Isolation of an Individual Allosteric Interaction in Tetrameric Phosphofructokinase from *Bacillus stearothermophilus*†

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ABSTRACT: Phosphofructokinase from *Bacillus stearothermophilus* (BsPFK) is a model allosteric enzyme system in which the interactions between substrates and allosteric effectors have been extensively studied. However, the oligomeric nature of BsPFK has made it difficult to determine the molecular basis of the allosteric regulation because of the multitude of different types of heterotropic and homotropic interactions that are possible between the four active sites and four allosteric sites in the native tetramer. In an attempt to alleviate the complexity of the system and thereby allow the quantitation of a single interaction between one active site and one allosteric site, site-directed mutagenesis has been coupled with a hybrid-forming scheme to create and isolate a tetramer of BsPFK in which only a single active site and a single allosteric site are capable of binding their respective ligands with high (i.e., near wild type) affinity. Characterization of this single allosteric interaction indicates that the free energy involved in the inhibition by the allosteric effector phosphoenolpyruvate (PEP) is 1.48 ± 0.15 kcal/mol compared to the 3.58 ± 0.02 kcal/mol measured for the enzyme.

Phosphofructokinase from *Bacillus stearothermophilus* (BsPFK)[1] is a homotetramer with each subunit having a molecular mass of 34 kDa (*1*, *2*). The enzyme displays little homotropic cooperativity in the absence of allosteric ligands, but it is heterotropically inhibited or activated by PEP or MgADP, respectively, effects that are manifested primarily by altering the binding affinity of the substrate Fru-6-P. A single BsPFK subunit has on average one active site and one allosteric site. However, neither of these sites is completely contained within a subunit. Instead, each binding site occurs at a subunit interface so that each subunit encodes two separate halves of each site that must be combined with the complementary half on another subunit to creat a complete functional binding site. Four subunits combine in this fashion to form an active tetramer with four identical active sites and four identical allosteric sites located along the two different dimer−dimer interfaces, respectively (*3*, *4*). All of these sites can potentially interact with each other by means of either three homotropic interactions between the active sites, three homotropic interactions between the allosteric sites, or four different heterotropic interactions between the active and allosteric sites. Such extensive communication throughout the tetramer makes resolving the molecular basis for allosteric behavior in this enzyme extremely complex.

To enable the study of a single heterotropic allosteric interaction between the substrate Fru-6-P and the allosteric inhibitor PEP, we have endeavored to construct a functional tetramer of BsPFK in which only a single active site and a single allosteric site are capable of binding their respective ligands with high affinity. This system would eliminate the homotropic interactions and reduce the number of heterotropic interactions to one. Constructing such a tetramer required that we first identify mutations that would greatly diminish or eliminate ligand binding, without otherwise disrupting tetramer assembly, and then introduce those mutations selectively to only three of the four total binding sites of each type.

To meet the first requirement, we have taken advantage of the fact that each type of binding site is lined with arginine or lysine residues (*3*, *4*) which undoubtedly serve to promote the binding of the respective negatively charged ligands. By introducing mutations in which one or more of these positively charged residues are changed to negatively charged glutamate residues, the binding affinity of Fru-6-P and PEP was found to be substantially diminished. These mutations were then introduced into only three of the four subunits of a tetramer by forming a mixture of hybrid tetramers between the mutant subunits and the wild-type enzyme. The hybridization procedure employed is similar to that described previously for *Escherichia coli* PFK (*5*−*7*) in which KSCN is used to reversibly dissociate the tetramer. Separation of the 1:3 hybrid (wild type:mutant) from hybrids of other compositions was accomplished by ion-exchange chromatography and facilitated by incorporating additional "charge-tag" mutations in the mutant subunits. These charge tags

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[1] Abbreviations: BsPFK, phosphofructokinase from *Bacillus stearothermophilus*; Fru-6-P, fructose 6-phosphate; PEP, phosphoenolpyruvate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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consist of lysine to glutamate mutations at positions 90 and 91, positions which are located on the surface of each subunit far removed from either subunit−subunit contacts or ligand binding sites according to the crystal structure (*3*, *4*).

A similar approach has been utilized to study lactate dehydrogenase from *Bificobacterium longum* (*8*). That tetrameric enzyme contains two allosteric sites and four active sites, but the symmetry of the enzyme results in two unique heterotropic interactions that were investigated. In this study a single allosteric interaction within the complex BsPFK tetramer has been isolated as outlined above by incorporating R162E to lower the affinity of the active sites and R211E and K213E to lower the affinity of the allosteric sites in the mutant subunits. The native active and allosteric sites that remain in the 1:3 hybrid are located 21 Å from each other, and significant allosteric communication is retained between these high-affinity sites.

