Allosteric Properties of Muscle Phosphofructokinase. I. Binding of Magnesium Adenosine Triphosphate to the Inhibitory Site*

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ABSTRACT: Magnesium ions plus adenosine 5′-triphosphate protected the most reactive thiol group of rabbit skeletal muscle phosphofructokinase from reaction with dithiobis(2-nitrobenzoic acid).

The reactivity of this thiol group appeared to be an indicator for the binding of magnesium adenosine 5′-triphosphate to the inhibitory site on the enzyme. Employing thiol reactivity as a means of measuring the relative saturation of the enzyme, the following properties of the magnesium adenosine 5′-triphosphate binding site were observed. (1) The dissociation constant for magnesium adenosine 5′-triphosphate was lower than 10 μM. The affinity of the enzyme for adenosine triphosphate alone at the site being monitored was more than 100 times less than that for the metal complex and the affinity for magnesium inosine 5′-triphosphate was approximately 25 times less than that for magnesium adenosine 5′-triphosphate. (2) Adenosine 5′-monophosphate, adenosine 3′,5′-cyclic monophosphate, and inorganic phosphate reduced the affinity of the enzyme for magnesium adenosine 5′-triphosphate. (3) Citrate increased the affinity of the enzyme for the metal complex. (4) The dissociation constant for magnesium adenosine 5′-triphosphate was much higher at pH 7.35 than at pH 7.0. (5) Magnesium β,γ-methyleneadenosine 5′-triphosphate was also capable of binding to the inhibitory site. In the presence of fructose 6-phosphate the binding of derivative was decreased. The results of these studies are discussed in relation to the kinetics of phosphofructokinase and in relation to present views of allosteric properties of enzymes.

In recent years the kinetics of phosphofructokinase from a number of sources have been studied extensively (see Lowry and Passonneau, 1966; Hofer and Pette, 1968, and references therein). The complex kinetics have generally been ascribed to be due to inhibition by ATP at a regulatory site distinct from the catalytic site. Although it has been observed that the phosphofructokinase protomer can bind 3 moles of ATP (Kemp and Krebs, 1967), no direct evidence has been presented that describes an interaction of ATP at a site distinct from the catalytic site.

In a recent report from this laboratory it was noted that modification of the single most reactive thiol group of the enzyme results in a dramatic decrease in apparent cooperative substrate interactions (Forest and Kemp, 1968). In the present communication, evidence is presented that the reactivity of this one thiol group with DTNB[1] directly indicates the interaction of MgATP with the inhibitory site on the enzyme. This provides a means of determining those factors that influence the binding of the regulator to the inhibitory site.

Materials and Methods

ATP, ITP, AMP, and 3′,5′-cyclic AMP were obtained from P-L Biochemicals. DTNB, fructose-6-P (Sigma Grade 1), and the sodium salt of β-glycerophosphate were purchased from Sigma Chemical Co. and β,γ-methyleneadenosine triphosphate from Miles Laboratories, Inc.

Phosphofructokinase was prepared from rabbit skeletal muscle by the procedure of Kemp and Forest (1968) and was recrystallized three times. On the day of each experiment, the crystals were collected by centrifugation in the cold and dissolved in a buffer consisting of 25 mM glycylglycine–25 mM sodium glycerophosphate–1 mM EDTA (pH 7.2). The enzyme was dialyzed for 2 hr against this buffer and, to remove ATP, was passed through a 20 × 5 mm column containing a mixture of acid-washed charcoal and powdered cellulose in 1:1 proportion. The protein concentration was measured at 279 mμ by employing the extinction coefficient of 10.2 for a 1% solution (Parmeggiani *et al.*, 1966). All of the above operations were carried out at cold room temperature.

Reactions of phosphofructokinase with DTNB were performed at 20° and the rate followed at 412 mμ with a Gilford Model 2000 spectrophotometer. Titrations were performed in a buffer consisting of 25 mM glycylglycine, 25 mM glycerophosphate, and 1 mM EDTA and were started by the addition of DTNB. The extinction coefficient of 13.6 (Ellman, 1959) at 412 mμ for the thionitrobenzoic acid ion of DTNB was employed. DTNB was prepared fresh daily and its concentration determined by titration with an excess of β-mercaptoethanol.

Paetkau *et al.* (1968) have proposed on the basis of amino acid analysis and sedimentation studies a protomer molecular weight of 93,200 for muscle phosphofructokinase. A similar value for a minimal molecular weight was determined by substrate binding (Kemp and Krebs, 1967) and thiol titration studies (Kemp and Forest, 1968). All titration studies reported here will be expressed as the number titrated per protomer of 93,200 daltons.

