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The effect of introducing small cavities on the allosteric inhibition of phosphofructokinase from *Bacillus stearothermophilus*☆

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ABSTRACT

The allosteric coupling free energy between ligands fructose-6-phosphate (Fru-6-P) and phospho(*enol*) pyruvate (PEP) for phosphofructokinase-1 (PFK) from the moderate thermophile, *Bacillus stearothermophilus* (BsPFK), results from compensating enthalpy and entropy components. In BsPFK the positive coupling free energy that defines inhibition is opposite in sign from the negative enthalpy term and is therefore determined by the larger absolute value of the negative entropy term. Variants of BsPFK were made to determine the effect of adding small cavities to the structure on the allosteric function of the enzyme. The BsPFK Ile → Val (cavity containing) mutants have varied values for the coupling free energy between PEP and Fru-6-P, indicating that the modifications altered the effectiveness of PEP as an inhibitor. Notably, the mutation I153V had a substantial positive impact on the magnitude of inhibition by PEP. Van't Hoff analysis determined that this is the result of decreased entropy-enthalpy compensation with a larger change in the enthalpy term compared to the entropy term.

1. Introduction

The enzymes that catalyze the commitment steps of metabolic pathways are subject to intense regulation via allosteric mechanisms. However, the molecular basis of allosteric regulation is still not well understood in most cases. Due to the key role these enzymes play, the ability to manipulate allosteric control mechanisms holds promise for drug design [1–6]. For this reason, an enhanced understanding of the molecular mechanisms behind the regulation of allosteric enzymes is a prerequisite for rational drug design.

Prokaryotic phosphofructokinase-1 (PFK) is one such enzyme that has been extensively studied and characterized, resulting in an abundance of kinetic, structural, and thermodynamic information [7–19]. PFK from *Bacillus stearothermophilus* (BsPFK) in particular serves as model allosteric enzyme to explore the molecular mechanisms of allosteric regulation as well as the thermodynamic basis of the allosteric coupling.

From the X-ray crystallography structures of BsPFK, one can identify an extensive hydrogen-bonding network that stretches through the region between the allosteric site and the closest active site that are roughly 22 Å apart. The allosteric coupling between these sites has been previously shown to make the largest contribution to the overall heterotropic allosteric coupling free energy in BsPFK [18]. We recently probed the role of the residues in this region in the allosteric interaction by examining the influence of 3 chimeric substitutions on the allosteric coupling in PFK from the extreme thermophile *Thermus thermophilus* (TtPFK) [20]. Interestingly, restoring the entire network of hydrogen-bonded residues apparently linking the 2 sites enhanced the strength of the allosteric communication in TtPFK to levels comparable to, or even greater than, those of BsPFK. However, this overall enhancement was achieved as the sum of enhancements (in free energy terms) realized from each modification individually suggesting that the basis of the interaction is derived from properties that are more complex than a single, continuous chain of interacting residues might suggest. One possibility is that it is the ligand binding perturbations of the thermodynamic stability of the protein structure in this region that are important so that each amino acid substitution can influence the overall coupling essentially independently without the 'all or none' feature that a quasi-mechanical linkage would require.

To further probe the relationship between the structural

Abbreviations: PFK, phosphofructokinase; BsPFK, *Bacillus stearothermophilus* phosphofructokinase; EcPFK, *Escherichia coli* phosphofructokinase; Fru-6-P, fructose-6-phosphate; PEP, phospho(enol)pyruvate.

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properties in this region and the strength of allosteric coupling, we investigated the sensitivity of the allosteric behavior of BsPFK to 3 isoleucine to valine substitutions at 3 different positions in this general region. The substitution effectively removes only a single methylene group, thus creating a small cavity at each of the positions. In this way we intended to modestly perturb the configurational entropy in the immediate vicinity of each substitution without otherwise altering the structure of the protein to a significant extent. We have previously observed that the coupling free energy between PEP and Fru-6-P in BsPFK, which quantitatively describes both the nature and the magnitude of the allosteric effect, is dominated by its constituent entropy component [21]. We have also discussed previously that ligand-induced perturbations in configurational degeneracy and/or protein dynamics may be a major cause of this type of allosteric behavior [22,23].

