Studies on the Mechanism of Adenosine 5′-Monophosphate Inhibition of Bovine Liver Fructose 1,6-Bisphosphatase†

Stuart R. Stone and Herbert J. Fromm*

ABSTRACT: Inhibition by adenosine 5′-monophosphate (AMP) of the forward reaction of fructose 1,6-bisphosphatase (FBPase) has been studied by means of progress curve analysis. The full time course of the FBPase reaction was followed by coupling the reaction to the enzymes phosphoglucoisomerase and glucose-6-phosphate dehydrogenase. The data were analyzed by using three different methods of regression and these methods are compared. During these progress curve experiments, it was noticed that fructose 1,6-bisphosphate (Fru-P_2) was not fully utilized in the presence of AMP. This anomaly could be best explained by proposing the formation of an AMP·Fru-P_2 complex that was inactive with FBPase. Besides reducing the level of substrate available to FBPase, AMP caused slope-parabolic, intercept-parabolic noncompetitive inhibition with respect to Fru-P_2. A kinetic model for AMP inhibition of the forward reaction of FBPase is presented. Initial rate kinetics were used to study the reverse reaction of FBPase; AMP was a slope-parabolic, intercept-parabolic noncompetitive inhibitor with respect to both fructose 6-phosphate and inorganic phosphate. Initial velocity experiments in which both fructose 6-phosphate and inorganic phosphate were varied were carried out in the absence and presence of AMP. The results of these experiments indicated to which reverse-reaction enzyme forms AMP was binding. The possible physiological significance of the AMP inhibition of FBPase and of the proposed AMP·Fru-P_2 complex is discussed.

Fructose 1,6-bisphosphatase (FBPase)[1] is an important enzyme in gluconeogenesis; in the presence of a divalent metal ion, it catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-P_2) to form fructose 6-phosphate (Fru-6-P) and inorganic phosphate (P_i). The enzyme from a wide variety of sources is subject to allosteric inhibition by AMP (Pontremoli & Horecker, 1971), and this inhibition is thought to be one of the important control mechanisms in carbohydrate metabolism (Newsholme & Start, 1973). A number of qualitative studies have been made of AMP inhibition of the forward reaction of FBPase (Pontremoli & Horecker, 1971). In addition, Nimmo & Tipton (1975b) have presented a detailed report on the interactions between FBPase, AMP, and the activating divalent cation. AMP is known to be a nonlinear noncompetitive inhibitor with respect to Fru-P_2 (Nimmo & Tipton, 1975a; Taketa & Pogell, 1965). Other than this, little is known about the interactions between the reactants, Fru-P_2, Fru-6-P, and P_i, the enzyme, and AMP. The present study investigates these interactions.

In recent studies (Casazza et al., 1979; S. R. Stone and H. J. Fromm, unpublished experiments), the authors have examined the kinetic mechanism of FBPase at saturating levels of Mg^{2+}. As a product inhibitor, Fru-6-P yielded linear noncompetitive inhibition with respect to Fru-P_2, whereas P_i was a linear competitive inhibitor with respect to the substrate. The FBPase reaction was shown to be kinetically reversible and displayed sequential Michaelis–Menten kinetics in the reverse reaction (Casazza et al., 1979). The kinetics of the reverse reaction were further investigated by using substrate analogues of Fru-6-P and P_i and a product analogue of Fru-P_2. The results were in harmony with a rapid-equilibrium random reaction mechanism and a rapid-equilibrium ordered mechanism in which P_i is bound first and a nonproductive enzyme·Fru-6-P complex forms (S. R. Stone and H. J. Fromm, unpublished experiments).

Information obtained in these previous investigations has been utilized in the current study of the mechanism of AMP inhibition. FBPase has an extremely low Michaelis constant for Fru-P_2 (Casazza et al., 1979; Dudman et al., 1978). A full time course assay has been used to obtain data at Fru-P_2 concentrations below the level of its Michaelis constant, and the data have been analyzed on the basis of the integrated rate equation. Certain anomalies were detected during these experiments, and these anomalies could best be explained in terms of an AMP·Fru-P_2 complex. Results from the forward and reverse reactions indicated that AMP was an S-parabolic, I-parabolic noncompetitive inhibitor with respect to Fru-P_2, Fru-6-P, and P_i. The possible physiological significance of AMP inhibition and of the proposed AMP·Fru-P_2 complex is discussed.

Experimental Procedure

Materials

Phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim, whereas aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and all other biochemicals used in enzyme assays were purchased from Sigma. Other chemicals were high-purity preparations available from commercial sources. Concentrations of Fru-6-P were determined spectrometrically by using phosphoglucoisomerase and glucose-6-phosphate dehydrogenase.

