Isolation of a Single Activating Allosteric Interaction in Phosphofructokinase from *Escherichia coli*†

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ABSTRACT: *Escherichia coli* phosphofructokinase 1 (EcPFK) is a homotetramer with four active and four allosteric sites. Understanding of the structural basis of allosteric activation of EcPFK by MgADP is complicated by the multiplicity of binding sites. To isolate a single heterotropic allosteric interaction, hybrid tetramers were formed between wild-type and mutant EcPFK subunits in which the binding sites of the mutant subunits have decreased affinity for their respective ligands. The 1:3 (wild-type:mutant) hybrid that contained only one native active site and one native allosteric site was isolated. The affinity for the substrate fructose-6-phosphate (Fru-6-P) of a single wild-type active site is greatly decreased over that displayed by the wild-type tetramer due to the lack of homotropic activation. The free energy of activation by MgADP for this heterotropic interaction is −0.58 kcal/mol at 8.5 °C. This compares to −2.87 kcal/mol for a hybrid with no homotropic coupling but all four unique heterotropic interactions. Therefore, the isolated interaction contributes 20% of the total heterotropic coupling. By comparison, wild-type EcPFK exhibits a coupling free energy between Fru-6-P and MgADP of −1.56 kcal/mol under these conditions, indicating that the effects of MgADP are diminished by a homotropic activation equal to −1.3 kcal/mol. These data are not consistent with a concerted allosteric mechanism.

Phosphofructokinase (PFK[1]) catalyzes the transfer of a phosphate group from MgATP to fructose-6-phosphate (Fru-6-P) with the production of MgADP and fructose-1,6-bisphosphate. This reaction is a major regulation point of the glycolytic pathway. The enzyme from *Escherichia coli* (EcPFK) is inhibited by PEP and activated by MgADP when either ligand binds to the same allosteric site (*1*). Since PEP and MgADP cause a shift in affinity for Fru-6-P without affecting the maximal activity, both are classified as K-type effectors of the enzyme (*2*). MgADP also decreases the homotropic cooperativity displayed by Fru-6-P binding in the presence of MgATP (*2*). MgADP activation therefore involves both a direct effect on the binding of Fru-6-P and an alleviation of the MgATP antagonism of Fru-6-P binding. The purpose of this investigation was to establish the means for discovering the structural basis for these behaviors.

EcPFK is a homotetramer (*1*, *3*) in which each subunit (M_r = 34 000) contributes one active site and one allosteric site. The four subunits are assembled as a dimer of dimers (222 symmetry), and the four active sites are formed at one dimer−dimer interface while the four allosteric sites are formed at the other dimer−dimer interface (*1*, *3*). The activation by MgADP of Fru-6-P binding therefore derives from a total of four bound MgADP ligands potentially interacting with each of four Fru-6-P equivalents on a particular tetramer. A first step in understanding the basis for MgADP activation is to isolate a single Fru-6-P−MgADP activating interaction. To do this we set about to construct a hybrid EcPFK tetramer with one wild-type subunit and three mutated subunits of EcPFK in which the mutated subunits have substantially reduced affinity for both Fru-6-P and MgADP. Our strategy to reduce the affinity of these negatively charged ligands was to modify one or more positively charged residues in the binding sites that appeared, on the basis of the X-ray structure (*1*, *3*), to promote binding. Assuming the effects of these mutations were localized to the binding site, we would anticipate that hybrids between these mutant enzmes and wild-type PFK would closely resemble the structure of the wild-type enzyme. Consequently, any allosteric properties displayed by such a hybrid enzyme would be the result of a single heterotropic interaction between the single equivalent each of Fru-6-P and MgADP that bind to the respective native binding sites. We have recently employed a similar strategy for constructing hybrids of PFK from *Bacillus stearothermophilus* (BsPFK) for studying a single Fru-6-P−PEP inhibitory interaction (*4*).

In this case the binding of Fru-6-P to the single native site is noncooperative as expected, even in the presence of MgATP. Therefore, the actions of MgADP are not complicated by the ability of Fru-6-P to mimic the ability of MgADP to counteract the inhibitory effects of MgATP. Consequently, the current study also has revealed the contribution made by the homotropic interactions to the heterotropic couplings in the wild-type tetramer.

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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*.

