Allosteric Properties of Muscle Phosphofructokinase. III. Thiol Reactivity as an Indicator of Conformational State*

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ABSTRACT: In the absence of added effectors, a single thiol group of rabbit muscle phosphofructokinase displays unusual reactivity with fluorodinitrobenzene. The pH dependence of the arylation reaction suggests that one or more dissociable groups with pK(s) near pH 7.0 are responsible for the high reactivity of the thiol group. The reactivity of this thiol group acts as an indicator of the conformational state of the enzyme. Activators of the enzyme such as adenosine 3′,5′-cyclic monophosphate, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), fructose 6-phosphate, and fructose 1,6-diphosphate cause an increase in the reactivity of the thiol group when present at low concentrations, whereas magnesium adenosine 5′-triphosphate (MgATP) inhibits thiol reactivity. These changes in thiol reactivity are interpreted in terms of an interconversion between active and inactive conformers of the enzyme. The binding of magnesium complexes of other nucleoside triphosphates leads to decreases in thiol reactivity, but MgATP is the most effective in driving the enzyme into the inactive conformer. Although low concentrations of AMP and ADP increase thiol reactivity, high concentrations decrease the reactivity; an indication that these ligands are capable of interacting at both activating and inhibiting sites. Magnesium ion increases the affinity of the enzyme for ATP at the site that results in inhibition. The affinity of the inhibitory site decreases in the order ATP > ADP > AMP. Phosphocreatine, citrate, and phosphoenolpyruvate promote the conversion to the inhibited conformation at high concentrations and are effective at low concentrations in the presence of subsaturating levels of MgATP. The results are discussed in relation to the present views of allosteric properties of enzymes. A theoretical calculation is provided that demonstrates that a single ligand capable of interacting with both activating and inhibiting sites on an enzyme can displace the equilibrium toward either an active or an inactive conformation depending on the concentration of the ligand.

The thiol groups of muscle phosphofructokinase have been the subject of a number of investigations in recent years (Younathan *et al.*, 1968; Kemp and Forest, 1968; Kemp, 1969a,b; Chapman *et al.*, 1969; Hofer, 1970). On the basis of their reactivity with DTNB[1] the sulfhydryl groups of the enzyme have been classified into five groups (Kemp and Forest, 1968). The first (class I) consists of one cysteinyl residue that is exceedingly reactive and is protected by MgATP. The second class of thiol groups (class II) consists of two cysteinyl groups that are somewhat less reactive and are protected from reaction with DTNB by fructose-6-P and adenine nucleotides. Class III has one thiol group and class IV comprises five whose reactivities are highly dependent upon pH and two of which are protected from reaction with DTNB by fructose-1,6-P_2. The remaining thiol groups react extremely slowly in the native enzyme. This classification scheme has been confirmed by the work of Hofer (1970) who studied the thiol reactivity with *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, tetranitromethane, and FDNB.

The class I thiol group was of particular interest because of the observation that the protection by MgATP is due to binding at the ATP inhibitor site and not at the catalytic site (Kemp, 1969a). It appeared likely that a conformational change was being indicated by the changes in thiol reactivity, but it was impossible to study this phenomenon in detail because the DTNB reaction in the absence of MgATP was too fast to measure. For this reason, FDNB was chosen as indicator of thiol reactivity since this reagent reacts at a rate slow enough to permit a reasonably accurate quantitative determination of increases as well as decreases in reactivity. Such an approach permits an examination of the specificity of the adenine nucleotide inhibitor site. Furthermore, one can consider whether activators of the enzymic reaction such as fructose-6-P, fructose-1,6-P_2, AMP, ADP, and 3′,5′-AMP bind to one form of the enzyme while inhibitors such as MgATP, citrate, creatine phosphate, and phosphoenolpyruvate bind to another. The technique thus provides a means of investigating the effects of substrates and allosteric effectors on the conformation of phosphofructokinase and it provides a basis for discussing the properties of the enzyme in the context of models that have been proposed to account for the kinetic behavior of allosteric enzymes (Monod *et al.*, 1965; Koshland *et al.*, 1966).

Materials and Methods

All nucleotides used in this work were obtained from P-L Biochemicals. Fructose-6-P, fructose-1,6-P_2, phosphocreatine, phosphoenolpyruvate, and the sodium salt of β-glycerophosphate were purchased from Sigma Chemical Co. FDNB and glycylglycine were products of Calbiochem.

α-Glycerophosphate dehydrogenase and aldolase were prepared from rabbit skeletal muscle as described previously (Kemp, 1969b). The specific activities of the dehydrogenase

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDNB, fluorodinitrobenzene; 3′,5′-cAMP, adenosine 3,′5′-cyclic monophosphate; 2′,3′-cAMP, adenosine 2′,3′-cyclic monophosphate.

and aldolase at pH 7.5 and 25° were 150 and 12, respectively. Triosephosphate isomerase was purchased from Calbiochem. Immediately preceding their use in assays, these three enzymes were dialyzed to remove ammonium sulfate. Phosphofructokinase was prepared from rabbit skeletal muscle as described by Kemp and Forest (1968) and was recrystallized three times. On the day of each experiment, the crystals were collected by centrifugation and were dissolved. Ammonium sulfate and ATP were removed as described previously (Kemp, 1969b), and the protein concentration was determined at 279 mμ as described by Parmeggiani *et al.* (1966).

