Fructose Diphosphatase from Rabbit Liver. X. Isolation and Kinetic Properties of the Enzyme–Adenosine Monophosphate Complex*

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ABSTRACT: The formation at pH 7.5, in the absence of either substrate or cation, of a fructose diphosphatase–adenosine monophosphate complex has been described. Four sites are available on the enzyme for the binding of adenosine monophosphate. The four sites are functionally equivalent and not interacting, the association constant for the formation of the enzyme–adenosine monophosphate complex being 0.5×10^5 M^{-1}. In the presence of 1 mM fructose 1,6-diphosphate the association constant is increased and a positive interaction between the adenosine monophosphate binding sites is induced. Mg^{2+} and Mn^{2+} influence the binding and the inhibition by adenosine monophosphate. In the case of Mg^{2+} the effect is fully explained by the decrease in the concentration of free adenosine monophosphate which results from its removal as the adenosine monophosphate–metal complex. In the case of Mn^{2+} two additional factors must be considered: (1) a competition between adenosine monophosphate and Mn^{2+} for sites of the enzyme and (2) the induction of a positive interaction between the adenosine monophosphate binding sites.

Since the first reports on the inhibition of fructose diphosphatase by AMP[1] (Taketa and Pogell, 1963; Newsholme, 1963), many laboratories have published similar observations and it is now clear that fructose diphosphatases from a number of widely different biological sources share the property of being regulated by AMP (Mendicino and Vasarhely, 1963; Rosen *et al.*, 1965; Gancedo *et al.*, 1965; Krebs and Woodford, 1965; Fraenkel *et al.*, 1966; Opie and Newsholme, 1967).

In the case of the enzyme from yeast (Rosen and Rosen, 1966), liver (Taketa and Pogell, 1965; Pontremoli *et al.*, 1966a), and kidney (Marcus, 1968), the inhibition by AMP has been characterized as "allosteric," since in each case it was possible to modify the enzyme so as to reduce or abolish the sensitivity toward AMP, without decreasing the catalytic activity.

The inhibition by AMP is noncompetitive with respect to fructose 1,6-diphosphate and competitive with respect to Mg^{2+} and Mn^{2+} (Taketa and Pogell, 1965; Opie and Newsholme, 1967). The inhibitory effect of AMP increases with decreasing pH and with decreasing temperature (Taketa and Pogell, 1965) and is more pronounced in the assay with Mg^{2+} than with Mn^{2+} (Pontremoli *et al.*, 1966a; Opie and Newsholme, 1967).

The kinetic studies so far available, carried out with crude extracts or partially purified enzyme preparations, have shown that plots of percentage inhibition against AMP concentration are either hyperbolic or sigmoid, depending upon the source of the enzyme used (Gancedo *et al.*, 1965; Taketa and Pogell, 1965; Opie and Newsholme, 1967). The experiments of Taketa and Pogell (1965) with the rat liver enzyme led them to propose the existence in the enzyme of three to four interacting sites for the binding of AMP. Recently, Watanabe *et al.* (1968) have reported that fructose 1,6-diphosphatase from liver requires fructose 1,6-diphosphate for the binding of AMP.

We have previously described the preparation of crystalline fructose diphosphatase from rabbit liver and we have now utilized this preparation for the direct measurement of the binding of AMP by the enzyme. The results of our studies on the effect of fructose 1,6-diphosphate, Mg^{2+}, and Mn^{2+} on the binding of AMP to the enzyme are presented. They provide explanation of data previously reported by other authors on the inhibition of fructose diphosphatase by AMP.

Experimental Procedures

Materials. Fructose diphosphatase was prepared as previously described (Pontremoli *et al.*, 1965a,b) to remove traces of contaminating metals, and concentrated solutions of the enzyme were incubated for 30 min at 2° in the presence of 1 mM EDTA (pH 7.5) and then dialyzed for 4 hr at 2° against triethanolamine–HCl buffer (0.02 M, pH 7.5) or passed through a column of Sephadex G-25 equilibrated with the same buffer. The specific activity was 20 IU/mg of protein. The molecular weight was taken to be 130,000 (Pontremoli *et al.*, 1965a). Glucose 6-phosphate dehydrogenase and glucose 6-phosphate isomerase were obtained from

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[1] See *Biochemistry 5*, 1445 (1966).

