Phosphofructokinase from the Liver Fluke *Fasciola hepatica*

II. Kinetic Properties of the Enzyme

DEBORAH BENNETT STONE[1] AND TAG E. MANSOUR

Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California 94304

(Received October 25, 1966)

SUMMARY

This paper describes the kinetics of phosphofructokinase from the liver fluke *Fasciola hepatica*. Activation of the enzyme was accompanied by a marked increase in its affinity for both fructose-6-P and ATP. Results with the activated enzyme showed that each of the three ligands (fructose-6-P, ATP and cyclic 3′,5′-AMP) could alter the saturation function of the other. The enzyme was strongly inhibited by ATP in the presence of low fructose-6-P concentrations. Both cyclic 3′,5′-AMP and fructose-6-P could increase the concentrations of ATP required to produce 50% inhibition. Possible interaction among the substrates and the effector ligands on the enzyme was considered in terms of the Monod-Changeux-Wyman model for allosteric proteins. Saturation curves for fructose-6-P indicated cooperative homotropic interactions between multiple binding sites for this substrate. ATP at high concentration increased the homotropic interactions while cyclic 3′,5′-AMP had the opposite effect. The saturation function of cyclic 3′,5′-AMP was also shown to be influenced by both enzyme substrates. Fructose-6-P at high concentration caused a decrease in the half-saturation point for cyclic 3′,5′-AMP while ATP had the opposite effect. Saturation curves for Mg^{++} were also altered by a change in concentration of fructose-6-P, ATP or cyclic 3′,5′-AMP. The possible role of these kinetic properties on the fine regulation of activated phosphofructokinase was discussed.

INTRODUCTION

Phosphofructokinase isolated from the liver fluke *Fasciola hepatica* was essentially inactive when assayed under a variety of conditions (1–3). Experiments reported in the preceding paper (1) demonstrated that a partially purified preparation of this enzyme can be activated by incubation with cyclic 3′,5′-AMP[2] and a thermostable fraction containing adenine nucleotides, Mg^{++}, and inorganic phosphate. In the present paper the effect of varying the concentrations of fructose-6-P and ATP on the activity of phosphofructokinase before and after activation was investigated. In addition, studies were carried out with the activated enzyme to determine possible kinetic interactions between the substrate, fructose-6-P, the inhibitor, ATP, and the activator, cyclic 3′,5′-AMP. These kinetic results will be discussed in terms of the Monod, Changeux, and Wyman model for allosteric enzymes (4, 5).

MATERIALS AND METHODS

Activation and assay of phosphofructokinase activity. Inactive phosphofructokinase was isolated from liver fluke homogenates by differential centrifugation as described previously (1). The sedimented material collected between 80,000 *g* and 150,000 *g* (150-sediment fraction) was used for all experiments reported below. The 150-sediment enzyme was activated as

[1] Present address: Cardiovascular Research Institute, University of California School of Medicine, San Francisco, California 94122.

[2] The following abbreviation is used: cyclic 3′,5′-AMP, adenosine 3′,5′-phosphate.

described before (1): Activation mixtures containing 0.02 ml 150-sediment, 0.065 ml thermostable fraction, and 10^{-4} M cyclic 3′,5′-AMP in a final volume of 0.10 ml were incubated for 12 min at 30°. In some experiments for activation of phosphofructokinase the thermostable fraction was replaced by a nucleotide–Mg^{++}–inorganic phosphate mixture of similar composition: potassium glycylglycine buffer, 50 mM, pH 7.5; ATP, 3.6 mM; ADP, 3.5 mM; AMP, 2.1 mM; $MgCl_2$, 8 mM; and KH_2PO_4, 6.9 mM. Activation of 150-sediment phosphofructokinase by the nucleotide–Mg^{++}–inorganic phosphate mixture was indistinguishable from that produced by the thermostable fraction. Following phosphofructokinase activation, aliquots of the activation mixture were diluted and assayed spectrophotometrically for phosphofructokinase activity. Methods for enzyme dilution and assay (procedure B) were described before (1). The 150-sediment was diluted 500–1000 times in the final assay reaction mixture.

In the results reported below, the term "inactive phosphofructokinase" refers to the enzyme as isolated in the 150-sediment without activation. The term "inactive" is used here in the sense that enzyme activity could be measured only in the presence of extremely high concentrations of both substrates. The term "activated phosphofructokinase" refers to the enzyme after incubation with cyclic 3′,5′-AMP and other required components for activation as described above.

Since the usual Michaelis-Menten formulation could not be applied to the kinetics described below, the affinity of the enzyme for its substrate as well as other ligands was calculated from linear plots as the concentration required for 50% of the maximal observed velocity. This concentration is referred to as the half-saturation point.

