Interaction of Inhibitors with Muscle Phosphofructokinase*

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The interaction of several inhibitors with muscle phosphofructokinase has been studied by both equilibrium binding measurements and kinetic analysis. At low concentrations of citrate a maximum of 1 mol is bound per mol of enzyme protomer. Tight binding requires MgATP and very weak binding is observed in the absence of either magnesium ion or ATP. ITP at low concentrations cannot replace ATP. In the presence of MgATP and at pH 7.0, the dissociation constant for the enzyme-citrate complex is 20 \(\mu\text{M}\). At 50 \(\mu\text{M}\) citrate and excess magnesium ion, the concentration of ATP required to give half-maximal binding of citrate is approximately 3 \(\mu\text{M}\). Both P-enolpyruvate and 3-P-glycerate compete for the binding of citrate and the estimated \(K_i\) values are 480 and 52 \(\mu\text{M}\), respectively. Creatine-P, another inhibitor of muscle phosphofructokinase, does not compete with the binding of citrate.

Measurement of the equilibrium binding of ATP shows that citrate, 3-P-glycerate, P-enolpyruvate, and creatine-P all increase the affinity of enzyme for MgATP with the concentration required to give an effect increasing in the order given. In kinetic studies, citrate, 3-P-glycerate and P-enolpyruvate each act synergistically with ATP to inhibit the phosphofructokinase reaction. This is indicated by the observation that the three metabolites do not inhibit the enzyme with ITP as the phosphoryl donor and that they inhibit at ATP concentrations that are not themselves inhibitory. Furthermore, the sensitivity to the inhibitors increases with increasing ATP concentrations.

Stirking differences in the extent of inhibition can be seen by varying the order of addition of assay components. Preincubation of the enzyme with ATP and citrate, 3-P-glycerate, or P-enolpyruvate results in greater inhibition than when the inhibitor is added after the reaction is started with fructose-6-P. Furthermore, the inhibition is reversed partially 10 to 15 min after the addition of fructose-6-P. This phenomenon is particularly striking with creatine-P as the inhibitor. Very high concentrations of this inhibitor are required to show any effect if the inhibitor is added after fructose-6-P. These effects are interpreted as reflecting slow conformational changes between an active form with high affinity for fructose-6-P and an inactive, or less active, conformation that binds the inhibitors.

Citrate, 3-P-glycerate, P-enolpyruvate, and creatine-P increase the rate of the phosphofructokinase at subsaturating concentrations of MgITP.

The results indicate a common binding site on the enzyme for citrate, 3-P-glycerate, and P-enolpyruvate that is distinct from the ATP inhibitory site. An additional site (or sites) for creatine-P is indicated. All four inhibitors act synergistically with ATP by increasing the affinity of the enzyme for MgATP at an inhibitory site. The inhibitors appear also to increase the affinity of the catalytic nucleoside triphosphate site for substrate.

The kinetic behavior of skeletal muscle phosphofructokinase is influenced by a large number of metabolites, suggesting the possibility of many highly specific binding sites on the enzyme surface (see Ref. 1, for review). The molecular weight of the protomer is in the range of 8 to 9 \(\times\) 10^4 g/mol (2-5) and each protomer of muscle phosphofructokinase possesses a site for fructose-6-P and another site capable of binding adenine nucleotides, with cyclic AMP being most tightly bound (6).

These two sites have also been described for sheep heart phosphofructokinase by Setlow and Mansour (7). A second adenine nucleotide site binds MgATP with high affinity and is thought to be an inhibitory site (8), and an additional nucleoside triphosphate site presumably represents the catalytic site (6, 8). Kinetic studies have suggested at least two sites for monovalent cations (9) and a divalent cation site has been described by Mathias (10). Other effectors of enzyme activity include inorganic phosphate, fructose-1,6-P, citrate, 3-phosphoglycerate, 2,3-bisphosphoglycerate, P-enolpyruvate, and creatine-P. Of these only citrate has been studied from the point of view of defining binding sites. The equilibrium binding studies of Lorenson and Mansour (11) concluded that citrate competes with the binding of ATP. On the other...
hand, on the basis of indirect measurements, we have suggested that ATP increases the affinity of the enzyme for citrate indicating a unique site for citrate (6, 12). In examining molecular models of several effectors, structural similarities in terms of charge distribution can be seen among the inhibitors; citrate, 3-P-glycerate, P-enolpyruvate, and creatine-P. The present study examines by kinetic analysis and by equilibrium binding the role of these four inhibitors in the regulation of phosphofructokinase and provides evidence that citrate, 3-P-glycerate, and P-enolpyruvate bind at a common site distinct from the ATP binding sites, and that all four effectors act synergistically with ATP.