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MATERIALS AND METHODS

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

Mutagenesis. pGDR16 (*5*), containing the EcPFK in pAlter I (Promega), was used with the Altered Sites II in vitro Mutagenesis System (Promega) to construct mutations according to manufacturer's instructions. The following mutagenesis primers were synthesized by the Gene Technologies Laboratory of the Institute of Developmental and Molecular Biology at Texas A&M University:

R21A	5′-CAGCGCAGAACGAACAACCCCAGCAATTGCGGCGTTCATGCCTGG-3′
R25A	5′-CAGACCTTCTGTCAGCGCAGAAGCAACAACCCCGCGAATTGCGGC-3′
R162E	5′-CATCACTTCCACCACGGAAATTTCCTGGTGAGAAGAAGAGGTGTCAC-3′
R243E	5′-GATGTGGCCCAGCACAGTTGCTTCGGTTTCACGACCGGT-3′
K2E/K3E	5′-CCGCTTGTCAACACACCGATTTCCTCAATCATGACTACCTCTGAAGC-3′

Plasmid DNA was isolated using Wizard spin preps (Promega). The DNA was sequenced across the mutated site to confirm the desired mutation. Plasmids containing wild-type and mutant EcPFK genes were transformed into DF1020 cells for protein expression (*6*, *7*).

Protein Purification. Wild-type and mutant EcPFK proteins were purified from DF1020 cells expressing the desired plasmid construct using a modified method of Kotlarz and Buc (*8*) as described by Johnson et al. (*5*). Protein concentration was determined by absorbance readings in the absence of nucleotides using $\in_{278} = 0.6$ cm^2 mg^{-1} (*9*).

Hybrid Formation and Separation. Hybrid tetramers were formed by mixing the two parent proteins in Buffer A with 2 mM DTT. Buffer A contains 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM EDTA. KSCN was added to a final concentration between 0.4 and 0.6 mM, depending on the hybrid being formed. Hybrid formation was relatively insensitive to total protein concentration and hybrids were successfully formed using total protein concentrations between 0.4 and 9 mg/mL. The hybridization reaction was incubated for 80 min at room temperature after KSCN addition and then dialyzed at 4°C into Buffer A containing 2 mM DTT and 2 mM Fru-6-P. Relative amounts of hybrid species were controlled by altering the ratio of parent proteins in the hybridization reaction. 2 mM Fru-6-P was required in all buffers after hybrid formation to prevent the dimer exchange observed in the absence of Fru-6-P (data not shown). Hybrid species were separated by applying to a Mono Q HR 10/10 anion-exchange column (Pharmacia) for FPLC. Hybrid species were eluted with a linear NaCl gradient. The slope of the gradient was optimized for separation of hybrid species of each hybridization reaction. Hybrid peaks were assigned by relative positions and confirmed by native polyacrylamide gel electrophoresis (see Figure 2) as described previously (*4*, *5*). Fractions were dialyzed into Buffer A with 2 mM Fru-6-P.

Kinetic Assays. Activity measurements were carried out in 1 mL of a 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, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, 5 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 (*10*), MgADP was added as a solution of equal molar MgADP/MgATP. In the absence of MgADP, 1 mM creatine phosphate and 10 μg/mL creatine kinase were added to convert MgADP to MgATP (*2*). A 10 μL sample 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. (*11*).

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 in which the Fru-6-P concentration dependence was saturable were fit to the Hill equation (*12*):

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

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 Fru-6-P titrations that did not exhibit saturation because the concentration of Fru-6-P was low relative to $K_{0.5}$, the data were fit to the following equation:

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

The effect of MgADP on the activity of modified or native EcPFK was evaluated by fitting to the following equation (*13*):

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

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. $K^o_{app} = K_{app}$ when [MgADP] = 0, $K^o_{ix/b}$ = 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.

$Q_{ax/b}$ is related to the coupling free energy, $\Delta G_{ax/b}$, between Fru-6-P and MgADP in the saturating presence of MgATP by (*10*):

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

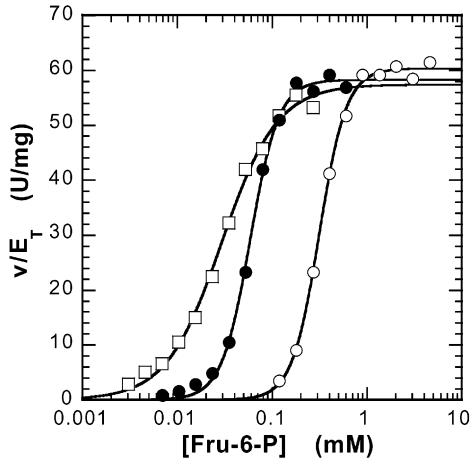

FIGURE 1: Influence of MgADP on EcPFK specific activity at 8.5 °C with [MgATP] equal to 3 mM. Control activity (○) was obtained in the presence of a creatine kinase coupling system that removes MgADP from the assay. Data obtained for MgADP concentration equal to 0.02 (●) and 3.5 mM (□) are also presented. The latter concentration fully saturates the allosteric site of the enzyme while the former amount represents the concentration of MgADP that is present due to contamination of the MgATP in the absence of the creatine kinase coupling system.

