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Redefining the Role of the Quaternary Shift in *Bacillus stearothermophilus* Phosphofructokinase

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Abstract

Bacillus stearothermophilus PFK (BsPFK) is a homotetramer that is allosterically inhibited by phosphoenolpyruvate (PEP), which binds along one dimer-dimer interface. The substrate, fructose 6-phosphate (Fru-6-P), binds along the other dimer-dimer interface. Evans et al., observed that the inhibitor, phosphoglycolate, bound structure, when compared to the substrate and activator bound structure of wild-type BsPFK, exhibits a 7° rotation about the substrate-binding interface, termed the quaternary shift [Schirmer, T., and Evans, P. R. (1990) *Nature* 343, 140-145]. We report that the variant D12A BsPFK exhibits a 100-fold increase in the binding affinity for PEP, a 50-fold decrease in the binding affinity for Fru-6-P, but an inhibitory coupling comparable to wild type. Crystal structures of the apo and PEP bound forms of D12A BsPFK have been determined (Protein Data Bank ID codes 4I36 and 4I7E, respectively), and both indicate a shifted structure similar to the inhibitor-bound structure of wild type. D12 does not directly bind to either substrate or inhibitor and is located along the substrate-binding interface. A conserved hydrogen bond between D12 and T156 forms across the substrate-binding subunit-subunit interface in the substrate-bound form of BsPFK. The variant T156A BsPFK, when compared to wild-type, shows a 30-fold increase in PEP binding affinity, a 17-fold decrease in Fru-6-P binding affinity, and an estimated coupling that is also approximately equal to wild-type. In addition, the T156A BsPFK crystal structure bound to PEP is reported (Protein Data Bank ID code 4I4I), and it exhibits a shifted structure similar to D12A BsPFK and the inhibitor-bound structure of wild type. The results suggest that main role of the quaternary shift may be to influence ligand binding and not to cause the heterotropic allosteric inhibition per se.

Phosphofructokinase-1 (PFK)¹ is a highly regulated enzyme that catalyzes the first committed step of glycolysis in prokaryotes. It is one of two non-homologous gene products that catalyze the transfer of the γ -phosphate of MgATP to fructose-6-phosphate (Fru-6-P) producing fructose-1, 6-bisphosphate and MgADP. PFK from the moderate thermophile, *Bacillus stearothermophilus* (Bs), is a homotetramer of 34 KDa per subunit that forms a dimer of dimers creating two unique subunit-subunit interfaces. One subunit-subunit interface contains four identical binding sites for Fru-6-P per homotetramer. The other subunit-subunit interface contains the four identical binding sites for the allosteric ligands

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¹Abbreviations: PFK, phosphofructokinase; Fru-6-P, fructose 6-phosphate; F1,6BP, fructose-1,6-bisphosphate; BsPFK, phosphofructokinase from *Bacillus stearothermophilus*; PEP, phospho(eno)pyruvate; PGA, phosphoglycolate; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, dithiothreitol; EPPS, N-(2-hydroxy-ethyl)piperazine-N'-(3-propanesulfonic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RMSD, root-mean-square deviation.

per homotetramer. The allosteric binding sites physiologically are capable of binding either MgADP or phosphoenolpyruvate (PEP). For BsPFK, MgADP serves as a very weak activator and PEP is a very strong inhibitor at 25°C. Both PEP and MgADP alter the affinity BsPFK displays for Fru-6-P without changing the enzyme's maximal activity; therefore, both allosteric ligands are considered to be K-type effectors.

The regulation of PFK has been the subject of study for nearly five decades since Blangy et al. first characterized the kinetics of PFK from *E. coli* and explained the behavior using a 2-state model (1) in which the 2 states undergo a concerted transition in accordance with the model proposed by Monod, Wyman, and Changeux (2). Schirmer and Evans compared the crystal structure of PFK bound to the inhibitor analog phosphoglycolate (PGA) (PDB accession code 6PFK) (3) with that obtained with BsPFK bound to the Fru-6-P and the activator MgADP (PDB accession code 4PFK) (4, 5) and found a substantial conformational difference. The conformational change includes a difference in the quaternary structures of the two enzyme forms. The quaternary structure of the inhibitor bound BsPFK has undergone a 7° rotation about the substrate binding interface, termed the quaternary shift, when compared to the substrate plus activator bound BsPFK. The conformational change also includes the movement of residues E161 and R162 in the active site. When Fru-6-P is bound to the enzyme, the positively charged side chain of R162 interacts with the negatively charged phosphate group of Fru-6-P. In the PGA bound structure, R162 effectively replaces the negatively charged side chain of E161. Given the structural differences seen between the substrate and activator bound and inhibitor bound structures of BsPFK, Schirmer and Evans proposed that these 2 structures corresponded to the 2 states that Blangy et al. had invoked to explain the allosteric behavior of PFK (3). Since 1990, BsPFK often has been used as a textbook example of the structural basis for allosteric regulation (6, 7).