MATERIALS AND METHODS

Materials. All chemical reagents used for protein purification and kinetic assays were of analytical grade and were purchased from Sigma-Aldrich, Bio-Rad, or Fisher. The Matrex Blue A−agarose resin used for purification of BsPFK was purchased from Amicon Corp. The coupling enzymes aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were purchased as ammonium sulfate suspensions from Boehringer Manheim. Prior to use in kinetic assays, the coupling enzymes were dialyzed extensively against 50 mM MOPS−KOH, pH 7.0, 100 mM KCl, 14 mM $MgCl_2$, and 0.1 mM EDTA. Creatine kinase, creatine phosphate, NADH, and sodium salts of Fru-6-P and PEP were purchased from Sigma. Bicinchoninic acid reagents used in determining protein concentration were purchased from Pierce. Site-directed mutagenesis was carried out using the Promega Altered Sites In Vitro Mutagenesis System. T4 DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Promega. Ampicillin repair and mutagenesis oligonucleotides were synthesized at the Gene Technologies Laboratory at the Institute of Developmental and Molecular Biology at Texas A&M University. Sequencing of plasmids to confirm the formation of mutants was also performed at the Gene Technology Laboratory. Deionized distilled water was used throughout.

Site-Directed Mutagenesis. Site-directed mutagenesis to create all of the mutants described in this study was formed using a plasmid containing the gene for BsPFK in pAlter under the lac promoter and the Promega Altered Sites In Vitro Mutagenesis System as described previously (*9*). To construct the mutant of BsPFK which contains the surface charge-tag substitutions (K90,91E), a single active site substitution (R162E), and two allosteric site substitutions (R211E/K213E), the gene for BsPFK containing the charge-tag substitutions was excised from its pALTER vector containing ampicillin resistance and subcloned into a new pALTER vector containing ampicillin sensitivity. This new plasmid was then used to construct new single-stranded DNA that was subsequently used to incorporate the active and allosteric site substitutions as described previously (*9*). All mutants were verified by sequencing the region of the BsPFK gene containing the substitution(s). Mutants were expressed in *E. coli* DF1020, a strain that produces no wild-type phosphofructokinase (*10*, *11*).

Protein Purification. Purification of wild type and all mutant forms of BsPFK was performed as described earlier with minor modifications (*9*, *12*).

Enzymatic Activity Assays. Activity measurements of BsPFK were conducted by coupling the reaction catalyzed by BsPFK to the oxidation of NADH and monitoring the corresponding decrease in the absorbance at 340 nm. Assays were carried out in 1.0 mL reaction volumes containing storage buffer (50 mM EPPS−KOH, 100 mM KCl, 14 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0), 0.2 mM NADH, 2 mM dithiothreitol, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, and 5 μg of triosephosphate isomerase adjusted to pH 8.0 at 25 °C. Creatine kinase and creatine phosphate were added to regenerate MgATP from MgADP to alleviate the activation of BsPFK by MgADP. The concentration of MgATP was kept constant and equal to 3 mM in all assays, and the concentrations of Fru-6-P and the inhibitor PEP were as indicated. Assays were initiated by the addition of 10 μL of BsPFK that had been appropriately diluted so as not to exceed a change of 0.1 absorbance unit at 340 nm per minute. One unit of activity is defined as the amount of enzyme needed to produce 1 μmol of fructose-1,6-bisphosphate per minute. Activity measurements were conducted on Beckman Series 600 spectrophotometers using a linear regression calculation to convert change in absorbance at 340 nm to enzyme activity.

Hybrid Formation. Five milligrams of wild-type BsPFK and 15 mg of mutant BsPFK were incubated together in 2 M KSCN for 30 min at room temperature to facilitate the dissociation of the parental tetramers. A greater amount of mutant protein was used to yield greater formation of the 1:3 (wild type:mutant) hybrid tetramer. After incubation, the sample was dialyzed against 20 mM Tris-HCl, pH 9, for 1 h at room temperature prior to being loaded on a Pharmacia Mono-Q HR 10/10 FPLC anion-exchange column previously equilibrated with 20 mM Tris-HCl, pH 9. Elution of the different hybrid compositions was accomplished using a linear salt gradient of 3 mM NaCl/mL at 0.5 mL/min. Fractions of 1.5 mL were collected, and their absorbance at 280 nm was analyzed to determine which fractions contained the hybrids. To provide supplemental evidence for the presence of the hybrids, activity assays (as described above) were performed on the fractions whose absorbance at 280 nm indicated that protein was present. However, these assays were performed in the presence of 20 mM Fru-6-P so as to detect some activity from the subunits exhibiting low affinity for Fru-6-P contained within the various hybrids.

