Allosteric Properties of Muscle Phosphofructokinase. II. Kinetics of Native and Thiol-Modified Enzyme*

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ABSTRACT: The kinetics of native skeletal muscle phosphofructokinase were compared with the kinetic properties of phosphofructokinase that had one thiol group modified per protomer by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Native phosphofructokinase displayed the following properties. (1) As the concentration of adenosine 5′-triphosphate was increased in the assay, the Hill interaction coefficient and the concentration of fructose 6-phosphate necessary to achieve half-maximal velocity also increased. No cooperative interactions were observed with inosine 5′-triphosphate as the phosphoryl donor. (2) At pH 7.35, the interaction coefficient and concentration of fructose 6-phosphate necessary for half-maximal velocity were much lower than that observed at pH 7.0. (3) In the presence of adenosine 5-monophosphate or inorganic phosphate, both the interaction coefficient and the half-maximal velocity concentration of fructose 6-phosphate were decreased. The following results were obtained with studies of the thiol-modified enzyme. (1) Maximum activity was decreased by about two-thirds. (2) The pH–activity profile was unchanged by the modification. (3) Modification had no influence on the interaction of fructose 6-phosphate or nucleoside triphosphate with the catalytic site on the enzyme as determined with inosine 5′-triphosphate as the phosphoryl donor. (4) With adenosine 5′-triphosphate as the phosphoryl donor, modification greatly reduced the observed interaction coefficient but did not significantly reduce the half-maximal velocity concentration of fructose 6-phosphate. As a result, the modification activates the enzyme under the assay conditions of high concentration of adenosine 5′-triphosphate and low concentrations of fructose 6-phosphate. The kinetics of native and thiol-modified phosphofructokinase are discussed in relation to a model that proposes two conformational states for the enzyme, and this model is compared with present views of allosteric enzymes. It is proposed that thiol modification does not restrict interaction of magnesium adenosine 5′-triphosphate with the inhibitory site but instead limits the complete conformational expression of that interaction.

It is generally accepted that phosphofructokinase plays an important role in the regulation of glycolysis (Lowry *et al.*, 1964) and that the regulatory role is due to its complex kinetic properties (Lowry and Passonneau, 1964). Hofer and Pette (1968) have provided the most extensive analysis to date of the regulatory properties of skeletal muscle phosphofructokinase.

Several laboratories have investigated the changes in activity and regulatory properties of heart and skeletal muscle phosphofructokinase that occur upon reaction of sulfhydryl groups with a variety of reagents (Younathan *et al.*, 1968; Kemp and Forest, 1968; Hofer and Pette, 1968; Froede *et al.*, 1968). To limit the changes that can occur upon extensive thiol modification, it was decided to react selectively only one thiol group per protomer with DTNB[1] and to compare the regulatory properties of this enzyme with native phosphofructokinase. In a preliminary account of this approach it was shown that modification of one thiol group resulted in an almost complete loss of the kinetic expression of cooperative interactions (Forest and Kemp, 1968). Of particular interest to this approach is the recent observation that the reactivity of this particular thiol group acts as an indicator for the binding of MgATP to the inhibitory site on phosphofructokinase (Kemp, 1969). In the present study, a more complete study of the regulatory properties of the native and thiol-modified enzymes is presented to provide some insight into the complex allosteric properties of phosphofructokinase.

Materials and Methods

ATP, ITP, AMP, and DPNH were obtained from P-L Biochemicals. DTNB, fructose-6-P (Sigma Grade I), and the sodium salt of β-glycero-P were purchased from Sigma Chemical Co.

α-Glycero-P dehydrogenase and aldolase were prepared from rabbit skeletal muscle as follows. Rabbit muscle was extracted and a 1.7–2.7 M ammonium sulfate fraction was prepared as described by Telegdi (1964). The ammonium sulfate fraction was dissolved and dialyzed against a buffer at pH 7.5 consisting of 5 mM potassium phosphate and 0.5 mM EDTA. The dialyzed fraction was placed on a column of DEAE-Sephadex equilibrated with the same buffer. The initial effluent contained aldolase which was further purified by an ammonium sulfate fractionation procedure similar to that of Beizenherz *et al.* (1953). The dehydrogenase was eluted from the column by the addition of NaCl to a concentration of 70 mM in the elution buffer. DPNH was added to the pooled elute to a concentration of 10 μM and ammonium sulfate was slowly added to a final concentration of 2.0 M.

