Mechanism of Substrate Inhibition in *Escherichia coli* Phosphofructokinase†

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ABSTRACT: Phosphofructokinase from *Escherichia coli* (EcPFK) is a homotetramer with four active sites, which bind the substrates fructose-6-phosphate (Fru-6-P) and MgATP. In the presence of low concentrations of Fru-6-P, MgATP displays substrate inhibition. Previous proposals to explain this substrate inhibition have included both kinetic and allosteric mechanisms. We have isolated hybrid tetramers containing one wild type subunit and three mutated subunits (1:3). The mutated subunits contain mutations that decrease affinity for Fru-6-P (R243E) or MgATP (F76A/R77D/R82A) allowing us to systematically simplify the possible allosteric interactions between the two substrates. In the absence of a rate equation to explain the allosteric effects in a tetramer, the data have been compared to simulated data for an allosteric dimer. Since the apparent substrate inhibition caused by MgATP binding is not seen in hybrid tetramers with only a single native MgATP binding site, the proposed kinetic mechanism is not able to explain this phenomenon. The data presented are consistent with an allosteric antagonism between MgATP in one active site and Fru-6-P in a second active site.

Phosphofructokinase-1 from *Escherichia coli* (EcPFK)[1] is a homotetramer, which contains four active sites. The two substrates, fructose-6-phosphate (Fru-6-P) and MgATP, have a number of effects on the titration profiles of one another (*1*). In the absence of MgATP, Fru-6-P binds hyperbolically, but in the presence of saturating MgATP, Fru-6-P binds sigmoidally. In the absence and saturating presence of Fru-6-P, MgATP titrations to EcPFK are hyperbolic. However, at low concentrations of Fru-6-P, MgATP displays apparent substrate inhibition.

Several authors have proposed mechanisms to explain the substrate inhibition by MgATP at low Fru-6-P concentrations. Zheng and Kemp (*2*) concluded that substrate inhibition could be explained by the existence of two kinetic pathways in a random, nonrapid equilibrium system. The first pathway would bind MgATP first and Fru-6-P second. The second pathway would bind Fru-6-P first and MgATP second and would be kinetically faster than the first pathway, and hence preferred. At high MgATP and low Fru-6-P concentrations, the first pathway (slow) would be used, and at low MgATP and high Fru-6-P concentrations the lower pathway (fast) would be used. The mechanism assumes that at low Fru-6-P concentrations, the kinetic pathway used would switch from the preferred, fast pathway to the slower pathway as the MgATP concentration is increased. Support for the kinetic mechanism has been offered in the correlations between the lack of substrate inhibition and decreased k_{cat} (and therefore an assumed approach of rapid equilibrium) in a number of kinetic studies with alternate substrates and mutant proteins (*2, 3*).

Berger and Evans (*4*) suggested that MgATP binds at a second binding site in EcPFK distant from the active site and causes allosteric inhibition in the active site. However, Johnson and Reinhart (*1*) offered an explanation based on allosteric interactions between MgATP of one active site and Fru-6-P in a second active site, therefore eliminating the need to presume a new, unidentified MgATP binding site.

The EcPFK enzyme shares 55% identity with phosphofructokinase 1 from *Bacillus stearothermophilus* (BsPFK), which is also a homotetramer. Like EcPFK, BsPFK displays apparent substrate inhibition by MgATP at low Fru-6-P. Byrnes et al. (*5*) concluded that a kinetic mechanism, like that described by Zheng and Kemp (*2*), would explain the substrate inhibition observed for BsPFK. However, Byrnes et al. (*5*) concluded that the BsPFK and EcPFK proteins do not function in a similar manner since EcPFK shows cooperativity in Fru-6-P binding at saturating MgATP and BsPFK does not.

To discriminate between the various proposed explanations of MgATP substrate inhibition, the current study uses hybrid EcPFK tetramers that are constructed with one wild type subunit and three mutated subunits (1:3). In these hybrids, the three mutated subunits contain mutations that decrease affinity for Fru-6-P (R243E) or MgATP (F76A/R77D/R82A). The 1:3 hybrids have allowed for systematic removal of Fru-6-P/MgATP interactions. If the kinetic mechanism described above were able to explain the apparent substrate inhibition, the phenomenon would be observed in all kinetically active hybrids. Since the 1 wild type:3 F76A/R77D/R82A hybrid lacks substrate inhibition, the kinetic mechanism is not supported. Therefore, the results obtained in the current study are explained as they relate to an allosteric mechanism. The

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[1] Abbreviations: PFK, phosphofructokinase; DTT, dithiothreitol; EPPS, *N*-(2-hydroxy-ethyl)piperazine-*N*′-(3-propanesulfonic acid); Fru-6-P, fructose 6-phosphate; PEP, phosphoenolpyruvate; EcPFK, phosphofructokinase I from *Escherichia coli*; BsPFK, phosphofructokinase I from *Bacillus stearothermophilus*.

