Reaction Path of Phosphofructo-1-kinase Is Altered by Mutagenesis and Alternative Substrates†

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ABSTRACT: *Escherichia coli* phosphofructokinase (PFK) has been proposed to have a random, nonrapid equilibrium mechanism that produces nonallosteric ATP inhibition as a result of substrate antagonism. The consequences of such a mechanism have been investigated by employing alternative substrates and mutants of the enzyme that produce a variety of nonallosteric kinetic patterns demonstrating substrate inhibition and sigmoid velocity curves. Mutations of a methionine residue in the sugar phosphate binding site produced apparent cooperativity in the interaction of fructose 6-phosphate. Cooperativity could also be seen with native enzyme using a poorly binding substrate, fructose 1-phosphate. With an alternative nucleotide, 1-carboxymethyl-ATP, coupled with a mutation that introduced a negative charge in the nucleotide binding site, one could observe substrate inhibition by fructose 6-phosphate and apparent cooperativity in the interaction with nucleotide. Furthermore, the use of a phosphoryl donor, γ-thiol-ATP, which greatly reduced the catalytic rate, apparently facilitated the equilibration of all binding reactions and eliminated ATP inhibition. These unusual kinetic patterns could be interpreted within the random, steady-state model as reflecting changes in the rates of particular binding and catalytic events.

Escherichia coli phosphofructokinase (PFK)[1] is recognized as a complex regulatory enzyme that is activated by GDP and inhibited by PEP as a result of interaction of these effectors at a single site distinct from the catalytic site (*1*). In addition to the allosteric regulation of the enzyme by GDP and PEP that can be explained by a transition between R and T states, other apparent cooperative interactions do not easily fit the concerted transition allosteric model. In particular, the enzyme is inhibited by ATP in a process that appears to be mechanism-based as opposed to allosteric. Deville-Bonne et al. (*2*) suggested that ATP and Fru-6-P bind at random, but not independently, and that there is a negative interaction between the two substrates. These observations were confirmed by additional kinetic studies that suggested the two substrates antagonize each other in a saturable manner (*3*, *4*). The phenomenon of substrate antagonism was studied at the protein level as well by measuring interactions of substrates by intrinsic fluorescence studies (*5*, *6*), equilibrium binding (*6*), and protection against proteolysis (*7*). However, such interactions would not lead to substrate inhibition in a random mechanism as long as the catalytic reaction is sufficiently slow to permit equilibration during substrate binding. Kinetic studies with a PFK mutant that had a decreased maximal velocity suggested that ATP inhibition of the wild-type enzyme results from a random mechanism wherein the binding of the first substrate lowers the affinity for the second substrate and with the rate of catalysis too high to permit rapid equilibrium in substrate binding and release (*4*). Byrne et al. (*8*) reached a similar conclusion in describing the inhibition of *Bacillus stearothermophilus* PFK inhibition by ATP. This phenomenon has been observed by others where a different interpretation has been applied. Auzat et al. (*9*) reported that saturation by Fru 6-P was no longer cooperative either when γ-thio-ATP is used with wild-type enzyme or with the T125S mutant of PFK. Their interpretation was that substitution of sulfur in ATP or the loss of the methyl group of threonine locked PFK in its active conformation. An alternative interpretation of these data is the phosphoryl transfer from thio-ATP is much slower than with ATP, which allows substrate equilibration and loss of mechanism-based, nonhyperbolic behavior. In the current study, not only does one observe a mechanism-based inhibition of the enzyme by ATP, but mechanism-based cooperativity with sugar phosphate, inhibition by Fru 6-P, and cooperativity with the nucleotide phosphoryl donor with the alternative substrates or with site-directed mutants with altered substrate affinity. Furthermore, mutants or alternative substrates that decrease the catalytic step abolish this apparent cooperativity, supporting the mechanism-based interpretation of these phenomena.

