Allosteric Inhibition of Rat Liver Fructose 1,6-Diphosphatase by Adenosine 5'-Monophosphate*

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The two major sites for glycogenogenesis in mammals are liver and kidney (2, 3). Both of these organs contain the specific enzyme, fructose 1,6-diphosphatase, which catalyzes the following reaction (4-6).

\[
\text{d-Fructose 1,6-diphosphate} \quad \rightarrow \quad \text{d-fructose 6-phosphate} \quad + \text{inorganic phosphate}
\]

This enzymatic step is presumed to be obligatory for glycogenesis in order to bypass the highly exergonic phosphofructokinase reaction (7). A sensitive mechanism for the regulation of fructose 1,6-diphosphatase activity would be of importance in control of the direction of flow of carbohydrate metabolism in these organs, i.e. catabolism via glycolysis or glycogenolysis versus the anabolic process of glycogenogenesis. We have recently reported three possible mechanisms for the regulation of liver fructose 1,6-diphosphatase activity under physiological conditions (8): (a) the specific and reversible inhibition by adenosine 5'-monophosphate, (b) the reversible inhibition of the enzyme by substrate at concentrations greater than 0.1 mM, and (c) the reversible inactivation of fructose 1,6-diphosphatase by reaction with ATP or ADP. This latter process could be clearly distinguished from inhibition by AMP, since the inactivation did not occur in the presence of either substrate, phosphate, or ethylenediaminetetraacetate and was not reversible by either dilution or overnight dialysis at 4°C. Our detailed studies on the first two of these mechanisms is the subject of the present report.

Recent reports from several other laboratories have confirmed the widespread role of both AMP and fructose 1,6-diphosphatase as inhibitors of fructose 1,6-diphosphatase from different sources. Weber (9) first reported inhibition of rat liver fructose 1,6-diphosphatase by excess substrate, and Newsholme and Krebs* have obtained results similar to ours with rat liver extracts. Mendicino and Vasarhely (6) and Salas, Vitiello, and Sol's (11) have shown that both rat kidney and frog muscle contain specific enzymes for hydrolysis of fructose 1,6-diphosphate and that both AMP and substrate acted as inhibitors. Williams, Wilson, and Utter also have isolated a specific fructose 1,6-diphosphatase from yeast extracts which was inhibited by AMP. It is of interest that the fructose 1,6-diphosphatase activity induced in Escherichia coli by growth on noncarbohydrate precursors (12) was not inhibited by either AMP or high substrate concentrations.8 The specific "neutral" fructose 1,6-diphosphatase of Euglena gracilis (13), which was still present in extracts of a streptomycin-bleached strain of the protozoan and is presumably involved in glycogenogenesis, was inhibited by excess substrate but not by AMP.

EXPERIMENTAL PROCEDURE

Materials—Adenosine 5'-monophosphate, TPN+, and fructose 1,6-diphosphate were obtained as the sodium salts from Sigma Chemical Company; phosphohexoisomerase (yeast) and glucose-6-P dehydrogenase (yeast) from either Sigma or California Corporation for Biochemical Research; and twice crystallized papain suspensions from Worthington Biochemical Corporation.

Preparation of Fructose 1,6-Diphosphatase—All procedures were carried out at 0-4°C except where indicated otherwise. Normal, well fed rats, weighing from 150 to 200 g, were killed by blows on the head and exsanguinated. The livers were quickly removed, homogenized for 1 minute in either a Waring Blender or a glass homogenizer in 4 volumes of ice-cold 0.154 M KCl per g of tissue, and centrifuged for either 90 minutes at 20,000 x g or 60 minutes at 60,000 x g. An aliquot of this high speed supernatant was dialyzed overnight against glass-distilled water at 4°C. The dialyzed rat liver supernatant was further purified by (a) acidifying to pH 4.5 with glacial acetic acid and removing the precipitate by centrifugation and (b) heating this supernatant for 5 minutes with stirring in a water bath kept at 55°C, cooling in ice, neutralizing with 10 N NaOH, and removing the precipitate by centrifugation. The heated fructose 1,6-diphosphatase preparation thus obtained, about 5- to 10-fold purified, had no aldolase activity and could be stored at -20°C for more than 4 months without any appreciable loss of enzyme activity. No evidence for AMP deamination or hydrolysis was found under our assay conditions with high speed supernatant. Rat liver has been reported to contain small amounts of AMP deaminase (14).

All reagents for enzyme analyses were made in glass-distilled water.