Methods

Enzyme Purification. Fructose 1,6-bisphosphatase was purified from fresh beef liver by using a modification of the

† From the Biochemistry and Biophysics Department of Iowa State University, Ames, Iowa 50011. *Received September 7, 1979.* Supported in part by Research Grant No. 10546 from the National Institutes of Health, U.S. Public Health Service, and by Grant PCM 77-09018 from the National Science Foundation. This is Journal Paper No. J-9632 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011, Project 2244.

[1] Abbreviations used: AMP, adenosine 5′-monophosphate; FBPase, fructose 1,6-bisphosphatase; Fru-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; P_i, inorganic phosphate.

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procedure of Geller & Byrne (1975). All dialyses were performed by using a rocking dialyzer, and all buffers contained 1 mM phenylmethanesulfonyl fluoride to guard against proteolysis. The fresh liver (200 g) was homogenized and then fractionated by using methanol and $(NH_4)_2SO_4$ as described by Geller & Byrne (1975). The 50–65% $(NH_4)_2SO_4$ precipitate was suspended in 20 mM Tris buffer, pH 7.3, containing 70 mM NaCl, 0.5 mM EDTA, 1.5 mM $MgSO_4$, and 1 mM phenylmethanesulfonyl fluoride (buffer A). The suspension was centrifuged for 20 min at 40000*g*, and the supernatant fluid was placed on a 5.0 × 68 cm column of Sephacryl S-200 previously equilibrated with buffer A. The sample was eluted with the same buffer, and the fractions that contained the bulk of the fructose 1,6-bisphosphatase activity were pooled. The pooled fractions were brought to 3.0 mM with respect to EDTA and chromatographed on phosphocellulose as described by Geller & Byrne (1975). FBPase prepared in this manner had a specific activity of 19.3 IU/mg of protein as determined by using the standard P_i release assay (Geller & Byrne, 1975). Protein was assayed with bovine serum albumin as a standard (Lowry et al., 1951). The pH 6.5/9.0 activity ratio of this preparation was 2.0. The final preparation displayed only one protein band upon disc gel electrophoresis.

Progress Curve Analysis of the Forward Reaction. Progress curve data were collected by using a continuous spectrophotometric assay in which the formation of Fru-6-P was coupled to the phosphoglucoisomerase and glucose-6-phosphate dehydrogenase reactions. Assay mixtures contained, in a final volume of 1.0 mL, 25 mM Tris–25 mM histidine buffer (pH 6.5), 5.0 mM $MgSO_4$, 0.15 mM NADP, 0.1 mM EDTA, 1.0–5.0 μM Fru-P_2, AMP as indicated, glucose-6-phosphate dehydrogenase (4.0 units), and phosphoglucoisomerase (6.0 units). The coupling enzymes were exhaustively dialyzed to remove $(NH_4)_2SO_4$. The assay mixtures were incubated for 5 min at 28 °C before the assays were started by the addition of FBPase. The temperature was maintained at 28 °C during the assay. Upon addition of FBPase, the chart was started so as to establish zero time. FBPase was diluted for assay into a solution of 25 mM Tris–25 mM histidine buffer (pH 6.5) containing 5.0 mM $MgSO_4$, 0.1 mM EDTA, 1.4 mM dithiothreitol, and bovine serum albumin (2 mg/mL). The amount of FBPase added was adjusted such that the reaction was completed within 20 min. The course of the reaction was followed as the increase in absorbance at 340 nm by using a Cary 118 spectrophotometer on the 0.02-absorbance full-scale setting. The concentration of Fru-6-P at various time intervals was read off the chart tracing and transferred to computer cards for analysis. Data from progress curves obtained in the absence and presence of AMP at several enzyme concentrations indicated that there was no time-dependent inactivation of FBPase (Selwyn, 1965). Additionally, these experiments demonstrated that there was no significant lag period in the production of Fru-6-P.

Analysis of progress curve data was performed essentially as described by Duggleby & Morrison (1977). Individual progress curves were fitted by nonlinear regression to

$$Vt = P - K_a \ln (1 - P/A_0) \quad (1)$$

where t, A_0, P, V, and K_a represent time, the initial concentration of Fru-P_2, the concentration of Fru-6-P, the apparent maximum velocity, and the apparent Michaelis constant, respectively. Data points were collected for values of P from 0.1 to 0.95 times A_0. Nonlinear regression analysis was performed on the equally weighted points by using the Gauss–Newton method or the steepest descent gradient method (SAS Institute Inc., Raleigh, NC). If it was essential to know the exact origin of the progress curve, the data were fitted to

