose-2,6-P₂, absorbance maxima were observed at 288 and 282 nm and minima at 303 and 240 nm. The absorbance maxima were increased upon the addition of increasing fructose-2,6-P₂ (Fig. 10). A final concentration of 25 𝜇M fructose-2,6-P₂ elicited the maximum increase in the molar absorbance at 288 and 282 nm. When the molar absorption at 288 nm was plotted versus the free fructose-2,6-P₂ concentration, a hyperbolic plot was obtained (Fig. 10, inset).

The UV difference spectra indicated that approximately 85% of the change in conformation was induced at a fructose-2,6-P₂ concentration at which 1 mol of fructose-2,6-P₂ would be bound per mol of fructose-1,6-bisphosphatase tetramer. This suggests an extreme form of negative subunit interaction, where the binding of the first mole of fructose-2,6-P₂ elicits a conformational change and the binding of the second, third, and fourth moles of fructose-2,6-P₂ do not alter the conformation further (80). In this case, the free fructose-2,6-P₂ concentration which elicited a half-maximal change in molar absorbance at 289 nm was 3.75 𝜇M. These absorption spectroscopy measurements reaffirm our conclusions on the negative cooperative nature of the fructose-2,6-P₂ binding to rat liver fructose 1,6-bisphosphatase.

**Fig. 10. Ultraviolet difference spectra induced by fructose-2,6-P₂.** Ultraviolet difference spectra were recorded on the 0.02 absorbance scale (period 2.5, spectral band width 2.0, and scan rate 0.5). The enzyme concentration was 10 𝜇M. Fructose-2,6-P₂ was added at the following concentrations: 1.25 𝜇M (a), 2.5 𝜇M (b), 3.75 𝜇M (c), 5.0 𝜇M (d), 7.5 𝜇M (e), 10 𝜇M (f), 12.5 𝜇M (g), 15 𝜇M (h), 20 𝜇M (i), 25 𝜇M (j), and 30 𝜇M (k). The dashed line represents recording in the absence of any additions. Inset, the change in extinction coefficient at 288 nm is plotted versus the free fructose-2,6-P₂ concentration.

In a previous publication (5), AMP-induced perturbations in UV spectra indicated that maxima were increased at 288 and 279 nm and that minima were increased at 253 nm. These absorbance changes were saturable, and additions of AMP above 150 𝜇M did not elicit further changes in the UV difference spectra. A plot of the change in molar absorption at 279 nm versus the AMP concentration yielded a sigmoidal curve consistent with positive cooperativity in AMP interaction with the enzyme. The AMP concentration which gave a half-maximal change in extinction coefficient at 279 nm was approximately 40 𝜇M. This is consistent with both steady state kinetic determinations of Kᵣ as well as multiple equilibrium determinations of the AMP concentration which gives half-maximal binding.

**DISCUSSION**

The majority of studies on the binding of ligands to hepatic fructose 1,6-bisphosphatase have been done with the "alkaline" rabbit liver enzyme (26, 27). This enzyme has an alkaline pH optimum because an NH₂-terminal Mᵣ = 6000 fragment has been cleaved from the enzyme during the purification scheme (31), Pontremoli et al. (27) found that 4 mol of fructose 1,6-bisphosphate bound to the alkaline form of the rabbit liver enzyme and that this binding exhibited positive cooperative interactions. Sarngadharan et al. (28), on the other hand, found no evidence for cooperative interaction between substrate sites. Recent studies with the native rabbit liver enzyme have shown fructose 1,6-bisphosphate binding which was consistent with the presence of one binding site/subunit and a negative cooperative interaction between the sites (28).

These results suggest important differences in ligand binding between the proteolytically modified and the native enzyme. Furthermore, our finding that the type of cooperativity induced by the binding of fructose-2,6-P₂ to the enzyme is pH-dependent (Figs. 1 and 7) suggests that slight differences in binding conditions could account for the diverse results reported in the literature for fructose-1,6-P₂ binding. Detailed analysis of the binding of other ligands to a native form of fructose 1,6-bisphosphatase has not been heretofore reported. The rat liver enzyme employed in the present studies had not undergone proteolytic cleavage as indicated by a ratio of activity at pH 7.4 to that at pH 9.2 of 3, a molecular weight of the subunit of 41,000 (32), and NH₂-terminal analysis indicating a blocked NH₂ terminus (data not shown). We have been unable to demonstrate binding of substrate to the rat liver enzyme with the flow dialysis method due to the rapid hydrolysis of fructose-1,6-P₂. However, we were able to demonstrate fructose 1,6-bisphosphatase interaction with the enzyme by employing UV difference spectra (Fig. 9). The concentration of fructose-1,6-P₂ required to elicit half-maximal molar absorption changes at 297 nm (146 𝜇M) was much higher than the Kᵣ for fructose-1,6-P₂. It is possible that the UV difference spectra induced by substrate not only reflect conformational changes induced by fructose-1,6-P₂ interaction at the catalytic site, but also reflect fructose-1,6-P₂ interaction with a postulated inhibitory allosteric site (25).