RESULTS

Effect of Fructose-6-P Concentration on Phosphofructokinase Activity

Experiments were carried out to determine the effect of substrate concentration on the inactive and activated forms of the enzyme (see under Materials and Methods). Typical fructose-6-P saturation curves are shown in Fig. 1. In this experiment, the concentration of ATP was 0.6 mM and the ATP/Mg^{++} ratio was held constant at 1. The saturation curve for the activated form of the enzyme had a sigmoidal shape, while the curve for the inactive enzyme showed a more gradual, linear increase in activity with increasing fructose-6-P concentration. At 10 mM fructose-6-P, a concentration sufficient to saturate the activated enzyme, the inactive enzyme showed only one-fifth the activity of the activated enzyme. Even at fructose-6-P concentrations as high as 30 mM, the activity of the inactive enzyme was significantly less than that of the activated enzyme. The concentration of fructose-6-P required for half saturation of the enzyme was 3.8 mM for the activated enzyme and 13 mM for the inactive enzyme. These results indicated that activation of liver fluke phosphofructokinase caused an increase in both the V_{max} and the affinity of the enzyme for fructose-6-P.

Since previous experiments had demonstrated that the inactive enzyme could be activated by high concentrations of inorganic polyvalent anions added directly to the assay reaction mixture, it seemed possible that the activity displayed by the "inactive" enzyme in the presence of fructose-6-P concentrations as high as 30 mM might be due to an effect of this polyvalent anion on the enzyme rather than to a kinetic property of the inactive enzyme itself. However, no evidence to support this hypothesis has been found. Addition of closely related compounds such as inorganic phosphate or glucose-6-P to the assay reaction mixture in concentrations up to 20 mM did not alter the fructose-6-P saturation curve of the inactive enzyme. If fructose-6-P at high concentrations was increasing enzyme activity because of its anionic property, closely related compounds might be expected to mimic its action resulting in a reduction in the fructose-6-P concentration required for half-saturation of the enzyme.

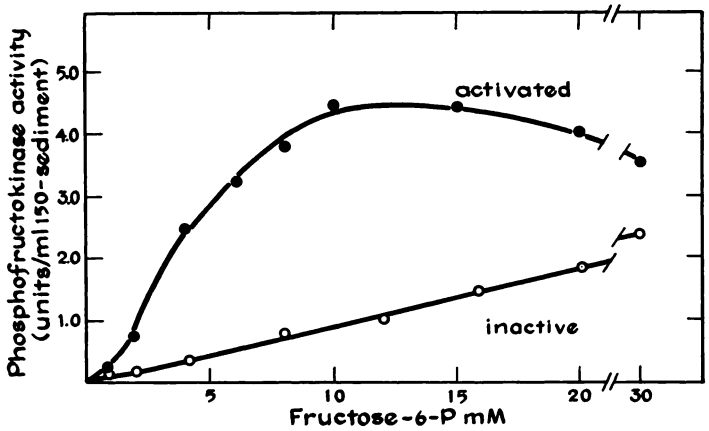

FIG. 1. *Effect of fructose-6-P concentration on liver fluke phosphofructokinase*

Enzyme activation and assay were carried out as described under Materials and Methods. ATP concentration in the assay mixture was 0.6 mM; ATP/Mg^{++} ratio was maintained at 1. Fructose-6-P concentrations ranged from 0 to 30 mM. The enzyme was assayed before (*inactive*) and after (*activated*) activation.

Effect of ATP Concentration on Phosphofructokinase Activity

The saturation curves for the other substrate, ATP, are given in Fig. 2. When assayed at a fructose-6-P concentration of 2 mM, the activated enzyme showed maximal activity at approximately 0.1 mM ATP (Fig. 2a). At higher concentrations of ATP the enzyme was inhibited. At an ATP concentration of 2 mM the enzyme activity was reduced to approximately 5% of the maximal activity. In order to measure the kinetics of the inactive enzyme with respect to ATP, it was found necessary to use fructose-6-P concentrations as high as 20 mM (10 times the concentration used for the activated enzyme). In the presence of such high concentrations of fructose-6-P, the ATP saturation curve for the inactive enzyme closely resembled that for the activated enzyme (Fig. 2b). Under these conditions, the inactive enzyme displayed maximal activity at an ATP concentration of 0.5 mM. The concentration of ATP required for half-saturation occurred at 0.084 mM ATP which was significantly greater than that for half-saturation of the activated enzyme (0.016 mM). At concentrations of ATP higher than 0.5 mM the inactive enzyme was inhibited significantly. Thus, phosphofructokinase was found to be sensitive to ATP inhibition both before and after activation.

