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Regulation of Rabbit Liver Fructose-1,6-bisphosphatase by Metals, Nucleotides, and Fructose 2,6-Bisphosphate As Determined from Fluorescence Studies†

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Received May 19, 1986; Revised Manuscript Received July 18, 1986

ABSTRACT: The fluorescent nucleotide analogue formycin 5′-monophosphate (FMP) inhibits rabbit liver fructose-1,6-bisphosphatase (I_{50} = 17 μM, Hill coefficient = 1.2), as does the natural regulator AMP (I_{50} = 13 μM, Hill coefficient = 2.3), but exhibits little or no cooperativity of inhibition. Binding of FMP to fructose-1,6-bisphosphatase can be monitored by the increased fluorescence emission intensity (a 2.7-fold enhancement) or the increased fluorescence polarization of the probe. A single dissociation constant for FMP binding of 6.6 μM (4 sites per tetramer) was determined by monitoring fluorescence intensity. AMP displaces FMP from the enzyme as evidenced by a decrease in FMP fluorescence and polarization. The substrates, fructose 6-phosphate and fructose 1,6-bisphosphate, and inhibitors, methyl α-D-fructofuranoside 1,6-bisphosphate and fructose 2,6-bisphosphate, all increase the maximal fluorescence of enzyme-bound FMP but have little or no effect on FMP binding. Weak metal binding sites on rabbit liver fructose-1,6-bisphosphatase have been detected by the effect of Zn^{2+}, Mn^{2+}, and Mg^{2+} in displacing FMP from the enzyme. This is observed as a decrease in FMP fluorescence intensity and polarization in the presence of enzyme as a function of divalent cation concentration. The order of binding by divalent cations is Zn^{2+} = Mn^{2+} > Mg^{2+}, and the K_d for Mn^{2+} displacement of FMP is 91 μM. Methyl α-D-fructofuranoside 1,6-bisphosphate, as well as fructose 6-phosphate and inorganic phosphate, enhances metal-mediated FMP displacement from rabbit liver fructose-1,6-bisphosphatase. In the presence of the α-methyl substrate analogue the K_d's for Mn^{2+} and Mg^{2+} are 14 μM and 0.8 mM, respectively. The K_d for Mn^{2+} binding to 4 enzyme sites per tetramer obtained by fluorescence experiments in the presence of methyl α-D-fructofuranoside 1,6-bisphosphate strongly indicates that divalent cation binding to the catalytic metal site (K_m for Mn^{2+} is 15 μM) is responsible for FMP displacement. It is concluded that catalytic metal and nucleotide binding is competitive, an observation sufficient to explain the effect of AMP on substrate turnover. Fructose 2,6-bisphosphate does not enhance metal ion mediated displacement of FMP. Mn^{2+} and Zn^{2+} concentrations sufficient to displace a significant proportion of enzyme-bound FMP had no effect on the fluorescence of the enzyme–FMP complex when saturating fructose 2,6-bisphosphate was present. Fructose 2,6-bisphosphate either blocks catalytic metal binding or permits concomitant binding of FMP and catalytic metal ion. In light of the results of other investigators, the former hypothesis is a more plausible interpretation. A model for the molecular regulation of fructose-1,6-bisphosphatase monomer by metal, AMP, and fructose 2,6-bisphosphate is presented.

Hydrolysis of D-fructose 1,6-bisphosphate (Fru-1,6-P_2)[1] to D-fructose 6-phosphate and inorganic phosphate is catalyzed by fructose-1,6-bisphosphatase (EC 3.1.3.11). The rabbit liver enzyme is a tetramer of M_r 140 000 (Traniello et al., 1971, 1972). Fructose-1,6-bisphosphatase from mammalian liver requires the presence of divalent metal ions such as Zn^{2+}, Mn^{2+}, Mg^{2+}, or Co^{2+} for catalytic activity (Frey et al., 1976; Tejwani et al., 1976; Pedrosa et al., 1977; Kirtley & Dix, 1971). The affinity of fructose-1,6-bisphosphatase for these divalent cations decreases in the following order: Zn^{2+} > Mn^{2+} > Mg^{2+} (Benkovic et al., 1978a,b). Because of the high affinity for Zn^{2+}, Benkovic et al. (1978b) have proposed that fructose-1,6-bisphosphatase may function in vivo as a zinc metalloenzyme. Rabbit liver fructose-1,6-bisphosphatase contains 8 divalent cation sites per molecule of enzyme (two per subunit) (Benkovic et al., 1978a,b; Libby et al., 1975). The high-affinity metal ion site on each subunit is referred to as the "structural"[2] metal ion site. The low-affinity sites have been dubbed "catalytic" because the dissociation constants for

† This research was supported in part by Research Grant 10546 from the National Institutes of Health, U.S. Public Health Service, and Grant DMB-8502211 from the National Science Foundation. This is Journal Paper J-12296 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 2575.

[1] Abbreviations: AMP, adenosine 5′-monophosphate; α-Me-Fru-1,6-P_2, methyl α-D-fructofuranoside 1,6-bisphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; FMP, formycin A 5′-monophosphate; FBPase, fructose-1,6-bisphosphatase; PMSF, phenylmethanesulfonyl fluoride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] While the terms "structural" and "catalytic" denote specific functions for the two metal ions associated with each fructose-1,6-bisphosphatase subunit, direct evidence for the participation of either metal ion in the catalytic mechanism is not available. However, we have chosen to use the usual convention of referring to the high-affinity metal sites as structural and the low-affinity metal sites, which require the presence of substrate for binding, as catalytic.