$$Vt = P - x - K_a \ln [1 - (P - x)/A_0] \quad (2)$$

where x is the apparent displacement of P at zero time and all other terms are defined as for eq 1. For AMP inhibition studies, in which data from several progress curves were used, the data for each curve were adjusted such that all curves had the same value for A_0; then, the data were fitted to eq 1. The results of these regressions were used to fit the data to a number of kinetic models; three different methods were used. In the first method, all the points used in the original analyses were weighted according to the reciprocal of the mean square error of the analysis from which they were obtained. The weighted points were then fitted to an integrated rate equation for inhibition by using nonlinear regression. The second method was essentially the same as the first except that the data from each curve were compressed into three "idealized" points as recommended by Duggleby & Morrison (1978). For fitting data to an integrated rate equation for inhibition, the steepest descent gradient method was found superior to the Gauss–Newton method of nonlinear regression. With the Gauss–Newton method, the values of inhibition constants estimated by regression were strongly dependent upon the starting values provided for these constants. In the third method, apparent values of $1/V$ and K_a/V were obtained by fitting each curve to eq 1. These values were weighted according to the reciprocal of their variances. Weighted linear regression was then used to determine how the apparent values of $1/V$ and K_a/V varied with the concentration of AMP. In all three methods, analysis of variance was used to test whether the inclusion of additional parameters gave a significantly better fit. All three methods chose the same equation of best fit and gave essentially the same values for the parameters of this equation. However, the first two methods, which used nonlinear regression, yielded unreasonable standard errors for the estimates of the parameters. The standard errors obtained for the estimates of the maximum velocity and the Michaelis constant for Fru-P_2 were often as large as the estimates, whereas the standard errors of the inhibition constant were always less than 1% of the estimates of these parameters. In contrast, the third method, which used a combination of nonlinear and weighted linear regression, yielded reasonable standard errors for all the parameters in the equation of best fit; therefore, this method was the preferred method of analysis.

Initial Rate Kinetic Studies of the Reverse Reaction. A coupled spectrophotometric assay was used to measure the rate of the reverse reaction of FBPase at 28 °C. The assay mixture contained, in a final volume of 1.0 mL, 25 mM Tris–25 mM histidine buffer (pH 6.5), 50 μM NADH, 8.0 mM $MgSO_4$, 0.1 mM EDTA, aldolase (8.0 units), α-glycerophosphate dehydrogenase (8.0 units), triosephosphate isomerase (61 units), P_i, and Fru-6-P. The coupling enzymes were dialyzed exhaustively to remove $(NH_4)_2SO_4$. The assay mixtures were incubated at 28 °C for 5–10 min before the reactions were started by the addition of FBPase. Stock solutions of enzyme were diluted to the desired concentration by using 25 mM Tris–25 mM histidine buffer (pH 6.5) containing 8.0 mM $MgSO_4$, 0.1 mM EDTA, and bovine serum albumin (2 mg/mL). When P_i or Fru-6-P was used to initiate the reaction, the initial rates were identical with those obtained by using FBPase to initiate the reaction. Ionic strength was maintained constant when P_i was varied through appropriate additions of KCl. The enzyme-dependent rate of decrease of absorbance was measured by using a Cary 118 spectrophotometer on a full-scale setting of 0.02 or 0.05 optical density units. Cells

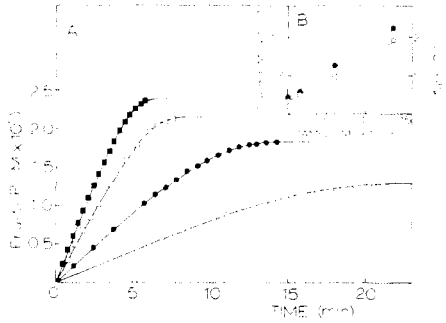


FIGURE 1: (A) Time course for the conversion of Fru-P_2 into Fru-6-P in the presence of 0.0 (■), 12.3 (□), 24.5 (●), and 36.8 μM (○) AMP. Assays were performed as described under Experimental Procedure. The data were fitted to eq 2, and the results of these analyses were used to draw the curves illustrated. The assays containing 0.0 and 12.3 μM AMP were started by the addition of 6.3×10^{-4} IU of FBPase, whereas assays carried out in the presence of 24.5 and 36.8 μM AMP were started with 1.25×10^{-3} IU of FBPase. The data were normalized to an FBPase level of 1.25×10^{-3} IU. (B) The apparent values of K_a/V and $1/V$ derived from each curve in Figure 1A are plotted against $(AMP)^2$. Apparent values for K_a/V (●) and $1/V$ (○) were obtained from the curves illustrated in Figure 1A, and weighted linear regression was used to determine how these values varied with the concentration of AMP as described under Experimental Procedure. The best fit was obtained by assuming that the apparent values of K_a/V and $1/V$ varied in proportion to $(AMP)^2$; (B) illustrates this fit.