A key distinction that should be made when assessing the allosteric action of a ligand, such as the inhibitor PEP, is between the affinity it displays for the enzyme and the effectiveness with which it inhibits once it binds. The latter attribute is quantitatively conveyed by the coupling between the inhibitor and substrate (8). We have previously shown that the repositioning of R162 and E161 has a surprisingly small role to play in the inhibitory coupling between Fru-6-P and PEP (9). The present investigation suggests that the quaternary shift similarly does not appear to be the basis for the coupling between Fru-6-P and PEP.

A significant structural perturbation associated with the quaternary shift is the disruption of several inter-subunit hydrogen bonds involving residue D12. Previous work performed by Ortigosa, et al. noted the significance of D12 when constructing certain hybrid forms of BsPFK (10). In that study, a D12A mutation was made in concert with other mutations in order to allow the proper exchange of subunits when making hybrids. The characteristics of the single mutant were never fully investigated. We report herein the properties of D12A that include the X-ray crystal structures of D12A BsPFK with and without PEP bound (PDB accession numbers 4I36 and 4I7E, respectively). Both structures exhibit a quaternary structure similar to that introduced by the binding of PGA to the wild type enzyme. While D12A alters the binding of PEP and Fru-6-P, it has very little effect on the allosteric coupling.

In an effort to further understand the role of D12, a residue that is 100% conserved in over 150 prokaryotic PFK-1 sequences, we also have examined the behavior of variants of BsPFK in which those residues with which D12 interacts across the interface have been changed to alanine. Generally these variants also exhibit comparable or greater coupling between Fru-6-P and PEP. The structure of one of these variants, T156A, bound to PEP has

also been determined (PDB Accession number 4I4I), and not surprisingly it also exhibits the quaternary shift.

It is of note that both D12A and T156A allowed for the first time structures of BsPFK to be solved in which the native inhibitor, PEP, is bound.

Materials and Methods

Materials

All chemical reagents used in buffers, protein purifications, and enzymatic assays were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Creatine kinase and the ammonium sulfate suspension of glycerol-3-phosphate dehydrogenase were purchased from Roche (Indianapolis, IN). The ammonium sulfate suspensions of aldolase and triosephosphate isomerase, and the sodium salts of phosphocreatine, ATP, and PEP were purchased from Sigma-Aldrich. The coupling enzymes were extensively dialyzed against 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 0.1 mM EDTA before use. The sodium salt of Fru-6-P was purchased from Sigma-Aldrich or USB Corporation (Cleveland, OH). NADH and DTT were purchased from Research Products International (Mt. Prospect, IL) and the crystallization materials were purchased from Hampton Research (Aliso Viejo, CA). DE52 and Memetic Blue 1 resin used in protein purifications were purchased from Whatman (Maidstone, England) and Promatic BioSciences (Rockville, Maryland), respectively. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis System from Stratagene (La Jolla, CA). Oligonucleotides were synthesized and purchased from Integrated DNA Technologies, Inc (Coralville, IA). DNA modifying enzymes and dNTP's were purchased from Stratagene (Cedar Creek, TX), New England Biolabs (Ipswich, MA), or Promega (Madison, WI). Deionized distilled water was used throughout.

Site-directed Mutagenesis

The plasmid pBR322/BsPFK (11) contains the gene for BsPFK behind the native *Bacillus stearothermophilus* promoter and was received as a generous gift from Simon H. Chang (Louisiana State University). Mutagenesis was performed following the QuikChange Site-Directed Mutagenesis protocol. Two complementary oligonucleotides were used to make the mutant genes and only the template oligo is shown below. The underlined bases designate the codon for the alanine that replaced the specific residue indicated.

D12A: G TTG ACA AGC GGC GGC GCC TCG CCG GGA ATG

T156A: C GAC AAA ATC CGC GAC GCC GCG ACG TCG

T158A: CGC GAC ACG GCG GCC TCG CAC GAG

S159A: GAC ACG GCG ACG GCC CAC GAG CGG AC

H160A: CG GCG ACG TCG GCC GAC CGG ACG TAC G

Wild-type and all mutant BsPFKs were expressed in *E.coli* RL257 cells (12), which is a strain of *E.coli* lacking both the PFK-1 and PFK-2 genes.

Protein Purification for Wild-type, T158A, S159A, and H160A BsPFK

The purification of both wild-type and variant BsPFKs was performed as described previously, with a few modifications (13). RL257 cells containing the appropriate plasmid were grown at 37°C for 16-18 hours in LB (Lysogeny Broth) ampicillin (Tryptone 10 g/L, yeast extract 5 g/L, and Sodium Chloride 10 g/L, ampicillin 100 ug/mL). Cells were pelleted and frozen for at least 12 hours. The cells were resuspended in purification buffer (10 mM

Tris-HCl, 1 mM EDTA pH 8.0) and sonicated at 0°C in 15 second pulses for 8 minutes with a Fisher 550 Sonic Dismembrator. The crude lysate was centrifuged using a Beckman model J2-21 centrifuge equipped with a JA20 rotor at $22,500 \times g$ for one hour at 4°C. The clear supernatant was heated at 70°C for 15 minutes, cooled on ice for 15 minutes, and centrifuged as before. The diluted supernatant for the wild-type protein was loaded onto a Mimetic Blue 1 column. The column was equilibrated with purification buffer before loading the supernatant containing the protein of interest. The column was washed with at least 5 bed volumes of purification buffer and the enzyme was eluted with a 0 – 1 M NaCl gradient. Enzyme containing fractions were pooled and dialyzed into 20 mM tris-HCl pH 8.5 and loaded onto a Pharmacia Mono-Q anion exchange column. The enzyme was eluted with a 0 – 1 M NaCl gradient and PFK containing fractions were combined, concentrated, then dialyzed into EPPS Buffer (50 mM EPPS, 10 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA pH 8.0). Concentrated enzyme was stored in EPPS buffer at 4°C. The final enzyme was determined to be pure by SDS-PAGE and the concentration was ascertained using the absorbance at 280 nm ($\epsilon = 18910 \text{ M}^{-1}\text{cm}^{-1}$ (14)).