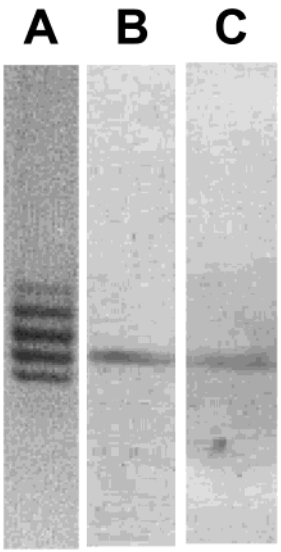

FIGURE 2: Lane A: native polyacrylamide gel electrophoresis of hybrid mixture containing all 5 possible hybrid combinations, including wild-type (top band) and mutant enzyme (bottom band) formed after dialysis of KSCN as described in Materials and Methods. Lane B: isolated 1:3 hybrid after 15 days of storage at 4 °C in the presence of Fru-6-P. Lane C: isolated 1:3 hybrid after 30 days of storage under the same conditions. Note the absence of appreciable re-hybridization under these conditions.

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

RESULTS

Activation by MgADP. Under conditions of saturating MgATP concentration, MgADP activates EcPFK by reducing the $K_{0.5}$ for Fru-6-P while simultaneously eliminating the positive cooperativity evident in Fru-6-P saturation profiles. This effect can be seen in Figure 1, in which saturating MgADP reduces $K_{0.5}$ to a value 6% that of wild type in the absence of MgADP and the Hill Coefficient to a value of 1.1 from 3.8 in the absence of MgADP. Note that these data, along with those presented below, were obtained at 8.5 °C. At this temperature MgADP and Fru-6-P exhibit higher binding affinities for EcPFK compared to the affinities displayed at 25 °C. We performed assays at this colder temperature in an effort to promote binding of Fru-6-P to hybrids containing a single native active site that otherwise exhibited relatively low affinity due to the loss of positive homotropic cooperativity (see below).

Hybrid Formation. The goal of this investigation was to isolate a single heterotropic coupling interaction between the substrate, Fru-6-P, and the allosteric activator, MgADP, within EcPFK. The approach involved constructing hybrid tetramers of the enzyme containing two different types of subunits, in a 1:3 combination, that differ by the presence or absence of various mutations including charge tag mutations. The charge tag mutations facilitate the separation, by anion exchange chromatography and native gel electrophoresis, of the 1:3 hybrid from the hybrids with different subunit compositions. A similar approach was recently employed to monitor subunit exchange in EcPFK (*5*) and to isolate a single inhibitory interaction in PFK from *B. stearothermophilus* (*4*). In the present study, three of the subunits contained the dual modification K2E/K3E as a charge tag. K2 and K3 are located on the surface of the enzyme away from binding sites and subunit interfaces. The choice of a positive to negative change was made because it augmented the small chromatographic separation caused by the other mutations contained in these subunits as described below. The E195K/E199K charge tag, previously reported (*5*), does not provide adequate separation in the presence of the binding site mutations.

Hybrids were formed by treating a mixture of both variants of EcPFK with KSCN to dissociate the enzymes, followed by dialysis to remove the KSCN and to promote reassociation as described in the Materials and Methods. Reversible subunit dissociation of EcPFK subunits by KSCN was first demonstrated by Deville-Bonne, et al. (*14*). KSCN treatment has been used to form hybrid tetramers in both *E. coli* and *B. stearothermophilus* PFK isozymes (*4*, *5*, *15*). Neither the KSCN treatment nor the K2E/K3E mutation exhibited kinetic and regulatory properties significantly different from those displayed by untreated wild type (data not shown). The typical separation of the hybrid mixtures on a Pharmacia Mono Q anion exchange column was sufficient to allow for the unambiguous identification and isolation of the 1:3 hybrid. The 1:3 hybrid, once isolated, was stable against rehybridization for 30 days provided it was stored in 2 mM Fru-6-P at 4 °C (Figure 2).

Active Site and Allosteric Site Mutations. Our strategy further required the identification of mutations that would diminish the binding affinity for Fru-6-P and MgADP at the active site and allosteric site, respectively. Positively charged arginine residues occur on either side of the subunit interface of each binding site, where they presumably promote the binding of the respective negatively charged ligands. Consequently these residues were the target of our efforts to compromise the binding of Fru-6-P and MgADP in their respective binding sites.