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[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

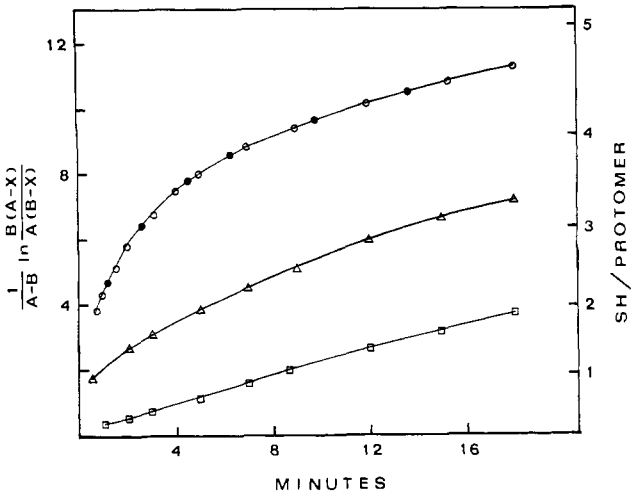


FIGURE 1: Inhibition of thiol reactivity by MgATP. Reactions carried out at 20° with a protein concentration of 0.21 mg/ml and a DTNB concentration of 33 μM in a buffer consisting of 25 mM glycylglycine, 25 mM glycerophosphate, and 1 mM EDTA (pH 7.0). The points shown were calculated from a recording of the reaction rates with the following additions: (○) no additions; (●) plus 6 mM $MgCl_2$; (△) plus 2 mM ATP; (□) plus 6 mM $MgCl_2$ and 2 mM ATP.

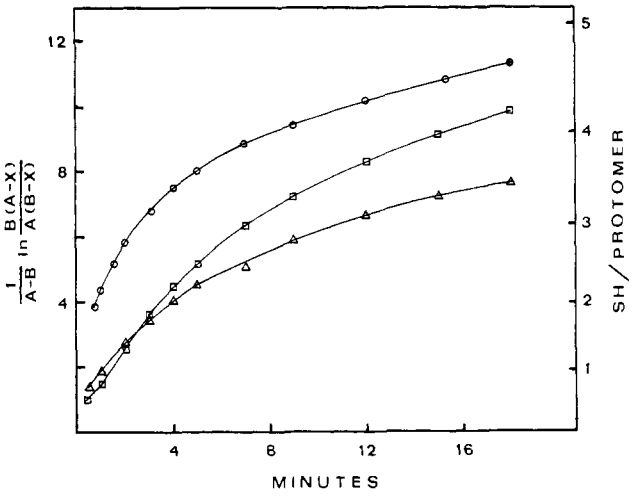


FIGURE 2: Thiol reactivity in the presence of MgITP. Buffer, protein concentration, and DTNB concentration identical with Figure 1. (○) No additions; (△) plus 2 mM ITP; (□) plus 2 mM ITP and 6 mM $MgCl_2$.

Results

Effect of MgATP on Thiol Reactivity. Previous studies of the titration of native and denatured phosphofructokinase indicated that the reaction was first order with respect to each reactant, that is, protein thiol groups and the reagent DTNB (Kemp and Forest, 1968). The data obtained from titration studies may be plotted as a second-order reaction of time *vs.* $1/(A - B) \ln B(A - X)/A(B - X)$, where A = total thiol content of the protein and B = the amount of DTNB added. Figure 1 describes a second-order plot of the initial reaction of DTNB with phosphofructokinase in the presence and absence of ATP and Mg^{2+}. In the absence of any additions or in the presence of Mg^{2+} alone, three to four thiol groups reacted rapidly with DTNB. ATP alone did not influence the most reactive sulfhydryl group (class I)[2] but did reduce the rate of titration of the two less reactive class II thiol groups. These results are in agreement with data published previously (Kemp and Forest, 1968). In the presence of both ATP and Mg^{2+} no highly reactive thiol groups were detected. It thus appeared that the binding of MgATP by the enzyme either directly or indirectly blocked the most reactive sulfhydryl group on the enzyme.

Because modification of the class I thiol group results in a dramatic decrease in the apparent cooperative interactions displayed by the enzyme (Forest and Kemp, 1968), it appeared possible that the reactivity of this sulfhydryl group measures the binding of MgATP to an inhibitory site on the enzyme. In that case, MgITP which is an effective substrate but not an inhibitor (Uyeda and Racker, 1965) should be ineffective in blocking the reaction of DTNB with the class I thiol group. The results given in Figure 2 show that this was the case. MgITP afforded a small degree of protection of the reactive thiol group while MgATP at the same concentration was a very effective inhibitor of the reaction. ITP alone also provided a small degree of protection of the class I thiol group.