MATERIALS AND METHODS

Materials. *E. coli* strains used in these studies were DF 1020 [*pro*-82, Δ*pfk*201, *recA*56, Δ(*rha-pfkA*)200, *endA*1, *hsdR*17, *supE 44*] and XL1-Blue MRF′ supercompetent cell [Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *recA*, *lacI*^{q}ZΔ*M15*-F′] from Stratagene (La Jolla, CA). The plasmid bearing the PFK gene, pRZ3, has been described previously

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[1] Abbreviations: PFK, phosphofructo-1-kinase; Fru 6-P, fructose 6-phosphate; Fru 1,6-bisP, fructose 1,6-bisphosphate; γ-S-ATP, γ-thio-ATP; cmATP, 1-carboxymethyl-ATP; PCR, polymerase chain reaction.

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(*4*). The following mutants used in this study have been described previously: R111E, M169A, and M169L (*10*, *11*). All reagents were purchased from Sigma Chemical Co. unless otherwise indicated. The concentration of nucleoside triphosphates and sugar phosphate substrates were determined spectrophotometrically in PFK coupled assays with limiting concentrations of the analyzed substrate. One of the substrates, γ-thio-ATP (γ-S-ATP), has a considerable contamination by ADP. It should be noted that ADP should have no effect on any of the kinetics described in the following because of the presence in all assays of saturating GDP.

PCR Mutagenesis. An additional mutant (R25S) was prepared using a modified two-stepped PCR protocol (*12*). Two PCR reactions were performed in the first step. One contained a mutagenic antisense primer and a second sense primer that was upstream from the *Hin*dIII site that flanks the 5′ end of the gene. The second reaction contained a sense mutagenic primer and an antisense primer downstream from the *Bgl*II site within the gene. The PCR products were purified with a Prep-A-Gene DNA Purification Kit (Bio-Rad, Hercules, CA). The second round PCR was performed using the first round products as template. This PCR product was purified as before and digested with *Hin*dIII and *Bgl*II. The digested product was again purified with the DNA Purification Kit and subsequently ligated with pRZ3, and transformed into XL1-Blue MRF′ supercompetent cells. The modified pRZ3 then was purified and sequenced to verify the mutation using the ThermoSequenase cycle sequencing kit from Amersham (Cleveland, OH). After confirmation of the mutation site, the entire gene was sequenced in one direction at the University of Iowa sequencing facility.

Expression and Purification of the Enzymes. The mutated *E. coli* PFK was expressed in DF 1020. The bacteria were grown in LB media containing 100 ng/mL ampicillin. The wild-type and several high activity mutants were purified to homogeneity by the method of Kotlarz and Buc (*13*) as modified by Banas et al. (*14*) using Blue Sepharose CL-6B affinity chromatography washed with 50 mM Tris/acetate, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.4 (buffer A) containing 0.6 M KCl, then eluted with 1 mM ATP and 2.5 mM $MgCl_2$ in the washing solution. The mutant R111E, which was bound weakly by the column, was purified to more than 95% purity using Blue Sepharose CL-6B that was washed extensively with buffer A without added KCl and eluted with 1 mM ATP and 2.5 mM $MgCl_2$ in buffer A. The enzymes were subsequently purified to homogeneity by ion-exchange chromatography using Uno-Q (Bio-Rad, Hercules, CA). The purified enzymes were stored in the presence of 2 mM ATP and 50% glycerol in − 20 °C. Before use, the enzymes were dialyzed against assay buffer. All mutant preparations were judged to be homogeneous by SDS−PAGE.

To verify the integrity of the overall structure, circular dichroism was employed. Spectra were determined using Jasco 700 spectropolarimeter with a 2-mm path at room temperature and a time constant of 1 s at 50 nm/min. Native and modified enzymes were dialyzed against 20 mM potassium phosphate at pH 7.2 prior to spectral determinations.