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rate equation for an allosteric tetramer has not been solved. As a result, experimental data will be compared with simulated data for an allosteric dimer (*6*).

MATERIALS AND METHODS

Materials. All chemical reagents were analytical grade, purchased from Fisher or Sigma. Mimetic Blue 1 agarose resin from Prometic Biosciences was used in protein purification. Sephacryl S-300 resin from Pharmacia was used for size exclusion. Creatine phosphate and the sodium salt of Fru-6-P were purchased from Sigma. The sodium salt of ATP was obtained from Boehringer Mannheim. Creatine kinase, aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were obtained from Boehringer Mannheim.

Mutagenesis, Protein Purification, Hybrid Formation, and Hybrid Isolation. The K2E/K3E/R243E mutant EcPFK was previously constructed (*7*). pGDR16 (*8*), containing the EcPFK in pAlter I (Promega), was used with the Altered Sites II In Vitro Mutagenesis system (Promega) to construct the F76A/R77D/R82A mutation according to manufacturer's instructions. The mutagenesis primer F76A/R77D/R82A (5′-CACGGCGGCGATGTTCTCGTCGTCGGCTTCCGG-3′) was synthesized by the Gene Technologies Laboratory of the Institute of Developmental and Molecular Biology at Texas A&M University.

Plasmid DNA was isolated using Wizard spin preps (Promega). The DNA was sequenced across the mutated site to confirm the desired mutation. In addition, the entire gene was sequenced to confirm that no other mutations were introduced. Plasmids containing wild type and mutant EcPFK genes were transformed into DF1020 cells for protein expression (*9*, *10*). Protein purification of wild type and the K2E/K3E/R243E mutated EcPFKs out of DF1020 cells has previously been described (*8*). The F76A/R77D/R82A mutant protein did not bind to the Mimetic Blue 1 agarose resin, consistent with previous findings (*11*). Therefore, after sonication and centrifugation as previously described (*8*), an ammonium sulfate cut of 39 to 53% saturation at 4 °C precipitated the F76A/R77D/R82A mutant protein. The pellet was dissolved in minimal buffer A (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM EDTA) and further purified by size exclusion using a Sephacryl S-300 column. Active fractions were pooled and loaded onto a Mono-Q 10/10 (Pharmacia) FPLC ion exchange column. Elution from the ion exchange column was with a linear NaCl gradient. Using this described purification for the F76A/R77D/R82A mutant protein yielded a single band on SDS−PAGE (data not shown). Protein concentration was determined by reading the absorbance in the absence of nucleotides using $\epsilon_{278} = 0.6$ cm^2 mg^{-1} (*12*).

The methodology for hybrid formation and separation has been previously discussed (*7*, *13*). These methods include subunit exchange in the presence of KSCN and separation of hybrid species with a Mono-Q 10/10 (Pharmacia) FPLC ion exchange column. The charge differences between wild-type subunits and F76A/R77D/R82A mutant subunits were sufficient to allow separation of the different hybrid species. However, the K2E/K3E surface charge tag was added to the R243E mutated subunits to allow separation of hybrid species between these subunits and wild-type subunits (*7*). Subunit exchange of isolated 1:3 hybrids was prevented by adding 2 mM Fru-6-P to the buffers used in hybrid separation and storage (*7*).

Kinetic Assays. Activity measurements were carried out in 600 μL of an EPPS buffer containing 50 mM EPPS−KOH (pH 8.0 at 8.5 °C), 10 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 150 μg of aldolase, 30 μg of glycerol-3-phosphate dehydrogenase, 30 μg of triosephosphate isomerase, 12.5 mM creatine phosphate, and 80 μg/mL creatine kinase. Fru-6-P and MgATP concentrations were varied as indicated. Ten microliters of EcPFK was used to initiate the enzymatic reaction, which was monitored at 340 nm over time. One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol of fructose-1,6-bisphosphate/min.