It appeared desirable to provide some quantitative measure of the ability of the Mg–nucleoside triphosphate complexes to block the reaction of the class I thiol group with DTNB. To slow down the reaction as much as possible, low concentrations of DTNB had to be used in this type of experiment. The reaction of DTNB with the enzyme was studied over a range of ATP concentrations while holding $[Mg^{2+}]$ constant and in excess. These results are presented in Figure 3. The rate of the reaction in the absence of added ATP was too fast to measure with accuracy and is not shown. From these and similar data, one can plot the data as a second-order reaction and determine the rate constants for the reaction of DTNB with the most reactive thiol group. Second-order plots of these data gave reasonably straight lines up to about two-thirds of completion. A plot of rate constant *vs.* the concentration of ATP is shown in Figure 4. If one can assume that the reaction being measured was that of DTNB with free enzyme, then the dissociation constant for MgATP must be below 10 μM. Without a value for the rate of reaction in the absence of MgATP it is not possible to carry out a direct calculation of the dissociation constant. The calculations are based upon the assumption that all of the ATP exists as the MgATP complex. At 6 mM Mg^{2+}, this is close to being the case and no corrections were employed in the present data presented in Figure 4.

Similar studies were carried out with MgITP and the second-order rate constants obtained are shown by the curve to the right in Figure 4. A much higher concentration of ITP was needed to achieve the same degree of inhibition of rate of the thiol reaction. If, for example, one determines the concentration of nucleoside triphosphate required to slow down the rate of the reaction to 2×10^5 M^{-1} min^{-1}, one can estimate from Figure 4 an ATP concentration of 12 μM and an ITP concentration of 300 μM. In other words, MgATP is 25 times more effective in blocking the titration of the class I thiol group than is MgITP.

[2] Designation of thiol groups as class I and class II taken from the classification of Kemp and Forest (1968).

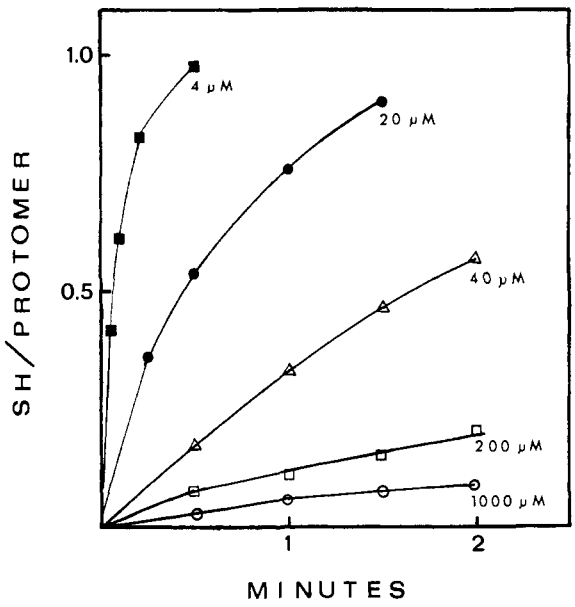


FIGURE 3: Inhibition of thiol reactivity by varying concentrations of MgATP. Reactions followed at 20° with a protein concentration of 0.36 mg/ml and a DTNB concentration of 7.2 μM in a buffer consisting of 25 mM glycylglycine, 25 mM sodium glycerophosphate, 6 mM $MgCl_2$, and 1 mM EDTA (pH 7.0). Concentrations of ATP are indicated in the figure.

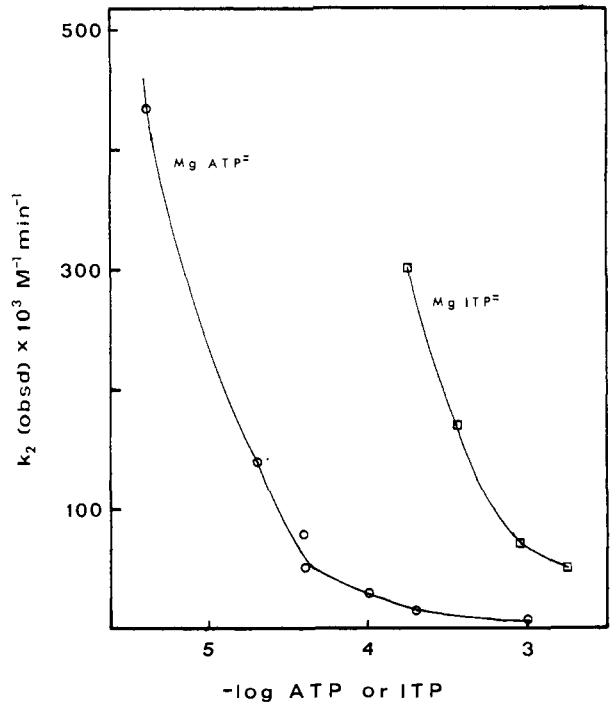


FIGURE 4: Variation of the second-order rate constant with the concentration of ATP and ITP. Reactions carried out under the conditions described in Figure 3 and with concentrations of nucleoside triphosphate indicated in the figure. Data obtained from the reactions were plotted to obtain second-order rate constants.

