# Interactions between Citrate and Nucleoside Triphosphates in Binding to Phosphofructokinase†

Yasushi Ogawa‡ and Daniel E. Atkinson*

*Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024*
*Received May 23, 1984*

ABSTRACT: Interrelationships between the binding by rabbit muscle phosphofructokinase of citrate, ATP, GTP, and adenyl-5′-yl imidodiphosphate (AMP-PNP) were investigated. To allow measurements at 25 °C, pyruvate kinase and phosphoenolpyruvate were included in the dialysis media to rephosphorylate ADP formed by the weak ATPase action of phosphofructokinase. Binding of citrate was enhanced by GTP nearly as much as by ATP, although GTP does not inhibit the catalytic activity of the enzyme. The results are consistent with the interpretation that binding of GTP, and, by analogy, ATP, at the catalytic site enhances the binding of citrate. AMP-PNP also enhanced citrate binding. Both ATP and GTP appear to bind at three sites per enzyme subunit, with the apparent third site binding relatively weakly. The estimated dissociation constants for the first two sites, about 33 μM for both for ATP compared with 3 and 280 μM for GTP, are consistent with kinetic results that imply lack of effective competition by GTP for the inhibitory site. When a compound binds at two or more sites on a macromolecule, the position and shape of the binding curve are sensitive to the geometric mean of the binding constants but quite insensitive to the magnitudes of the individual constants; thus, binding affinities cannot be estimated with confidence in such cases.

The reaction catalyzed by phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) appears to be a major regulatory step in glycolysis. The activity of this enzyme is affected by a number of modifiers, by association/dissociation of the subunits (Lad et al., 1973), and by hormones (Richards & Uyeda, 1980). Phosphofructokinase is also inhibited by ATP, one of its substrates. Each subunit of the enzyme appears to contain both the catalytic site and a regulatory site where binding of ATP causes inhibition of the enzyme activity (Colombo et al., 1975). The effect of ATP is to decrease the affinity for fructose 6-P at the catalytic site (Mansour, 1963; Ramaiah et al., 1964). Some other modifiers, such as AMP and citrate, seem to act indirectly by affecting the binding of ATP at the regulatory site (Colombo et al., 1975).

We studied binding of modifiers to rabbit muscle phosphofructokinase and the effect of the binding of one modifier on another and related the results of these binding studies to the kinetic behavior of the enzyme. Where appropriate, phosphoenolpyruvate and pyruvate kinase were added to rephosphorylate ADP formed by the minor ATPase side reaction catalyzed by phosphofructokinase.

## Materials and Methods

Tissue solubilizer NCS and isotopically labeled ATP, GTP, and citric acid were purchased from Amersham Corp. Enzymes and other nucleotides and substrates were from Sigma Chemical Co. Spectraphor-2 membrane tubing was obtained from Spectrum Medical Industries.

Phosphofructokinase was purified from fresh rabbit muscle as described by Kemp (1975) and was stored in crystalline form at 4 °C and pH 7.2 in 50 mM β-glycerophosphate, 2.0 mM ATP, and 2.0 M $(NH_4)_2SO_4$. The purified enzyme had a specific activity of 170–180 units/mg of protein.

*Equilibrium Dialysis.* A suspension of crystalline phosphofructokinase was sedimented by centrifugation, dissolved in 25 mM glycylglycine, 25 mM β-glycerophosphate, 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 4.0 mM magnesium acetate, and 0.1 mM ATP at pH 7.0, and dialyzed against the same buffer at 4 °C to remove ammonium sulfate. To remove bound ATP, the dialyzed enzyme was mixed with acid-washed charcoal (2 mg/mg of protein) suspended in the same buffer. Charcoal was removed by centrifugation, and the supernatant was passed through a column (4 × 20 mm) containing equal weights of Whatman cellulose powder CF 1 (long fibers) and acid-washed charcoal. The absorbance ratio, $A_{280}/A_{260}$, of the charcoal-treated enzyme varied between 1.4 and 1.65. A value of 1.7 would indicate that all ATP had been removed (Parmaggiani et al., 1966).