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

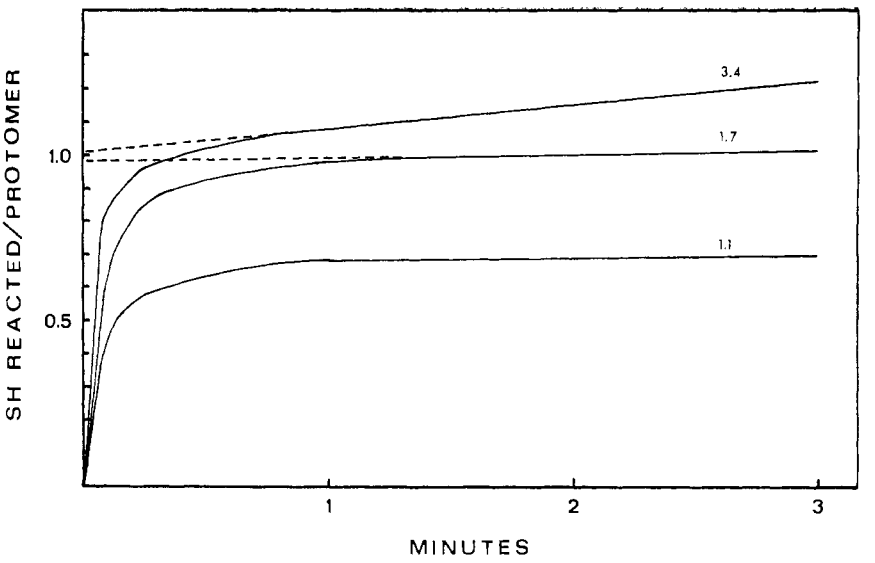

FIGURE 1: Effect of variation of DTNB concentration on the extent of the disulfide interchange reaction. Reactions carried out at 26° with a protein concentration of 1 mg/ml and DTNB concentrations of 11.8 μM (1.1 equiv), 18.2 μM (1.7 equiv), and 36.4 μM (3.4 equiv). The buffer at pH 7.2 contained glycylglycine, 25 mM; glycero-P, 25 mM; EDTA, 1 mM; fructose-6-P, 0.4 mM; and ATP, 0.1 mM. Reactions were started by the addition of DTNB and were followed at 412 mμ in a spectrophotometer.

The solution was stirred overnight and the crystals were collected by centrifugation and dissolved. The concentrated enzyme in the presence of DPNH could be recrystallized by the addition of ammonium sulfate to a concentration of 1.6 M. The specific activity of the aldolase and the dehydrogenase at pH 7.5 and 25° were 12 and 150, respectively. Triose phosphate 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 in the cold and dissolved in a buffer consisting of 25 mM glycylglycine, 25 mM sodium glycero-P, and 1 mM EDTA (pH 7.2). The enzyme was dialyzed for 3 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 performed at cold room temperature. DTNB was prepared fresh for each experiment and its concentration determined by titration with an excess of β-mercaptoethanol. The millimolar extinction coefficient of 13.6 (Ellman, 1959) at 412 mμ for the thionitrobenzoate ion of DTNB was employed.