**EXPERIMENTAL PROCEDURE**

Enzyme—Aldolase was prepared from frozen rabbit muscle as described previously (13). Glycerol-3-P dehydrogenase and triose phosphate isomerase were purchased from Sigma Chemical Co. Preceding their use in assays, these enzymes were dialyzed for 24 hours against 25 mM Na-glycero-P/25 mM glycglycine/1 mM EDTA, all at pH 7.0, to remove ammonium sulfate. Phosphofructokinase was prepared in this laboratory from frozen rabbit skeletal muscle (Pei-Freez, Rogers, Ark.) by the procedure of Kemp (14) and was crystallized three times. On the day of each experiment, the crystals were collected by centrifugation and dissolved in a buffer (pH 7.0) consisting of 25 mM Na-glycero-P, 25 mM glycglycine, 1 mM EDTA, 0.1 mM ATP, and 0.1 mM dithiothreitol, and the solution was dialyzed against the same buffer for 2 to 3 hours at 4°C. For equilibrium binding studies it was necessary to remove ATP. This was accomplished by passing the dialyzed enzyme solution through a column (20 x 4 mm) containing a mixture of 1 part of acid-washed charcoal and 1 part of coarse powdered cellulose. An A$_{280}$/A$_{410}$ ratio of above 1.6 indicated that ATP had been removed from the enzyme (15).

**Kinetic Analysis**—Kinetic analyses were performed on a Gilford spectrophotometer at 30°C and at pH 7.0 in 3 ml of a medium containing 50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 0.1 mM dithiothreitol, 0.15 M KCl, 1 mM EDTA, 0.6 unit of aldolase, 0.3 unit of triose-P isomerase, 0.3 unit of glycero-P dehydrogenase, and 0.2 mM NADH. The concentrations of ATP or ITP, and fructose-6-P were as indicated under "Results and Discussion." MgCl$_2$ was added in an amount 5 mM in excess of the concentration of nucleoside triphosphate. Phosphofructokinase was diluted to the desired concentration in 25 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, for determination of phosphofructokinase and provides evidence that citrate, 3-P-glycerate, and P-enolpyruvate bind at a common site distinct from the ATP binding sites, and that all four effectors act synergistically with ATP.

**RESULTS AND DISCUSSION**

**Binding of [14C]Citrate by Muscle Phosphofructokinase**—Previous studies from this laboratory have suggested that the site of interaction of citrate on skeletal muscle phosphofructokinase was distinct from the ATP inhibitory site and that indeed the affinity for citrate was increased in the presence of MgATP. Such conclusions were based upon indirect observations on the conformation of the enzyme as monitored by thiol reactivity with dithionitrobenzoic acid (8) and with fluorodinitrobenzene (12). The converse had been demonstrated directly, that is, the affinity of the enzyme for ATP was increased in the presence of citrate (6); but direct binding studies of citrate by the skeletal muscle have not been performed. Citrate binding by sheep heart phosphofructokinase has been investigated by Mansour and co-workers (1, 11) who concluded ATP and citrate bind to the same site on the enzyme although in those studies the binding of citrate was always measured in the presence of low concentrations of ATP. In the rabbit, and in several other mammals as well, we have concluded that the skeletal muscle enzyme and the major isozyme of heart were identical (17); and thus the finding that the citrate and ATP binding were competitive was surprising in view of the studies of muscle phosphofructokinase. We thus decided to investigate directly the binding of citrate by skeletal muscle phosphofructokinase and to determine the role of ATP in citrate binding.

The binding studies were carried out by the Hummel and Dreyer technique (16) as described under "Experimental Procedure." A glycglycine/glycero-P buffer was used for these studies as it was in previous binding studies (6) for the reason that complete recovery of the protein from Sephadex columns could be obtained with this buffer. With other buffer systems in the absence of added effectors, protein aggregation occurred and subsequently precipitated on the column. This phenomenon was also observed by Lorensen and Mansour (11) in studies of equilibrium binding by heart phosphofructokinase in imidazole buffer. Glycglycine/glycero-P buffer is not completely inert in that it does change the kinetics as well as enhance the stability of the enzyme (18). However, the enzyme in this buffer does show all of the usual kinetic properties of phosphofructokinase as indicated in earlier studies, although kinetic parameters may be shifted from what is observed in N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, for instance. For binding studies, it was obviously an advantage to use a buffer in which the enzyme was stable in the presence of added effectors.

