Equilibrium Binding Studies of a Tryptophan-Shifted Mutant of Phosphofructokinase from *Bacillus stearothermophilus*†

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ABSTRACT: A tryptophan-shifted mutant of phosphofructokinase (PFK) from *Bacillus stearothermophilus* has been constructed. This mutant, which is functionally similar to wild-type, provides the opportunity to examine the allosteric properties of PFK under equilibrium conditions. The unique fluorescence properties of the tryptophan-shifted mutant enzyme, W179F/F230W, have been utilized to deduce the thermodynamics of ligand binding and the allosteric perturbations in the absence of catalytic turnover. Specifically, phospho(enol)pyruvate (PEP) and MgADP binding to the mutant PFK can be directly observed using tryptophan fluorescence, and dissociation constants for these ligands have been measured to be equal to 2.71 ± 0.04 and 90.4 ± 3.5 μM, respectively. In addition, the homotropic couplings for the allosteric ligands have been assessed for the first time. PEP binds cooperatively with a Hill number of 2.9 ± 0.3, while MgADP binding is not cooperative. The equilibrium couplings between these ligands and the substrate fructose 6-phosphate (Fru-6-P) have also been determined and follow the same trends with temperature observed under steady-state kinetic assay conditions using wild-type PFK, indicating that the presence of bound MgATP has little influence on the allosteric interactions. Like wild-type PFK, the coupling free energies for the mutant result from largely compensating enthalpy and entropy components at 25 °C. Furthermore, the sign of each coupling free energy, which signifies the nature of the allosteric effect, is opposite that of the enthalpy contribution and is therefore due to the larger absolute value of the associated entropy change. This characteristic stands in direct contrast to the thermodynamic basis of the allosteric response in the homologous PFK from *E. coli* in which the sign of the coupling free energy is established by the sign of the coupling enthalpy.

Phosphofructokinase (PFK)[1] catalyzes the phosphorylation of fructose 6-phosphate (Fru-6-P) by MgATP to produce fructose 1,6-bisphosphate and MgADP. This reaction is the first committed step of glycolysis and is allosterically regulated by phospho(enol)pyruvate (PEP) and MgADP. PFK from *B. stearothermophilus* is a homotetramer with each subunit having a molecular weight of 34 000. Like *E. coli* PFK, *B. stearothermophilus* PFK contains one tryptophan per subunit, but unlike *E. coli* PFK, the fluorescence of this tryptophan is largely unresponsive to ligand binding (*1*). While studies of the intrinsic fluorescence of *E. coli* PFK have allowed us to study the allosteric properties under equilibrium conditions, such investigations are for the most part impossible with wild-type *B. stearothermophilus* PFK. The tryptophan of *B. stearothermophilus* PFK is immobile and inaccessible to bulk solvent and quenchers such as acrylamide (*1*). X-ray crystallographic studies confirm that the single tryptophan is buried in the core of the protein (*1*, *2*).

In this study we have constructed a "tryptophan-shifted" mutant whose fluorescence emission properties are responsive in unique ways to the binding of the allosteric ligands. In this mutant, the tryptophan at position 179 has been changed to a phenylalanine, and the phenylalanine at position 230 has been changed to a tryptophan (W179F/F230W). According to the crystal structure of wild-type *B. stearothermophilus* PFK (*2*, *3*), the new position of the tryptophan is approximately 15 Å from the Fru-6-P binding site and 20 Å from the allosteric site, assuming minimal disturbance in the structure by the amino acid substitutions. The new position is directly across the monomer from the native position and is possibly solvent-exposed. This mutant is readily expressed and purified, and the fluorescence is particularly responsive to the binding of allosteric ligands. These properties have been successfully utilized to characterize for the first time the allosteric properties of *B. stearothermophilus* PFK under equilibrium conditions in the absence of turnover. The results demonstrate unequivocally that the allosteric behavior of both ADP and PEP derives from changes in the entropy of the system at 25 °C, in contrast to results previously obtained for *E. coli* PFK (*4*, *5*) but in agreement with results previously inferred from steady-state kinetics (*6*). In addition, the homotropic interactions associated with allosteric ligand binding have been assessed directly for the first time. PEP binds with considerable positive cooperativity

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[1] Abbreviations: PFK, phosphofructokinase; PEP, phospho(enol)pyruvate; Fru-6-P, fructose 6-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BCA, bicinchinic acid; DTT, dithiothreitol; PCR, polymerase chain reaction.