2. Materials and methods

2.1. Materials

All chemical reagents used in buffers for protein purification, enzymatic assays, and fluorescence assays were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), or Research Products International (Mt. Prospect, IL). Deionized distilled water was used throughout. Lyophilized creatine kinase, the ammonium sulfate suspension of glycerol-3-phosphate dehydrogenase, and the potassium salt of phospho(*enol*)pyruvate were purchased from Roche (Indianapolis, IN). The ammonium sulfate suspensions of aldolase, the ammonium sulfate suspension of triosephosphate isomerase, the disodium salt of fructose-6-phosphate, and the disodium salt of phosphocreatine were purchased from Sigma-Aldrich (St. Louis, MO). The coupling enzymes were extensively dialyzed against 50 mM EPPS pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EDTA before use. NADH and DTT were purchased from Research Products International (Mt. Prospect, IL). Mimetic Blue 1 A6XL resin used in protein purification was purchased from Promatic BioSciences (Rockville, MD). The Mono-Q HR anion exchange column used in protein purification was purchased prepacked for FPLC use from Pharmacia (currently GE Healthcare, Uppsala, Sweden). Amicon Ultra centrifugal filter units (spin concentrators) were from Millipore Corporation (Billerica, MA) and poly(ethylene glycol)-3000 was from Sigma-Aldrich (St. Louis, MO). 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 dNTPs were purchased from Stratagene (Cedar Creek, TX), New England Biolabs (Ipswich, MA), or Promega (Madison, WI).

2.2. Site-directed mutagenesis

The plasmid p-ALTER1/BsPFK contains the gene for BsPFK. Mutagenesis was performed on this plasmid following the protocol outlined in the QuikChange II Site-Directed Mutagenesis System from Stratagene. Two complementary oligonucleotides were designed to target the sequence surrounding the codon for each of the mutated amino acids; the template oligonucleotides are shown below:

I150V-BsPFK: 5′— ATA CGG TCA TTG ATG CCG TCG ACA AAA TCC GCG AC —3′.

I53V-BsPFK: 5′— GCC ATC GAC AAA GTG CGC GAC ACG G —3′.

I234V-BsPFK: 5′— GAC TTC GGC CGG CAA GTG CAG GAA G —3′.

2.3. Protein purification of wild type, I150V, I153V, and I234V-BsPFK

The plasmid p-ALTER1/BsPFK contains either the gene for BsPFK or the mutated genes. Expression from this plasmid occurs via the tac promoter. Wild-type BsPFK was expressed in *E. coli* RL257 cells [24], which is a strain lacking both the pfkA and pfkB genes. The purification of BsPFK was performed as described previously [25], with a few modifications. RL257 cells containing the plasmid p-ALTER1/BsPFK were grown at 37 °C for 16–18 h in Lysogeny Broth with tetracycline (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and tetracycline 15 μg/mL). Induction of expression with 1 mM IPTG was carried out at the beginning of the growth. Cells were harvested by centrifugation and frozen at −20 °C for a minimum of 12 h. The cell pellet was resuspended in 60 mL of purification buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and sonicated using a Fisher 550 Sonic Dismemberator at 0 °C in 15-s pulses at setting six for 8–12 min. The crude lysate was centrifuged at 22,500 × g for 30 min at 4 °C. The clarified supernatant was incubated in a 70 °C water bath for 15 min, cooled on ice for 15 min, and centrifuged again at 22,500 × g for 30 min at 4 °C. The supernatant was diluted to at least 500 mL and then loaded onto a 100 mL Mimetic Blue 1 A6XL column that was previously equilibrated with purification buffer. The column was washed with purification buffer until the A_{280} reached a baseline, and the enzyme eluted with a 0–1.5 M NaCl gradient. Fractions containing enzyme activity were pooled and dialyzed into 20 mM Tris-HCl pH 8.5 and loaded to a Pharmacia/GE healthcare Mono-Q HR anion exchange column that was pre-equilibrated with the same buffer. The enzyme was eluted with a 0–1 M NaCl gradient, and fractions containing pure BsPFK were combined, concentrated with either a spin concentrator or poly(-ethylene glycol)-3000, and then dialyzed into EPPS buffer (50 mM EPPS pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mM EDTA). The final enzyme was determined to be pure by SDS-PAGE, and stored at 4 °C. Protein concentration was determined by measuring absorbance at 280 nm ($\varepsilon = 18910\ M^{-1}cm^{-1}$) [16].