Fig. 1 (lower curve) shows a double reciprocal plot of the binding of citrate by muscle phosphofructokinase in the presence of 5 mM MgCl$_2$ and 20 μM ATP. A dissociation constant of approximately 20 μM is calculated from these data, and the presence of 1 mol per protomer is indicated. The presence of ATP is indeed necessary to show significant binding of citrate in agreement with previous studies on the effect of citrate on thiol reactivity (12). In the presence of magnesium ion the concentration of ATP required to enhance citrate binding is very low as indicated in Fig. 2, which shows the effect of increasing concentrations of MgATP on the binding of citrate at a concentration of 50 μM by muscle phosphofructokinase. The half-maximal concentration of ATP is near 3 μM. It is seen that in the absence of MgATP the binding is barely measurable. This binding is either the result of residual ATP...
Drever (16) as described under "Experimental Procedure." In all cases, protein varied from 3.1 to 9.0 mg/ml, plus 2 mM MgCl₂ and the indicated concentration of citrate. Protein was examined in the ultracentrifuge under conditions which were used in the binding studies. For this reason, the enzyme varies with the varying protein and citrate concentrations that were used in the binding studies. It is likely that in the absence of fructose-6-P, which decreases ATP binding at the inhibitory site (8), the affinity for MgATP at the inhibitory site is greater than at the catalytic site, a situation which is obviously reversed in the presence of fructose-6-P.

Aggregation State of Enzyme in Presence of Citrate—It should be noted that no cooperativity was observed in the binding of citrate (Fig. 1). In our earlier studies (8), binding of fructose-6-P and mono- and diphosphonucleosides also exhibited hyperbolic binding isotherms. Hill and Hamnes (19) have noted that the binding of fructose-6-P and fructose 1,6-biphosphate show little or no cooperativity when the enzyme is associated to aggregates larger than the tetramer but that negative cooperativity is observed with binding by phosphofructokinase dimers. At the concentrations of enzyme employed in Fig. 1 (3.1 to 9 mg/ml) it might be expected that higher aggregates would predominate. Lad et al. (20), however, have shown that high concentrations of citrate stabilize smaller aggregates, presumably dimers. It is possible that in the binding studies of Fig. 1 small aggregates are present or, to make interpretation more complex, the aggregation state varies with the varying protein and citrate concentrations that were used in the binding studies. For this reason, the enzyme was examined in the ultracentrifuge under conditions which approximate the conditions of the binding studies. In Fig. 3, protein concentrations varied from 3.1 to 9.0 mg/ml. To approximate the extremes of the binding study, the distribution of aggregates of phosphofructokinase at 3 and 9 mg/ml with and without low concentrations of citrate was studied and the results at a single time point of sedimentation are shown in Fig. 3. Calculation of sedimentation coefficient indicated that under all conditions the major species was a 32 S component which represents the tetramer, octomer, and hexadecamer. The results of Fig. 3 show that low concentrations of citrate do not influence the aggregation state of enzyme at the high levels of protein employed in the binding study. It is obvious that dissociation of the enzyme to dimers is not required for the tight binding ($K_a = 20 \mu M$) of citrate.

Compensation for Citrate Site by Other Inhibitors of Phospho-
the citrate site, the apparent $K_i$ can be calculated from the
presumably equal to the $K_i$ for 3-P-glyceric acid. If one
assumes that P-enolpyruvate was also binding competitively at
the slope of plot of the citrate binding data. This value is
taken from the increase in

\[ K_i = \frac{K_{citrate}(I)}{r_{max}(citrate)} - \frac{[citrate] + K_{citrate}}{r_{max}} \]

where $r$ = moles of citrate bound per protomer, $r_{max}$ = 1, (I) =
concentration of the competing molecule, and $K_{citrate} = 20 \mu$M.

| Addition            | Citrate bound | $K_i$ |
|---------------------|---------------|-------|
| None                |               | 0.150 |
| P-Enolpyruvate (3 mM) | 0.021         | 420   |
| P-Enolpyruvate (2 mM) | 0.033         | 480   |
| P-Enolpyruvate (1 mM) | 0.054         | 480   |
| 3-P-Glycerate (1.4 mM) | 0.007         | 60    |
| 3-P-Glycerate (0.3 mM) | 0.026         | 54    |
| 3-P-Glycerate (0.14 mM) | 0.046         | 53    |
| Creatine-P (5 mM)   | 0.155         |       |

For single point competition experiments of Table I (see equation
shown in table).