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Mn^{2+} and Zn^{2+} binding are approximately equal to their respective K_m's for catalysis (Benkovic et al., 1978b; Libby et al., 1975; Pontremoli et al., 1978). Binding of metal ions to the catalytic sites requires the presence of substrates or substrate analogues (Benkovic et al., 1978a,b; Libby et al., 1975; Pontremoli et al., 1978).

Fructose-1,6-bisphosphatase is a key control point in gluconeogenesis via its role in the futile cycle of Fru-1,6-P_2 synthesis and degradation (Marcus, 1981; Tejwani, 1983; Hers et al., 1982). AMP and Fru-2,6-P_2 are recognized as physiologically significant regulators of fructose-1,6-bisphosphatase activity. The mechanism of Fru-2,6-P_2 inhibition has been attributed to active site binding (Ganson & Fromm, 1982, 1985; Kitajima & Uyeda, 1983) leading to competitive inhibition (Ganson & Fromm, 1982; Gottschalk et al., 1982; Pontremoli et al., 1982). Other investigators have proposed that Fru-2,6-P_2 binds at an allosteric site (Pilkis et al., 1981a; Hue & Bartrons, 1985) because Fru-2,6-P_2 changes the Fru-1,6-P_2 concentration dependence from hyperbolic to sigmoidal (Van Schaftingen et al., 1981; Pilkis et al., 1981a).

AMP is clearly recognized as an allosteric inhibitor of fructose-1,6-bisphosphatase. The inhibition is noncompetitive with respect to Fru-1,6-P_2 for the enzyme from a variety of sources (Pontremoli et al., 1970; Pontremoli & Horecker, 1971; Horecker et al., 1975). Chemical modification of several different amino acid residues of fructose-1,6-bisphosphatase from yeast, liver, and kidney results in reduced or abolished sensitivity to inhibition by AMP with little or no effect on catalytic activity (Benkovic & deMaine, 1982). Covalent modification of kidney FBPase with the photoaffinity reagents 8-azido-AMP (Marcus & Haley, 1979) and 2-azido-AMP (Riquelme & Czarnecki, 1983) results in a loss of AMP binding and catalytic activity, both of which are linearly related to the extent of incorporation of the label.

Unfortunately, most of the investigations of AMP inhibition and binding employed alkaline fructose-1,6-bisphosphatase, a partly degraded form of the enzyme, which exhibits maximal activity at alkaline pH (Nakashima & Horecker, 1971; Sarngadharan et al., 1972). The proteolyzed alkaline enzyme exhibits decreased sensitivity to inhibition by AMP (Traniello et al., 1971) in comparison with neutral fructose-1,6-bisphosphatase, which is maximally active at pH 7.5. Binding studies on the alkaline form of the rabbit liver enzyme have shown the presence of 4 binding sites for AMP per molecule of fructose-1,6-bisphosphatase (Sarngadharan et al., 1969; Pontremoli et al., 1968). Fru-1,6-P_2 enhances AMP binding and induces positive cooperativity among AMP sites (Sarngadharan et al., 1969; Pontremoli et al., 1968). AMP does not affect Fru-1,6-P_2 binding, suggesting that the inhibitory effect is on turnover of bound substrate rather than substrate binding (Benkovic & deMaine, 1982).

The effect of metal ions on AMP inhibition is unclear. Pontremoli et al. (1979) have reported that very low concentrations of Zn^{2+} (<0.4 μM) enhance nucleotide inhibition. Mg^{2+} reduces both AMP binding and inhibition (Watanabe et al., 1968; Opie & Newsholme, 1967; Sarngadharan et al., 1969; Pontremoli et al., 1968). This effect has been attributed to formation of the Mg^{2+}–AMP chelate. AMP inhibition is greater with Mg^{2+} as the catalytic metal ion than with Mn^{2+} (Pontremoli et al., 1966; Opie & Newsholme, 1967). Pontremoli et al. (1968) could not account for the decreased sensitivity to AMP in the presence of Mn^{2+} by Mn^{2+}–AMP complex formation and proposed that Mn^{2+} and AMP binding is competitive. Mn^{2+} binding to the alkaline form of FBPase is approximately 1 order of magnitude weaker than binding to the neutral enzyme (Pontremoli et al., 1969; Libby et al., 1975).

The interaction of substrates, metal ions, and AMP with fructose-1,6-bisphosphatase is complex. To study the interaction of these ligands and Fru-2,6-P_2 with rabbit liver fructose-1,6-bisphosphatase, FMP was employed as a fluorescent probe. The inhibition of fructose-1,6-bisphosphatase by FMP and its displacement from the enzyme by AMP indicate that it is a suitable nucleotide analogue for examining the effect of substrates, substrate analogues, and metal ions on the interaction of AMP with fructose-1,6-bisphosphatase. The results presented here indicate that binding of Mg^{2+}, Mn^{2+}, or Zn^{2+} at the catalytic metal site is competitive with respect to FMP binding. In addition, divalent cations bind to the catalytic metal site in the absence of substrates, albeit weakly. α-Me-Fru-1,6-P_2 and Fru-6-P enhance metal binding by approximately 1 order of magnitude. In contrast, Fru-2,6-P_2 does not enhance metal binding at the catalytic site and may prevent binding. The results of this investigation provide a model for fructose-1,6-bisphosphatase regulation that integrates the effects of AMP, Fru-2,6-P_2, and catalytic metal ion.

