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Enhancing Allosteric Inhibition in *Thermus thermophilus* Phosphofructokinase

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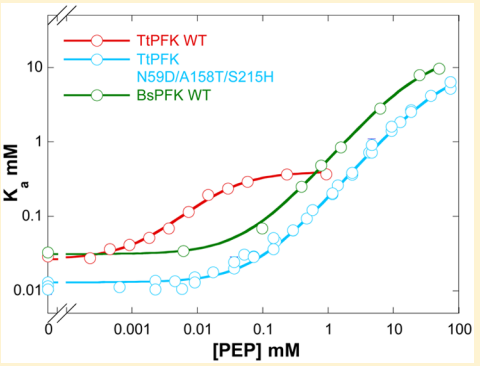
ABSTRACT: The coupling between the binding of the substrate Fru-6-P and the inhibitor phospho(*enol*)pyruvate (PEP) in phosphofructokinase (PFK) from the extreme thermophile *Thermus thermophilus* is much weaker than that seen in a PFK from *Bacillus stearothermophilus.* From the crystal structures of *Bacillus stearothermophilus* PFK (BsPFK) the residues at positions 59, 158, and 215 in BsPFK are located on the path leading from the allosteric site to the nearest active site and are part of the intricate hydrogen-bonding network connecting the two sites. Substituting the corresponding residues in *Thermus thermophilus* PFK (TtPFK) with the amino acids found at these positions in BsPFK allowed us to enhance the allosteric inhibition by PEP by nearly 3 kcal mol^{-1} (50-fold) to a value greater than or equal to the coupling observed in BsPFK. Interestingly, each single variant N59D, A158T, and S215H produced a roughly 1 kcal mol^{-1} increase in coupling free energy of inhibition. The effects of these variants were essentially additive in the three combinations of double variants N59D/A158T, N59D/S215H, and A158T/S215H as well as in the triple variant N59D/A158T/S215H. Consequently, while the hydrogen-bonding network identified is likely involved in the inhibitory allosteric communication, a model requiring a linked chain of interactions connecting the sites is not supported by these data. Despite the fact that the allosteric activator of the bacterial PFK, MgADP, binds at the same allosteric site, the substitutions at positions 59, 158, and 215 do not have an equally dramatic effect on the binding affinity and the allosteric activation by MgADP. The effect of the S215H and N59D/A158T/S215H substitutions on the activation by MgADP could not be determined because of a dramatic drop in MgADP binding affinity that resulted from the S215H substitution. The single variants N59D and A158T supported binding but showed little change in the free energy of activation by MgADP compared to the wild type TtPFK. These results support previous suggestions that heterotropic inhibition and activation occur by different pathways prokaryotic PFK.



Phosphofructokinase (PFK) from the extreme thermophile *Thermus thermophilus* (TtPFK) is allosterically inhibited by phospho(*enol*)pyruvate (PEP) and activated by MgADP,[1] as are PFKs isolated from virtually all other prokaryotic organisms. These effects are manifested by a change in the affinity for the substrate, fructose 6-phosphate (Fru-6-P), upon binding of the allosteric ligand. The nature and magnitude of this effect are quantified by the coupling free energy between Fru-6-P and the allosteric ligand.[2] Similar to *Thermus thermophilus*, *Bacillus stearothermophilus* is a moderately thermophilic organism, and the PFK isolated from *B. stearothermophilus* (BsPFK) shares a 57% sequence identity and a 70% sequence similarity with TtPFK. Moreover, both TtPFK and BsPFK share the interesting property that the coupling free energies describing both the inhibition by PEP and the activation by MgADP are entropy driven.[1,3] The latter refers to the fact that the entropy component of the coupling free energy, rather than the enthalpy, is responsible for the nature of the allosteric effects at 25 °C in contrast to the allosteric effects in *Escherichia coli* PFK, for which the opposite is true. In addition, the Michaelis constants for Fru-6-P are nearly equal. Although k_{cat} for TtPFK is nearly 3-fold lower than that for BsPFK at 25 °C, this difference is likely minimized physiologically due to the higher temperature experienced by TtPFK *in vivo*.

