Disentangling the Web of Allosteric Communication in a Homotetramer: Heterotropic Activation in Phosphofructokinase from *Escherichia coli*†

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ABSTRACT: Phosphofructokinase from *Escherichia coli* (EcPFK) is a homotetramer with four active sites and four allosteric sites. Understanding the allosteric activation of EcPFK by MgADP has been complicated by the complex web of possible interactions, including active site homotropic interactions, allosteric site homotropic interactions, and heterotropic interactions between active and allosteric sites. The current work has simplified this web of possible interactions to a series of single heterotropic interactions by forming and isolating hybrid tetramers. Each of the four unique heterotropic interactions have independently been isolated and compared to a control that has all four of the unique heterotropic interactions. If the interactions are labeled with the distances between interacting ligands, the 45-Å interaction contributes 20% ± 1%, the 33-Å interaction contributes 34% ± 1%, the 30-Å interaction contributes 21% ± 1%, and the 23-Å interaction contributes 25% ± 1% with respect to the total free energy of MgADP/fructose 6-phosphate (Fru-6-P) activation in the control. The free energies of the isolated interactions sum to 100% ± 2% of the total. Therefore, the four unique interactions are all contributors to activation, are nonequivalent, and are additive.

We have recently employed a hybridization strategy for isolating the individual heterotropic inhibitory interactions in phosphofructokinase from *Bacillus stearothermophilus* (BsPFK)[1] (*1*, *2*). That approach, which entailed the construction of hybrid tetramers of wild-type and mutant proteins, resulted in the observation that each interaction contributes uniquely to the overall inhibitory effect. Furthermore, the results indicated that heterotropic allosteric communication in that tetramer results from the sum of the individual unique pairwise interaction free energies once the homotropic effects were removed.

Phosphofructokinase from *Escherichia coli* (EcPFK) catalyzes the same reaction and is very similar both structurally and functionally to BsPFK. Both enzymes are subject to inhibition of Fru-6-P binding by phospho(enol)pyruvate (PEP), they contain 54% amino acid sequence identity and 73% amino acid sequence similarity, and their three-dimensional structures are highly similar (*3*−*7*). However, EcPFK exhibits much more significant activation of Fru-6-P binding by MgADP at ambient temperatures compared to BsPFK (*3*, *8*, *9*). In addition, EcPFK shows apparent positive homotropic cooperativity with respect to Fru-6-P binding in the presence of MgATP, and this cooperativity is removed in the presence of MgADP (*10*, *11*). The purpose of this report is to delineate the contributions of heterotropic interactions to the activation by MgADP of EcPFK.

EcPFK, like BsPFK, is a homotetramer with four active sites and four allosteric sites (*5*−*7*). The four subunits are assembled as a dimer of dimers with two different types of subunit interfaces. The four active sites are at one type of subunit interface with residues from both subunits interacting with Fru-6-P. Allosteric sites are formed at the second type of subunit interface. The allosteric inhibitor PEP and the allosteric activator MgADP compete for binding at each allosteric site. As with the Fru-6-P binding site, residues from both subunits interact with MgADP when it is bound to the allosteric site (Figure 1) (*5*).

Even with linked-function analysis as a tool (*12*, *13*), disentangling the web of potential communication pathways that contribute to the total allosteric regulation of EcPFK is difficult. These potential communication pathways include homotropic interactions between active sites, homotropic interactions between allosteric sites, and heterotropic interactions between active sites and allosteric sites. For allosteric activation of Fru-6-P binding by MgADP in a wild-type EcPFK tetramer, there are 28 possible interactions between the four Fru-6-P and four MgADP ligands. However, there are only three unique Fru-6-P homotropic interactions, three unique MgADP homotropic interactions, and four unique heterotropic interactions. Linkage analysis argues that determining the values of each of the couplings associated with these unique interactions is the key to understanding the entire network that is responsible for the allosteric response (*13*). In this report, we will address specifically the heterotropic couplings that contribute to the activation of EcPFK by MgADP.