2.4. Protein purification of EcPFK

The purification of wild type EcPFK protein followed the protocol of Johnson et al. [11] with a few modifications, and is the same as above with the following exceptions. RL257 cells containing the p-ALTER1/EcPFK plasmid were grown to $OD_{600} = 0.6$ and then induced with 1 mM IPTG in Lysogeny Broth containing 100 μg/mL ampicillin at 37 °C. After induction the cells were grown until $OD_{600} = 1.2$ and harvested by centrifugation. Instead of a heat step, the supernatant after sonication and clarification was incubated with DNase at 37 °C for 15 min and then centrifuged for thirty minutes to remove the remaining cellular debris. The supernatant, containing EcPFK, was then further purified as above with a Mimetic Blue 1 A6XL column followed by an anion exchange step. Protein determined pure by SDS-PAGE was then concentrated and dialyzed into EPPS buffer (50 mM EPPS pH 8.0, 10 mM $MgCl_2$, 10 mM NH_4Cl, and 0.1 mM EDTA). Protein concentration was calculated by measuring absorbance at 278 nm ($\varepsilon = 0.6\ mg^{-1}cm^2$) [26].

2.5. Steady-state kinetic assays

Activity measurements for PFK were carried out using a coupled enzyme system [27,28] in a 0.6 mL reaction volume of EPPS buffer containing 50 mM EPPS pH 8.0, 5 mM $MgCl_2$, 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, and 5 μg of triosephosphate isomerase at 25 °C unless otherwise noted. Creatine kinase (40 μg/mL) and phosphocreatine (4 mM) were added as

an ATP regenerating system to avoid the accumulation of MgADP, which is an activator. Temperature was controlled using a NESLab RTE-111 circulating water bath. Fru-6-P and PEP were added at varied concentrations as indicated. Assays were started by the addition of 10 μL of appropriately diluted PFK and the reaction was monitored as the absorbance at 340 nm decreased over time. The rate of the reaction was measured on Beckman Series 600 spectrophotometers using a linear regression calculation to convert change in absorbance at 340 nm to PFK activity. One unit of PFK activity is described as the amount of enzyme needed to produce 1 μmol of fructose-1,6-bisphosphate per minute.

2.6. Quantifying allosteric couplings

The allosteric coupling free energy that describes the allosteric inhibition of PFK by PEP (ΔG_{ay}), is determined from the coupling parameter (Q_{ay}) which is equal to the ratio of the dissociation constant for Fru-6-P in the absence of PEP (K_{ia}^{0}) to the dissociation constant for Fru-6-P in the saturating presence of PEP (K_{ia}^{∞}) as shown in Equations (1) and (2):

$$Q_{ay} = \frac{K_{ia}^{0}}{K_{ia}^{\infty}} \tag{1}$$

$$\Delta G_{ay} = -RTln(Q_{ay}) \tag{2}$$

Following this definition, ΔG_{ay} is the standard free energy for the following disproportionation equilibrium [29]:

$$\text{PFK : Fru6P} \; + \; \text{PEP : PFK} \leftrightarrow \; \text{PFK} \; + \; \text{PEP : PFK : Fru6P} \tag{3}$$

where PFK:Fru6P represents PFK with Fru-6-P bound, PEP:PFK represents PFK with PEP bound and PEP:PFK:Fru6P represents the ternary complex with both ligands bound at the same time, and PFK represents the enzyme with neither ligand bound. Since ΔG_{ay} describes quantitatively both the nature and the magnitude of the allosteric effect, understanding the basis for allosteric action requires one to understand why the disproportionation equilibrium achieves a particular value as defined by Equations (1) and (2) [29].

2.7. Data analysis

Data were fit using the non-linear least-squares fitting analysis option in Kaleidagraph software version 4.5 (Synergy). For the steady-state kinetic assays the initial velocity data were plotted against concentration of Fru-6-P and fit to the Hill Equation [30]:

$$v = \frac{V[A]^{n_H}}{K_a^{n_H} + [A]^{n_H}} \tag{4}$$

where, v is the initial velocity, $[A]$ is the concentration of the substrate Fru-6-P, V is the maximal velocity, and n_H is the Hill coefficient. K_a is defined as the concentration of Fru-6-P at which the enzymes activity is half-maximal. Assuming the binding of Fru-6-P achieves a rapid equilibrium in the steady state, which was shown to be valid in EcPFK and BsPFK using a steady-state kinetic method [31], K_a is equivalent to the dissociation constant for Fru-6-P from the binary enzyme-substrate complex [14,19,31]. Values of K_a obtained from the initial velocity experiments were plotted against the concentration of opposing ligand and fit according to:

$$K_a = K_{ia}^{o}\left(\frac{K_{iy}^{o} + [Y]}{K_{iy}^{o} + Q_{ay}[Y]}\right) \tag{5}$$

where K_{ia}^{o} is the dissociation constant for Fru-6-P in the absence of PEP, K_{iy}^{o} is the dissociation constant for PEP in the absence of Fru-6-P, and Q_{ay} is the coupling coefficient defined in Equation (1) [29,32,33]. By definition, Q_{ay} represents the equilibrium constant for the disproportionation equilibrium (Equation (3)). At any given temperature the coupling free energy (ΔG_{ay}) is related to entropy ($T\Delta S_{ay}$) and enthalpy (ΔH_{ay}) components through the relationship:

$$\Delta G_{ay} = \Delta H_{ay} - T\Delta S_{ay} \tag{6}$$

ΔG_{ay} represents a standard free energy, although the superscript "o" is dropped from the designation to avoid confusion with the use of that superscript for other purposes in our notation [32]. The coupling entropy and enthalpy components were determined by measuring the coupling constant as a function of temperature and then fitting the data to the basic van't Hoff equation:

$$log(Q_{ay}) = \frac{\Delta S_{ay}}{R \times 2.303} - \frac{\Delta H_{ay}}{R \times 2.303}\left(\frac{1}{T}\right) \tag{7}$$

where T is absolute temperature in kelvin and R is the Gas Constant (1.99 cal $K^{-1}mol^{-1}$).

3. Results

PFK catalyzes the phosphorylation of Fru-6-P by MgATP to form fructose-1,6-bisphosphate and MgADP in the first committed step of glycolysis. This crucial glycolytic reaction is allosterically inhibited by the downstream pathway intermediate, PEP, and activated by MgADP, both of which compete for the same effector binding site. These K-type allosteric effects are manifested by a change in the enzyme's affinity for its substrate, Fru-6-P, upon binding of the allosteric effector. The nature and magnitude of this effect are unambiguously quantified by the allosteric coupling free energy between Fru-6-P and the effector ligand [29].

Three BsPFK variants, each containing a single amino acid substitution of isoleucine to valine, were constructed that removed a single $-CH_3$ group and replaced it with a $-H$, thus creating a small cavity at a specific location in the enzyme. Turnover numbers for each variant and Hill Coefficients exhibited only small variation from the corresponding values for wild type (Table 1). To establish the magnitude of PEP inhibition in the wild type and cavity containing variants of BsPFK, the apparent dissociation constants for Fru-6-P were determined as a function of PEP concentration using steady-state kinetics. The apparent dissociation constants for Fru-6-P were obtained from individual titration curves fit to Equation (4) for a range of PEP concentrations. The apparent dissociation constants obtained from these fits were then plotted as a function of PEP concentration and fit to Equation (5) as shown in Fig. 1. From these fits, the apparent dissociation constants for Fru-6-P in the absence of PEP (K_{ia}^{o}), the apparent dissociation constants for PEP in the absence of Fru-6-P (K_{iy}^{o}), and the allosteric coupling parameters (Q_{ay}) were obtained. The data for the allosteric coupling parameters were used to calculate the standard allosteric coupling free energies, ΔG_{ay} according to Equation (2). The resulting values of K_{ia}^{o}, K_{iy}^{o}, Q_{ay}, and ΔG_{ay} are also presented in Table 1 for wild-type BsPFK along with each of the three variants.

Of particular note, one of the BsPFK mutants, I153V, exhibits an allosteric coupling free energy that is 0.8 ± 0.1 kcal/mol greater than that for wild type BsPFK, indicating that PEP inhibits this enzyme almost 4-fold more strongly. The I153V variant of BsPFK also displays an increased affinity for both the substrate Fru-6-P and inhibitor PEP, by 1.7- and 3.8-fold respectively, compared to wild-type. The other two BsPFK variants did not exhibit comparable

Table 1
Ligand dissociation constants and coupling constants from wild type and variant forms of *B. stearothermophilus* PFK at 25 °C.