Protein Purification for D12A and T156A BsPFK

Protein purification for D12A BsPFK and T156A BsPFK were carried out as described above, with the following exceptions. T156A BsPFK lost a significant amount of activity during the heating step unless 1 mM Fru-6-P was added to the supernatant before heating. Fru-6-P was not added to any other buffer for the duration of the purification for T156A BsPFK. D12A BsPFK and T156A BsPFK did not respond to the Mimetic Blue 1 resin the same as wild-type so that the supernatant was loaded onto a DE52 column. The DE52 column was prepared and treated the same as the Mimetic Blue 1 column.

Crystallization and Data Collection

The apo and the PEP bound D12A BsPFKs and the T156A BsPFK were crystallized using the hanging drop vapor diffusion method at 16°C. Apo D12A BsPFK was obtained through extensive dialysis of the mutant enzyme in EPPS buffer containing 50 mM EPPS, 10 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, 10 mM Fru-6-P pH 8.0 followed by extensive dialysis in Fru-6-P free EPPS buffer. The crystallization condition for the Apo D12A BsPFK structure was a 4 μL drop consisting of 2 μL of solvent (0.2 M magnesium chloride hexahydrate, 0.1 M tris hydrochloride pH8.5, and 30% w/v polyethylene glycol 4,000) and 2 μL of protein (stock concentration of apo D12A BsPFK was 35 mg/mL stored in EPPS buffer). PEP bound D12A BsPFK was obtained through purification of the enzyme as directed above. The PEP bound to the enzyme is indigenous *Escherichia coli* PEP. The crystallization condition for the PEP bound D12A structure was a 6 μL drop consisting of 2 μL of solvent (0.1 M sodium citrate tribasic dehydrate pH 5.6, 20% v/v 2-propanol, 20% w/v polyethylene glycol 4,000) and 4 μL of protein (stock concentration of D12A BsPFK was 34 mg/mL stored in EPPS buffer). The crystallization condition for the T156A BsPFK structure was either 2 μL of solvent (0.1M HEPES sodium pH 7.5 and 1.4 M Sodium citrate tribasic dehydrate) and either 3 or 4 μL protein (stock concentration of T156A BsPFK was 13 mg/mL). The T156A BsPFK crystal structure is bound to four PEP molecules, just as the PEP bound D12A BsPFK. And just as the D12A BsPFK is bound to the indigenous *Escherichia coli* PEP, so is the T156A BsPFK. Numerous attempts were made to crystallize T156A BsPFK without the inhibitor bound; however, none were successful. In addition, attempts were made to crystallize the variants T158A, S159A, and H160A BsPFKs. All proteins yielded crystals, but the quality of the crystals were insufficient and did not produce quality data.

Within 2-3 days crystals formed for both species (D12A and T156A BsPFK) of enzyme. Variant BsPFK crystals were briefly soaked in cryogenic mother liquor containing 30%

ethylene glycol and then flash-frozen in a liquid N₂ stream at 100 K. Diffraction data for the apo and PEP bound D12A BsPFK crystals were collected on an Advanced Photon Source (APS) beam line 23-ID (insertion device) using a MAR 300 CCD detector (MarMosaic from Marresearch-Charged Coupled Device) (Rayonix LLC, Evanston, IL). The high resolution data of T156A BsPFK was collected at beamline 19-ID on an Area Detector Systems Corporation Q315 area detector at the Advanced Photon Source, Argonne National Laboratory. The HKL2000 program package (HKL Research, Inc., Charlottesville, VA) (15) was used for integration and scaling of the PEP bound D12A crystals, while d*TREK (Rigaku Americas, Woodlands, TX) was used for integration and scaling of the apo D12A and T156A crystals (15, 16). Data collection details are summarized in Table 2.

Structure Determination and Refinement

Molecular replacement program PHASER (University of Cambridge, UK) (17) was used to solve the structure of the apo and PEP bound BsPFKs using the phosphate bound crystal structure of BsPFK (PDB accession code 3PFK) (5) with waters and ions removed as the search model. Rigid body refinement followed by simulated annealing refinement at 5000K was carried out using Phenix (Python-based Hierarchical Environment for Integrated Xtallography, Berkely, CA) (18) for the apo structure while CCP4 Refmac (York Structural Biology Laboratory, University of York, Heslington, UK) with TLS refinement (translation, libration and screw-rotation) (Science and Technology Facilities Council, Daresbury, UK) were used for the refinement of the PEP bound protein (19, 20). Subsequently, refinement was carried out in alternating cycles of manual model building in COOT (Crystallographic Object-Orientated Toolkit, Oxford, UK) (21) followed by refinement until the R-factors converged. The stereochemistry of the final models of the BsPFK enzymes were verified by MolProbity (Duke University, Durham, NC) (22).