One can compare the relative affinity of this site on the enzyme for ATP alone in a similar manner. In the presence of 4 mM ATP an apparent second-order rate constant of 7.6×10^4 M^{-1} min^{-1} was obtained. The corresponding concentration of MgATP to achieve this effect is 0.03 mM. Thus MgATP binds more than 100 times more tightly to the enzyme at this site than does ATP alone.

Effect of pH on MgATP Binding. The work of Hofer and Pette (1968) showed that inhibition of phosphofructokinase by ATP is much less apparent at pH 7.3 than at pH 7.1. Thus it might be expected that the affinity of the inhibitory site of the enzyme for MgATP would be decreased at higher pH and this would be reflected in the reactivity of the class I thiol group. This prediction is borne out by the data presented in Figure 5. The upper curve describes the variation in the second-order rate constant determined at pH 7.35 in the presence of varying concentrations of MgATP. The lower curve is that taken from Figure 4 of rate constants obtained at pH 7.0. Interpretation of these data is not as simple as it would first appear. Thiol titrations of native and denatured phosphofructokinase indicated that in the disulfide interchange reaction with DTNB the observed rate constant increased with higher pH due to the fact that the thiolate ion is the reactive species (Kemp and Forest, 1968). The rate constant should vary according to the relationship $k_{2\,obsd} = k_2(K/(K + [H^+]))$, where K equals the dissociation constant for the thiol group. If one is working far below the pK for the sulfhydryl group, then a 2.23-fold decrease in $[H^+]$ (an increase of 0.35 pH unit) will lead to 2.23-fold increase in the observed rate. One can then calculate a theoretical curve from the data obtained at pH 7.0 for the rate constants that would be obtained in presence of MgATP at pH 7.35 if the rate constants were determined only by the decrease in $[H^+]$. This theoretical curve is plotted in Figure 5 as a dashed line and it falls below the actual data obtained for the titration at pH 7.35. Thus the data support the concept that the binding of MgATP at pH 7.35 is much less than that observed at pH 7.0. The difference between the reactivities at pH 7.0 and pH 7.35 is probably greater than that indicated by the actual data for pH 7.35 and the theoretical curve. As indicated in an earlier publication (Kemp and Forest, 1968), the high reactivity of the class I thiol group is probably due to its possessing an unusually low pK, and if the pK is close to 7, then from the relationship between $k_{2\,obsd}$ and pK given above the rate would increase much less than the relative decrease in $[H^+]$.

Other Effectors of Enzyme Activity. Effect on MgATP Binding. If indeed the diminished thiol group reactivity is due to the binding of MgATP to an inhibitory site on the enzyme, then the activators of phosphofructokinase should reverse the MgATP effect and make the class I thiol group available for reaction with DTNB. To examine this possibility, phosphofructokinase was treated with the thiol reagent in the presence of a concentration of MgATP that would partially block the disulfide interchange reaction. The rate constant was then determined from second-order plots of the course of the reaction. In parallel experiments, the various effectors of the enzyme were included with MgATP in the reaction and the rate constants were again determined.

5′-AMP, 3′,5′-cyclic AMP, P_i, and citrate have no observable effect on the reactivity of the class I thiol group when they are present alone with the enzyme (Kemp and Forest, 1968). The results presented in Table I are in agreement with the argument above. Activators of phosphofructokinase, the adenosine monophosphates and P_i, increased

TABLE I: Effect of Activators and Inhibitors on Thiol Reactivity.[a]

Concn of ATP (mM)	Addn	Concn of Addn (mM)	$k_{2\,obsd}$ ($\times 10^3$ M^{-1} min^{-1})
			>500
0.02			130
0.02	Citrate	0.5	36
0.04			78
0.04	Citrate	0.5	21
0.04	5′-AMP	1	290
0.04	3′,5′-AMP	1	329
0.04	HPO_4^{2-}	12	144
0.04	NH_4^+	12	78

[a] Thiol titration contained the following at pH 7.0 and 20°: 0.16 mg/ml of phosphofructokinase, 8.2 μM DTNB, 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA, 6 mM $MgCl_2$, and additions as indicated. The values for $k_{2\,obsd}$ were obtained from second-order kinetic plots of the titration data.