	n_H	k_{cat} (sec^{-1})	K^o_{ia} (mM)	K^o_{iy} (mM)	Q_{ay}	ΔG_{ay} (kcal/mol)
WT	1.1 ± 0.1	126 ± 3	0.039 ± 0.002	0.063 ± 0.004	0.0021 ± 0.0002	3.66 ± 0.06
I150V	1.5 ± 0.1	110 ± 3	0.027 ± 0.001	0.074 ± 0.005	0.0042 ± 0.0003	3.25 ± 0.04
I153V	1.3 ± 0.1	100 ± 1	0.024 ± 0.001	0.017 ± 0.001	0.00054 ± 0.00007	4.46 ± 0.08
I234V	1.8 ± 0.2	129 ± 8	0.033 ± 0.002	0.057 ± 0.004	0.0024 ± 0.0002	3.58 ± 0.05

increases in allosteric coupling free energy. In fact, the coupling free energy for I150V-BsPFK was diminished by 0.4 ± 0.1 kcal/mol while the coupling free energy of I234V-BsPFK was unchanged within error. The other variants displayed slightly lower K^o_{ia} values than, and K^o_{iy} values very similar to, wild type.

In order to further explore the thermodynamic basis for both of the changes observed in the allosteric couplings, we examined the temperature dependence of the coupling parameters. ΔH_{ay} and ΔS_{ay} components of ΔG_{ay} were obtained by determining Q_{ay} as a function of temperature ranging from 10 to 40 °C, followed by van't Hoff analysis. The allosteric coupling free energy remains entropy driven for all of the variants, as indicated by the positive slopes of all lines in Fig. 2. In addition, all of the data for each variant are well described by straight lines, signifying that there are not detectable changes in heat capacity, ΔC_p, over this range of temperatures. The values of ΔH_{ay}, $T\Delta S_{ay}$ and ΔG_{ay} are listed in Table 2.

It is evident in Fig. 2 that although the coupling of I234V at 25 °C is comparable to that of wild type, it varies significantly from wild type at other temperatures giving rise to a slope, and hence ΔH_{ay}, that is considerably less negative. ΔS_{ay}, obtained from the difference between ΔG_{ay} and ΔH_{ay}, is also clearly smaller in absolute magnitude. However, since both ΔH_{ay} and $T\Delta S_{ay}$ have decreased by 12.1 kcal/mol at 25 °C, ΔG_{ay} remains unchanged at that temperature.

A comparison of the effects of the other 2 mutations is of interest because they have opposite effects on the magnitude of the allosteric inhibition at 25 °C, with I150V causing the maximal extent of inhibition to decrease (ΔG_{ay} decreases) and I153V causing the maximal extent of inhibition to increase (ΔG_{ay} increases). Ironically, the component ΔH_{ay} and $T\Delta S_{ay}$ absolute values change in opposite directions to ΔG_{ay} in each case. The absolute values of both ΔH_{ay} and $T\Delta S_{ay}$ increase relative to wild type values for I150V and both become less negative for I153V. In each case, however, the absolute value of the change in ΔH_{ay} is greater than the absolute value of the change in $T\Delta S_{ay}$. In the case of I150V the values of ΔH_{ay} and $T\Delta S_{ay}$ both become more negative and consequently the difference, i.e. the amount of enthalpy-entropy compensation, becomes smaller and hence ΔG_{ay} decreases. For I153V, the values become less negative, so the amount of enthalpy-entropy compensation increases and ΔG_{ay} increases, signifying a greater extent of inhibition.

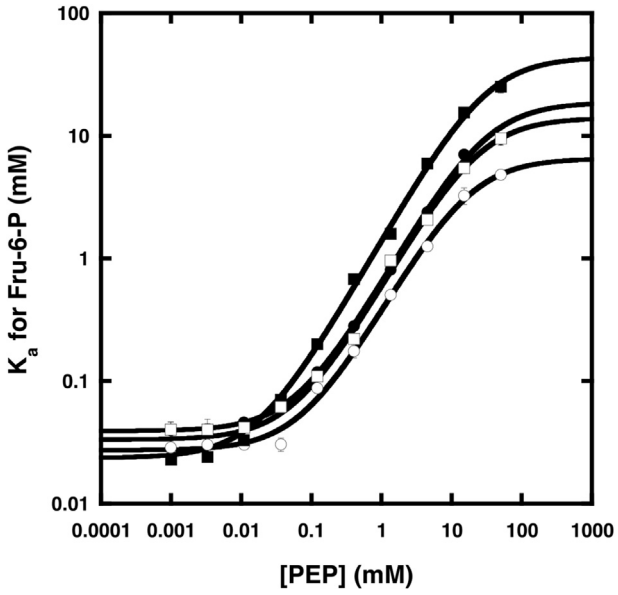

Fig. 1. The log of the apparent K_a for Fru-6-P as a function of the log of [PEP] for wild type (filled circles), I150V (open circles), I153V (filled squares), and I234V (open squares) variants of BsPFK.