Fractions comprising a single absorbance and/or activity peak were pooled together, and 1 mL was loaded into a Millipore Corp. Amicon Bioseparations Centricon YM-10 and centrifuged in a Beckman Model J-6B centrifuge at 3000*g* for 10 min to concentrate the protein. Fifteen microliters of this concentrated sample was loaded onto a 4% stacking/10% resolving native polyacrylamide gel (*13*) and run for 3 h at 100 V in an ice bath using the Bio-Rad Mini-Protean II electrophoresis system. After electrophoresis, gels were stained in 0.1% Coomassie blue for approximately 30 min prior to destaining and analysis.

Data Analysis. All data analysis was performed on either a Power Macintosh 7100/80AV or a Macintosh G4 using Kaleidagraph 3.08 (Synergy Software) to fit to the following equations. The concentration of Fru-6-P which resulted in

half-maximal activity, $K_{1/2}$, was determined using the Hill equation:

$$v = \frac{V_{max}[A]^{n_H}}{(K_{1/2})^{n_H} + [A]^{n_H}} \quad (1)$$

where v equals the steady-state rate of turnover, [A] represents Fru-6-P concentration, V_{max} represents the maximal specific activity, $K_{1/2}$ is the concentration of Fru-6-P giving a rate equal to one-half V_{max}, and n_H is the Hill coefficient. The variation in $K_{1/2}$ as a function of PEP concentration was fit to the equation:

$$K_{1/2} = K_{ia}{}^{0}\left(\frac{K_{iy}{}^{0} + [Y]}{K_{iy}{}^{0} + Q_{ay}[Y]}\right) \quad (2)$$

where [Y] represents the concentration of the allosteric inhibitor PEP (*14*−*16*). Therefore, $K_{ia}{}^{0}$ is the dissociation constant for the substrate Fru-6-P in the absence of any allosteric ligand, $K_{iy}{}^{0}$ is the dissociation constant for the allosteric ligand in the absence of substrate Fru-6-P, and Q_{ay} is the coupling parameter describing the extent to which the binding of the allosteric ligand affects the binding of substrate and vice versa as defined by the equation:

$$\frac{K_{ia}{}^{0}}{K_{ia}{}^{\infty}} = \frac{K_{iy}{}^{0}}{K_{iy}{}^{\infty}} = Q_{ay} \quad (3)$$

where $K_{ia}{}^{\infty}$ and $K_{iy}{}^{\infty}$ represent the dissociation constants for A and Y, respectively, in the saturating presence of the other ligand. By resolving both of the terms $K_{iy}{}^{0}$ and Q_{ay}, eq 2 allows separate quantification of both effector binding affinity and its action once bound, respectively.

The coupling parameter, Q_{ay}, describes both the nature and magnitude of the effect that allosteric ligands have on the binding of substrate. If $Q_{ay} < 1$, the allosteric ligand is an inhibitor, and if $Q_{ay} > 1$, the allosteric ligand is an activator. If $Q_{ay} = 1$, then the allosteric ligand has no effect on the binding of substrate. In the case of the inhibitor PEP, the smaller the value of Q_{ay}, the greater the extent of inhibition of substrate binding.

The coupling parameter can also be used to calculate the free energy associated with the interaction between substrate and allosteric effector, provided the rapid equilibrium assumption is valid as it is for BsPFK (*17*), using the equation:

$$\Delta G_{ay} = -RT \ln(Q_{ay}) \quad (4)$$

where ΔG_{ay} is the free energy of inhibition by PEP, R is the gas constant expressed in kcal/(deg·mol), and T is absolute temperature in kelvin. Allosteric inhibition is defined by a ΔG_{ay} value greater than zero while allosteric activation results in a ΔG_{ay} value less than zero. When no coupling between the ligands occurs, $\Delta G_{ay} = 0$.

RESULTS

The purpose of the mutations K90,91E used in the charge tag is to alter the chromatographic properties of the mutated subunits, and ideally they should not affect the kinetic or thermodynamic characteristics of the enzyme. Indeed, the turnover number, the K_m for MgATP, the $K_{1/2}$ for Fru-6-P, and the dependence of $K_{1/2}$ for Fru-6-P as a function of PEP determined from steady-state kinetics for both wild type and the mutant K90,91E are the same (data not shown). The charge tag does successfully alter the affinity of the enzyme on an FPLC Mono-Q anion-exchange column, however. Under typical elution conditions wild type is eluted from the column at a salt concentration of approximately 250 mM NaCl. However, nearly 400 mM NaCl is required to elute K90,91E from the column. This difference in chromatographic behavior is sufficient to separate not only mixtures of wild-type and charge-tagged proteins but also hybrid tetramers containing any combination of wild-type and mutant subunits (see below).