Experimental Procedures

Materials. Fructose-1,6-bisphosphatase was purified from frozen rabbit liver (Pel-Freez Biologicals) by using a modification (Benkovic et al., 1974) of the method of Ulm et al. (1975). PMSF (1 mM) was included in all buffers until the final elution from CM-cellulose. Fructose-1,6-bisphosphatase activity was measured by using a coupled spectrophotometric assay (Ulm et al., 1975). Purified neutral fructose-1,6-bisphosphatase had a specific activity of approximately 22 units/mg (pH 7.5). The pH 7.5/pH 9.6 activity ratio was 4.5–5.5 and remained constant throughout purification. For fluorescence experiments, protein concentrations were determined from the optical absorption at 280 nm ($\epsilon_{0.1\%}$ = 0.89; Ulm et al., 1975).

Stock solutions of ligands and enzyme were treated with Chelex 100 (Bio-Rad Laboratories) before fluorescence experiments. The concentrations of Fru-6-P, Fru-1,6-P_2, and Fru-2,6-P_2 (purchased from Sigma Chemical Co.) in stock solutions were determined enzymatically as described by Ganson and Fromm (1985). α-Me-Fru-1,6-P_2 was synthesized by phosphorylating methyl α-fructofuranoside (Benkovic et al., 1971; Hartman & Barker, 1965) by Dr. Nancy J. Ganson. AMP was purchased from Sigma Chemical Co. Calbiochem was the supplier of FMP. Nucleotides were quantitated in stock solutions by using the following molar absorption coefficients (pH 7.0): AMP, ϵ^{259} = 15 400 M^{-1} cm^{-1}; FMP, ϵ^{295} = 10 500 M^{-1} cm^{-1}. Distilled deionized water was used throughout, and all reagents were of the highest purity available commercially.

Fluorescence Measurements. Tris·HCl (25 mM, pH 7.5) containing 0.15 M KCl was used to buffer fluorescence samples. Fluorescence measurements (25 °C) were carried out on a Fluorolog Series 111C spectrofluorometer (Spex Industries, Inc.) equipped with a 150-W xenon lamp and computer-controlled data accumulation and processing. Fluorescence emission spectra were corrected for fluorescence of the fluorescent ligands, enzyme, and reagents, where necessary.

Methods. The enhancement factor is defined as

$$EF = \frac{F_{max} \text{ of enzyme–ligand complex}}{F_{obsd} \text{ of free ligand}} \quad (1)$$

for the same concentration of ligand.

The fluorescent ligand was titrated with increasing concentrations of fructose-1,6-bisphosphatase to determine the

stoichiometry and affinity of the ligand–protein interaction from fluorescence intensity data. The number of ligand binding sites on the enzyme and the dissociation constant for the enzyme–ligand complex (K_d) were calculated from

$$(1/f) = (n/K_d)([E_T]/b) - ([L_T]/K_d) \quad (2)$$

$[E_T]$ and $[L_T]$ are the total enzyme subunit and ligand concentrations, respectively; f and b refer to the fraction of free ligand and fraction of bound ligand, respectively; n is the number of ligand binding sites per protein subunit; and K_d is the dissociation constant for a single binding site. This method of analysis is comparable to that of Scatchard (1949) as described by Tanford (1961) except that b and f refer to bound and free ligand rather than protein. Extrapolation of $1/F_{obsd}$ vs. $1/[E_T]$ to infinite protein concentration allowed evaluation of F_{max}, the fluorescence intensity of the ligand when fully bound to the enzyme. The fractions of ligand bound (b) and free (f) were calculated from $b = (F_{obsd} - F_0)/(F_{max} - F_0)$ and $f = 1 - b$, where F_{obsd} is the fluorescence intensity of the enzyme–ligand mixture and F_0 is the ligand fluorescence in the absence of protein.

Dissociation constants for ligands that displace FMP were calculated from the fluorescence intensity at 359 nm at various ligand concentrations by using

$$K^{app}_{d,E\cdot L} = \frac{[E\cdot FMP][K_{d,E\cdot FMP}][L_T]}{[FMP_f][E_T] - [K_{d,E\cdot FMP}][E\cdot FMP] - [E\cdot FMP][FMP_f]} \quad (3)$$

$[E\cdot FMP]$ and $[FMP_f]$ are the concentrations of bound and free FMP, $[E_T]$ is the total enzyme subunit concentration, $K_{d,E\cdot FMP}$ is the dissociation constant for the enzyme–FMP complex, $[L_T]$ refers to the total concentration of the displacing ligand (AMP or divalent cation), and $K^{app}_{d,E\cdot L}$ is the apparent dissociation constant for the displacing ligand at each ligand concentration. Bound and free FMP at various ligand concentrations were calculated from the fluorescence intensity as described. The validity of this equation requires that $[L_T] > [E_T] > [FMP]$. The derivation of eq 3 is similar to the procedure described by Brandt et al. (1967) for determining the dissociation constants for amide substrates that displace proflavin from chymotrypsin.

Fluorescence polarization is defined as $P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, where $I_\parallel$ and $I_\perp$ are the intensities observed parallel and perpendicular to the polarization of the exciting light, respectively, and P is polarization. Polarization was measured by using the L-format method. To calculate the actual intensity ratio ($I_\parallel/I_\perp$), the ratio of the observed intensities was corrected by the measured G factor, which is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (Lakowicz, 1983).