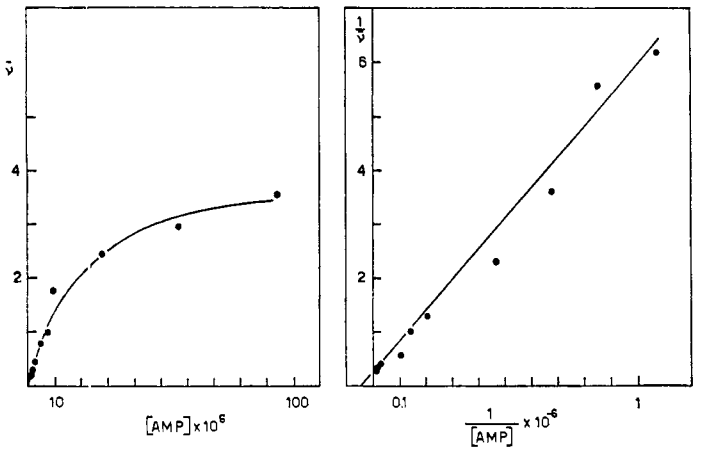

FIGURE 1: Effect of AMP concentration on the number of equivalents bound per mole of enzyme ν. Fructose diphosphatase, dissolved in 0.5 ml of 0.02 M triethanolamine–HCl buffer (pH 7.5), was filtrated as described under Methods through Sephadex G-50 columns equilibrated with the same buffer containing [^{14}C]AMP as indicated. The quantity of protein was 10 mμmoles except for the columns with the two highest AMP concentrations where it was 20 mμmoles. In A the data were plotted directly; in B as the reciprocal.

Boehringer und Soehne, Germany. Fructose 1,6-diphosphate and TPN^+ (sodium salts) were obtained from Sigma Chemical Corp., St. Louis. Sephadex G-50 (coarse) and Sephadex G-25 were supplied by Pharmacia, Uppsala, Sweden. [^{14}C]AMP (sp act. 3.2×10^5 cpm per μmole) was purchased from the Radiochemical Centre, Amersham, England.

Methods. The determination of specific activity of the enzyme preparations was performed spectrophotometrically as previously described (Pontremoli *et al.*, 1968). For the experiments reported here, fructose diphosphatase activity was estimated from the rate of appearance of inorganic phosphate using the method of Fiske and Subbarow (1925). This method had the advantage that it did not require the addition of compounds other than the enzyme, substrate, and buffer. The incubation was performed at 2° and pH 7.5 (the pH being measured at this temperature). The mixtures (0.5 ml) contained 0.02 M triethanolamine–HCl buffer, 1 mM fructose 1,6-diphosphate, and $MgCl_2$ or $MnCl_2$ as indicated for the individual experiments. Only the linear part of the hydrolysis curves was employed for the calculations. The reaction was stopped by the addition of 0.1 ml of 5 N H_2SO_4.

Protein concentration was calculated from the absorbance at 280 mμ and standardized against a known dry weight of dialyzed enzyme. A solution containing 1 mg/ml yielded an absorbance of 0.83 at 280 mμ.

Sephadex G-50 filtration was performed at 2° and pH 7.5 on columns (1.2 × 37 cm) equilibrated with 0.02 M triethanolamine–HCl buffer containing [^{14}C]-AMP at various concentrations; when indicated $MgCl_2$ or $MnCl_2$ was added. The flow rate was 4 ml/min and 1-ml fractions were collected for the first 22 ml after which the volume of the fractions was 5 ml. Each fraction was analyzed for protein (280-mμ absorbance) and AMP (radioactivity). At high concentrations of AMP, it was necessary to subtract the absorbance at 280 mμ due to this substance before calculating the protein concentration.

Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter in 10 ml of Bray's (1960) solution. The bound AMP was calculated from the difference in radioactivity between the fractions with and without fructose diphosphatase.

The constants for binding of Mn^{2+} by AMP and fructose 1,6-diphosphate were measured by the method of Waalas (1958) and for Mg^{2+} by the method of Burton (1959). The measurements were made at 2° in 0.02 M triethanolamine–HCl buffer (pH 7.5). In each case association constants for the complexes were calculated assuming 1:1 complexes. For the AMP–Mn^{2+} complex an association constant of 200 M^{-1} was estimated at an AMP concentration of 0.1 mM in the range of 0.2–2 mM $MnCl_2$. For the fructose 1,6-diphosphate–Mn^{2+} complex, with 0.1 mM fructose 1,6-diphosphate and 0.2–2 mM $MnCl_2$, the association constant was approximately 1800 M^{-1}. In the case of Mg^{2+}, difficulties were encountered since the ionic strength of the medium changed as the $MgCl_2$ concentration increased from 1 to 20 mM. At low $MgCl_2$ concentration, the association constant for the AMP–Mg^{2+} complex was approximately 100, while at higher concentrations the association constant was 70 M^{-1}. The average value of 85 M^{-1} was used in the calculations.

Results

Measurements of AMP Binding to Fructose 1,6-Diphosphatase. This was studied at pH 7.5 employing columns of Sephadex G-50 which had been equilibrated with various concentrations of [^{14}C]AMP. The sample containing fructose diphosphatase was placed on the column and eluted with a solution containing the same concentration of AMP. At the highest concentration of AMP employed, the number of equivalents of AMP bound approached 4. The binding of AMP to the enzyme was found to follow the mass law expression even at very low AMP concentration (Figure 1), and the double-reciprocal plot yielded a straight line with an intercept on the ordinate of 0.25; thus, at infinite AMP concentration, the number of equivalent bound ap-

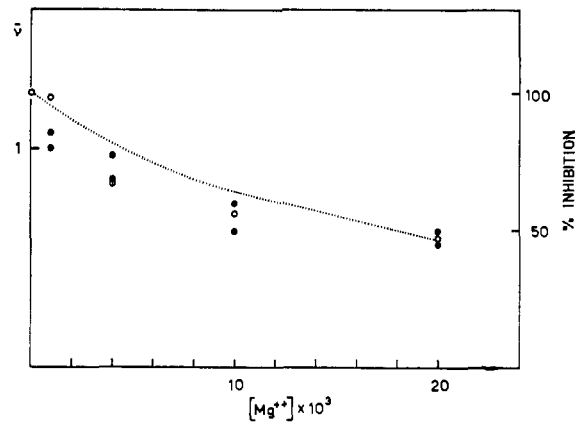

FIGURE 2: Effect of Mg^{2+} concentration on the formation of the enzyme–AMP complex and on the inhibition by AMP. The open circles represent moles of AMP bound per mole of enzyme. The closed circles are per cent inhibition by AMP. The dotted line represents the predicted values for bound AMP calculated on the basis of an association constant of 85 M^{-1} for the 1:1 AMP–Mg^{2+} complex and of 0.5×10^5 M^{-1} for the enzyme–AMP complex. The enzyme–AMP complex was prepared as described under Methods by filtration of fructose diphosphatase (1.3 mg) dissolved in 0.5 ml of 0.02 M triethanolamine–HCl buffer (pH 7.5) through a Sephadex G-50 column equilibrated with 10^{-5} M [^{14}C]AMP. $MgCl_2$ was added, as indicated, to the buffer–AMP solutions employed to prepare the columns and elute the enzyme. The fractions containing enzyme were analyzed for protein content and for radioactivity associated with the protein. For evaluation of the inhibition by AMP, reference samples, without AMP, were prepared for each concentration of $MgCl_2$ employed and submitted to exactly the same treatment as the samples with AMP. Catalytic activity was then assayed directly, without dilution, on the protein-containing fractions eluted from the columns. The reactions were started by addition of 0.1 M fructose 1,6-diphosphate to a final concentration of 1 mM.

proached the value of 4. The association constant for the binding of AMP to the enzyme was calculated to be 0.5×10^5 M^{-1}.