Radioactive FDNB labeled with tritium (1.55 Ci/mmole) was obtained from New England Nuclear. This material was diluted with unlabeled FDNB to a specific activity of 15.5 mCi/mmole. The specific radioactivity was checked by the synthesis, purification, and quantitation of DNP-glycine as described by Pontremoli *et al.* (1965). The specific activity determined by this method was within 4% of that calculated from a dilution of the commercial sample based on assumptions that the commercial tritiated FDNB was radiochemically pure and the unlabeled FDNB was chemically pure.

Arylation was carried out in a shaking water bath at 20° with 0.15 mg of enzyme in 1 ml of 25 mM glycylglycine–25 mM glycerolphosphate–1 mM EDTA at pH 7.0, and with the additions indicated in the Results. The reaction was started by the addition of 24 nmoles of FDNB in 20 μl of ethanol. Arylation was stopped by the addition of 0.1 ml of bovine serum albumin (20 mg/ml) immediately followed by the addition of 1 ml of cold 10% trichloroacetic acid. The precipitate was collected and washed eight times with ether. The washed protein was dissolved in 1 ml of 20% formic acid. Scintillation fluid was added and the sample was counted in a scintillation spectrometer employing a quench correction determined by the use of an external standard. A small amount of apparently nonspecifically bound radioactivity could be washed out of the pellet only by exhaustive either washing. To correct for this residual radioactivity after the washing procedure described above, the following control was employed. To one protein sample in each experiment, serum albumin and trichloroacetic acid were added prior to the addition of tritiated FDNB. This sample was washed and counted as described above. The radioactivity that carried through this procedure was 0.09 ± 0.01 mole/mole of enzyme protomer for more than 30 separate determinations. All data presented in the results are corrected for this nonspecific binding. The phosphofructokinase protomer weight of 93,000 was used for all calculations (Paetkau *et al.*, 1968).

To prove that FDNB labels only cysteine the enzyme was subjected to total acid hydrolysis after a 10-min incubation with a 15-fold excess of [^{14}C]FDNB at pH 7.0. The dinitrophenylated amino acids were extracted as described by Fraenkel-Conrat *et al.* (1955). The hydrolysate was made 1 N in HCl, extracted several times with ether, and evaporated to dryness and the residue was then dissolved in water. This fraction was subjected to paper chromatography in butanol–acetic acid–water (4:1:5, v/v). At least 95% of the radioactivity was localized in a spot that moved identically with a marker *S*-2,4-dinitrophenylcysteine.

Enzyme assays were performed with a Gilford Model 2000 spectrophotometer at 26° and the indicated pH in 3 ml of a medium containing 25 mM glyclyglycine, 25 mM glycerophosphate, 1 mM EDTA, 6 mM $MgCl_2$, 0.2 mM DPNH, 0.6 unit of aldolase, 0.3 unit or triosephosphate isomerase, 0.3 unit of α-glycerol phosphate dehydrogenase, and other additions as indicated. Reactions were started by the addition of fructose-6-P.

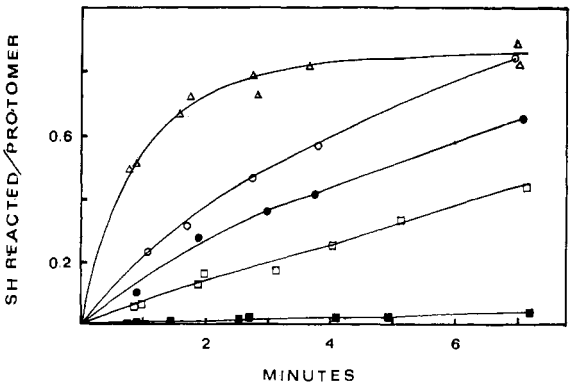

FIGURE 1: Effect of substrates on the dinitrophenylation of the class I thiol group. Reaction conditions and the procedure for counting samples were as described in Methods. Arylation was carried out with (○) no additions, (●) 6 mM $MgCl_2$, (□) 0.5 mM ATP, (■) 0.5 mM ATP and 6 mM $MgCl_2$, (△) 1 mM fructose-6-P.

Results and Discussion

Reactivity of the Class I Thiol Group in the Presence of Substrates. The class I thiol group of muscle phosphofructokinase is readily arylated at pH 7.0 with FDNB as shown in Figure 1. Under the conditions of the reaction the most reactive thiol group was fully titrated with FDNB in 10 min. Arylation of the class II thiol groups proceeded relatively slowly and the remaining thiol groups did not react at a measurable rate. In agreement with data obtained with DTNB titrations (Kemp, 1969a), in the presence of both ATP and Mg^{2+} no reactive sulfhydryl was detected. Mg^{2+} alone had a slight inhibitory effect and no further inhibition could be achieved with higher concentrations of this ion. ATP alone also provided some protection of the thiol group but as will be shown subsequently relatively high concentrations of the nucleotide alone are required to give complete blockade. In contrast, very low concentrations of MgATP are effective. In all cases the data fit curves for pseudo-first-order rates when the concentration of FDNB was in great excess.