Results

FMP Binding to Rabbit Liver Fructose-1,6-bisphosphatase. FMP is a fluorescent structural analogue of AMP (Robins et al., 1966; Koyama et al., 1966) that substitutes efficiently for AMP in a variety of enzymatic reactions (Ward et al., 1969a). The fluorescence properties of FMP at neutral pH and 20 °C have been characterized (Ward et al., 1969b). The fluorescence emission spectrum of FMP, when excited at 308 nm, exhibits a maximum in intensity at 344 nm (Figure 1, curve A). Binding of FMP to rabbit liver fructose-1,6-bisphosphatase is evident from the enhancement observed in the fluorescence emission upon addition of the enzyme (Figure 1, curve B). A shift in the maximum fluorescence to a shoulder at 359 nm is also seen. The enhanced fluorescence of the

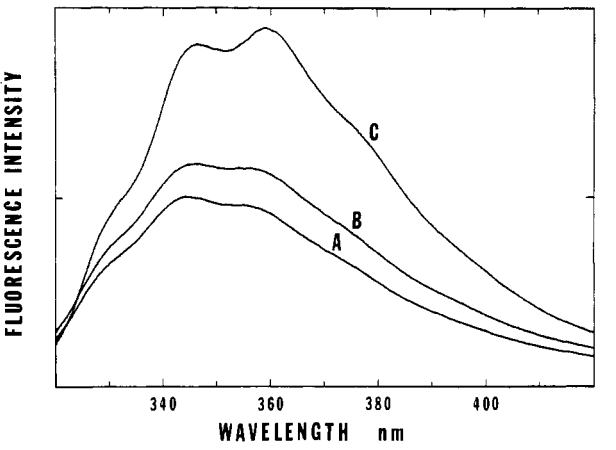


FIGURE 1: Fluorescence emission spectrum (uncorrected) of FMP bound to fructose-1,6-bisphosphatase. Fluorescence excitation was at 308 nm. Fluorescence emission spectra are as follows: (A) 5.2 μM FMP, in 25 mM Tris·HCl buffer (pH 7.5) containing 0.15 M KCl; (B) after addition of 10 μM fructose-1,6-bisphosphatase; (C) in the presence of 10 μM enzyme and 100 μM Fru-1,6-P_2.

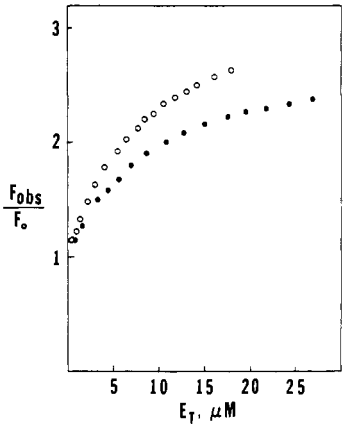


FIGURE 2: Titration of FMP with fructose-1,6-bisphosphatase. The fluorescence enhancement (intensity of observed fluorescence/FMP fluorescence intensity in the absence of enzyme) of 5 μM FMP at increasing concentrations of fructose-1,6-bisphosphatase (●) and in the presence of 0.22 mM α-Me-Fru-1,6-P_2 (○) is shown.

nucleotide analogue in the presence of fructose-1,6-bisphosphatase provides a measurement for determining the extent of FMP binding. The stoichiometry and binding affinity for FMP were determined by titrating a solution of the fluorescent ligand with enzyme. Figure 2 shows the data from a typical enzyme titration experiment. The number of binding sites for FMP, calculated by using eq 2, is 1 per enzyme subunit ($n = 1.2$), with a single dissociation constant of 6.6 μM. The fluorescence emission enhancement factor, determined by extrapolation to infinite enzyme concentrations at which all of the FMP is bound, is 2.7. The quantum yield of formycin nucleotides is similarly increased upon going from an aqueous medium to an 80% aqueous–ethanol solvent (Ward et al., 1969b).

In view of the binding of FMP to fructose-1,6-bisphosphatase, the effect of the fluorescent nucleotide analogue on enzyme activity was subsequently examined (Figure 3). FMP inhibits the rabbit liver enzyme, with a concentration of 17 μM required for 50% inhibition as compared with a value of 13 μM for AMP. The Hill coefficients determined for AMP and FMP inhibition are 2.3 and 1.2, respectively. To determine whether FMP is binding at the allosteric inhibitory site on fructose-1,6-bisphosphatase, which is specific for AMP, the effect of AMP on the fluorescence of the enzyme–FMP complex was examined (Figure 4A). Titration with AMP results

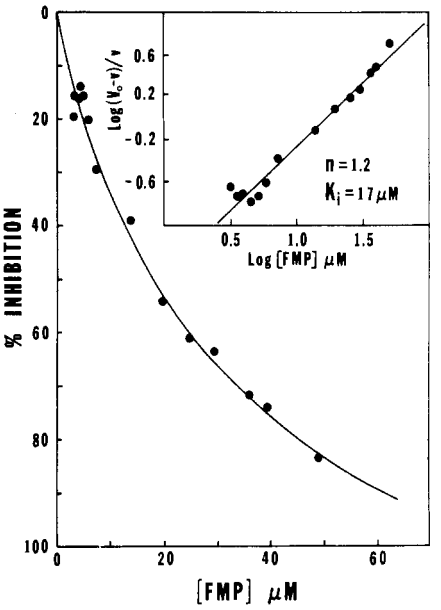


FIGURE 3: Inhibition of rabbit liver fructose-1,6-bisphosphatase by FMP. Enzyme activity was measured spectrophotometrically by using a coupled assay as described by Ulm et al. (1975). The inset shows the Hill plot of the FMP inhibition data. K_i refers to the concentration of FMP at 50% inhibition, and n represents the slope of the line.