Despite these similarities, TtPFK and BsPFK differ substantially in their respective responses to the allosteric ligands. TtPFK binds MgADP and PEP roughly 50−60-fold more tightly than BsPFK. However, PEP inhibits BsPFK 30-fold more strongly, while MgADP activates both enzymes with nearly equal effectiveness.[1] In an attempt to pinpoint the basis of the weaker coupling between PEP and Fru-6-P exhibited by TtPFK, we have analyzed the available structures, determined by X-ray crystallography, of BsPFK in the apo form,[4] BsPFK bound to the inhibitor phosphoglycolate,[5] and BsPFK bound to both the substrate, fructose 6-phosphate (Fru-6-P) and the allosteric activator (ADP).[6] From these structures one can identify a series of residues involved in an extensive hydrogen-bonding network that extends from the allosteric site to the closest active site. The allosteric influence between these sites was previously shown to make the strongest contribution to the

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overall heterotropic coupling free energy in PFK from both *E. coli* and *B. stearothermophilus*.[7,8]

Each of the four possible single pairwise heterotropic allosteric interactions in BsPFK occurs within each individual subunit, based on the interfacial nature of the allosteric and substrate binding sites. This observation was confirmed by the allosteric properties of 1:3 hybrid tetramers to which only a single allosteric ligand and a single substrate ligand were able to bind with native affinity.[7,8] However, the intrasubunit nature of these heterotropic interactions does not imply that no residues from other subunits can be involved in an intrasubunit allosteric interaction—rather that the residues that might be involved in this manner are not perturbed by ligands binding to the other subunits in a way that would influence the intrasubunit coupling. When one examines the residues that lie directly between the closest pair of Fru-6-P and PEP binding sites, one finds six interacting side chains that seem to link the two sites. As shown in Figure 1, three of these residues, T156, T158, and

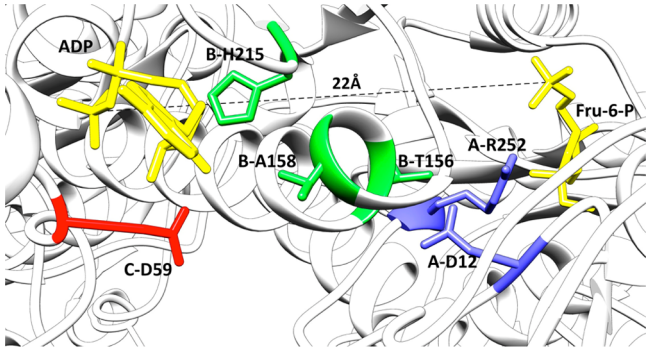

Figure 1. Crystal structure of Fru-6-P and ADP-bound BsPFK.[6] One of the 22 Å interactions is shown measured from the phosphate of ADP to the phosphate of Fru-6-P; both molecules are highlighted in yellow. Residues highlighted (left to right) are D59 in red (subunit C), H215, T158, and T156 in green (subunit B), and R252, and D12 in blue (subunit A).

H215, are contributed by the parent subunit (B) formally containing the interaction and are shown in green. The other residues are contributed by each of two neighboring subunits: D59 from subunit C shown in red, and D12 and R252 from subunit A, shown in blue. Arginine 252 directly coordinates Fru-6-P and was shown to be crucial for allosteric coupling in *E. coli* PFK.[9] The backbone of D59 interacts with the allosteric ligand, and the side chain carboxyl forms a hydrogen bond with R154 (Figure 2) in both the PG-bound and the Fru-6-P (+ ADP) bound states. The backbone of R154 forms a hydrogen bond with the T158 in apo and Fru-6-P (+ ADP) bound form. In the Fru-6-P (+ ADP) bound structure T158 interacts with H215 and connects though a water molecule to the side chain of the D59 hydroxyl. By contrast, T158 undergoes a large displacement in the inhibitor-bound structure due to the unwinding of the helix containing it. T158 instead forms a hydrogen bond with D12 across the active site interface. T156 located on the same helix is moved closer to the allosteric site and replaces T158 as a hydrogen-bond partner for H215.

