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Binding of Metabolites by Phosphofructokinase*

Robert G. Kemp† and Edwin G. Krebs

ABSTRACT: The binding of metabolites by skeletal muscle phosphofructokinase has been studied by the gel filtration technique. Adenosine 5′-diphosphate (ADP), adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP), and AMP were bound competitively with dissociation constants of 0.5, 0.6, and 1.8 μM, respectively. Competition studies using a variety of compounds indicated a high specificity for adenine derivatives at this binding site. The dissociation constant for cyclic 3′,5′-AMP was decreased in the presence of fructose-6-P and fructose-1,6-diP. At pH 6.95, the dissociation constant for fructose-6-P with P-fructokinase was 17 μM; the constant decreased at higher pH and in the presence of AMP, inorganic phosphate, and ammonium ion. Adenosine 5′-triphosphate (ATP) inhibited the binding of fructose-6-P and this inhibition was released by inorganic phosphate. Extrapolation of the data for the binding by P-fructokinase of fructose-6-P, AMP, ADP, and cyclic 3′,5′-AMP to maximum binding indicated a molar binding unit of 90,000 g for these compounds, but 3 moles of ATP was found to be bound by this amount of enzyme. Dissociation constants for the ATP binding sites were in the range of 5–15 μM. Citrate increased the affinity of P-fructokinase for ATP and decreased the affinity of the enzyme for AMP and fructose-6-P. The results of the equilibrium binding studies are discussed in relation to the kinetic behavior of P-fructokinase.

Phosphofructokinase represents one of the most complex enzymes in regard to the regulation of its activity by interaction with a variety of compounds. The kinetics of P-fructokinase from a great number of sources have been studied extensively (Mansour, 1963; Passonneau and Lowry, 1962, 1963; Atkinson and Walton, 1965; Lowry and Passonneau, 1966; Uyeda and Racker, 1965; Underwood and Newsholme, 1965; Viñuela *et al.*, 1963). This work has indicated that under appropriate conditions the enzyme is inhibited by a substrate for the reaction, adenosine 5′-triphosphate (ATP),[1] and by citrate and Mg^{2+}. The activity is enhanced by the other substrate, fructose-6-P, by the products of the reaction, fructose 1,6-diphosphate and ADP, and by AMP, cyclic 3′,5′-AMP, inorganic phosphate, and various cations. The sigmoid response of the enzyme to fructose-6-P has prompted Monod *et al.* (1965) to include P-fructokinase in the growing list of allosteric enzymes. Recently, Lowry and Passonneau (1966), in a thorough study of brain P-fructokinase, have suggested that there are at least seven and possibly 12 kinetically important binding sites for substrates, inhibitors, and deinhibitors. Garfinkel (1966), from a computer analysis of the kinetic data of Passonneau and Lowry (1962, 1963) for skeletal muscle P-fructokinase, has indicated that 1 mole of enzyme binds 2 moles of fructose-6-P, 3 moles of ATP, 3 moles of AMP, 5 moles of inorganic phosphate, and 1 mole of citrate.

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[1] Abbreviations used in this work: AMP, ADP, and ATP, adenosine 5′-mono-, -di-, and -triphosphates; cyclic 3′,5′-AMP, adenosine 3′,5′-monophosphate; IMP and ITP, inosine 5′-mono-, and -triphosphates; UMP and UTP, uridine 5′-mono-, and -triphosphates; XMP, xanthosine 5′-monophosphate; CMP, cytidine 5′-monophosphate; GMP, guanosine 5′-monophosphate; glycero-P, β-glycerophosphate; FDP, fructose diphosphate.

With the availability of crystalline P-fructokinase (Parmeggiani and Krebs, 1965), it appeared of interest to study the binding of the various effectors directly in an attempt to shed more light on these complex interactions. It should be noted, however, that the binding studies to be reported here have been carried out with amounts of enzyme far in excess of those used in kinetic studies. Furthermore, the dissociation constants obtained under the conditions defined are not necessarily identical with Michaelis constants obtained in kinetic studies since the latter values may be dependent upon rate constants other than those involved in association and dissociation of the particular effector or substrate.

Materials

The sodium salt of glycero-P was obtained from Nutritional Biochemicals and was twice crystallized to remove contaminating inorganic phosphate. ATP, ITP, UTP, ADP, AMP, IMP, XMP, CMP, GMP, and cyclic 3′,5′-AMP were purchased from P-L Biochemicals. Adenosine 3′-(2′)-phosphate, adenosine, adenine, 2-deoxyadenosine, 6-*N*-dimethyladenine, and adenosine 5′-sulfate were purchased from the California Corp. for Biochemical Research. Caffeine was obtained from Nutritional Biochemicals Corp. The nucleotides and sugar phosphates were purified by ion-exchange chromatography employing techniques similar to those described by Bartlett (1959). ATP was purified on Dowex 1 (formate) with a linear gradient of ammonium formate from 0 to 2 M at pH 6.6. ADP and fructose 1,6-diphosphate (Sigma) were similarly purified with an ammonium formate gradient from 0 to 2 M at pH 4.0. Ammonium formate was removed from the eluted nucleotide fractions by filtration through Bio-Gel P-2. Removal of ammonium formate from FDP fractions was achieved by passing the material through Dowex 50 (H^+) followed by extraction of formic acid with ether. Fructose 6-phosphate, grade B (Calbiochem), and adenosine 5′-sulfate were purified on Dowex (formate) with a linear gradient from 0 to 2 M formic acid. [^{14}C]Fructose (Calbiochem) was converted to [^{14}C]fructose 6-phosphate in the presence of yeast hexokinase, ATP, and Mg^{2+}. The product was purified on Dowex 1 (formate) and identified by demonstrating that in the presence of P-fructokinase, ATP, and Mg^{2+} it could be completely converted to fructose 1,6-diphosphate as indicated by a shift in the elution pattern from an anion-exchange resin. [^{14}C]ADP, [^{14}C]AMP, and cyclic 3′,5′-[^{3}H]AMP were purchased from Schwarz BioResearch, Inc., and were purified by thin layer chromatography on cellulose (Randerath, 1963). [γ-^{32}P]ATP was prepared by a procedure combining features of the methods of Tanaka *et al.* (1959) and Jones (1962), and purified by the method of Hurlbert *et al.* (1954). Final purification was achieved by thin layer chromatography on cellulose. Two- or three-times crystallized skeletal muscle P-fructokinase was prepared by the method of Parmeggiani and Krebs (1965). Crystalline yeast hexokinase was the generous gift of Dr. S. P. Colowick.

Methods

The binding of nucleotides and sugar phosphates to P-fructokinase was measured by filtration of the protein through Sephadex G-50 equilibrated with the isotopically labeled small molecule under study. This method was introduced by Hummel and Dreyer (1962) and recently described in detail by Fairclough and Fruton (1966).