Modification of the most reactive thiol group of phosphofructokinase by DTNB was carried out at protein concentration of 1 mg/ml. To assure the stability of the diluted enzyme, ATP and fructose-6-P were added to final concentrations of 0.1 and 0.4 mM, respectively. In a previous publication it was assumed that when 1 equiv of reagent/equiv of protomer was present the reaction would be quantitative (Forest and Kemp, 1968). Further analysis showed that this was not correct. Although it was observed that 1 equiv of DTNB reacted rapidly with the enzyme, the reaction was not stoichiometric as determined from the extinction coefficient of thionitrobenzoate ion and a protomer molecular weight for phosphofructokinase of 93,200 daltons. The results of adding varying amounts of DTNB to the enzyme are described in Figure 1. When 1.1 equiv of DTNB was added to phosphofructokinase, approximately 0.7 equiv of thionitrobenzoate ion was produced. If 1.7 equiv of DTNB/protomer of phosphofructokinase were added, 1 equiv reacted rapidly. If the concentration of DTNB was increased to 3.4 equiv, again only one thiol group reacted rapidly followed by a very slow reaction with the remaining thiol groups of enzyme. This was consistent with the earlier observation that DTNB reacted with a single thiol group of phosphofructokinase much more rapidly than with any of the remaining thiol groups (Kemp and Forest, 1968). The procedure adopted for thiol modification was as follows. At 26° and pH 7.2, DTNB was added to a solution of phosphofructokinase to achieve the following final concentrations: phosphofructokinase, 1 mg/ml (10.7 μM in protomer); DTNB, 18.2 μM; fructose-6-P, 0.4 mM; ATP, 0.1 mM; glycylglycine, 25 mM; glycero-P, 25 mM; and EDTA, 1 mM. The ratio of DTNB to phosphofructokinase protomer was 1.7 to 1. After 2.5 min, the reaction mixture was diluted by the addition of four volumes of ice-cold buffer consisting of glycylglycine, glycero-P, and EDTA at the concentrations given above. The preparation was stored on ice and further dilutions were carried out immediately before assay. Phosphofructokinase used in control experiments was handled in an identical manner except that DTNB was excluded from the preincubation. Assays were performed with a Gilford Model 2000 spectrophotometer at 26° and the indicated pH in 3 ml of a medium containing 25 mM glycylglycine, 25 mM glycero-P, 1 mM EDTA, 6 mM $MgCl_2$, 0.2 mM DPNH, 0.6 unit of aldolase, 0.3 unit of triose phosphate isomerase, 0.3 unit of α-glycero-P-dehydrogenase, ATP or ITP as indicated in the Results, fructose-6-P as indicated, and a final phosphofructokinase concentration of 0.33 μg/ml. It was necessary to use the same concentration of enzyme for all reactions since it was found that assays at low concentrations of fructose-6-P were not linear with enzyme concentration. Under these conditions a doubling of the protein concentration led to a more than twofold increase in rate. This was shown to be not due to instability of the diluted enzyme but may be a reflection of the state of aggregation of the enzyme. Reactions were started by the addition of fructose-6-P.

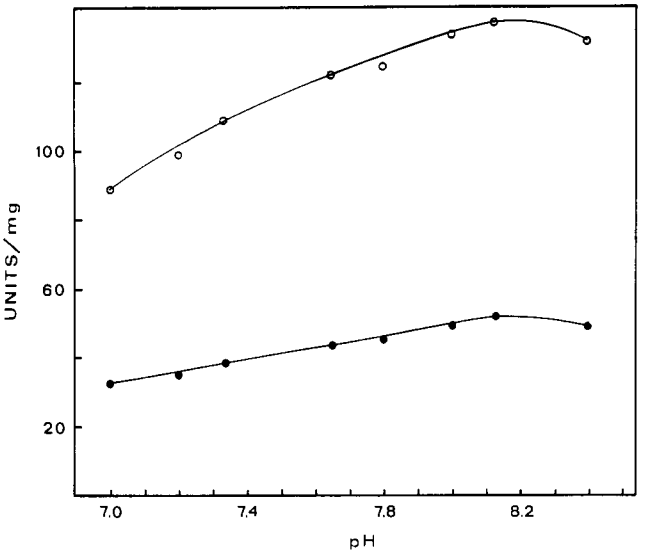

FIGURE 2: Effect of thiol modification on the pH–maximum activity profile. Rates were determined at varying pH with native (○) and thiol-modified enzyme (●) in the presence of 1 mM ATP, 1 mM fructose-6-P, and other conditions described in the Materials and Methods section.

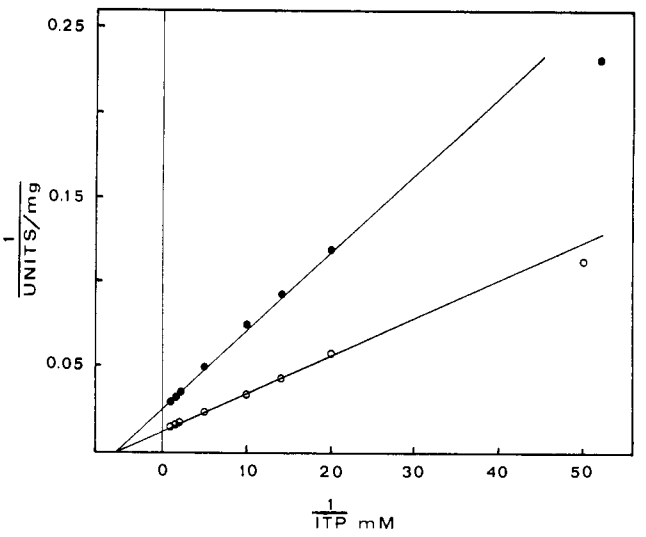

FIGURE 4: Effect of thiol modification on ITP affinity. Reactions carried out at pH 7.0 with native (○) and thiol-modified enzyme (●) in the presence of 1 mM fructose-6-P, ITP as indicated, and other conditions described in the Materials and Methods section.