In TtPFK these interactions are not possible because different amino acids occupy key positions, namely, asparagine for aspartate at position 59[a], serine for histidine at position 215, and alanine for threonine at position 158. It has been proposed previously that the lack of an interaction between N59 and S215 would lead to destabilization of the effector site interface,

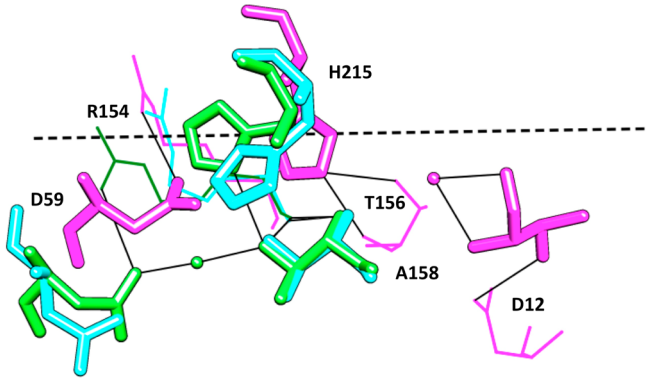

Figure 2. Alignment of crystal structures of BsPFK in apo (cyan),[4] PG- (magenta),[5] and Fru-6-P and ADP-bound (green)[6] forms. Residues 59, 215, and 158 are shown as sticks. Residues R154, T156, and D12, which are involved in hydrogen bond interactions with the side chains of 59, 215, and 158, are shown as wires. The hydrogen bonds are shown as solid lines. Dotted line represents the distance between the closest allosteric and active sites defines as the 22 Å interaction.

and the lack of an interaction between A158 and D12 would weaken the allosteric site interface.[10] We wondered, given the location of these residues between the closest allosteric and active sites and the importance of R252 in the propagation of the allosteric response,[9] whether the ensuing disruption in the putative pathway of allosteric communication between the two sites would result in a weaker heterotropic coupling between PEP and Fru-6-P binding. Furthermore, this possibility, if true, suggests that recreating this network would result in an increase in coupling, i.e., a strengthening of the allosteric inhibition by PEP in TtPFK. To test this hypothesis we made single, double, and triple chimeric substitutions at positions 59, 158, and 215 to the corresponding amino acids in BsPFK. We report herein the couplings between PEP and Fru-6-P for each if these modified enzymes. The results reveal the limitations of modeling allosteric interactions as simple steric conflicts linking one site to another.

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). The sodium salt of Fru 6-P was purchased from Sigma-Aldrich or USB Corporation (Cleveland, OH). NADH and dithiothreitol were purchased from Research Products International (Mt. Prospect, IL). Creatine kinase and the ammonium sulfate suspension of glycerol-3-phosphate dehydrogenase were purchased from Roche Applied Sciences (Indianapolis, IN). The ammonium sulfate suspensions of aldolase and triosephosphate isomerase, as well as, the sodium salts of phosphocreatine and PEP were purchased from Sigma-Aldrich. The sodium salt of ATP was purchased from Sigma-Aldrich and Roche Applied Sciences. The experiments involving quantifying the allosteric response of TtPFK to MgADP were conducted using sodium salt of ATP purchased from Roche Applied. The coupling enzymes were dialyzed extensively against 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EDTA before use.

Mutagenesis. The pALTER plasmid with the wild type TtPFK gene was used as the starting template for mutagenesis.[1] For double and triple substitutions, the plasmid containing the

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gene with the single or double was used as a template. The mutations were introduced using QuikChange (Stratagene, La Jolla, CA) using a pair of complementary primers. The template primer for each mutation is shown below with the substitution underlined:

N59D: GCGGGACGTGGCCGATATCATCCAGCGGGG

A158T: GGGACACCGCGACGAGCCACGAGCG

S215H: GAGGCGGGGGAAGAAGCATTCCATCGTGGTGGTGG

The resulting sequences were verified via DNA sequencing at the Gene Technology Laboratory at Texas A&M University.

Protein Expression and Purification. The RL257 cells[11] containing the plasmid with the TtPFK gene were induced with IPTG at the beginning of growth and grown at 30 °C for 18 h in LB (Luria−Bertani media: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride) 15 μg/mL tetracycline. The cells were harvested by centrifugation in a Beckman J6 at 4000 rpm and frozen at −80 °C for at least 2 h before lysis. The cells were resuspended in purification buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and sonicated using the Fisher 550 Sonic Dismembrator at 0 °C for 8−10 min using 15 s pulse/45 s rest sequence. The crude lysate was centrifuged using a Beckman J2−21 centrifuge at 22500*g* for 30 min at 4 °C. The supernatant was heated at 70 °C for 20 min, cooled, and centrifuged for 30 min at 4 °C. The protein was then precipitated using 35% ammonium sulfate at 0 °C and centrifuged. The pellet was dissolved in minimal volume of 20 mM Tris-HCl, pH8 and dialyzed several times against the same buffer. The protein was then applied to a MonoQ column (GE Life Sciences), which was equilibrated with the purification buffer (20 mM Tris-HCl, pH8) and eluted with a 0 to 1 M NaCl gradient. Fractions containing PFK activity were analyzed for purity using SDS-PAGE, pooled, and dialyzed against the same buffer and stored at 4 °C. The protein concentration was determined using the BCA assay (Pierce).