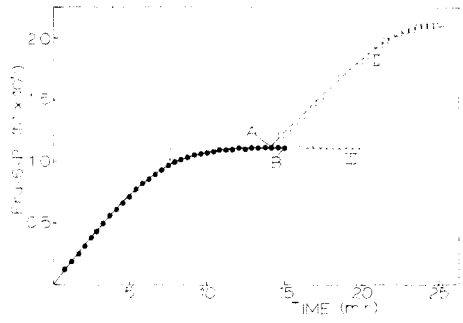


FIGURE 2: Time courses for the conversion of Fru-P_2 into Fru-6-P in the presence of 25.7 μM AMP. At zero time, the reaction, which contained 1.58 μM Fru-P_2, was started by the addition of 7.2×10^{-4} IU of FBPase (curve I, ●). After the reaction had reached completion, further additions were made to the assay as follows: either, at point A, a further 1.58×10^{-9} mol of Fru-P_2 was added (curve II, □) or, at point B, a further 7.2×10^{-4} IU of FBPase was added (curve III, ○). The data points in curves II and III were corrected for the dilutions that occurred upon the addition of Fru-P_2 and FBPase, respectively. The data were fitted to eq 2, and the results of these analyses were used to draw the curves.

of 1.0-cm path length were used. The rate of decrease in absorbance was linear for at least 5 min. Two controls were performed at the highest concentration of AMP used to make certain that AMP was not affecting the coupling system. Firstly, a check was made to ensure that increased levels of coupling enzymes did not result in increased initial rates. Secondly, the assay was performed at different levels of FBPase to test whether the initial rate was proportional to the amount of FBPase used. Initial rate data were analyzed by using a computer program written in OMNITAB II language; an α value of 2.0 was used for the weighting factor (Siano et al., 1975). Velocities are reported as M min^{-1}.

Results

Inhibition by AMP of the Forward Reaction of FBPase. AMP inhibition of the forward reaction of FBPase was studied by analyzing the full progress curve of the reaction. Because the product Fru-6-P is removed by the coupling enzymes, the reaction should proceed until all the Fru-P_2 present is utilized. In the presence of AMP, however, the reaction reached an end point before all the Fru-P_2 was hydrolyzed. A smaller amount of the total Fru-P_2 was hydrolyzed with increasing levels of AMP, as shown in Figure 1A. Similar results were obtained with two different lots of AMP. The data presented in Figure 2 indicate that the decreased utilization of Fru-P_2 in the presence of AMP was not due to enzyme inactivation. After the reaction in the presence of AMP was allowed to reach apparent completion (curve I, Figure 2), more FBPase was added (point B, Figure 2). This additional enzyme did not produce any further increase in the amount of product formed. If more Fru-P_2 was added to the seemingly completed reaction instead of FBPase (point A, Figure 2), additional product was formed (curve II, Figure 2). Once again, however, incomplete utilization of the added Fru-P_2 was observed. The method of Selwyn (1965) indicated the absence of time-dependent enzyme inactivation in the absence or presence of AMP. When adenosine, adenosine 5′-diphosphate, adenosine 5′-triphosphate, or adenosine 3′,5′-monophosphate, at a concentration of 100 μM, was added to the reaction mixtures instead of AMP, all the Fru-P_2 present was hydrolyzed. In addition to being dependent on the concentration of AMP, the level of unreacted Fru-P_2 also was dependent on the initial concentration of Fru-P_2 present in the reaction mixtures. On the other hand, the concentration of Mg^{2+} in the reaction mixture did not seem to affect the level of unreacted Fru-P_2. These results can be explained by proposing the formation of an AMP·Fru-P_2 complex that is inactive with FBPase. We currently are attempting to characterize this complex by using phosphorus-31 nuclear magnetic resonance spectroscopy.

In contrast to FBPase, aldolase was able to fully utilize Fru-P_2 in the presence of AMP. Similarly, adenylate kinase could fully utilize AMP in the presence of Fru-P_2. These enzymes were used to further investigate the proposed AMP·Fru-P_2 complex. After the FBPase reaction in the presence of AMP reached completion, aldolase was added to the assay mixture. Aldolase was able to metabolize all the unreacted Fru-P_2. If adenylate kinase and an excess of adenosine 5′-triphosphate (1.0 mM) were added to the assay mixture instead of aldolase, it was possible for FBPase to hydrolyze the previously unreacted Fru-P_2. These results indicate that the unreacted Fru-P_2 is still present as Fru-P_2 but in a form that is inactive with FBPase. Moreover, once AMP is removed, the unreacted Fru-P_2 is once again active with FBPase.

Theoretically, as the FBPase reaction removes free Fru-P_2 from solution, the AMP·Fru-P_2 complex should break down to yield more free Fru-P_2; thus, the reaction should proceed until all the Fru-P_2 present initially is utilized. In practice, however, the rate of breakdown of the complex could be extremely slow compared with the rate of the FBPase reaction such that data similar to those presented in Figures 1 and 2 would be obtained. In analyzing data from progress curves obtained in the presence of AMP, it was assumed that the proposed AMP·Fru-P_2 complex did not break down during the course of the assay and that the amount of free Fru-P_2 present at the start of the assay was equal to the total amount of Fru-6-P formed during the assay. Excellent fits were obtained on the basis of these assumptions, as shown by Figures 1 and 2.