8 M. F. Utter, personal communication.
9 B. M. Pogell, unpublished observations.

* E. A. Newsholme and H. A. Krebs, personal communication.
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Fructose 1,6-diphosphatase activity was determined spectrophotometrically by following the rate of TPNH formation at 340 nm in the presence of excess phosphohexoisomerase and glucose-6-P dehydrogenase. Accurate measurements with low substrate concentrations were obtained with a Gilford multisample absorbance recording system set at maximum sensitivity so that full scale deflection of the 10-inch recording chart corresponded to an absorbance of 0.1. The usual test system contained 0.1 mM FDP, 50 mM Tris-HCl (pH 7.5), 0.15 mM TPN+, 20 mM 2-mercaptoethanol, 10 mM MgSO4, excess phosphohexoisomerase (2 units per ml) and glucose-6-P dehydrogenase (0.3 unit per ml), and fructose 1,6-diphosphatase in a final volume of 1.0 ml. Assays were conducted at 30°. Fructose 1,6-diphosphatase was added to the complete system minus substrate, and the reaction was started by addition of FDP. With dialyzed preparations of fructose 1,6-diphosphatase, complete reaction mixtures minus substrate were incubated for 10 minutes at 30° before the addition of FDP in order to obtain a maximum activity of the diluted enzyme (from 1:500 to 1:2500 final dilution of liver). In the assay with phosphate buffer, a relatively large amount of glucose-6-P dehydrogenase was required to saturate the system because of the inhibitory effect of phosphate on this enzyme. Except for an initial lag period of less than 1 minute, all reaction rates were constant over the time period studied.

For fructose 1,6-diphosphatase assays by the measurement of inorganic phosphate liberation, the routine spectrophotometric assay system was employed with the omission of TPN+, phosphohexoisomerase, and glucose-6-P dehydrogenase. After 10 to 30 minutes of incubation at 30°, the reaction was stopped by the addition of 0.2 ml of 30% trichloroacetic acid, and the precipitate was removed by centrifugation. The inorganic phosphate in the supernatant was determined according to the micromethod described by Chen, Toribara, and Warner (15).

RESULTS

Effect of Substrate Concentration on Rat Liver Fructose 1,6-Diphosphatase—Concentrations of FDP greater than 0.1 mM were found to inhibit the enzyme activity markedly at physiological pH. In Tris buffer at pH 7.5, the inhibitions were 58 and 82% with 1 and 20 mM FDP, respectively (Fig. 1). Similar results were obtained with either phosphate or glycylglycine buffers at pH 7.5. Determination of the $K_m$ by a Lineweaver-Burk plot from data in the range of noninhibitory substrate concentrations gave a value of $5.5 \times 10^{-4}$ M with crude rat liver supernatant fluid, which still retained aldolase activity (Fig. 1, inset). With an aldolase-free preparation, a lower $K_m$ of 1 to 3 $\times 10^{-4}$ M was found (see Fig. 4). The small variation in this value was due to inaccuracies in our assays at very low FDP concentrations and also to some variation with different Mg++ concentrations (5 and 10 mM). It is of interest that the total activity and $K_m$ values of aldolase in rat liver were lower but of the same order of magnitude as fructose 1,6-diphosphatase.

The pH optimum for fructose 1,6-diphosphatase in the presence of 0.1 mM FDP and 10 mM Mg++ at 30° was 7.3 (Fig. 2). Maximal inhibition by high substrate concentrations was also found at this pH (Fig. 2). At pH values greater than 9.0 or lower than 6.0, there was no inhibition by 1 mM FDP. In fact, the abbreviation used is: FDP, D-fructose 1,6-diphosphate.
the rate of enzyme activity continued to rise with increasing sub-
strate concentration at pH 9.3 in the presence of excess Mg++. The facts that a plot of activity against log [FDP] gave a
symmetrical bell-shaped curve (see Fig. 4) and that maximum
inhibition by substrate was found at the pH for maximum activ-
ity support a mechanism of inhibition by FDP due to competitive
binding of 2 identically charged substrate molecules to the active
site. If one assumes that the binding of 2 FDP molecules per
active site results in an inactive complex, then the first binding
constant for FDP in the activable position would be much
smaller than the binding constant for the second substrate
molecule. The calculated ratio, $K_{FDPR}:K_{FDPII}$, was 0.1 (16).

**Effect of Temperature on Fructose 1,6-Diphosphatase Activity**

The effect of temperature on the rate of enzyme activity at pH
7.3 is shown in Fig. 3. A linear Arrhenius plot was found over
the temperature range of 2°–46°. The assays were made at each
temperature in the presence of excess phosphohexoisomerase
and glucose-6-P dehydrogenase, and in all cases there was no
change in reaction rate during the course of the analyses. The
$Q_10$ varied from 2.84 (0–10°) to 2.14 (40–50°), and the calculated
activation energy, $E_a$, was 15.9 kcal per mole.

**Inhibition by Adenosine 5'-Monophosphate—**AMP was found
to be a reversible, noncompetitive, and specific inhibitor of rat
liver fructose 1,6-diphosphatase. The reversibility of the
inhibition was shown by the fact that treatment of the enzyme
with 5 mM AMP, a concentration more than sufficient to cause
complete inhibition, followed by dilution resulted in complete
restoration of activity. Similar results were obtained by treat-
ment of the inhibited system with purified AMP deaminase.

The data shown in Fig. 4 indicate that the inhibition by AMP
was noncompetitive, even at high substrate concentrations.
Thus, with 10 mM FDP, which inhibited the enzyme 72%, the
further addition of 0.16 mM AMP increased the inhibition to
93%. The fraction of activity inhibited by AMP was inde-
pendent of substrate concentrations ranging from $5 \times 10^{-5}$ to
$10^{-3}$ M. Reciprocal plots of velocity against FDP concentration
in the range in which there was no substrate inhibition had a
common intercept on the negative $x$ axis (Fig. 4, inset). These
data correspond to the case expected for “perfectly noncompeti-
tive” inhibition where $\alpha = 1$, i.e. where the inhibitor has no
effect on $K_m$ (17).