Kinetic Assays

Activity measurements for PFK were carried out in a 0.6 mL reaction volume of EPPS buffer containing 50 mM EPPS, 10 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 3 mM ATP, 250 µg aldolase, 50 µg of glycerol-3-phosphate dehydrogenase, 5 µg of triosephosphate isomerase, 40 µg/mL of creatine kinase and 4 mM phosphocreatine at pH 8.0 and 25 °C. Fru-6-P and PEP were added at varied concentrations as indicated. Assays were started by the addition of 6 µL of appropriately diluted PFK and the reaction was monitored as the absorbance at 340 nm decreased over time. Dilution of T156A BsPFK in EPPS buffer resulted in significant loss of activity; therefore, all dilutions of T156A BsPFK were done in EPPS buffer containing 1 mM Fru-6-P. The addition of Fru-6-P resulted in T156A BsPFK remaining stable throughout the duration of the assays. The rate of the reaction was measured on Beckman Series 600 spectrophotometers. One unit of PFK activity is described as the amount of enzyme needed to produce 1 µmol of F16BP per minute.

Data Analysis

Data were fit to the following equations using the least-squares fitting analysis of Kaleidagraph software (Synergy). Initial velocity activities, that were measured in the kinetic assays where the Fru-6-P concentration was saturable, were fit to the Hill equation (23):

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

where v is the initial rate, E_T is the total enzyme active site concentration, $[A]$ is the concentration of the substrate Fru-6-P, k_{cat} is the turnover number, $K_{1/2}$ is the concentration of Fru-6-P that gives one-half the maximal specific activity, and nH is the Hill coefficient. In the D12A BsPFK kinetic assays, the $K_{1/2}$ cannot be determined because its value is too large; therefore, the data were fit to the following equation:

$$\frac{v}{E_T} = \frac{k_{cat}}{K_{1/2}} [A]. \quad (2)$$

Analysis of Allosteric Response

The heterotropic allosteric responses of BsPFK to PEP were quantified with reference to the single substrate, single modifier mechanism as described previously (8, 24, 25). For an enzyme like BsPFK, that exhibits heterotropic allosteric effects exclusively as a perturbation of the apparent affinity for the substrate, Fru-6-P, the allosteric response can be quantified by 2 parameters: the dissociation constant for PEP in the absence of Fru-6-P, K_{iy}^0 , and the coupling parameter, Q_{ay} . Q_{ay} is defined by the following:

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

where K_{ia}^0 equals the dissociation constant for Fru-6-P in the absence of PEP, K_{ia}^∞ equals the dissociation constant for Fru-6-P in the saturating presence of PEP, K_{iy}^∞ equals the dissociation constant for PEP in the saturating presence of Fru-6-P, and the second equality is required by the principle of thermodynamic linkage. Equation 3 indicates that Q_{ay} quantitatively reveals both the nature of the allosteric effect (values less than 1 imply inhibition and values greater than one indicate activation) and the magnitude of the allosteric effect.

In practice, K_{iy}^0 and Q_{ay} are determined by plotting either the $K_{1/2}$ from Equation 1 or the reciprocal of $k_{cat} / K_{1/2}$ from equation 2 versus the PEP concentration and fitting these data to the following equation as previously described (8):

$$K_a = K_{ia}^0 \frac{K_{iy}^0 + [Y]}{K_{iy}^0 + Q_{ay}[Y]} \quad (4)$$

where K_a is equal to $K_{1/2}$ when data were fit to Equation 1 or $K_{1/2} / k_{cat}$ when data were fit to Equation 2, and $[Y]$ is the concentration of PEP.

Results

As documented by Shirmer and Evans (3), BsPFK undergoes a quaternary shift of about 7° about the dimer-dimer interface that contains the substrates binding sites upon the binding of the inhibitor phosphoglycolate, PGA. This shift causes a concomitant unwinding of the last turn of helix 6 as shown in Figure 1. The loop that forms when PGA is bound contains residues 156-160. In the Fru-6-P and MgADP bound BsPFK crystal structure, D12 interacts with T156, S159, and H160 in the adjacent subunit when it is in the helix configuration (Figure 1A). In the PGA bound BsPFK structure, D12 interacts with only T158 across the interface and not with the other residues of the loop (Figure 1B). Thus D12, which is 100% conserved in bacteria containing PFK-1, exhibits a complete change in its interacting partners when the inhibitor binds. T156, also 100% conserved, experiences a change in

interacting partners as well, from D12 when Fru-6-P and MgADP are bound to Y164 (an intra-subunit interaction not shown in Figure 1) when the inhibitor is bound.