Second or third crystals of P-fructokinase were collected by a brief centrifugation in the cold and dissolved in buffer consisting of 25 mM glycylglycine, 25 mM Na-glycero-P, 1 mM EDTA, and 5 mM mercaptoethanol. This buffer, which was adjusted to pH 6.95 $\pm$ 0.02 with HCl, provided a degree of stability to the enzyme without interfering with the various kinetic parameters (*e.g.*, ATP inhibition and AMP activation) as determined by independent kinetic studies. The enzyme solution was dialyzed in the cold for several hours against the buffer and, to remove bound ATP, was passed through a 20 $\times$ 4 mm column containing a mixture of one part of acid-washed charcoal and one part of coarse powdered cellulose. A 280:260 mμ ratio of 1.68 or greater indicated that ATP had been removed from the enzyme (Parmeggiani *et al.*, 1966). The protein concentration was determined at 279 mμ by using the extinction coefficient of 10.2 for a 1% solution (Parmeggiani *et al.*, 1966). Preparations were used for binding studies within 30 min of the time they were freed of ATP. A column with a diameter of 1.5 cm and containing 25–35 ml of Sephadex G-50 Fine (Pharmacia) was previously equilibrated with buffer containing the radioactive nucleotide or sugar ester, the binding of which was to be studied. A small disk of glass filter paper was used to stabilize the surface of the gel. P-Fructokinase in 1.5 ml of the solution used to equilibrate the column was applied to the top of the column and allowed to enter the gel. The column was eluted with more of the same solution and fractions of 1–1.2 ml were collected. Flow rates of 12–20 ml/hr were employed. All studies were carried out at 23°. Five-tenths milliliter of each fraction was counted in a Packard Tri-Carb scintillation spectrometer employing a scintillant solution containing 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene dissolved in 1 l. of dioxane. In early studies, those fractions-containing protein yielded radioactivity measurements which were consistently low, possibly owing to coprecipitation of bound nucleotide when the protein was placed in the scintillant solution. This problem was obviated by denaturing the protein prior to removal of the sample by heating those fractions containing protein for 10 min in a boiling water bath.

The results of a typical experiment demonstrating the binding of cyclic 3′,5′-[^{3}H]AMP to skeletal muscle P-fructokinase is shown in Figure 1. The attainment of equilibrium during gel filtration is indicated by the return of the base-line concentration of the small molecule to its initial value after the emergence of the enzyme and after the appearance of the trough in the

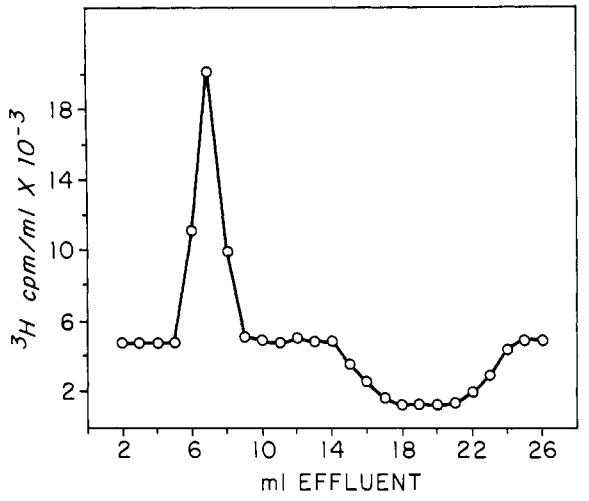

FIGURE 1: Representative elution profile for measurement of the binding of cyclic 3′,5′-AMP by P-fructokinase. Concentration of cyclic 3′,5′-[^{3}H]AMP, 0.1 μM; amount of protein, 0.34 mg; temperature, 23°; pH 6.95; flow rate, 15 ml/hr. In addition to cyclic 3′,5′-[^{3}H]AMP, the buffer contained 25 mM glycylglycine, 25 mM glycero-P, 5 mM mercaptoethanol, and 1 mM EDTA.

elution profile. The amount of radioactivity bound by P-fructokinase during such an experiment was determined from an average of the areas corresponding to the protein peak and the trough in the elution diagram. The agreement between these two areas was always better than 10%. The value for moles bound as determined from this average and from the specific radioactivity of the small molecule was related to the amount of protein employed in the experiment.

The influence of other compounds on the binding of the small molecules to the protein was carried out by adding, in addition to the radioactive compound under study, an unlabeled small molecule. The binding of the radioactive molecule under these conditions was then related to binding in the absence of added effector.

Results

Binding of Cyclic 3′,5′-AMP, AMP, and ADP by Phosphofructokinase. A double-reciprocal plot of cyclic 3′,5′-[^{3}H]AMP binding by phosphofructokinase at pH 6.95 is indicated in Figure 2. Over an 800-fold range in nucleotide concentration the data fit a straight line indicating a lack of cooperative interactions in the binding of this nucleotide. At a single concentration of the cyclic nucleotide (0.2 μM), the amount of enzyme was varied from 0.1 to 1 mg with no effect on the molar binding ratio. The data shown in Figure 2 represent results from a large number of P-fructokinase preparations since usually the preparations were characterized both by their specific activity in a kinetic assay and by their ability to bind cyclic 3′,5′-AMP. The dissociation constant determined from a least-squares evaluation of the reciprocal plot was 0.6 μM and maximum binding observed in the presence of 50 mM cyclic 3′,5′-AMP indicated 1 mole bound/92,000 g of enzyme. The intercept on the ordinate indicates maximum binding of 1 mole/90,000 g of enzyme. Binding studies with varying pH suggested that the affinity of the enzyme for the nucleotide decreased only by about 10% in the range from pH 6.6 to 8.7. In the presence of 6 mM $MgCl_2$, the dissociation constant was increased to about 1 μM.

Figure 3 shows a similar plot for the binding of [^{14}C]AMP and [^{14}C]ADP by P-fructokinase. The dissociation constant for AMP was 1.8 μM, about three

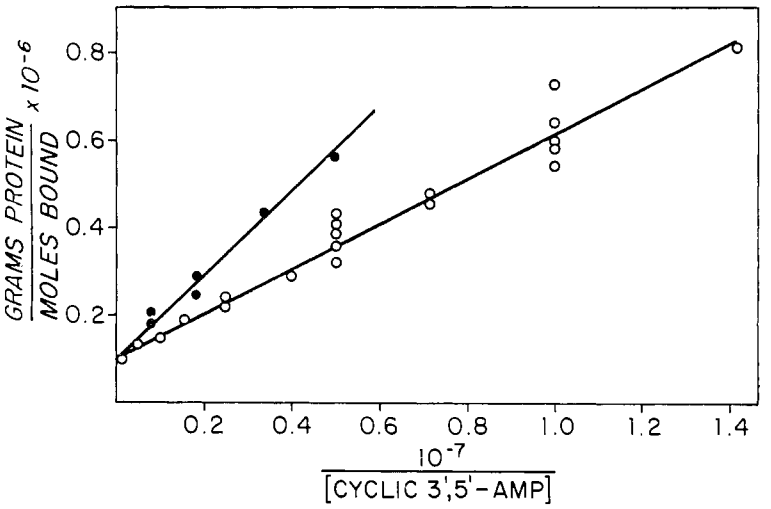

FIGURE 2: Reciprocal plot for the binding of cyclic 3′,5′-[^{3}H]AMP by P-fructokinase. O, cyclic 3′,5′-[^{3}H]AMP; ●, cyclic 3′,5′-[^{3}H]AMP plus 6 mM $MgCl_2$. Amount of protein varied from 0.2 to 7 mg. Other conditions are described in Figure 1.