The effect of cyclic 3′,5′-AMP was tested on both forms of the enzyme at different concentrations of ATP. In the presence of 0.1 mM cyclic 3′,5′-AMP the V_{max} for both forms of the enzyme was increased and inhibition by ATP was relieved (Fig. 2a,b). The concentration of ATP required to produce 50% inhibition of the activated enzyme was increased from 0.48 mM in the absence of cyclic 3′,5′-AMP to 0.93 mM in the presence of the cyclic nucleotide. The concentration of ATP necessary to produce 50% inhibition in the case of the inactive enzyme was increased from 7.0 mM to 9.3 mM in the presence of cyclic 3′,5′-AMP. The cyclic adenylic nucleotide, however, did not change significantly the saturation curves at low concentrations of ATP with either form of the enzyme.

Other compounds were tested for their ability to relieve ATP inhibition of the activated enzyme. 5′-AMP in a concentration of 0.1 mM was found to be as effective as cyclic 3′,5′-AMP. Neither ADP nor inorganic phosphate (potassium salt) was effective in relieving ATP inhibition of the liver fluke enzyme when tested at a concentration of 0.1 mM.

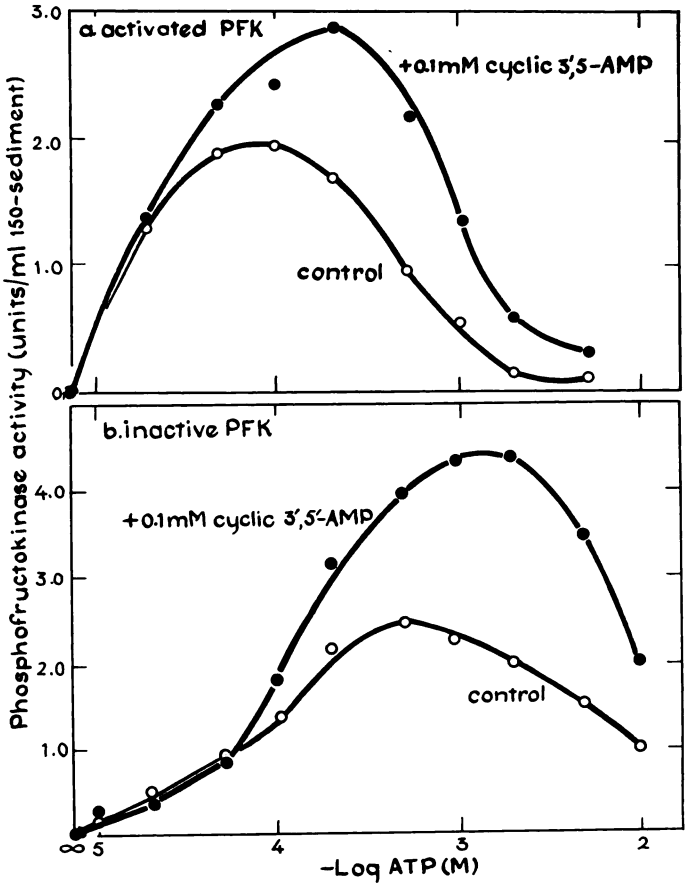

FIG. 2. *Effect of ATP concentration on liver fluke phosphofructokinase (PFK) in the presence and absence of cyclic 3',5'-AMP*

The enzyme was assayed after (a, *activated*) and before (b, *inactive*) activation. Enzyme activation and assay were carried out as described under Materials and Methods, except that the nucleotide–Mg^{++}–phosphate mixture contained no ATP and no AMP. Fructose-6-P concentration in the assay mixture was 20 mM for the inactive enzyme and 2 mM for the activated enzyme. ATP concentrations ranged from 0 to 5 mM. The ATP/Mg^{++} ratio was maintained at 1. Where indicated cyclic 3',5'-AMP was added to the assay reaction mixture in a final concentration of 0.1 mM.

Enzyme–Substrate–Effector Interactions

The fact that the saturation curve for fructose-6-P was not hyperbolic as well as the observed relief of ATP inhibition by cyclic 3',5'-AMP suggested possible interactions among the three ligands. In order to investigate this phenomenon more extensively, studies were carried out to determine the effect on the activated enzyme of varying concentrations of fructose-6-P, ATP, and cyclic 3',5'-AMP. These results are summarized in Figs. 3 through 7. The effect of cyclic 3',5'-AMP and ATP on the saturation function for fructose-6-P is given in Fig. 3. At a noninhibitory concentration of ATP (0.1 mM), the saturation curve for fructose-6-P was not hyperbolic in shape, indicating that the kinetics are not of the Michaelis-Menten type. The half-saturation point occurred at a fructose-6-P concentration of 2.8 mM. In the presence of 1.0 mM cyclic 3',5'-AMP, a concentration which is near saturation, the rate-concentration curve for fructose-6-P was hyperbolic in shape. The concentration of fructose-6-P required for half-saturation