Dialysis cells were assembled by clamping Spectraphor-2 membranes between blocks of Plexiglas containing matching holes 8 mm in diameter and 1.6 mm deep. A hole about 0.6 mm in diameter was drilled from the surface of the block into each half-cell to allow for additions through a syringe needle. A solution of phosphofructokinase (50 μL, containing 50–60 μg of enzyme) was injected into half of each cell. An equal volume of a solution containing the labeled ligand and, in some experiments, a modifier in the same buffer was injected into the other half-cell. The injection holes were sealed with tape, and the cells were submerged in a water bath at 25 °C and rotated for 3.5–4 h. Control experiments showed that virtual equilibrium across the membrane was reached in about 2.5 h under those conditions. Samples (40 μL) were removed and carefully mixed with 250 μL of NCS tissue solubilizer and then with 4.0 mL of toluene-based scintillation fluor for determination of radioactivity.

For analysis of the binding results, we assumed no interaction between sites binding the same ligand. Values of dissociation constants were estimated by fitting the results to eq 1, where $r$ is the number of moles of ligand bound per mole of enzyme subunit and [S] is the concentration of unbound

† This work was supported by National Institutes of Health Grant AM09863 and National Science Foundation Grant PCM76-18321.
‡ Present address: Cardiovascular Research Institute, University of California, San Francisco, CA 94143.

0006-2960/85/0424-0954$01.50/0 

$$r = [S]/(K_1 + [S]) + [S]/(K_2 + [S]) + [S]/(K_3 + [S]) \quad (1)$$

ligand. The dissociation constants $K$ were varied to obtain the best fit to the observed binding. The resulting estimates are apparent values (Adair constants) and are not necessarily true intrinsic dissociation constants. The lengths of error bars in the figures represent standard errors of the means.

Muscle phosphofructokinase catalyzes the hydrolysis of ATP or GTP (Uyeda, 1970). This hydrolysis, although extremely slow in comparison with the phosphorylation activity of the enzyme, proceeds far enough in a few hours to render the results of binding experiments ambiguous. Therefore, when the binding of ATP or GTP was to be studied, pyruvate kinase and phosphoenolpyruvate were included in the medium to rephosphorylate any diphosphate formed.

In Tris buffer, muscle phosphofructokinase is somewhat unstable at room temperature and loses a significant fraction of its activity during an equilibrium dialysis run of several hours. Therefore, the enzyme was dissolved in glycylglycine/β-glycerophosphate buffer for the dialysis experiments. Under these conditions there was little or no loss of activity during dialysis. In this buffer, phosphofructokinase is somewhat less sensitive to inhibition by ATP than in Tris buffer (Colombo et al., 1975) and the affinity for fructose 6-P is somewhat greater. However, the kinetic behavior of the enzyme is generally similar in both buffers.

*Assays for Phosphofructokinase Activity.* Phosphofructokinase activity was assayed as described by Kemp (1971) with modifications. A unit of activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of fructose bisphosphate/min at pH 8.0 and 37 °C. Crystalline enzyme was dissolved in 50 mM β-glycerophosphate, 0.1 mM EDTA, 0.2 mM dithiothreitol, and 0.1 mM ATP at pH 8.0 to a final concentration of 2–3 units/mL. The assay mixture contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 3.0 mM $(NH_4)_2SO_4$, 4.0 mM magnesium acetate, 0.16 mM NADH, 2.0 mM ATP, 2.0 mM fructose 6-P, 1.0 unit of aldolase, 3.0 units of α-glycerophosphate dehydrogenase, 34 units of triosephosphate isomerase, and 0.01–0.015 unit of phosphofructokinase. The reaction was started by addition of fructose 6-P after a 2-min preincubation at 37 °C. Enzyme activity was calculated from the rate of change in absorbance at 340 nm.