In particular, R21 and R25 are each approximately 3 Å from the nearest oxygen of the β-phosphate of MgADP bound in the allosteric site (*1*). R21A and R25A were each tested for their ability to disrupt MgADP binding at the

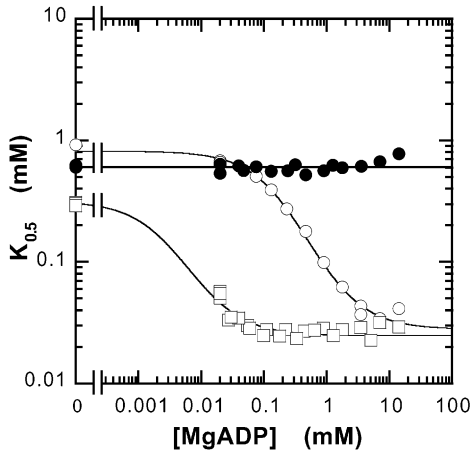

FIGURE 3: $K_{0.5}$ for Fru-6-P as a function of MgADP concentration for wild type (□), R25A (○), and R21A (●). $K_{0.5}$ values were obtained by fitting the dependence of specific activity on Fru-6-P concentration to eq 1 as described in the text. Curves shown through data for wild type and R25A represent the best fit of these data to eq 3.

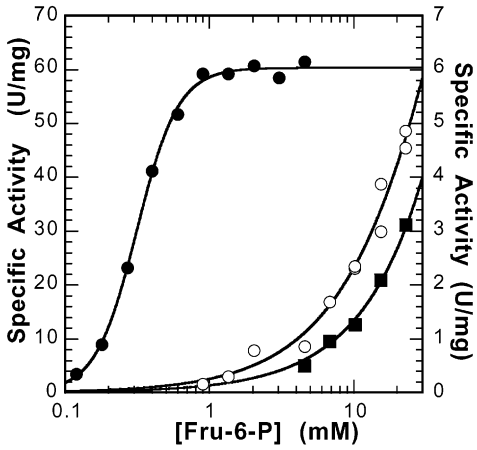

FIGURE 4: Specific activity vs Fru-6-P concentration for wild type (●, left vertical axis), R162E (○, right vertical axis), and R243E (■, right vertical axis). Solid lines represent the best fit of the data through either eq 1 (wild type) or eq 2 (R162E and R243E).

allosteric site (Figure 3). This evaluation was performed by examining the ability of MgADP to decrease $K_{1/2}$ for Fru-6-P. The $K^{o}_{ix/b}$ for the R25A mutant was over 100-fold greater than that of wild-type EcPFK; however, the R21A mutation exhibited no apparent activation over the entire range of ADP concentrations examined. Lau and Fersht also reported that R21A caused a greater decrease in MgADP binding affinity than R25A (*16*). R21A was used in subsequent experiments to reduce MgADP affinity.

Similarly, both R162E and R243E mutations were analyzed for their ability to decrease the affinity of EcPFK for Fru-6-P. R162 is 2.9 Å, and R243 is 3.3 Å away from the 6′ phosphate of fructose 1,6-bisphosphate bound in the active site in the X-ray structure determined by Shirakihara and Evans (*1*). Berger and Evans have previously reported that the R162S and R243S decreased Fru-6-P affinity (*17*). The R162E and R243E mutations were so successful at decreasing Fru-6-P binding affinity that saturation could not be observed with up to 20 mM Fru-6-P (Figure 4). We utilized R243E in subsequent experiments. Despite the lack of saturation, R243E clearly decreases the binding affinity for Fru-6-P by well over 2 orders of magnitude.