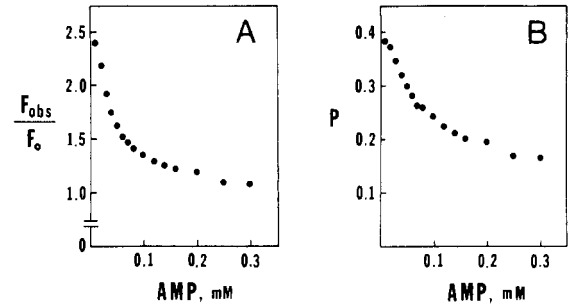


FIGURE 4: Effect of AMP on the fluorescence of the fructose-1,6-bisphosphatase–FMP complex. The sample contained 20 μM fructose-1,6-bisphosphatase and 5 μM FMP. (A) Variation in fluorescence enhancement (expressed as the observed fluorescence of the enzyme + FMP solution relative to the FMP fluorescence in the absence of enzyme) as a function of AMP concentration. (B) Decreasing FMP polarization (P) with increasing [AMP]. The polarization of free FMP is 0.102.

in a decrease in the fluorescence emission intensity at 359 nm and a shift in the fluorescence maximum to that of free FMP (344 nm). The displacement of FMP from the enzyme by AMP is clearly demonstrated by the observed decrease in the fluorescence polarization of the enzyme–FMP complex as shown in Figure 4B. At high AMP concentrations, the observed fluorescence polarization approached that of free FMP. The binding of AMP monitored by decreased polarization of FMP was in agreement with the binding of AMP monitored by decreased fluorescence of FMP. The apparent binding constants for AMP were calculated from the displacement of FMP as monitored by the decreased fluorescence emission at 359 nm (Figure 4A) by using eq 3 and a K_d for the enzyme–FMP complex of 6.6 μM. The apparent binding constants obtained at various AMP concentrations were used to calculate bound and free AMP concentrations, and the data were then plotted according to the Scatchard equation. Because this method requires that the concentration of AMP exceed that of the enzyme, a complete binding titration was not possible; however, the Scatchard plot is linear for the AMP concentrations examined in Figure 4. The single K_d for AMP determined by FMP displacement is 19.9 μM, n is 1.05 sites

Table I: Dissociation Constants for FMP Binding to Fructose-1,6-bisphosphatase[a]

complex	[ligand] (mM)	K_d (μM)	n	EF
enzyme		6.59 ± 0.58	1.19 ± 0.11	2.68
+Fru-6-P	0.20	5.06 ± 0.26	1.00 ± 0.05	3.00
+α-Me-Fru-1,6-P_2	0.22	6.31 ± 0.50	1.23 ± 0.10	3.17
+Fru-1,6-P_2	0.20	6.03 ± 0.41	1.33 ± 0.09	2.96
+Fru-2,6-P_2	0.10	6.34 ± 0.40	1.18 ± 0.08	3.06

[a] Dissociation constants were determined by titrating 5 μM FMP with fructose-1,6-disphosphatase as described under Methods. The excitation wavelength was 308 nm, and fluorescence emission was followed at 359 nm. The enhancement factor (EF) was calculated from the maximal fluorescence of FMP when all the ligand is bound (F_b), which was determined by extrapolation to infinite enzyme concentration. n is the number of divalent cation binding sites per enzyme subunit. Each of the ligand concentrations was sufficient to saturate the enzyme.

per subunit, and the binding does not seem to be cooperative.

Ligand Effects on FMP Binding. Having established that FMP probably binds to the same site on fructose-1,6-bisphosphatase as AMP, the effects of various ligands on FMP fluorescence and binding were examined. The effect of Fru-1,6-P_2 on the fluorescence emission of the fructose-1,6-bisphosphatase–FMP complex is shown in Figure 1, curve C. The increase in fluorescence at 359 nm, upon Fru-1,6-P_2 addition, reflects conformational differences in the ternary and binary enzyme complexes. Titration of a solution of FMP with enzyme, in the presence of a saturating concentration of Fru-1,6-P_2, yielded a dissociation constant for FMP from the enzyme–substrate complex of 6.0 μM and a 10% increase in the enhancement factor for the enzyme–substrate–FMP complex relative to the enzyme–FMP complex (Table I). The stoichiometry of FMP binding to the enzyme–substrate complex was 1 molecule per fructose-1,6-bisphosphatase subunit (n = 1.3). α-Me-Fru-1,6-P_2 (enzyme titration shown in Figure 2), Fru-6-P, and Fru-2,6-P_2 also increase the enhancement factor for the enzyme–FMP complex and have little or no effect on the dissociation constant for FMP, as summarized in Table I.

Effect of Metal Ions on FMP Binding. The effect of metal ions on the fluorescence of the fructose-1,6-bisphosphatase–FMP complex is exemplified by Mn^{2+}. The fluorescence decreases, and the emission maximum shifts toward that of free FMP with increasing concentrations of divalent cations. The fluorescence enhancement of a solution of 5 μM FMP due to the presence of 30 μM fructose-1,6-bisphosphatase decreases by approximately 30% in the presence of 0.5 mM Mn^{2+}. The addition of EDTA restores the fluorescence intensity to that observed in the absence of added metal ions (data not shown). The effectiveness of various metal ions in reducing the enzyme–FMP fluorescence decreases in the order $Zn^{2+} = Mn^{2+} > Mg^{2+}$. The concentrations of the divalent metal ions effective in reducing the enzyme–FMP fluorescence are sufficiently low to preclude the possibility of FMP–metal complex formation. By assuming that Mg^{2+} and Mn^{2+} are displacing FMP from the enzyme, dissociation constants for these divalent cations were calculated in the same manner as previously described for AMP.[3] The K_d's for Mg^{2+} and Mn^{2+} are 10.4 mM and 91.3 μM, respectively (Table II). The effect of metal

[3] Using the protocol for fluorescence studies described in this report, it is not possible to distinguish between mutually exclusive binding and the case where the dissociation constant is very high for dissociation of metal ions from the ternary complex of enzyme, metal, and FMP. For simplicity we have assumed the former case even though there is a suggestion from Figure 5 that ternary complex formation may occur.