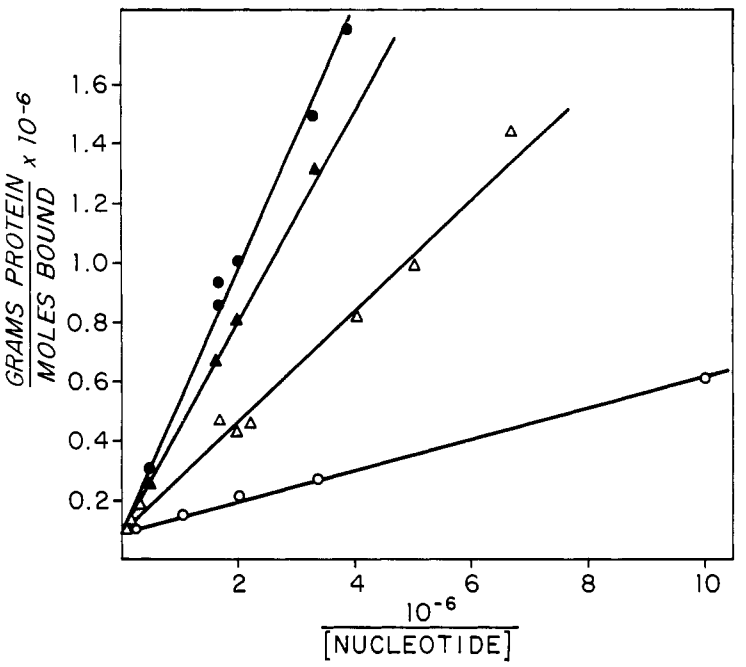

FIGURE 3: Reciprocal plot for the binding of [^{14}C]AMP and [^{14}C]ADP by P-fructokinase. △, [^{14}C]AMP; ▲, [^{14}C]AMP plus 6 mM $MgCl_2$; ○, [^{14}C]ADP; ●, [^{14}C]ADP plus 6 mM $MgCl_2$.

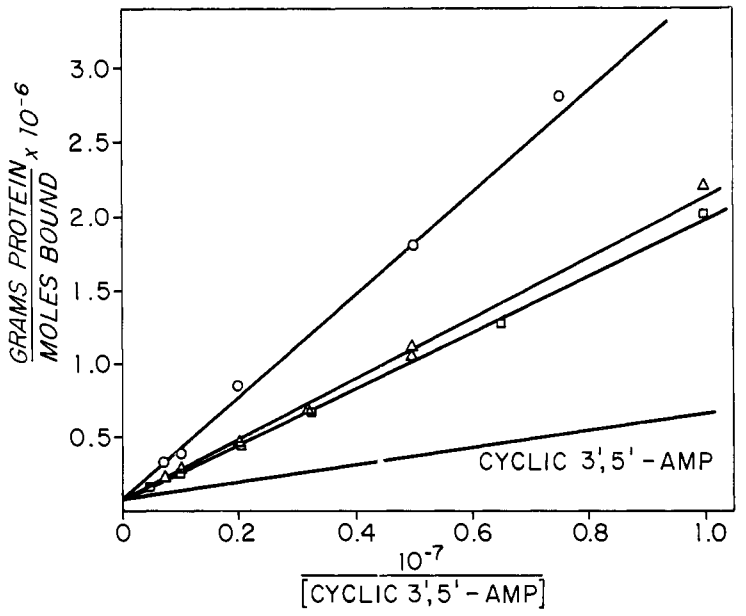

FIGURE 4: Competition by AMP, ADP, and ATP for cyclic 3′,5′-[^{3}H]AMP binding by P-fructokinase. Lower line represents plot taken from Figure 2. Δ, cyclic 3′-5′-[^{3}H]AMP plus 4 μM AMP; ○, cyclic 3′,5′-[^{3}H]AMP plus 2 μM ADP; □, cyclic 3′,5′-[^{3}H]AMP plus 20 μM ATP. Protein varied from 0.5 to 1.5 mg.

times higher than that for cyclic AMP. The binding of AMP was also shown to be relatively insensitive to pH in the range of 6.6–8.4. The dissociation constant for ADP was found to be 0.5 μM, very close to that for cyclic 3′,5′-AMP. Extrapolation of the plots to the ordinate indicated a binding unit of approximately 90,000 g for both AMP and ADP. In the presence of 6 mM $MgCl_2$, the dissociation constant of AMP was raised to 4 μM.

Competition by Other Nucleotides for Cyclic 3′,5′-AMP Binding. To investigate the possibility of a direct competition for the same binding site among the

adenine nucleotides, the binding of cyclic 3′,5′-AMP was studied over a wide concentration range in the presence of AMP, ADP, and ATP; and, as shown in Figure 4, the intersection of the lines on the axis indicates competitive inhibition. K_i values calculated for AMP and ADP were found to be 2 and 0.5 μM, respectively. That these values are in excellent agreement with the dissociation constants obtained by direct binding studies of AMP and ADP indicates a common binding site for cyclic 3′,5′-AMP, AMP, and ADP on P-fructokinase. The K_i for ATP was calculated to be approximately 10 μM. Although the ATP was purified before use, a small contamination with ADP could give a value for the K_i for ATP that is too low. Further evidence for the competitive nature of the inhibition is indicated by the fact that the binding of the cyclic nucleotide is completely blocked if AMP, ADP, or ATP are present at high concentrations.