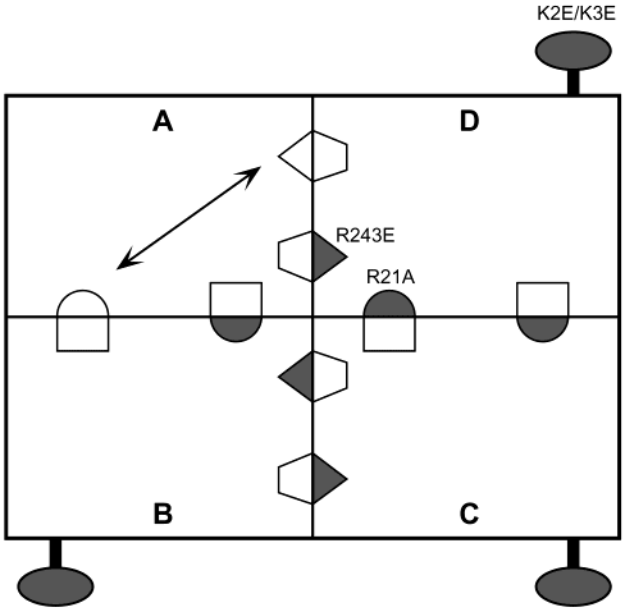

FIGURE 5: Schematic of the 1|1 hybrid tetramer PFK. The four subunits are labeled A−D, the four active sites lie along the vertical dimer−dimer interface, and the four allosteric sites lie along the horizontal dimer-dimer interface, consistent with the X-ray crystal structure determined by Shirakihara and Evans (*1*). Subunits B−D are depicted as containing the R243E mutation in the active site and the R21A mutation in the allosteric site, while subunit A represents wild type. Both mutations effectively block ligand binding at their respective sites as demonstrated in Figures 3 and 4. Subunits B−D also contain the surface charge modification of K2E/K3E that facilitates the chromatographic separation of this hybrid from other hybrids as described in the text. Note that only one active site and one allosteric site remain unmodified, giving rise to a single potential allosteric interaction, denoted by the double-headed arrow.

Experimental and Control Hybrids. A 1:3 hybrid of PFK was formed between one subunit of wild type and three subunits of a mutant EcPFK containing R243E, R21A, and K2E/K3E according to the procedures described above. As illustrated in Figure 5, this hybrid contains one native active site and one native allosteric site. To distinguish this hybrid enzyme from those discussed below, we introduce the following notation: *x*|*y* where *x* equals the number of native substrate binding sites (i.e. active sites) and *y* equals the number of native allosteric sites in the tetramer. This hybrid is therefore designated 1|1, whereas wild-type PFK would be designated 4|4. Provided that the low-affinity binding by either Fru-6-P or MgADP to the mutated binding sites can be ignored or accounted for, the 1|1 hybrid isolates a single potential allosteric interaction between Fru-6-P and MgADP binding to their respective native binding sites. A similar strategy has recently been used to isolate a single inhibitory heterotropic interaction for PFK from *B. stearothermophilus* (*4*).

The 1|1 hybrid not only isolates a unique heterotropic interaction but also eliminates all homotropic interactions between multiple Fru-6-P sites. To evaluate the effect of the loss of the homotropic interactions exclusively, a hybrid tetramer with all four native allosteric sites but only one native Fru-6-P binding site was constructed by forming a 1:3 hybrid between wild type and a mutant containing only the R243E and the K2E/K3E charge tags. This hybrid, termed the 1|4 control, primarily allows for the assessment of the

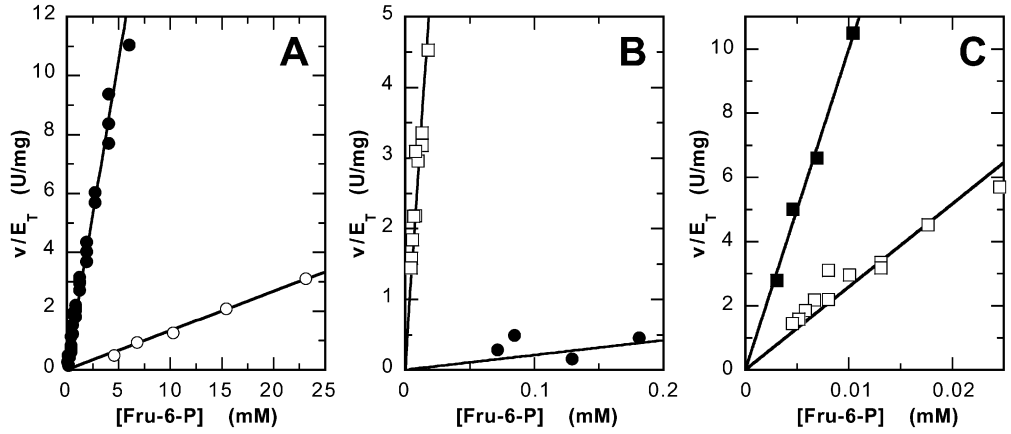

FIGURE 6: (A) Dependence of the specific activity of the 1|4 hybrid (●) and the R243E mutant enzyme, designated 0|4 (○) on [Fru-6-P] under first-order conditions. Slopes of the corresponding lines gives k_{cat}/K_m according to eq 2 described in the text. (B) Dependence of the specific activity of the 1|4 hybrid on [Fru-6-P] under first-order conditions with (□) and without (●) saturating [MgADP]. (C) Comparison of the 1|4 hybrid (□) to wild-type EcPFK, designated 4|4, (■) both in the presence of saturating [MgADP].

allosteric behavior of the enzyme in the absence of homotropic interactions between multiple Fru-6-P binding events.