Kinetic Assays. Initial velocity measurements were carried out in 600 μL of buffer containing 50 mM EPPS-KOH, pH 8, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, 5 μg of triosephosphate isomerase, and 0.5 mM ATP. 40 μg/mL of creatine kinase and 4 mM phosphocreatine were present in all assays performed in the absence of MgADP. The amount of Fru-6-P and PEP or MgADP used in any given assay varied. When measuring the activation by MgADP, phosphocreatine and creatine kinase were excluded from the assay mix, and equimolar MgATP was added with MgADP to avoid competition at the active site. The reaction was initiated by adding 10 μL of TtPFK appropriately diluted into 50 mM EPPS (KOH) pH 8, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA. The conversion of Fru-6-P to fru 1,6-BP was coupled to the oxidation of NADH, which resulted in a decrease in absorbance at 340 nm. The rate of the decrease in A_{340} was monitored using a Beckman Series 600 spectrophotometer.

Data analysis. Data were fit using the nonlinear least-squares fitting analysis of Kaleidagraph software (Synergy). The initial velocity data were plotted against concentration of Fru-6-P and fit to the following equation:

$$v^\circ = \frac{V[\mathrm{A}]^{n_\mathrm{H}}}{K_\mathrm{a}^{n_\mathrm{H}} + [\mathrm{A}]^{n_\mathrm{H}}} \quad (1)$$

where v° is the initial velocity, [A] is the concentration of the substrate Fru-6-P, V is the maximal velocity, n_H is the Hill coefficient, and K_a is the Michaelis constant defined as the concentration of substrate that gives one-half the maximal velocity. For the reaction in rapid equilibrium, K_a is equivalent to the dissociation constant for the substrate from the binary enzyme−substrate complex.

The K_a and K_y values obtained from the initial velocity and fluorescence experiments were plotted against effector or substrate concentrations and fit to eq 2:

$$K_\mathrm{a} = K_\mathrm{ia}^\circ\left(\frac{K_\mathrm{iy}^\circ + [\mathrm{Y}]}{K_\mathrm{iy}^\circ + Q_\mathrm{ay}[\mathrm{Y}]}\right) \quad (2)$$

where K_ia° is the dissociation constant for Fru-6-P in the absence of allosteric effector, Y is PEP, K_iy° is the dissociation constant for PEP in the absence of Fru-6-P, and Q_ay is the coupling coefficient.[2,12,13] When eq 3 is applied to the allosteric action of MgADP, the subscripts are changed from "y" to "x", and MgADP is designated as "X", to be consistent with the notation we have used previously.[14]

Q_ay is defined as the coupling constant, which describes the effect of allosteric effector on the binding of the substrate (and vice versa) and is defined by eq 3:

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

where K_ia° and K_ia^∞ represent the dissociation constants for the substrate in the absence and saturating presence of the allosteric effector, respectively, and K_{iy}° and K_{iy}^∞ represent the dissociation constants for the allosteric effector in the absence and saturating presence of the substrate, respectively.

The coupling constant Q_ay is related to the coupling free energy (ΔG_ay) and its enthalpy (ΔH_ay) and entropy (ΔS_ay) components through the following relationship:[15]

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

The coupling entropy and enthalpy components were determined by measuring the coupling constant as a function of temperature and the data were fit to eq 5:

$$\ln Q_\mathrm{ay} = \frac{\Delta S_\mathrm{ay}}{R} - \frac{\Delta H_\mathrm{ay}}{R}\left(\frac{1}{T}\right) \quad (5)$$

where ΔG_ay is the coupling coefficient, ΔS_ay is the coupling entropy, ΔH_ay is coupling enthalpy, T is absolute temperature in K, and R is gas constant (R = 1.99 cal K^{-1} mol^{-1})

Crystal Structure Analysis. The analysis of crystal structures of apo,[4] phosphoglycolate-,[5] and Fru-6-P and ADP-bound[6] BsPFK was done using UCSF CHIMERA software.