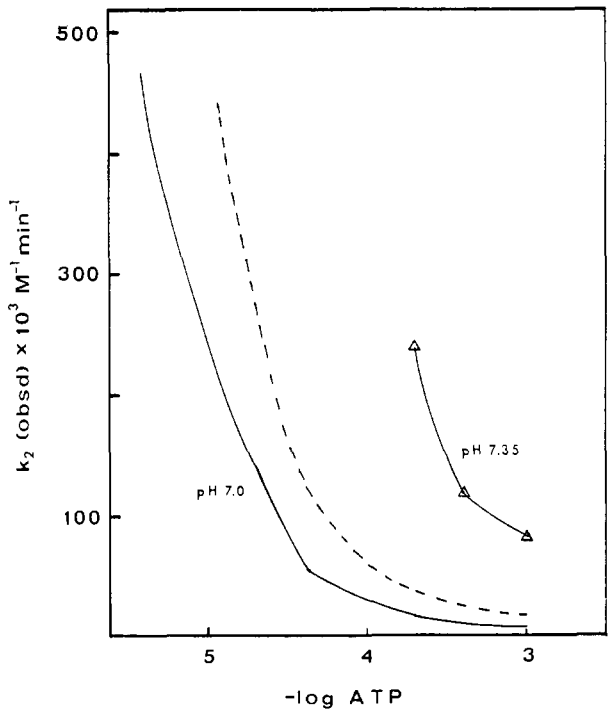


FIGURE 5: Effect of pH on ability of MgATP to block the thiol group. The curve to the left was taken from Figure 4. The dashed line represents the theoretical increase in thiol reactivity if the increase were due only to base catalysis (see Results). The triangles represent second-order rate constants obtained from reactions at pH 7.35 with all other conditions identical with those described in Figure 4.

the reactivity of the class I thiol group indicating a displacement of MgATP from the enzyme. Citrate, by comparison, is an inhibitor of the enzymic reaction and the results indicate that the rate constant for the disulfide interchange reaction is decreased. This suggests that the affinity of the enzyme for MgATP is increased in the presence of citrate ion. The only unexpected result was that NH_4^+, also an activator of the enzyme, did not influence thiol reactivity. It is possible that the protonated amino group of glycylglycine present in the buffer fully saturated the receptor site for the ammonium ion and thus no additional effect by NH_4^+ was observed.

Binding of β,γ-Methylene-ATP and Its Reversal of Fructose-6-P. Kinetic studies of the phosphofructokinase reaction have indicated inhibition by ATP could be overcome by increasing the concentration of fructose-6-P (Hofer and Pette, 1968). This could imply that high concentrations of fructose-6-P displace MgATP from the inhibitory site on the enzyme and therefore increase the reactivity of the class I thiol group with DTNB. Studies of this phenomenon demand that all participants in the reaction, ATP, fructose-6-P, and Mg^{2+}, be present. This, of course, is impossible because these substrates would be immediately converted to their respective products. To overcome this limitation, a derivative of ATP was employed that could not participate in the reaction; namely, β,γ-methylene-ATP. Figure 6 compares the rates of the reaction of DTNB and phosphofructokinase in the absence of added factors, in the presence of MgATP, and in the presence of Mg^{2+} and the methylene derivative of ATP. The results show that the derivative reduced the rate of the reaction with the class I thiol group. Relatively high concentrations of the derivative are required to achieve this effect, and it is thus apparent that the enzyme has less affinity for the derivative than it does for MgATP. Figure 7 describes the results of the DTNB reaction with phosphofructokinase in the presence of the methylene derivative of ATP and Mg^{2+} with and without added fructose-6-P. An increase in the reactivity of the class I thiol group in the presence of fructose-6-P is shown suggesting a displacement of the ATP derivative from the enzyme. In Table II are presented the rate constants for the thiol reaction obtained from similar experiments. As expected, increasing the concentration of the methylene-ATP led to a further reduction in the rate of the reaction. The addition of fructose-6-P partially released the inhibition of the protein thiol–DTNB reaction.

Discussion

Previous studies of the kinetics of phosphofructokinase have been interpreted to indicate the presence of an inhibitory as well as a catalytic site for ATP on the enzyme. Studies of the equilibrium binding of ATP by phosphofructokinase indicated the presence of three sites per protomer that could bind ATP (Kemp and Krebs, 1967). From those experiments, however, one was unable to determine which if any of the three sites represented a catalytic or regulatory site for the nucleoside triphosphate. Furthermore, the equilibrium binding studies suffered from the disadvantage that magnesium ion was not present.

The results of the present communication show that the most reactive thiol group of phosphofructokinase is protected from reaction with DTNB by MgATP. The mechanism whereby the nucleoside triphosphate–metal complex protects this thiol group cannot be determined. Several possibilities exist. The thiol group may be at the MgATP site or