Data Analysis. Data were fit to appropriate equations using the nonlinear least-squares fitting analysis of Kaleidagraph (Synergy) software. Initial rates obtained from kinetic assays of varying Fru-6-P concentration were fit to the Hill equation (*14*):

$$\frac{v}{E_T} = \frac{k_{cat}[\text{Fru-6-P}]^{n_H}}{(K_{0.5})^{n_H} + [\text{Fru-6-P}]^{n_H}} \quad (1)$$

where v = initial rate, E_T = total enzyme active site concentration, k_{cat} = turnover number, $K_{0.5}$ = the concentration of Fru-6-P that yields a rate equal to one-half the maximal specific activity, and n_H = the Hill coefficient. The effect of MgATP on the $K_{0.5\ (Fru\text{-}6\text{-}P)}$ of mutated or wild-type EcPFK was evaluated by fitting to the following equation (*15*):

$$K_{0.5\ (F6P)} = K^{\circ}_{ia}((K^{\circ}_{ib} + [\text{MgATP}])/(K^{\circ}_{ib} + Q_{ab}[\text{MgATP}])) \quad (2)$$

where $K^{\circ}_{ia} = K_{0.5\ (F\text{-}6\text{-}P)}$ when [MgATP] = 0, K°_{ib} = the dissociation constant for MgATP when [Fru-6-P] = 0, and Q_{ab} = the coupling constant between MgATP and Fru-6-P.

RESULTS

The current study has used hybrid tetramers to systematically decrease the possible allosteric interactions between the two substrates Fru-6-P and MgATP. In this approach, hybrid tetramers were isolated which contain one wild-type subunit and three mutated subunits. The mutated subunits of the current work have either decreased affinity for Fru-6-P or decreased affinity for MgATP.

Our strategy requires that a mutation or mutations be identified that decrease affinity for Fru-6-P and that a mutation or mutations be identified that decrease affinity for MgATP. Previously, we have characterized the R243E mutation, which resulted in a decreased affinity for Fru-6-P by well over 2 orders of magnitude relative to wild type (*7*). To decrease MgATP affinity, we have combined three of the mutants previously introduced by Wang and Kemp, F76A, R77D, and R82A (*11*). Of special note is that Fru-6-P binds in rapid equilibrium (*16*), but MgATP does not (*1*). Therefore, in Figure 1 the affinity of Fru-6-P has been followed as a function of MgATP as a method of determining the apparent binding affinity of MgATP. As designed, the F76A/R77D/R82A triple mutation shows a greatly decreased affinity for MgATP, and saturation by MgATP is not achieved with up to 50 mM MgATP.

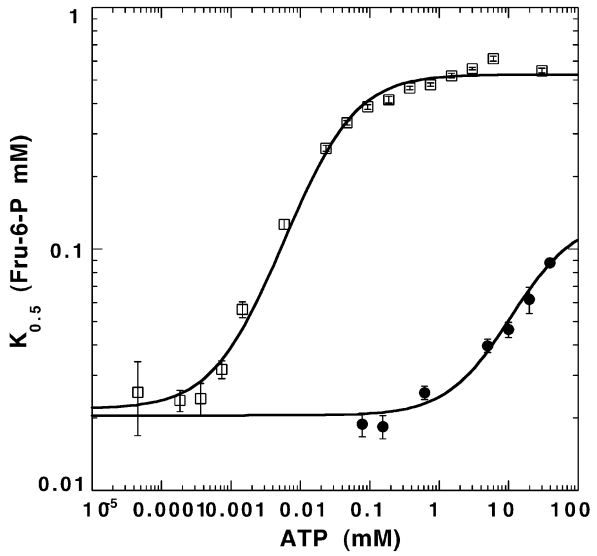

FIGURE 1: $K_{0.5}$ for Fru-6-P as a function of MgATP concentration for wild type (□) and F76A/R77D/R82A (●) tetramers. $K_{0.5}$ values were obtained by fitting the dependence of specific activity on Fru-6-P concentration to eq 1 as described in the text. Curves shown through data for wild type represent the best fit of these data to eq 2.

Figure 2A displays the MgATP substrate inhibition of wild-type EcPFK that has been observed at concentrations of Fru-6-P that are low (but not zero) relative to the $K_{0.5}$ for Fru-6-P. For comparison, the hybrid tetramer with one wild type subunit and three subunits containing the R243E mutation was isolated. This hybrid tetramer contains all four native MgATP binding sites, but only a single native Fru-6-P binding site and the response of this hybrid to MgATP concentration is shown in Figure 2B. These data demonstrate that at a low concentration of Fru-6-P the hybrid tetramer continues to display MgATP substrate inhibition. Next, a hybrid tetramer with one wild-type subunit and three subunits containing the triple F76A/R77D/R82A mutation was isolated. This hybrid tetramer contains a single native MgATP binding site but retains all four native Fru-6-P binding sites. As shown in Figure 2C, this hybrid does not exhibit MgATP substrate inhibition at concentrations up to 0.01 mM MgATP.