Results

pH–Activity Profile of Thiol-Modified Enzyme. Pontremoli *et al.* (1965) have shown that modification of the thiol groups of another regulatory enzyme, fructose diphosphatase, altered the pH optimum of the enzyme. For this reason, the activities of native and thiol-modified phosphofructokinase were studied at different hydrogen ion concentrations. The results are shown in Figure 2. The assays were performed at 1 mM ATP and 1 mM fructose-6-P, concentrations that were optimal as determined in preliminary experiments. Thiol modification led to a decrease in activity at all pH values, but the shape of the activity–pH profile was identical for the two enzymes.

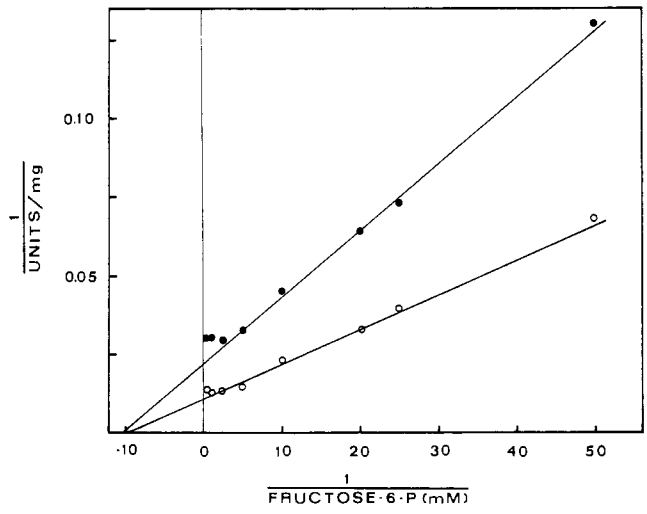

FIGURE 3: Effect of thiol modification on the affinity for fructose-6-P. Reactions carried out at pH 7.0 with native (○) and thiol-modified enzyme (●) in the presence of 2 mM ITP, fructose-6-P as indicated, and other conditions described in the Materials and Methods section.

Effect of Thiol Modification on Substrate Affinity. To determine whether or not thiol modification influences the interaction of substrates with the enzyme, the kinetics of the native and modified enzyme were studied with ITP as the phosphoryl donor. The choice of ITP was based on the observation by Uyeda and Racker (1965) that this nucleoside triphosphate is an effective phosphoryl donor but not an effective inhibitor of the enzyme. Furthermore, it has been shown recently that the inhibitory site on the enzyme has an affinity for MgITP that is approximately $^1/_{25}$ that for MgATP (Kemp, 1969). Enzyme velocities were measured at pH 7.0 with 2 mM ITP and varying concentrations of fructose-6-P. Figure 3 shows a double-reciprocal plot obtained for the reaction of both native and thiol-modified enzyme under these conditions. Modification resulted in a 65% decrease in V_{max} but did not significantly change the K_m for fructose-6-P. A K_m of 80–100 μM was calculated for both the native and the modified enzyme preparations. A small degree of substrate inhibition was indicated as shown by the slight upward curvature of the plot at high concentrations of fructose-6-P. Similar studies were carried out at pH 7.35. Under those conditions, the K_m for both enzyme preparations was decreased to approximately 50 μM. Substrate inhibition by fructose-6-P was not observed at pH 7.35. It is evident that the thiol modification does not influence the affinity of the enzyme for fructose-6-P at the catalytic site. To determine whether the thiol modification influences the enzyme affinity for the second substrate, the nucleoside triphosphate, the reaction was studied at a saturating concentration of fructose-6-P (1 mM) and with varying concentrations of ITP. The data obtained were plotted as reciprocals and are shown in Figure 4. Although a 60–70% decrease in maximal velocity resulted from the modification of the most reactive thiol group, the K_m for ITP was identical for the native and the thiol-modified enzyme (180 μM). It can be concluded that

thiol modification does not affect substrate binding by the catalytic site of phosphofructokinase.