Thus, the four binding sites for AMP appear to be equivalent and no cooperation was detectable under the conditions described.

Effect of Fructose 1,6-Diphosphate on the Binding of AMP to Fructose Diphosphatase. As shown in Table I, more AMP was bound to the enzyme in the presence than in the absence of the substrate. The effect was particularly evident at high (1 mM) substrate concentration.

Analysis of the data of Table I, where the binding of AMP to the enzyme as a function of AMP concentration and in the presence of 1 mM fructose 1,6-diphosphate was studied, showed that the shape of the saturation curve, which in the absence of the substrate is hyperbolic, became sigmoid in the presence of fructose 1,6-diphosphate.

Inhibition of Fructose Diphosphatase by AMP in the Presence of Mg^{2+}. The result of the experiment reported in Figure 2 shows that, when the concentration of Mg^{2+} was increased, both the binding of AMP and the inhibition of catalytic activity decreased in a parallel manner. The effect of Mg^{2+} was almost precisely what would have been predicted on the basis of binding of

TABLE I: Effect of AMP and Fructose 1,6-Diphosphate Concentrations on the Number of Equivalents Bound per Mole of Enzyme.[a]

Expt	AMP (μM)	Fructose 1,6-Diphosphate (mM)	Moles of AMP Bound/Mole of Enzyme
1	10.0	0.0	1.65
2	10.0	0.1	2.16
3	10.0	1.0	2.95
4	1.4	1.0	0.56
5	2.5	1.0	1.33
6	5.1	1.0	2.36
7	9.0	1.0	2.80

[a] The experiments were performed as described under Methods. Fructose diphosphatase was 10 mμmoles. The Sephadex G-50 columns were equilibrated with AMP and fructose 1,6-diphosphate at the concentrations indicated.

AMP by Mg^{2+}, assuming an association constant of 85 M^{-1}, and an enzyme–AMP association constant of 0.5×10^5 M^{-1}.

In other experiments we have shown that the inhibition by AMP, as a function of the Mg^{2+} concentration, followed the same pattern when 0.2 mM fructose 1,6-diphosphate was employed instead of 1 mM; and the concentration of fructose diphosphatase was decreased to 0.012 from 0.5 mg/ml.

Thus the effect of Mg^{2+} can be fully accounted for in term of the formation of an AMP–Mg^{2+} complex and there is no evidence for a competition between Mg^{2+} and AMP for the enzyme. This was further substantiated by the fact that inhibition by AMP did not depend upon Mg^{2+} concentration provided correction for binding of AMP by Mg^{2+} was made (Figure 3).

Stoichiometry of AMP Binding and Inhibition of Catalytic Activity in the Presence of Mg^{2+}. In order to correlate the binding of AMP and the inhibition of catalytic activity, the following parameters must be considered: the effect of Mg^{2+} on the free AMP concentration and the effect of fructose 1,6-diphosphate on the binding of AMP to the enzyme.

The samples utilized for the determination of the residual catalytic activity in the experiment illustrated in Figure 2 contained, in 0.5 ml, 4 mμmoles of fructose diphosphatase. The amount of AMP bound to the enzyme was 4.8 mμmoles in the case of 1 mM $MgCl_2$ and 2.4 mμmoles in the case of 20 mM $MgCl_2$. The amount of AMP, free or bound to Mg^{2+}, was 5 mμmoles in the entire range of Mg^{2+} concentrations studied.

When, for the assay of the catalytic activity, 1 mM fructose 1,6-diphosphate was added, the association constant of the enzyme–AMP complex was increased but, since the amount of AMP present was small, the

TABLE II: Effect of Mn^{2+} on Binding of AMP and Inhibition of Enzyme Activity.