The specific activities of wild-type and D12A BsPFK, are shown Figure 2 as a function of Fru-6P concentration. Equation 1 was used to fit these data. However, the apparent dissociation constant for Fru-6-P ($K_{1/2}$) from D12A BsPFK is substantially increased compared to that of wild-type, which makes performing complete Fru-6-P titrations in the presence of inhibitor impossible due to the inability to achieve saturation by substrate. Therefore, to measure the nature and magnitude of the allosteric effect between Fru-6-P and PEP for D12A BsPFK and other variants exhibiting low affinity, only data in the linear region of the Fru-6-P titration curves at low concentrations of Fru-6-P were examined and fit to Equation 2. The slope of this linear region is equal to $k_{cat}/K_{1/2}$ and reflects the first binding event of Fru-6-P to the enzyme.

Figure 3 compares plotting $K_{1/2}$ or $K_{1/2}/k_{cat}$ versus the PEP concentration for wild-type BsPFK in order to demonstrate the validity of using $K_{1/2}/k_{cat}$ in Equation 3. The apparent dissociation constant for PEP in the absence of Fru-6-P, K_{iy}^0 , and the coupling parameter, Q_{ay} , determined from both methods agree within error as can be seen by the similar dependence of the transition on PEP concentration and the similar distance between the plateau values, respectively (8). The apparent dissociation constant for Fru-6-P, K_{ia}^0 , is the only parameter of Equation 3 that is not comparable between the two techniques. However, by simply fitting Equation 1 to a Fru-6-P titration curve in the absence of PEP, K_{ia}^0 can be approximated as being equal to $K_{1/2}$ from this fit. Subsequent $K_{1/2}$ values as a function of PEP concentration can then be calculated for comparison purposes by normalizing to this value, assuming that k_{cat} remains relatively constant. This analysis assumes that the rapid equilibrium assumption with respect to Fru-6-P binding remains valid as PEP concentration is varied as we have found to be the case for the wild type BsPFK (28).

Figure 4A illustrates the normalized $K_{1/2}$ values versus PEP concentration for D12A BsPFK versus wild-type. Kinetic and coupling parameters derived from these data are presented in Table 1. D12A BsPFK exhibits a dissociation constant for Fru-6-P, K_{ia}^0 , of 1.4 ± 0.1 mM, which is almost 50-fold greater than the dissociation constant for substrate from wild-type BsPFK. By contrast, D12A BsPFK has a dissociation constant for PEP, K_{iy}^0 , equal to 0.45 ± 0.01 μ M, which is about 100-fold smaller than K_{iy}^0 for wild-type. Most striking, however, is that despite these large differences in binding affinities, the coupling parameter between Fru-6-P and PEP for D12A is remarkably similar to that of wild-type.

The $K_{1/2}$ versus the PEP concentration for wild-type and the other variants of BsPFK are also shown in Figures 4A and 4B, and the kinetic and coupling parameters for these forms are given in Table 1. The dissociation constants for Fru-6-P from T158A, S159A, and H160A are comparable to the comparable dissociation constant of wild-type BsPFK.

However, the K_{ia}^0 for T156A is 0.52 ± 0.005 mM, which represents a 17-fold decrease in the substrate binding affinity when compared to wild-type. The PEP binding affinities of the mutant BsPFKs are a bit more varied. T156A, S159A, and H160A all exhibit enhanced PEP binding affinity, though to different degrees. T158A, unlike the other variants, exhibits a diminished PEP binding affinity of almost 2-fold compared to that of wild-type. D12A and T156A BsPFKs are the only two variants that show a significant enhancement in PEP binding affinity while also exhibiting a diminished Fru-6-P binding affinity.

The coupling constants of the variants of BsPFK vary dramatically depending on the mutation. T158A is the only mutant BsPFK that shows a significant decrease in coupling, as

conveyed by an increase in the value of Q_{ay} . Both S159A and H160A BsPFKs exhibit enhanced coupling, when compared to wild-type, while the coupling parameters for T156A and D12A BsPFK are remarkably similar to those of wild-type and D12A BsPFKs.

Diffraction quality crystals of D12A bound to PEP were obtained and a structural model was created consistent with data obtained by X-ray crystallography. The PEP binding of this mutant is enhanced to such a degree that the endogenous PEP stayed bound to the enzyme throughout the extensive purification and dialysis processes. The crystals belong to the space group $P2_12_12_1$ with unit cell dimensions of $a=96.65$, $b=112.97$, and $c=131.04$. The asymmetric unit consists of one tetramer. The final structure was refined to R-factor/R-free values of 18.86%/23.48% using diffraction data to 2.0 Å resolution. Given the kinetic and coupling similarities between D12A BsPFK and T156A BsPFK, diffraction quality crystals of T156A BsPFK were also obtained and a model of the structure was determined. The crystal structure of T156A BsPFK also has four PEP molecules bound to the effector binding sites. The crystals belong to the same space group as PEP bound D12A BsPFK crystals and are similar. The final structure was refined to R-factor/R-free values of 16.6%/23.78% to a resolution of 2.5 Å. The details describing the diffraction data and refinement statistics are displayed in Table 2. Coordinates for these structures have been deposited in the Protein Data Bank, access numbers 4I7E and 4I4I, respectively.