Our present experiments on AMP binding to the enzyme confirm the results of others (26) investigating the rabbit liver enzyme; they have reported the temperature-dependent formation of an enzyme-AMP complex which contains 4 mol of AMP/mol of tetrameric enzyme and which exhibits positive cooperativity. Our results are at variance with those of Tejwani et al. (33), who used a homogeneous preparation of the rat liver enzyme which had not been modified by proteolysis. These workers found that AMP binding was absolutely dependent on the presence of fructose-1,6-P₂ and that approximately 2.0 mol of AMP were bound per mol of enzyme.
We found that AMP bound to the enzyme in the absence of substrate. It is unlikely that AMP binding observed was due to trace amounts of fructose-1,6-P₂ since the enzyme was first incubated with Mg²⁺ to hydrolyze the fructose-1,6-P₂ and then dialyzed extensively. No fructose-1,6-P₂ could be detected by spectrophotometric assay. Furthermore, if the enzyme was incubated with Mg²⁺ and then subjected to (NH₄)₂SO₄ precipitation followed by extensive dialysis, the binding of AMP was unaltered (data not shown). We concluded that fructose-1,6-P₂ was necessary to observe AMP binding to the enzyme. Inhibition of the enzyme by AMP is greatly diminished at pH 9.2 (1). At pH 9.2, only 2 mol of AMP bind to the enzyme and the primary intrinsic binding constant is greatly decreased. It is reasonable to postulate that at alkaline pH the enzyme assumes a conformation such that it exhibits “half-sites” reactivity, an extreme form of negative cooperativity, thus accounting for the diminished inhibition.

At pH 7.4, rat liver fructose 1,6-bisphosphatase appears to exhibit both positive and negative cooperativity, positive cooperativity in response to AMP and negative cooperativity in response to fructose-2,6-P₂. This indicates that effector-fructose 1,6-bisphosphatase interactions may best be described by the sequential model of Koshland, Nemethy, and Filmer (see Ref. 30). The fact that fructose-2,6-P₂ binding is characterized by negative cooperativity is consistent with the report that fructose-1,6-P₂ and the nonhydrolyzable fructose-1,6-P₂ analogs, α-methyl D-fructofuranoside-1,6-P₂ and β-methyl D-fructofuranoside-1,6-P₂, bind in a negatively cooperative manner (28). The present studies are also consistent with the idea that fructose-2,6-P₂ binds at the active site.

A number of findings suggest that fructose-2,6-bisphosphate binds only to the catalytic site of the rat liver enzyme. 1) Only 1 mol of fructose-2,6-bisphosphate binds per mol of enzyme subunit; 2) when the catalytic site is acetylated, fructose-2,6-P₂ does not bind, whereas, when the active site is protected by fructose-1,6-P₂, against acetylation, binding is retained; and 3) competitive binding studies with α-methyl D-fructofuranoside-1,6-P₂ and β-methyl D-fructofuranoside-1,6-P₂ indicate that these compete with fructose-2,6-P₂ for binding to rat liver fructose 1,6-bisphosphatase. Taken together, these data confirm earlier statements from this laboratory that fructose-2,6-P₂ binds at the catalytic site of fructose 1,6-bisphosphatase (5). This position has been corroborated by Gottschalk et al. (34) who found that fructose-2,6-P₂ behaves similarly to fructose-1,6-P₂ in protecting the active site of pig kidney fructose 1,6-bisphosphatase from modification by pyridoxal 5'-phosphate. The active site of rat liver fructose 1,6-bisphosphatase is also protected against inactivation by ace-tylimidazole in the presence of fructose-2,6-P₂ (5). A further indication that fructose-2,6-P₂ acts at the active site has been provided by Ganson and Pronm (35) who have shown that fructose-2,6-P₂ inhibits the reverse reaction of rabbit liver fructose 1,6-bisphosphatase. Their initial velocity studies indicated that fructose-2,6-P₂ competes with both fructose 6-phosphate and P₃ in the kinetically reversible reaction (35). Most recently, Pontremoli et al. (36) have reported that fructose-2,6-P₂ is a competitive inhibitor with respect to substrate of the rabbit liver enzyme. Thus, there is at present no evidence that fructose-2,6-P₂ interacts with any site other than the catalytic site.