Expt	Total Mn^{2+} Concn (mM)	Total AMP Concn (μM)	Inhibn (%)[a]	AMP Bound[b] (equiv/mole of enzyme)
1	0.00	10		1.4
	0.02	10	15	1.26
	0.05	10	13	1.02
	1.00	10	3	1.00
	2.00	10	2	0.72
2	0.1	10	15	1.0
	0.3	10	5	1.0
	1.0	10	3	0.9
3	1.0	5	1	0.4
	1.0	10	3	0.9
	1.0	30	20	1.8
	1.0	60	37	3.1
	1.0	90	48	3.3

[a] The experimental conditions are those reported in the legend of Figure 4 except that in the expt 1 and 2 0.2 mM fructose 1,6-diphosphate was used. [b] Experimental conditions are as described in the legend of Figure 2 except that $MnCl_2$ was substituted for $MgCl_2$.

effect of the shift in the association constant must have been limited. A rough estimate of this change can be made utilizing the data of Table I. From the calculation and taking account for the AMP–Mg^{2+} complex concentration, it can be shown that, at 1 mM $MgCl_2$ where the inhibition is 85%, the number of moles of AMP bound per mole of enzyme could be increased from 1.25 to a maximum of 1.72.

It seems therefore that the binding of AMP to some of the four sites affects the catalytic activity of all the subunits.

Fructose Diphosphatase Inhibition by AMP in the Presence of Mn^{2+}. The inhibition of enzyme activity and the binding of AMP in the presence of Mn^{2+} were studied with the same techniques as were employed for Mg^{2+}.

The addition of Mn^{2+} decreased the extent of binding of AMP to the enzyme (Table II, expt 1). The effect on binding, however, could not be attributed to a decrease in the concentration of free AMP resulting from the formation of an AMP–Mn^{2+} complex (association constant 200 M^{-1}); at 0.05 mM Mn^{2+}, where the amount of binding was decreased by 30%, the amount of AMP removed by Mn^{2+} would not be significant. Thus an additional factor, such as a competition between AMP and Mn^{2+} for site(s) on the enzyme, must be operating. Such competition influences the catalytic activity, since the inhibition by AMP was higher at low Mn^{2+} concentration (Figure 4, Table II). As the concentration

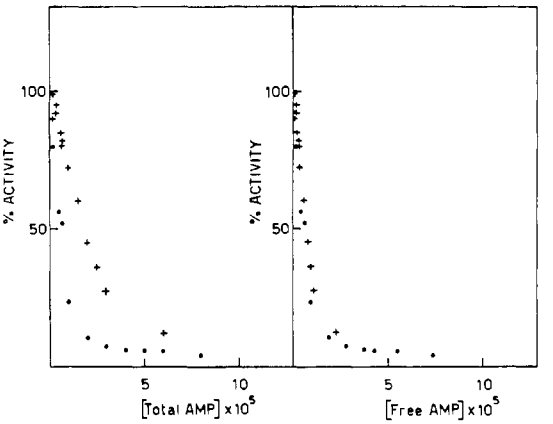

FIGURE 3: Inhibition of fructose diphosphatase by AMP in the presence of Mg^{2+}. The incubation mixtures (0.5 ml) contained fructose diphosphatase (5.85 μg), either 1 mM $MgCl_2$ (●) or 20 mM $MgCl_2$ (+), 1 mM fructose 1,6-diphosphate, 0.02 M triethanolamine–HCl buffer (pH 7.5), and AMP as indicated in the figure. The mixtures were incubated for 10 min at 2°, the reaction was stopped by addition of 0.1 ml of 5 N sulfuric acid, and the orthophosphate formed was determined. In A the residual activity is plotted against total AMP concentration. In B the concentration of free AMP was calculated on the basis of an association constant of 85 M^{-1} for the AMP–Mg^{2+} complex. The effect of fructose 1,6-diphosphate on the concentration of Mg^{2+} was neglected.