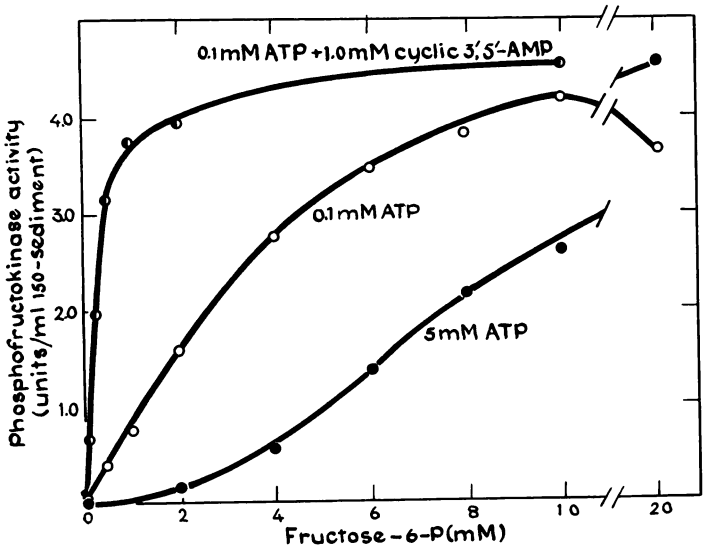

FIG. 3. *Effect of cyclic 3',5'-AMP and ATP on the saturation function for fructose-6-P*

Enzyme activation and assay were carried out as described under Materials and Methods. The ATP concentrations are indicated on the curves. The ATP/Mg^{++} ratio was maintained at 1. Where indicated, cyclic 3',5'-AMP was added to the assay reaction mixture in a final concentration of 1 mM. Fructose-6-P in the assay mixture was varied from 0 to 20 mM.

of the enzyme was reduced nearly 10-fold to 0.3 mM. Increasing the ATP concentration to 5 mM resulted in a sigmoidal fructose-6-P saturation curve. At this inhibitory level of ATP the concentration of fructose-6-P required for half-saturation of the enzyme was increased to 8.6 mM. Neither cyclic 3',5'-AMP, nor ATP, caused a significant change in the maximum velocity of the reaction; only the half-saturation point for fructose-6-P was altered.

The altered shape of the fructose-6-P saturation curve in the presence of an effector indicated a change in the interactions among several binding sites for fructose-6-P on the enzyme, or, in the terminology of Monod *et al.* (5), in the "co-operative homotropic interactions" of fructose-6-P. ATP, in inhibitory concentrations increases the cooperative homotropic interactions, while cyclic 3',5'-AMP tends to reduce them. The degree of cooperation of the several binding sites for fructose-6-P under different conditions may be estimated by the use of the Hill equation for oxygen binding by hemoglobin. (6). This equation can be expressed in the following form:

$$\log \frac{v}{V_{max} - v} = n \log S - \log K \quad (1)$$

where S is substrate (fructose-6-P) concentration, K is a constant, and n stands for the power of the substrate concentration (4). The maximum velocity observed experimentally was taken as V_{max}. If the logarithm of $v/(V_{max}-v)$ is plotted against the logarithm of the substrate concentration, the slope of the curve gives an estimate of n. An increase in the value for n can mean an increase in the interaction of the catalytic sites. In Fig. 4, the data from Fig. 3 have been plotted according to the Hill equation. In the presence of 0.1 mM ATP, n increased from a value of approximately 1 at low fructose-6-P concentrations, to 2.45 at near saturating fructose-6-P concentrations. This indicates that the binding of one molecule of fructose-6-P to the enzyme increased the affinity for the next molecule. In the presence of either cyclic 3',5'-AMP or inhibitory concentrations of ATP, straight-line plots were obtained. In the presence of cyclic 3',5'-AMP, n had a value of 1.4 indicating a decrease in the cooperative interaction