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

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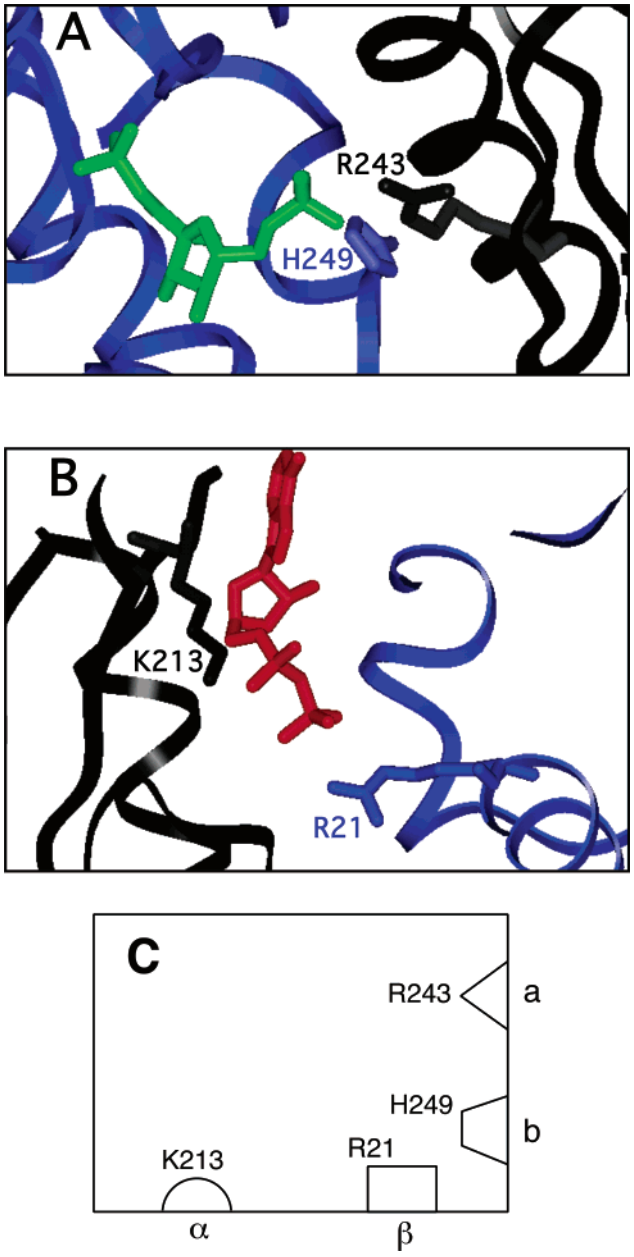

FIGURE 1: The interfacial nature of both the Fru-6-P (A) and MgADP (B) binding sites and a schematic of the subunit structure (C) of EcPFK as revealed by the structure determined by Shirakihara and Evans (*5*). The two subunits that form a binding site are in blue and black ribbon. Residues that are mutated in the current study to reduce affinity are as stick models and colored according to their respective subunits. In panel A, Fru-1,6-BP is shown as a green stick model. In panel B, ADP is shown in an orange stick model. In panel C, the two complementary halves of the Fru-6-P binding site are labeled a and b, and the two complementary halves of the allosteric binding site are labeled α and *β*. The amino acids that were mutated to reduce the respective ligand affinity are depicted.

A method for isolating single heterotropic MgADP/Fru-6-P interactions in EcPFK has previously been reported (*14*). An individual heterotropic interaction is isolated in a hybrid tetramer created between a wild-type subunit and three subunits with mutated Fru-6-P and MgADP binding sites (*14*, *15*). Subunit exchange between parent tetramers is facilitated by KSCN, and a surface charge tag (K2E/K3E) is added to mutated subunits to aid in separation of all possible hybrid forms on an ion exchange column. Neither the KSCN treatment nor the K2E/K3E charge tag altered kinetic or coupling parameters.

Hybrids are made between two different proteins, one of which is usually (but not always) wild-type. Consistent with the nomenclature previously introduced (*14*), the subunit composition will be denoted by two numbers separated by a colon, the first number representing the protein with the fewest mutations. In addition, the number of native binding sites will be denoted as follows: no. of native active sites|no. of native allosteric sites. Therefore, a 1:3 hybrid tetramer between wild-type and a mutant protein that results in a single native active site and a single native allosteric site is denoted 1|1. Such a hybrid would isolate a single heterotropic interaction between the native active and allosteric sites.

A 1|1 hybrid tetramer not only isolates a single heterotropic interaction but also lacks the possible homotropic Fru-6-P interactions and the possible homotropic MgADP interactions of a wild-type tetramer. Homotropic interactions in MgADP binding have not been reported, and MgADP binding monitored by changes in intrinsic fluorescence does not exhibit cooperativity (*9*). However, in the presence of saturating MgATP, apparent homotropic interactions between Fru-6-P binding sites have been well documented in wild-type EcPFK (*10*, *11*). Therefore activation by isolated heterotropic interactions cannot directly be compared to the overall activation measured for a wild-type tetramer since the latter includes homotropic effects. Instead, activation of isolated heterotropic interactions must be compared to a hybrid EcPFK tetramer that lacks homotropic interactions between Fru-6-P binding sites but has all four of the unique heterotropic MgADP/Fru-6-P interactions. This control has previously been characterized and is referred to as the 1|4 control (*14*).