To isolate a single substrate and inhibitor binding site, the affinity of the remaining six sites must be decreased to the extent that the high-affinity sites can be saturated before significant binding occurs in the low-affinity sites. The goal was to decrease the affinity of the mutated sites by at least 1000-fold. This decrease would ensure that even in the saturating presence of an antagonistic allosteric ligand the binding of the substrate would occur solely in the high-affinity site before occurring in the low-affinity sites.

The strategy we employed exploited the fact that both the Fru-6-P binding site and the allosteric binding site contain several positively charged residues, predominantly arginines, undoubtedly to promote the binding of the respective negatively charged ligand (*3*, *4*). Figure 1A illustrates the result of incorporating an R162E mutation in the Fru-6-P binding site. As desired, the affinity of Fru-6-P is diminished approximately 3 orders of magnitude relative to that of the wild type. Significantly, however, Fru-6-P not only demonstrates a low affinity for this mutated binding site but also supports enzymatic turnover, indicating that the overall structure of the active site has not been radically perturbed.

The consequences of incorporating two mutations, R211E and K213E, in the allosteric site are summarized in Figure 1B. Since it is difficult to follow PEP binding directly, we have assesssed the impact of these mutations indirectly by observing the ability of PEP to inhibit the binding of Fru-6-P. As indicated in Figure 1B, BsPFK containing these mutations requires roughly 3 orders of magnitude more PEP to begin to increase the $K_{1/2}$ for Fru-6-P. The fact that $K_{1/2}$ does increase at least a fewfold at high PEP concentration suggests that allosteric communication has not been completely disrupted with these mutations, but PEP affinity has clearly been diminished substantially. Table 1 summarizes the kinetic and allosteric properties of wild type and these two mutant forms of BsPFK.

After these mutations were identified, they were combined with the charge-tag mutations to form a single modified enzyme containing the charge-tag mutations (K90,91E) and both the active site (R162E) and the allosteric site (R211E/K213E) mutations. This combined mutant should not only exhibit low binding affinity for Fru-6-P and PEP but also be chromatographically separable from wild type. Moreover, hybrids between this mutant and wild type should contain high-affinity binding sites in direct proportion to the number of wild-type subunits. Consequently, a 1:3 hybrid (wild type:mutant) between wild-type BsPFK and this mutant BsPFK would generate a tetrameric enzyme with only one native active site and one native allosteric site that can be isolated

Table 1: Steady-State Kinetic and Thermodynamic Coupling Parameters for Wild Type, the Active Site Mutant R162E, and the Allosteric Site Mutant R211E/K213E at 25 °C with [MgATP] = 3 mM

enzyme	V_{max} (units/mg)	K_{ia}^0 (mM)	K_{iy}^0 (mM)	Q_{ay}	Hill coefficient[a]
wild type	169 ± 1	0.035 ± 0.001	0.062 ± 0.001	0.0024 ± 0.0001	1.10 ± 0.05
R162E	146 ± 6	21.2 ± 1.9	ND	ND	1.15 ± 0.06
R211E/K213E	109 ± 1	0.058 ± 0.001	22 ± 17	0.47 ± 0.14	1.12 ± 0.08

[a] Pertaining to Fru-6-P saturation profiles.