RESULTS

To establish the magnitude of PEP inhibition in the single, double, and triple variants of TtPFK, the apparent dissociation constants for Fru-6-P were determined as a function of PEP concentration. The individual titration curves were fit to eq 1 to obtain the dissociation constant for Fru-6-P as well as the specific activity and the Hill number (Table 1). The specific activities of all single, double, and triple mutants assayed are comparable to that of wild type TtPFK. No decrease in specific activity was observed in any of the mutants with the addition of PEP. An increase in the homotropic cooperativity with the

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Table 1. Specific Activities and Hill Numbers for Single, Double, and Triple Revertant Variants of TtPFK at pH 8, 25°C[a]

	SA (U/mg)[b]	Hill no.
wild type[c]	41 ± 2	1.6 ± 0.1
N59D	33 ± 2	1.2 ± 0.1
A158T	40 ± 2	1.6 ± 0.2
S215H	36 ± 1	1.8 ± 0.3
N59D/A158T	23 ± 1	1.0 ± 0.1
N59D/S215H	41 ± 1	1.8 ± 0.2
A158T/S215H	26 ± 1	1.1 ± 0.1
N59D/A158T/S215H	46 ± 1	1.0 ± 0.1

[a]The error represents the standard error calculated for the fit of the data to eq 1. [b]1 U equals the turnover of 1 μmol/min. [c]From ref 1.

addition of PEP was seen in all mutants, similar to what is seen in wild type TtPFK.[1]

The data for the apparent dissociation constants as a function of PEP concentration were fit to eq 2 to obtain the coupling parameter (Q_{ay}) and the dissociation constants for Fru-6-P in the absence of PEP (K°_{ia}) and for PEP in the absence of Fru-6-P (K°_{iy}). These parameters were used to calculate the standard coupling and binding free energies, ΔG_{ay}, ΔG_{a}, and ΔG_{y}, respectively, reported in Figure 3A–C. It is interesting to note that each of the mutations produced roughly 1 kcal mol^{-1} increase in the coupling free energy. The substitution of N59D also resulted in a large decrease in PEP binding affinity without a major effect on the Fru-6-P binding affinity. A158T resulted in a slight increase in F6P binding and a slight decrease in PEP binding. S215H did not have a significant effect on PEP or Fru-6-P binding. The fact that N59D produced an increase in coupling while making the PEP binding weaker, and A158T and S215H produced a similar increase while having very modest or no effect on the PEP binding, suggests that the binding of the inhibitor and the actual inhibition are independent of one another as we have observed previously.[16,17]

Each combination of the double substitutions N59D/A158T, N59D/S215H, and A158T/S215H produced a further increase in coupling free energy (Figure 3A). The overall change in the coupling free energy for each of the double mutants appears to be roughly equal to the sum of the changes resulting from the constituent individual mutations, suggesting that these residues act independently in increasing the inhibitory response of the enzyme. Each of the double substitutions also retains the ligand binding features of the single mutations it contains. For example, both combinations containing N59D show a much weaker PEP binding, while those containing A158T show a slightly improved Fru-6-P binding (Figure 3B,C).

The TtPFK variant containing N59D/A158T/S215H shows an even further increase of the coupling free energy between PEP and Fru-6-P and produced an inhibition that is 50-fold greater than that observed for wild type. Indeed, this variant is inhibited 1.75 times the inhibition experienced by BsPFK (Table 2). These values, expressed as coupling free energies, are present in Figure 3. This variant also shows weaker PEP binding similar to that displayed by the N59D variant and a slightly stronger Fru-6-P binding than that seen in the A158T variant (Figure 3B,C). For comparison, previously reported variants of TtPFK, L313W, and C11F/A273P, exhibited virtually no effect on the coupling free energy between PEP and Fru-6-P and only minor effects (less than 3-fold) on the binding interactions of Fru-6-P and PEP individually.[1]