Loss of Fru-6-P cooperativity also causes a greatly decreased Fru-6-P binding affinity. Quantifying the apparent binding constant for hybrids with a single native active site is not possible due to the inability to saturate with Fru-6-P. However, we have used the apparent first-order rate constant, k_{cat}/K_m, revealed by the slope of the linear dependence of EcPFK activity on substrate concentrations that are low relative to $K_{0.5}$, to obtain information regarding the MgADP-dependent changes in Fru-6-P binding affinity (*14*). This interpretation of relative changes in k_{cat}/K_m is justified if two assumptions are true: k_{cat} is independent of effector concentration, and substrate binds in rapid equilibrium. Both assumptions are valid in the wild-type enzyme (*3*, *16*) and are presumably also true within any given hybrid construct (*14*).

The current study takes advantage of the interfacial nature of both the Fru-6-P and MgADP binding sites (*5*) to independently isolate all four of the unique heterotropic activating interactions in EcPFK (see below). To distinctly identify each of the four interactions, the distance between the 6′-phosphate of Fru-1,6-BP and the *β*-phosphate of MgADP in an EcPFK/MgADP/Fru-1,6-BP structure (*5*) has been determined. The four unique heterotropic interactions will therefore be referred to as the 45-Å, 33-Å, 30-Å, and 23-Å interactions. The previously reported hybrid EcPFK tetramer that isolated a single heterotropic interaction between Fru-6-P and MgADP is the 45-Å interaction (*14*).

MATERIALS AND METHODS

Materials. All chemical reagents were analytical grade, purchased from Fisher or Sigma-Aldrich. Matrex Gel Blue A-agarose resin from Amicon Corporation or Mimetic Blue 1 agarose resin from Prometic Biosciences was used in protein purification. Creatine phosphate and the sodium salts of Fru-6-P and ADP were purchased from Sigma. The sodium salt of ATP was obtained from Roche Applied Science or Sigma-Aldrich. Creatine kinase, aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were obtained from Boehringer Mannheim.

Mutagenesis, Protein Purification, Hybrid Formation, and Hybrid Isolation. pGDR16 (*17*), containing the EcPFK in pAlter I (Promega), was used with the Altered Sites II in vitro mutagenesis system (Promega) to construct mutations according to manufacture's instructions. Mutagenesis primers were synthesized by the Gene Technologies Laboratory of the Institute of Developmental and Molecular Biology at Texas A&M University and are as follows: K2E/K3E, 5′-CCGCTTGTCAACACACCGATTTCCTCAATCATGAC-TACCTCTGAAGC-3′; R21A, 5′-CAGCGCAGAACG-AACAACCCCAGCAATTGCGGCGTTCATGCCTGG-3′; K213E, 5′-GGTAATCGCCACGATCGCGTGTTTTTCAC-CTTTCGCGATACCCGC-3′; R243E, 5′-GATGTGGCCCAG-CACAGTTGCTTCGGTTTCACGACCGGT-3′; H249E, 5′-CCACCGCGCTGGATTTCGCCCAGCACAGTTGCGC-3′.

Plasmid DNA was isolated using Wizard spin preps (Promega). The DNA was sequenced across the modified site to confirm the desired mutation. Plasmids containing wild-type and mutant EcPFK genes were transformed into DF1020 cells for protein expression (*18*, *19*). Protein purification out of DF1020 cells was as previously described (*17*). The methodology for hybrid formation and separation were previously discussed. These methods include subunit exchange in the presence of KSCN and the addition of a surface charge tag (K2E/K3E) to mutated subunits to increase separation when eluting with a linear NaCl gradient from a Mono-Q 10/10 (Pharmacia) FPLC ionic exchange column (*14*). 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 unless noted.

Since each of the 1:3 hybrid tetramers isolated in this study included different mutations in the mutated subunits, methods to isolate each hybrid varied slightly. The KSCN concentration used to promote monomer exchange, as well as the linear NaCl gradient used for eluting from the ion exchange column, was optimized for each hybrid. The 45-Å 1:3 hybrid was stabilized against subunit exchange at 4 °C during hybrid formation and isolation. Only very small quantities of the parent mutant protein used to construct the 23-Å 1:3 hybrid were obtained from the standard purification. Therefore multiple protein preparations of this mutant protein were combined before hybridization with wild-type. Subunit exchange in the 30-Å and 33-Å 1:3 hybrids was not completely prevented by 2 mM Fru-6-P. Therefore, Fru-6-P was added to a final 20 mM immediately after elution from the ion exchange column. Both the 30-Å and 33-Å 1:3 hybrids were stable against exchange in the higher Fru-6-P concentration.