The demonstration that fructose 2,6-bisphosphate binds only to the catalytic site and the direct demonstration that the fructose bisphosphate enhances the enzyme's affinity for AMP strongly suggest that fructose bisphosphate binding brings about a conformational change which facilitates AMP binding. Also compatible with this hypothesis is the finding that fructose-2,6-P₂ and AMP both induce similar UV difference spectra with saturable absorbance maxima (Ref. 5 and Fig. 10). This suggests that fructose-2,6-P₂ binding may induce a conformational change in the enzyme similar to that induced by AMP interaction with the allosteric site. Thus, the allosteric inhibition of the enzyme by AMP can be mediated by the binding of a substrate analog (fructose-2,6-P₂) to the catalytic site. It is not clear how such an active site-mediated modulation of the allosteric properties of the enzyme is brought about, but consistent with this idea are the results of NMR and EPR studies which indicate that there is a close proximity between the catalytic and AMP sites on the subunit of the rabbit liver enzyme (29).

The results of this study allow us to make certain estimates of the free versus bound fructose-2,6-P₂ levels in liver cytosol and to evaluate the role of AMP and fructose-2,6-P₂ in regulating enzyme activity. The concentration of fructose-1,6-bisphosphatase subunit in rat liver cytosol is about 20 μM. The concentration of fructose-2,6-P₂ in liver from fed rats is about 8 nmol/g or 16 μM assuming 0.5 ml of H₂O/g (8, 37). From the intrinsic association constants it can be calculated that approximately 60% of fructose-2,6-P₂ is bound to fructose 1,6-bisphosphatase in rat liver cytosol. It is possible that the high affinity of the enzyme for the binding of the first mole of fructose-2,6-P₂ to the enzyme ensures that this first mole will bind, while the weaker affinity for the second, third, and fourth moles would ensure some rapid turnover of fructose-2,6-P₂ in the cell. This rationale for negative cooperativity was suggested by Levitzki and Koshland (30) for the negative cooperativity observed for NAD binding to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. At a given enzyme concentration, the concentration of free fructose-2,6-P₂ which results in the binding of 1 mol of fructose-2,6-P₂/mol of enzyme will, according to UV difference spectral measurements, induce approximately 85% of the change in molar absorbance difference (Fig. 10, inset). This may indicate that fructose-2,6-P₂ inhibition is primarily regulated by the binding of the first mole of fructose-2,6-P₂.

The role of AMP in the regulation of rat liver fructose 1,6-bisphosphatase is uncertain. AMP concentrations in the liver are not altered during fluctuations in the glycolytic/gluconeogenic pathways except during anoxia. Furthermore, concentrations of AMP in rat liver cytosol are much higher than those which have been determined to give half-maximal inhibition, 600 μM for the former as opposed to 20 μM for the latter. However, computer-simulated estimations of adenine nucleotide levels in rat liver cytosol, which take into account the compartmentation as well as the protonation and chelation equilibria of adenine nucleotides (38), indicate that the free concentration of AMP is approximately 25 μM (39). This is significantly less than the total AMP concentration and indicates that the free cytosolic AMP concentration is within the range of the I₅₀, and therefore within a range where AMP inhibition can be regulated. When the dissociation constants for AMP are determined from the intrinsic association constants reported here, the dissociation constant for the first mole of AMP is 200 μM. This would indicate that at a cytosolic AMP concentration of 25 μM and an enzyme concentration of 5 μM, 10% of the low affinity site would be present as an E-AMP complex. As we have reported, fructose-2,6-P₂ increases the affinity of the enzyme for AMP. The dissociation constant is correspondingly decreased to 50 μM in the presence of fructose-2,6-P₂. This would indicate that, under the above conditions, the enzyme would be predominantly in the E-AMP complex.
conditions, 32% of the low affinity site would be present as an E-AMP complex. This effect of fructose-2,6-P₂ could then be amplified due to the positive cooperativity characteristic of AMP inhibition. The effect of fructose-2,6-P₂ upon AMP binding to rat liver fructose 1,6-bisphosphatase may provide a fine tuning mechanism for AMP inhibition. In the absence of fructose-2,6-P₂, the Kᵣ for AMP binding is ~37 μM and the Kᵣ' is low (Kᵣ' = 5.0 × 10^4), whereas in the presence of fructose-2,6-P₂, the Kᵣ for AMP binding is decreased to ~20 μM and the Kᵣ' is increased by an order of magnitude (Kᵣ' = 2.0 × 10⁶) (Table III). It is expected then that when fructose-2,6-P₂ concentrations are elevated, fructose 1,6-bisphosphatase synergistically. In the reverse situation, when the concentration of fructose-2,6-P₂ is low, for example in starvation (37), in diabetes (40), or in states of glucagon excess (7, 41), AMP inhibition would be attenuated due to the decrease in affinity of the enzyme for AMP.

Acknowledgments—We gratefully acknowledge Dr. T. H. Claus, Lederle Laboratories, for his assistance in preparation of the manuscript and Dr. S. P. Colowick, Dr. D. W. Regen, and Dr. C. R. Park for their critical reading of the manuscript. We are also grateful to Dr. B. Hughes for conducting the atomic absorption spectrophotometry.

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J. Biol. Chem. 1983, 258:10445-10454.

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