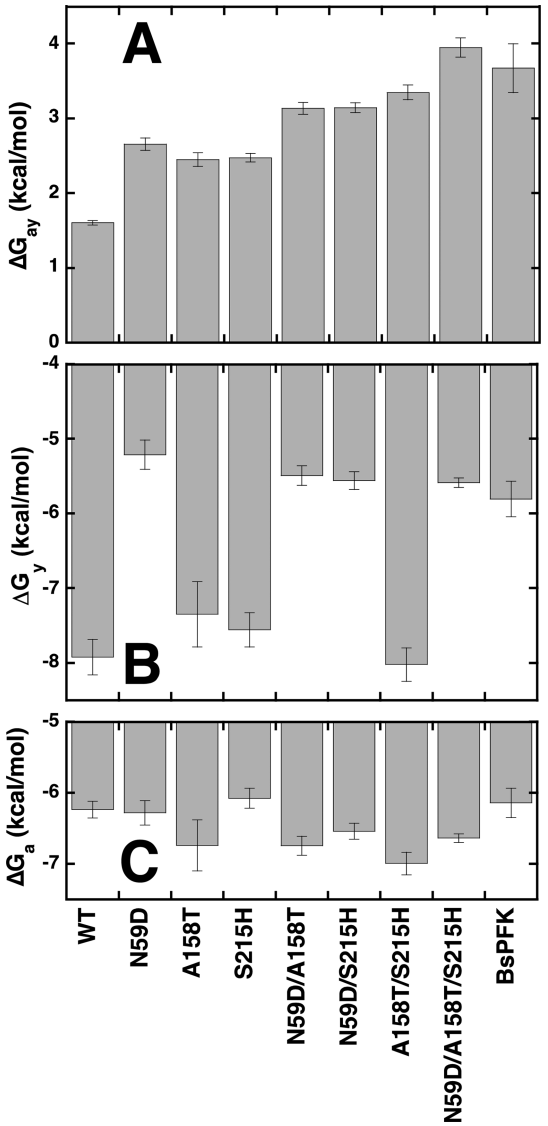

Figure 3. (A) Diagram summarizing the coupling free energies for binding of PEP and Fru-6-P to wild type TtPFK and BsPFK and the revertant mutants of TtPFK. (B) Diagram summarizing the binding free energies for PEP in wild type TtPFK and BsPFK and the revertant mutants of TtPFK. (C) Diagram summarizing the binding free energies for Fru-6-P in wild type TtPFK and BsPFK and the revertant mutants of TtPFK.

Since the triple mutant produced such a significant increase in coupling free energy of inhibition, we wanted to evaluate which thermodynamic components of coupling free energy were affected and to what extent. To assess the dependence of coupling on temperature and establish the entropic and enthalpic contributions to the PEP inhibition in N59D/A158T/S215H TtPFK, we analyzed the coupling parameter as a function of temperature (Figure 4). The values for ΔH_{ay} and $T\Delta S_{ay}$ were determined to be −11.0 ± 0.5 kcal mol^{-1} and −14.9 ± 0.5 kcal mol^{-1}, respectively. It is significant that both the entropy and enthalpy of PEP inhibition of the triple variant are much closer to the values obtained for wild type BsPFK than to those for wild type TtPFK (Table 2).[18]

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Table 2. Summary of Kinetic and Thermodynamic Parameters for Wild Type TtPFK, BsPFK, and TtPFK N59D/A158T/S215H Revertant Mutant at pH 8 and 25 °C[a]

	TtPFK[b]	TtPFK N59D/A158T/S215H	BsPFK[c]
K_{ia}° (μM)	27.0 ± 0.6	13.0 ± 0.2	31 ± 2
K_{ix}° (μM)	0.4 ± 1	ND	19 ± 2
Q_{ax}	1.6 ± 0.1	ND	1.70 ± 0.01
K_{iy}° (μM)	1.58 ± 0.07	79 ± 0.2	93 ± 6
Q_{ay}	0.067 ± 0.002	0.0012 ± 0.0007	0.0021 ± 0.0003
ΔG_{ay} (kcal mol^{-1})	1.60 ± 0.02	3.95 ± 0.03	3.67 ± 0.1
ΔH_{ay} (kcal mol^{-1})	−7.5 ± 0.3	−11.0 ± 0.5	−10 ± 1
$T\Delta S_{ay}$ (kcal mol^{-1})	−9.1 ± 0.3	−14.9 ± 0.5	−14 ± 1

[a]Subscripts 'a', 'x', and 'y' represent Fru-6-P, MgADP and PEP, respectively. [b]From ref 1 except for ΔH_{ay} and $T\Delta S_{ay}$. $T\Delta S_{ay}$ was calculated using the values for ΔG_{ay} and ΔH_{ay}. [c]From ref 12.