The effects of modifiers on phosphofructokinase activity were studied at pH 7.0 and 25 °C. Aldolase, α-glycerophosphate dehydrogenase, and triosephosphate isomerase were dialyzed against 50 mM Tris-HCl, 0.1 mM DTT, and 0.1 mM EDTA at pH 7.5 to remove ammonium sulfate. Crystalline phosphofructokinase was dissolved in 50 mM β-glycerophosphate, 0.1 mM EDTA, 0.2 mM DTT, and 0.1 mM ATP at pH 8.0 to a final concentration of 2–3 units/mL. The assay mixture contained 25 mM glycylglycine, 25 mM β-glycerophosphate, 0.1 mM EDTA, 0.1 mM DTT,[1] 50 mM KCl, 4.0 mM $Mg(OAc)_2$, 0.16 mM NADH, 1.0 unit of aldolase, 3.0 units of α-glycerophosphate dehydrogenase, 24 units of triosephosphate isomerase, various concentrations of ATP and fructose 6-P, 0.005–0.015 unit of phosphofructokinase, and modifiers at pH 7.0. The reaction was initiated by addition of fructose 6-P after incubation for 2 min at 25 °C.

[1] Abbreviations: AMP-PNP, adenyl-5′-yl imidodiphosphate; DTT, dithiothreitol; $S_{0.5}$, concentration of substrate at which the reaction velocity is half of the maximal rate or concentration of ligand at which half of a class of binding sites are occupied; $I_{0.5}$, concentration of inhibitor at which half-maximal inhibition is observed.

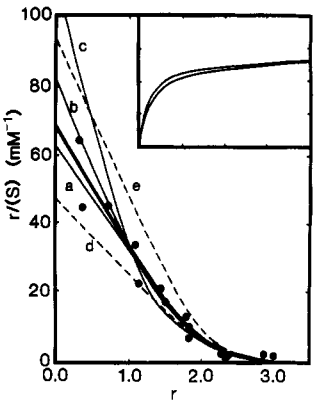


FIGURE 1: Binding of ATP by rabbit muscle phosphofructokinase. In this Scatchard plot, $r$ is the number of moles of [U-$^{14}$C]ATP bound per mole of enzyme subunit and (S) is the concentration of free ATP. Binding was determined at pH 7.0 and 25 °C in the presence of 1.0 mM phosphoenolpyruvate and 0.5 unit of pyruvate kinase. The concentration of phosphofructokinase was 1.3 mg/mL. The curves in the figure are obtained from the equation for simple Michaelis binding at three noninteracting sites (see the text). The heavy line is for the dissociation constants that gave the best fit to the experimental results: 21, 53, and 1110 μM. For the other curves, the dissociation constant for the third site was left at 1110 μM and the others were varied to test the sensitivity of the fit to variation in assigned values of the constants. For the solid curves, the first two sites were varied so as to hold their geometric mean approximately constant: a, 33 and 33; b, 15 and 74; c, 10 and 113. The broken curves are for two sites with identical dissociation constants: d, 22 and 22; e, 44 and 44. All dissociation constants are expressed in micromolar units. Inset: Direct plot of $r$ as a function of (S). The upper curve corresponds to both the heavy curve and curve a of the main figure, and the lower curve corresponds to curve c.

*Purification of AMP-PNP.* The triethylammonium salt of AMP-PNP was purified by a modification of the procedure of Yount et al. (1971a). The nucleotide (20–30 μmol) was applied to a column (0.4 × 25 cm) packed with DEAE-Sephadex ($HCO_3^-$). The column was washed with 200 mL of water, and AMP-PNP was eluted with 500 mL of a linear gradient from 0 to 1.0 M triethylammonium bicarbonate, pH 7.5. The peak fractions containing AMP-PNP were pooled and lyophilized. The final product was dissolved in 1–2 mL of water, divided into 200-μL portions, and stored at −70 °C. This product was at least 99% pure as determined by high-pressure liquid chromatography and was stable for at least 1 month at −70 °C.