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in the absence of other ligands, whereas MgADP binds essentially uncooperatively.

MATERIALS AND METHODS

Materials. All chemical reagents used in buffers, *B. stearothermophilus* PFK purification, and fluorescence and enzymatic assays were of analytical grade, and purchased from either Sigma, Fisher, or Aldrich. The Matrex Gel Blue A-agarose resin for affinity chromatography was purchased from Amicon Corp. The coupling enzymes aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were purchased as ammonium sulfate suspensions from Boehringer Mannheim. The coupling enzymes were dialyzed extensively against a buffer consisting of 50 mM MOPS−KOH, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA at pH 7.0 before use. Creatine phosphate, creatine kinase, and sodium salts of Fru-6-P, ATP, ADP, and PEP were purchased from Sigma. Site-directed mutagenesis was carried out using the Altered Sites In Vitro Mutagenesis System which was purchased from Promega and included pALTER vector, pALTER control vector, and ampicillin repair and control oligonucleotides. Other oligonucleotides were synthesized using an Applied Biosystems 392 DNA/RNA synthesizer. DNA modifying enzymes and restriction endonucleases were purchased from either New England Biolabs or Promega. The plasmid pBR322/Bs-pfk (*7*), which contains the gene for *B. stearothermophilus* PFK, was obtained from Dr. Simon Chang (Louisiana State University). The cell strain DF1020 [a *rec*A derivative of DF1010 (8): *pro*-82, Δ*pfk*B201, *rec*A56, Δ(*rha-pfk*A)200, *end*A1, *hds*R17, *sup*E44] used to express wild-type and mutant *B. stearothermophilus* PFKs was obtained from Dr. Robert Kemp (Chicago Medical School). Deionized distilled water was used throughout.

Site-Directed Mutagenesis. In addition to containing the gene for *B. stearothermophilus* PFK, the plasmid pBR322/Bs-pfk also contained approximately 1 kilobase of the native promoter that was found to complicate our mutagenesis strategy (F. Janiak-Spens, unpublished result). PCR was used to construct a *Hin*dIII site approximately 70 bases upstream from the start codon. The resulting PCR fragment was digested with *Hin*dIII, which now had sites on both ends of the fragment, and ligated into the *Hin*dIII site of pALTER. The ligation reaction was transformed into JM109 competent cells, plated on tetracycline plates that had previously been treated with IPTG and X-gal, and incubated overnight at 37 °C. White colonies were screened for proper insertion of the fragment by checking for overexpression of *B. stearothermophilus* PFK. The resulting plasmid contained the gene for *B. stearothermophilus* PFK in pALTER under the lac promoter (pMRLBsPFK).

Mutagenesis was performed in accordance with the protocol for the Altered Sites In Vitro Mutagenesis System as provided by Promega. Oligonucleotides for site-directed mutants were as follows:

W179F: GCC-AGC-CCC-GA<u>A</u>-<u>A</u>AT-AAG-GCG-ATG-TCG-CC

F230W: GAA-TTT-GCC-GGC-C<u>CC</u>-AGT-CGA-CGC-CGC-TTC-C

The underlined bases indicate those that were used to introduce mutations. Plasmid from selected ampicillin resistant colonies was sequenced by the Sanger dideoxy method (*9*) using an ABI sequencer and dye-labeled terminators to screen for the desired mutations and to verify the sequence of the mutated DNA. The new plasmid containing the mutations for amino acid residues 179 and 230 was designated pMRL05.