To further examine the specificity of the cyclic 3′,5′-AMP binding site, a series of mononucleotides was equilibrated with a single concentration of radioactive cyclic 3′,5′-AMP and the enzyme. These data are presented in Table I. The apparent dissociation constants

TABLE I: Inhibition of Cyclic 3′,5′-[^{3}H]AMP Binding by 5′-Mononucleotides.[a]

Added Compd	Concn (μM)	Cyclic 3′,5′-AMP Bound (moles/10^5 g)	App K_i[b] (μM)
No addition, expt 1		0.16	
No addition, expt 2		0.15	
AMP	4	0.049	1.7
IMP	500	0.095	800
UMP	500	0.10	900
XMP	100	0.18	$>10^4$
CMP	100	0.17	$>10^4$
GMP	100	0.17	$>10^4$

[a] Columns were equilibrated with buffer containing 0.1 μM cyclic 3′,5′-[^{3}H]AMP and the added mononucleotide. P-Fructokinase (0.2–0.5 mg) in 1.5 ml of the same buffer was added to each column. [b] Apparent K_i values for the added mononucleotides were calculated assuming competitive inhibition (see text).

for the mononucleotides determined from the single-point measurements were calculated by assuming competitive inhibition at the cyclic 3′,5′-AMP binding site. Calculations were made using a rearrangement of the Michaelis–Menten equations for competitive inhibition to obtain $K_i = K_{\text{cyclic 3',5'-AMP}}\,(I)\,\bar{v}/(\bar{v}_{max}(\text{cyclic 3',5'-AMP}) - [(\text{cyclic 3',5'-AMP}) + K_{\text{cyclic 3',5'-AMP}}]\bar{v})$ where $\bar{v}$ = moles of cyclic 3′,5′-AMP bound/10^5 g, $\bar{v}_{max}$ = 1.11 moles/10^5 g, (I) = concentration of the competing molecule, and $K_{\text{cyclic 3',5'-AMP}} = 0.6$ μM.

The results indicate AMP as the only mononucleotide effective in reducing the amount of bound radioactive cyclic 3′,5′-AMP. The apparent K_i for AMP calculated for the single-point inhibition study is in good agreement with the value determined from Figure 4. Inosine 5′-phosphate, which differs from AMP only in the substitution on the 6 position of the purine ring, is about 1/500th as effective as AMP in reducing the amount of bound cyclic 3′,5′-AMP. This suggests a high specificity for the adenine moiety.

The apparent specificity of this binding site for adenine nucleotides was investigated further by measuring competition for cyclic 3′,5′-AMP binding by a variety of adenine derivatives. The results presented in Table II show that all of the adenine derivatives tested

TABLE II: Inhibition of Cyclic 3′,5′-[^{3}H]AMP Binding by Adenine Derivatives.[a]

Added Compd	Concn (μM)	Cyclic 3′,5′-AMP Bound (moles/10^5 g)	App K_i[b] (μM)
No addition, expt 1		0.24	
No addition, expt 2		0.26	
Adenosine 5′-sulfate	4	0.11	2
	50	<0.01	
Adenosine 3′-(2′)-phosphate	400	0.040	50
Adenosine	400	0.037	50
Adenine	100	0.1	50
	1000	<0.02	
2-Deoxyadenosine	100	0.08	50
6-*N*-Dimethyladenine	500	0.1	200

[a] Columns were equilibrated with buffer containing 0.2 μM cyclic 3′,5′-[^{3}H]AMP and the added derivative. P-Fructokinase (0.2–0.6 mg) in 1.5 ml of the same buffer as added to each column. [b] Apparent K_i values for the added derivatives were calculated assuming competitive inhibition (see text).

compete for the cyclic 3′,5′-AMP binding site. Although adenosine 5′-sulfate has one less negative charge than AMP at pH 7, it has an apparent dissociation constant similar to that nucleotide. That the actions of adenosine 5′-sulfate and adenine are competitive is indicated by the fact that higher concentrations of these compounds lead to almost complete inhibition of cyclic 3′,5′-AMP binding. While adenosine exhibits fairly tight binding to the enzyme, the nucleosides, uridine, cytidine, and guanosine, had no effect on cyclic 3′,5′-AMP binding when they were included at a concentration of 1 mM. Although methylation of the 6 nitrogen of adenine led to a lower affinity of the purine ring for the enzyme, 6-*N*-dimethyladenine was a more effective inhibitor than the nucleotide inosine 5′-

phosphate (Table I). The only pyrimidine or purine structure other than adenine derivatives that effectively inhibited cyclic 3′,5′-AMP binding was caffeine which can also compete with AMP binding to phosphorylase *b* (Alpers *et al.*, 1963; Kihlman and Overgaard-Hansen, 1955). Although not as effective as adenine, 0.5 mM caffeine inhibited the binding of 0.2 μM cyclic 3′,5′-AMP by 60%; and at 1 mM caffeine, the inhibition was approximately 90%.

Cyclic 3′,5′-AMP Binding. Effect of Other Activators and Inhibitors. To test the effect of other compounds which influence P-fructokinase kinetics, the enzyme was equilibrated with a single concentration of the cyclic nucleotide and several concentrations of the effectors. These results are summarized in Table III. Ammonium ion and inorganic phosphate, both of which increase the velocity of P-fructokinase, had no influence on the binding of cyclic 3′,5′-AMP. Uyeda and Racker (1965) have reported that UTP shows inhibitory effects similar to those observed with ATP while ITP is not strongly inhibitory. In regard to their ability to inhibit cyclic adenylate binding, ITP and UTP were both about one-tenth as effective as ATP.

Citrate decreased the affinity of the enzyme for cyclic 3′,5′-AMP; this inhibition did not appear to be competitive as indicated by the fact that 4 mM citrate was only slightly more inhibitory than 1 mM citrate with less than complete inhibition. At lower concentrations (0.2 mM) citrate caused less inhibition. Citrate was shown to similarly decrease the affinity of the enzyme for AMP and ADP. The combination of citrate and ATP elicited a much stronger inhibition of the binding of the cyclic nucleotide than would be expected from additive effects of the two compounds. As will be shown later, this effect is due to an increased affinity of the enzyme for ATP in the presence of citrate, thereby making ATP a better competitor for the cyclic 3′,5′-AMP binding site.

In the presence of fructose 6-phosphate the affinity of the enzyme for cyclic 3′,5′-AMP is increased. Raising the concentration of fructose 6-phosphate above 0.1 mM did not result in a further increase in the affinity for the cyclic nucleotide, which would suggest that in this concentration range the enzyme is saturated with fructose 6-phosphate. To eliminate the possibility that the increased binding was due to the opening of another site on the enzyme, the binding was studied over a wide concentration range of cyclic 3′,5′-AMP (Figure 5). In the presence of a "saturating" amount of fructose 6-phosphate (0.1 mM), the reciprocal plot indicated that the increase in cyclic nucleotide binding was due to a decrease in the dissociation constant from 0.6 to 0.2 μM. At lower concentrations of fructose 6-phosphate the affinity is increased to a lesser degree. Extrapolation of the data to the ordinate yielded the same intercept in the presence and absence of the sugar ester. It should be noted that in all cases, the data for cyclic 3′,5′-AMP binding yield straight lines in reciprocal plots. In the presence of fructose-1,6-diP the affinity for cyclic 3′,5′-AMP is increased in a manner similar to that observed with fructose 6-phosphate (Table III).