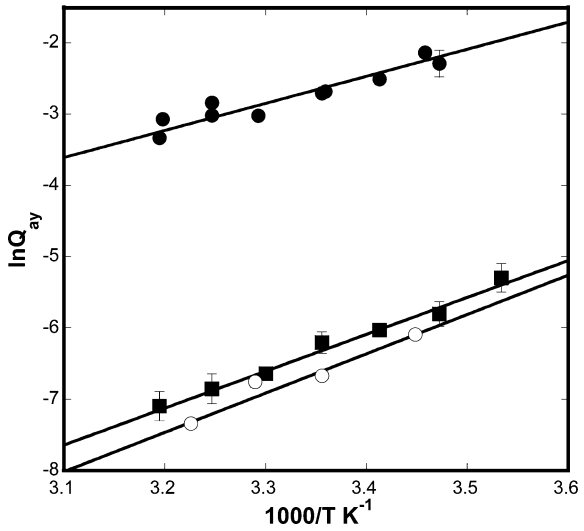

Figure 4. Van't Hoff plots of ln Q_{ay} as a function of temperature. The data are shown in closed circles for wild type TtPFK, in open circles for N59D/A158T/S215H, and in solid squares for wild type BsPFK. The data were fit to eq 5 (solid line) to obtain the enthalpy component of coupling free energy at 25 °C.

After achieving such a large increase in the coupling free energy of inhibition upon the introduction of the N59D/A158T/S215H revertant mutations into TtPFK, it was of interest to see if these mutations would have a similar effect on the coupling free energy describing the activation by MgADP. To establish the effect of the N59D/A158T/S215H mutations on the binding and coupling of MgADP, the dissociation constants for Fru-6-P were measured as a function of the concentration of MgADP. Equimolar MgATP was added to avoid competitive inhibition at the active site. The data for the apparent dissociation constants as a function of MgADP concentration were fit to eq 2, which yielded a coupling free energy of 0 (data not shown), suggesting that either MgADP does not bind to this variant or that MgADP does bind but elicits no allosteric response. To verify whether the MgADP is able to bind to the allosteric site of this variant, the PEP binding was measured as a function of MgADP concentration. (MgADP and PEP bind to the same allosteric site in prokaryotic PFK.[6]) PEP binding was not affected when up to 1 mM MgADP was added to the assays, suggesting that MgADP does not bind to the N59D/A158T/S215H variant of TtPFK at physiological concentrations. To determine which of the point mutations may be responsible for diminished MgADP binding, we measured the effect of the N59D, A158T, and S215H individually on the binding and coupling of MgADP. Similar to the triple variant, the S215H variant did not bind MgADP, suggesting that the perturbations caused by introducing a histidine at position 215 greatly impair the ability of MgADP to bind to TtPFK. As shown in Figure 5A, both N59D and A2158T variants yielded an increase in the absolute value of the respective coupling free energies compared to wild type TtPFK.

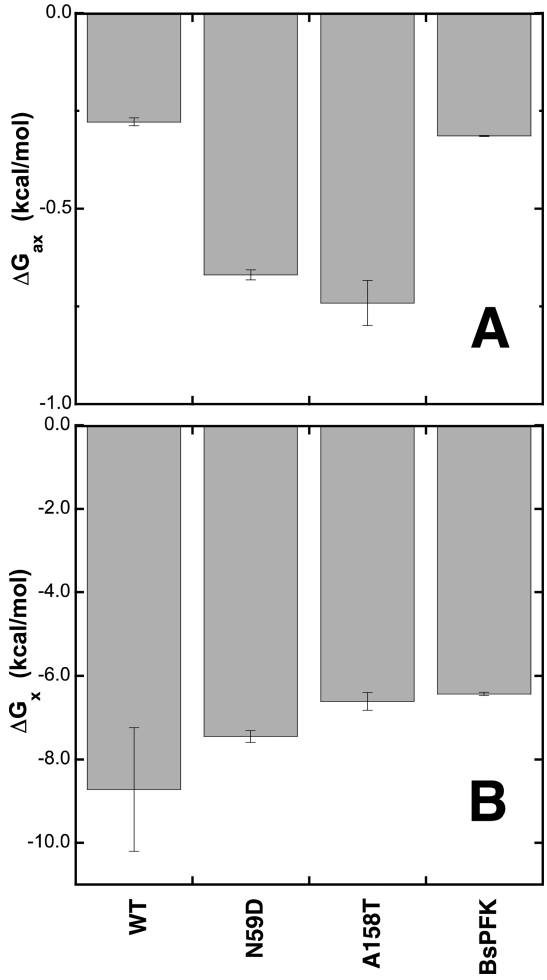

Figure 5. (A) Diagram summarizing the coupling free energies for binding of Fru-6-P and MgADP for wild type TtPFK as well as N59D and A158T revertant mutants. (B) Diagram summarizing the binding free energies for MgADP for wild type TtPFK as well as N59D and A158T revertant mutants.