If the kinetic mechanism proposed by Zheng and Kemp (*2*), and later by Byrnes et al. (*5*), explained the apparent MgATP substrate inhibition, then this substrate inhibition should remain in all native active sites that support activity. Activity in the hybrid tetramers originates only from the single native active sites that bind both MgATP and Fru-6-P. However, the hybrid that has no other MgATP binding sites does not exhibit MgATP substrate inhibition in the same concentration range as do the other enzyme forms. Therefore, the kinetic mechanism used to explain the MgATP substrate inhibition is not supported. Furthermore, removal of three MgATP substrate binding sites removes the substrate inhibition. Therefore, these data do not support a need to assume a new unidentified MgATP allosteric site, which has also been previously proposed (*4*). Sequencing confirmed that no unplanned mutations could be the cause of this result.

The data presented in Figure 2 are most consistent with MgATP inhibition occurring as a consequence of allosteric interactions between MgATP binding in one active site and Fru-6-P binding in a different active site. The possible interactions between the substrates for the wild type and hybrid tetramer enzymes are depicted in Figure 3. Only in wild type and the 1:3 hybrid with R243E do potential interactions between Fru-6-P and MgATP at different active sites exist.

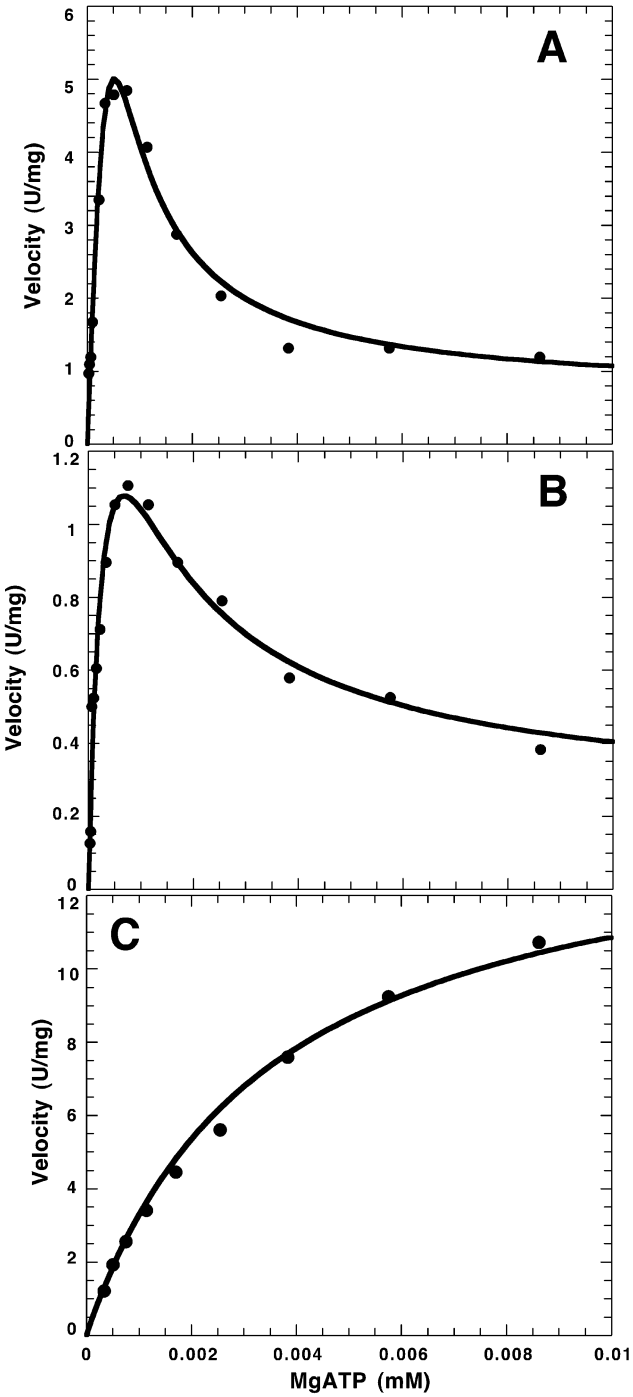

FIGURE 2: Activity of EcPFK tetramers as a function of MgATP concentration and at 55 μM Fru-6-P. Lines represent data trends for the wild type tetramer data (A), the 1 wild type:3 R243E hybrid tetramer (B), and the 1 wild type:3 F76A/R77D/R82A hybrid tetramer (C).