Effect of Thiol Modification on Substrate Cooperativity. In contrast to the studies of enzyme velocities with ITP as the phosphoryl donor, strong cooperativity with regard to fructose-6-P was observed with ATP as the phosphoryl donor. Figure 5 describes a Hill plot (Hill, 1910; Monod *et al.*, 1965) of data obtained from reactions with native enzyme carried out at pH 7.0 and at three different concentrations of ATP. Increasing concentrations of ATP increased the slope of the plotted data and increased the $s_{0.5}$ for fructose-6-P (see Koshland *et al.*, 1966, for the use of $s_{0.5}$ instead of apparent K_m). The slope of the line will be referred to as the "interaction coefficient" and is presumed to be determined by the number of interacting sites for fructose-6-P and the strength of interaction among them. The data obtained from this plot are given in Table I. At high concentrations of

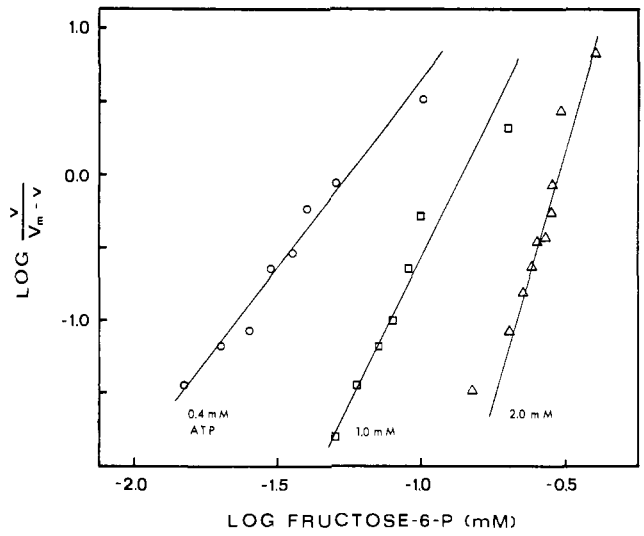

FIGURE 5: Hill plot of data obtained with native phosphofructokinase and varying concentrations of ATP. Data obtained from assays of native enzyme at pH 7.0 with the concentrations of ATP indicated on the plot and fructose-6-P concentrations given on the abscissa. Other conditions are described in the Materials and Methods section.

TABLE I: Effect of Thiol Modification on the Kinetic Properties of Phosphofructokinase.[a]

Enzyme	Concn ATP (mM)	F-6-$P_{0.5}$ (mM)	V_m (units/mg)	Interaction Coeff
Native	0.4	0.054	78	2.6
Modified	0.4	0.042	25	1.5
Native	1.0	0.14	89	5–5.5
Modified	1.0	0.10	31	1.5
Native	2.0	0.30	75	6.5–8.5
Modified	2.0	0.28	38	1.7

[a] Kinetic parameters were obtained from Figures 5 and 6.

ATP the reaction rate was extremely sensitive to small concentration changes of fructose-6-P in the half-maximal velocity region of the substrate saturation curve. For this reason it is impossible to measure accurately the slope of lines obtained in the Hill plot. It should be noted that the high Hill coefficients are only obtained when the same concentration of enzyme is used in all assays. In an earlier report from this laboratory (Forest and Kemp, 1968), a lower Hill coefficient was noted. Higher concentrations of enzyme were used at low substrate concentrations to obtain convenient reaction rates. Because velocities are not linear with enzyme concentration under these conditions, the true dependence of velocity upon substrate concentration was not observed in those experiments.

The effect of the modification of one thiol group is shown in Figure 6 and is summarized in Table I. Although modification decreased the maximal velocity by more than 60%, activation in the range of eightfold was observed at very low concentrations of fructose-6-P. This was due to decreased cooperativity. As seen in Table I, at all concentrations of ATP employed the slope of the lines in the Hill plot was reduced to about 1.5. On the other hand, F-6-$P_{0.5}$ was not greatly influenced by the modification and only small decreases were observed in all cases. Thus, the effect of increasing concentrations of ATP on the interaction coefficient was abolished by thiol modification whereas the effect of increasing concentrations of ATP on the apparent affinity of the enzyme for fructose-6-P was not influenced by the modification.