Protein Expression. The plasmids pBR322/Bs-pfk and pMRL05, which encode for wild-type and W179F/F230W *B. stearothermophilus* PFK, respectively, were transformed into competent *E. coli* DF1020 cells (*10*). Both enzymes are constitutively expressed from their respective plasmids. However, wild-type *B. stearothermophilus* PFK is expressed by its native promoter, while W179F/F230W *B. stearothermophilus* PFK is expressed from the lac promoter.

Protein Purification. Purification of wild-type and W179F/F230W *B. stearothermophilus* PFKs was carried out as described previously with a few modifications (*11*). DF1020 cells containing the appropriate plasmid were grown 18−24 h in Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L potassium phosphate dibasic, 2.2 g/L potassium phosphate monobasic, and 4 mL/L glycerol at pH 7.2) containing 100 μg/mL ampicillin at 37 °C. Cells were pelleted, resuspended in sonication buffer (50 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA), and sonicated at 0 °C in 15 s pulses for 10 min. The crude lysate was centrifuged at 12000*g* for 60 min at 4 °C. The supernatant that contained the soluble proteins was heated to 70 °C for 15 min, cooled on ice, and centrifuged as before. The supernatant was loaded onto a Matrex Gel Blue A-agarose resin affinity column which had been equilibrated with sonication buffer + 0.1 M NaCl. The column was washed with at least 5 bed volumes of sonication buffer + 0.1 M NaCl and the enzyme eluted with a gradient from 0.1 to 1.5 M NaCl. Wild-type and W179F/F230W mutant enzymes eluted at approximately 1.1 M NaCl. Fractions which contained the enzyme were pooled, dialyzed into MOPS buffer (50 mM MOPS, pH 7, 14 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA), and concentrated using an Amicon ultrafiltration apparatus with a molecular weight cutoff of 10 000. Concentrated enzyme was stored at 4 °C. The final enzyme preparation was pure as estimated by SDS−PAGE (*12*) stained with Coomassie Blue. Protein concentrations were determined by using BCA protein assay reagent (*13*) or by absorbance assuming ϵ_{280} equal to 18 910 M^{-1} cm^{-1}. ϵ_{280} was calculated based on the number of tyrosine and tryptophan residues (*14*). The concentrations calculated by both methods agreed very well with one another.

Enzymatic Activity Determination. Activity measurements were carried out in a 1.0 mL reaction volume of a MOPS buffer adjusted to pH 7.0 at the appropriate temperatures containing 50 mM MOPS−KOH, 100 mM KCl, 14 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, and 5 μg of triosephosphate isomerase. When necessary, creatine kinase and creatine phosphate were added to regenerate ATP and to avoid the accumulation of ADP. Assays were initiated by the addition of 10 μL of PFK that had been appropriately diluted. Activity measurements were conducted on Beckman Series 600 spectrophotometers using a linear regression calculation to convert the change in absorbance at 340 nm to enzyme activity. A unit of activity

is defined as the amount of enzyme required to produce 1 μmol of fructose 1,6-bisphosphate per minute.

Steady-State Fluorescence. Steady-state fluorescence intensity was measured using an SLM-4800 fluorometer upgraded with photon counting electronics and instrument control software from I.S.S., Inc. (Champaign, IL). Samples were excited at 300 nm using a xenon arc lamp as the source. Emission was collected either through an emission monochromator or through a Schott WG-345 cut-on filter. Ligand titrations into a solution of W179F/F230W *B. stearothermophilus* PFK were carried out in an initial volume of 2 mL in 50 mM MOPS, pH 7.0, 14 mM $MgCl_2$, 100 mM KCl with the protein concentration ranging from 1 to 5 μM. All emission values were corrected for dilution and blank contributions, obtained from otherwise identical samples with PFK omitted.