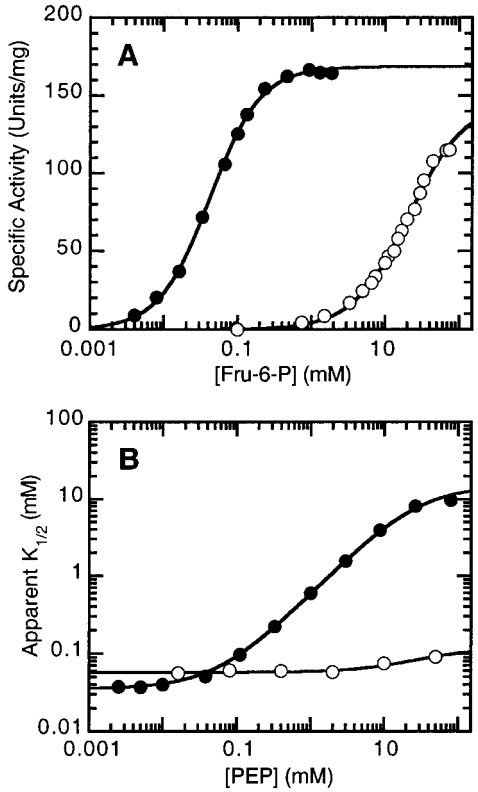

FIGURE 1: Steady-state kinetic experiments indicating the effects of active and allosteric site mutations on the binding of the substrate Fru-6-P and the inhibitor PEP, respectively. (A) Fru-6-P saturation profiles for wild type (●) and the active site mutant R162E (○) at 25 °C, pH 8.0, in the absence of PEP. The MgATP concentration was 3 mM. The curves correspond to the best fit of these data to eq 1 as described in the text. (B) Dependence of the apparent $K_{1/2}$ for Fru-6-P on increasing concentrations of the inhibitor PEP for wild type (●) and enzyme containing the allosteric site modifications R211E/K213E (○) at 25 °C, pH 8.0. Curves correspond to the best fit of these data to eq 2 as described in the text. The resulting parameters are presented in Table 1.

from the other hybrids using anion-exchange chromatography.

Hybrids between the two parental enzymes [wild-type BsPFK and the combined mutant BsPFK (containing the mutations K90,91E/R162E/R211E/K213E)] were formed following the KSCN-induced dissociation and reassociation protocol described above. Figure 2 illustrates the elution profile of hybrids formed from 5 mg of wild-type BsPFK and 15 mg of mutant BsPFK and separated using an FPLC MonoQ anion-exchange column. Five peaks are clearly evident, a result in agreement with the expected number of combinations possible between the two proteins. Wild-type BsPFK elutes identically with the first peak, and the mutant BsPFK coelutes with the last peak (data not shown). The three peaks that elute between these extremes correspond to 3:1, 2:2, and 1:3 (wild type:mutant) hybrids, respectively, with the 1:3 hybrid indicated by the arrow. These peaks can

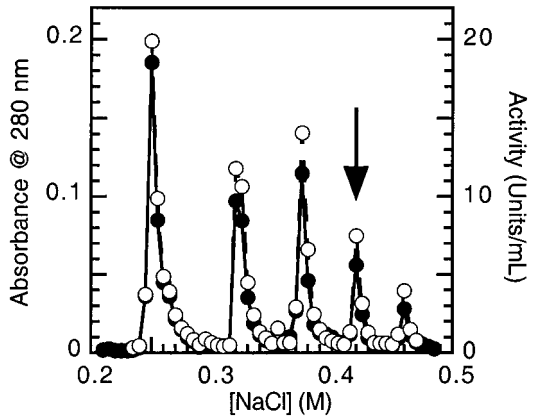

FIGURE 2: Elution profile from a Mono-Q anion-exchange column for hybrids between wild type and the combined mutant of BsPFK. Both the absorbance at 280 nm (●) and the activity (○) of the collected fractions were measured. The left peak corresponds to wild type, the right peak corresponds to the mutant, and the peak indicated by the arrow is the 1:3 (wild type:mutant) hybrid. Activity assays were performed with high concentrations of Fru-6-P to reveal enzyme forms with poor Fru-6-P binding affinities.

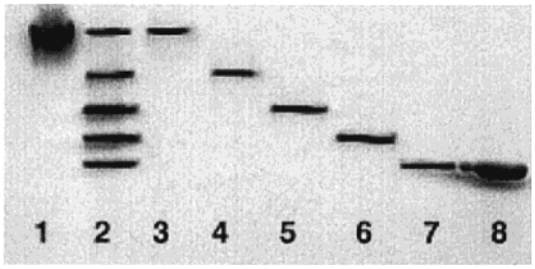

FIGURE 3: Native PAGE gel confirming the identification of the five peaks (numbered left to right) obtained in Figure 2. Lane assignments: (1) control sample of wild-type BsPFK, (2) sample of the hybrid mix prior to separation on the anion-exchange column, (3) peak 1 corresponding to wild type (4:0 hybrid), (4) peak 2 corresponding to the 3:1 hybrid, (5) peak 3 corresponding to the 2:2 hybrids, (6) peak 4 corresponding to the 1:3 hybrid, (7) peak 5 corresponding to mutant BsPFK, and (8) control sample of mutant BsPFK.

be detected both by absorbance at 280 nm and by enzymatic activity, although high concentrations of Fru-6-P were required to detect the mutant forms. Figure 3 shows a native PAGE gel that confirms the presence of five enzyme species after the hybridization procedure is performed and the ability of the Mono-Q column to isolate each of the resulting species cleanly. Once isolated, the hybrids are stable against rehybridization for at least 4 weeks as confirmed by similar native PAGE experiments (data not shown).