Enzyme Assays. Enzyme activity for both native and modified *E. coli* PFK was assayed at 30 °C and at pH 7.2 in a Gilford *Response* spectrophotometer. For standard assays, the medium contained 100 mM Tes/KOH, 1 mM EDTA, 0.2 mM NADH, 1 mM GDP, 4 mM $MgCl_2$, the indicated concentrations of MgATP, Fru 6-P or alternative substrates, 1 mM dithiothreitol, 0.6 unit of aldolase, and 0.3 unit each of triose-phosphate isomerase and glycerophosphate dehydrogenase. Because ATP was added as an equimolar mixture of $MgCl_2$ and ATP, the magnesium ion concentration was kept 4 mM higher than the concentration of ATP and exceeded by 2 mM the combined concentrations of ATP, EDTA, and GDP to ensure that virtually all of the ATP existed as the MgATP complex in all reactions. Reactions were initiated by the addition of Fru-6-P. All reagents and assay auxiliary enzymes were purchased from Sigma.

Analysis of kinetic parameters was carried out using the GraFit graphical analysis program. Fru-6-$P_{0.5}$ was obtained using the Hill equation and the K_m for ATP using the Michaelis−Menten equation.

Other Methods. An image of the *E. coli* PFK was viewed on a Silicon Graphics 4 Indigo^2 R10,000 running version 96.0516 of Quanta. The Brookhaven Protein Databank (Brookhaven ID 1PFK) provided the initial ATP−PFK crystal structure coordinates of the R state complex with Fru 1,6-bisP, ADP, and magnesium ion (*1*).

The concentration of protein was determined by Bradford's dye binding assay with bovine serum albumin as the standard (*15*). Gel electrophoresis of proteins was carried out using 12% polyacrylamide support according to the system of Laemmli (*16*).

1-Carboxymethyl-ATP was synthesized as described by Lindberg and Mosbach (*17*) and purified by column chromatography on DEAE-Sephadex with a linear gradient to 1 M ammonium bicarbonate. The nucleotide was desalted by column chromatography in water on Sephadex G-10.

RESULTS AND DISCUSSION

Studies with an ADP-Insensitive Mutant. One possible explanation for the observed ATP inhibition of bacterial PFK is that ATP interacts with the PEP/ADP allosteric site to produce an inhibited conformation. Lau and Fersht (*18*) showed that mutation of Arg25 to Ala reduced binding of GDP by 30-fold and abolished PEP binding. This residue is thought to be involved in the binding of the α- and β-phosphate groups of ADP or GDP. One would presume that possible binding to this site by ATP would also be disrupted. In the current study Arg25 was converted to Ser, which was found to eliminate GDP activation of the enzyme. This mutant enzyme showed a pattern of ATP inhibition nearly identical to that of wild-type enzyme (data not shown), suggesting that binding of ATP to the allosteric site is not the mechanism of ATP inhibition. These data are similar to those obtained with *B. stearothermophilus* by Byrnes et al. (*8*) who showed that a mutant that was incapable of reversing PEP inhibition by ADP continued to display ATP inhibition.

Kinetic Model for PFK. Shown in Scheme 1 is a kinetic model for the forward reaction that has been proposed for *E. coli* PFK (*4*) and *B. stearothermophilus* PFK (*8*) in the presence of GDP, which should ensure that the enzyme is in the fully active R State.

In a random, nonrapid equilibrium mechanism the rate constant k_9 is greater than any other forward rate constant. Thus, the rate-limiting step is not the catalytic transformation