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, 3 μg of triosephosphate isomerase, and 3 mM ATP. Fru-6-P and MgADP concentrations were varied as indicated. To prevent competition between MgADP and MgATP in the active site (*9*), MgADP was added as a solution of equal molar MgADP/MgATP. In the absence of MgADP, 4 mM creatine phosphate and 40 μg/mL creatine kinase were added to convert MgADP contamination from substrate stocks to MgATP (*11*). Six microliters of EcPFK was used to initiate the enzymatic reaction, which was monitored at 340 nm over time. A unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol of fructose 1,6-bisphosphate per minute. MgADP contamination in MgATP was quantified using the method of Jaworek et al. (*20*).

Data Analysis. Data were fit to appropriate equations using the nonlinear least-squares fitting analysis of Kaleidagraph (Synergy) software. Tetramers with sufficient affinity for Fru-6-P to achieve saturation were fit to the Hill equation (*21*):

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

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. For hybrids with a single native active site, the linear region of the Fru-6-P saturation curves were fit to

$$\frac{v}{E_T} = \left(\frac{k_{cat}}{K_m}\right)[\text{Fru-6-P}] \quad (2)$$

Parameters obtained from these fits were then fit to

$$K_{app} = K^{\circ}_{app}\left(\frac{K^{\circ}_{ix/b} + [\text{MgADP}]}{K^{\circ}_{ix/b} + Q_{ax/b}[\text{MgADP}]}\right) \quad (3)$$

where $K_{app} = K_{0.5}$ when data were fit to eq 1 and K_{app} = the reciprocal of k_{cat}/K_m when data were fit to eq 2, as discussed in the text. $K^{\circ}_{app} = K_{app}$ when [MgADP] = 0, $K^{\circ}_{ix/b}$ = the dissociation constant for MgADP when [Fru-6-P] = 0 and the cosubstrate MgATP is saturating, and $Q_{ax/b}$= the coupling constant between MgADP and Fru-6-P with MgATP saturating (*12*).

$Q_{ax/b}$ is a measure of the influence MgADP has on Fru-6-P binding and Fru-6-P has on MgADP binding and is equal to the ratio of K°_{app} to K^{∞}_{app} where K^{∞}_{app} equals K_{app} when MgADP is saturating. Furthermore, $Q_{ax/b}$ can be related to the coupling free energy, $\Delta G_{ax/b}$, by the following (*9*):

$$\Delta G_{ax/b} = -RT\ln(Q_{ax/b}) \quad (4)$$

where R = the gas constant and T = absolute temperature in kelvin.

The $Q_{ax/b}$ determined for 1|1 hybrids were corrected for contributions from the mutated allosteric sites. This correction was accomplished by dividing $[1/(k_{cat}/K_m)]$ for the 1|1 hybrids

by $[1/(k_{cat}/K_m)]$ for the respective 1|0 control. The corrected data were then fit to eq 3.

RESULTS

Strategy. The current study has used hybrid tetramers to independently isolate each of the four unique heterotropic interactions between Fru-6-P and MgADP in EcPFK. EcPFK hybrid tetramer formation and isolation has previously been described (*14*). A hybrid tetramer with one wild-type subunit and three subunits with decreased Fru-6-P and MgADP affinities (1:3) contains a single native active site and a single native allosteric site (1|1) and therefore a single isolated heterotropic interaction. Isolating each of the four unique heterotropic interactions is dependent on the interfacial nature of both the Fru-6-P binding site and the allosteric site (Figure 1).

The key to this strategy is the ability to greatly diminish ligand binding to a site by modifying contact residues from only one side of the interface. Consistent with the notation utilized with BsPFK (*2*), we designate each side of the active site as "a" and "b", respectively. Similarly the sides of the allosteric site are designated "α" and "*β*", respectively. Previously we have shown that R243E nearly prevents binding of Fru-6-P to the active site (*14*). This represents an a-side mutation, as defined in Figure 1C. Additionally, R21A, a *β*-side mutation, inhibited the binding of MgADP to the allosteric site (*14*).

Provided that it can be demonstrated, as we do below, that mutations from the b-side of the active site and the α-side of the allosteric site similarly block binding, four different mutant proteins can be generated in which binding to active sites and allosteric sites is blocked. These four proteins can be designated [aα], [a*β*], [bα], and [b*β*] to indicate the location of the site-blocking mutations. Separately combining each of these four mutated proteins in 1:3 hybrids with wild-type creates four different 1|1 tetramers, each of which isolates a different interaction between the single native active site and the single native allosteric site as shown in Figure 2.