*Purity of Nucleotides and [$^{14}$C]Citrate.* The purities of ATP, ADP, AMP, AMP-PNP, [$^{14}$C]ATP, and [$^{14}$C]GTP were assayed by chromatography on polyethylenimine–cellulose thin-layer plates with 0.65 M LiCl as the elution solvent and by high-pressure liquid chromatography in an anion-exchange column (Whatman Partisil-10 SAX) with a linear elution gradient of phosphate buffer from 1 mM, pH 3.35, to 700 mM, pH 4.5.

The purity of [$^{14}$C]citrate was determined by chromatography on cellulose thin-layer plates with phenol/water/formic acid (82/17/1) as the elution solvent (Myers & Huang, 1969). The plate was sprayed with bromcresol green to locate carboxylic acids.

*Other Methods.* The concentration of phosphofructokinase was estimated spectrophotometrically by assuming an extinction coefficient at 280 nm of 1.02 mL mg$^{-1}$ cm$^{-1}$ (Parmaggiani et al., 1966). The amount of protein was determined by the method of Schaffner & Weissman (1973).

## RESULTS

*Binding of ATP and GTP.* A Scatchard plot of the results of an equilibrium dialysis measurement of the binding of ATP

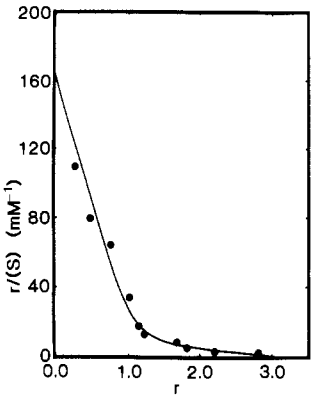


FIGURE 2: Binding of GTP by rabbit muscle phosphofructokinase. Binding of [U-$^{14}$C]GTP was determined in the presence of 1.0 mM phosphoenolpyruvate and 0.5 unit of pyruvate kinase at pH 7.0 and 25 °C. The concentration of phosphofructokinase was 1.4 mg/mL.

by phosphofructokinase (Figure 1) suggests that two sites bind with high affinities and that there is a third site at which ATP is bound more weakly. The best fit of these results to eq 1 was obtained with dissociation constants of 21 $\mu$M, 53 $\mu$M, and 1.11 mM (Figure 1, heavy line). However, when two sites bind with comparable affinities, it is impossible to estimate their individual strengths with any precision. The binding curve corresponding to any such pair will be nearly indistinguishable from that for any other pair with the same or a closely similar geometric mean if the ratio of the dissociation constants does not exceed about 10 (Figure 1, light curves and inset). When the geometric means differ by even a relatively small amount, however, the resulting curves are readily distinguishable, as illustrated by the broken lines in Figure 1. Thus, it may be concluded from these results that the dissociation constants for the two tightly binding sites do not differ widely and that their geometric mean is not far from 33 $\mu$M. Although the results suggest the existence of a third weakly binding site, the possibility of still weaker binding at more than one site cannot be excluded. In any case we doubt that this binding corresponding to the third apparent dissociation constant has any kinetic significance.

The binding of GTP to phosphofructokinase is shown as a Scatchard plot in Figure 2. Again the results suggest binding at three sites, but in this case the apparent affinity of the first site is much greater than that of the second. Best-fit values of apparent dissociation constants derived from these results are 6.3 $\mu$M, 280 $\mu$M, and 1.04 mM.

*Binding of Citrate.* Under the conditions of our experiments, phosphofructokinase binds citrate only weakly in the absence of ATP (Figure 3). In the presence of ATP, the amount of citrate bound is a hyperbolic function of the concentration of citrate (Figure 3); a Scatchard plot of the results (not shown) is linear, indicating no cooperativity. The $S_{0.5}$ value for citrate in the presence of 200 $\mu$M ATP is about 70 $\mu$M (estimated from a Scatchard plot of the results presented in Figure 3). This result is in reasonably good agreement with the observation that citrate inhibition of the catalytic actiity of phosphofructokinase with ATP as the phosphoryl donor was half-maximal at a concentration of about 65 $\mu$M (Figure 4). The observation that one molecule of citrate is bound per subunit is in agreement with results from other laboratories (Colombo et al., 1975; Lorenson & Mansour, 1969).