Binding of ATP by P-fructokinase. Studies of the binding of ATP by P-fructokinase indicate some of the difficulties which may be encountered in this technique owing to the fact that the compounds which significantly contaminate ATP, *i.e.*, ADP and AMP, bind more tightly to the enzyme than ATP itself. In all experiments, commercial samples of radioactive nucleotide were diluted to a convenient specific activity at the desired concentration by the addition of unlabeled nucleotide. The presence of a greater contamination in the radioactive sample than in the unlabeled carrier would result in a higher specific activity for the contaminant and magnify the error due to binding by this compound. Initially, studies with ATP labeled with ^{14}C in the nucleoside portion of the molecule suggested a very large number of molecules bound by the enzyme. This binding could be greatly reduced by the addition of a small amount of unlabeled ADP, suggesting that much of the observed binding was due to contamination by [^{14}C]ADP This problem was eliminated by the use of [γ-^{32}P]ATP. ADP and AMP formed from the breakdown of ATP thus no longer contained radioactivity. The [γ-^{32}P]ATP was purified on thin layer chromatography and was used within 1 week of the final purification step. A radioautogram indicated the absence of radioactive ADP under conditions which would have detected 0.1% contamination. When the

TABLE III: Effect of P-Fructokinase Activators and Inhibitors on Cyclic 3′,5′-[^3H]AMP Binding.[a]

Added Compd	Concn (μM)	Cyclic 3′,5′-AMP Bound (moles/10^5 g)
No addition, expt 1		0.26
No addition, expt 2		0.24
HPO_4^{2-}	2000	0.24
NH_4^+	2000	0.23
ATP	20	0.08
UTP	200	0.08
ITP	200	0.10
Citrate	200	0.20
	1000	0.10
	4000	0.09
ATP–citrate	20:1000	<0.005
F-6-P	2	0.29
	10	0.39
	100	0.49
	400	0.48
FDP	0.5	0.25
	5	0.3
	50	0.4
	500	0.5

[a] Columns were equilibrated with buffer containing 0.2 μM cyclic 3′,5′-[^3H]AMP and the added effector. P-Fructokinase (0.2–0.6 mg) in 1.5 ml of the same buffer was added to each column.

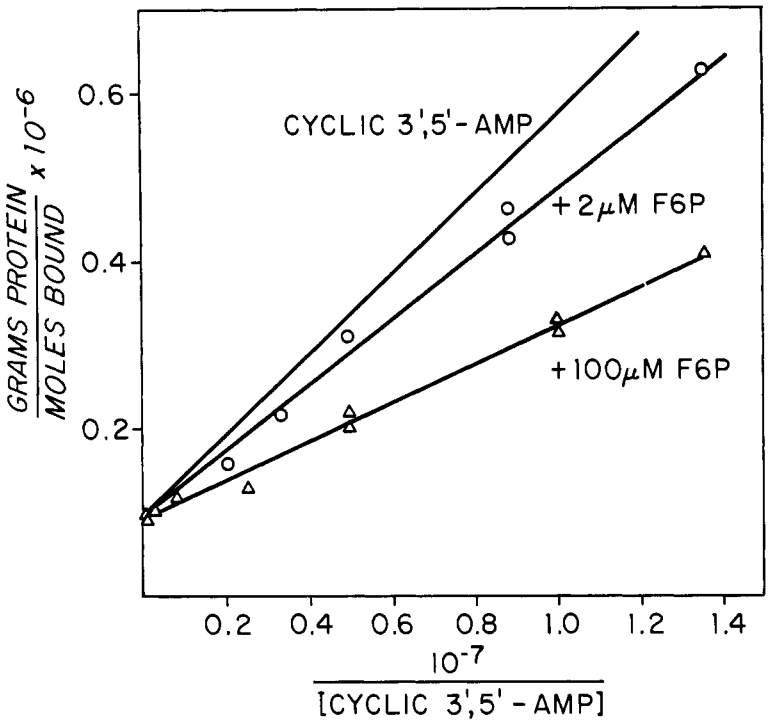

FIGURE 5: Effect of fructose 6-phosphate on cyclic 3′,5′-[^{3}H]AMP binding by P-fructokinase. Upper line represents the plot taken from Figure 2. O, cyclic 3′,5′-[^{3}H]AMP plus 2 μM fructose 6-phosphate; Δ, cyclic 3′,5′-[^{3}H]AMP plus 100 μM fructose 6-phosphate. Protein varied from 0.1 to 5 mg.

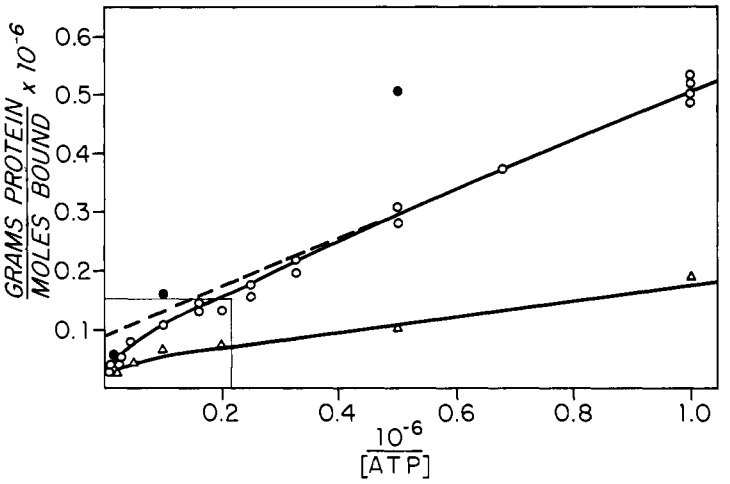

FIGURE 6: Binding of [γ^{32}P]ATP by P-fructokinase. O, [γ^{32}P]ATP; Δ, [^{32}P]ATP plus 0.5 mM sodium citrate; ●, [γ^{32}P]-ATP plus 0.1 mM cyclic 3′,5′-AMP. Protein varied from 1.3 to 10 mg. The portion of the curve in the lower right-hand corner that is enclosed in a box is shown in Figure 7.

[γ-^{32}P]ATP was converted to ADP in the presence of glucose and hexokinase, the ADP contained less than 2% of the ^{32}P which was initially present in the triphosphonucleoside.