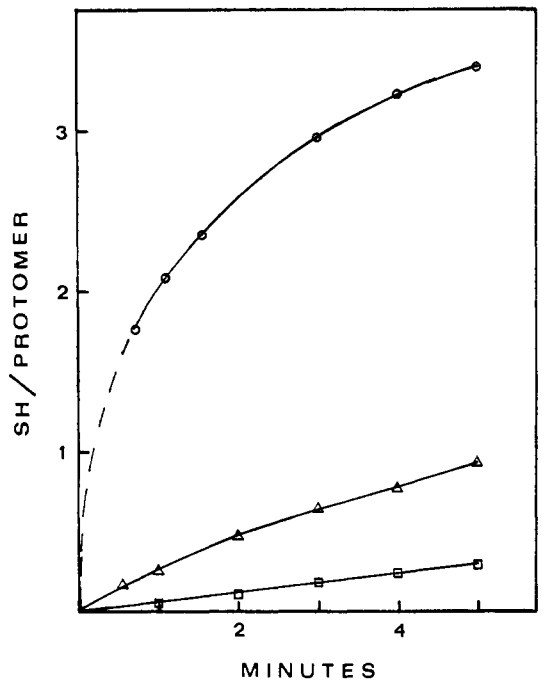


FIGURE 6: Inhibition of thiol reactivity by Mg-β,γ-methylene-ATP. Reactions followed at 20° with a protein concentration of 0.22 mg/ml and a DTNB concentration of 29 μM in a buffer consisting of 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA, and 6 mM $MgCl_2$ (pH 7.0). (○) No additions; (△) 4 mM β,γ-methylene-ATP; (□) 2 mM ATP.

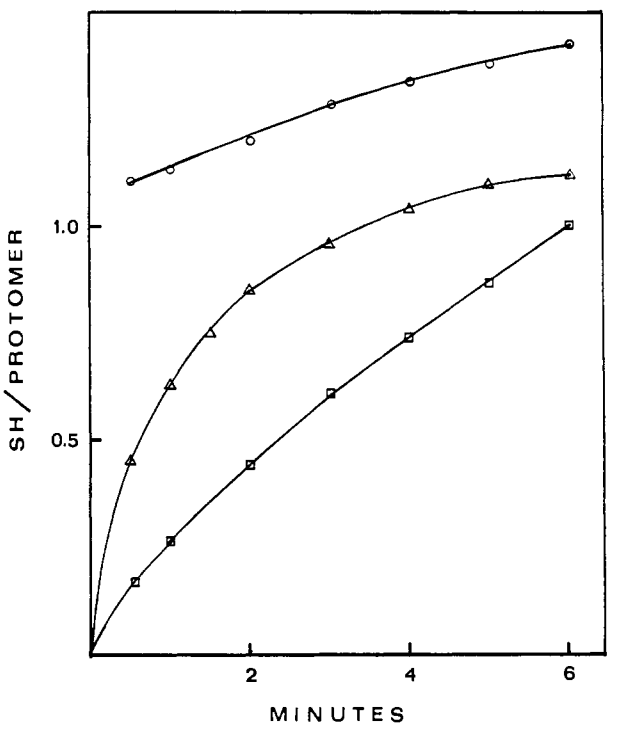
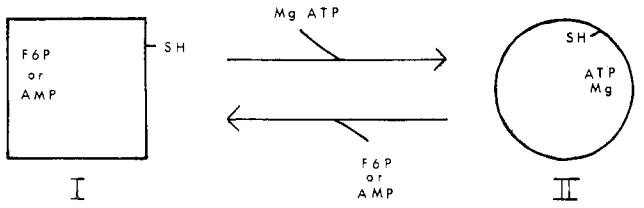


FIGURE 7: Reversal of the effect of β,γ-methylene-ATP by fructose-6-P. Conditions are identical with those described in Figure 6: (○) plus 1 mM fructose-6-P; (□) plus 4 mM β,γ-methylene-ATP; (△) plus 4 mM β,γ-methylene-ATP and 1 mM fructose-6-P.

actually involved in the binding of MgATP. A more likely possibility is that the binding of MgATP results in a conformational change that either buries the thiol group in the protein or disrupts a particular structural relationship that confers the high reactivity to the thiol group. In regard to this latter case, a proton-withdrawing functional group of an amino acid may be located in such a position as to confer greater nucleophilicity to the class I thiol group (Kemp and Forest, 1968). The binding of MgATP may disrupt this particular configuration. In any case the reactivity of the thiol group in question appears to act as a monitor for the binding of MgATP at a given site on the enzyme. It was shown that ATP alone in high concentration shows some tendency to block the disulfide interchange reaction. It is possible that the binding site being monitored is identical with one of the three previously described by equilibrium binding measurements (Kemp and Krebs, 1967), or that it represents still a fourth binding site for ATP on the phosphofructokinase protomer. In the equilibrium binding studies, the site with the weakest affinity for ATP had a dissociation constant of approximately 15 μM. In the experiments described here, it can only be said that the dissociation constant for MgATP is lower than 10 μM. The dissociation constant for ATP alone was shown to be at least 100 times higher than the dissociation constant for MgATP.