**Specificity of Inhibition—**The following compounds, when
added after substrate at a final concentration of 1 mM, had no
inhibitory effect on fructose 1,6-diphosphatase activity at pH
7.5: 2'-AMP, 3'-AMP, UMP, CMP, GMP, IMP, ATP, GTP,
UTP, CTP, UDP, GDP, CDP, DPN+, pyrophosphate, adenine,
ribose-5-P, adenosine, guanosine, uridine, and cytidine. Cyclic
3',5'-AMP also was without effect at 0.5 mM. ADP was no
longer inhibitory when added to the purified, myokinase-free
rabbit liver fructose 1,6-diphosphatase (8).

Deoxyadenosine 5'-monophosphate was the only other com-
ponent which produced a significant inhibition of the enzyme,
being almost as potent as AMP. A $K_i$ of 0.13 mM was found
for deoxy AMP at 30° and pH 7.5, a value almost identical with
the $K_i$ of 0.11 mM found for AMP under these conditions.

This information indicates that the 6-amino group of the
$^5K_i$ denotes concentration of inhibitor necessary for 50% inhibi-

![Fig. 3. Effect of temperature on fructose 1,6-diphosphatase (FDPase) activity. Crude rat liver supernatant was incubated at different temperatures with the routine assay system with 10 mM phosphate buffer, pH 7.5, in place of Tris. The final pH of the system was 7.3. After temperature equilibration, reactions were started by addition of FDP. For the experiment at 2°, dialyzed supernatant was used, and this value was corrected for the small loss of activity upon dialysis by measurement of enzyme activity at 20° before and after dialysis. Dialysis was necessary because of the significant inhibition by endogenous AMP at low temperatures. For assay of fructose 1,6-diphosphatase at 2°, the reaction chamber was cooled by circulating ice water and equilibrated at 2°. Reaction mixtures kept in ice water were transferred to the chamber after addition of substrate.](http://www.jbc.org/)

![Fig. 4. Inhibition of fructose 1,6-diphosphatase by AMP at different substrate concentrations. ()--(), fructose 1,6-diphosphatase activities of heated, aldolase-free enzyme preparation were assayed at different FDP concentrations with or without added AMP. The reaction was started by addition of FDP, fol-
lowed immediately by AMP. Other assay conditions were as described in “Experimental Procedure.” ○---○, percentage inhibition of fructose 1,6-diphosphatase by 0.08 mM AMP; Δ---Δ, percentage inhibition by 0.16 mM AMP. The inset shows double reciprocal plots of the data in this figure for the range of noninhibitory substrate concentrations.](http://www.jbc.org/)
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Phosphate buffer (10 mM, pH 7.5) was used in place of Tris, and the final pH of the reaction mixture was 7.3. The reaction was started by addition of FDP, followed immediately by AMP. Other conditions of assay were the same as those in "Experimental Procedure." For the experiment at 2°, dialyzed supernatant was used (see legend to Fig. 3). At 30°, identical inhibitions by AMP were observed with both undialyzed and dialyzed preparations. Final dilutions of liver in the reaction mixtures were 1:500 (2°), 1:5000 (46°), and 1:2500 (other temperatures). (At 2°, there was a time lag of about 10 minutes before maximal inhibition by AMP was attained. This observation suggests that AMP binding may produce a conformational change of the enzyme molecule, which occurs at a slower rate at lower temperatures. Newsholme and Krebs have postulated a similar conformational change from kinetic studies at higher temperatures.)

purine base and a 5'-monophosphate group are essential for inhibitory activity, whereas the 2-hydroxyl group of the ribose is apparently not involved.

Nature of AMP Inhibition—The effect of varying AMP concentration on fructose 1,6-diphosphatase activity did not obey simple mass action theory. Plots of relative enzyme activity against inhibitor concentration (Fig. 5) gave sigmoid-shaped curves rather than rectangular hyperbolas, suggesting that more than one AMP molecule per enzyme molecule participated in the formation of inactive enzyme-inhibitor complex. Moreover, the extent of inhibition by AMP increased with decreasing temperature.

If one assumes the following over-all reaction,

$$E + n \text{AMP} \rightleftharpoons E-\text{AMP}_n$$

then

$$K = \frac{[E-\text{AMP}_n]}{[E][\text{AMP}]^n}$$

and

$$\log \frac{[E-\text{AMP}_n]}{[E]} = \log K + n \log [\text{AMP}]$$

where

$$E = \text{active fructose 1,6-diphosphatase}$$

$$E-\text{AMP}_n = \text{inactive enzyme-inhibitor complex}$$

$$n = \text{apparent number of AMP molecules reacting per enzyme molecule to form an inactive enzyme complex, assuming one active site per enzyme molecule}$$