When the concentration of citrate is held constant, the amount of that modifier bound is a hyperbolic function of the concentration of ATP (Figure 5A). A Scatchard plot of these results yields an estimate of 36 $\mu$M for the concentration of ATP having a half-maximal effect, in agreement with the

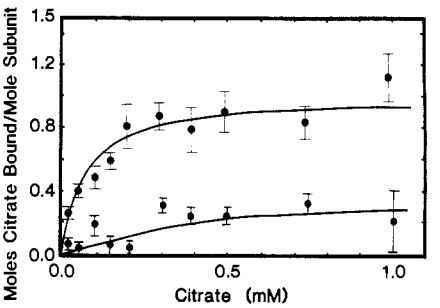


FIGURE 3: Binding of citrate by rabbit muscle phosphofructokinase; effect of ATP. The binding of [1,5-$^{14}$C]citrate was determined at pH 7.0 and 25 °C in the presence of 1.0 mM phosphoenolpyruvate and 0.5 unit of pyruvate kinase. Upper curve, ATP present at 200 $\mu$M; the concentration of phosphofructokinase was 1.1 mg/mL. Lower curve, no ATP; the concentration of phosphofructokinase was 0.8 mg/mL.

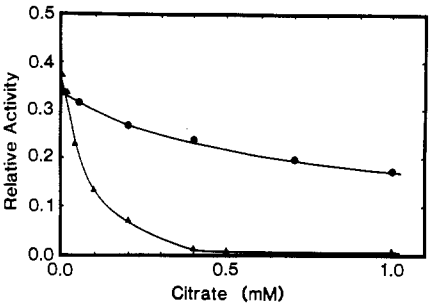


FIGURE 4: Inhibition of catalytic activity of phosphofructokinase by citrate; dependence on phosphate donor. The assay mixture contained 200 $\mu$M fructose 6-P, 1.0 mM phosphoenolpyruvate, 3.0 units of pyruvate kinase, and standard components as described under Materials and Methods. The assay was run at pH 7.0 and 25 °C. The phosphate donor was 1.0 mM GTP for the upper curve and 1.0 mM ATP for the lower curve.

dissociation constants estimated by equilibrium dialysis.

The enhancement of citrate binding by ATP is consistent with the interactions of citrate and ATP as synergistic inhibitors of the catalytic activity of phosphofructokinase. Since GTP does not inhibit the enzyme, it might be expected also not to affect the binding of citrate. However, citrate binding was found to be markedly enhanced by GTP (Figure 5B). The enhancement does not seem to be hyperbolic, and no valid estimate of binding parameters can be obtained from the nonlinear Scatchard and double-reciprocal plots. However, it is evident that the magnitude of the effect is comparable to that of ATP, and the sensitivity of the response appears to exceed that of ATP. No ATP contamination of the GTP used in these studies was found by HPLC analysis, where ATP contamination of about 0.5% should have been detected.

In view of the pronounced effects of GTP on citrate binding, it seems surprising that citrate inhibits the catalytic activity of phosphofructokinase only very weakly when GTP is the phosphate donor (Figure 4).

*Effects of Other Modifiers on the Binding of Citrate.* As seen earlier (Figure 3), phosphofructokinase binds citrate only weakly in the absence of ATP. This weak binding is not affected significantly by the other substrate, fructose 6-P, or by ADP or AMP, which are activators of phosphofructokinase activity (results not shown). It is not possible to measure the effects of fructose 6-P or ADP on the enhanced binding of citrate caused by ATP because of the reaction that would occur if both fructose 6-P and ATP were present simultaneously and because pyruvate kinase cannot be used to stabilize the concentration of ATP if effects of ADP are to be studied. Therefore, the ATP analogue AMP-PNP (Yount et al.,