The lack of inhibition of citrate binding by creatine phosphate indicated that this effector may be binding at a different site on the enzyme. It was possible that the binding was very weak, however, and an additional competition experiment was carried out under conditions more favorable for competition by creatine phosphate. Labeled citrate was present at 1 $\mu$M and creatine phosphate at 10 $\mu$M. With no creatine phosphate present 0.058 mol of citrate/mol of protomer was bound and this was reduced to 0.058 in the presence of creatine phosphate. A reduction of this modest magnitude, even if significant, would indicate a $K_i$ of greater than 1 M if indeed creatine phosphate binds at the citrate binding site. Such a value is not consistent with kinetic data and it must be concluded that creatine phosphate does not interact at the citrate site. As will be shown in the following section, binding must certainly occur under the foregoing conditions, but it must occur independently of the binding of citrate.

Effect of Inhibitors on ATP Binding—It is possible that
creatine phosphate binds directly at the ATP inhibitory site or that it enhances ATP binding. Previous studies from this laboratory have indicated that citrate enhanced the binding of ATP by phosphofructokinase (6, 8). If, as suggested by the data of Table I, P-enolpyruvate and 3-phosphoglyceric acid bind at the citrate site, these metabolites should also increase ATP binding. The four inhibitors, citrate, P-enolpyruvate, 3-P-glycerate, and creatine phosphate, were examined for their ability to increase the binding of ATP by the enzyme. The results are described in Table II. In this experiment a low level of ATP was used (1 $\mu$M) and increases in affinity could be detected in the presence of all four inhibitors. The species of ATP that is binding in this instance is MgATP because Mg$^{2+}$ was present at 5 mM. A possible complication in such an experiment is that an ATPase activity has been described for muscle phosphofructokinase (23). Such an activity could lead to destruction of ATP during the binding run and invalidate the data. In the previous study of the ATPase activity (23), an activity of 0.075 unit/mg was observed at pH 8.1 and 1 mM ATP. The phosphofructokinase used in the present study also showed ATPase activity, but the specific activity was slightly less than

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**Table I**

**Competition with citrate binding**

Gel filtration binding studies carried out at pH 6.9 in the presence of
3.5 $\mu$M [14C]citrate, 2 mM MgCl$_2$, 20 $\mu$M ATP, 25 mM glycylglycine, 25 mM Na-glycero-P, 1 mM dithiothreitol, and the indicated metabolite. $K_i$ values were calculated assuming competitive inhibition from the rearrangement of the Michaelis-Menten equation as follows:

$K_i = K_{citrate}(I)/r_{max}(citrate) - ([citrate] + K_{citrate})/r_{max}$

where $r$ = moles of citrate bound per protomer, $r_{max}$ = 1, (I) = concentration of the competing molecule, and $K_{citrate} = 20 \mu$M.

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**Fig. 3.** Representative schlieren patterns of the sedimentation of phosphofructokinase. Pictures taken at 23 min with phase plate
analyzer angle of 65°. Runs were performed at 20' and 40,000 rpm in a
buffered solution at pH 7.0 consisting of 25 mM glycylglycine, 25 mM
Na-glycero-P, 1 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl$_2$, 0.05
mM ATP, and with or without 0.1 mM sodium citrate. The upper figure
describes a paired run with (lower) and without (upper) citrate at 9
mg/mL of phosphofructokinase. The lower figure describes paired runs
at 3 mg/mL of enzyme with (lower) and without (upper) citrate.

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**Table II**

**Effect of Inhibitors on ATP Binding**

Gel filtration binding studies carried out at pH 6.9 in the presence of
3.5 $\mu$M [14C]citrate, 2 mM MgCl$_2$, 20 $\mu$M ATP, 25 mM glycylglycine, 25 mM Na-glycero-P, 1 mM dithiothreitol, and the indicated metabolite. $K_i$ values were calculated assuming competitive inhibition from the rearrangement of the Michaelis-Menten equation as follows:

$K_i = K_{citrate}(I)/r_{max}(citrate) - ([citrate] + K_{citrate})/r_{max}$

where $r$ = moles of citrate bound per protomer, $r_{max}$ = 1, (I) = concentration of the competing molecule, and $K_{citrate} = 20 \mu$M.
ently inhibit the enzyme by increasing the affinity of the enzyme for MgATP at the inhibitory site, but they do so as the result of interactions at sites differing from one another and from the MgATP site. As will be indicated below, the inhibitors appear to increase the affinity of the enzyme for nucleoside triphosphate not only at the inhibitory site but at the catalytic site as well.