In contrast to the influence of MgATP, fructose-6-P, which is considered to be both a substrate and an activator of the phosphofructokinase reaction, increased the rate of dinitrophenylation. These results suggest that an equilibrium between two forms of the enzyme exists in the absence of added metabolites. This equilibrium is displaced by the effectors of the enzyme: MgATP has a greater affinity for the inhibited state of the enzyme and so displaces the equilibrium toward that form, whereas fructose-6-P displaces the equilibrium toward the active conformation of the enzyme. To study the dependence of the rate of arylation on the concentration of fructose-6-P, the reaction was measured in the presence of varying concentrations of the effector. This was done by obtaining the initial rate of FDNB incorporation; that is, the amount of isotope incorporated after a 1-min incubation in the presence of different concentration of the effector (Figure 2). Such data for extent of incorporation at early times should be roughly proportional to true rates. The concentration of fructose-6-P that gives a half-maximum increase in thiol reactivity (approximately 50 μM) is in reasonable agreement with the dissociation constant of fructose-6-P obtained from equilibrium binding studies (Kemp and Krebs, 1967). The decrease

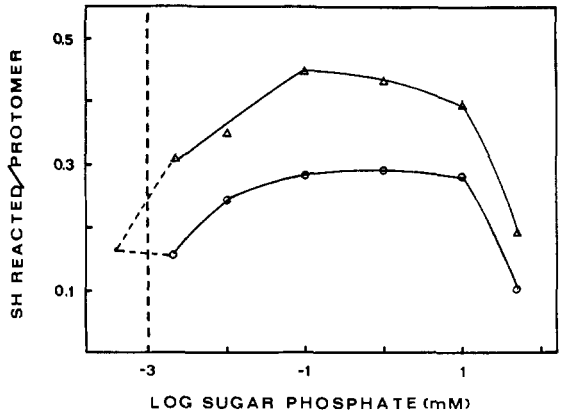

FIGURE 2: Influence of varying concentrations of fructose-6-P and fructose-1,6-P_2 on thiol reactivity. Reactions were carried out as described in Methods. The point on the left of the figure represents the SH reacted/protomer in 1 min, with no additions, (△) fructose-6-P, (○) fructose-1,6-P_2. The points in the figure represent the mean of four determinations. The standard error obtained in each case was less than ±0.03.

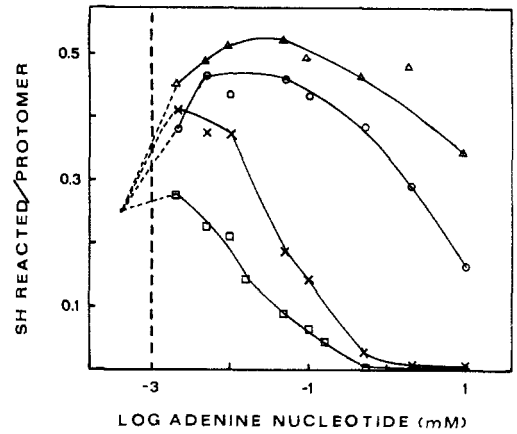

FIGURE 3: Effect of varying concentrations of different adenine nucleotides on the arylation of phosphofructokinase. The point on the left of the figure represents the extent of arylation in 1 min with no additions. Other symbols are (□) ATP, (×) ADP, (○) AMP, and (△) 3′,5′-cAMP. Each point is the mean of six determinations. The standard deviation of the arithmetic mean was not greater than ±0.03.

in thiol reactivity at very high concentrations of fructose-6-P suggests that the substrate may be an inhibitor of the reaction catalyzed by phosphofructokinase when present at high levels. A direct observation of this inhibition has been made in phosphofructokinase assays with fructose-6-P at concentrations higher than 1 mM (Kemp, 1969b). The substrate inhibition is most likely the result of binding at some secondary site at higher concentrations of substrate. Fructose-1,6-P_2 is not only a product of the reaction catalyzed by phosphofructokinase but it is also a potent activator. This effector also increased the thiol group reactivity (Figure 2), but an important difference was noted between the effects of fructose-6-P and fructose-1,6-P_2. Although the concentration required to give a half-maximal activation of the arylation was similar to that observed with fructose 6-phosphate, fructose-1,6-P_2 did not increase reactivity to the same extent. Under the conditions of the arylation experiment described in Figure 2, fructose-1,6-P_2 increased the initial rate of arylation by a factor of two, whereas fructose-6-phosphate more than tripled the initial rate of uptake. The sites of interaction are undoubtedly different; this is also indicated by the observation that the effects of 1 mM fructose-6-phosphate and 1 mM fructose-1,6-P_2 on the arylation rate were synergistic. In the presence of both sugar phosphates, more than 0.6 thiol group/mole of protomer was arylated in 1 min. That a somewhat different conformational change as well as different sites are involved was previously suggested by the actions of these two effectors on more slowly reacting thiol groups (Younathan *et al.*, 1968; Kemp and Forest, 1968). Fructose-6-P blocks the class II thiol groups while fructose-1,6-P_2 blocks two of the thiol groups in class IV. Although it might appear that these two effectors displace an equilibrium to a more active conformer that is characterized by increased thiol reactivity, it is likely that there is more than one active conformer on the basis of the differential effects of the effectors on both the class I and other less reactive thiol groups.