On the basis of this allosteric explanation of the MgATP substrate inhibition, the following observations were made. The F76A/R77D/R82A triple mutation decreases affinity for MgATP as demonstrated in Figure 1. However, MgATP still binds with a very low affinity to the subunits with the triple mutation. Therefore, MgATP inhibition should be observed

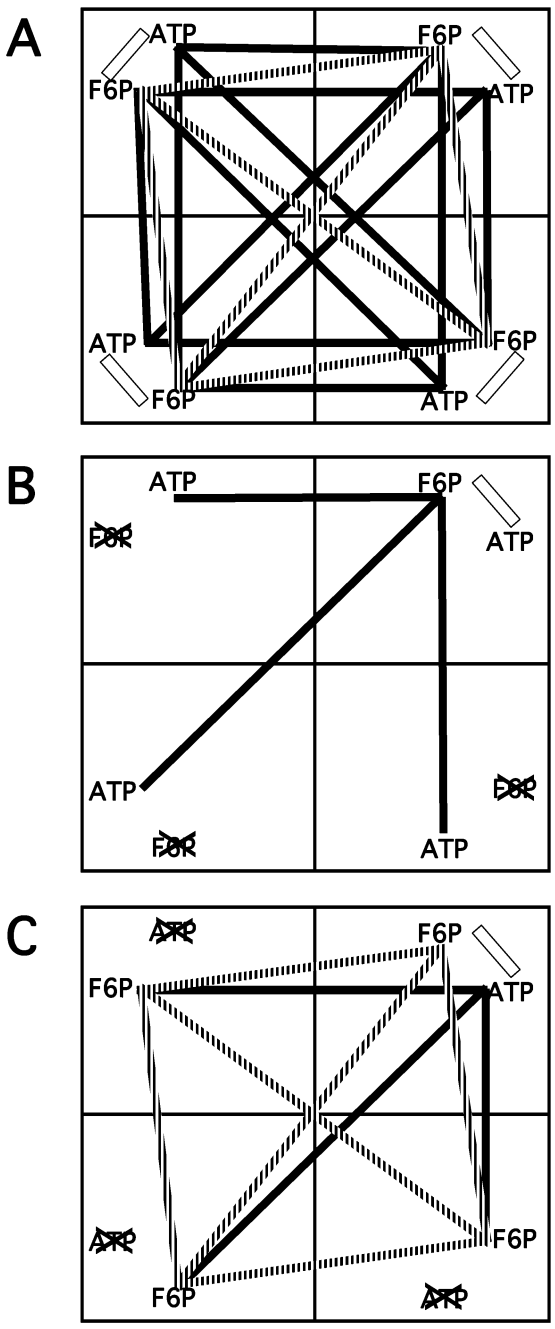

FIGURE 3: Schematics of the possible allosteric interactions between substrates. The three tetramers A, B, and C represent the three tetramers assayed in panels A, B, and C of Figure 2, respectively. Fru-6-P and MgATP are represented in the schematics as F6P and ATP, respectively. Within the tetramers, striped lines represent Fru-6-P to Fru-6-P homotropic interactions (cooperativity; $Q_{aa/b}$), solid lines represent heterotropic interactions between Fru-6-P in one active site and MgATP in a second active site (Q_{ab2}), and unfilled lines represent Fru-6-P to MgATP heterotropic interactions within the same active site (Q_{ab1}).

in this 1:3 hybrid (wild type:F76A/R77D/R82A) at very high concentrations of MgATP. Figure 4 shows that at very high concentrations of MgATP, the 1:3 hybrid (wild type:F76A/R77D/R82A) demonstrates the substrate inhibition as predicted. This supports that the lack of the substrate inhibition at low MgATP concentrations is not an artifact of the introduced mutations.