4. Discussion

BsPFK is qualitatively very similar, both structurally and functionally, to other prokaryotic PFKs, the canonical example of which is PFK from *E. coli* (EcPFK). An important difference, however, – one shared with TtPFK – is the response of the allosteric coupling to variations in temperature. As illustrated in Fig. 3, the response of BsPFK to increasing temperature is opposite that of EcPFK. For

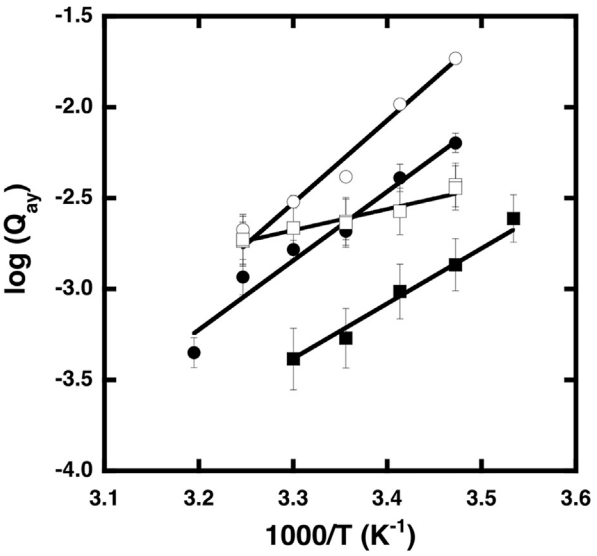

Fig. 2. The variation in the log of Q_{ay} verses reciprocal temperature of wild type (filled circles), I150V (open circles), I153V (filled squares), and I234V (open squares) variants of BsPFK.

Table 2
Thermodynamic parameters underlying the inhibition by PEP of wild type and variant BsPFK and wild type EcPFK at 25 °C.

	ΔH_{ay} (kcal/mol)	$T\Delta S_{ay}$ (kcal/mol)	ΔG_{ay} (kcal/mol)
WT-BsPFK	−17.4 ± 1.3	−21.0 ± 1.3	+3.66 ± 0.06
I150V-BsPFK	−20.8 ± 0.7	−23.9 ± 0.7	+3.25 ± 0.04
I153V-BsPFK	−13.9 ± 1.3	−18.2 ± 1.3	+4.46 ± 0.08
I234V-BsPFK	−5.3 ± 0.9	−8.9 ± 0.9	+3.58 ± 0.05
WT-EcPFK	+16.2 ± 1.7	+13.7 ± 1.7	+2.50 ± 0.05

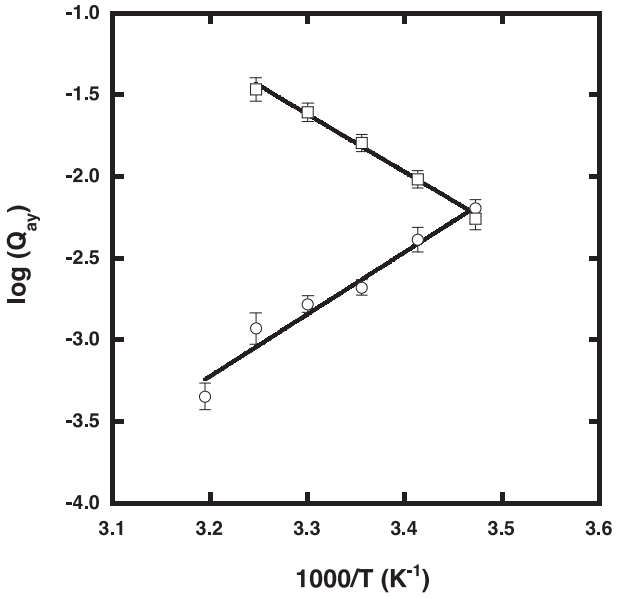

Fig. 3. Van't Hoff plots of the coupling parameter, Q_{ay}, quantifying the allosteric inhibition by PEP of BsPFK (circles) and EcPFK (squares).