Influence of pH on the Kinetic Parameters of Native and Modified Enzyme. Hofer and Pette (1968) noted the striking increase in activity of phosphofructokinase when assays were carried out at pH 7.3 *vs.* 7.1 at low substrate concentration. Furthermore, it has been shown that the inhibitory site on phosphofructokinase has much less affinity for MgATP at pH 7.35 than it does at pH 7.0 (Kemp, 1969). Table II summarizes the kinetic data obtained at pH 7.0 and 7.35 with native and thiol-modified enzyme. With native enzyme, both

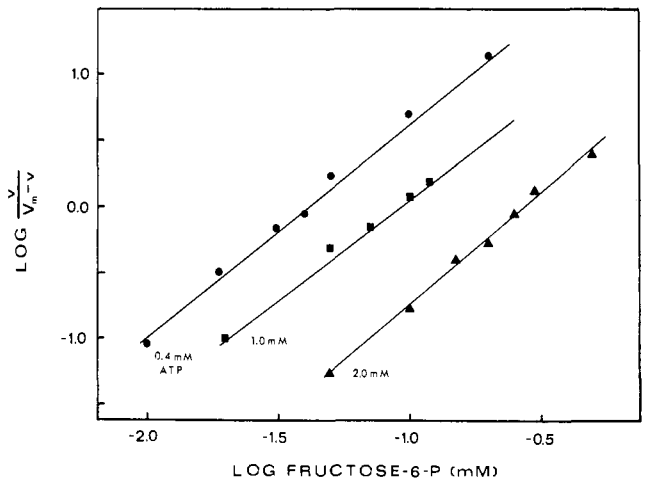

FIGURE 6: Hill plot of data obtained with thiol-modified phosphofructokinase and varying concentrations of ATP. Data obtained from assays of thiol-modified enzyme at pH 7.0 with the concentrations of ATP indicated on the plot and fructose-6-P concentrations given on the abscissa. Other conditions are described in the Materials and Methods section.

TABLE II: Influence of pH on Native and Modified Phosphofructokinase.[a]

Enzyme	pH	F-6-$P_{0.5}$ (mM)	V_m (units/mg)	Interaction Coeff
Native	7.0	0.30	75	6.5–8.5
Modified	7.0	0.28	38	1.7
Native	7.35	0.079	110	1.9
Modified	7.35	0.060	35	1.4

[a] Assays were performed at 2 mM ATP and the conditions described in the Methods section. Data for individual assays were plotted as log $V/V_m - V$ *vs.* log F-6-P to obtain the indicated parameters. Data for assays at pH 7.0 were taken from Table I.

TABLE III: Influence of Thiol Modification on the Action of Activators of Phosphofructokinase.[a]

Enzyme	Addition	F-6-$P_{0.5}$ (mM)	V_m (units/mg)	Interaction Coeff
Native	None	0.30	75	6.5–8.5
Modified	None	0.28	38	1.7
Native	0.1 mM AMP	0.10	71	2.9
Modified	0.1 mM AMP	0.09	30	1.5
Native	6 mM P_i	0.16	73	4.4
Modified	6 mM P_i	0.14	30	1.7

[a] Assays at pH 7.0 were performed at 2 mM ATP and other conditions described in the Methods section. Data for assays were plotted as log $V/V_m - V$ *vs.* log S to obtain the indicated parameters. Data for assays with no additions were taken from Table I.

the F-6-$P_{0.5}$ and the Hill coefficient were substantially lower at the more alkaline pH. This was consistent with the reported decrease in binding of MgATP at pH 7.35. The pH effects contrasted the results of thiol modification where only the interaction coefficient was greatly effected. This suggests that the modification of the thiol group may not be reducing the affinity of the enzyme for MgATP at the inhibitory site but only suppressing the full expression of the interaction. Assays of thiol-modified enzyme at pH 7.35 showed further small reductions in both F-6-$P_{0.5}$ and the interaction coefficient (Table II).

Influence of Thiol Modification on the Effect of the Activators of Phosphofructokinase. AMP and P_i are known to be activators of muscle phosphofructokinase (Lowry and Passonneau, 1964). This appears to be due to their effect of directly or indirectly displacing MgATP from the inhibitory site on the enzyme (Kemp, 1969). Table III summarizes the effects of AMP and P_i on native and thiol-modified enzyme. With native phosphofructokinase, both AMP and P_i decreased both the interaction coefficient and F-6-$P_{0.5}$. Thiol modification did not influence the expression of the action of these effectors. Modification did not cause a further decrease in the F-6-$P_{0.5}$, but did decrease the interaction coefficients to approximately the same levels as that observed with modified enzyme in the absence of positive effectors.