The dependence of enzyme activity on Fru-6-P concentration for wild-type BsPFK, the mutant form of BsPFK, and the 1:3 hybrid is shown in Figure 4. Uniquely, the Fru-6-P dependence of the 1:3 hybrid is clearly biphasic, illustrating both high- affinity and low-affinity binding sites for the substrate Fru-6-P, as would be expected if the one native

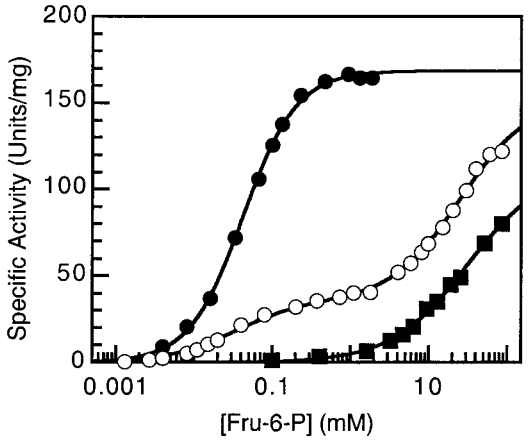

FIGURE 4: Fru-6-P saturation profiles for wild type (●), the combined mutant (■), and the 1:3 (wild type:mutant) hybrid (○) of BsPFK at 25 °C, pH 8.0. MgATP and PEP concentrations were kept constant at 3 and 0 mM, respectively. Curves correspond to the best fit of these data to either eq 1 or eq 5 as described in the text.

Table 2: Steady-State Kinetic Parameters for Wild Type, the Combined Mutant, and the 1:3 (Wild Type:Mutant) Hybrid of BsPFK at pH 8, 25 °C, and [MgATP] = 3 mM[a]

enzyme	V_{max} (units/mg)	$K_{1/2}$ (mM)	V_{max}' (units/mg)	$K_{1/2}'$ (mM)
wild type	169 ± 1	0.042 ± 0.001		
mutant[b]			112 ± 9	31 ± 6
1:3 hybrid	36 ± 1	0.034 ± 0.004	90 ± 3	25 ± 2

[a] Wild-type and mutant parameters were obtained by fitting the data shown in Figure 4 to eq 1 with the Hill Coefficient, n_H, equal to 1 while the hybrid parameters were obtained by fitting to eq 5 as described in the text. Values pertaining to the mutant are listed in the right two columns to facilitate their comparison to the corresponding hybrid parameters. [b] The combined mutant contains the mutations K90,91E, R162E,R211E, and K213E.

binding site retained its wild-type affinity for Fru-6-P. An analysis of each set of data confirms this to be the case. Data pertaining to wild type and mutant were fit to eq 1 (although the Hill coefficient did not prove to be significantly different from 1) while the data from the 1:3 hybrid were fit to the equation:

$$v = \left[\left(\frac{V_{max}[A]}{K_{1/2} + [A]}\right) + \left(\frac{V_{max}'[A]}{K_{1/2}' + [A]}\right)\right] \quad (5)$$

The curves in the figure correspond to the best fit of the respective data to the corresponding equations, and the parameter values recovered from these fits are presented in Table 2. The $K_{1/2}$ value for the high-affinity interaction closely agrees with the $K_{1/2}$ for the wild-type enzyme whereas the low-affinity $K_{1/2}$ is comparable to that of the mutant enzyme. Also, the maximal specific activity for the high-affinity interaction within the 1:3 hybrid is nearly one-fourth that of the wild-type tetramer as expected for a species containing one-fourth the number of native binding sites. Likewise, the maximal activity for the three low-affinity binding sites is approximately three-fourths that of the mutant tetramer.

Figure 5A shows the dependence of $K_{1/2}$ for Fru-6-P as a function of PEP concentration for wild-type BsPFK, the mutant form of BsPFK, and the high-affinity $K_{1/2}$ of the 1:3