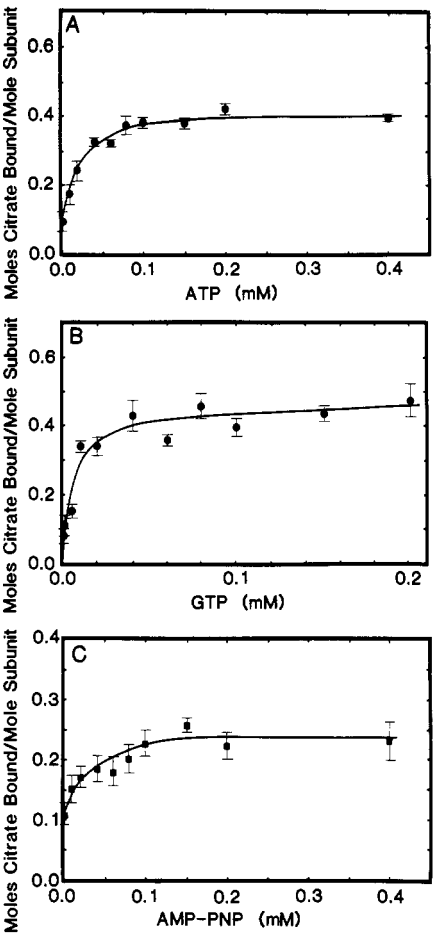


FIGURE 5: Binding of citrate by rabbit muscle phosphofructokinase as a function of the concentration of nucleoside triphosphates. The binding of [1,5-$^{14}$C]citrate was determined at pH 7.0 and 25 °C in the presence of 1.0 mM phosphoenolpyruvate and 0.5 unit of pyruvate kinase. (A) ATP was present at concentrations indicated on the abscissa. The concentration of phosphofructokinase was 1.2 mg/mL. (B) GTP was present at concentrations indicated. The concentration of phosphofructokinase was 0.78 mg/mL. (C) AMP-PNP was present at concentrations indicated. The concentration of phosphofructokinase was 1.0 mg/mL.

1971a,b) was used to simulate the ATP enhancement of citrate binding. This analogue is not a substrate for phosphofructokinase, but it has been shown to bind to the enzyme (Wolfman et al., 1978; Pettigrew & Frieden, 1979) and inhibits its catalytic activity competitively with ATP (Barzu et al., 1977; Lad et al., 1977).

Before using the analogue as a substitute for ATP, it was necessary to know whether it binds to the catalytic site, the regulatory site, or both. Since the inhibitory effect of ATP is exerted by decreasing the affinity for fructose 6-P at the catalytic site, inhibition can be minimized by use of a high concentration of fructose 6-P. Inhibition is also decreased by unphysiologically high levels of $Mg^{2+}$ (Hofer & Pette, 1968; Mansour & Ahlfors, 1968; Otto et al., 1974). At pH 7, in the presence of a high concentration (2 mM) of fructose 6-P and 5 mM magnesium acetate, the rate of the reaction is a nearly normal hyperbolic (Michaelis) function of ATP concentration. Double-reciprocal plots yielded estimates of 44 $\mu$M for the $S_{0.5}$ for ATP and 13 $\mu$M for the $I_{0.5}$ for AMP-PNP. At pH 8.0, where ATP has been reported not to inhibit (Hanson et al., 1973), the same concentrations of fructose 6-P and $Mg^{2+}$ gave estimates of 85 $\mu$M and 79 $\mu$M, respectively, for those parameters. Thus, it appears that AMP-PNP is bound at the catalytic site with an affinity equal to or greater than that of ATP.

To test for binding of the analogue at the regulatory site, the rate of reaction as a function of fructose 6-P concentration with GTP as the phosphate donor was measured in the absence and in the presence of AMP-PNP. Competitive binding at the catalytic site will contribute to the observed inhibition, but any effects on the shape of the curve and on the apparent affinity for fructose 6-P can be attributed to binding at the regulatory site. The addition of 400 $\mu$M AMP-PNP converted the response curve as a function of fructose 6-P concentration from hyperbolic to sigmoidal and raised the value of $S_{0.5}$ for fructose 6-P from 90 $\mu$M to about 500 $\mu$M. Since these are the same effects as are caused by ATP at the regulatory site, it may be concluded that AMP-PNP mimics ATP both in binding at that site and in the effects of such binding on affinities at the catalytic site.