If the breakdown of the complex is very slow compared with the FBPase reaction, the amount of unhydrolyzed Fru-P_2 will represent the amount of Fru-P_2 bound to AMP. Thus, it should be possible to calculate a stability constant for the proposed AMP·Fru-P_2 complex from progress curve data similar to those presented in Figure 1. If it was assumed that

Table I: Kinetic Constants for the Inhibition by AMP of the FBPase Reaction[a]

constant	enzyme interaction	eq	value
	Forward Reaction		
K_a	$E + A \rightleftharpoons EA$	3	$(2.50 \pm 0.08) \times 10^{-7}$ M
K_a'	$EI_2 + A \rightleftharpoons EAI_2$	$K_a' = K_a K_{ii}/K_i$	$(2.73 \pm 0.21) \times 10^{-7}$ M
K_i	$E + 2I \rightleftharpoons EI_2$	3	$(3.17 \pm 0.03) \times 10^{-10}$ M^2
K_{ii}	$EA + 2I \rightleftharpoons EAI_2$	3	$(2.90 \pm 0.20) \times 10^{-10}$ M^2
	Reverse Reaction		
K_i	$E + 2I \rightleftharpoons EI_2$	5	$(5.65 \pm 0.31) \times 10^{-10}$ M^2
K_{ii}	$EP + 2I \rightleftharpoons EPI_2$	5	$(3.69 \pm 0.09) \times 10^{-10}$ M^2
K_{iii}	$EPQ + 2I \rightleftharpoons EPQI_2$	5	$(8.52 \pm 0.44) \times 10^{-10}$ M^2

[a] The constants presented in the table have been classified in terms of dissociation constants for various enzyme-substrate-inhibitor interactions. For these interactions E, A, P, Q, and I represent the enzyme, Fru-P_2, Fru-6-P, P_i, and AMP, respectively. The values of the kinetic constants for the forward and reverse reactions were calculated from the results presented in Figure 1 and Table II, respectively, together with relationships derived from the equations indicated.

the unhydrolyzed Fru-P_2 was bound to AMP in a 1:1 ratio, the value of the stability constant varied with the AMP concentration: for example, on the basis of a 1:1 ratio, the data in Figure 1 yielded values for the stability constant of 8.8×10^3, 1.2×10^4, and 2.0×10^4 M^{-1} at 12.3, 24.5, and 36.8 μM AMP, respectively. However, if it was assumed that AMP was bound to Fru-P_2 in a 2:1 ratio, the data in Figure 1 yielded values for the stability constant of 7.7×10^8, 5.2×10^8, and 7.0×10^8 M^{-2} at 12.3, 24.5, and 36.8 μM AMP, respectively. On the basis of a 2:1 ratio, data obtained at different initial concentrations of Fru-P_2 yielded a value for the stability constant of $(6.0 \pm 0.7) \times 10^8$ M^{-2}. The stoichiometry of binding is being further investigated.

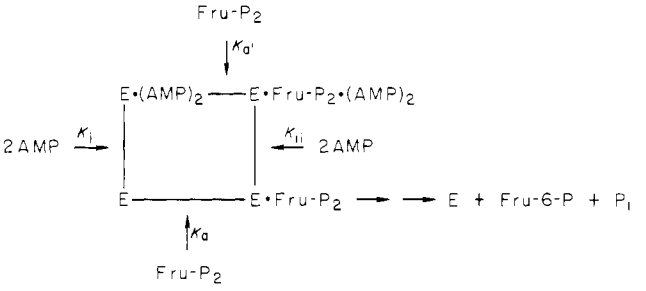
For assessment of whether the AMP·Fru-P_2 complex inhibited FBPase, a series of reactions were carried out at a high, constant level of AMP (37 μM), and the initial concentration of Fru-P_2 was varied over a threefold range from 1.2 to 3.6 μM. Under these conditions, the inhibition caused by AMP should be relatively constant but the concentration of the AMP·Fru-P_2 complex should vary with the initial concentration of Fru-P_2. In these experiments, the kinetic parameters of the progress curves did not vary with the initial concentration of Fru-P_2, suggesting that the AMP·Fru-P_2 complex does not inhibit FBPase.