**Kinetic Studies: General Considerations**—Without employing sophisticated techniques such as stop-flow, it is not possible to study the kinetics of phosphofructokinase at the enzyme concentrations employed in the earlier studies of protein conformation (8, 12) or in the studies reported here of binding and of sedimentation studies. For this reason it is not possible to make direct comparisons of quantitative kinetic data. Certain qualitative questions may be considered, however. Are the actions of citrate, P-glycerate, P-enolpyruvate, and creatine-P synergistic with ATP? Is the ranking of effectiveness, in terms of concentration, identical with that seen in the binding studies? In addition, experiments are presented to show that these same inhibitors, at identical concentrations used in inhibition studies, may act as activators at low concentrations of nucleoside triphosphate, suggesting enhanced affinity at the catalytic site for phosphorly donor.

**Effect of Order of Addition of Reaction Components**—Kinetic studies of phosphofructokinase usually have been complicated by lags in the initial phases of the reaction. Part, but not all of this lag can be attributed to the approach to steady state of the auxiliary enzymes. In practice one usually measures rates achieved 2 or 3 min after starting the reaction at which time the velocity is usually constant. We have observed under certain conditions, however, that the lag can be 10 to 15 min and the results observed 3 min after starting the reaction can be quite misleading. This phenomenon is shown in Fig. 4A. The upper curve shows the potent inhibition of phosphofructokinase by 0.1 mM citrate when the enzyme was preincubated for 4 min with all components except fructose-6-P. After the inhibition decreased, the final rate achieved was about 30 to 30% less than the assay performed without citrate (lower curve). A similar result was obtained if the citrate and fructose-6-P were added together. The middle curve shows the result of adding 0.1 mM citrate 4 min after the reaction had started. It was observed in this case that the inhibition occurred rapidly and that the extent of inhibition was similar to that observed after the reaction had started. In Fig. 4B is shown the extent of citrate inhibition when determined by the two different assay methods. The open circles describe the effect of citrate when the citrate was added before starting the reaction with fructose-6-P and the rate was determined 3 to 4 min after the reaction started. In this situation the response to increasing citrate concentrations occurred over a very narrow range and if one attempts to analyze the data by Hill-type plots, an extremely high n value is observed. The closed circles describe the citrate effect when it was added 4 min after starting the reaction. In this case the inhibition occurred rapidly and that the extent of inhibition was similar to that observed after the reaction had started. In Fig. 4B is shown the extent of citrate inhibition when determined by the two different assay methods. The open circles describe the effect of citrate when the citrate was added before starting the reaction with fructose-6-P and the rate was determined 3 to 4 min after the reaction started. In this situation the response to increasing citrate concentrations occurred over a very narrow range and if one attempts to analyze the data by Hill-type plots, an extremely high n value is observed. The closed circles describe the citrate effect when it was added 4 min after the reaction had started. The rate was determined subsequently 3 to 4 min after citrate addition. A Hill plot of these data gave an n value of approximately 4. The lag could also be produced by preincubation of the enzyme with 3-P-glycerate, P-enolpyruvate, or 2,3-bisphosphoglycerate, further supporting the idea that these inhibitors and citrate act in an identical manner.

**Preincubation with creatine-P** also produced the lag, an effect which may be related to its influence on the binding of MgATP (Table II). Prolonged preincubation with MgATP did not produce the lag periods described in Fig. 4A.

**There are several possible explanations for these phenomena based upon previous information on phosphofructokinase.** It is possible that the products generated in the first several

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### Table II

| Addition      | Concentration | Moles ATP bound per mole phosphofructokinase protomer |
|---------------|---------------|-----------------------------------------------------|
| None          | 0.001         | 0.16 ± 0.02*                                        |
| Citrate       | 0.04          | 0.34                                                |
|               | 0.08          | 0.48                                                |
|               | 0.10          | 0.53                                                |
|               | 0.3           | 0.60                                                |
| 3-P-Glycerate | 0.3           | 0.45                                                |
|               | 0.5           | 0.68                                                |
| P-Enolpyruvate| 1.0           | 0.28                                                |
|               | 2.0           | 0.41                                                |
| Creatine-P    | 4.0           | 0.44                                                |
|               | 8.0           | 0.59                                                |