The results shown in Figure 6 indicate that P-fructokinase binds more ATP than ADP, AMP, or cyclic 3′,5′-AMP. Extrapolation of the binding data (Figure 7) indicates maximum binding to be 1 mole/30,000 g of enzyme. This would suggest that 3 moles of ATP can bind to each subunit of 90,000 g as defined by AMP binding. In these experiments the highest concentration of ATP employed was 0.4 mM (with 15.6 mg of protein), and it would not have been feasible to go higher since the amount of enzyme required to bind a significant

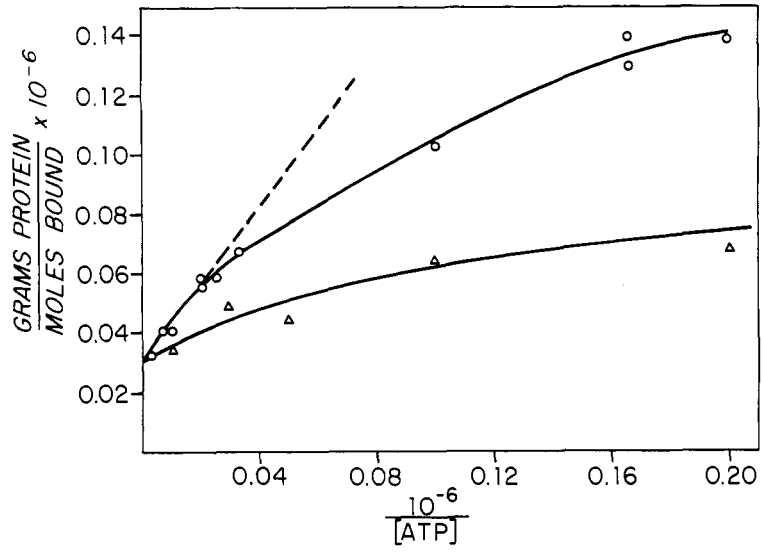

FIGURE 7: Binding of [γ^{32}P]ATP by P-fructokinase (taken from Figure 6).

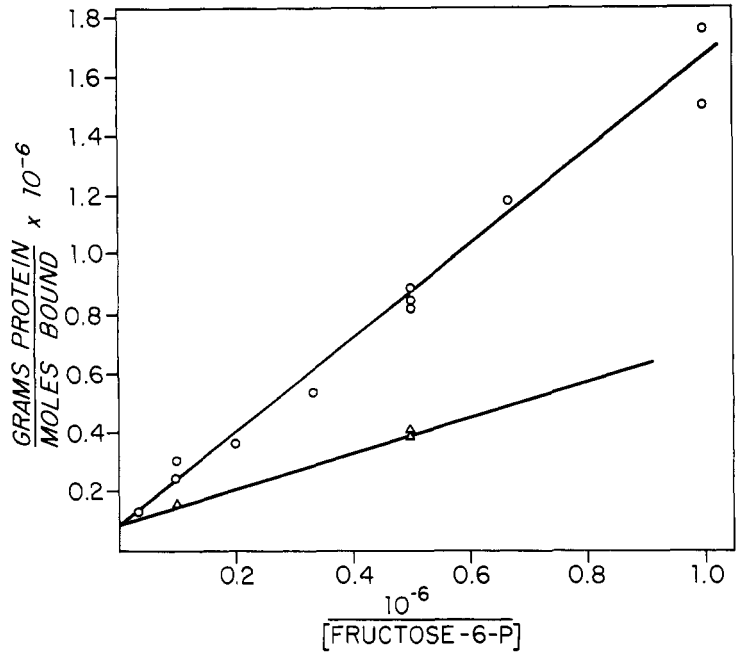

FIGURE 8: Binding of [^{14}C]fructose 6-phosphate by P-fructokinase. O, [^{14}C]fructose 6-phosphate alone at pH 6.95; Δ, [^{14}C]fructose 6-phosphate plus 10 μM AMP at pH 6.95. Protein varied from 1.5 to 13 mg.

fraction of the nucleotide would have been prohibitive from an experimental standpoint. Thus, if a binding site were present with a dissociation constant appreciably above 0.1 mM, this would not have been detected. The break in the double-reciprocal plot (Figures 6 and 7) suggests that all of the detected binding sites do not have identical affinities for ATP. The data for the binding site with the highest affinity for ATP (upper part of Figure 6) extrapolates to approximately 1 mole bound/90,000 g and yields a dissociation constant of 4–5 μM. The site with the lowest affinity for ATP (Figure 7) yields a dissociation constant of about 13 μM when the contribution to the total amount of bound nucleotide by the other two sites is subtracted. The dissociation constant for the remaining site would fall somewhere between 5 and 13 μM.

As indicated in Figure 6, the addition of sodium citrate (0.5 mM) to the column buffer increased the affinity of the enzyme for ATP. In the presence of citrate a break in the double-reciprocal plot (Figures 6 and 7)

is less obvious and the affinity of all ATP binding sites (based on the AMP binding unit of 90,000) would appear to be increased with dissociation constants estimated to be in the range of 1.5–3 μM.

The effect of cyclic 3′,5′-AMP on the binding of ATP is complex. It was previously shown that ATP competes with the binding of cyclic 3′,5′-AMP with an apparent K_i of approximately 10 μM (Figure 4). Presumably this competition is direct, and in the converse experiment shown here (Figure 6), the cyclic nucleotide would be expected to compete with ATP binding at the site with a dissociation constant in this range. This result, however, is not apparent from the limited amount of data presented. Because of the presence of more than one binding site for ATP, competition by the cyclic nucleotide for the binding of ATP at the site(s) with low affinity for ATP (above 5 μM) could not be easily measured. On the other hand, it appears that the cyclic nucleotide does reduce the binding of ATP at the site with the highest affinity for ATP but in a noncompetitive manner. This is indicated by the fact that at a concentration of cyclic 3′,5′-AMP which would be saturating (0.1 mM) the binding of ATP is not completely inhibited. Increasing the concentration of the cyclic nucleotide to 0.7 mM (not shown) led to no further decrease in ATP binding. Ammonium chloride (3 mM) had no effect on the binding of ATP when tested at several concentrations of this nucleotide.

Binding of [^{14}C]Fructose 6-Phosphate by Phosphofructokinase. Figure 8 illustrates a reciprocal plot of the data obtained with [^{14}C]fructose 6-phosphate. A dissociation constant of 17 μM and a binding unit of 90,000, identical with that obtained with AMP, ADP, and cyclic 3′,5′-AMP was calculated from these data. In the presence of 10 μM AMP, under which conditions the AMP site should be 85% saturated, the dissociation constant for fructose 6-phosphate was decreased to 7 μM. Binding studies of fructose 6-phosphate at varying pH (Figure 9) indicate that the amount of sugar phosphate bound at a concentration of 2 μM increases more than twofold in the pH range from 6.7 to 8.0.