$$K = \text{apparent overall association constant}$$

From the inhibition data obtained at various temperatures, log \( [E-\text{AMP}_n]/[E] \) was plotted against log [AMP], and the family of straight lines shown in Fig. 6 was obtained. The slope of each curve equals \( n \), and log \( K \) is the intercept on the ordinate where log [AMP] = 0. It may be seen that a constant value for \( n \) of 2.3 was found over the temperature range 2°–46°. The deviation of the value of \( n \) from that of a whole integer was too large to be explained by experimental error and suggested, in direct analogy with the binding of oxygen to hemoglobin (18, 19), that the binding of each AMP molecule influenced the binding constants of subsequent inhibitor molecules. Similar observations have been made recently for other allosteric binding sites (20). Values of the apparent association constant, \( K \), were obtained by measuring log \( [E-\text{AMP}_n]/[E] \) at log [AMP] = −3. Assuming that the average slope was 2.3, then log \( K_{[\text{AMP}=1]} = \log K_{[\text{AMP}=0.01]} + 0.9 \). It may be seen in Table I that the apparent association constant increased as the temperature decreased. The change in log \( K \) per unit reciprocal of absolute temperature remained constant throughout the temperatures studied, except for a slight decrease at 2° (Fig. 7). The molar enthalpy change, \( \Delta H \), calculated from the slope of this line according to the equation \( \Delta H = -\text{slope} \times 2.303 R \), was −42.6 kcal per mole. The thermodynamic parameters, \( \Delta F \) and \( \Delta S \), were calculated from this information by using the equations.
### TABLE I

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_a$ (mM)</th>
<th>$K_	ext{a}$ (mM)</th>
<th>$\Delta F$ (kcal/mole)</th>
<th>$\Delta S$ (e.u./mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$30^\circ$</td>
<td>$1.5 \times 10^2$</td>
<td>0.0055</td>
<td>-15.2</td>
<td>-100</td>
</tr>
<tr>
<td>$46^\circ$</td>
<td>$1.6 \times 10^4$</td>
<td>0.11</td>
<td>-12.8</td>
<td>-90</td>
</tr>
<tr>
<td>$38^\circ$</td>
<td>$2.2 \times 10^4$</td>
<td>0.24</td>
<td>-11.9</td>
<td>-90</td>
</tr>
<tr>
<td>$20^\circ$</td>
<td>$4.9 \times 10^4$</td>
<td>0.46</td>
<td>-11.2</td>
<td>-98</td>
</tr>
</tbody>
</table>

* $K_a$ denotes concentration of AMP necessary for 50% inhibition.

$\Delta F = -RT \ln K$ and $\Delta S = (\Delta H - \Delta F)/T$. $\Delta F$ decreased from -15.2 to -11.2 kcal per mole as the temperature was increased from $2^\circ$ to $46^\circ$, and the over-all reaction had a large entropy decrease of -99 e.u. per mole. These data are summarized in Table I. The experimental values of $K_a$ (AMP concentration needed to give 50% inhibition) as a function of temperature are also tabulated. These values were identical with those calculated from the $K$ values assuming $K_1 = 1/K_1^{1/2}$.

The over-all sequence involved in the formation of the inactive fructose 1,3-diphosphatase-AMP complex is characterized by a highly exothermic and exergonic reaction with a relatively large negative entropy change, presumably representing the summation of several equilibria. A similar increase in the association constant with lowering of temperature has been reported by Roughton, Otis, and Lyster (21) for the binding of oxygen to hemoglobin. For the over-all reaction, hemoglobin $+ 4 O_2 \rightarrow$ hemoglobin ($O_2$)$_4$, they have reported a total enthalpy change of $-43.6$ kcal per mole. From their data, the over-all entropy change was calculated to be $-159$ e.u. per mole.

A sigmoid curve similar to those shown in Fig. 5 was found with dAMP as inhibitor. The value of $n$ was 2.2, and an apparent over-all association constant of $3.4 \times 10^8$ was found at $30^\circ$ and pH 7.5. The extent of inhibition by mixtures of dAMP and AMP was additive.

**Evaluation of "Actual n" by Theory of Pauling**—The $n$ value obtained above does not necessarily represent the actual number of molecules participating in the over-all reaction for cases involving several sequential steps, in which considerable amounts of intermediates are present. For the case of hemoglobin, in which it is known experimentally that 4 $O_2$ are bound to four heme sites on the protein, Adair (18) has assumed that there are four sequential reactions for the binding of $O_2$, each with a different association constant, $K_1$, $K_2$, $K_3$, and $K_4$. Pauling (19) and Coryell, Pauling, and Dodson (22) proposed a modified explanation which involves only a two-constant equation by assuming that the free energy change accompanying each $O_2$ addition to hemoglobin is reduced by $RT \ln \alpha$ for each $O_2$ molecule already bound. The factor $\alpha$ was defined as the interaction constant.

We have adapted this latter theory to the present situation in order to predict the actual number of AMP molecules bound per enzyme molecule. For the case in which the actual $n = 4$, according to Pauling, see Equation 1, where $y = \text{fractional saturation of enzyme molecules with AMP}$. If the number of AMP molecules bound per enzyme molecule is a direct measure of the extent of enzyme inhibition, i.e. if an AMP:E ratio of 1 inhibits 25%, a ratio of 2 inhibits 50%, a ratio of 3 inhibits 75%, and a ratio of 4 inhibits 100%, then $y = \text{fraction of enzyme inhibited by AMP}$. The equation would be the same for the physical situation of four active sites and four cooperative AMP-binding sites per enzyme molecule.