Table II: Dissociation Constants for Metal Ion Binding to Fructose-1,6-bisphosphatase Determined by FMP Displacement in the Absence and Presence of Active-Site Binding Ligands

complex	[ligand] (mM)	metal	K_d (M)	n^a
enzyme		Mg^{2+}	$(1.04 \pm 0.09) \times 10^{-2}$	1.01 ± 0.11
		Mn^{2+}	$(9.13 \pm 0.36) \times 10^{-5}$	0.65 ± 0.03
+Fru-6-P	0.20	Mn^{2+}	$(4.18 \pm 0.14) \times 10^{-5}$	0.75 ± 0.03
+α-Me-Fru-1,6-P_2	0.22	Mg^{2+}	$(8.14 \pm 0.52) \times 10^{-4}$	1.00 ± 0.07
		Mn^{2+}	$(1.39 \pm 0.05) \times 10^{-5}$	0.90 ± 0.04
		Zn^{2+}	$(2.86 \pm 0.15) \times 10^{-5}$	1.06 ± 0.07

[a] n refers to the total number of FMP binding sites per enzyme subunit.

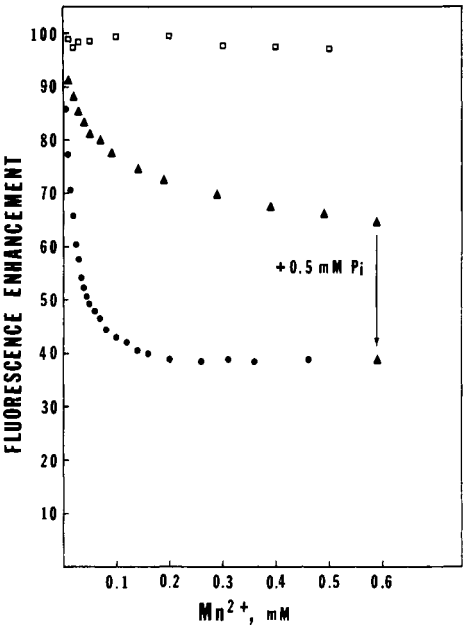


FIGURE 5: Effect of Mn^{2+} on the fluorescence emission of the fructose-1,6-bisphosphatase–FMP complex in the presence of α-Me-Fru-1,6-P_2, Fru-6-P, and Fru-2,6-P_2. One hundred percent fluorescence enhancement is defined as the increase in fluorescence emission intensity relative to free FMP (in the absence of enzyme) when no Mn^{2+} is present ($F_{obsd} - F_0$). Each sample contained 5 μM FMP and 20 μM enzyme. The following ligands were present at saturating concentrations: (●) 0.216 mM α-Me-Fru-1,6-P_2; (▲) 0.197 mM Fru-6-P; (□) 0.206 mM Fru-2,6-P_2. Addition of 0.5 mM P_i to the sample containing Fru-6-P further reduced the fluorescence to the level indicated.

ions was also examined in the presence of α-Me-Fru-1,6-P_2 (Figure 5) since this substrate analogue is known to enhance the binding of divalent cations to fructose-1,6-bisphosphatase. This experiment could not be performed with Fru-1,6-P_2 inasmuch as the substrate would be hydrolyzed by the enzyme in the presence of metal ions. The Mn^{2+} concentrations required to reduce the enzyme–FMP fluorescence are significantly lower in the presence of α-Me-Fru-1,6-P_2 (Figure 5). The binding of Mn^{2+} to α-Me-Fru-1,6-P_2 is too weak to account for the decreased fluorescence (Libby et al., 1975). To determine whether metal ion binding results in the displacement of FMP from rabbit liver fructose-1,6-bisphosphatase, the fluorescence polarization of the enzyme–FMP–α-Me-Fru-1,6-P_2 complex was examined in the presence of increasing concentrations of Mn^{2+}. The titration curve for the reduction in fluorescence polarization due to Mn^{2+} in the presence of α-Me-Fru-1,6-P_2 is similar to the titration curve obtained by monitoring the fluorescence emission at 359 nm (data not shown). It is concluded that the effect of the divalent cations is to displace FMP from the enzyme and that Mn^{2+} is not quenching FMP fluorescence due to the paramagnetic effect of this metal ion. By use of the apparent K_d's obtained from the data in Figure 5 for Mn^{2+} displacement of FMP from fructose-1,6-bisphosphatase in the presence of α-Me-Fru-1,6-P_2, a linear Scatchard plot was obtained. A single dissociation constant for Mn^{2+} of 13.9 μM was calculated, and the intercept (0.90) is indicative of 1 metal binding site per enzyme subunit (Table II). The experiments show that the divalent cation responsible for FMP displacement is binding at the catalytic metal site in the presence of α-Me-Fru-1,6-P_2. Dissociation constants for Mg^{2+} and Zn^{2+} binding to the enzyme–α-Me-Fru-1,6-P_2 complex were also obtained by FMP displacement and are listed in Table II. Fru-6-P also enhances Mn^{2+} binding to fructose-1,6-bisphosphatase (Figure 5), and the addition of P_i seems to further enhance metal binding (Figure 5). The K_d for Mn^{2+} binding to the enzyme–Fru-6-P complex is 41.8 μM (Table II).