To ascertain the extent to which the mutated R21A allosteric sites in the 1|1 hybrid might still be contributing to the observed coupling, a 1|0 control hybrid also was constructed in which one subunit contained R21A and three contained R21A, R243E, and K2E/K3E. This control has one native active site and no native allosteric sites. Any heterotropic allosteric effects exhibited by this hybrid would have to arise from the binding of MgADP to the mutated allosteric sites.

Functional Properties of the Hybrids. Wild-type EcPFK exhibits positive cooperativity when interacting with Fru-6-P. All hybrids that contain only a single Fru-6-P binding site lack cooperativity and therefore exhibit a low binding affinity. Binding to this isolated native site is so low that it cannot be saturated. However, the interactions of Fru-6-P with single native sites and sites containing the R243E mutation can be compared via the parameter k_{cat}/K_m, obtained by fitting data at low degrees of saturation to eq 2. In Figure 6A a comparison of k_{cat}/K_m for the 1|4 hybrid with the R243E mutant enzyme (0|4) is presented. Note that these data depend linearly on Fru-6-P concentration, indicating that k_{cat}/K_m can be readily obtained from the slope according to eq 2. Introducing 1 native site to the mutant tetramer increases k_{cat}/K_m by 15-fold. This effect could be due to an increased catalytic potential of the native site over the mutated site and/or an increase in the affinity for Fru-6-P displayed by the native site relative to the mutated site. Regardless, it is clear that k_{cat}/K_m predominantly reflects the behavior of the native site in enzyme tetramers containing a mixture of native active sites and those containing the R243E modification.

The single native site present in the 1|4 hybrid tetramer remains very responsive to the activator MgADP as shown in Figure 6B. In the presence of a saturating concentration of MgADP, the k_{cat}/K_m increases by 155-fold. This activation is much greater than the 16-fold activation experienced by wild-type PFK when one considers the change in $K_{0.5}$ (Figure 1). The difference in the extent of maximal activation between wild type and the 1|4 hybrid tetramer likely reflects the absence of positive homotropic cooperativity in Fru-6-P binding exhibited by the 1|4 hybrid (see discussion below).

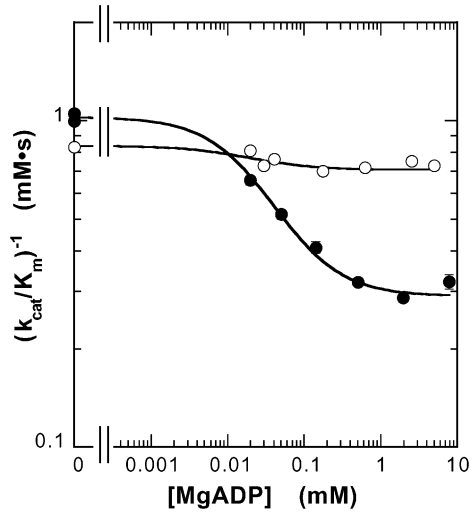

FIGURE 7: Influence of MgADP on the reciprocal of k_{cat}/K_m for the 1|4 hybrid (●) and the 1|0 control hybrid (○). Curves represent the best fit of each set of data to eq 3 described in the text. When not evident, error bars are smaller than the symbols.

This explanation is supported by the data presented in Figure 6C in which the 1|4 hybrid is compared to 4|4 (wild-type EcPFK) under conditions of saturating MgADP concentration. As noted in Figure 1, wild-type EcPFK loses its positive cooperativity when MgADP is saturating. Consequently, a plot of specific activity versus Fru-6-P concentration should be linear at low concentrations of Fru-6-P for 4|4 as well as 1|4 as is evident in Figure 5C. The resulting value of k_{cat}/K_m (given by the slope) for 4|4 is 3.9 times greater than k_{cat}/K_m for 1|4. This is very nearly equal to the value of 4 which would be expected if k_{cat} for each active site in 4|4 were equal to k_{cat} for the native site on 1|4.