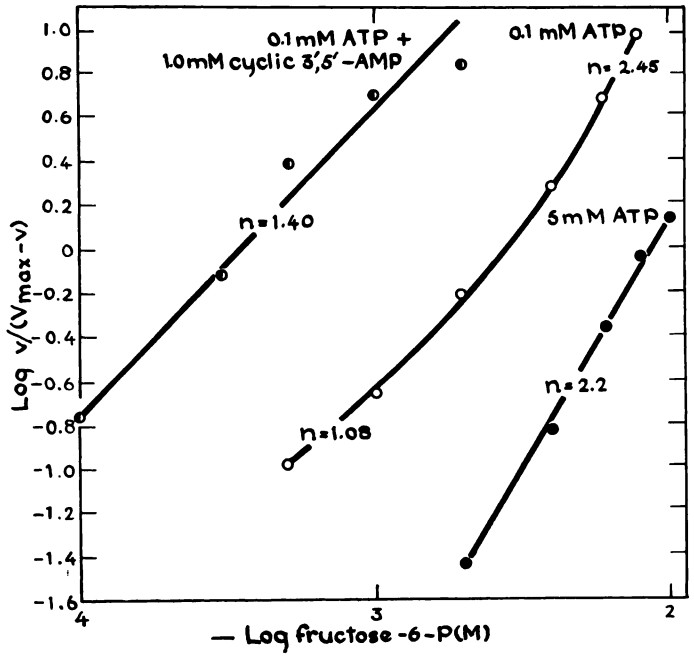

FIG. 4. *Dependence of the reaction velocity on the power of fructose-6-P concentration in the presence and absence of effectors*

The data from Fig. 3 are plotted according to Eq. (1) (see text).

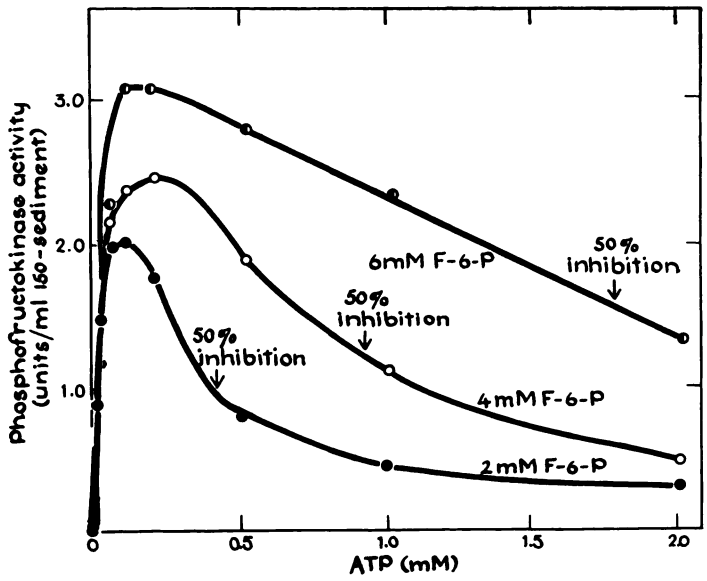

FIG. 5. *Effect of fructose-6-P concentration on the saturation function for ATP*

Enzyme activation and assay were carried out as described under Materials and Methods except that the nucleotide–Mg^{++}–phosphate mixture contained no ATP and no AMP. ATP concentration in the assay mixture ranged from 0 to 2 mM; the ATP/Mg^{++} ratio was maintained at 1. The fructose-6-P (F-6-P) concentrations are indicated on the curves. The arrows mark the concentration of ATP required for 50% inhibition of the enzyme.

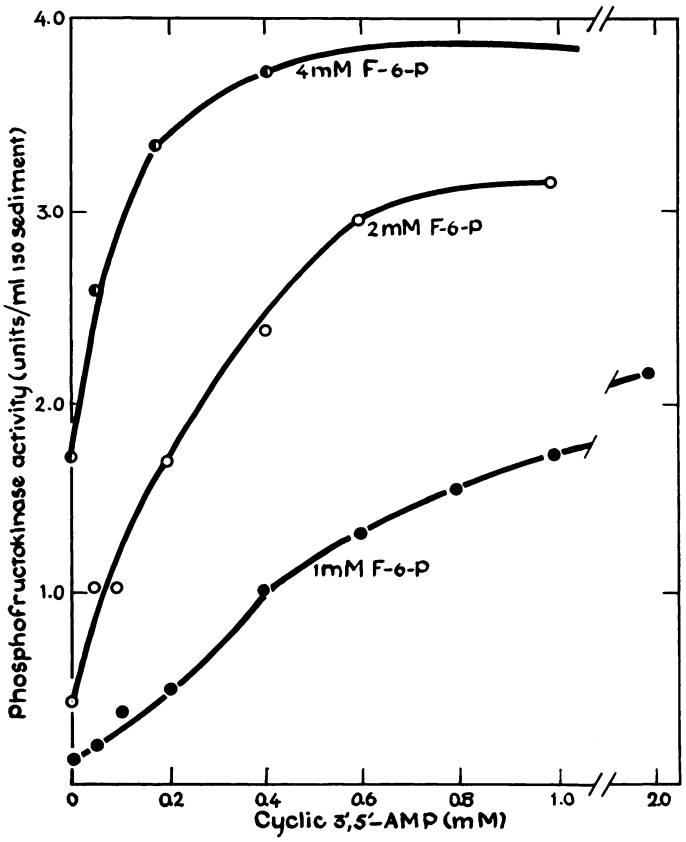

FIG. 6. *Effect of fructose-6-P concentration on the saturation function for cyclic 3',5'-AMP*