Data Simulations. The rate equation of a symmetrical tetramer with two different substrate binding sites per subunit

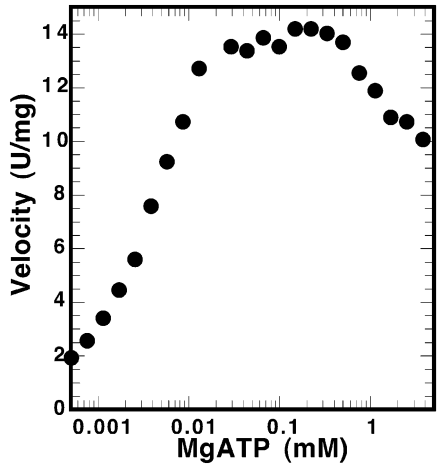

FIGURE 4: Activity of the 1 wild type:3 F76A/R77D/R82A hybrid tetramer as a function of MgATP and 55 μM Fru-6-P. Higher MgATP concentrations display substrate inhibition. At still higher MgATP, velocity increases due to catalytic activity in the mutated active sites (data not shown).

is too complex to be derived explicitly. Johnson and Reinhart previously compared experimental results with simulated data obtained from a symmetrical dimer, the equation for which follows, assuming no homotropic cooperativity in MgATP binding (*1*):

$$v/V = \{Q_{ab2}^2 Q_{ab1}^2 Q_{aa/b}^2 B^2 A^2 + Q_{ab2} Q_{ab1} Q_{aa/b} B A^2 + Q_{ab2} Q_{ab1} B^2 A + Q_{ab1} AB\} / \{(Q_{ab2}^2 Q_{ab1}^2 Q_{aa/b}^2 B^2 + 2Q_{ab2} Q_{ab1} Q_{aa/b} B + 1)A^2 + 2(Q_{ab2} Q_{ab1} B^2 + [Q_{ab2} + Q_{ab1}]B + 1)A + B^2 + 2B + 1\} \quad (3)$$

where $A = [\text{Fru-6-P}]/K_{ia}^{\circ}$, $B = [\text{MgATP}]/K_{ib}^{\circ}$, and Q_{ab1} and Q_{ab2} are the two types of heterotropic interactions between Fru-6-P and MgATP. Q_{ab1} is the heterotropic coupling between Fru-6-P and MgATP binding to the same active site. Q_{ab2} is the heterotropic coupling between Fru-6-P binding in one active site and MgATP binding to a different active site. Equation 3 allows for Fru-6-P cooperativity but not for MgATP cooperativity. If one assumes typical values for the magnitude of heterotropic and homotropic interactions ($Q_{ab1} = Q_{ab2} = 0.1$, $Q_{aa/b} = 10$), eq 3 predicts the curve shown in Figure 5A. As noted by Johnson and Reinhart (*1*), a symmetrical dimeric enzyme can exhibit substrate inhibition, analogous to the MgATP inhibition exhibited by wild-type PFK tetramer, in the absence of any additional allosteric interactions not related to substrate binding sites.

The following equation pertains to an unsymmetrical dimer in which one substrate, A, can only bind at one active site, while the other substrate, B, can bind at both active sites. This dimeric situation would be analogous to the 1:3 hybrid with the R243E mutant protein that can bind an MgATP on each subunit but can bind Fru-6-P at only one active site.

$$v/V = \frac{Q_{ab1} AB + Q_{ab2} Q_{ab1} B^2 A}{2B + A + Q_{ab1} AB + B^2 + Q_{ab2} AB + Q_{ab2} Q_{ab1} B^2 A + 1} \quad (4)$$