Data Analysis. Data analysis was performed on a Power Macintosh 7100/80AV using Kaleidagraph 3.08 (Synergy Software) to fit to the following equations. The concentration of Fru-6-P which resulted in half-maximal activity, $K_{1/2}$, along with the maximum velocity, V_{max}, and Hill coefficient, n_H, were determined using the Hill equation (*15*):

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

where v is the steady-state rate of turnover, E_T is the total amount of enzyme active sites, k_{cat} is the turnover number, and n_H is the Hill coefficient. The variation in $K_{1/2}$ as a function of PEP concentration was fit to the following equation:

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

where K_{ia}^{o} is the dissociation constant for the substrate Fru-6-P in the absence of allosteric ligand, K_{iy}^{o} is the dissociation constant for the allosteric ligand in the absence of substrate, and Q_{ay} is equal to the coupling parameter describing the extent to which the binding of the allosteric ligand affects the binding of substrate and vice versa as defined by the following expression:

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

where K_{ia}^{∞} and K_{iy}^{∞} are the dissociation constants for A and Y, respectively, in the saturating presence of the other ligand[2] (*16*). To be consistent with previously adopted conventions (*5, 6, 17*), A represents Fru-6-P and Y represents PEP. By resolving K_{iy}^{o} and Q_{ay}, eq 2 provides separate quantification of both PEP binding affinity and its action once bound, respectively.

Data collected using fluorescence assays were analyzed using the following equation that is analogous to eq 1:

$$F = \frac{\Delta F[A]^{n_H}}{(K_{1/2})^{n_H} + [A]^{n_H}} + F_o \quad (4)$$

where F is the steady-state fluorescence emission, F_o is equal to F when [A] = 0, and ΔF is equal to the limiting value of $F - F_o$ when [A] is saturating.

The thermodynamic parameters governing the interaction between Fru-6-P and PEP were determined using van't Hoff analysis. The coupling constant, Q_{ay}, was measured as a function of temperature using either kinetic or fluorescence assays. The thermodynamic components were then determined by the following relationship:

$$\Delta G_{ay} = -RT \ln Q_{ay} = \Delta H_{ay} - T\Delta S_{ay} \quad (5)$$

Therefore, ΔH_{ay} and ΔS_{ay} can be derived from a plot of log Q_{ay} vs $1/T$ where the slope is $\Delta H_{ay}/R$ and the intercept is $\Delta S_{ay}/R$.

RESULTS

To determine whether the conformation of W179F/F230W has been altered to a significant degree, the functional properties of the modified enzyme were assessed and compared to those of wild-type. The basic kinetic parameters were determined by measuring the catalyzed rate as a function of varying Fru-6-P concentration and fitting the data to eq 1. MgATP was maintained at a saturating concentration for these experiments, and the results are presented in Table 1. In addition, the dependence of the rate on MgATP concentration at saturating concentration of Fru-6-P was also determined. Since MgATP binds noncooperatively, these data were fit to the Michaelis−Menten equation (i.e., eq 1 with $n_H = 1$), and the K_m for MgATP is reported in Table 1. The catalytic rate constant, k_{cat}, is the same for both the wild-type and mutant enzymes, indicating that the mutations have done little to disturb enzyme catalysis (Table 1). Further comparison of the $K_{1/2}$ for Fru-6-P and the K_m for MgATP shows that neither of the apparent substrate binding affinities are compromised to a significant degree as a result of the modifications, although the small degree of cooperativity associated with Fru-6-P binding is abolished in the mutant (Table 1). These results are consistent with the mutations being relatively far removed from the active site and causing minimal structural perturbation.