The large activation by MgADP exhibited by the 1|4 hybrid is substantially diminished if the number of native allosteric sites is reduced from 4 to 1 as shown in Figure 7, where k_{cat}/K_m for the 1|1 hybrid as a function of MgADP concentration is presented. MgADP produces only a 3-fold increase in k_{cat}/K_m. This small effect results almost exclusively from the native allosteric site and not from MgADP binding to the modified allosteric sites as indicated by the lack of response of the 1|0 hybrid to comparable concentra-

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

enzyme	$K^{o}_{ix/b}$ (mM)	$Q_{ax/b}$	$\Delta G_{ax/b}$ (kcal/mol)
wild type	0.040 ± 0.003	16.1 ± 0.6	−1.56 ± 0.02
1|4 hybrid	ND[a]	155 ± 6[b]	−2.87 ± 0.02
1|1 hybrid	0.043 ± 0.006	3.2 ± 0.1	−0.66 ± 0.02
1|1 hybrid, corrected	0.041 ± 0.004	2.8 ± 0.1	−0.58 ± 0.01

[a] Not determined, although $K^{o}_{ix/b}$ can be estimated from the value of $Q_{ax/b}$ and the data in Figure 6C to be equal to approximately 0.055 mM. [b] Determined from the data in Figure 6B as described in the text.

tions of MgADP, also shown in Figure 7. Note in particular that the effect of MgADP on $(k_{cat}/K_m)^{-1}$ is saturable. The data in Figure 7 fit well to eq 3, which allows for the estimation of the dissociation constant for MgADP ($K^{o}_{ix/b}$) as well as the maximum of extent of activation by MgADP. Assuming the effects of MgADP can be attributed to K_m and not k_{cat} and that the rapid-equilibrium assumption is valid, the maximum extent of activation is equivalent to the coupling parameter, $Q_{ax/b}$, that would have been measured in more conventional ways had the binding of Fru-6-P to these species proven to be saturable. Values derived for $K^{o}_{ix/b}$, $Q_{ax/b}$ and $\Delta G_{ax/b}$ are presented in Table 1. The small effect of MgADP attributable to the modified allosteric sites, estimated by the apparent $Q_{ax/b}$ for the 1|0 control (1.15 ± 0.05) can be accounted for in these values by dividing the values of $(k_{cat}/K_m)^{-1}$ for the 1|1 hybrid by $(k_{cat}/K_m)^{-1}$ for the 1|0 control. These corrected values are also presented in Table 1.

DISCUSSION

Understanding the basis for allosteric behavior in an enzyme as simple as a homotetramer with a single active site and a single allosteric site per subunit represents a formidable challenge. Four different heterotropic coupling interactions between pairs of different ligands, combined with six different homotropic interactions between pairs of identical ligands, must be understood. We have recently approached this problem in PFK from *B. stearothermophilus* through the construction of a hybrid enzyme in which the allosteric behavior results from only a single heterotropic interaction (*4*). In this report we follow a similar approach with EcPFK to isolate a single activating interaction. However, challenges unique to the *E. coli* enzyme present themselves.

First, tetramers of EcPFK are less stable than their BsPFK counterparts. While isolated 1:3 hybrids of BsPFK are stable for several weeks, similar hybrids of EcPFK will begin to rehybridize to form other species if left for several hours at room temperature in the absence of ligands. Fru-6-P and low temperature will stabilize the hybrids, however, so that no evidence of subunit exchange is evident over 3 weeks as indicated in Figure 2; sufficient time for further characterizations to be performed.

Second, unlike BsPFK, EcPFK exhibits positive cooperativity in Fru-6-P binding (*2*). Consequently, a single native active site exhibits low affinity for Fru-6-P in the absence of the self-activation associated with subsequent Fru-6-P interactions. It is impossible to quantify unambiguously the binding constant for Fru-6-P to either a single native or mutated active site because saturation cannot be demonstrated. However, we have utilized k_{cat}/K_m to provide insight into the binding interactions of Fru-6-P to the hybrid and mutant species we have constructed. k_{cat}/K_m is the apparent first-order rate constant associated with substrate combination with an enzyme, and it is revealed by the linear dependence of the enzyme catalyzed reaction rate when substrate concentration is very low relative to K_m. Although k_{cat}/K_m is a rate parameter, a change in k_{cat}/K_m conveys information regarding the relative change in substrate binding affinity provided the change does not also produce a change in k_{cat} and provided that the rapid equilibrium assumption is valid with respect to substrate binding. The latter assumption has been addressed and found to be valid with respect to wild-type EcPFK (*18*) and would almost certainly then be also true for enzyme forms displaying even weaker binding affinity than wild type. Regarding the effects of MgADP on k_{cat}/K_m, all data in the literature (e.g., ref *2*) pertaining to wild-type EcPFK and the data obtained under our assay conditions as shown in Figure 1, indicate that MgADP has very little if any effect on k_{cat}. Therefore, we feel reasonably confident that the changes we observe in k_{cat}/K_m for the hybrid enzymes, that are due to the action of MgADP, reflect changes MgADP is having on the affinity of the enzyme for Fru-6-P. Consequently, changes in k_{cat}/K_m can lead to the quantification of the coupling free energy between Fru-6-P and MgADP.