Enzyme activation and assay were carried out as described under Materials and Methods. Cyclic 3',5'-AMP in concentrations ranging from 0 to 2 mM was included in the assay reaction mixture. ATP concentration was 1 mM; ATP/Mg^{++} ratio was maintained at 1. The fructose-6-P (F-6-P) concentrations are indicated on the curves.

between substrate binding sites at high concentrations of fructose-6-P. At inhibitory levels of ATP (5 mM), n had a value of 2.2 indicating an increased interaction between fructose-6-P binding sites at low concentrations of fructose-6-P.

In Fig. 5 the effect of fructose-6-P concentration on the saturation curve for ATP is given. While fructose-6-P had only a slight effect on the concentration of ATP required for half-saturation, it caused a significant increase in its 50% inhibitory concentration. An increase in the concentration of fructose-6-P from 2 mM to 6 mM caused a 4-fold increase in the 50% inhibitory concentration of ATP (Fig. 5). Thus, fructose-6-P had an effect on the saturation curve for ATP similar to that of cyclic 3',5'-AMP shown in Fig. 2.

Finally, the saturation function of cyclic 3',5'-AMP was found to be influenced by both substrates, fructose-6-P and ATP. In Fig. 6 the effect of increasing fructose-6-P concentration on the saturation curves for cyclic 3',5'-AMP is shown to have two effects: the shape of the curve approached a hyperbola and the half-saturation point was displaced to lower concentrations of cyclic 3',5'-AMP. The results summarized in Fig. 7 show that increasing the concentration of ATP from 1 mM to 10 mM increased the concentration of cyclic 3',5'-AMP required for half-saturation. Levels of ATP which were not inhibitory had

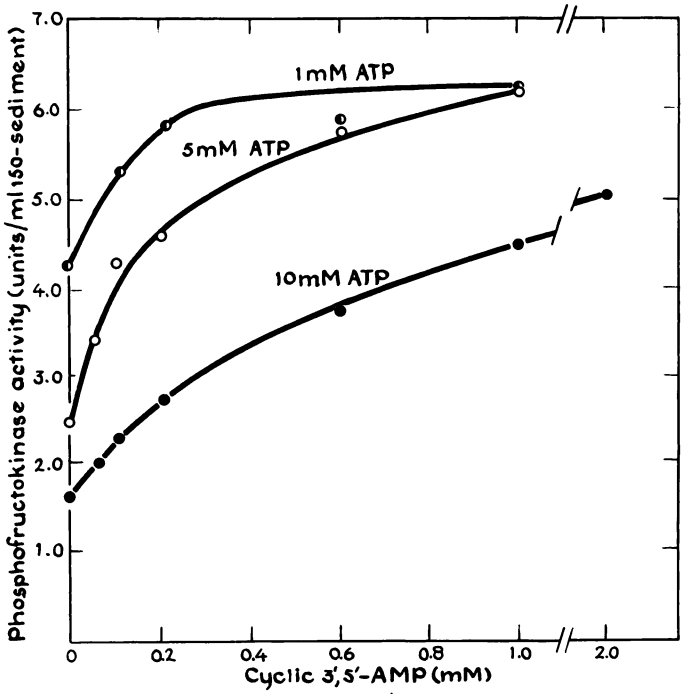

FIG. 7. *Effect of inhibitory concentrations of ATP on the saturation function for cyclic 3',5'-AMP*

Enzyme activation and assay were carried out as described under Materials and Methods except that the nucleotide–Mg^{++}–phosphate mixture contained no ATP and no AMP. Cyclic 3',5'-AMP in concentrations ranging from 0 to 2 mM was included in the assay reaction mixture. Fructose-6-P concentration was 10 mM (saturating). The ATP concentrations are indicated on the curves. The ATP/Mg^{++} ratio was maintained at 1.

little effect on the saturation function for cyclic 3',5'-AMP.

In the above studies, a constant ATP/Mg^{++} ratio of 1 was maintained. In order to determine the effect of Mg^{++} concentration on the enzyme activity, experiments were carried out in which the ATP concentration was held constant and the Mg^{++} concentration varied. Because of the presence of Mg^{++} in the enzyme preparation after activation, the lowest concentration of this cation which could be studied was 0.04 mM (the amount carried over from the activation step to the assay reaction mixture). In the presence of 2 mM fructose-6-P and 0.2 mM ATP, maximal enzyme activity occurred at a Mg^{++} concentration of 2 mM and a Mg^{++}/ATP ratio of 10 (Fig. 8, *control*). An increase in the fructose-6-P concentration or the addition of cyclic 3',5'-AMP to the assay reaction mixture resulted in an increase in the V_{max} of the enzyme reaction as well as a decrease in the concentration of Mg^{++} required for half-saturation (from 0.3 mM for the control condition to approximately 0.1 mM with 4 mM fructose-6-P and 0.06 mM in the presence of cyclic 3',5'-AMP). At higher levels of ATP (1 mM) the shape of the saturation curve for Mg^{++} became nonhyperbolic with a lower slope. The point of half-saturation was increased to 2 mM Mg^{++}; however maximal enzyme activity still occurred at a Mg^{++}/ATP ratio of 10.