Table IV indicates the effect of various P-fructokinase activators and inhibitors on the binding of fructose 6-P by the enzyme. Ammonium ion and inorganic phosphate, both of which are considered deinhibitors of P-fructokinase, slightly increase the affinity of the enzyme for fructose 6-phosphate. Although the combination of these two factors plus AMP (10 μM) does not appear to increase greatly the binding of fructose 6-phosphate over that seen with AMP alone, a slight additional increase in the amount of bound fructose 6-phosphate was observed. As would be expected from the fact that AMP and ADP bind at the same site on the enzyme, ADP also increases the affinity of the enzyme for fructose 6-phosphate. A decrease in the affinity of the enzyme for fructose 6-phosphate was observed in the presence of citrate.

ATP also decreased the affinity of the enzyme for fructose 6-phosphate. Figure 10 describes the reduction in the amount of fructose 6-phosphate bound at a single concentration due to increasing levels of ATP.

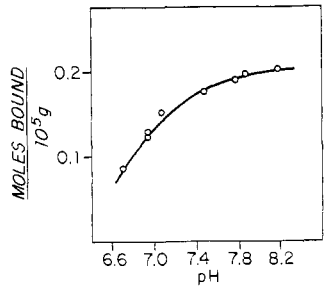

FIGURE 9: pH dependence of [^{14}C]fructose 6-phosphate binding by P-fructokinase. Protein varied from 4 to 6 mg. The concentration of [^{14}C]fructose 6-phosphate was 2 μM for all points.

TABLE IV: Effect of P-Fructokinase Activators and Inhibitors on [^{14}C]Fructose 6-Phosphate Binding.[a]

Added Compd	Concn (mM)	Fructose 6-Phosphate Bound (moles/10^5 g)
No addition, expt 1		0.114
No addition, expt 2		0.122
No addition, expt 3		0.126
NH_4^+	3	0.14
		0.16
NH_4^+	6	0.15
HPO_4^{2-}	3	0.17
HPO_4^{2-}	6	0.16
ADP	0.1	0.25
AMP	0.01	0.26
		0.25
AMP–NH_4^+–HPO_4^{2-}	0.01:6:6	0.28
Citrate	0.5	0.056
ATP	0.04	0.079
ATP–HPO_4^{2-}	0.04:5	0.127

[a] Columns were equilibrated with buffer at pH 6.95 containing 2 μM [^{14}C]fructose 6-phosphate and the added effector. P-Fructokinase (2–6 mg) in 1.5 ml of the same buffer was added to each column.

The inhibition is not competitive as indicated by the fact that the inhibition is constant at concentrations of ATP above 0.4 mM. Maximum inhibition of binding is about 55%. The inhibition is half-maximal at about 10–20 μM ATP which corresponds to one of the binding sites for ATP observed in the direct binding of [γ-^{32}P]-ATP. Inorganic phosphate (5 mM) completely reversed the inhibition by ATP (Table IV).

Discussion

The foregoing equilibrium binding studies have indicated the great complexity of the interactions involved

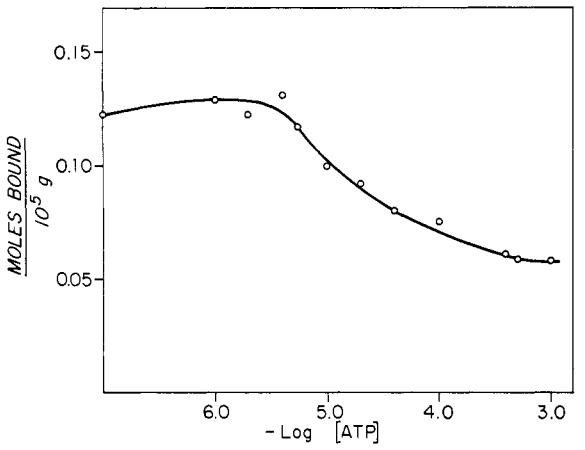

FIGURE 10: Effect of ATP on the binding of [^{14}C]-fructose 6-phosphate. Columns were equilibrated with buffer at pH 6.95 containing [^{14}C]fructose 6-phosphate plus the indicated concentration of ATP. Protein varied from 5 to 6 mg. The concentration of [^{14}C]fructose 6-phosphate was 2 μM for all points.

in the binding of substrates and various metabolites known to effect P-fructokinase activity. Effects of several types were observed. The case of simple competition for a single binding site is illustrated by the competition between cyclic 3′,5′-AMP, AMP, ADP, and ATP. Inhibition of a type which is not competitive is indicated by the effect of citrate on AMP binding and of citrate and ATP on fructose 6-phosphate binding. Enhancement of the binding of one molecule due to the presence of a second is best illustrated by the effect of citrate on ATP binding and the reciprocal relationship between the AMP binding site and the fructose 6-phosphate binding site.

The results have indicated the presence of a binding site on P-fructokinase that is highly specific for adenine derivatives. This site binds ADP, cyclic 3′,5′-AMP, AMP, and ATP with dissociation constants 0.5, 0.6, 1.8, and approximately 10 μM, respectively. The relative strength of binding of these nucleotides is not reflected in the amount of negative charge that they carry. The relatively high binding energy of AMP to the protein and thus the specificity of nucleotide binding appears to be the result of the adenine ring structure itself. On the basis of its dissociation constant, the free energy for the dissociation of AMP was calculated to be about 7 kcal. The competition studies with adenine would indicate that only two kilocalories are lost owing to the absence of a negative charge at the 5′-hydroxyl. The specificity for the charge being at this position is indicated by the observation that adenosine 2′-(3′)-phosphate is no more effective in inhibiting the binding of cyclic 3′,5′-AMP than adenosine. Sulfate could be substituted for phosphate at the 5′-hydroxyl without significantly changing the binding properties. A primary amino function on the 6 position of purine ring is not necessary as indicated by the fact that less than 1 kcal is lost by substitution of the hydrogens on this nitrogen by methyl groups.

The binding properties of caffeine to P-fructokinase are puzzling in that its structure bears comparatively little similarity to adenine. Previously, caffeine has been shown to act competitively with AMP in the phosphorylase *b* reaction (Alpers *et al.*, 1963) and with cyclic 3′,5′-AMP in the cyclic 3′,5′-AMP phosphodiesterase reaction (Butcher and Sutherland, 1962). The inhibitory properties of caffeine may be due to an ability to bind fairly nonspecifically to proteins. In studies on the ability of purines to solubilize small amounts of hydrocarbon, caffeine was found to be exceedingly effective in the solubilization of pyrene, presumably the result of polarization interactions (Boyland and Green, 1962). This may suggest the ability of caffeine to interact with hydrophobic regions on proteins.