That the MgATP binding site being monitored is the inhibitory site on the enzyme is supported by the following observations. (1) MgITP, while serving as an effective phosphoryl donor in the catalytic reaction of phosphofructokinase, is not an inhibitor of the enzyme when present at those concentrations of ATP that are inhibitory (Uyeda and Racker, 1965). As shown in the present studies, MgITP is a much less effective inhibitor of the reaction of the class I thiol group with DTNB than is MgATP. (2) The inhibition by ATP of the catalytic reaction is much less pronounced at more alkaline pH (Hofer and Pette, 1968). As shown here, the ability of MgATP to block the class I thiol group is greatly reduced if the pH of the disulfide interchange reaction is carried out at pH 7.35 instead of pH 7.0. This increase in thiol group reactivity at any given concentration of MgATP cannot be explained simply by the base catalysis of the interchange reaction. (3) 5′-AMP, 3′,5′-AMP, and P_i are all deinhibitors of the phosphofructokinase reaction (Lowry *et al.*, 1966). As shown in the data presented in this communication, these compounds reverse the thiol titration inhibition by MgATP. This can be best interpreted as a direct or indirect displacement of MgATP from the enzyme. (4) Citrate is an inhibitor of the phosphofructokinase reaction (Passonneau and Lowry, 1963). Citrate, while exerting no influence of its own on thiol reactivity (Kemp and Forest, 1968), caused a further reduction in the reactivity of the class I thiol group in the presence of a less than saturating concentration of MgATP. It can be interpreted that at least part of inhibitory action of citrate on phosphofructokinase is due to its ability to increase the affinity of the enzyme for MgATP. This effect cannot be due to a magnesium ion chelating role by citrate because of the fact that the citrate effect was observed in the presence of a more than tenfold excess of magnesium ion. Furthermore, it has been previously reported that citrate increases the affinity of the enzyme for ATP in the absence of divalent metal ion (Kemp and Krebs, 1967). (5) High concentrations of fructose-6-P overcome the inhibition by ATP of the phosphofruc-

SCHEME I



tokinase reaction (Lowry and Passonneau, 1966). In the studies reported here, fructose-6-P increased the rate of reaction of the class I thiol group with DTNB in the presence of inhibitory concentrations of magnesium ion and β,γ-methylene-ATP. (6) Blocking the class I thiol group by treating it with DTNB results in a decrease in the inhibition of the catalytic reaction by ATP (Forest and Kemp, 1968). This is consistent with a proposal that the modification results in the complete or partial inhibition of MgATP binding to the inhibitory site on the enzyme.

The model in Scheme I is suggested to explain the results reported in the present communication. Two conformational states of phosphofructokinase are proposed. For simplification, the forms are shown as protomers while in reality the enzyme exists as a tetramer or octomer (Paetkau and Lardy, 1967). Although the diagrams show grossly different structures, the conformational changes may be quite subtle. In the presence of magnesium ion and ATP conformation species I is converted into form II that has no available thiol groups. This change has been also interpreted to result in the displacement of fructose-6-P or AMP from the enzyme. Conversely, in the presence of high concentrations of fructose-6-P or AMP, MgATP would be displaced from the enzyme converting it into form I. That fructose-6-P and AMP induce a similar conformational change is also suggested by the fact that these two metabolites protect the same two thiol groups (class II) from reaction with DTNB (Kemp and Forest, 1968). Phosphate also converts the enzyme from form II into I, but this compound does not influence thiol reactivity when studied in the absence of MgATP. Fructose-1,6-diP protects still a different more slowly reacting set of two thiol groups (Kemp and Forest, 1968; Younathan *et al.*, 1968). It is thus possible that other structural changes take place but the net result should be those indicated in Scheme I; that is, a form with a reactive thiol group and no bound MgATP and a form with MgATP bound and without a reactive thiol group. Not shown in Scheme I is the MgATP catalytic site; this may be present on both forms I and II. The active, uninhibited species of the enzyme would, by this scheme, be conformational form I; and form II would represent the inhibited state of the enzyme.

There are two ways of interpreting the mechanism by which these conformational changes take place. First, one may consider a natural equilibrium exists among forms I and II. Fructose-6-P would have a higher affinity for form I and would displace the equilibrium toward that form. MgATP would necessarily have a higher affinity for form II and thus displace the equilibrium toward that form when present in high concentrations. This idea, of course, encompasses some of the features of the Monod–Wyman–Changeux model for allosteric transitions (Monod *et al.*, 1965). To explain the phenomenon of cooperativity with fructose-6-P as the substrate it is necessary to propose that the enzyme exists in solution as a polymer of the protomeric forms shown in Scheme I. This is undoubtedly the case as stated earlier. A puzzling result of the equilibrium binding studies of Kemp and Krebs (1967) was the observation that no cooperativity was detected in the binding of fructose-6-P. This observation demands that the equilibrium in the absence of additions greatly favors form I. A second interpretation of the mechanism of the conformation changes is the "induced fit" theory of Koshland (Koshland *et al.*, 1966). This proposes that the conformational change results from the binding of ligand to form I, thus inducing the formation of form II. There is no need for a natural equilibrium among the two forms. Interactions between protomers of the polymeric enzyme would allow for the cooperative kinetics of the enzyme reaction.