*Mean and standard deviation for five separate determinations.*
minutes without citrate, or in 10 to 15 min with citrate, relieve or prevent the citrate inhibition. This does not appear to be the case because the products generated are ADP, NAD, and glycerol-3-P, and the addition of these products at concentrations that could be generated during the lag to the preincubation with citrate did not prevent the long lag period. Fructose 1,6-bisphosphate, a known activator of phosphofructokinase, would also accumulate to a steady state level during the assay. El-Badry et al. (24) have described the inhibition of phosphofructokinase by aldolase and fructose-1,6-bisphosphatase that appeared to be due to depletion of the sugar phosphate. To test for an effect on the length of lag phase in the presence of citrate, fructose 1,6-bisphosphate was added after the addition of substrate to the assay. Following a short burst in NADH oxidation due to the presence of fructose 1,6-bisphosphate the rate decreased to the rate observed in the absence of fructose 1,6-bisphosphate. The lag was not eliminated by this treatment. It was further reasoned that if the lag were due to fructose 1,6-bisphosphate accumulation, then increasing the concentration of auxiliary enzymes in the assay should prolong the lag by reducing the steady state level of fructose 1,6-bisphosphate. However, the lag phase was not prolonged by the presence of a 4-fold higher concentration of aldolase, triose phosphate isomerase, and the dehydrogenase.

Another possible explanation for this phenomenon is that it results from slow conformational changes, termed hysteresis by Frieden (25). Previous work from this laboratory has provided evidence for at least two conformational states for the enzyme (12). Citrate and ATP have higher affinity for the inhibited conformation (or conformations) and fructose-6-P and activators have higher affinity for the active conformation. Hulme and Tipton (26) have noted that the kinetics of heart phosphofructokinase are influenced by protein concentration, an effect which they interpreted from the viewpoint of an associating-dissociating system. They further noted that citrate exacerbated the nonlinear response of the enzyme activity to protein concentration and that activators relieved the effect. The importance of the state of aggregation in phosphofructokinase activity was first suggested by Mansour (27) in studies of the heart enzyme. Earlier work with the rabbit liver enzyme from this laboratory (28) has noted variations in initial velocities depending upon the order of addition of reactants. The initial burst of reaction rate followed by a gradual decrease when reactions were started by the addition of enzyme was thought to reflect the high activity of the aggregated enzyme which decreased following dilution in the assay mixture. Ramaiyah and Tejwani (29) have examined more extensively this phenomenon with liver phosphofructokinase and have concluded that the enzyme occurs in interconvertible forms.

Lad et al. (20) have shown that dilution of muscle phosphofructokinase to a concentration of 0.15 mg/ml at pH 7.0 and 5°C results in a depolymerization of tetramer to dimers with reaction half-time of 1.5 hours, and that the depolymerization is associated with a loss of activity as measured at pH 8.0. These workers (20) have demonstrated further that the presence of high concentrations of citrate (5 mM) greatly enhance the rate of dissociation of muscle phosphofructokinase to inactive dimers. The other inhibitor, ATP, does not produce this effect but Bock and Frieden (30) have indicated that ATP enhances the rate of pH-dependent dissociation. Presumably, the presence of low concentrations of MgATP would have permitted Lad et al. (20) to observe the citrate effect at low concentrations in their studies, based upon the results of Fig. 2 as well as previous data from this laboratory (12). Frieden (31) has shown that the activity changes that accompany association-dissociation phenomena due to pH changes are relatively slow. The recovery of activity 10 to 15 min after the addition of fructose-6-P (Fig. 4) may be the result of a slow fructose-6-P-induced association to a new equilibrium with the accompanying recovery of activity. Lad et al. (20) demonstrated that fructose-6-P opposes the action of citrate and that mixtures of citrate and fructose-6-P stabilized aggregates that are of intermediate size. In the case of the upper curve in Fig. 4A where citrate and ATP were incubated with the enzyme prior to the addition of fructose-6-P, one might predict that the inactive form, which is probably a depolymerized form, was stabilized. Upon addition of fructose-6-P, the achievement of the new equilibrium of active and inactive conformers was slow.