BsPFK, PEP becomes a better inhibitor at higher temperature (within the range examined) whereas for EcPFK, PEP becomes a weaker inhibitor as temperature is increased. A thermodynamic analysis of these data indicates that sign of the coupling free energy between PEP and Fru-6-P, which is indicative of the binding antagonism between these two ligands, is established by the constituent coupling entropy for BsPFK and instead of the constituent coupling enthalpy as is the case for EcPFK. We have previously suggested that configurational degeneracy may be an important contributor to this dominant entropy term in enzymes like BsPFK [21–23].

It is tempting when considering this possibility to predict that structural perturbations that increase configurational flexibility may have an effect on the strength of allosteric communication that is analogous to that which temperature does; i.e. increasing flexibility might increase the strength of PEP inhibition as does increasing temperature. Indeed, the notion that an allosteric protein transitions between "Relaxed" and "Tense" forms is as old as the first mechanistic explanation of allosteric behavior and the associated 2-state models that followed [34]. The experiments reported herein were performed in an effort to test this idea.

In the present study, three different isoleucine-to-valine mutations were made to the BsPFK enzyme in an attempt to make a small change to counter the thermophilic stability of the BsPFK structure. We then determined the associated coupling parameters to assess whether these modifications modified the extent of inhibition by PEP. Each modification in BsPFK represented the loss of a single methylene group from the native structure, leaving a small cavity at the affected location. The isoleucine residues that were modified in this study were selected for modification because they are in close proximity to the region directly between these sites, the center of which is depicted as a dashed line in Fig. 4.

B. stearothermophilus is a moderate thermophile that grows natively at 55 °C, and BsPFK is correspondingly temperature-stable. Many studies have attempted to uncover the source of increased thermal stability in thermophilic enzymes, but it appears that there is not a single molecular or thermodynamic explanation, although the added stability is generally evident at all temperatures [35]. In the case of BsPFK, Kim et al. showed that the native tryptophan position is quite rigid [36]. Additional fluorescence studies using tryptophan-shifted mutants have revealed that a majority of the BsPFK structure is rigid [16,37,38].

In structural analyses of protein stability, Matthews observed that 9 different Ile → Val variants of lysozyme changed the denaturation free energy between WT and the mutants such that their stability decreased from 0.5 to 1.8 kcal/mol, with an average ΔΔG of 1.3 ± 0.4 kcal/mol [39]. In another study, the thermodynamic parameters for the denaturation of five Ile → Val lysozyme mutants determined by scanning calorimetry showed decreased stability compared to wild-type lysozyme in all cases [40]. X-ray crystallography studies of these lysozyme variants revealed identical overall structures and only small structural rearrangements were observed locally around some mutation sites.

Consequently, we presume that the removal of a methylene

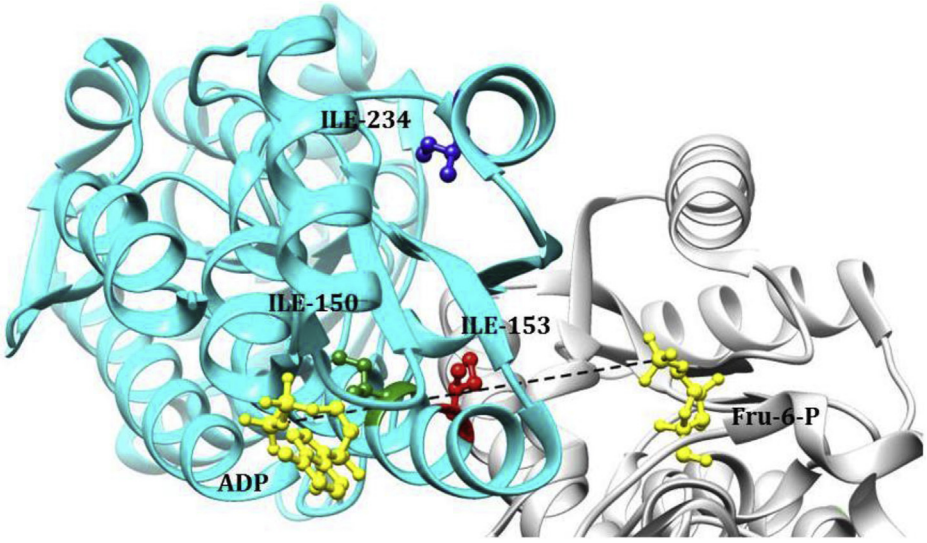

Fig. 4. X-ray crystallography structure (PDB accession code 4PFK) showing, in ball-and-stick, the locations of I150 (green), I153 (red), and I234 (blue) at which individual mutations to valine were constructed. ADP and Fru-6-P are shown in the allosteric and active site, respectively (yellow). ADP binds to the same allosteric site as PEP. Cyan depicts one subunit of the active homotetramer.