DISCUSSION

The kinetic effects described above for the activated liver fluke phosphofructokinase can well be explained by the Monod *et al.* model (4, 5). Each of the three ligands (fructose-6-P, ATP, and cyclic 3',5'-AMP) could alter the saturation curve of the other. The saturation curve for fruc-

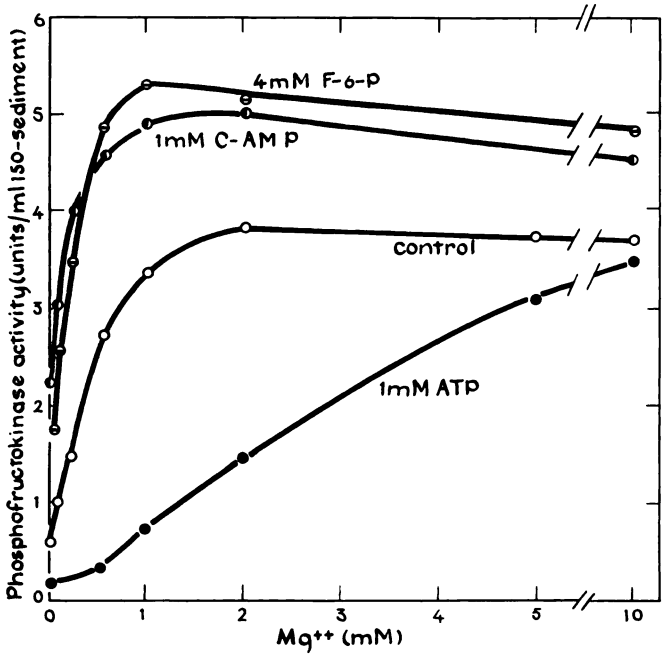

FIG. 8. *Effect of Mg^{++} concentration on liver fluke phosphofructokinase*

Enzyme activation and assay were carried out as described under Materials and Methods except that the nucleotide-Mg^{++}-phosphate mixture contained no ATP and no AMP. Mg^{++} concentration ranged from 0.04 to 10 mM. For the *control* condition, the activated enzyme was assayed at 2 mM fructose-6-P and 0.2 mM ATP. The enzyme was also assayed at 4 mM fructose-6-P (F-6-P), 1 mM ATP, and in the presence of 1 mM cyclic 3',5'-AMP (C-AMP), as indicated on the appropriate curves.

tose-6-P was sigmoidal (Fig. 3) suggesting that more than one fructose-6-P molecule per enzyme molecule participated in the reaction. Furthermore, the affinity of the enzyme for fructose-6-P was markedly altered in the presence of cyclic 3',5'-AMP or inhibitory levels of ATP. The "degree of cooperation" of different binding sites for fructose-6-P on the enzyme was further illustrated when the saturation curves were plotted according to the Hill equation (Fig. 4). These plots gave n values which varied from 1 to 2.45. ATP at high concentration increased the homotropic interactions as judged by the high n value for the Hill slope, and cyclic 3',5'-AMP had the opposite effect (Fig. 4). Similar results were reported with other enzymes with allosteric binding sites (5). According to Monod *et al.* (5) two types of allosteric systems may be distinguished by measurements of V_{max} and apparent affinity constants. The liver fluke enzyme appears to obey the "K system" since the presence of an effector (cyclic 3',5'-AMP or ATP in inhibitory concentrations) modified the apparent affinity of the protein for fructose-6-P without altering the V_{max} (Fig. 3) as would be expected in a "V system."

The data reported above indicate that saturation curves for the cationic cofactor of liver fluke phosphofructokinase, Mg^{++}, are influenced by ATP, fructose-6-P, and cyclic 3',5'-AMP. In the presence of low fructose-6-P concentrations and inhibitory levels of ATP, the curve was close to a straight line. With increase in the concentration of fructose-6-P, decrease in the inhibitory level of ATP, or addition of cyclic 3',5'-AMP, the curves approached an hyperbola. Similar experiments on the kinetics for Mg^{++} have recently been described for the transferase I kinase which converts the glucose-6-P independent form

of (UDPG: α-1,4-glucan α-4-glucosyl) transferase to the dependent form (7). Like phosphofructokinase, this enzyme is activated by cyclic 3′,5′-AMP. The cyclic adenylic nucleotide was shown also to cause a decrease in the concentration of Mg^{++} required for half saturation. However, in contrast to the phosphofructokinase system, cyclic 3′,5′-AMP did not appreciably alter the shape of the Mg^{++} saturation curve of the transferase I kinase; only the affinity of the enzyme for Mg^{++} appears to be altered.