To further characterize the integrity of the modified enzyme, the allosteric response of the enzyme with respect to the inhibitor PEP was evaluated. Figure 1 shows the dependence of the $K_{1/2}$ for Fru-6-P on PEP concentration at pH 7 and 25 °C for wild-type and W179F/F230W PFKs. Both wild-type and mutant PFKs display a decreased affinity for Fru-6-P as the concentration of PEP increases. Table 2 presents a comparison of the coupling parameters between Fru-6-P and PEP for wild-type and W179F/F230W determined using kinetics. The dissociation constants for Fru-6-P and PEP are comparable for the mutant and wild-type, indicating that the affinity the enzyme displays for these ligands is relatively unchanged. However, W179F/F230W PFK has a smaller coupling constant, Q_{ay}, than wild type, indicating that PEP is a somewhat better inhibitor of the mutant. In wild-type PFK, PEP inhibits the binding of Fru-6-P by 120-fold, whereas in W179F/F230W, PEP inhibits 600-fold.

[2] This notation is a modification of the notation proposed by Cleland (*16*) in which the terms K_a and K_{ia} were used to distinguish between the Michaelis constant and the thermodynamic dissociation constant, respectively, of substrate "A".

Table 1: Kinetic Parameters for Wild Type and the W179F/F230W Mutant of *B. stearothermophilus* PFK at 25 °C, pH 7.0

	wild type	W179F/F230W
k_{cat} (s^{-1})	95 ± 1	96 ± 1
$K_{1/2}$ for Fru-6-P (μM)[a]	27 ± 1	34 ± 2
n_H for Fru-6-P[a]	1.48 ± 0.08	1.08 ± 0.06
K_m for MgATP (μM)[b]	43 ± 2	52 ± 2

[a] [MgATP] = 3 mM. [b] [Fru-6-P] = 2 mM.

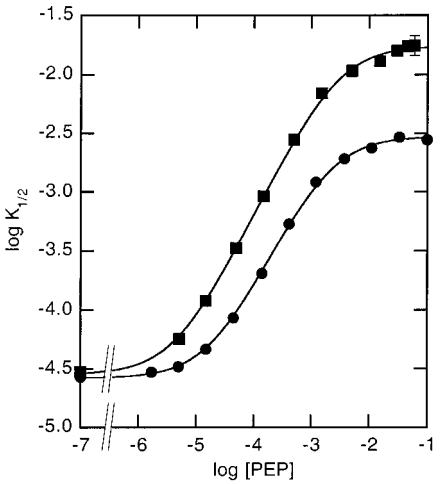

FIGURE 1: Logarithm of the apparent $K_{1/2}$ for Fru-6-P as a function of [PEP] in the saturating presence of MgATP for (●) wild-type and (■) W179F/F230W PFK. $K_{1/2}$ values at each PEP concentration were determined as described under Materials and Methods. The solid line represents the best nonlinear regression fit of the data to eq 2. MgATP concentration was held constant at 3 mM. Error bars are shown and appear smaller than the symbols.

Table 2: Coupling Parameters for the Interaction between Fru-6-P and PEP As Determined in the Presence and Absence of Saturating MgATP for Wild-Type and W179F/F230W Mutant *B. stearothermophilus* PFK at 25 °C, pH 7.0

	wild-type kinetics	W179F/F230W kinetics	W179F/F230W fluorescence
K^o_{ia} (μM)	25 ± 1	29 ± 1	0.91 ± 0.02
K^o_{iy} (μM)	17 ± 1	11 ± 1	2.7 ± 0.1
Q_{ay} (×100)	0.84 ± 0.02	0.22 ± 0.01	0.17 ± 0.04

To further verify that the mutations have not substantially changed the allosteric nature of PFK, van't Hoff analysis was used to determine the thermodynamic components associated with the coupling between Fru-6-P and PEP. Figure 2 shows the van't Hoff plots for both wild-type and W179F/F230W PFK derived from steady-state kinetics assays, and the corresponding thermodynamic parameters extracted from these data are presented in Table 3. Although the absolute values for ΔH_{ay} and $T\Delta S_{ay}$ for both enzyme forms are slightly different, the most important result to note is that, for both the wild-type and mutant enzymes, the entropy and enthalpy contributions have the same sign and are largely compensating, with the positive sign for ΔG_{ay} being determined by the negative entropy component as previously noted for wild-type (*6*). These results contrast with those previously observed for *E. coli* PFK (*5*) in which the sign of the positive coupling free energy is established by the positive coupling enthalpy component (see Figure 2).