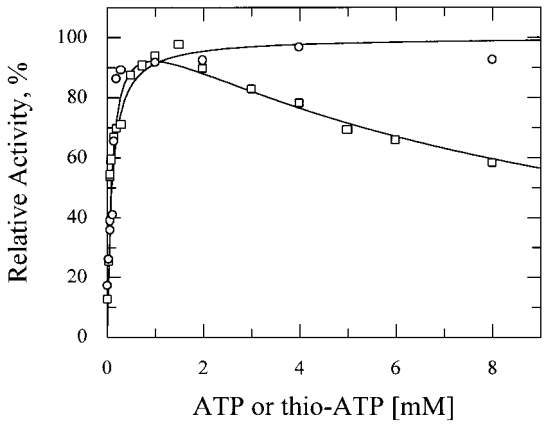
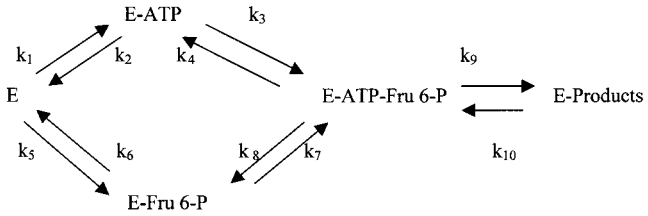


FIGURE 1: γ-Thio-ATP does not show substrate inhibition. Data are expressed as relative activity to maximal activity under these conditions where k_{cat} was 49 s^{-1} with ATP (squares) and 0.016 s^{-1} with γ-thio-ATP (circles). The assay was performed at pH 7.2 with 0.1 mM Fru 6-P and 1mM GDP and other conditions described in the Material and Methods.

Scheme 1



but one of the binding events. Of the four binding reactions, E to E-Fru 6-P is the fastest followed by E to E-ATP. The binding of the second substrate in each case is slower, consistent with the previously discussed substrate antagonism. Inhibition by ATP occurs at low concentrations of Fru 6-P when the high concentration of ATP forces the reaction into the slower E to E-ATP path and the high rate of catalytic transformation prevents equilibration of the binding events.

In the experiments described below, the features of this mechanism will be examined by altering the rates of the E to E-Fru 6-P and E to E-ATP pathways by using alternative substrates and *E. coli* PFK mutants that change the relative rates of the binding and catalytic events. Note that in all experiments, GDP is present at 1.0 mM to force the enzyme into the active conformation, and any observed cooperative phenomena should be mechanistic rather than allosteric.

γ-Thio-ATP, a Substrate with Reduced Rate of Phosphoryl Transfer. γ-Thio-ATP has been shown to be an alternative phosphoryl donor for PFK with a greatly reduced rate of catalysis (*9*). These results were confirmed here. When assayed at subsaturating Fru 6-P (0.1 mM), the observed k_{cat} with γ-S-ATP was 0.016 s^{-1} compared to 49 s^{-1} with ATP, a reduction of more than 3000-fold. On the other hand, the apparent K_ms under these conditions were very similar; 0.1 mM for ATP and 0.096 for γ-S-ATP. The apparent K_ms for Fru 6-P when assayed at 2 mM nucleotide were 0.1 mM with ATP as the second substrate and 0.14 mM with γ-S-ATP as the second substrate. However, the more significant difference in the kinetics with the two substrates is illustrated in Figure 1. With γ-S-ATP, substrate inhibition is not observed at high concentrations of the nucleotide. The explanation for this phenomenon is that lowering of the rate of the catalytic step (k_9 in Scheme 1) has made it the rate-limiting reaction. This now produces equilibration of the binding events and permits flux through the faster E to E-Fru 6-P path even at high nucleotide concentration.

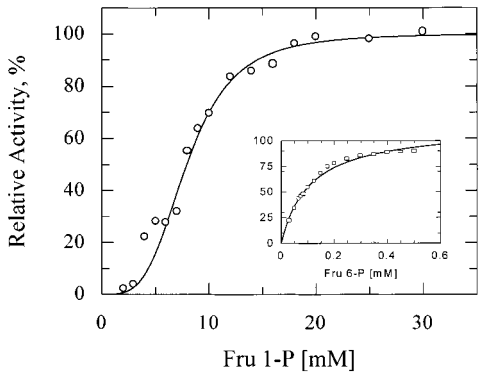


FIGURE 2: Cooperative kinetics with Fru 1-P. Data are expressed as relative activity to maximal activity with each substrate. For Fru 6-P (shown in inset) k_{cat} was 88 s^{-1}. With Fru 1-P, k_{cat} was 1.4 s^{-1}. The assay was performed at pH 7.2 with 1.0 mM ATP and 1mM GDP and other conditions described in the Material and Methods.