The fact that PEP remained bound to the mutant enzymes after purification from an *E. coli* lysate demonstrates the extremely tight PEP binding of D12A and T156A BsPFKs. The purification process included two columns, one of which is a strong anion exchange column, as well as numerous dialysis steps. The binding antagonism between Fru-6-P and PEP to BsPFK was exploited in order to remove the PEP from the D12A BsPFK. Specifically, D12A BsPFK was dialyzed against a solution containing Fru-6-P for over 48 hours, followed by dialysis for at least 24 hours against buffer containing no ligand. Diffraction quality crystals of apo D12A BsPFK were obtained and showed similar packing to the PEP-bound crystals. The asymmetric unit consists of a single tetramer. The final structure was refined to R-factor/R-free values of 20.07%/25.39% to a resolution of 2.3 Å. The details describing the diffraction data and refinement statistics are displayed in Table 2 and the coordinates have been deposited in the Protein Data Bank (accession number 4I36). Several attempts using the same strategy were made to crystallize an apo form of T156A BsPFK. However, every crystal form contained PEP, which might suggest that apo T156A is unstable.

No large structural differences between the apo and PEP forms of D12A BsPFK and the PEP bound form of T156A are evident. Figure 5 compares the D12 region of these structures. All three structures exhibit the quaternary shifted position closely resembling that found in the PGA-bound wild-type BsPFK structure as revealed by residues 156-160 forming a loop rather than a helix as shown in Figure 1B. In addition, the active sites are comparable among the three inhibited forms of BsPFK (data not shown).

Figure 6A is an overlay of the residues in the effector binding site for the apo and PEP structures of D12A BsPFK. In the PEP bound structure of D12A, K214 forms a hydrogen bond with Y69, a hydrogen bond that does not exist in the apo version of D12A. The hydrogen bond formed between K214 and Y69 pulls K214 out of the binding pocket and causes H215 to tilt towards the CH₂ group of PEP. The change in the orientation of D59 observed between the apo and bound D12A structures is a result of the ligand binding since the same residue overlays perfectly with the PGA bound structure of wild-type. Figure 6B is an overlay of the effector site residues in the PEP bound D12A structure and in the PGA bound wild-type structure. The positions of the residues are very similar between the inhibitor bound proteins except for K214 and H215. The imidazole ring of H215 is closer to

the CH₂ group of PEP in the D12A structure, and in the PGA structure the ring is orientated away from the ligand.

Comparisons of the B-factor distribution for the variant BsPFK crystal structures suggest that the dynamics of these structures are remarkably similar to each other as well (data not shown). Overall, the B factors of the variant structures resemble those of apo wild-type BsPFK (26).

Discussion

The mechanism proposed by Schirmer and Evans to explain the allosteric behavior of BsPFK attributes the PEP inhibition to the conformational changes observed between the two crystal structures of BsPFK, the Fru-6-P and MgADP bound structure and the PGA bound structure (3). In contrast, the current study, as well as previous studies conducted with BsPFK (9, 10, 27, 28), provides evidence that is difficult to reconcile with this proposal. The crystal structure of the apo D12A BsPFK indicates that the enzyme has undergone the quaternary shift previously associated with the binding of the inhibitor; however, analysis of its coupling parameters shows that the mutant enzyme exhibits an extent of inhibition by PEP that is nearly equivalent to that of wild-type. If the quaternary shift and the associated conformational changes including the unwinding of the 156-160 helical turn are responsible for PEP inhibition, then D12A BsPFK should not exhibit further inhibition upon the binding of PEP. In essence, one would expect the mutant enzyme to exhibit characteristics similar to those of the wild-type enzyme that had already been saturated by inhibitor. Indeed, D12A BsPFK exhibits a binding for Fru-6-P in the absence of PEP that is comparable to the Fru-6-P binding of wild-type when PEP is saturating. However, even with the poor Fru-6-P binding that D12A BsPFK exhibits, the mutant enzyme still shows further heterotropic inhibition by PEP of a magnitude that is comparable to that of wild-type.

Given that D12 potentially interacts with multiple residues across the substrate interface, the role of each potential interaction was assessed through mutagenesis, followed by kinetic and coupling analysis. T158 is the only residue across the substrate interface from D12 that seems to interact with D12 when the inhibitor is bound to the enzyme, and it is the only potential D12 interaction that is not highly conserved. T158A is also the only mutant enzyme tested that showed a significant enhancement in Fru-6-P binding affinity and a diminished PEP binding affinity. In addition, T158A is the only mutation that showed a diminished coupling parameter. These data suggest that the potential hydrogen bond formed between D12 and T158 when PEP is bound to the enzyme is not responsible for the D12A BsPFK binding and coupling characteristics.

Likewise, the binding characteristics of S159A BsPFK and H160A BsPFK do not follow the trends seen with D12A BsPFK. While S159A does cause a slight enhancement in PEP binding affinity, the Fru-6-P binding remains comparable to that of wild-type. H160A does enhance PEP affinity to nearly the same level as does D12A, but the Fru-6-P affinity is within a factor of 2 of wild-type. Interestingly, the coupling coefficients for S159A BsPFK and H160A BsPFK show much greater inhibition than wild-type. In fact, the coupling of H160A BsPFK cannot be accurately estimated because fully saturating amounts of Fru-6-P and PEP cannot be achieved. Unfortunately, all crystals grown for H160A BsPFK so far have diffracted to only low resolution.