Influence of Adenine Nucleotides on Thiol Reactivity. Since fructose-6-P and fructose-1,6-P_2 proved to be activators of the dinitrophenylation reaction, it was of interest to know if other positive modifiers of the reaction catalyzed by phosphofructokinase would give a similar effect. When arylation was carried out in the presence of AMP and ADP, it was found that low concentrations of these nucleotides increased the rate of arylation; but, surprisingly, higher concentrations had an inhibitory effect. This led to a detailed study of the influence of varying concentrations of different adenine nucleotides on the FDNB reaction (Figure 3). Low concentrations of ADP, AMP, and cAMP increased the arylation rate and high concentrations of ATP, ADP, and AMP inhibited arylation. The data that indicate a slight increase in arylation rate caused by the lowest concentration of ATP are not statistically significant. These results suggest that two types of nucleotide binding sites are involved. One site with high affinity for the nucleotides increases the arylation rate and a second site with lower affinity inhibits arylation. Affinity for the activating site decreases in the order cyclic AMP $\geq$ ADP $>$ AMP $\gg$ ATP. The site appears to be identical with the binding site specific for adenine nucleotides, indicated by gel filtration binding studies, which bound ADP, 3′,5′-cAMP, AMP, and ATP with dissociation constants of 0.5, 0.6, 1.8, and 10 μM (Kemp and Krebs, 1967). This site will be referred to as the activator adenine nucleotide site because binding to this site increases the affinity of the enzyme for fructose-6-P (Kemp and Krebs, 1967). The low-affinity site will be referred to as the inhibitor site. Binding of low concentrations of adenine nucleotides may cause activation by the displacement of the conformational equilibrium toward the form with the reactive thiol group whereas at higher concentrations the equilibrium is displaced toward the inactive form of the enzyme.

An identical series of experiments was repeated with the addition of 6 mM $MgCl_2$. The control in Figure 4 shows that in 3 min, in the presence of 6 mM $MgCl_2$, 0.4 mole/mole of the class I thiol was titrated. Mg^{2+} seems to decrease the affinity of the adenine nucleotides for the site that increases thiol reactivity and to increase the binding of ATP to the inhibitor site. It was shown previously by gel filtration binding that Mg^{2+} decreased the affinity of adenine nucleotides for the activator site (Kemp and Krebs, 1967). MgATP is much more effective in blocking the reaction of the thiol group than is ATP and no binding of MgATP to the activator site was indicated from the rates of arylation.

To examine further the specificity of adenine nucleotide

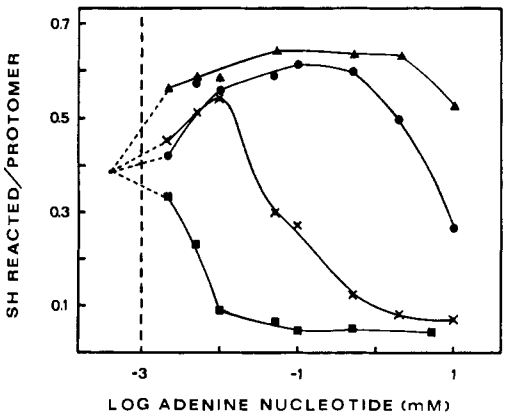

FIGURE 4: Thiol reactivity in the presence of varying concentrations of magnesium–adenine nucleotide complexes. $MgCl_2$ at 6 mM was present in each experiment. The point on the left of the figure represents the extent of arylation in 3 min with no additions. Other symbols are (■) ATP, (×) ADP, (●) AMP, and (▲) 3′,5′-cAMP. Each point is the mean of four determinations. The standard deviation was not greater than ±0.03.

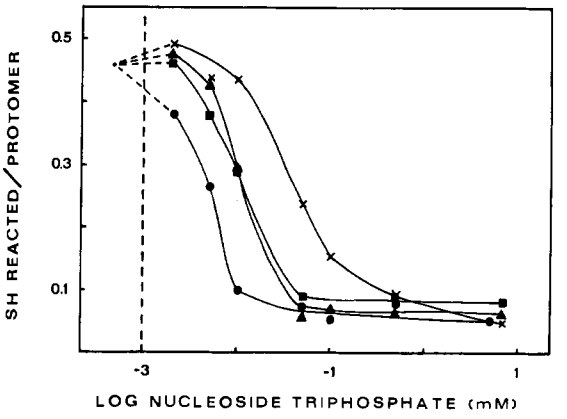

FIGURE 5: Inhibition of thiol reactivity by different nucleotide triphosphates. The point on the left of the figure represents the extent of arylation in 3 min with 6 mM $MgCl_2$ and no further additions. Other symbols are (●) ATP, (■) CTP, (▲) UTP, (×) ITP. The results represent the mean of five experiments; av std dev = ±0.04.

binding to the activator site and to the inhibitor site, the effects on thiol reactivity of 2′-AMP, 3′-AMP, and 2′,3′-cAMP were compared to those of 5′-AMP and 3′,5′-cAMP. To facilitate a qualitative survey, only two concentrations of nucleotides were tested based upon the results obtained with AMP and ADP shown in Figure 4: 0.1 mM at which concentration compounds with high affinity for the activator site should be detected, and at 10 mM, where the lower affinity inhibitor site should be detected. The results suggested that, in addition to 5′-AMP, 3′-AMP and 2′-AMP bind to both activator and inhibitor sites. Both high and low concentrations of the cyclic nucleotides increased thiol reactivity, but high concentrations of the cyclic nucleotides did not increase thiol reactivity to the extent that low concentrations did, suggesting that a very weak binding may be occurring at the inhibitor site in addition to the tight binding at the activator site. 2-′AMP, 3′-AMP, and 2′,3′-AMP interacted at the activator site, but the increases in thiol reactivity were much less than that seen with 5′-AMP and 3′,5′-cAMP. This is most likely due to a lower affinity of the enzymes for the 2′- and 3′-nucleotides rather than a case of incomplete conversion to the active conformer when the site is occupied with these nucleotides. Gel filtration binding studies have shown that the activator site is highly specific for adenine compounds and has a relatively low affinity for 3′(2′)-AMP (Kemp and Krebs, 1967).