A polymerization reaction should be concentration-dependent and the effect of varying protein concentration on the length of the lag phase was examined. Assays were performed at pH 7.0 in the presence of 1 mM ATP, 1 mM fructose-6-P, and 0.1 mM citrate with phosphofructokinase concentrations in the assay varying in the range of 0.06 to 0.6 µg/ml. Under these assay conditions, lags of less than 1 min were observed at 0.6 µg/ml and more than 30 min at 0.06 µg/ml. Intermediate concentrations gave intermediate lag times, but the length of the time was not directly proportional to the dilution. More extensive studies over a broader range of concentrations would be necessary to determine a quantitative relationship between the degree of dilution and the length of lag. If the lag is due to a dimerization, one might predict that lag should vary as the square of the degree of dilution. It is important to note the final
rate achieved after the lag varied in direct proportion to the protein concentration as did the rate achieved if the citrate was added subsequent to starting the reaction with fructose-6-P. In all of the inhibition studies to be described, the assays were performed by adding the inhibitor 4 min after the reaction was started with fructose-6-P and subsequently measuring rates 3 to 4 min after inhibitor addition. The linearity of the rate with time and protein concentration obtained with these conditions indicated that the assay was being performed under steady state conditions. Phosphofructokinase was present in the subsequent assays at a concentration of 0.5 to 0.4 μg/ml.

Synergism between ATP and Citrate—ITP is an efficient substrate for phosphofructokinase but it acts only very weakly as an inhibitor. MgITP has been shown to bind poorly to the inhibitory ATP site (12). As shown in Fig. 2, low concentrations of ITP do not promote citrate binding by the enzyme. Because low concentrations of citrate interact with phosphofructokinase only in the presence of ATP, then citrate inhibition should not be seen when ITP is used as the phosphoryl donor in the reaction. This is demonstrated by the data of Fig. 5. In Fig. 5A, the saturation curves for ATP and ITP are shown, and in Fig. 5B is described the action of citrate at three concentrations of ATP. No inhibition by citrate was observed with ITP as substrate, whereas employing concentrations of ATP that were not inhibitory alone, i.e. 0.7 to 1.5 mM, potent inhibition was observed only in the presence of citrate. In other words, citrate had no effect alone under these conditions, but was very potent when present together with ATP and the potency increased at higher levels of ATP. It should be noted that the inhibition could be relieved by increasing the concentration of fructose-6-P or by the addition of activators such as AMP.

Synergism and 3-P-Glycerate, P-Enolpyruvate, and Creatine Phosphate—The effective concentrations of P-glycerate and P-enolpyruvate were in the order observed for their ability to enhance the binding of MgATP by phosphofructokinase; that is, the required concentrations of 3-P-glycerate were slightly higher than those used with citrate, whereas P-enolpyruvate was effective only at concentrations above 1 mM. These results are shown in Fig. 6. Increasing the level of ATP increased the effectiveness of the metabolites as inhibitors of enzyme activity. As in the case of citrate, the two inhibitors had no effect alone with ITP as the phosphoryl donor, and the inhibition could be relieved by the addition of AMP.

Creatine-P was a very poor inhibitor of phosphofructokinase when the assay was carried out in the prescribed manner; that is, when the inhibitor is added 4 min after the addition of the substrate, fructose-P. For example, at 1 mM ATP and the other conditions of Fig. 6, creatine phosphate at 40 mM inhibited the enzyme less than 50%. It has been shown by a number of workers, including ourselves, that creatine-P is an effective inhibitor at quite low concentrations (1). In work from this laboratory (18), we reported that at pH 7.1 with ATP at 0.2 mM and fructose-6-P at 0.4 mM the concentration of creatine-P required to give 50% inhibition was only 1.9 mM. However, in those studies as well as those from other laboratories, the enzyme was assayed in the conventional way of starting reactions by the addition of substrate. Under the conditions of Fig. 6, creatine-P was strongly inhibitory (about 90% inhibition at 3 mM) if it was preincubated with the enzyme for several minutes and the reaction started with fructose-6-P. After 12 to 15 min, however, the rate began to slowly increase and approach that of the uninhibited enzyme. In other words, this is the same phenomenon, probably one related to slow conformational changes, that was discussed previously with regard to citrate inhibition (Fig. 4). The phenomenon observed with creatine phosphate was much more pronounced in that the inhibition was practically eliminated if creatine phosphate was added several minutes after the reaction had started. It should be noted that the relative effect of changing the order of addition with 3-P-glycerate and P-enolpyruvate as inhibitors was similar to that seen with citrate, which is consistent with these two inhibitors binding to the same site that citrate occupies.