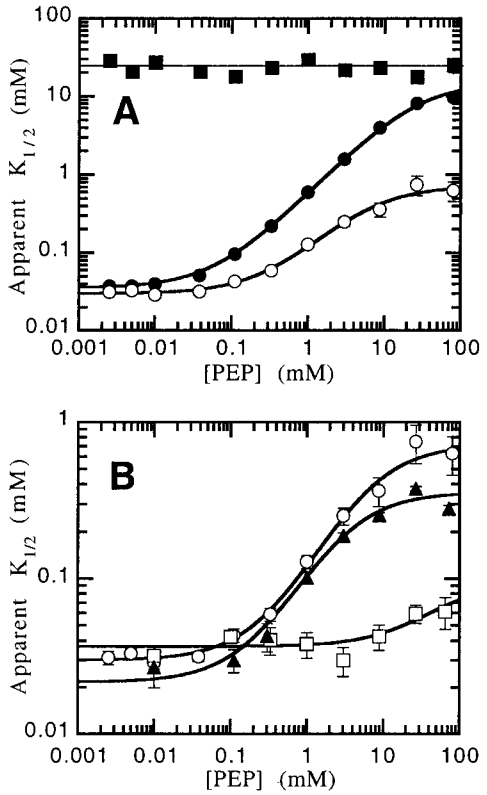

FIGURE 5: Dependence of the apparent $K_{1/2}$ for Fru-6-P on increasing concentrations of the inhibitor PEP. (A) PEP inhibition for wild type (●), the BsPFK mutant (■), and the 1:3 hybrid (○). (B) PEP inhibition for the uncorrected hybrid (○), the control hybrid (□), and the corrected 1:3 hybrid (▲). Error bars represent ± standard error and are smaller than the symbol when not evident.

hybrid. The $K_{1/2}$ for Fru-6-P of the mutant form of BsPFK does not change as a function of PEP concentration over the range examined, while PEP does increase both the $K_{1/2}$ for wild type and the high-affinity $K_{1/2}$ of the hybrid in a saturable manner. It is clear from these data, when interpreted in conjunction with those of Figure 4, that the binding of Fru-6-P to the single native active site in the 1:3 hybrid is subject to inhibition by PEP. Analysis of this inhibition, provided by fitting the data to eq 2, indicates that both the magnitude of the inhibition (as revealed by the coupling parameter Q_{ay}) and the affinity of PEP for the enzyme (as revealed by the dissociation constant K_{iy}^0) are both diminished in the hybrid relative to the wild type. The values of these parameters are presented in Table 3.

To confirm that the inhibition in the hybrid is due to the native allosteric site and not to inhibition derived from weak binding to the mutated allosteric sites, a different hybrid was constructed to serve as a control. This control hybrid was formed as outlined above except that, instead of incorporating a single wild-type subunit, the control hybrid contained a single subunit of BsPFK containing the allosteric site mutations R211E/K213E. This control hybrid tetramer therefore contained one native Fru-6-P binding site, three damaged Fru-6-P binding sites, and four damaged allosteric binding sites. The binding of Fru-6-P to this control hybrid was biphasic and similar to the 1:3 wild-type:mutant hybrid binding presented in Figure 4. The response of the high-affinity $K_{1/2}$ for Fru-6-P to increasing PEP concentration for the control hybrid is presented in Figure 5B and contrasted to that of the experimental hybrid containing a wild-type

Table 3: Thermodynamic Coupling Parameters for Wild Type, the Uncorrected 1:3 Hybrid, and the Corrected 1:3 Hybrid at 25 °C, pH 8.0

enzyme	K_{ia}^0 (mM)	K_{iy}^0 (mM)	Q_{ay}	ΔG_{ay} (kcal/mol)
wild type	0.035 ± 0.001	0.062 ± 0.001	0.0024 ± 0.0001	3.58 ± 0.02
1:3 hybrid (uncorrected)	0.030 ± 0.001	0.27 ± 0.04	0.042 ± 0.008	1.88 ± 0.11
1:3 hybrid (corrected)	0.031 ± 0.002	0.22 ± 0.07	0.081 ± 0.02	1.48 ± 0.15

subunit discussed above. In contrast to the experimental hybrid, the control hybrid does not exhibit significant inhibition by PEP except for a slight effect at very high PEP concentration. We attribute this small effect to the inhibition of Fru-6-P binding to the native binding site by PEP binding to the damaged, low-affinity allosteric sites. This effect, though small, would be contributing to the inhibition observed in the experimental hybrid containing a single native PEP binding site. The contribution to the inhibition by the mutated allosteric sites within the original 1:3 hybrid can be deleted from the observed inhibition, leaving only the contribution from the single native allosteric site, using the equation:

$$K_{1/2\ \text{corrected}} = \frac{K_{1/2}(\text{hybrid containing one native allosteric site})}{K_{1/2}(\text{hybrid containing zero native allosteric sites})} \quad (6)$$

Figure 5B also illustrates the dependence of the corrected $K_{1/2}$ values for Fru-6-P as a function of PEP concentration for the isolated single allosteric interaction between the native Fru-6-P binding site and the native allosteric site in the experimental hybrid. The thermodynamic parameters obtained after this correction are also presented in Table 3. The corrected results indicate that the single native allosteric site induces an approximately 12-fold decrease in the single active site's affinity for Fru-6-P, which corresponds to a 41% contribution (1.48 ± 0.15 kcal/mol) to the overall coupling free energy determined for the wild-type tetramer.