Similarly, for the case in which the actual $n = 3$, the relative amounts of the four molecular species would be as shown in Scheme 1. In this scheme, the large circle enclosing three smaller circles denotes fructose 1,6-diphosphatase, each inner circle representing an AMP-binding site, and

$$K_i = \frac{[E-AMP]}{[E][AMP]}$$

Then

$$y = \frac{K_i[AMP] + 2\alpha K_i'[AMP]^2 + \alpha' K_i''[AMP]^3}{1 + 3K_1[AMP] + 3\alpha K_i'[AMP]^2 + \alpha' K_i''[AMP]^3}$$

For the case in which the actual $n = 2$, the apparent $n$ can never be greater than 2 (19). This situation, therefore, is incompatible with our measurements, in which the apparent $n$ was always greater than 2.

![Figure 7](http://www.jbc.org/doi-fig)
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FIG. 8. Comparison of experimental data with two theoretical curves for three- and four-site models of AMP binding to fructose 1,6-diphosphatase. •, observed values at 30°. Heated, aldolase-free enzyme was fully reactivated by preincubation as in the experiment of Fig. 2. The reaction was started by addition of substrate, followed immediately by AMP. The assay conditions were identical with those in “Experimental Procedure” except that Tris buffer, pH 7.3, was employed.

The relationship between the apparent \( n \) and \( \alpha \) for the two cases, actual \( n = 4 \) and 3, respectively, was determined by the procedure of Coryell (23). A family of curves, \( \log \frac{y}{1 - y} \) versus \( \log [\text{AMP}] \), was drawn for varied \( \alpha \) values by assigning an arbitrary value for \( K \). As shown by Coryell, the proportionality between \( n \) and \( \alpha \) is independent of \( K \). The average slope, \( n \), for each \( \alpha \) was determined graphically between the points for 10 and 90% inhibition of activity. The \( n \) values thus obtained were plotted against \( RT \ln \alpha \). From these curves, the respective \( \alpha \) values for our experimental \( n \) were determined for the two models. Furthermore, since \( 1/[\text{AMP}] = K \times \alpha \) at 90% inhibition in both models, it was possible to determine \( K \) from our data. With this information, theoretical curves were drawn of \( \log \frac{y}{1 - y} \) versus \( \log [\text{AMP}] \) for the two models and compared with our experimental data at 30°, as is shown in Fig. 8. It may be seen that our data fit best the model in which 3 AMP molecules combine per enzyme molecule. For values of actual \( n \) greater than 4, there would be even more deviation from our data. Unfortunately, the greatest differences between the “3” and “4” model occur in the range of less than 10% and more than 90% inhibition of fructose 1,6-diphosphatase, but even in this range our experimental error was too small to allow for the “4” model.

If we assume that the model in which actual \( n = 3 \) is correct, the first association constant may be determined as a function of temperature, and the thermodynamic properties of the reaction, \( E + \text{AMP} \rightleftharpoons E \cdot \text{AMP} \), determined. At 30°, \( K_1 = 990 \text{ M}^{-1} \), \( \Delta H_1 = -16.3 \text{ kcal per mole}, \Delta F_1 = -4.2 \text{ kcal per mole}, \) and \( \Delta S_1 = -40 \text{ e.u. per mole} \).

Removal of AMP Inhibition by Papain Treatment—We have previously reported a 2- to 4-fold increase in the total fructose 1,6-diphosphatase activity at pH 9.5 of both rat and rabbit liver extracts upon proteolysis with either liver cathepsins or crystalline papain (24, 25). When the effect of papain digestion on AMP inhibition and activity at pH 7.5 was tested, the results shown in Fig. 9A were obtained. There was almost complete relief from inhibition by AMP before any significant decrease in activity occurred at neutral pH. The time course of removal of AMP inhibition and increase in activity at pH 9.5 (not shown in Fig. 9A) closely paralleled one another.

The presence of 0.5 mM AMP in the incubation mixture for papain digestion markedly reduced the rate of papain action on the enzyme (Fig. 9A). This experiment was identical with

Fig. 9. A, removal of AMP inhibition of fructose 1,6-diphosphatase (FDPase) by treatment with papain. Heated, aldolase-free extract (0.3 ml) was incubated at 38° in a final volume of 0.4 ml containing 50 mM malonate, pH 6.0, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, and 0.0092 mg of crystalline papain. At timed intervals, aliquots were assayed for fructose 1,6-diphosphatase activity at 30° and pH 7.5 with or without the addition of 0.5 mM AMP. As described under “Experimental Procedure,” each aliquot was preincubated for 10 minutes at 30° in the complete reaction mixture minus FDP. The reactions were started by the addition of substrate, followed immediately by AMP. B, effect of AMP upon rate of removal of AMP inhibition by papain. Assay conditions were identical with those in A except for the presence of 0.5 mM AMP in the initial digestion mixture. The final dilution of enzyme was sufficient so that there was negligible inhibition produced by the AMP present in the initial digestion mixture.
that shown in Fig. 9A except for the presence of AMP during papain treatment. The relative rates of removal of AMP in inhibition and inactivation of fructose 1,6-diphosphatase at pH 7.5 were 8 and 27% of the respective control rates without AMP. AMP had no effect upon general proteolysis of the liver extract by papain under these conditions, and AMP at the same concentration did not alter the rates of papain action on fructose 1,6-diphosphatase. This information would suggest that AMP binding to the fructose 1,6-diphosphatase molecule specifically protects the enzyme against papain digestion and preferentially protects the sites involved in AMP inhibition.