Attempts were made to fit the data presented in Figure 1 to a variety of kinetic equations; the best fit was obtained with a rate equation for S-parabolic, I-parabolic noncompetitive inhibition, viz.

$$v = \frac{V_1 A}{A(1 + I^2/K_{ii}) + K_a(1 + I^2/K_i)} \quad (3)$$

where A, I, V_1, K_a, K_i, and K_{ii} represent the concentration of uncomplexed Fru-P_2, the concentration of AMP, the maximum velocity of the forward reaction, the Michaelis constant for Fru-P_2, the inhibition constant for the interaction of AMP with the free enzyme, and the inhibition constant for the interaction of AMP with the enzyme·substrate complex, respectively. Other rate equations that described noncompetitive inhibition and contained polynomials up to the fourth power in I were tested, but they did not describe the inhibition as well as eq 3. The values for the kinetic constants of eq 3 were determined from the plots presented in parts A and B of Figure 1 as described under Experimental Procedure, and these values are given in Table I. The model shown in Scheme I will yield eq 3. It was not necessary to include terms in I as well as I^2 to describe the parabolic nature of the inhibition. This indicates that the complexes enzyme·AMP and enzyme·Fru-P_2·AMP are kinetically unimportant. AMP inhibition of FBPase was also studied by using initial velocities obtained with the $^{32}P_i$ release assay (Casazza et al., 1979). The results of these initial velocity experiments were in agreement with the results of the progress curve experiments illustrated in Figure 1.

Scheme I



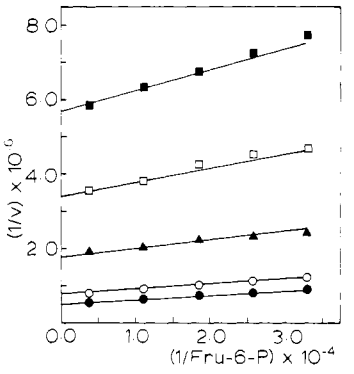


FIGURE 3: Plot of the reciprocal of initial velocity vs. the reciprocal of the molar concentration of Fru-6-P in the presence of 0.0 (●), 25.0 (○), 50.0 (▲), 75.0 (□), and 100.0 μM (■) AMP. Assays were performed as outlined under Experimental Procedure with a fixed concentration of 25.0 mM P_i. Each assay contained 0.29 IU of FBPase.

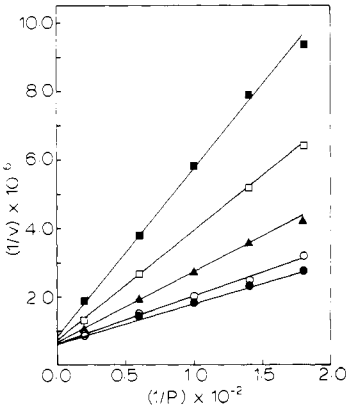


FIGURE 4: Plot of the reciprocal of initial velocity vs. the reciprocal of the molar concentration of P_i in the presence of 0.0 (●), 12.5 (○), 25.0 (▲), 37.5 (□), and 50.0 μM (■) AMP. Assays were performed as outlined under Experimental Procedure with a fixed concentration of 0.543 mM Fru-6-P. Each assay contained 0.29 IU of FBPase.

Inhibition by AMP of the Reverse Reaction of FBPase. AMP was found to be an S-parabolic, I-parabolic noncom-

Table II: Effect of AMP upon the Values of the Kinetic Coefficients for the Reverse Reaction of FBPase[a]

AMP (μM)	coeff values $1/V$ (min $M^{-1} \times 10^{-5}$)	K_q/V (min $\times 10^{-3}$)	K_p/V (min $\times 10^{-2}$)	$K_{iq}K_p/V$ (M min)
0	2.78 ± 0.17	9.46 ± 0.26	2.05 ± 0.10	3.34 ± 0.15
37	14.6 ± 1.0	127 ± 2	1.83 ± 0.52	28.2 ± 1.2
t values[b]	51.7[c]	232[c]	−1.87	95.1[c]

[a] The values of the kinetic coefficients were obtained from two initial velocity experiments which were conducted under the conditions described under Experimental Procedure at the concentrations of AMP indicated. In both experiments, the concentration of P_i was varied from 5.6 to 50.0 mM, whereas the concentration of Fru-6-P was varied from 0.28 to 2.51 mM. Each assay contained 0.33 IU of FBPase. A t test was used to evaluate the significance of the difference between the coefficient values obtained in the two experiments. [b] df = 21. [c] These t values are significant at the 1% level of significance.

petitive inhibitor with respect to both Fru-6-P and P_i, as shown in Figures 3 and 4, respectively. The data of Figures 3 and 4 were fitted to a number of kinetic equations, and the best fit was obtained by using an equation identical in form with eq 3. In this case, however, A, I, V_1, K_a, K_i, and K_{ii} in eq 3 represented the concentration of the varied substrate, the concentration of AMP, the apparent maximum velocity of the reverse reaction, the apparent Michaelis constant of the varied substrate, the slope inhibition constant, and the intercept inhibition constant, respectively. As with the AMP inhibition of the forward reaction, it was not necessary to include terms in I as well as I^2 to describe the parabolic inhibition. Once again, rate equations for inhibition that contained polynomials up to the fourth power in I were tested. In addition, the data from Figures 3 and 4 were fitted to equations for uncompetitive and competitive inhibition, respectively. In each analysis, however, the goodness of fit was found inferior to that obtained with eq 3. Analysis of the data in Figure 3, according to eq 3, in which Fru-6-P was the varied substrate and P_i was held constant at 25.0 mM, yielded values for K_i and K_{ii} of $(2.76 \pm 0.71) \times 10^{-9}$ and $(9.37 \pm 0.60) \times 10^{-10}$ M^2, respectively. When P_i was the varied substrate with Fru-6-P held constant at 0.54 mM, the values for K_i and K_{ii} were $(8.14 \pm 0.47) \times 10^{-10}$ and $(6.7 \pm 2.5) \times 10^{-9}$ M^2, respectively.