Active and Allosteric Site Mutations. A decrease in Fru-6-P affinity caused by R243E has previously been shown to be sufficient to resolve Fru-6-P binding to a single native active site in a 1|1 hybrid. In addition, the R21A mutation has been shown to eliminate MgADP binding in all attainable MgADP concentrations (*14*). Like R243, H249 interacts with the 6′-phosphate of bound Fru-6-P but from the b-side of the interface (*5*). However, as shown in Figure 1A, H249 and R243 in the same Fru-6-P binding site reside on different subunits. The H249E mutation reduces affinity for Fru-6-P well over 3 orders of magnitude. Indeed, the affinity is so poor, that V_{max} could not be determined even with 20 mM Fru-6-P (Figure 3A). Therefore, either R243E or H249E (a-side and b-side mutations, respectively) can be used in 1|1 a hybrid without Fru-6-P binding to the sites containing either of these mutations contributing to the measured heterotropic interactions.

As with the R21A mutation previously characterized (*14*), no allosteric activation was observed in the K213E (α-side mutant) protein, although the affinity for Fru-6-P in the absence of MgADP increases slightly, as is evident from the failure of $K_{0.5}$ for Fru-6-P to decrease with MgADP

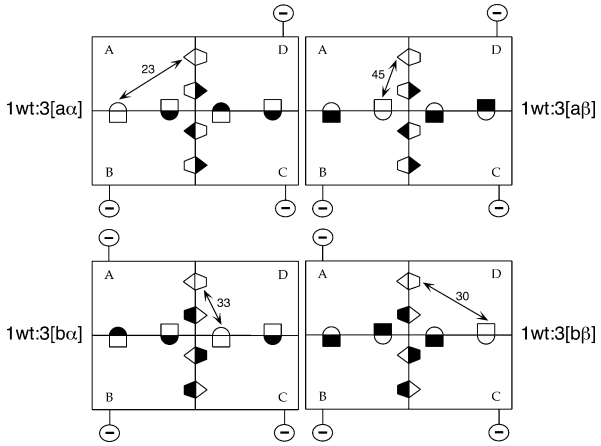

FIGURE 2: A schematic for the four hybrid tetramers that isolate each of the four unique heterotropic interactions contained in wild-type EcPFK. The K2E/K3E charge tag was added to facilitate separation of hybrid species and is indicated as a surface charge. In each tetramer, the four subunits are labeled A, B, C, and D. Symbol shapes and the specific residues mutated are as defined in Figure 1C. Filled symbols represent mutated portions of binding sites that confer reduced ligand affinity to the entire site in each case. The four active sites are located along the vertical dimer−dimer interface, and the four allosteric sites are located along the horizontal dimer−dimer interface in this depiction. Each binding site consists of two complementary sides as described in Figure 1 and in the text. The 23-Å and 45-Å interactions are depicted within the A subunit, and the 30-Å and the 33-Å interactions are depicted within subunit D, for reasons of pictorial clarity only. Since all subunits are identical, all four interactions can be depicted in any subunit. The lengths of arrows in this depiction are not proportional to true distance in the three-dimensional structure.

(Figure 3B). This result is consistent with the previously reported observation that K213A also does not exhibit activation by MgADP (*22*). The lack of allosteric effect in both the R21A and K213E mutations do not distinguish between the absence of MgADP binding and failure of the allosteric site to communicate with the Fru-6-P binding site. For our purposes, either property is sufficient.

Control Hybrids. Since hybrid tetramers with single isolated heterotropic interactions have a single native active site, these tetramers lack homotropic cooperativity in Fru-6-P binding. Therefore, heterotropic activation measured in hybrid tetramers with isolated heterotropic interactions cannot be quantitatively compared with the overall activation exhibited by wild-type tetramers. Instead, 1|1 hybrids must be compared to a control that has all four possible unique heterotropic interactions but no homotropic interactions (Figure 4). The construction and isolation of this control have previously been described, and it is designated the 1|4 control (*14*). Allosteric activation exhibited by this control is listed in Table 1 for comparison.

To ensure that the possible binding of ligands to the mutated R21A or K213E allosteric sites in the hybrid tetramers does not contribute to the observed activation of the experimental hybrids, two 1|0 control hybrids were constructed (Figure 4). The first 1|0 control is a 1:3 hybrid tetramer with one R21A subunit and three K2E/K3E/R21A/R243E mutated subunits. This hybrid tetramer, termed the $1|0_{21}$ control, serves as a control for the 45-Å and 30-Å interactions, both of which contain R21A mutations. The $1|0_{21}$ control has one native active site, three mutated active sites and four R21A mutated allosteric sites. The $1|0_{21}$ control