After prolonged treatment of fructose 1,6-diphosphatase with papain, there was still some inhibition by much higher AMP concentrations, but the Kᵢ had increased 82-fold and the apparent number of AMP molecules combining per enzyme molecule was reduced from 2.3 to 1 (Fig. 10).

Neither autolysis at pH 4.5 nor trypsin treatment at pH 7.5 increased the activity of fructose 1,6-diphosphatase in the presence of AMP. Urea and citrate increased the amount of inhibition observed with AMP. In the presence of 5 mM LiBr, the inhibition by 0.1 mM AMP was reduced from 45 to 7%. However, the fructose 1,6-diphosphatase activity was 35% of the control level.

**Effect of Metabolic Intermediates on AMP Inhibition**—The following compounds produced no significant inhibition or reversal of inhibition of fructose 1,6-diphosphatase by AMP: glucose-6-P (5 mM), 3 fructose-6-P (5 mM), 4 fructose-1-P (5 mM), L-alanine (10 mM), L-glycine (10 mM), or-ketoglutarate (1 mM), fumarate (1 mM), succinate (10 mM), glycerate (1.2 mM), 2-P-glycerate (1 mM), 3-P-glycerate (1.2 mM), L-α-glycerol-P (10 mM), L-α-ketogluceraldehyde-3-P (2.2 mM), 3-P-glycerate (1 mM), 2-P-glycerate (1 mM), L-α-ketogluceraldehyde (10 mM), or γ-lysine (0.5 mM), n-glucosamine (1 mM), 3-P-glycerate (0.654 mM) + DPNH (0.054 mM), 3-P-glycerate (1.2 mM), 2-P-glycerate (1 mM), P-enolpyruvate (5 mM), L-lactate (1 mM), acetyl-CoA (1 mM), oxaloacetate (0.8 mM), α-ketoglutarate (1 mM), fumarate (1 mM), succininate (10 mM), L-glycine (10 mM), L-alanine (10 mM), L-glutamate (10 mM), NH₄Cl (1 mM), cyclic 3',5'-AMP (0.5 mM), 2'-AMP (1 mM), 3'-AMP (1 mM), P₃ (10 mM), L-kynurenine (0.5 mM), and p-hydroxyphenylpyruvate (1 mM).

Neither corticosterone acetate (0.020 mM) nor hydrocortisone acetate (0.025 mM) or succinate (0.021 mM) had any effect on fructose 1,6-diphosphatase activity with or without addition of AMP.

Pyruvate protected the enzyme from inhibition to some extent when it was present in the reaction system before addition of AMP. The amount of inhibition by 0.1 mM AMP was reduced from 57 to 41% by pyruvate concentrations of 5 and 10 mM, respectively. However, there was no effect when pyruvate was added after AMP. Malate produced a similar but lesser relief of inhibition.

The addition of 1 mM ATP in the presence of substrate lowered the inhibition by 0.1 mM AMP. The reduction of inhibition was time-dependent, and the extent of recovery was limited. This effect of ATP can most likely be attributed to the lowering of the AMP concentration by the action of myokinase present in the dialyzed liver supernatant.

**Effect of pH and Mg²⁺ on AMP Inhibition**—The extent of inhibition of fructose 1,6-diphosphatase by AMP was found to decrease with increasing pH. The reduced inhibition was characterized by both an increased Kᵢ and a lack of complete inhibition, even at high AMP levels. Maximal inhibition was observed at pH 7.0 in the presence of 10 mM Mg²⁺, but measurements at lower pH values were inaccurate because of the low enzyme activity.

At pH 7.5, increasing the Mg²⁺ concentration produced some relief of AMP inhibition; the Kᵢ increased from 0.19 to 0.31 mM when the Mg²⁺ concentration was increased from 10 to 25 mM.

The apparent n value was not altered by the change in Mg²⁺ concentration.

**DISCUSSION**

The generalized concept of allosteric sites on proteins and their interactions with effector molecules has been elegantly discussed by Monod, Changeux, and Jacob (20). In our view, the simplest definition of “allosteric inhibition” would be the interaction of an enzyme protein with an inhibitor at a site on the enzyme different from the catalytic site with a resultant loss of enzyme activity. Our present observations (a) that there is a specific and noncompetitive inhibition of fructose 1,6-diphosphatase by AMP, a compound bearing no obvious structural relationship to the substrate; (b) that it is possible by treatment with papain to remove the AMP inhibition while retaining enzyme activity; and (c) that a conformational change of the fructose 1,6-diphosphatase molecule probably occurs in the presence of AMP, all support our contention that AMP interacts with this enzyme at an allosteric site. In addition, the cooperative interaction of AMP molecules upon the enzyme is a phenomenon associated with most systems involving allosteric interactions and is obviously of importance in terms of physiological control processes.