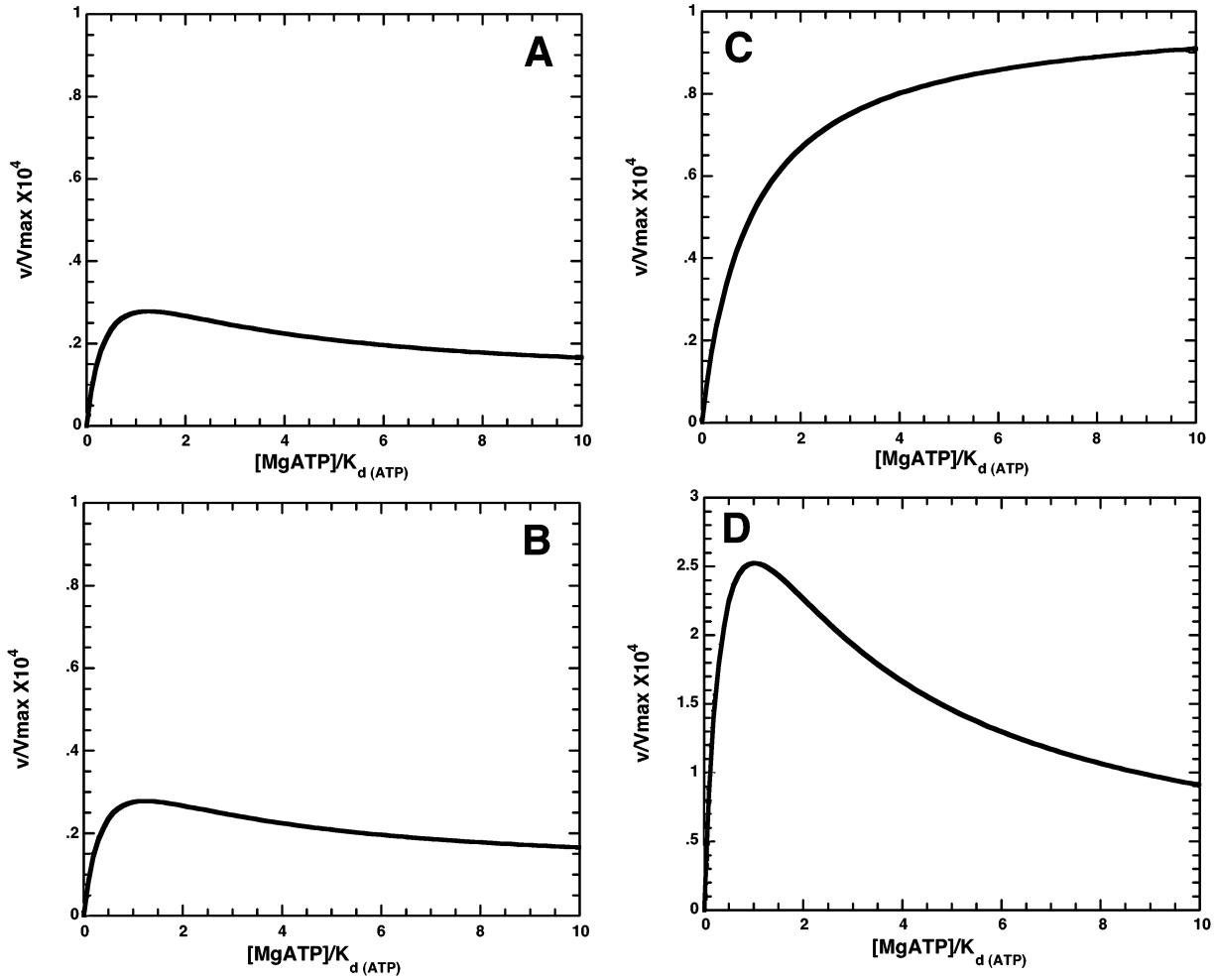

FIGURE 5: Data simulations with a dimer model. Data simulations in panels A, B, and C are the dimer approximations of the three tetramers represented in panels A, B, and C of Figures 2 and 3. In panels A, B, and C, [Fru-6-P]/$K_{d\ Fru-6-P} = 1 \times 10^{-3}$, $Q_{ab1} = Q_{ab2} = 0.1$, and when applicable, $Q_{aa/b} = 10$. (A) Simulations for a symmetrical dimer with two different substrates binding to each subunit as described in eq 3. (B) Simulations for a dimer that binds Fru-6-P in one subunit and Fru-6-P and MgATP in the second subunit as described in eq 4. (C) Simulations for a dimer that binds MgATP in one subunit and Fru-6-P and MgATP in the second subunit as described in eq 5. (D) Simulated data for a dimer that binds Fru-6-P in one subunit and Fru-6-P and MgATP in the second subunit as described by eq 4, but which lacks coupling between Fru-6-P and MgATP within the same active site ($Q_{ab1} = 1$). All other parameters in panel D are as for panels A, B, and C.

Parameters are as described for eq 3. Note that there are no coupling terms for homotropic interactions in eq 4 because there are no possible Fru-6-P homotropic interactions and wild type EcPFK does not display cooperativity in MgATP binding (*1*). When typical values of 0.1 are once again assumed for Q_{ab1} and Q_{ab2}, eq 4 predicts the curve shown in Figure 5B. The simulated data continue to display apparent substrate inhibition by MgATP. This is qualitatively consistent with the experimental results of the 1 wild type:3 R243E hybrid tetramer shown in Figure 2B.