The specificity of the inhibitory site for nucleoside triphosphates was also examined (Figure 5). MgATP was the most effective blocker of the arylation reaction and MgITP was the least effective. The effects of the nucleotides on thiol reactivity are directly comparable to the action of the various nucleoside triphosphate as inhibitors of the reaction catalyzed by phosphofructokinase (Uyeda and Racker, 1965). Enzymic assays carried out at pH 7.0 and 0.5 mM fructose-6-P showed that ATP was the strongest inhibitor and ITP was the weakest, even at concentrations as high as 4 mM. A small discrepancy was observed in the relative effects of UTP and CTP. As thiol blockers their effects were very similar while in kinetics of the phosphorylation of fructose-6-P, UTP was effective at concentrations about 2.5 times lower than that required for CTP. It is likely that the technique of measuring conformational changes based on thiol reactivity is not sensitive enough to detect such small differences.

From the data presented on the effects of various adenine nucleotides certain conclusions can be drawn regarding the adenine nucleotide activator site and the ATP inhibitor site. For binding to the latter site of phosphofructokinase, the following properties were noted. (a) Adenosine phosphate derivatives are bound more tightly than other purine or pyrimidine nucleotides. The specificity for adenine derivatives is not as striking as was noted previously for the activator site (Kemp and Krebs, 1967). (b) The binding of nucleotides to this site is proportional to the magnitude of charge they carry. Singly charged cyclic nucleotides are bound weakly or not at all. (c) There is not a strong specificity for the position of the charge on the ribose. In experiments not shown here, the inhibitory action of 2-dATP paralleled that of ATP, indicating the lack of need for a hydroxyl group at the 2′ position.

Mg^{2+} increases the inhibitory effect of ATP but apparently has little or no effect on ADP, AMP, and 3′,5′-cAMP binding. The formation of a Mg^{2+} complex of ATP apparently arranges the phosphate arm of the nucleotide in some favorable conformation for binding at the inhibitor site. This result is in contrast to the binding properties of the activator site where Mg^{2+} decreases the affinity for 3′,5′-cAMP, AMP, and ADP (Kemp and Krebs, 1967).

Thiolysis. One possible explanation for the blockade of thiol arylation by MgATP is that the thiol group is directly involved in the binding of the nucleotide–metal complex. Such a possibility has been suggested by Hofer (1970). If this is correct, then the arylated protein should be incapable of binding MgATP at the inhibitory site. To test this possibility, the rate of thiolysis of the arylated protein was studied in the presence and absence of MgATP. Studies of creatine kinase (Mahowald and Agodoa, 1969) and glyceraldehyde-3-phosphate dehydrogenase (Shaltiel and Soria, 1969) have indicated that the dinitrophenol groups can be removed from cysteinyl residues by incubation with excess 2-mercaptoethanol. Similarly, 2-mercaptoethanol removes the aryl group from phosphofructokinase and an even faster rate of thiolysis can be achieved with dithiothreitol. Results of thiolysis with dithiothreitol are shown in Figure 6. The experiment was carried out by first incubating the enzyme with FDNB, as described in Methods, then passing it through a Sephadex G-25 column

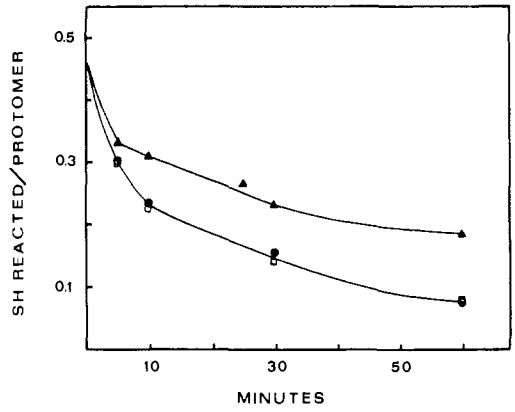

FIGURE 6: Thiolysis of DNP-phosphofructokinase. The abscissa represents time after the addition of 0.2 M dithiothreitol. The data was not corrected for the nonspecific binding of 0.09 ± 0.01 mole/mole enzyme as was described in Methods. Thiolysis was carried out at pH 7.0 with 6 mM $MgCl_2$ (●), with 1 mM 3′,5′-cAMP (□), and with 1 mM ATP and 6 mM $MgCl_2$ (▲). For conditions used in the preparation of DNP-phosphofructokinase, see text.