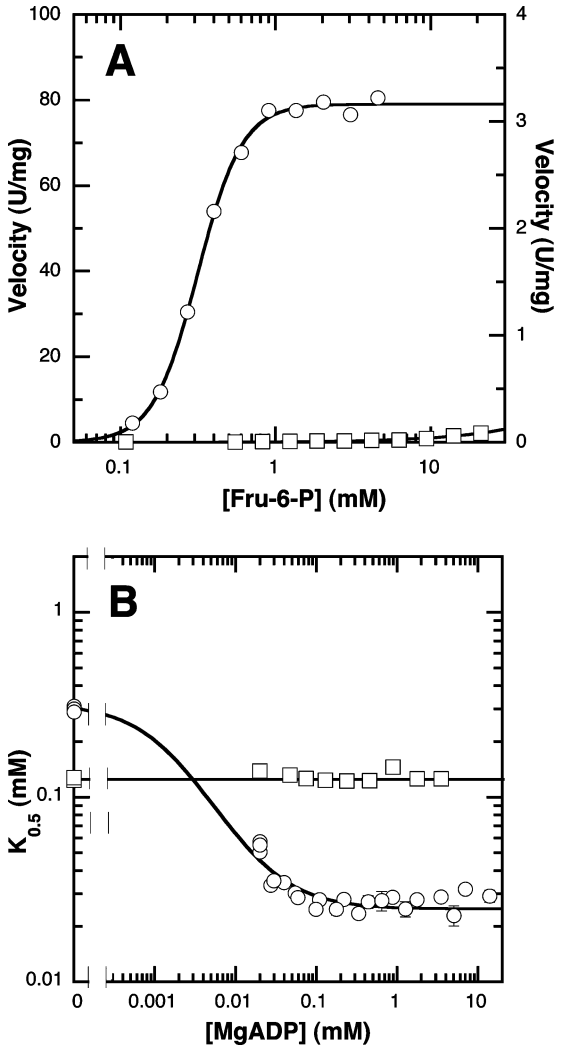

FIGURE 3: Panel A presents Fru-6-P titrations of activity for wild-type (○; left *y*-axis) and H249E (□; right *y*-axis). Lines represent best fits to eq 1 or 2. Panel B presents the apparent binding constant for Fru-6-P vs MgADP concentration for wild-type (○) and K213E (□). The curve through wild-type data represents the best fit to eq 3.

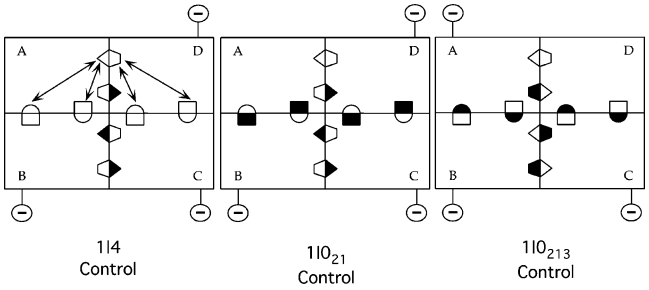

FIGURE 4: A schematic of the controls used in this study. Symbol shapes and the specific residues mutated are as defined in Figure 1C. Filled symbols represent mutated binding sites with reduced ligand affinity. The R243E mutation was used to decrease Fru-6-P binding in these controls. The R21A mutation was used to decrease MgADP binding in the 1|0_{21} control. The K213E mutation was used to decrease MgADP binding in the 1|0_{213} control.

has previously been characterized (*14*). Even though $Q_{ax/b}$ of this control is near 1 (1.15 ± 0.05), the coupling measured for both the 45-Å and 30-Å isolated interactions was corrected to account for this small contribution.

Table 1: Parameters from Linkage Analysis Obtained at 8.5 °C

enzyme	$Q_{ax/b}$	$\Delta G_{ax/b}$ (kcal/mol)	% contribution[a]
wild-type	16.1 ± 0.6	−1.56 ± 0.02	
1|4 control	155 ± 6	−2.87 ± 0.02	100
45-Å interaction	2.8 ± 0.1	−0.58 ± 0.01	20 ± 1
33-Å interaction	5.7 ± 0.3	−0.99 ± 0.03	34 ± 1
30-Å interaction	2.9 ± 0.2	−0.61 ± 0.03	21 ± 1
23-Å interaction	3.6 ± 0.2	−0.73 ± 0.04	25 ± 1

[a] Percent contribution with respect to $\Delta G_{ax/b}$ at 8.5 °C determined for the 1|4 control.