**Fructose 1,6-diphosphatase activities were measured by determination of the phosphate liberated.**
It would appear that papain treatment removes the effect of at least two of the AMP-binding sites. On the other hand, change in pH does not alter the apparent n value. The large negative enthalpy change observed for the association reaction of enzyme and AMP and the decreased inhibition at higher pH values would be in agreement with the involvement of a positively charged imidazole group of the protein in the binding of AMP. In addition, the large negative entropy change for this inhibition reaction would predict that a large conformational change of the fructose 1,6-diphosphatase molecule occurs, even when only 1 AMP molecule is bound to the enzyme.

**Possible Physiological Significance of AMP Inhibition**—There is a variety of information in the recent literature to suggest that an immediate and primary effect of glucocorticoid hormones occurs before increased enzyme synthesis de novo at the level of interconversion of FDP to fructose-6-P. Segal and López (26) recently reported a 7- to 9-fold increase in the rate of liver interconversion of FDP to fructose-6-P. Segal and López (26) inhibition reaction would predict that a large conformational change of the fructose 1,6-diphosphatase molecule occurs, even when only 1 AMP molecule is bound to the enzyme.

A rapid formation of glucose-6-P from noncarbohydrate precursors would be consistent with this assumption. The specific inhibition of fructose 1,6-diphosphatase by AMP could well serve as a "fine metabolic valve" for regulation of glyconeogenesis. Thus, a change in AMP concentration at 38° and pH 7.3 from 0.1 to 0.6 μM is sufficient to reduce the enzyme activity from 90 to 10% of the maximal level (Fig. 5). In addition, phosphofructokinase is markedly activated by AMP under suitable conditions in vitro in liver (31) and other mammalian tissues (32-35). It is therefore probable that small changes in the level of this nucleotide may control the net flux between FDP and fructose-6-P in vivo and thus be an important mechanism in control of the direction of carbohydrate metabolism. Some evidence for the possible physiological role of AMP in control of glyconeogenesis comes from the recent studies of Krebs, Dierks, and Gascoyne (36) on the formation of glucose from lactate in pigeon liver homogenates. Net carbohydrate production by this system was completely inhibited by the addition of 0.32 mM AMP.

One factor that we have not yet ascertained is the concentration of AMP necessary to inhibit fructose 1,6-diphosphatase at the protein concentrations present in intact liver. The levels of enzyme would be 2500 times higher than the amount used in the normal assay system. A slight increase in Kᵢ at 30° from 0.13 to 0.19 mM did occur when the total dilution of liver extract was decreased from 1:5000 to 1:80.

It is also possible that a change in the intracellular pH or Mg⁺⁺ concentration of liver in vivo could alter the extent of inhibition by AMP to some degree. The levels of FDP in liver extracts from normal and adrenalectomized rats have varied from 0.030 to 0.070 μmole per g, wet weight, values well below the concentration of substrate which significantly inhibit fructose 1,6-diphosphatase. However, it is possible that physiological conditions may exist under which control of fructose 1,6-diphosphatase activity by substrate inhibition is of importance.

Mammalian regulation of fructose 1,6-diphosphatase activity by AMP and substrate inhibitions can be considered to be directly analogous to the immediate control provided by "negative feedback" inhibition in microorganisms. Similarly, the increase in total liver fructose 1,6-diphosphatase produced by glucocorticoid administration, would be analogous to induction or derepression in bacteria. This latter process requires 4 to 5 days to occur in mammals and represents synthesis de novo of enzyme protein (37).

**SUMMARY**

1. Rat liver fructose 1,6-diphosphatase was markedly inhibited by substrate concentrations greater than 0.1 mM. In the range of noninhibitory substrate concentrations, a pH optimum of 7.3 and Kᵢ of 1 to 3 x 10⁻⁶ M were observed. Maximum inhibition also occurred at pH 7.3.

2. Adenosine 5'-monophosphate (or deoxyadenosine 5'-monophosphate) specifically, reversibly, and noncompetitively inhibited the enzyme. Plots of percentage inhibition against inhibitor concentration gave sigmoid-shaped curves. The apparent number of AMP molecules bound per enzyme molecule was found to be 2.3, and, from theoretical considerations, the actual number was estimated to be 3 (or 4).

3. Lowering of the temperature of the system markedly increased the inhibition by AMP, the Kᵢ dropping from 0.46 mM at 46° to 0.0055 mM at 2°. The apparent n value of 2.3 was not altered by the change in temperature, and the over-all association constant increased with decreasing temperature. The over-all association reaction of inactive fructose 1,6-diphosphatase-AMP complex formation was found to have both a large negative enthalpy and entropy change of -42.6 kcal per mole and -99 e.u. per mole, respectively.

4. Papain treatment almost completely removed the inhibition by AMP and reduced the apparent n value to 1. The selective removal of the AMP inhibition by treatment with papain, together with the specificity and noncompetitive nature of the inhibition, strongly support the idea that binding of AMP occurs at an allosteric site.