TABLE I [a]

Additions	SH Reacted/Protomer
None	0.564 ± 0.02
6 mM $MgCl_2$	0.434 ± 0.03
0.5 mM ADP	0.255 ± 0.01
0.5 mM ADP + 0.1 mM cAMP	0.256 ± 0.02
0.02 mM ATP + 6 mM $MgCl_2$	0.224 ± 0.02
0.02 mM ATP + 6 mM $MgCl_2$ + 0.1 mM cAMP	0.487 ± 0.03

[a] Determination of the extent of the reversibility of the inhibition of thiol reactivity, produced by ADP and MgATP. The extent of arylation in 2 min was determined under the conditions described under Methods. The mean of 4 determinations and the standard deviation of the mean has been given for each experiment.

to remove excess FDNB. The rate of thiolysis was determined by following the decrease in protein bound DNP resulting from the addition of 0.2 M dithiothreitol. In the presence of 1 mM MgATP, the rate of thiolysis was retarded. Such an inhibition was not observed in the presence of 1 mM 3′,5′-cAMP indicating that the decrease in the rate of thiolysis was not due to the binding of ATP by the adenine nucleotide activator site. Thus, the cysteinyl residue in question is undoubtedly not involved directly in the binding of ATP but its accessibility is reduced by the conformational change that results from MgATP binding. That the thiol group is not directly involved in binding was also suggested by the earlier kinetic studies of thionitrobenzoate modified phosphofructokinase (Kemp, 1969b).

Implications of Two Sites. If the concentration of nucleotide is sufficiently high, the enzyme assumes the inhibited conformation. In relating this to the kinetic pattern of phosphofructokinase, how does AMP activate a molecule that has been forced into the inhibited conformation by MgATP? Does MgATP occupy both activator and inhibitor sites in this situation? To illustrate this problem the experiment described in Table I was performed. Although these data are presented as single time points, in several experiments samples were taken at various times and the results suggested a simple pseudo-first-order reaction rate. A concentration of ADP (0.5 mM) sufficient to force the enzyme into the inhibited conformation was employed (see Figure 4) and a slow arylation rate was observed. The addition of a low concentration of cAMP (0.1 mM) that alone gives a potent increase in arylation rate did not change the rate of arylation in the presence of high ADP. This suggests that ADP was occupying both sites and, being competitive with cAMP, did not permit displacement of the equilibrium to the active conformer. A different result is observed in the presence of MgATP which apparently has little or no affinity for the activator site. cAMP was capable of increasing the arylation rate when the enzyme was displaced to the inhibited conformer by MgATP (Table I). Thus, when the inhibitor has less affinity for the active conformer the inhibition is reversible.

The results have been presented from the viewpoint of an equilibrium between two states; that is, in the language of the Monod–Wyman–Changeux model for allosteric transitions (Monod *et al.*, 1965). It was of interest to demonstrate with the equations of that model a theoretical situation where a single substance binding to both active and inactive conformers at different sites could both increase and decrease the fraction of molecules in the active state. The principle feature of such a calculation is that because inhibitor and substrate or activator are identical, the relative concentration of inhibitor can be defined as some fraction of the relative concentration of activator. The fractional value is proportional to the ratio of affinity of the active state for activator to the affinity of the inhibited state for inhibitor.[2] Employing a variety of constants a number of theoretical curves can be generated that resemble the experimental data of Figures 3 and 4. Some examples are given in Figure 7. This figure shows the effect of varying the number of protomers that make up the active molecule and the effect of varying the relative affinity of the two states for the ligand. In the latter case, as the differences in affinities of the two states for the ligand becomes greater, the concentration of ligand necessary to decrease the fraction of molecules in the active state is greater.

Other Inhibitors of Enzyme Activity. Phosphofructokinase is inhibited by phosphoenolpyruvate, phosphocreatine, and citrate and all three are capable of partially blocking the reactive thiol group at high concentrations (Figure 8). In the presence of a very low concentration of MgATP (5 μM) that by itself only partially blocks the thiol group, these three compounds are effective at much lower concentrations (Figure 8). Apparently the inhibitors bind to the inactive conformer and act synergistically with MgATP in displacing the equilibrium to the inactive form. Citrate is the most effective of the three in acting with MgATP to displace the equilibrium. This is

[2] The function of state equation for an allosteric protein in the presence of both an activator and inhibitor is according to the Monod–Wyman–Changeux Model (Monod *et al.*, 1965): $\bar{R} = [(1+\alpha)^n / L(1-\beta)^n(1+C\alpha)^n + (1+\alpha^n)]$, where L = the equilibrium constant for the $R \rightleftharpoons T$ transition, R represents the active conformer, $\bar{R}$ = the fraction of molecules in the active conformational state, n = the number of protomers in the oligomeric protein, $(K_R/K_T) = C$ = the ratio of dissociation constants of the ligand for the activator site, $\alpha = (F/K_R)$ = ligand concentration relative to its dissociation constant, $\beta = (i/K_i)$ = concentration of inhibitor relative to its dissociation constant. For the case of a single ligand being both the activator and inhibitor, $\beta = (F/K_i) = (K_R/K_i)\alpha$.

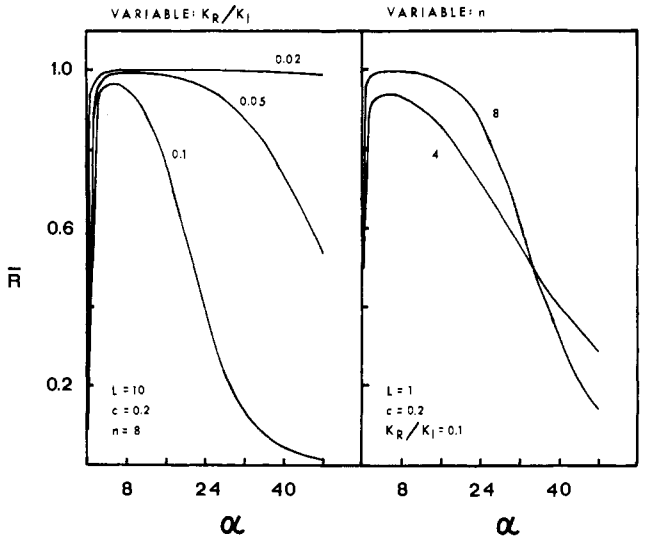

FIGURE 7: Theoretical curves for the fraction of molecules in the active conformer for the Monod–Wyman–Changeux model assuming a single effector binding to both activator and inhibitor sites. The values for the different functions are given on the figure and the definitions of these functions are given in footnote 2.