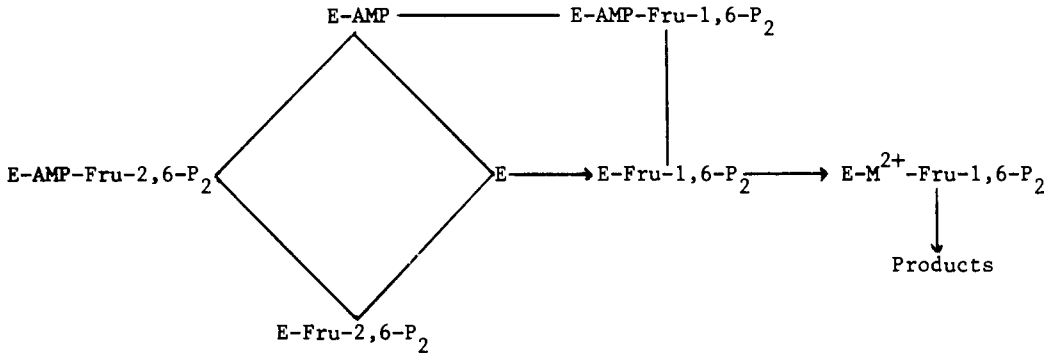
In contrast to the effect of Fru-6-P and α-Me-Fru-1,6-P_2, Fru-2,6-P_2 does not enhance divalent cation mediated displacement of FMP from fructose-1,6-bisphosphatase (Figure 5). Millimolar concentrations of Zn^{2+} or Mn^{2+} had no effect on enzyme–FMP fluorescence in the presence of Fru-2,6-P_2, although these same concentrations significantly reduced the fluorescence emission in the absence of the inhibitor.

Discussion

AMP and Fru-2,6-P_2 are physiological regulators of fructose-1,6-bisphosphatase (Marcus, 1981; Hers et al., 1982; Tejwani, 1983). The interaction of these inhibitors with the enzyme from rabbit liver has been studied by using the fluorescent ligand FMP. This nucleotide analogue inhibits fructose-1,6-bisphosphatase at the same concentrations required for AMP inhibition. The interaction of FMP with the enzyme does not entirely mimic that of the natural allosteric inhibitor. FMP inhibition is not significantly cooperative, and the binding of 4 molecules of FMP per enzyme tetramer as monitored by fluorescence is linear in the absence and presence of substrates and substrate analogues (K_d = 6.6 μM). Nevertheless, FMP provides a fluorescent probe for the AMP sites on fructose-1,6-bisphosphatase. This approach is justified by the inhibition observed with FMP and its displacement from rabbit liver fructose-1,6-bisphosphatase by AMP. Using the displacement of FMP to monitor AMP binding in fluorescence experiments yielded a single K_d for AMP of 20 μM. The binding of AMP to 4 noninteracting sites on alkaline rabbit liver fructose-1,6-bisphosphatase as measured by gel filtration (Pontremoli et al., 1968) agrees with the fluorescence results.

The inhibition of fructose-1,6-bisphosphatase by AMP is highly specific. The 6-amino group and 5′-monophosphate are essential for binding, whereas the ribose 2-hydroxyl is not (Taketa & Pogell, 1965). The photoaffinity reagents 2-azido-AMP and 8-azido-AMP inhibit pig kidney fructose 1,6-bisphosphatase with Hill coefficients of 2.7 (Riquelme & Czarnecki, 1983) and 2.0 (Marcus & Haley, 1979), respectively. The weak inhibition by 8-azido-AMP (K_i = 0.48 mM) has been attributed to the syn preferred orientation of this molecule (Riquelme & Czarnecki, 1983). The conformation of FMP is also more syn than anti (Prusiner et al., 1973; Sundaralingam, 1975), yet FMP inhibits the rabbit liver enzyme with an I_{50} of 17 μM. Thus, blocking of the adenine C-8 position may reduce nucleotide binding. Distance measurements from paramagnetic Mn^{2+} at the structural metal site to the nucleotide and ribose protons of AMP by nuclear magnetic relaxation (NMR) techniques suggest that AMP bound to rabbit liver fructose-1,6-bisphosphatase assumes an anti conformation (Cunningham et al., 1981). However,

Scheme I



conditions of fast exchange may not pertain for each of the protons examined (Cunningham et al., 1981; Scheffler and Fromm, unpublished observations), and in light of the tight binding by FMP, this assignment is open to further investigation.

AMP binding to rabbit liver fructose-1,6-bisphosphatase is enhanced by Fru-1,6-P_2 as well as by Fru-6-P and P_i, which also induce positive cooperativity among nucleotide sites (Pontremoli et al., 1968; Benkovic et al., 1978b). Recent ^1H NMR studies with fructose-1,6-bisphosphatase have shown that both Fru-1,6-P_2 and Fru-2,6-P_2 enhance nucleotide binding as observed by a decrease in the k_{off} from ≥ 3500 s^{-1} to 200 s^{-1} (Scheffler and Fromm, unpublished results). In contrast, FMP binding is not enhanced by active-site binding ligands, nor is it cooperative, although conformational differences between the enzyme–FMP complex and the enzyme–substrate–FMP species are detected as an increase in the maximum fluorescence of the bound probe.