The analogue AMP-PNP also resembles ATP in enhancing the binding of citrate by phosphofructokinase (Figure 5C). The change in affinity for citrate is not as pronounced as that caused by ATP, as shown by the smaller amount of citrate bound at 100 $\mu$M in the presence of saturating levels of the analogue as compared to ATP, but the affinity for AMP-PNP at the regulatory site must resemble that for ATP, as shown by the similar ranges of concentrations of the two nucleotides over which the affinity for citrate is affected. The effect was estimated from a Scatchard plot to be half-maximal at an AMP-PNP concentration of about 22 $\mu$M.

Both ADP and AMP decreased the binding of citrate in the presence of AMP-PNP. When the analogue was present at 200 $\mu$M, the enhancement of binding was nearly abolished by 50 $\mu$M ADP or by 200 $\mu$M AMP. Under similar conditions 500 $\mu$M fructose 6-P caused a decrease in citrate binding of about 25% (results not shown).

## Discussion

Enhancement by ATP of the binding of citrate by phosphofructokinase has been previously reported (Colombo et al., 1975). This interaction is consistent with the synergistic inhibition of the catalytic activity of this enzyme by ATP and citrate. The effect of ATP on citrate binding was half-maximal at about 36 $\mu$M, which agrees well with the dissociation constants that we observed for ATP binding. It follows from the calculated curves of Figure 1 and the corresponding discussion in the text that it is not possible from the results of binding studies to determine whether enhancement of citrate binding results from binding of ATP at the site with the highest or second-highest affinity for ATP. Colombo et al. (1975) interpreted their results as indicating that in the presence of fructose 6-P the regulatory site has a lower dissociation constant for ATP (about 13 $\mu$M) than the catalytic site. The reason for the discrepancy between their results and ours is not clear.

It would be expected that the synergistic inhibitory interaction between ATP and citrate would result from binding of ATP at the regulatory site. The similar affinities for ATP of the catalytic and regulatory sites make it difficult to test this prediction directly by binding studies. However, our results are consistent with the interpretation that the enhancement by GTP of citrate binding results from interaction of GTP with the highest affinity site. Half-maximal enhancement of citrate binding is seen at a GTP concentration of about 10 $\mu$M (Figure 5B). This value resembles the lowest binding constant for GTP, 3 $\mu$M, much more closely than the second constant, 280 $\mu$M (Figure 2), and is also similar to the kinetic Michaelis constant for GTP as phosphoryl donor, 14 $\mu$M (results not shown). These results suggest that binding of GTP at the catalytic site enhances the affinity of the enzyme for citrate.

By analogy, the effect of ATP on affinity for citrate might result from binding at the catalytic site (or at both sites). This is consistent with the finding that when the concentration of ATP is near or below the kinetic Michaelis constant citrate increases the rate of the phosphofructokinase reaction (Kemp et al., 1976; our similar results not shown). A possible interpretation is that binding of ATP at the catalytic site increases the affinity of the enzyme for citrate and that citrate binding increases the affinity of both sites for ATP.

Wolfman et al. (1978) reported that the binding of AMP-PNP at the regulatory site was increased by the presence of citrate. Since the plot of rate as a function of fructose 6-P concentration with GTP as phosphoryl donor was converted from hyperbolic to sigmoidal on the addition of AMP-PNP, that ATP analogue appears to bind at the regulatory site and mimic the effect of ATP at that site. Wolfman et al. (1978) observed high- and low-affinity binding sites for AMP-PNP with dissociation constants for approximately 1 and 100 $\mu$M at 4 °C. The differences between their results and ours may result at least in part from the differences in temperatures and buffers employed.

The binding results presented here are in general agreement with the kinetic behavior of the enzyme. Inhibition by ATP is counteracted by ADP and AMP; these interactions result in a response to variation in the adenylate energy charge of the type normally seen in ATP-regenerating pathways (Atkinson, 1977). ADP and AMP also decrease the inhibitory effect of citrate (Kemp, 1971; Passonneau & Lowry, 1963; Tornheim & Lowenstein, 1976). The decrease in citrate binding observed on addition of ADP or AMP is consistent with those kinetic effects.