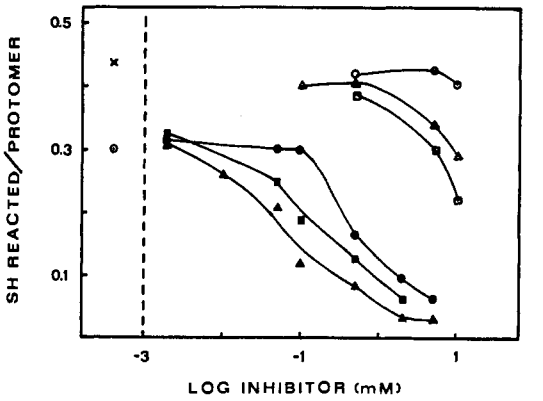

FIGURE 8: Effect of phosphoenolypruvate, creatine phosphate, and citrate on the arylation of the class I thiol group. $MgCl_2$ at 6 mM was present in all reactions. The X on the left of the figure represents the incorporation of isotope with no additions and the circled point at the left shows incorporation in the presence of 5 μM ATP. The symbols are as follows: (○) creatine phosphate, (△) citrate, (□), phosphoenolpyruvate, (●) creatine phosphate and 5 μM ATP, (▲) citrate and 5 μM ATP, and (■) phosphoenolpyruvate and 5 μM ATP. The results represent the mean of six determinations, av std dev = ±0.04.

consistent with the comparative effects of these three inhibitors on the muscle phosphofructokinase reaction (Kemp, 1971).

The observation that citrate increased the ability of Mg-ATP to block the reactive thiol group is also consistent with the gel filtration data of Kemp and Krebs (1967) which indicated an increased affinity of the enzyme for ATP in the presence of citrate. On the other hand, Lorenson and Mansour (1969) have proposed a different mechanism for the action of citrate based upon gel filtration binding studies with heart phosphofructokinase. They concluded that citrate inhibits the enzyme by binding at the same site that ATP occupies and that there is an apparent competition between citrate and ATP for binding. Their results are not entirely clear, however, because they measured citrate binding in the presence of ATP and ATP binding in the presence of citrate. This unusual procedure was employed to avoid the heavy losses of protein that were observed during the gel filtration studied. Furthermore, their data do not suggest a true competition as indicated by the observation that the dissociation constants for ATP were identical in the presence of 20 and 200 μM citrate. On the other hand, it is possible that high concentrations of ATP could inhibit citrate binding as suggested by Lorenson and Mansour (1969), although it is difficult to interpret binding data obtained in nonequilibrium conditions that exist in a system where extensive losses of protein occur during the course of equilibration.

Effect of pH on Dinitrophenylation. Previous studies of the thiol groups of phosphofructokinase have suggested that the high reactivity of the class I thiol group was due to its very low apparent p*K* (Kemp and Forest, 1968). The relatively slow rate of the thiol arylation of FDNB was thought to provide a better approach to estimating the pH dependence of the thiol group's unusual reactivity. The reaction was found to be highly dependent upon pH in the range of 6.0–8.4. To obtain values for isotope incorporation that were less than 0.5 mole/mole of protomer, it was necessary to employ reaction times as short as 15 sec at higher pH values. Based upon the assumption that a single thiol group was reacting and that the reaction was pseudo-first order under all conditions, first-order rate constants could be calculated. The pseudo-first order rate assumption was only proven at pH 7.0 and it is quite likely that other thiol groups may be reacting to some extent at high pH. Other amino acid side chains such as amino or imidazole groups may also be reacting with FDNB. Recognizing the limitations of such calculations, the first-order rate constants were plotted as a function of pH (Figure 9). The two solid lines in that figure are the lines obtained for the theoretical titration of dissociable groups with p*K*'s of 6.7 and 7.1. The closeness of the fit suggests that the reactivity of the class I thiol group is indeed dependent upon dissociable groups with p*K* in this range. This may be interpreted as the intrinsic reactivity of the thiol group itself or a measure of the pH dependence of a change in protein conformation that influences the availability of the thiol group for reaction.

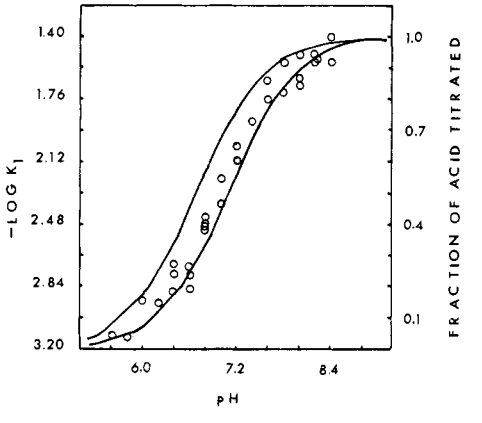

FIGURE 9: Effect of pH on the dinitrophenylation of the class thiol group. Pseudo-first-order rate constants (K_1) were calculated from the equation $K_1 = (1/t)\{\ln [a/(a - x)]\}$, where x, the number of thiol groups reacted/protomer in t sec, never exceeded 0.5 mole/mole and $a = 1$. (○) Experimental values obtained. The two theoretical curves represent the fraction of acid titrated, obtained from the Henderson–Hasselbalch equation, using p*K* values of 6.7 and 7.1.