Weak metal ion binding sites on rabbit liver fructose-1,6-bisphosphatase have been detected by the resultant decrease in FMP fluorescence, which evidently is due to its displacement from the enzyme. The order of effectiveness of metal ions in competition with FMP is $Zn^{2+} = Mn^{2+} > Mg^{2+}$. In the presence of α-Me-Fru-1,6-P_2, or Fru-6-P and P_i, the concentration of each metal ion required to displace FMP is reduced by approximately 1 order of magnitude. The metal ion sites of fructose-1,6-bisphosphatase that are competitive with FMP binding are most likely the catalytic metal sites. Substrates and α-Me-Fru-1,6-P_2 are known to enhance catalytic divalent cation binding (Pedrosa et al., 1977; Benkovic et al., 1978a,b), and the effectiveness of different metals in displacing FMP is the same as observed for metal binding to the catalytic site. Catalytic metal binding has not previously been detected in the absence of active-site binding ligands. The binding of Mn^{2+} to fructose-1,6-bisphosphatase in the presence of α-Me-Fru-1,6-P_2 measured by fluorescence yields a dissociation constant of 14 μM. The dissociation constant for catalytic Mn^{2+} in the presence of α-Me-Fru-1,6-P_2 determined by electron paramagnetic resonance (EPR) is 50 μM (Libby et al., 1975), in agreement with the value obtained by Mn^{2+} displacement of FMP. The K_m's for Mn^{2+} and Mg^{2+} catalysis are 15 μM and 660 μM, respectively (Libby et al., 1975; Benkovic et al., 1978b; Pontremoli et al., 1978). Binding of metal ions at the structural divalent cation sites of fructose-1,6-bisphosphatase are 1 order of magnitude tighter than catalytic metal binding (Libby et al., 1975). The dissociation constant for structural Mn^{2+} binding to fructose-1,6-bisphosphatase in the presence of the substrate analogue is 1.1 μM.

The competitive binding of FMP and metal ions is not unique to this nucleotide analogue. Opie and Newsholme (1967) observed reduced AMP inhibition of skeletal muscle fructose-1,6-bisphosphatase in the presence of Mn^{2+} and Mg^{2+}. Pontremoli et al. (1968) reported that Mn^{2+} reduces AMP inhibition and binding to the alkaline rabbit liver enzyme. However, the effect of Mg^{2+} on AMP inhibition has been attributed to metal chelation of the nucleotide (Opie & Newsholme, 1967; Pontremoli et al., 1968). The fluorescence results indicate that Mg^{2+} is also a competitive binding ligand with respect to AMP.

The mechanism of Fru-2,6-P_2 inhibition has been attributed to both active-site binding (Ganson & Fromm, 1982, 1985; Kitajima & Uyeda, 1983) and binding at an allosteric site (Hers & Hue, 1983; Hue & Bartrons, 1985; Francois et al., 1983). Fru-1,6-P_2 and Fru-2,6-P_2 are mutually exclusive binding ligands as shown by kinetic and binding studies (Pontremoli et al., 1982; Ganson & Fromm, 1983; Kitajima & Uyeda, 1983). The distances measured by NMR from the phosphate groups of the substrate and inhibitor to the structural Mn^{2+} of fructose-1,6-bisphosphatase are identical (Gason & Fromm, 1985), and both ligands perturb the same observable aromatic resonances of the ^1H NMR spectrum of fructose-1,6-bisphosphatase (Scheffler & Fromm, 1986). Thus, the evidence for Fru-2,6-P_2 binding at the active site is considerable. Differences in the interaction of Fru-1,6-P_2 and Fru-2,6-P_2 with fructose-1,6-bisphosphatase are evident because the latter is not a substrate. Using FMP as a fluorescent probe for metal binding indicates that Fru-2,6-P_2 does not enhance metal ion mediated displacement of FMP. In fact, Fru-2,6-P_2 either blocks catalytic metal binding or induces a protein conformational change, allowing the mutual binding of FMP and Mn^{2+}. Kitajima and Uyeda (1983) have noted that Mn^{2+}, Co^{2+}, and, to a lesser extent, Mg^{2+} reduce Fru-2,6-P_2 binding. It seems likely that Fru-2,6-P_2 and catalytic metal ion binding are competitive.

The reaction catalyzed by fructose-1,6-bisphosphatase constitutes a control point in glycolysis and gluconeogenesis and is regulated by the action of AMP and Fru-2,6-P_2. Inhibition by these ligands is synergistic (Pilkis et al., 1981b; Van Schaftingen et al., 1981). On the basis of fluorescence studies with FMP, we have found that FMP and divalent metal ions, which are essential for catalysis, bind competitively. Fru-2,6-P_2 and the substrate, Fru-1,6-P_2, also bind in a mutually exclusive fashion, most likely at the active site. Substrate binding is required to promote catalytic metal binding. From ^1H NMR studies, we know that Fru-2,6-P_2 lowers the k_{off} for AMP; i.e., it makes the allosteric site for AMP more sticky. Thus, the relationship between the regulatory ligands is as follows: AMP displaces the catalytic metal from fructose-1,6-bisphosphatase, and its affinity for the enzyme is enhanced by Fru-2,6-P_2. In addition, Fru-2,6-P_2 prevents substrate binding by competition at the active site and also prevents catalytic metal binding. The net result is potent synergistic inhibition of fructose-1,6-bisphosphatase.

Scheme I summarizes our interpretation of the interaction

of AMP, Fru-2,6-P_2, "catalytic metal site" metal (M^{2+}), and fructose-1,6-bisphosphatase. The interaction pathway considers ligand binding only to the enzyme monomer and does not incorporate cooperative effects that are known to occur with the ligands illustrated in Scheme I (Pontremoli et al., 1968; Libby et al., 1975) nor does it allow for formation of ternary complexes of enzyme, metal, and AMP. Nevertheless, this pathway provides the basis for regulation of fructose-1,6-bisphosphatase at the molecular level.

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