The similarities between the kinetic properties of the liver fluke phosphofructokinase and those from mammalian sources have been previously emphasized (8, 9). Relief of ATP inhibition by cyclic 3′,5′-AMP and 5′-AMP, as well as by ADP and inorganic phosphate has been observed for phosphofructokinase isolated from guinea pig heart (8), brain (9) and rabbit muscle (10). In contrast to the enzyme from mammalian tissues and from the liver fluke, ATP inhibition of phosphofructokinase from both yeast (11) and *Escherichia coli* (12) is relieved by AMP, but not by cyclic 3′,5-′-AMP. This specificity is particularly striking since *E. coli* can synthesize cyclic 3′,5′-AMP (13), but yeast apparently cannot (11). Cyclic 3′,5′-AMP is synthesized by the liver fluke (14) as well as several mammalian tissues (15). As far as the fluke is concerned, it appears that while cyclic 3′,5′-AMP seems to be essential for the conversion of inactive to active phosphofructokinase and cannot be replaced by 5′-AMP (1), both nucleotides can relieve the enzyme from ATP inhibition.

The results reported in this paper as well as in the preceding paper (1) clearly show that phosphofructokinase is endowed with two main mechanisms for its control. The first is through conversion of an inactive enzyme to an active one. The inactive enzyme has an extremely low affinity for the substrates while the activated enzyme has a greater affinity. Thus, the inactive enzyme is "inactive" only in the sense that extremely high (and presumably unphysiological) levels of both substrates are required for activity. The second mechanism is through allosteric regulation of the activated enzyme. Thus, regulation of phosphofructokinase in the liver fluke ranges from complete absence of enzyme activity in the resting state to full activity modulated by a refined allosteric mechanism for its regulation. Cyclic 3′,5′-AMP seems to play an important role in controlling the enzyme by both mechanisms. The cyclic nucleotide was found to be essential for conversion of the inactive to the active enzyme (1, 2). Furthermore, cyclic 3′,5′-AMP can alter the kinetics of the enzyme when it is inhibited by ATP. Serotonin, which is known to increase the synthesis of cyclic 3′,5′-AMP (14) could therefore increase the activity of phosphofructokinase through the two mechanisms. The nucleotide 5′-AMP can also play a regulatory role as a modulator of the enzyme once it is activated in the presence of cyclic 3′,5′-AMP.

ACKNOWLEDGMENTS

This work was supported by Research Grant AI04214 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service; and a Research Career Development Award GM-K3-3848 from the Division of General Medical Sciences, U. S. Public Health Service.

REFERENCES

1. D. B. Stone and T. E. Mansour, *Mol. Pharmacol.* **3**, 161 (1967).
2. T. E. Mansour and J. M. Mansour, *J. Biol. Chem.* **237**, 629 (1962).
3. T. E. Mansour, *J. Pharmacol. Exptl. Therap.* **135**, 94 (1962).
4. J. Monod, J. P. Changeux and F. Jacob, *J. Mol. Biol.* **6**, 306 (1963).
5. J. Monod, J. Wyman and J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
6. J. Wyman, *Advan. Protein. Chem.* **4**, 407 (1948).
7. F. Huijing and J. Larner, *Proc. Natl. Acad. Sci. U. S.* **56**, 647 (1966).
8. T. E. Mansour, *J. Biol. Chem.* **238**, 2285 (1963).
9. J. V. Passonneau and O. H. Lowry, *Biochem. Biophys. Res. Commun.* **7**, 10 (1962).
10. K. Uyeda and E. Racker, *J. Biol. Chem.* **240**, 4682 (1965).
11. A. Ramaiah, J. A. Hathaway and D. E. Atkinson, *J. Biol. Chem.* **239**, 3619 (1964).

12. D. E. Atkinson and G. M. Walton, *J. Biol. Chem.* **240**, 757 (1965).
13. R. S. Makman and E. W. Sutherland, *Federation Proc.* **22**, 470 (1963).
14. T. E. Mansour, E. W. Sutherland, T. W. Rall and E. Bueding, *J. Biol. Chem.* **235**, 466 (1960).
15. E. W. Sutherland and G. A. Robison, *Pharmacol. Rev.* **18**, 145 (1966).