Evidence for the above-described AMP binding site being the one that is functional in deinhibiting P-fructokinase may be summarized as follows. (1) The binding specificity for AMP among the various mononucleotides is identical with that observed for deinhibition in kinetic studies of skeletal muscle P-fructokinase (Passonneau and Lowry, 1962). (2) Kinetically, cyclic 3′,5′-AMP is about three times as effective as AMP at low concentrations (Passonneau and Lowry, 1962). In the binding studies described above, cyclic 3′,5′-AMP is bound three times more tightly than AMP in the absence of $MgCl_2$ and about four times more tightly in the presence of magnesium ion. (3) Binding of nucleotides at this site increases by more than twofold the affinity of the enzyme for fructose 6-phosphate. This provides an explanation for the observation that the nucleotides increase the rate of the reaction at noninhibitory as well as at inhibitory concentrations of ATP (Passonneau and Lowry, 1964). (4) The binding of AMP reduces the amount of bound ATP at one and probably two of the three sites observed in ATP binding studies. Assuming one of these sites is the site which leads to inhibition by ATP, the data are consistent with AMP being a deinhibitor of P-fructokinase. (5) The data also support the recent results of Lowry and Passonneau (1966) who, from kinetic data, suggest that AMP, cyclic 3′,5′-AMP, and ADP bind at the same site and that this site is not influenced by NH_4^+ and inorganic phosphate.

The relative effects of magnesium ion on the binding of AMP, cyclic 3′,5′-AMP, and ADP are not identical. In the presence of 6 mM $MgCl_2$, the dissociation constants of the mononucleotides are increased by about twofold. This is very likely owing to the interaction of Mg^{2+} with the protein leading to some slight alteration in the nucleotide binding site. On the other hand, the dissociation constant of ADP is increased tenfold which indicates an additional influence of the metal ion, presumably due to direct interaction with the nucleotide. Under the conditions of the experiment ADP would be present as the $MgADP^-$ complex. Thus, it appears that the complex binds less tightly than free ADP.

The binding studies have indicated 1 site/90,000 g for fructose 6-phosphate. In the absence of added effectors the dissociation constant was 17 μM. If one assumes that this represents the catalytic site on the enzyme, several observations provide reasonable explanations for the previously reported kinetic phenomenon. A decrease in the dissociation constant for the enzyme was noted with increasing pH. AMP, and to a lesser extent NH_4^+ and inorganic phosphate, increase the affinity of the enzyme for this substrate. ATP increases the dissociation constant and this inhibition of fructose 6-phosphate binding by ATP is released by inorganic phosphate. Citrate also raises the dissociation constant of fructose 6-phosphate. Although these findings are consistent with kinetic studies, it should be noted that the degree of inhibition of the reaction by ATP is much greater than the observed reduction in the dissociation constant (Uyeda and Racker, 1965). Similarly, in kinetic studies, the effects of NH_4^+, inorganic phosphate, and AMP were more striking than those observed here (Lowry and Passonneau, 1966). It is probable that the presence of various effectors bound to the protein influences rate constants other than those involved in the association and dissociation of the substrates, and it would be unrealistic to expect quantitative agreement between kinetic studies and thermodynamic studies of binary and tertiary complexes alone. Furthermore, since these studies could not be carried out with the complete reaction requirements, magnesium ion was not included in studies of the interaction between ATP and fructose 6-phosphate. It is quite likely that the metal ion shifts the binding constants of one or both substrates. Lowry and Passonneau (1966) have observed greater inhibition of brain P-fructokinase by free ATP than by the $MgATP^{2-}$ complex.

Three moles of ATP were bound by P-fructokinase for every fructose 6-phosphate or AMP binding site. One of these binding sites is identical with the AMP binding site and at least one must represent the catalytic site. Two other effects of ATP have been reported by Parmeggiani *et al.* (1966). ATP appears to be obligatory for the crystallization of skeletal muscle P-fructokinase and is actually bound in the crystal lattice; and studies with the ultracentrifuge have indicated that ATP leads to a dissociation of the enzyme into a less highly aggregated form. That this latter effect is not due to binding at the AMP site is indicated by the observation that cyclic 3′,5′-AMP does not lead to dissociation of the enzyme and that cyclic 3′,5′-AMP at concentrations which would compete effectively with ATP at this binding site does not reverse the dissociating effect of ATP (R. G. Kemp and E. G. Krebs, unpublished results). Citrate reduces the dissociation constant for ATP at all three binding sites on the enzyme. P. J. Randle (personal communication) has found that with less than saturating levels of ATP, low concentrations of citrate increase the rate of P-fructokinase. This is consistent with the fact that citrate increases the affinity of the enzyme for ATP at the substrate binding site.

The effect of citrate on the enzyme appears to be expressed in several ways. Decreases in the affinity of the enzyme for fructose 6-phosphate and for the adenine mono- and diphosphonucleosides were observed. On the other hand, a striking increase in the affinity of the enzyme for ATP was noted. These findings along with the observed interplay between the AMP, ATP, and fructose 6-phosphate binding sites would indicate that structural changes take place on the enzyme owing to the binding of any one of these compounds leading to changes in the affinity of the enzyme for the other. The inhibition of enzyme activity by citrate is the result of two actions: a direct effect through inhibition of the binding of fructose 6-phosphate; and an indirect action due to an increase in the affinity of the enzyme for ATP which in turn decreases the affinity for fructose 6-phosphate. It should be noted that one can infer from the data a dissociation constant for citrate in the range of 0.1–0.5 mM.

The binding unit of 90,000 provides an estimate of a minimal molecular weight for P-fructokinase. Previously, Ling *et al.* (1965) have estimated a molecular weight of 360,000 from the sedimentation constant of the most slowly sedimenting species observed in their studies. Four AMP or fructose 6-phosphate binding units would make up a particle of this size. At low protein concentration and in the presence of ATP, Parmeggiani *et al.* (1966) have observed a sedimentation coefficient of 8.4 S which would be consistent with a molecular weight of about 200,000.

Although the foregoing observations provide possible mechanisms for the effects elicited by the large number of molecules which influence P-fructokinase, one important problem not explained is the apparent second order response of the enzyme to increasing concentrations of fructose 6-phosphate (Mansour, 1963). This has been explained previously by invoking cooperative binding between two fructose 6-phosphate sites on the enzyme (Monod *et al.*, 1965). Under no conditions, however, have such homotropic interactions been observed in the binding of either substrate or mononucleotides.

As indicated in the results, the amount of cyclic 3′,5′-AMP bound at a single concentration did not vary when such studies were carried out at enzyme concentrations (0.1–1 mg/ml) which result in different degrees of aggregation of the enzyme (Parmeggiani *et al.*, 1966). It should be stressed, however, that the equilibrium binding of the various molecules has been studied with concentrations of enzyme greater than those normally employed in kinetic studies, and that this may be highly significant with an enzyme such as P-fructokinase which displays concentration-dependent association phenomena. It is also realized that additional binding sites for substrates or effectors may be present on the enzyme which are not detectable by this technique. A binding site with a dissociation constant above 0.1 mM could not be measured, and binding dependent on the presence of combinations of factors (*e.g.*, ATP, Mg^{2+}, and fructose 6-phosphate) would go unobserved.

Acknowledgments

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