of Mn^{2+} was raised, the inhibition by AMP decreased, and under appropriate conditions could be abolished completely. When the Mn^{2+} concentration was maintained at 1 mM and the concentration of AMP was increased, there was an increase in the amount of AMP bound by the enzyme, but even with more than 3 equiv bound/mole of enzyme, only 50% inhibition was observed (Table II, exp 3). It is noteworthy that in the expt 1 and 2 (Table II) at 1 mM Mn^{2+}, there was little inhibition when 1 mole of AMP was bound/mole of enzyme. This is in contrast to the effect of AMP in the presence of Mg^{2+}.

The effect of Mn^{2+} was further investigated by direct

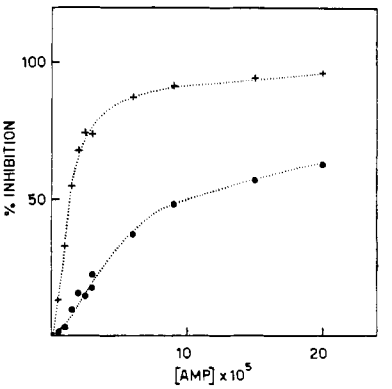

FIGURE 4: Inhibition of fructose diphosphatase by AMP in the presence of Mn^{2+}. The incubation mixtures (0.5 ml) contained fructose diphosphatase (5.85 μg), either 0.07 mM $MnCl_2$ (+) or 1 mM $MnCl_2$ (●), 1 mM fructose 1,6-diphosphate, 0.02 M triethanolamine–HCl buffer (pH 7.5), and AMP as indicated in the figure. The mixtures were incubated for 10 min at 2°, the reaction was stopped by addition of 0.1 ml of 5 N H_2SO_4, and the orthophosphate formed was determined.

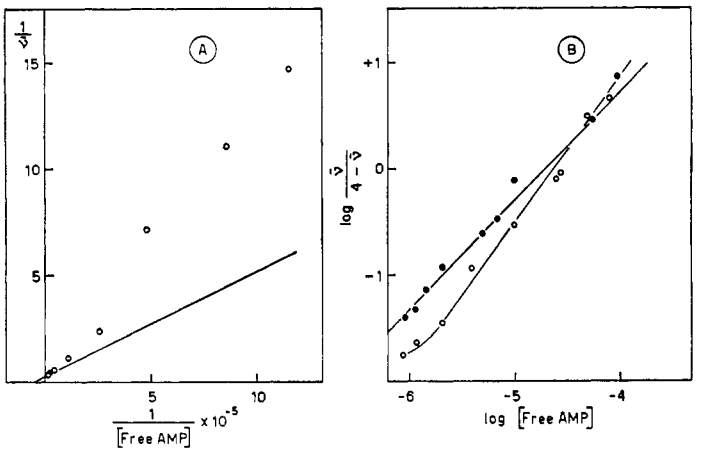

FIGURE 5: Effect of AMP and Mn^{2+} concentrations on the number of equivalents bound per mole of enzyme. Fructose diphosphatase, dissolved in 0.5 ml of 0.02 M triethanolamine–HCl buffer (pH 7.5), was filtrated as described under Methods through Sephadex G-50 columns equilibrated with the same buffer containing [^{14}C]AMP at the following concentrations: 1.04, 1.38, 2.52, 4.7, 10.0, 29.5, 58.6, and 94.0 μM. The quantity of protein was 10 mμmoles except for the columns with the two highest AMP concentrations, where it was 20 mμmoles. In A $1/\nu$ is plotted against 1/AMP in the presence of 1 mM $MnCl_2$ (O). The continuous line was reproduced from Figure 1. Free AMP concentration was calculated on the basis of an association constant of 200 for the AMP–Mn^{2+} complex. In B log $\bar{\nu}/(4 - \bar{\nu})$ is plotted against log AMP, in the absence (●), and in the presence (O) of 1 mM $MnCl_2$.