Discussion

In the first paper of this series, evidence was presented to support the existence of two conformational states of phosphofructokinase (Kemp, 1969). In the presence of MgATP the enzyme is converted to a form with no highly reactive thiol groups as determined by the reaction of DTNB with the enzyme. On the basis of the specificity for MgATP and of the pH dependence of MgATP effect, it was proposed that the inhibition of thiol reactivity was due to binding of MgATP to the inhibitory site on the enzyme. Of importance to this argument was the fact the activators of phosphofructokinase, fructose-6-P, AMP, and P_i, reversed the MgATP effect on thiol reactivity. It therefore appeared that phosphofructokinase can exist in at least two conformational states and that the enzyme with MgATP bound and without a reactive thiol group represented the inhibited conformation while the species with a reactive thiol group was the active conformation.

The kinetic data obtained with native phosphofructokinase are consistent with the foregoing model as indicated in the following. (1) As the concentration of ATP was increased the Hill interaction coefficient and the concentration of fructose-6-P necessary to achieve half-maximal velocity also increased. In terms of the model given above, the presence of higher concentrations of MgATP that tend to convert the enzyme to the inactive state required higher concentrations of fructose-6-P to drive the enzyme back to the active conformation. (2) No cooperative interactions were observed with ITP as the phosphoryl donor. This was consistent with the observation that MgITP at low concentrations does not bind to the inhibitory site (Kemp, 1969). (3) At pH 7.35, the F-6-$P_{0.5}$ and the Hill interaction coefficient were much lower than that observed at pH 7.0. It was previously shown that at this more alkaline pH MgATP binding at the inhibitory site was greatly reduced (Kemp, 1969). In earlier kinetic studies of muscle phosphofructokinase from several species, it was observed that at low concentrations of fructose-6-P the velocity of the enzyme was exceedingly dependent on pH in the region of pH 7.0 (Trivedi and Danforth, 1966; Hofer and Pette, 1968). From the data given here, this phenomena must be due to three factors: the decrease in F-6-$P_{0.5}$ at more alkaline pH; the decrease in the Hill interaction coefficient; and to a small extent, the increase in maximal velocity (Figure 2). (4) In the presence of activators of phosphofructokinase, AMP, and P_i, both F-6-$P_{0.5}$ and the interaction coefficient were significantly decreased. Thus, the conditions that are considered to lead to deinhibition may be interpreted as leading to the direct or indirect displacement of MgATP from the inhibitory site with the resulting conversion of the enzyme to the active conformation.

Because the reactivity of a thiol group acts as an indicator for the binding of MgATP and for the conformational state of the enzyme, one would predict that modification of that thiol group would influence either the binding of MgATP

by the enzyme or the conformational expression of that binding.

Kinetic analysis of the thiol-modified enzyme indicated the following. (1) The maximum activity manifested by the modified enzyme was about 30–40% of that given by native enzyme. (2) The pH–maximum activity profiles of the native and thiol-modified enzyme were identical. (3) Thiol modification had no influence on the interaction of fructose-6-P or nucleoside triphosphate with the catalytic site on the enzyme as determined from kinetic analysis with ITP as the phosphoryl donor. (4) Thiol modification did not influence the activating effect of AMP and P_i on the catalytic reaction. (5) With ATP as the phosphoryl donor, thiol modification only slightly lowered the concentration of fructose-6-P required to achieve half-maximal velocity under a variety of conditions. On the other hand, the Hill interaction coefficient was greatly reduced by thiol modification. The net result is a potent activation of the enzyme when assayed under the condition of high concentration of ATP and a low concentration of fructose-6-P.

It is thus apparent that those factors that normally regulate phosphofructokinase activity are expressed as changes in both F-6-$P_{0.5}$ and the interaction coefficient and that thiol modification influences only the interaction coefficient. The thiol-modified enzyme can still interact with MgATP but the complete expression of the interaction is suppressed by the presence of the thionitrobenzoate moiety attached by disulfide linkage to a cysteinyl residue of the enzyme. If the loss of reactivity of the thiol group is due to its being "buried" upon interaction with MgATP, then one can readily visualize the steric inhibition of the conformational change by the bulky thionitrobenzoate group.