Assuming that the reverse reaction of FBPase follows a rapid-equilibrium random mechanism (S. R. Stone and H. J. Fromm, unpublished experiments), the rate equation is given by

$$\frac{1}{v} = \frac{1}{V_2} + \frac{K_q}{V_2 Q} + \frac{K_p}{V_2 P} + \frac{K_{iq}K_p}{V_2 PQ} \quad (4)$$

where P and Q represent the concentrations of Fru-6-P and P_i, respectively, and other terms are as defined elsewhere for a rapid-equilibrium, random bireactant mechanism (Fromm, 1975). For eq 4, the free enzyme and the enzyme forms enzyme·Fru-6-P, enzyme·P_i, and enzyme·Fru-6-P·P_i contribute the terms $K_{iq}K_p/(V_2PQ)$, $K_q/(V_2Q)$, $K_p/(V_2P)$, and $1/V_2$, respectively.[2] For determination of which terms in eq 4 were affected by AMP, two initial velocity experiments were carried out: one in the absence of AMP and one in the presence of AMP. The results of these experiments, which are presented in Table II, indicate that the $1/V_2$, K_q/V_2, and $K_{iq}K_p/V_2$ coefficients were affected by the presence of AMP but that the K_p/V_2 coefficient was not affected. Thus, AMP seems to be binding to the free enzyme and the enzyme·Fru-6-P and the enzyme·Fru-6-P·P_i complexes but not to the enzyme·P_i complex. These results, together with the results presented in Figures 3 and 4, suggest that AMP inhibition of the reverse reaction of FBPase may be best described by

$$\frac{1}{v} = \frac{1}{V_2}\left[1 + \frac{I^2}{K_{iii}} + \frac{K_q}{Q}\left(1 + \frac{I^2}{K_{ii}}\right) + \frac{K_p}{P} + \frac{K_{iq}K_p}{PQ}\left(1 + \frac{I^2}{K_i}\right)\right] \quad (5)$$

where K_i, K_{ii}, and K_{iii} represent the dissociation constants for the enzyme·$(AMP)_2$, the enzyme·Fru-6-P·$(AMP)_2$, and the enzyme·Fru-6-P·P_i·$(AMP)_2$ complexes, respectively, and other terms are as defined for eq 4. Values for the kinetic constants of eq 5 were calculated from the results presented in Table II together with relationships derived from eq 5, and these values are listed in Table I.

Discussion

Most previous kinetic studies of the forward reaction of FBPase have used the coupled spectrophotometric assay and have measured initial rates (Nimmo & Tipton, 1975a,b; Marcus et al., 1973). This method of study has not proved entirely satisfactory. FBPase has an extremely low Michaelis constant for Fru-P_2 (0.25 μM, Table I), and the sensitivity of the coupled spectrophotometric assay is such that initial rates cannot be measured at levels of the substrate around its Michaelis constant. However, the coupled spectrophotometric assay does have adequate sensitivity if data points are collected over the entire time course of the FBPase reaction and are fitted to the integrated form of the rate equation. The coupled FBPase assay is ideally suited to study by means of progress curve kinetics. Because Fru-6-P is removed by the coupling enzymes, the reaction is irreversible. Moreover, the other product, P_i, is a very poor product inhibitor (Casazza et al., 1979), and its effects need not be considered. The irreversibility of the reaction and the lack of product inhibition greatly reduce the complexity of the equation to be used in fitting the data. One difficulty with progress curve analysis is found in obtaining realistic values for the standard errors of the parameters that are estimated (Cornish-Bowden, 1975). Duggleby & Morrison (1978) have tried to circumvent this problem in experiments involving more than one curve by compressing the data for each curve into three "idealized" points. In the present study, this method was unsatisfactory inasmuch as it still yielded unreasonable standard errors for the estimates of the parameters. It was found more satisfactory to summarize each progress curve in terms of its apparent maximum velocity and apparent Michaelis constant and then to fit functions of these apparent constants to various kinetic models by using weighted linear regression as outlined under Experimental Procedure.