DISCUSSION

Allosteric enzymes are usually oligomeric, which complicates the examination of the energetics underlying heterotropic interactions between allosteric and substrate ligands. This complication arises not only from the fact that multiple copies of fundamentally different heterotropic interactions exist even in a highly symmetric homooligomer but also because changes in homotropic interactions can contribute to the apparent overall heterotropic influence between ligands. It has been shown previously (*18*), in the case of a homodimeric allosteric enzyme with one active site and one allosteric site per monomer, precisely how these features can influence the magnitude of the apparent heterotropic interaction between the allosteric and substrate ligands. The situation in higher order oligomers composed of identical subunits can be expected to be analogous. In the face of these complications, it is difficult to clearly interpret experiments designed to address the structural basis for allosteric behavior.

Accordingly, the oligomeric nature of PFK from *B. stearothermophilus* has made the analysis of specific heterotropic interactions very difficult. This study describes a successful approach to transforming the native homotetramer from a complex system containing an intertwined web of both homotropic and heterotropic interactions into a simplified heterotetramer containing a single heterotropic allosteric interaction and no homotropic interactions. This transformation was accomplished by combining a scheme for isolating hybrid tetramers of defined composition with mutations that greatly diminish the binding affinity of Fru-6-P and PEP but otherwise have little effect on the integrity of the protein. The former capability was achieved by introducing altered surface charge modifications to the enzyme so that hybrids formed with the wild type would exhibit varying degrees of mobility in ion-exchange chromatography. The latter was achieved by modifying the positively charged residues lining the binding sites. Since both the active and allosteric sites within BsPFK lie across subunit interfaces, another common feature of allosterically regulated enzymes (*19*, *20*), it was important that only mutations on a single side of either an active site or an allosteric site render the sites sufficiently "damaged" for this study. A similar result was obtained with lactate dehydrogenase from *B. longum* in which mutating a single side of the binding site for the allosteric activator fructose-1,6-bisphosphate resulted in a mutant form of the enzyme insensitive to allosteric activation (*8*).

Kinetic characterization of the 1:3 hybrid indicates that the hybrid-forming procedure itself does not significantly alter the properties of the heterotetramer compared to that of the native homotetramer. Furthermore, thermodynamic characterization of the wild-type homotetramer isolated after the hybrid formation procedure results in values for substrate and inhibitor affinities and coupling parameters that are identical to those measured for stock wild-type enzyme that has not undergone denaturation by KSCN (data not shown). Schachman and co-workers have observed a similar resilience of oligomeric functional properties to dissociation and reassociation with aspartate transcarbamoylase (*21*).

As expected, the interaction of Fru-6-P with the 1:3 hybrid is clearly biphasic, with the maximal velocity for the high-affinity interaction close to the predicted 25% of the wild-type tetramer (36 units/mg compared to 169 units/mg). Evidence for PEP binding with two affinities comes from a comparison of the inhibition of the 1:3 hybrid with that produced by an otherwise identical hybrid containing no native allosteric sites (Figure 5B). The higher affinity PEP interaction evident in the hybrid with a native site is about 4-fold lower than that exhibited by the wild type (Table 3); however, this can be explained by the positive cooperativity that is evident in PEP binding to the wild type, deduced from binding studies to a tryptophan-shifted mutant recently described (*22*).

It is clear from these data that the single native active site and a single native allosteric site are still able to communicate with each other in the 1:3 hybrid. However, the magnitude of the inhibition is diminished from that of the wild type. Analysis of the 1:3 hybrid indicates that this single interaction contributes a little less than one-half of the overall coupling free energy (1.48 ± 0.14 kcal/mol) compared to that measured for the wild-type tetramer (3.58 ± 0.02 kcal/mol). This smaller magnitude can be due to either the modifications

introduced to the protein and/or the fact that we have isolated only one of the several interactions that can in principle contribute the overall inhibition in the wild-type tetramer.

Nonetheless, it is remarkable that, despite various structural insults, a substantial allosteric communication remains. It seems quite likely that the basis of this interaction would bear at least some resemblance to its counterpart in the wild type. Indeed, an analysis of the structure of the wild type reveals that the native binding sites that remain in this 1:3 hybrid are 21 Å apart in the native structure. Regardless of perturbations that have unavoidably been introduced, the single interaction present in the 1:3 hybrid presents a enzyme for which the measured energetics correspond to a single physical relationship. As such, a precise determination of the structure−function relationships that give rise to the regulatory properties of this particular enzyme construct would seem to be possible.

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