Although sufficient information is not available to fit phosphofructokinase quantitatively into the published models of allosteric enzymes, it is of value to compare qualitatively the native and modified enzyme with the Monod–Wyman–Changeux model for allosteric transitions (Monod *et al.*, 1965). In the model, two states, an active and inactive, are reversibly accessible to oligomers of the enzyme. To display cooperative interactions, the substrate would have significant affinity for only one of these states. In the case of phosphofructokinase, evidence has been presented for at least two conformational states of the enzyme (Kemp, 1969). Furthermore, as demanded by the model, the enzyme exists in solution as oligomers of a protomeric species of 93,200 daltons (Paetkau and Lardy, 1967), and each protomer is capable of binding 1 mole of fructose-6-P and 3 moles of ATP (Kemp and Krebs, 1967). If indeed an equilibrium exists between the two states, then in the absence of ATP, the active conformation must be favored, as indicated by the lack of cooperativity with ITP as a substrate. The inhibitor, MgATP, binds preferentially to the inactive form whereas fructose-6-P and the activators bind to the active form. In reality, one cannot distinguish from the given information between the possibilities that the equilibrium is displaced by ligand binding or that the binding induces a conformation change as suggested by Koshland *et al.* (1966). In either case the result of the binding can be interpreted as a displacement of the equilibrium between the two states with a resultant change in both $s_{0.5}$ and the Hill interaction coefficient. It is of interest that the interaction coefficient approached eight or more in the presence of high concentrations of ATP (Tables I and II). On the basis of the Monod–Wyman–Changeux model, the Hill coefficient never exceeds the number of interacting sites (Blangy *et al.*, 1968). To fit the model, the data demand that the enzyme be present in the assay as an octomer or higher polymer of the 93,000 molecular weight protomer. Paetkau and Lardy (1967), on the other hand, have shown that the tetramer represents the fully active enzyme. There is no immediate explanation for this apparent lack of correlation.

In the Monod–Wyman–Changeux model, increased affinity of substrate for the inhibited state or decreased affinity for the active state result in a decreased interaction coefficient and in a very small change in the value of $s_{0.5}$ (see Figure 1b, Monod *et al.*, 1965). This describes the case of thiol-modified phosphofructokinase. The affinity of fructose-6-P for the active conformation was not changed by thiol modification as indicated by the data obtained with ITP as a substrate. Hence, a change in the ratio of affinities for the two states must be the result of an increased affinity of fructose-6-P for the inhibited conformation. If the allosteric transition is indeed restricted by the thiol blocking group, the result would be an incomplete conversion to the inhibited conformation by MgATP and the conformation thus assumed could have significant affinity for fructose-6-P. Although this qualitative treatment explains most of the data obtained in these studies, it does not explain the rather large decrease in maximal velocity that resulted from thiol modification. It is possible that the modification also resulted in some disruption of the catalytic site without changing substrate affinity.

Recently two other investigations into the modification of phosphofructokinase kinetics have been reported. Although the work has been done with phosphofructokinase with slightly different properties, the results provide an interesting comparison for the thiol-modification studies presented here. Ahlfors and Mansour (1969) modified sheep heart phosphofructokinase by photooxidation in the presence of methylene blue. The modified enzyme had a decreased F-6-$P_{0.5}$ and a lowered Hill constant. In contrast to thiol modification, photooxidation appeared to destroy the ability of the enzyme to interact with MgATP at the inhibitory site. In a study of yeast phosphofructokinase, Salas *et al.* (1968) desensitized the enzyme to ATP by a limited trypsin attack. Although the kinetics of this preparation were not carefully studied, it appeared that this modification also destroys the MgATP inhibitory site.

It is interesting to speculate on a physiological role for the uniquely reactive thiol group of phosphofructokinase since its reversible reaction with disulfides presents a possible way of modulating enzyme activity within the cell. Active and inactive forms of phosphofructokinase have been proposed for several systems (Viñuela *et al.*, 1964; Mansour, 1966). Furthermore, Helmreich and Cori (1965) were unable to account for the large increase in flux through phosphofructokinase following electrical stimulation of frog muscle on the basis of changes in the concentrations of metabolites that effect its activity. This type of control could arise from the interaction of phosphofructokinase with small disulfides or with other soluble enzymes. In regard to the latter, Pogell *et al.* (1968) have shown that phosphofructokinase reverses the AMP inhibition of fructose 1,6-diphosphatase apparently by protein–protein interaction. The influence of the phosphatase on the kinetics of the kinase was not studied in those experiments. Phosphofructokinase may also interact with the

cell membrane or cellular organelles. Mansour *et al.* (1966) found that the phosphofructokinase of sheep heart homogenates was associated with particulate material and could be solubilized by incubation with mercaptoethanol and various metabolites. The thiol requirement of the solubilization suggests an interaction involving sulfhydryl groups. It is exceedingly difficult, however, to assess either the possibility or the role of such an interaction in the intact cell.

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