group, resulting in the addition of a small cavity, has not largely altered the overall structure of the enzyme, but rather would allow for an increase in the local flexibility and a small decrease in the stability of the BsPFK enzyme. The fact that k_{cat} is not affected significantly by these modifications supports this premise. The question was: would any of these small changes perturb the extent of PEP inhibition? The answer to this question is straightforward in each case, but together they suggest that the explanation of the response is more complex than just ascertaining whether transmission of the allosteric signal is positively or negatively affected by a more flexible structure. The magnitude of allosteric inhibition is given by the standard free energy, ΔG_{ay}, for the disproportionation equilibrium. Since each of the four species in Equation (3) that contribute to the poise of this equilibrium are potentially altered in response to the mutation, one cannot predict *a priori* how changes of this type might influence the magnitude of an allosteric effect.

The effects we observed on allosteric coupling that resulted from these small cavity-containing mutations ranged from no effect at all to substantially altering the allosteric coupling either positively or negatively at 25 °C. The modification I153V has the strongest effect represented by an almost 4-fold augmentation of inhibition and a 0.8 kcal/mol increase in the allosteric coupling free energy at 25 °C. Residue Ile-153 is located within an α-helix that passes directly between an active and allosteric binding site pair that are 22 Å away from each other. Residue Ile-150 is also located within this helix, but is positioned slightly further away from the region between the binding sites. The removal of a methylene group at this position led to a weakening of the allosteric coupling. The variant with no effect on the inhibition of PFK by PEP at 25 °C, I234V, is located within another α-helix 12 Å away from the helix containing the other mutations. These results imply that the allosteric coupling free energy can be perturbed significantly with even small changes in the enzyme's structure.

This sensitivity is evident in spite of the fact that the allosteric coupling free energy in BsPFK results from small imbalances between otherwise highly compensating larger entropy and enthalpy terms [21]. The effects on the ΔH_{ay} and $T\Delta S_{ay}$ components are indeed much larger than those for ΔG_{ay}. However the magnitude of the effects on ΔH_{ay} and $T\Delta S_{ay}$ do not correlate with the magnitude of the effects on ΔG_{ay}. While I234V exhibits the smallest change in ΔG_{ay}, it by far exhibits the largest absolute value changes in ΔH_{ay} and $T\Delta S_{ay}$. The direction of the changes in ΔG_{ay} also does not correlate with the direction of the changes in ΔH_{ay} and $T\Delta S_{ay}$. I153V increases the value of ΔG_{ay} despite decreased magnitudes of the absolute values of ΔH_{ay} and $T\Delta S_{ay}$, whereas I150V decreases the value of ΔG_{ay} with increased absolute values of ΔH_{ay} and $T\Delta S_{ay}$.

In conclusion, modifications were made to probe the effect of adding a small cavity in multiple positions in the vicinity of a region of the structure of BsPFK previously shown to be important to the transmission of allosteric signal. The mutations were designed to modify the local flexibility at the site of the modification without disrupting the overall structure of the protein. Of these mutations, two substantially altered the allosteric coupling free energy that quantifies the allosteric inhibition of BsPFK by PEP at 25 °C, while all three had much greater effects on both the entropy and enthalpy components of the coupling free energy. However, both increased inhibition and decreased inhibition were observed, which are the result of different *relative* changes in the ΔH_{ay} and $T\Delta S_{ay}$ components of the coupling free energy.

Previous studies utilizing BsPFK have demonstrated that temperature and pH also exert their influence on allosteric inhibition by changing the relative contributions made by the ΔH_{ay} and $T\Delta S_{ay}$ terms of the coupling free energy [21]. When the entropy and enthalpy terms change by a relatively large amount, there is ample opportunity for the balance between them to also change. Perhaps the ability to so easily modify the extent of allosteric inhibition in BsPFK has served an evolutionary advantage, allowing the enzyme to quickly adapt to varying environments. It also seems that the ability to alter the efficiency of inhibition of a major metabolic pathway with relative ease would be advantageous for drug design. On the other hand, these results also indicate that predicting *a priori* the nature of a structural perturbation required to modify the allosteric response in a particular manner may prove to be impossible, contrary to the general expectations suggested by a two-state, “Relaxed” versus “Tense” model of allosteric behavior.

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