By contrast, both T156 and D12 are located along the substrate-binding interface, directly across from each other when Fru-6-P and MgADP are bound. Both residues are 100% conserved among all available bacterial type-1 ATP-dependent PFK sequences. Neither residue directly interacts with a ligand. In addition, when each residue is replaced by an

alanine, the coupling and kinetic characteristics are also similar to each other. D12A and T156A enhance PEP binding 100- and 28-fold, respectively and diminish Fru-6-P binding 50- and 17-fold, respectively. However, the coupling parameters exhibited by D12A and T156A are each remarkably similar to that of wild-type.

While it is possible to rationalize why the disruption of the D12-T156 interaction could destabilize the subunit interface sufficiently to allow for the quaternary shift to occur, it is more difficult to understand why this significant conformational perturbation seems not to influence the allosteric coupling between Fru-6-P and PEP. One possibility is that the structures do not paint the whole picture. K-type allostery, by its very nature, is a thermodynamic phenomenon that is composed of both enthalpic and entropic components. Static structural models obtained by whatever means, though informative, do not directly address dynamics and should be considered to reveal but one aspect of a thermodynamic allosteric mechanism. Another possibility is that the allosteric communication occurs primarily between sites within individual subunits. Monomeric and single domain proteins have been shown to be allosteric (29-31). Quaternary structural changes need not be a requirement for allostery.

The diminished maximal specific activity of D12A BsPFK when compared to wild-type BsPFK is a potential complication to the interpretation of the functionality of this variant. It seems likely, however, that the lower k_{cat} is related to the interaction D12 makes with R252 rather than its interactions with residues 156-160. R252 forms intra-subunit interactions with both D12 and Fru-6-P when it is bound. Previous comparisons of the mutant enzymes R252A and R252A/D12A to each other and wild-type reveal that the introduction of the D12A mutation to the R252A variant did not substantially alter the maximal specific activity of the enzyme (10, 13). The D12A enzyme forms stable homotetramers, both in the presence and absence of PEP. A comparison of the active sites of the apo D12A and the apo wild-type BsPFKs shows a very small perturbation in the orientation of R252 when it interacts with Fru-6-P in the wild-type enzyme. The D12-R252 interaction, when completely removed in the double mutant, may allow for the proper orientation of the substrate in the binding pocket. However, if the negative charge of D12 is removed and the positive charge of R252 is thereby unrestrained, then an altered orientation of R252 may interfere with the binding of Fru-6-P in the proper orientation. In order to address this possibility, we have attempted to produce crystals of D12A BsPFK bound to Fru-6-P so far with no success.

Schirmer and Evans assumed that the PGA BsPFK structure is equivalent to the PEP-bound structure. Indeed, the structures of the PEP-bound variants of BsPFK and the PGA-bound wild-type BsPFK would suggest that this assumption was appropriate since the structures are very similar. In particular, the structure of the effector binding pockets of the PGA bound wild-type BsPFK, apo D12A BsPFK, and the PEP bound D12A BsPFK are quite analogous. It is also true, however, there are no obvious structural explanations within the binding site for why PEP binds to D12A BsPFK 100-fold tighter than PGA (and PEP) do to wild-type.

Given these observations, we feel it is possible that the quaternary shift, envisioned at one time as a key component to the mechanism for allosteric inhibition of BsPFK, may instead have an influence on ligand binding that is remote from the binding sites but does not influence allosteric inhibition *per se*. D12A BsPFK, and likely T156A BsPFK, which have undergone the quaternary shift, exhibit significant changes in ligand binding when compared to wild-type, yet they possess allosteric coupling constants that are comparable to that of wild-type. Further investigations into how the inhibition influences the structure and dynamics of BsPFK must be conducted before a comprehensive mechanism for allosteric inhibition can be proposed.

Possibly more to the point, from the definition given in equation 3 it can easily be shown that the coupling parameter, Q_{ay} , represents the equilibrium constant for the following disproportionation equilibrium:

where Y-E represents inhibitor bound enzyme, E-A represents substrate bound enzyme, E represents free enzyme, and Y-E-A represents the ternary complex of enzyme with both inhibitor and substrate bound. The structures considered by Schirmer and Evans, and indeed the structures presented in this report, all relate to the enzyme forms depicted on the left hand side of this equilibrium. However, the question of *why* PEP inhibits PFK must be answered by understanding why this equilibrium is poised in favor of those left-hand-side components. This answer cannot be formulated without an understanding of the features associated with those components on the right-hand-side. We have recently published the structure of apo BsPFK, i.e. E, that provides an important component of this information (26). However, it is structural information (including dynamics) regarding the ternary complex, Y-E-A, that will provide ultimately the key to unlock the answer to the question of why PEP inhibits the binding of Fru-6-P. Unfortunately so far, structural information pertaining to the ternary complex has remained elusive.

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Figure 1. Crystal structure of D12 region in wild-type BsPFK. A) Structure of wild-type BsPFK bound to Fru-6-P and MgADP. Structural data obtained from structure 4PFK in the PDB. B) Structure of wild-type BsPFK bound to PGA. Structural data obtained from structure 6PFK in the PDB. Distances indicated are in Å.