The catalytic activity of the enzyme is also strongly influenced by pH in this region. Kühn *et al.* (1969), in a study of the kinetics of rat erythrocyte phosphofructokinase, demonstrated a very sharp pH dependence of the concentration of fructose-6-P necessary to give half-maximal velocity. Their data suggested that several dissociable groups with pK's near pH 7 were involved in this phenomenon. Profound differences in the relative inhibition of the rabbit muscle enzyme activity by ATP between pH 7.1 and 7.3 have also been reported (Kemp, 1969b).

That the class I thiol group is indeed highly reactive can be shown by comparing arylation rates with those observed with other proteins that are thought to have thiol group of high reactivity. Such comparions may be made by calculating second-order rate constants. One must assume that the FDNB is completely dissolved and that its disappearance can be accounted for completely by the thiolation reaction; that is, there is negligible hydrolysis during the course of the reaction. With these assumptions one calculates a second-order rate constant of approximately $7 \times 10^4\ M^{-1}\ min^{-1}$ for the arylation of phosphofructokinase at pH 8.0. If similar calculations are made of second-order rate constants of FDNB arylations by employing the data of Gold (1968) for glycogen phosphorylase and Shaltiel and Soria (1969) for glyceraldehyde-3-phosphate dehydrogenase, values that are less than one-tenth that observed with phosphofructokinase are obtained.

A Displaced Equilibrium or an Induced Shift? The data have been presented from the viewpoint of the Monod–Wyman–Changeux model for allosteric transitions (Monod *et al.*, 1965). The motivation for choosing this particular model was the observation that in the absence of added effectors the thiol reactivity was intermediate between that observed with activators and that observed in the presence of inhibitors. This suggests that in the absence of additions a natural equilibrium exists between two conformers of the enzymes. It is obvious, however, that the assumption of only two conformers is an oversimplification. We know that in the absence of added effectors a pair of thiol groups, referred to as class II, are moderately reactive and that these are blocked both by adenine nucleotides and fructose 6-phosphate (Younathan *et al.*, 1968; Kemp and Forest, 1968). Furthermore, fructose-1,6-P_2 which also increases the reactivity of the class I thiol group does not block the class II thiol groups but instead blocks the reaction of two other thiol groups (Kemp and Forest, 1968). It appears then that the enzyme cannot be explained by two simple conformers, and if there is more than one active conformer, they apparently share the property of having a thiol group in a particular position with increased reactivity. In other words, at least for one portion of the molecule there is a common change. The data then support the idea of a readily measurable equilibrium between conformers only as it pertains to a portion of the molecule, this is indeed a strain of the original theory. However, one might visualize a situation described by aspects of both theories. Subtle, local changes in conformation might be induced in different areas of the molecule by different effectors that lead to changes in thiol reactivity, such as the effects of fructose-6-P on class II thiol groups. In addition to this, such interaction leads to displacement of conformational equilibrium leading to changes in reactivity of the class I thiol. Still another possibility is that an equilibrium exists among more than two conformational species. On the other hand the data can be explained on the basis of various conformational forms all of which are induced by the presence of various effectors (Koshland *et al.*, 1966). The reactivity of the class I thiol group remains, however, as an indicator of whether the form so induced is active or inactive.

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References

Chapman, A., Sanner, T., and Pihl, A. (1969), *Eur. J. Biochem. 7*, 588.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 376.

Gold, A. M. (1968), *Biochemistry 7*, 2106.

Hofer, H. W. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351*, 649.

Kemp, R. G. (1969a), *Biochemistry 8*, 3162.

Kemp, R. G. (1969b), *Biochemistry 8*, 4490.

Kemp, R. G. (1971), *J. Biol. Chem. 246*, 245.

Kemp, R. G., and Forest, P. B. (1968), *Biochemistry 7*, 2596.

Kemp, R. G., and Krebs, E. G. (1967), *Biochemistry 6*, 423.

Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

Kühn, B., Jacobasch, G., and Rapoport, S. (1969), *Acta Biol. Med. Germ. 23*, 1.

Lorenson, M. Y., and Mansour, T. E. (1969), *J. Biol. Chem. 244*, 6420.

Mahowald, T. A., and Agodoa, L. (1969), *Biochem. Biophys. Res. Commun. 37*, 576.

Monod, J., Wyman, J., and Changeux, J.P. (1965), *J. Mol. Biol. 12*, 88.

Paetkau, V., Younathan, E. S., and Lardy, H. A. (1968), *J. Mol. Biol. 33*, 721.

Parmeggiani, A., Luft, J., Love, D. S., and Krebs, E. G. (1966), *J. Biol. Chem. 241*, 4625.

Pontremoli, S., Luppis, B., Tranellio, S., Wood, W. A., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 3469.

Shaltiel, S., and Soria, M. (1969), *Biochemistry 8*, 4411.

Uyeda, K., and Racker, E. (1965), *J. Biol. Chem. 240*, 4682.

Younathan, E. S., Peatkau, V., and Lardy, H. A. (1968), *J. Biol. Chem. 243*, 1603.