Inhibitors as Activators—In equilibrium binding studies reported earlier (6), it was noted that citrate increased the affinity of at least two and possibly three of the sites capable of binding ATP. Randle et al. (32) demonstrated in kinetic studies that citrate increased the affinity of the enzyme for ATP at the catalytic site. This is confirmed in the upper part of Fig. 7. At low concentrations of ATP, citrate acts as an activator of the reaction whereas the well documented inhibition is shown at higher ATP concentrations. Activation by citrate is even more apparent with ITP as the phosphoryl donor as shown in the lower part of Fig. 7. MgITP is bound less tightly by the enzyme and cooperativity in kinetic behavior is indicated by the sigmoid nature of the substrate versus velocity curve. Similar cooperativity can be observed with ATP as a substrate but the apparent Km for ATP is quite low, less than 20 μM at pH 7.0. With ITP as phosphoryl donor the apparent Km for ITP is close to 1 mM and, in the presence of 60 μM citrate the Km is decreased to approximately 0.4 mM.

As would be expected from the presumption of a binding site that is identical with the citrate site, 3-P-glycerate, and P-enolpyruvate also increase the activity of phosphofructoki-
with other conditions of Fig. 5, citrate at 20 mM is an effective inhibitor. Citrate effects at much higher concentrations than the enzyme is less sensitive to ATP inhibition, such as creatine-P, which also increased the binding of MgATP (Table II) but did not bind at the citrate site (Table I), increased the activity of the enzyme at low substrate levels.

Some General Comments—The citrate effects described herein are those requiring a high affinity site. The equilibrium binding studies indicated a $K_d$ of 20 mM and from Fig. 5 this concentration was inhibitory, although the extent of inhibition was dependent upon the concentration of ATP. At ATP concentrations that are inhibitory, for example, 2 mM ATP with other conditions of Fig. 5, citrate at 20 mM is an even more effective inhibitor. Citrate effects at much higher concentrations may also be observed and may require a separate mode of action or an additional site. For example, under conditions where the enzyme is less sensitive to ATP inhibition, such as pH 8.0 or with high concentrations of fructose-6-P (18), citrate can inhibit by the complex formation of magnesium. The dissociation of muscle phosphofructokinase in the presence of citrate (20) has only been studied with relatively high concentrations of citrate and it would be of greater interest to study this phenomenon at citrate concentrations that are inhibitory (20 to 200 mM) in the presence of MgATP. In the equilibrium binding studies described here, citrate binding was observed under conditions where dissociation to units smaller than the tetramer was not occurring as observed in sedimentation runs in an analytical ultracentrifuge (Fig. 3). It is quite possible, however, that citrate binding results in a conformational change that may shift the equilibrium to favor dissociation. Dissociation may only occur at concentrations much lower than those of the binding study. On the other hand, although dissociation is obviously not required for citrate binding, it is possible that it may be necessary for enzyme inhibition. The possible importance of dissociation to citrate inhibition is suggested by the time-dependent phenomena in Fig. 4 and the dependence upon protein concentration of citrate inhibition. Further support for the role of dissociation comes from the recent observation of Lad and Hammes (33) that phosphofructokinase tetramers cross-linked with a bifunctional reagent are resistant to citrate inhibition.

**Acknowledgment**—We thank Miss Alice Hsu for excellent technical assistance.

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**TABLE III**

Effect of inhibitors on phosphofructokinase in presence of subsaturating ITP

| Addition      | Concentration | Activity |
|---------------|---------------|----------|
| None          | mM | activity |
| Citrate       | 0.02 | 90      |
|               | 0.05 | 94      |
|               | 0.3  | 73      |
| 3-P-Glycerate | 0.02 | 78      |
|               | 0.05 | 90      |
|               | 0.2  | 94      |
| P-Ethanolpyruvate | 0.5 | 78      |
|               | 2.0  | 81      |
| Creatine-P    | 3.5  | 93      |
|               | 10.5 | 101     |

phosphate kinase in the presence of subsaturating substrate concentrations of ITP. This is described in Table III. The effective ranges of concentration are similar to those required to give inhibition at high substrate levels. Creatine-P, which also increased the binding of MgATP (Table II) but did not bind at the citrate site (Table I), increased the activity of the enzyme at low substrate levels.

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**Figure 1**

Activation of phosphofructokinase by citrate. Assay at pH 7.0 as described under “Experimental Procedure” with (●) and without (○) 60 mM sodium citrate and the indicated concentration of ATP (upper) and ITP (lower). Reactions were started by the addition of fructose-6-P to a final concentration of 1 mM.
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