5. The extent of inhibition by AMP was markedly decreased with increasing pH.

6. It was not possible to reverse the inhibition by AMP with any known metabolite. The significance of intracellular levels of AMP in regulation of glyconeogenesis is discussed.
APPENDIX: GRAPHICAL ESTIMATION OF APPARENT BINDING RATIOS FOR INHIBITORS AND ACTIVATORS OF BIOLOGICAL SYSTEMS

There have been many recent reports of allosteric interactions with enzymes in which more than 1 inhibitor or activator molecule binds in a cooperative manner to the protein molecule with either resultant less active or more active enzyme catalysis. One important physiological consequence of such reactions, long evident for the case of oxygen binding to hemoglobin (38), is that the larger this apparent n value, the smaller the change in concentration necessary to produce a given effect. This phenomenon is illustrated graphically in Fig. 11 for the general case

\[ E + n \text{ inhibitor} = E\text{-inhibitor}_n \]

or

\[ E + n \text{ activator} = E\text{-activator}_n \]

where \( K_{assoc} = 1 \). It may be seen that the larger the \( n \) value is, the steeper the slope of the curve in the range important for controlling enzyme activity. Identical variations in slope are obtained for any chosen value of \( K \). Another way of expressing this difference is to compare the \( n \) value with the corresponding ratio of concentrations necessary to change activity from 90% to 10% of maximal. As is shown in Table II, the ratio sharply decreases with increasing \( n \). It should be noted that in experimental reaction systems, in which considerable amounts of intermediates exist, the apparent \( n \) value does not necessarily represent the actual binding ratio.

Often it would be advantageous to have a way of estimating the apparent \( n \) value without the necessity of the extensive data and calculations required for a log-log plot based on the Hill equation. Two graphical methods for estimation of \( n \) are given below. An empirical relationship exists between \( n \) and the ratio of concentrations of inhibitor or activator necessary to change inhibition or activation from 40% to 60% of maximal, which approaches a straight line on a log-log plot within \( n \) values of 2 to 6 (Fig. 12). There is also a linear relationship between the log of ratios of concentrations necessary to produce any given percentage change in inhibition or activation and \( 1/n \) (Fig. 13). If experimental values for the extent of inhibition of an enzyme are known for any two inhibitor concentrations, the apparent \( n \) may be calculated from the following equation.

\[
\frac{\log \left( \frac{\text{inhibited enzyme}}{\text{active enzyme}} \right)_{\text{at [inhibitor]}}} {\log \left( \frac{\text{inhibited enzyme}}{\text{active enzyme}} \right)_{\text{at [inhibitor]}}} = \frac{1}{n}
\]

Several apparent \( n \) values estimated from data available in the literature with the aid of Fig. 12 are summarized in Table III. It is also of interest that the inhibition of infectivity of Type 2 Dengue virus by an agar polysaccharide gives an apparent \( n \) value of 0.6 (44). Most values fall within the range of 1 < \( n < 4 \). This makes sense from a physiological standpoint, since values outside this range result in situations in which only very large changes in concentration or very small variations

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**Table II**

<table>
<thead>
<tr>
<th>( n )</th>
<th>Ratio of concentrations necessary to change activity from 90% to 10% of maximal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6560</td>
</tr>
<tr>
<td>1</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
</tr>
<tr>
<td>10</td>
<td>1.55</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>System</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of liver fructose 1,6-diphosphatase by 5'-AMP at 30º.</td>
<td>2.3*</td>
</tr>
<tr>
<td>Substrate activation (isocitrate) of isocitrate dehydrogenase (39)</td>
<td>1.9†</td>
</tr>
<tr>
<td>Substrate activation (aspartate) of aspartate transcarbamylase (40)</td>
<td>3.5</td>
</tr>
<tr>
<td>Substrate activation (threonine) of threonine deaminase (41)</td>
<td>2.8</td>
</tr>
<tr>
<td>Induction of ( \beta )-galactosidase in a cryptic E. coli mutant (42)</td>
<td></td>
</tr>
<tr>
<td>a. By thiomethyl ( \beta )-( \beta )-galactoside</td>
<td>1.5</td>
</tr>
<tr>
<td>b. By thioisopropyl ( \beta )-( \beta )-galactoside</td>
<td>2.6</td>
</tr>
<tr>
<td>Inhibition of UDP-N-acetylglucosamine 2-epimerase by CMP-N-acetylneuraminic acid (43)</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Reported, 2.36.
† Reported, 1.85.
Fig. 12. Plot of ratio of inhibitor or activator concentrations necessary to change enzyme activity from 40% to 60% of maximal versus n. The ordinate scale was obtained by plotting

\[ \frac{C_{\text{Act}}(60\%)}{C_{\text{Act}}(40\%)} \text{ or } \frac{C_{\text{Inh}}(60\%)}{C_{\text{Inh}}(40\%)} - 1 \]

on a log scale and arbitrarily adding 1 to each number.
Fig. 13. Plot of ratio of concentrations necessary to produce a given percentage change in activity versus $n$. 
produce marked changes in enzyme activity (see Table II). Thus, when $n$ is 10, only a 55% increase in inhibitor concentration is required to change the activity from 90% to the 10% level. Presumably such a system would be much too sensitive to normal metabolic variations.

Acknowledgment—It is a pleasure to acknowledge our indebtedness to Dr. Sidney P. Colowick for his initial suggestion that the inhibition by adenosine 5'-monophosphate might involve cooperative interactions, and for his continued advice and stimulation during the development of this problem.

REFERENCES

Allosteric Inhibition of Rat Liver Fructose 1,6-Diphosphatase by Adenosine 5'-Monophosphate
Kazuhsa Taketa and Burton M. Pogell