From the present state of knowledge of this enzyme we are unable to conclude which model if any of those proposed fits the case of phosphofructokinase. Further kinetic studies of the native enzyme and the enzyme with one thiol group modified are presently under way in this laboratory. Kinetic analysis and studies of equilibrium binding of metabolites by native and modified enzyme are necessary for further assessment of the complex interactions manifested by phosphofructokinase.

TABLE II: Effect of Methylene-ATP and Fructose-6-P on Thiol Reactivity.[a]

Concn of Methylene-ATP (mM)	Concn of Fructose-6-P (mM)	$k_{2\,obsd}$ ($\times 10^3$ M^{-1} min^{-1})
		>500
	1.0	>500
1.0		160
2.0		41
2.0	1.0	98
2.0	2.0	115

[a] Thiol titration contained the following at pH 7.0 and 20°: 0.15 mg/ml of phosphofructokinase, 8.2 μM DTNB, 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA, 6 mM $MgCl_2$, and additions as indicated. The values for $k_{2\,obsd}$ were obtained from second-order kinetic plots of the titration data.

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Studies on the Subunit Structure of Ovine Brain Glutamine Synthetase*

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ABSTRACT: Ovine brain glutamine synthetase is isolated in an octameric form exhibiting a sedimentation coefficient, $s_{20,w}$, of 15.0 S and possessing a molecular weight of 525,000 ± 25,000. In the presence of 2 M urea at 25°, 1 M urea at 35°, 20% dimethylformamide, 20% dimethyl sulfoxide, or at values of pH greater than 8.1 in low ionic strength media, the enzyme dissociates reversibly to a form, probably a tetramer, exhibiting a sedimentation coefficient of 8.6 S. Such dissociation is prevented by adenosine triphosphate and Mg^{2+}. Association of the 8.6S species to re-form the octamer is promoted by removal of the dissociating agent or by addition of adenosine triphosphate and Mg^{2+}. Reversible dissociation in 1 M urea is temperature dependent. Studies at various values of pH suggest that a functional group of the enzyme with a pK of about 8.1 is involved in the reversible dissociation phenomenon. The data suggest that dissociation of the octamer to the 8.6S form does not involve extensive alteration of the tertiary structure of the enzyme, and that certain reagents are quite specific for disrupting the linkage between tetramers. The findings are consistent with a model of the octameric enzyme in which two heterologously linked tetramers are held together by weaker isologous bonds. Treatment of the octameric enzyme with maleic anhydride, acetic anhydride, tetranitromethane, diazonium-1H-tetrazole, or adjustment of the pH to values greater than 9.8 yields a catalytically inactive monomeric species [$s_{20,w}$ = 2.8 S; mol wt 65,000].

Glutamine synthetase has been isolated from sheep brain as an apparently homogeneous preparation exhibiting a molecular weight of about 525,000 (Pamiljans *et al.*, 1962; Ronzio *et al.*, 1969a; Haschemeyer, 1965). The enzyme is composed of 8 apparently identical subunits with a cubelike morphological appearance (Haschemeyer, 1965, 1966). Electron microscope studies and considerations of symmetry have led to the formulation of a model for the enzyme which possesses D_4 symmetry (Haschemeyer, 1968). Inhibition of glutamine synthetase by methionine sulfoximine is associated with the tight binding to the enzyme of 8 moles of methionine sulfoximine phosphate together with an equivalent quantity of ADP (Ronzio and Meister, 1968; Ronzio *et al.*, 1969b). In addition, the enzyme can bind with high affinity 8 moles of ATP and with less affinity a total of 16 moles of ATP (Wellner and Meister, 1966; Ronzio *et al.*, 1969b). The available data indicate that enzymatic activity resides in the native octameric enzyme. In the investigations reported here, the subunit structure of the enzyme has been examined by application of a number of procedures which produce dissociation of the octamer. Evidence has been obtained that the enzyme can be reversibly dissociated to yield a form exhibiting a sedimentation coefficient of 8.6 S and irreversibly to a monomeric component ($s_{20,w}$ = 2.8 S). The findings support the model proposed by Haschemeyer (1968).

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Experimental Section

Materials

Glutamine synthetase was isolated from sheep brain as described by Ronzio *et al.* (1969a); the preparations used here exhibited specific activities in the range 170–200 units/mg, and were stored in the dry lyophilized state at −20°. Under these conditions, some preparations of the enzyme developed small amounts of catalytically inactive very high molecular weight material; when present, such aggregates were removed from solutions of the enzyme by high-speed centrifugation.

Ultrapure urea was obtained from Mann Chemical Co. Adenosine triphosphate, adenosine diphosphate, and *N*-ethylmaleimide were obtained from Sigma Chemical Co. Tetranitromethane, 5-amino-1H-tetrazole, maleic anhydride, and