In an earlier study, we showed that a PFK mutant with a decreased k_{cat} but with little change in apparent substrate affinity also showed no ATP inhibition (*4*). The results of the mutant studies can be interpreted in identical manner to the γ-S-ATP data as supporting a nonequilibrating model for the wild-type enzyme.

Fru 1-P as an Alternative Substrate. Substrate specificity studies of mammalian PFK have shown that Fru 1-P can serve as a substrate, but with an increased K_m and lowered catalytic activity (*19*). In the current study, Fru 1-P was examined as a substrate for *E. coli* PFK. Because the product of the reaction is Fru 1,6-bisP, it can be assayed in the same coupled assay as that used for Fru 6-P. The apparent k_{cat} with Fru 1-P was found to be 60-fold lower than that seen with Fru 6-P and the concentration necessary for half-maximal activity was 7.9 mM, much higher than that seen with Fru 6-P. As shown in Figure 2, the most striking result was the strong apparent cooperativity seen with Fru 1-P. Again, it must be emphasized that this is observed under conditions where allosteric cooperativity should not be seen; that is, in the presence of the potent allosteric activator, GDP. These results are nonetheless consistent with model described earlier if one concludes that the rate constants for the addition of Fru 1-P to the enzyme (k_5 in Scheme 1) and to the E−ATP complex (k_3) are slower than that seen with Fru 6-P. Because substrate binding is rate-limiting, the overall rate is reduced. Furthermore, at low Fru 1-P concentrations, the reaction will proceed by the slower E to E-ATP path, and to the faster E to E-Fru 1-P path at higher concentrations of sugar phosphate. Thus, the cooperative interaction with Fru 1-P is seen as described in Figure 2.

With Fru 1-P, the apparent affinity for ATP is high. In the presence of 10 mM Fru 1-P, half-saturation for ATP was achieved near 0.02 mM. Furthermore, ATP inhibition was seen. Under these conditions, ATP levels above 0.08 mM were inhibitory (data not shown).

Mutation That Lowers Fru 6-P Affinity. In an earlier study (*10*), we have shown that mutation of a methionine residue that is highly conserved in all ATP- and PPi-dependent PFKs produces a profound decrease in the activity and in the apparent affinity for Fru 6-P. As shown in Figure 3, the

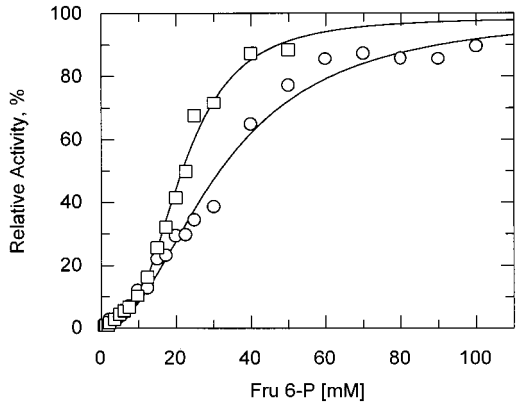


FIGURE 3: Effect of mutations that lower Fru 6-P affinity. Data are expressed as relative activity to maximal activity with each preparation. For M169L (squares) it was 14 s^{-1}, and for M169A (circles), k_{cat} was 1.4 s^{-1}. The assay was performed at pH 7.2 with 1.0 mM ATP, 1mM GDP and other conditions described in the Materials and Methods.