The calculated curves of Figure 1 show that when a substrate or other ligand binds to two or more sites with binding constants that are of the same order of magnitude, the results of kinetic or binding experiments are quite insensitive to the values of the individual binding constants. As is shown in the inset of Figure 1, a difference of 10-fold in binding constants would be very difficult to distinguish from equal affinities, even with highly precise binding measurements. That inherent insensitivity imposes a limitation on the accuracy of estimation of binding constants in such cases that we had not recognized and one that we have not seen previously commented on.

**Registry No.** ATP, 56-65-5; GTP, 86-01-1; AMP-PNP, 25612-73-1; citric acid, 77-92-9; phosphofructokinase, 9001-80-3.

## References


Atkinson, D. E. (1977) *Cellular Energy Metabolism and Its Regulation*, Academic Press, New York.

Barzu, O., Tilinca, R., Porutiu, D., Gorun, V., Jebeleanu, G., Ngoc, L. D., Kezdi, M., Goia, I., & Mantsch, H. H. (1977) *Arch. Biochem. Biophys. 182*, 42–51.

Colombo, G., Tate, P. W., Girotti, A. W., & Kemp, R. G. (1975) *J. Biol. Chem. 250*, 9404–9412.

Hanson, R. L., Rudolph, F. B., & Lardy, H. A. (1973) *J. Biol. Chem. 248*, 7852–7859.

Hofer, H. W., & Pette, D. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1378–1392.

Kemp, R. G. (1971) *J. Biol. Chem. 246*, 245–252.

Kemp, R. G. (1975) *Methods Enzymol. 17*, 67.

Kemp, R. G., Tsai, M. Y., & Colombo, G. (1976) *Biochem. Biophys. Res. Commun. 68*, 942–948.

Lad, P. M., Hill, D. E., & Hammes, G. G. (1973) *Biochemistry 12*, 4303–4309.

Lad, P. M., Wolfman, N. M., & Hammes, G. G. (1977) *Biochemistry 16*, 4802–4806.

Lorenson, M. Y., & Mansour, T. E. (1969) *J. Biol. Chem. 244*, 6420–6431.

Mansour, T. E. (1963) *J. Biol. Chem. 238*, 2285–2292.

Mansour, T. E., & Ahlfors, C. E. (1968) *J. Biol. Chem. 243*, 2523–2533.

Myers, W. F., & Huang, K. Y. (1969) *Methods Enzymol. 13*, 431.

Otto, M., Heinrich, R., Kuhn, B., & Jacobasch, G. (1974) *Eur. J. Biochem. 49*, 169–178.

Parmaggiani, A., Luft, J. H., Love, D. S., & Krebs, E. G. (1966) *J. Biol. Chem. 241*, 4625–4637.

Passonneau, J. V., & Lowry, O. H. (1963) *Biochem. Biophys. Res. Commun. 13*, 372–379.

Pettigrew, D. W., & Frieden, C. (1979) *J. Biol. Chem. 254*, 1887–1895.

Ramaiah, A., Hathaway, J. A., & Atkinson, D. E. (1964) *J. Biol. Chem. 239*, 3619–3622.

Richards, C. S., & Uyeda, K. (1980) *Biochem. Biophys. Res. Commun. 97*, 1535–1540.

Schaffner, W., & Weissman, D. (1973) *Anal. Biochem. 56*, 502–514.

Tornheim, K., & Lowenstein, J. M. (1976) *J. Biol. Chem. 251*, 7322–7328.

Uyeda, K. (1970) *J. Biol. Chem. 254*, 2268–2275.

Wolfman, N. M., Thompson, W. R., & Hammes, G. G. (1978) *Biochemistry 17*, 1813–1817.

Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971a) *Biochemistry 10*, 2484–2596.

Yount, R. G., Ojala, D., & Babcock, D. (1971b) *Biochemistry 10*, 2490–2496.