Normally one can have less confidence in attributing differences in k_{cat}/K_m values associated with different enzyme species to differences in binding exclusively, since k_{cat} may vary as well in these comparisons. Nonetheless, the data in Figure 6C indicate that k_{cat}/K_m in 1|4 in the presence of MgADP is almost exactly what one would expect based upon the change in stoichiometry of native active sites alone. Consequently it is likely that at least the 1|4 hybrid has a high degree of structural, as well as functional, similarity to wild-type EcPFK.

Notably, the coupling free energy between Fru-6-P and MgADP estimated via changes in k_{cat}/K_m for the 1|4 hybrid is substantially larger than the coupling free energy for 4|4 (wild-type EcPFK). Evidently the activation by subsequent Fru-6-P binding in wild type offsets the activation that might otherwise be realized by MgADP. Formally this involvement of homotropic interactions in an apparent heterotropic allosteric effect can be understood by referring to the linked-function origins of allosteric behavior of a homodimeric enzyme (*19*). The apparent coupling constant, Q, between an allosteric ligand, X, and a substrate, A, for a dimeric enzyme with two identical active sites and two identical allosteric sites is given by

$$Q = Q_{ax1}Q_{ax2}\left(\frac{Q_{aa/xx}}{Q_{aa}}\right)^{1/2}\left(\frac{Q_{xx/a}}{Q_{xx}}\right) \qquad (5)$$

where Q_{ax1} and Q_{ax2} are the two structurally different heterotropic couplings, Q_{aa} and Q_{xx} are the two homotropic couplings between A and X, respectively, and $Q_{aa/xx}$ and $Q_{xx/a}$ refer to the homotropic couplings between A and X after the other ligands are bound. The relevant point to be appreciated from this relationship is that if the homotropic coupling between the multiple substrates that bind diminishes when the allosteric ligand binds, as it does for wild-type EcPFK, then the overall apparent heterotropic coupling will be less than would otherwise be realized from the hetero-

tropic couplings themselves. In the 1|4 hybrid, where no homotropic coupling between multiple substrate interactions can occur since only binding to the single native site is being measured, the effect of the heterotropic couplings will not be diminished and a larger overall heterotropic coupling should be, and is, observed. Thus we can conclude that the homotropic activation in wild-type EcPFK is equal to −1.3 kcal/mol, obtained from the heterotropic MgADP activation of the 1|4 hybrid (−2.87 kcal/mol) minus the apparent heterotropic activation observed in wild-type EcPFK (−1.56 kcal/mol).

The coupling deduced for the 1|4 hybrid stands in stark contrast to that measured for the 1|1 hybrid, which is much smaller. Although small, the influence of MgADP in the 1|1 hybrid is clearly saturable, as expected for an allosteric effect between a single active site and a single allosteric site. The effect of MgADP on the 1|1 hybrid is nonetheless substantially greater than the effect of MgADP on the 1|0 hybrid, indicating that the allosteric effect stems from the binding of MgADP to the single native allosteric site. After correcting for the very small effect that is contributed by the mutated allosteric sites, the coupling free energy for the 1|1 hybrid is −0.58 ± 0.01 kcal/mol compared to −2.87 ± 0.02 kcal/mol for the 1|4 hybrid and −1.56 ± 0.02 kcal/mol for 4|4 (wild-type EcPFK). Thus, the single coupling represents approximately 37% of the wild-type coupling but only 20% of the 1|4 coupling. Since no Fru-6-P homotropic effects are involved in both the 1|1 and the 1|4 hybrids, 1|4 serves as the more appropriate basis of comparison than does 4|4.

Provided that we make the assumption that the mutations that were introduced to generate the 1:1 hybrid do not substantially alter the 3-dimensional structure of EcPFK, the results suggest that the binding of a single equivalent of MgADP is insufficient to achieve the maximum activation that can ultimately be realized by MgADP and Fru-6-P in wild-type EcPFK. Within the limits of that caveat, therefore, these results do not support a concerted mechanism of activation of EcPFK, which predicts that a single equivalent of activator is sufficient to produce the maximum extent of activation throughout the tetramer.

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