mutations of methionine 169 display strongly cooperative response to Fru 6-P concentration in the presence of the allosteric activator, GDP. The half saturation concentration for Fru 6-P for the M169L mutant is 23 mM as compared to 0.10 mM for the wild-type enzyme. The k_{cat} for this mutant is 14 s^{-1} compared to 88 s^{-1} for the wild-type enzyme when assayed with 2 mM ATP. Figure 3 also shows similar results with a different mutation of the critical methionyl residue. This mutant (M169A) had much lower activity with a k_{cat} of only 0.62 s^{-1}, but nonetheless showed strong cooperative effects of the residual activity. These results are again consistent with the model in Scheme 1 where the rate constants for the addition of Fru 6-P to the mutant enzyme (k_5 in the model) and to the E-ATP complex (k_3) are slower than that seen with wild-type enzyme. Under these conditions, the slower E to E-ATP path is favored at low substrate concentrations and the faster E-Fru 6-P path is taken only at higher concentrations of Fru 6-P. This will again produce apparent cooperative interactions.

ATP inhibition was also observed with the methionine mutants. In the presence of 1.0 mM Fru 6-P, ATP inhibition was observed with the M169L mutant when the ATP concentration exceeded 0.6 to 0.7 mM (data not shown).

1-Carboxymethyl-ATP (*cmATP*) *as an Alternative Substrate.* An attempt to alter the affinity of the phosphoryl donor involved introducing an additional negative charge in ATP. It was thought that substrate antagonism could be enhanced if that antagonism is due to repulsive charge interactions between ATP and Fru 6-P. An additional negative charge was introduced into the ATP by carboxymethylating the 1-position of the adenine ring. CmATP was a reasonably efficient substrate for PFK, giving a half-saturating substrate concentration of 0.6 mM as compared to 0.21 mM for ATP in the presence of 1.0 mM Fru 6-P. The modified nucleotide also showed substrate inhibition with a pattern similar to that with ATP. Furthermore, the maximal activity was only about 10% lower than that with ATP as substrate. In the presence of 2.0 mM cmATP, the half-saturation concentration for Fru 6-P was actually lower than that with 2.0 mM ATP as substrate (0.021 vs 0.10 mM). This is to be expected considering the antagonism between the two substrates and that the cmATP has slightly less affinity. Previous studies

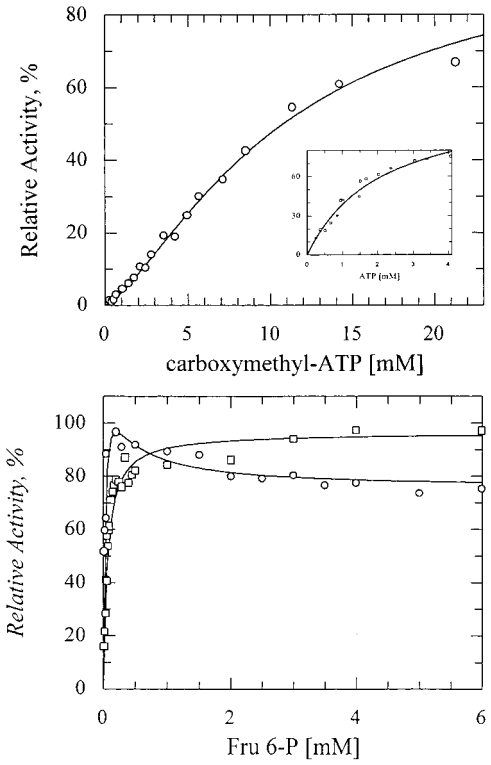


FIGURE 4: Altered reaction path with 1-carboxymethyl-ATP and mutant R111E. (A) Nucleotide concentration dependence. Data are expressed as relative activity to maximal activity with each substrate. The k_{cat} with ATP (shown in inset) was 59 s^{-1}. For R111E, k_{cat} was 64 s^{-1}. The assay was performed at pH 7.2 with 1.0 mM Fru 6-P, 1 mM GDP and other conditions described in the Material and Methods. (B) Fru 6-P dependence. ATP (squares) and cmATP (circles) were present at 2.0 mM. Because this concentration of cmATP was nonsaturating, maximum achieved activity was 12 s^{-1}.

have shown that increasing saturation with ATP increases the apparent K_m for Fru 6-P and vice versa (*4*). It is obvious that the use of the alternative substrate does not change the general pattern of the mechanism.