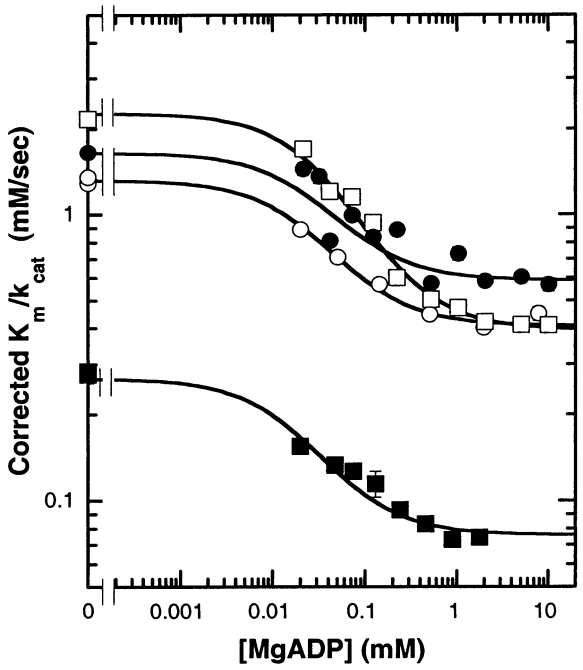

FIGURE 5: Corrected K_m/k_{cat} values from Fru-6-P titrations as a function of MgADP concentration. Data were corrected for possible contributions from mutated sites as described in the text. The 45-Å (○), the 33-Å (□), the 30-Å (●), and the 23-Å (■) heterotropic interactions are compared. Error bars are smaller than the symbols in most cases. Lines represent best fits to eq 3, and the resulting parameters are listed in Table 1.

The analogous 1|0_{213} control was made with one K213E subunit and three K2E/K3E/K213E/R243E mutated subunits (Figure 4). $Q_{ax/b}$ of the 1|0_{213} control was 1.06 ± 0.02 and the activation measured for both the 33-Å and 23-Å interactions were also corrected for this small contribution.

Hybrids Isolating Individual Heterotropic Interactions. A hybrid containing a single heterotropic interaction has previously been isolated and characterized by forming a mixed tetramer with one wild-type subunit and three K2E/K3E/R21A/R243E ([a,β]) modified subunits (*14*). The isolated 45-Å heterotropic interaction is contained in this hybrid tetramer. The 33-Å interaction was isolated by forming a 1:3 tetramer with one wild-type subunit and three K2E/K3E/K213E/H249E ([b,α]) mutated subunits. The 30-Å and 23-Å interactions were isolated in 1:3 hybrids with K2E/K3E/R21A/H249E ([b,β]) and K2E/K3E/R213E/R243E ([a,α]) mutated subunits, respectively.

Figure 5 shows a plot of the control corrected K_m/k_{cat} vs MgADP concentration for each of the four isolated heterotropic interactions. Lines in Figure 5 are best fits to eq 3. $Q_{ax/b}$ and $\Delta G_{ax/b}$, obtained from fits to eq 3, as well as percent contributions, are listed in Table 1. Of the four individual

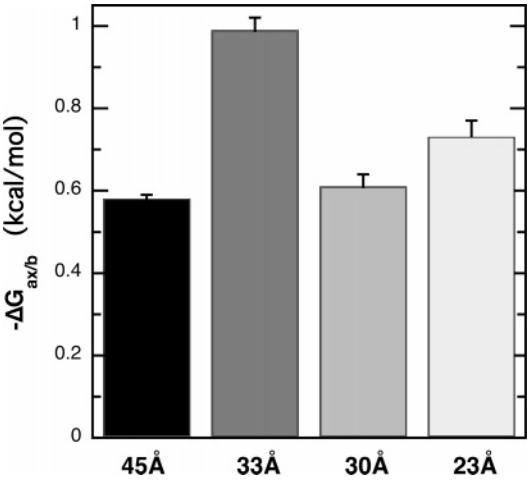

FIGURE 6: The coupling free energies determined for each of the isolated interactions.

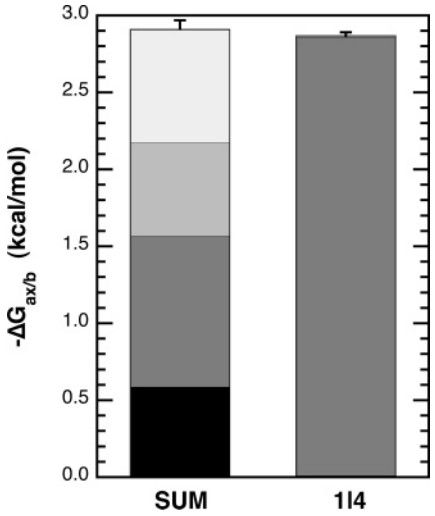

FIGURE 7: The sum of the coupling free energies determined for the isolated interactions compared to the total heterotropic coupling exhibited by the 1|4 control. Shadings are the same as those defined in Figure 6.

MgADP/Fru-6-P interactions, the 33-Å interaction contributes the largest percent, 34% of the total activation found in the 1|4 control. Both the 45-Å and 30-Å interactions contribute around 20% of the activation, and the 23-Å interaction, the shortest, contributes 25% of the total activation. Of particular interest is that three of the four individual interactions have a unique magnitude relative to the total observed interaction (Figure 6) and that the sum of the free energies of activation associated with the individual interactions is equivalent to that found in the 1|4 control (Figure 7).