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Both variants also showed a slight decrease in MgADP binding affinity (Figure 5B).

DISCUSSION

The large increase in the coupling free energy for inhibition upon introducing the triple chimeric substitution N59D/A158T/S215H (Figure 3A) appears at first glance to support the hypothesis that the seven residues that link the closest active and allosteric sites in BsPFK represent an important pathway for the influence of PEP binding to be transmitted to the Fru-6-P binding site. However, the results from the variants containing only the individual N59D, A158T, and S215H mutations, and their pair wise combinations, suggest that there is more to the story. If a complete network connecting the two sites is required, one would not have expected the single mutations to enhance the coupling in TtPFK as substantially as was observed. Rather one would have expected little effect as the individual mutations were introduced until all of the ostensibly required interactions had been restored. Instead, the addition of each successive chimeric mutation increased the coupling essentially additively. Since the absence of one or more individual H-bonding interactions does not appear to "break the chain" of interactions conducting the allosteric influence from one site to the other, the concept that a specific pathway is responsible for conveying the inhibitory impact of the binding of PEP to its binding site must be reconsidered.

One possible alternative explanation of why we observed such an improvement in coupling in the absence of a completely reconstructed network is to consider the entropic nature of the inhibition. The analysis of PEP coupling as a function of temperature shows that the inhibition by PEP is entropically driven in N59D/A158T/S215H TtPFK, just as it is in the wild type enzyme and BsPFK (Figure 4, Table 2). We observe a modest 3.5 kcal mol^{-1} decrease in enthalpy, which is offset with an even larger decrease in entropy, resulting in a larger overall ΔG_{ay}. If one assumes that the conformational entropy is a major contributor to these overall entropy estimates, it is possible to imagine a scenario where each of the three substitutions can independently modify motions to varying degrees in the various liganded states. Essentially such a thermodynamic mechanism would depend on the properties of each enzyme form as a whole and not necessarily be dependent on a particular localized network of interactions.

Another possible explanation for the substantial enhancements in allosteric effectiveness observed with the single and double variants is that these residues are in fact involved in multiple allosteric networks. We have shown that four unique pairwise allosteric interactions between PEP and Fru-6-P binding sites each contribute to the overall allosteric inhibition by PEP in the tetramer.[7,8] Although these residues were identified based upon their apparent linkage of the closest pair of sites in BsPFK, they may also be contributing to the coupling between the other geometric pairs of PEP and Fru-6-P sights.[7,8,19,20] Indeed, although the closest pair contributes the most to the overall coupling in BsPFK (and EcPFK), we have not made these measurements with TtPFK. It is possible that residues N59, A158, and S215 in particular belong to one or more allosteric networks that together define the overall allosteric response in the tetramer.

Although the effect of these mutations on the magnitude of inhibition by PEP is quite large, there is little change in the level of activation by MgADP displayed by the variants containing N59D and A158T (Figure 5A). This observation suggests that though N59 and A158 may play an important role in the inhibition by PEP, they are less involved in the allosteric activation by MgADP.

While the specific basis for the allosteric effects observed in TtPFK remain somewhat obscure, residues important to the high affinities for PEP and MgADP displayed by TtPFK have been clearly identified. N59D uniquely causes PEP binding affinity to decrease, while having little effect on the binding affinity of MgADP. By contrast, S215H strongly diminishes MgADP affinity while having virtually no effect on PEP affinity. Consequently, N59 and S215 must be considered crucial to the unusually tight binding of PEP and MgADP, respectively, to the single allosteric site of TtPFK.

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Notes

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ABBREVIATIONS

PFK, phosphofructokinase; TtPFK, *Thermus thermophilus* phosphofructokinase; BsPFK, *Bacillus stearothermophilus* phosphofructokinase; Fru-6-P, fructose-6-phosphate; PEP, phospho(*enol*)pyruvate

ADDITIONAL NOTE

[a]Because the TtPFK primary sequence contains additional residues compared to the BsPFK sequence, the TtPFK residues corresponding to the BsPFK D59, T158, and H215 are N59, A159, and S216, respectively. To avoid confusion when comparing the BsPFK and TtPFK, the remainder of this publication will refer to the BsPFK sequence even when referring to the TtPFK residue.

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