The use of cmATP with a mutant of PFK that has an additional negative charge in the ATP binding site produced more striking results. We have studied previously the properties of the R111E mutant of *E. coli* PFK (*11*), which results in a negative charge placed near the N-6 of the adenine ring of ATP. Those results showed that the mutant enzyme had an increased apparent K_m for ATP relative to wild-type (1.7 mM versus 0.21 mM in the presence of 1.0 mM Fru 6-P). Typical ATP inhibition was observed. The half-saturation concentration for Fru 6-P was somewhat lower than that for wild-type PFK (0.048 mM versus 0.10 mM) in the presence of 2.0 mM nucleotide, again reflecting less antagonism because of its lower affinity for ATP. With ATP as substrate, the kinetic pathway with the R111E mutant is similar to that seen previously with the native enzyme where the faster E to E-Fru 6-P pathway is favored at low concentrations of ATP and the slower E to E-ATP path is used at high ATP concentration.

When cmATP was used as the substrate with the R111E mutation, the relative rates of the binding events appeared to be considerably altered. As shown in Figure 4, the interaction of cmATP is now cooperative (4A), and Fru 6-P shows substrate inhibition at high concentrations (4B). The

$cmATP_{0.5}$ was 12 mM as compared to the apparent K_m of 0.60 with the wild-type enzyme. The velocity with respect to Fru 6-P was not obviously cooperative although the high apparent affinity made it difficult to determine velocities at substrate concentrations at or below half-saturation. Half saturation was achieved at Fru 6-P concentrations of 0.02 mM or lower with a subsaturating cmATP concentration of 2.0 mM. The interpretation of the cooperative kinetics with cmATP and the substrate inhibition by Fru 6-P with respect to the nonequilibrating random model is as follows. Despite the decreased affinity for cmATP, the pathway E to E-cmATP is the faster pathway to the ternary complex and is therefore utilized at high concentrations of cmATP. In the model of Scheme 1, this is described as $k_1 \cdot k_3 > k_5 \cdot k_7$. One possible explanation for the observed kinetic pattern is that k_7 in particular is decreased (addition of cmATP to the E-Fru 6-P complex) because of the strong repulsion of the additional negative charges with the combination of the Arg to Glu mutation and the addition of a carboxyl group to the ATP. At low concentrations of cmATP, the reaction proceeds by the slower E to E-Fru 6-P path, and to the faster E to E-cmATP path at higher concentrations of nucleotide, producing the apparent cooperativity. At high Fru 6-P concentrations, the reaction is forced through the now slower E to E-Fru 6-P path leading to substrate inhibition.

Conclusions. Although the foregoing results are consistent with the mechanism and the interpretations are consistent, some caution must be used in accepting these interpretations in the absence of determined rate constants for each of the steps with the mutants and alternative substrates. Nonetheless, the data described above confirm the previous explanation (*4*, *8*) for the phenomenon of mechanism-based ATP inhibition in the active conformation of the enzyme without invoking allosteric transitions. This mechanism is based on a nonequilibrating, steady state mechanism coupled with substrate antagonism. In addition to explaining ATP inhibition, the data demonstrate many of the apparent kinetic anomalies that can be generated in a steady state or nonequilibrating, random mechanism. A mutation in the sugar phosphate binding site produced apparent cooperativity in Fru 6-P interaction as did the use of a poorly binding substrate. With an alternative nucleotide, cmATP, coupled with a mutation in the active site, one could observe substrate inhibition by Fru 6-P and apparent cooperativity in the nucleotide interaction. Furthermore, the use of a phosphoryl donor that greatly reduced the catalytic rate facilitated the equilibration of binding steps and eliminated ATP inhibition. It should be noted that all of the unusual velocity curves observed here were predicted theoretically in an early treatise on kinetics by Segel (*20*).

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