Figure 2. Specific activity versus Fru-6-P concentration for wild-type and D12A BsPFK. Wild-type BsPFK is represented by open circles, and D12A BsPFK is represented by open squares. Solid lines represent the best fit of the data to Equation 1.

Figure 3.

Plots of $K_{1/2}/k_{cat}$ and $K_{1/2}$ vs. PEP concentration for wild-type BsPFK. $K_{1/2}/k_{cat}$ is shown with open circles and was determined as described in the text. $K_{1/2}$ values are indicated by closed circles. Solid lines represent the best fit of each data set to Equation 4.

Figure 4.

A) Influence of PEP on the $K_{1/2}$ for wild-type and the apparent $K_{1/2}$ for D12A and H160A variants of BsPFK. Wild-type is represented by open circles, D12A is represented by open squares, and H160A is represented by closed squares. B) Influence of PEP on $K_{1/2}$ of wild-type and the apparent $K_{1/2}$ for T156A, T158A, and S159A variants of BsPFK. Wild-type is represented by open circles, T156A is represented by open squares, T158A is represented by closed circles, and S159A is represented by closed squares. Solid lines represent the best fit to Equation 3.

Figure 5. Comparison of the D12 region of D12A and T156A variants of BsPfk. A) D12A apo. B) D12A with PEP bound. C) T156A with PEP bound. D12A BsPfk is shown in gray ribbon and T156A is depicted green ribbon. Residue D12 (or D12A) is blue, T156 (or T156A) is purple, T158 is pink, S159 is orange, and H160 is green. PEP is gold.

Figure 6.

A) Overlay of residues in the effector binding site of D12A BsPFK. Apo is in black and PEP bound structure is in light gray. PEP is shown in ball and stick mode. B) Overlay of residues in the effector binding site for inhibitor bound BsPFK. Wild-type PGA bound BsPFK in red, with PGA depicted in red and ball and stick. PEP bound D12A is in gray with PEP depicted in gray and ball and stick.

Table 1

Steady-state kinetic and coupling parameters for variant BsPFKs

Variant	Specific Activity (U/mg)	K_{ia}^0 (mM)	K_{iy}^0 (μ M)	Q_{ay}
wild-type	163 $\pm$ 3	0.030 $\pm$ 0.002	60 $\pm$ 4	0.0020 $\pm$ 0.0003
D12A	24 $\pm$ 1	1.4 $\pm$ 0.1	0.45 $\pm$ 0.01	0.0030 $\pm$ 0.0001
H160A	70 $\pm$ 2	0.056 $\pm$ 0.002	1.0 $\pm$ 0.1	<0.00001
T156A	50 $\pm$ 3	0.52 $\pm$ 0.01	2.1 $\pm$ 0.1	0.002 $\pm$ 0.001
T158A	70 $\pm$ 3	0.017 $\pm$ 0.001	98 $\pm$ 4	0.009 $\pm$ 0.001
S159A	140 $\pm$ 4	0.025 $\pm$ 0.003	12 $\pm$ 1	0.0005 $\pm$ 0.0001

Table 2Data collection and refinement statistics for D12A and T156A BsPFK structures^a

Data set	D12A apo	D12A-PEP	T156A-PEP
Unit cell (Å)	<i>a</i> = 96.05 <i>b</i> = 112.66 <i>c</i> = 129.73	<i>a</i> =96.65 <i>b</i> =112.97 <i>c</i> =131.04	<i>a</i> =96.405 <i>b</i> =111.575 <i>c</i> =129.448
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Number of molecules per asymmetric unit (Z)	4 monomers	4 monomers	4 monomers
Resolution (Å)	40-2.3	65-2.0	45-2.5
Completeness (%)	100.0(100.0)	97.0(78.2)	100.0(100.0)
<i>I</i> / <i>I</i>	12.2(3.1)	13.3(2.7)	36.49 (5.68)
<i>R</i> _{merge} ^b	7.3 (44.0)	5.3(38.9)	10.6 (46)
Refinement			
Resolution (Å)	40-2.3	50-2.00	45-2.5
Reflections (working/free)	63108 (3204)	89745(4715)	
<i>R</i> (%) ^c	20.07	18.86	16.6
<i>R</i> _{free} (%) ^c	25.39	23.48	23.78
Number of protein atoms/number of waters	9517/329	9574/626	9429/312
Average <i>B</i> factor (Å ²)	48.41	28.96	50.5
Average <i>B</i> factor for water molecules (Å ²)	44.21	43.53	53.1
Root mean square deviations	0.004	0.012	0.005
Rmsd Bond length (Å)			
Rmsd Bond angles (degrees)	0.517	1.355	0.637
Ramachandran statistics			
Most favored	97.3%	97.2%	97.79%
Allowed	2.5%	2.8%	0.08%

^aValues in parentheses are for high resolution shells.^b $R_{\text{sym}} = \frac{\sum_h \sum_i |I_{hi} - \langle I_h \rangle|}{\sum_h \sum_i I_{hi}}$ where I_{hi} is the i th observation of the reflection h , whereas $\langle I_h \rangle$ is the means intensity of reflection h .^c $R_{\text{cryst}} = \frac{|F_o| - |F_c|}{|F_o|}$. R_{free} was calculated with a fraction (5%) of randomly selected reflections excluded from refinement.