Using progress curve data, it was possible to show that AMP caused S-parabolic, I-parabolic noncompetitive inhibition with respect to Fru-P_2. AMP has previously been reported to cause nonlinear noncompetitive inhibition (Taketa & Pogell, 1965; Nimmo & Tipton, 1975b). The model that best fitted the data is shown in Scheme I. The fact that this model does not consider hybrid states involving enzyme complexes with one molecule of AMP is suggestive of strong cooperativity in the

[2] Substrate analogue inhibition, product analogue inhibition, and isotope partitioning data indicate that the mechanism of the reverse of FBPase is either rapid-equilibrium random or rapid-equilibrium ordered with P_i bound first and with the formation of an inactive enzyme·Fru-6-P complex (S. R. Stone and H. J. Fromm, unpublished experiments). The rate equation for this latter mechanism is identical in form with eq 4, and the same enzyme forms contribute the same terms to the rate equation.

binding of AMP or that these complexes exhibit high dissociation constants. This model is supported by data of other workers who reported a Hill coefficient of ~2.0 for AMP inhibition of FBPase (Nimmo & Tipton, 1975b; Taketa & Pogell, 1965; Marcus & Haley, 1979).

The values of the dissociation constants for the enzyme·$(AMP)_2$ and enzyme·Fru-P_2·$(AMP)_2$ complexes were not very different (Table I), indicating that the binding of AMP to the enzyme is not markedly affected by the presence of bound Fru-P_2. For the model presented in Scheme I, the following relationship holds: $K_a K_{ii} = K_{a'} K_i$. Because K_i and K_{ii} are approximately equal, it follows that K_a and $K_{a'}$ must also be approximately equal (Table I). Thus, the affinity of the enzyme for Fru-P_2 is not markedly affected by the presence of bound AMP. These conclusions are supported by the data of Nimmo & Tipton (1975b).

Previous studies on AMP inhibition of FBPase have only examined the forward reaction (Nimmo & Tipton, 1975a,b; Taketa & Pogell, 1965). By studying the reverse reaction, it has been possible to obtain much more information on the nature of the AMP inhibition. Moreover, by using previously obtained information on the mechanism of the reverse reaction (S. R. Stone and H. J. Fromm, unpublished experiments), we have been able to identify the enzyme forms to which AMP binds. The free enzyme, the enzyme·Fru-6-P complex, and the enzyme·Fru-6-P·P_i complex have a high affinity for AMP, whereas the enzyme·P_i complex does not seem to bind AMP. Thus, in contrast to the results obtained for the forward reaction, the affinity of the enzyme for AMP is affected by the presence of the substrates of the reverse reaction. The dissociation constant for the enzyme·$(AMP)_2$ complex obtained from forward reaction data differs from the value obtained from the reverse reaction data (Table I). This difference may be due to experimental error or to the slightly different level of Mg^{2+} used in the two studies. The binding of AMP to FBPase is affected by the level of Mg^{2+} present (Nimmo & Tipton, 1975b). Alternatively, the difference in dissociation constants may reflect isomerization of the free enzyme before binding Fru-P_2 and/or the substrates of the reverse reaction. Benkovic et al. (1979) have suggested that FBPase undergoes an isomerization step before binding Fru-P_2.

The progress curve experiments presented in Figures 1 and 2 suggested the formation of an AMP·Fru-P_2 complex. This complex may be of some physiological importance. The nonenzymic breakdown of the AMP·Fru-P_2 complex seems to be extremely slow, and FBPase is not able to hydrolyze Fru-P_2 when it is bound in the complex. On the other hand, aldolase seems to be able to utilize Fru-P_2 fully in the presence of AMP. Thus, the Fru-P_2 bound in the complex would be available for glycolytic metabolism but not for gluconeogenic metabolism. A significant amount of intracellular Fru-P_2 would be bound in this complex. The levels of AMP and Fru-P_2 in the cell have been estimated at ~100 (Blair et al., 1973; Start & Newsholme, 1968) and ~20 μM (Williamson et al., 1969), respectively. If AMP binds to Fru-P_2 in a 1:1 ratio with a stability constant of 2.0×10^4 M^{-1}, ~65% of the estimated 20 μM Fru-P_2 within the cell would be complexed to AMP. If, however, AMP binds to Fru-P_2 in a 2:1 ratio with a stability constant of 6.0×10^8 M^{-2}, almost all the Fru-P_2 present would be complexed. These calculations suggest that the level of free Fru-P_2 within the cell is extremely low. In addition, the concentration of aldolase within the cell is such that most of the Fru-P_2 in the cell would be bound to aldolase (Sols & Marco, 1970).

In conclusion, AMP seems to have two effects on the FBPase reaction. Firstly, as an inhibitor, AMP controls the activity of FBPase; at levels of AMP estimated to be found within liver cells, FBPase would exhibit less than 5% of its maximum activity. Secondly, by complexing with Fru-P_2, AMP affects the amount of substrate available to FBPase.

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