DISCUSSION

There are many possible through-protein, ligand−ligand interactions in an EcPFK tetramer with four Fru-6-P and four MgADP binding sites. There are, however, only four potentially unique heterotropic interactions between Fru-6-P and MgADP. Each of the four heterotropic interactions may uniquely contribute to the overall allosteric activation. In an effort to begin to disentangle the allosteric web of potential interactions, the current study has used hybrid tetramers to independently isolate each of the four different heterotropic interactions between Fru-6-P and MgADP. We have recently used the same approach to isolate the four different inhibiting interactions between Fru-6-P and PEP in BsPFK (*1*, *2*). This approach essentially examines the magnitude of activation felt at a single active site from the binding of a single equivalent of MgADP in turn to each of the four different allosteric sites that exist in the homotetramer.

The long-term goal of this project is to understand allosteric interactions in wild-type EcPFK. However, hybrid tetramers with isolated heterotropic interactions only have a single native active site. Since wild-type EcPFK is known to have homotropic Fru-6-P interactions in the presence of MgATP, allosteric activation in the isolated heterotropic interactions cannot directly be compared to the activation by MgADP of wild-type. Therefore a 1|4 control (Figure 4), which lacks homotropic Fru-6-P interactions but has all four possible heterotropic interactions, is the proper reference for comparison. Significantly, the total activation by MgADP of the 1|4 control is much larger than the activation of wild-type due to the absence of homotropic activation by Fru-6-P in the control.

In addition to the 1|4 control, two different 1|0 controls (Figure 4) were created to correct for any possible contribution to activation caused by binding of MgADP to the mutated allosteric sites in hybrid tetramers. Fortunately, the $Q_{ax/b}$ for both of the 1|0 controls is near one, indicating that there is little or no activation attributable to R21A or K213E mutated allosteric sites. For completeness, the respective $Q_{ax/b}$ for each of the isolated heterotropic interactions have been corrected for the very small contributions that might occur from the mutated allosteric sites.

The 45-Å, 33-Å, 30-Å, and 23-Å interactions each are capable of introducing between −0.5 and −1.0 kcal/mol of coupling free energy (Figure 6), which represents 20%, 34%, 21%, and 25% of the total activation found in the 1|4 control, respectively. These contributions sum to 100% of the activation found in the 1|4 control (Figure 7). Such a result is predicted by thermodynamic linkage (*13*). The 1|4 control has four native allosteric sites and therefore could in principle have homotropic interactions between bound MgADP ligands. Observed heterotropic interactions will reflect MgADP homotropic interactions if these homotropic interactions are Fru-6-P-dependent (*13*). Since the four isolated heterotropic interactions account for 100% of the heterotropic activation measured in the 1|4 control, there appears to be no Fru-6-P-dependent MgADP homotropic interactions between the allosteric sites in the 1|4 control. Directly measuring the MgADP homotropic interactions during turnover is not possible since the MgADP contamination in the MgATP substrate is almost saturating. In addition, the fact that the individual couplings account for all of the activation present in the 1|4 tetramer argues strongly that the values determined for the individual couplings are not unduly perturbed by the modifications required to produce the corresponding hybrid enzymes.

The results obtained for the four individual heterotropic interactions suggests that the allosteric activation of EcPFK cannot be adequately interpreted by the popular two-state models. Each of the four interactions contributes uniquely to the total activation. This is in contrast to the common implementation of the sequential model (*23*), which predicts that one interaction, but not the other three, will activate

substantially (*2*). Furthermore, since the free energy of activation for the four interactions sum to 100%, all four interactions are necessary to obtain the total observed activation. This observation does not support predications of the concerted model (*24*), which would predict that each interaction would produce 100% of the allosteric effect.

A comparison between two allosteric proteins examined with the same methodology is now possible since we have recently characterized the percent contributions of each of the four unique inhibiting PEP interactions found in BsPFK (*1*, *2*). The pattern of contribution found for MgADP activation in EcPFK is significantly different than the pattern of contribution found for PEP inhibition in BsPFK. Notably very little allosteric inhibition is conferred by the 45-Å interaction, and the largest contributor is the 23-Å interaction in contrast to the results for the MgADP activation of EcPFK summarized in Figure 6. This observation may suggest that MgADP activation and PEP inhibition use different communication pathways as previously suggested (*25*, *26*). A second possibility is that the differences are specific to each particular isozyme.

In summary, each of the individual heterotropic pairwise coupling free energies that are responsible for MgADP activation of Fru-6-P binding to EcPFK are unique in magnitude, and they combine additively to produce the total heterotropic effect observed in the tetramer once the homotropic Fru-6-P activation has been deleted.

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