Using the same notation, an unsymmetrical dimer that can bind two equivalents of A, but only one equivalent of B is modeled by the following equation:

$$v/V = \frac{Q_{ab1}AB + Q_{ab2}Q_{ab1}Q_{aa/b}A^2B}{2A + B + Q_{ab1}AB + A^2 + Q_{ab2}AB + Q_{ab2}Q_{ab1}Q_{aa/b}A^2B + 1} \quad (5)$$

This equation describes behavior that should be analogous to that of a hybrid tetramer between one wild type subunit and three F76A/R77D/R82A mutant subunits. This equation allows for homotropic interactions between the 2 equivalents of A that bind, consistent with the homotropic cooperativity observed in Fru-6-P binding (*1*). After assigning typical values of 0.1 for Q_{ab1} and Q_{ab2} and 10 for $Q_{aa/b}$, eq 5 predicts the behavior presented in Figure 5C. The simulated data predict the absence of inhibition by MgATP (denoted by B in eq 5), consistent with the behavior of the 1:3 hybrid shown in Figure 2C.

As previously noted, the coupling that is apparently required to give rise to the MgATP inhibition is between MgATP of a neighboring subunit to the Fru-6-P of the subunit with turnover. This coupling is designated as Q_{ab2}. To establish whether the intra-active site coupling between MgATP and Fru-6-P, designated Q_{ab1}, is also required for the substrate inhibition predicted by eq 4, the value of Q_{ab1} was set equal to 1. A coupling parameter equals 1 when there is no energetic influence of the binding of one ligand on the other. The resulting simulation is presented in Figure 5D. Substrate inhibition is clearly evident. On the other hand, if Q_{ab2} is set equal to 1, while Q_{ab1} is set equal to a value less

than 1, no substrate inhibition is predicted by eq 4 (data not shown). Hence we conclude that an antagonistic interaction between Fru-6-P binding at one active site and MgATP binding at a different active site is both necessary and sufficient to produce the substrate inhibition evident in Figure 2A.

DISCUSSION

We previously have used hybrid tetramers of EcPFK to characterize a unique heterotropic interaction in the allosteric MgADP activation of Fru-6-P affinity in EcPFK (*7*) as well as an allosteric PEP inhibition of Fru-6-P affinity in BsPFK (*13*). However, all of these characterizations were in the presence of saturating MgATP. In the current study, we have used the hybrid tetramer approach to systematically remove possible allosteric interactions between the two substrates Fru-6-P and MgATP. Using this hybrid approach, we have tested the different mechanisms proposed in the literature to describe the MgATP substrate inhibition at low Fru-6-P.

The substrate inhibition caused by MgATP binding is not seen in hybrid tetramers with only a single native MgATP binding site (Figure 2C). Therefore, the kinetic mechanism that has been proposed previously (*2*, *5*) is not able to adequately explain the observed phenomenon. The data collected for hybrid tetramers are consistent with an allosteric antagonism between substrates. MgATP in one active site behaves as an allosteric inhibitor of Fru-6-P affinity in a second active site.

The 1 wild type:3 R243E hybrid tetramer has only a single native Fru-6-P binding site and therefore lacks Fru-6-P cooperativity. However, this hybrid continues to display the apparent MgATP substrate inhibition. This result indicates that the MgATP inhibition is independent of Fru-6-P cooperativity. Since BsPFK exhibits MgATP inhibition but no Fru-6-P cooperativity, a likely explanation for the MgATP inhibition in BsPFK is also that MgATP in one active site behaves as an allosteric inhibitor of Fru-6-P affinity in a second active site. This rationale is different than that put forth by Byrnes et al. (*5*) who concluded different mechanisms were responsible for substrate inhibition in BsPFK and EcPFK. However, contributions of the three unique intrasubunit interactions (Q_{ab2}) to the total Fru-6-P/MgATP antagonism may be different in the two enzymes.

With the understanding that the MgATP inhibition is due to an allosteric mechanism, but not separate allosteric sites, we can speculate on why altered substrates and mutant proteins lack the effect (*2*, *3*). Mutations introduced into the tetrameric EcPFK have the potential of disrupting a number of through protein interactions between the substrates. On the other hand, altered substrates may have no coupling or altered coupling. The analogues may be efficient at binding to EcPFK, but binding does not guarantee that the same couplings exist since allosteric coupling is independent of ligand affinity (*15*, *17*−*19*).

In summary, we have adapted hybrid tetramer approaches previously used to isolate heterotropic Fru-6-P/MgADP and Fru-6-P/PEP interactions (*7*, *13*) to systematically remove possible intersubunit substrate interactions in EcPFK. Using this approach, we have been able to test a number of mechanisms proposed to explain the apparent MgATP substrate inhibition of EcPFK. Our data are consistent with an allosteric inhibition between MgATP in one active site of the tetramer and Fru-6-P in a different active site.

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