measurements of the formation of the AMP–enzyme complex in the presence of Mn^{2+} (Figure 5A). At low concentration of AMP, Mn^{2+} clearly influenced the binding to the enzyme, although the effect could not be explained in terms of either full or partial competition. Analysis of the data of Figure 5A by the Hill plot showed that the addition of Mn^{2+} resulted in a positive interaction between the binding sites for AMP (n = 1.42) (Figure 5B). Thus, Mn^{2+} affected the binding of AMP to the enzyme in two ways: (1) it decreased the binding of AMP to the enzyme, and (2) it induced a positive interaction between the AMP binding sites.

Discussion

In the preceding paper of this series (Pontremoli *et al.*, 1968) we reported that fructose diphosphatase binds four molecules of substrate at pH 7.5 in the absence of the activating cations Mg^{2+} or Mn^{2+}. In the case of fructose 1,6-diphosphate the binding occurs with positive interaction between the four sites. The enzyme has previously been reported to have a molecular weight of 130,000 (Pontremoli *et al.*, 1965a) and to dissociate into halves at pH 2 (Pontremoli *et al.*, 1965b) and into quarters on succinylation and carboxymethylation. Thus the enzyme appears to be composed of four subunits, possibly nonidentical.

In the present paper we have found that the enzyme also binds 4 equiv of AMP at pH 7.5. In this case, however, there is no evidence of interaction between the binding sites, which appear to have identical association constants whose value is 0.5×10^5 M^{-1}.

At subsaturating concentration of AMP, less AMP is bound in the presence of Mg^{2+} than in its absence, but this effect of the cation is fully explained in terms of the decrease in the concentration of free AMP which results from its removal as the AMP–metal complex. The observed effects of Mg^{2+} on inhibition of enzyme activity by AMP may also be explained in this way. Of particular interest is the observation that in the presence of Mg^{2+} the binding of much less than 4 equiv of AMP is sufficient to inhibit enzymic activity almost completely.

The results obtained in the presence of Mn^{2+} are more complex and suggest a competitive interaction between the binding sites for AMP and Mn^{2+}. When the concentration of Mn^{2+} is high, there is little inhibition by AMP, and the binding of AMP is reduced. At the highest concentrations of AMP employed, nearly 4 equiv was bound/mole of enzyme, but the activity was inhibited by only 50%. In addition to these competitive effects Mn^{2+} also induced a positive interaction between the AMP binding sites, which partially counteracted the effect of this ion on the affinity of AMP for the enzyme.

Watanabe *et al.* (1968) have recently reported the requirement of substrate for AMP binding to liver fructose diphosphatase. We have found no evidence for such a requirement; we have shown, however, that fructose 1,6-diphosphate promotes a definite increase in the binding of AMP to the enzyme and changes the shape of the saturation curve from hyperbolic to sigmoid. This effect increases by increasing substrate concentration, but is clearly detected only with fructose 1,6-diphosphate concentrations 1000- to 10,000-fold larger than the K_m. The influence of the substrate on AMP binding is quite surprising since AMP inhibition is noncompetitive with respect to fructose 1,6-diphosphate. The conclusion seems inescapable that no simple relation exists between binding of AMP to the enzyme and inhibition of catalytic activity.

The previous estimate of Taketa and Pogell (1965) of three to four binding sites for AMP is in agreement with our results. We can also explain the data first reported by these authors of a sigmoid kinetic obtained in plots of inhibition *vs.* AMP concentration. The sig-

moid kinetic, when catalytic activity is assayed in the presence of Mg^{2+}, depends upon the influence of the substrate on the AMP binding. In the presence of Mn^{2+}, both substrate and cation contribute to induce a positive interaction between the AMP binding sites.

The binding of 4 equiv each of substrate and allosteric effector suggests that each subunit may contain both types of binding sites. Preliminary evidence (Pontremoli, 1965) suggests that the enzyme contains at least two different types of subunits and further interpretation must await isolation and characterization of these subunits.

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