The primary purpose of making W179F/F230W was to create a form of *B. stearothermophilus* PFK in which the intrinsic tryptophan fluorescence would be responsive to conformational changes in the protein and at the same time retain all the allosteric properties of wild-type PFK. As shown by the data presented above, W179F/F230W is very similar to wild-type with respect to activity and allosteric response. Figure 3A shows a comparison between wild-type and W179F/F230W emission spectra at 25 °C and pH 7 (λ_{ex} = 300 nm). The spectra were normalized to 1.0 mg/mL protein. The new position of the tryptophan causes a dramatic decrease in the fluorescence intensity and a slight blue shift relative to wild-type. As shown previously (*1*), the only fluorescence change observed with wild-type *B. stearothermophilus* PFK with respect to ligand binding is a 10% decrease in fluorescence intensity when PEP binds (Figure 3B).[3] However, with W179F/F230W, a 35% increase in

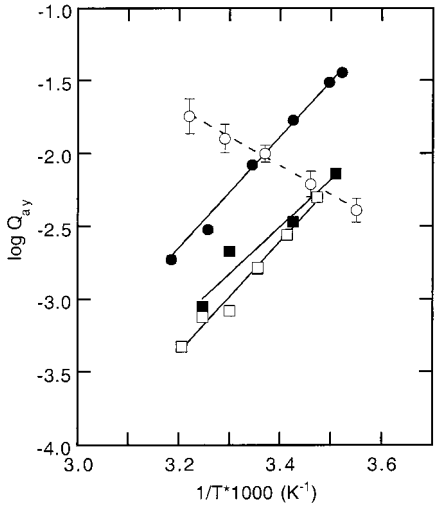

FIGURE 2: van't Hoff analysis of the coupling interaction between Fru-6-P and PEP. The symbols represent wild-type *B. stearothermophilus* PFK in the presence of MgATP (●), wild-type *E. coli* PFK in the absence of MgATP (○), W179F/F230W *B. stearothermophilus* PFK in the presence of MgATP (■), and W179F/F230W *B. stearothermophilus* PFK in the absence of MgATP (□). Results in the absence of MgATP were obtained with fluorescence titrations while those in the presence of MgATP were from steady-state kinetic assays. The solid and dashed lines represent the best fits to eq 5. Error bars represent ± standard error and are smaller than the symbol when absent. Assays of *B. stearothermophilus* PFK were performed as described in the text. *E. coli* PFK data are previously published (*5*), and fluorescence titrations of *E. coli* PFK were performed in the following solution conditions: 50 mM EPPS−KOH (pH 8.0), 10 mM $MgCl_2$, 10 mM NH_4Cl, 0.2 mM EDTA with PFK concentration between 4.2 and 5.7 μM as described by Johnson and Reinhart (*5*).

Table 3: Thermodynamic Parameters for the Interaction between Fru-6-P and PEP As Determined from van't Hoff Analysis for *B. stearothermophilus* PFK at 25 °C

kcal/mol	wild type (kinetics)	W179F/F230W (kinetics)	W179F/F230W (fluorescence)
ΔG_{ay}	+2.8 ± 0.3	+3.6 ± 0.4	+3.8 ± 0.7
ΔH_{ay}	−17.8 ± 0.2	−14.5 ± 0.3	−17.7 ± 0.5
$T\Delta S_{ay}$	−20.6 ± 0.2	−18.1 ± 0.3	−21.5 ± 0.5

[3] Although this change can be sufficient to follow PEP binding under some circumstances, at low protein concentration the water Raman peak (occurring at 334 nm when exciting at 300 nm) contributes a substantial background signal, necessitating the use of filters (e.g., Schott WG345) to block all but the long-wavelength portion of the emission where the quenching due to PEP binding is much less than 10%.

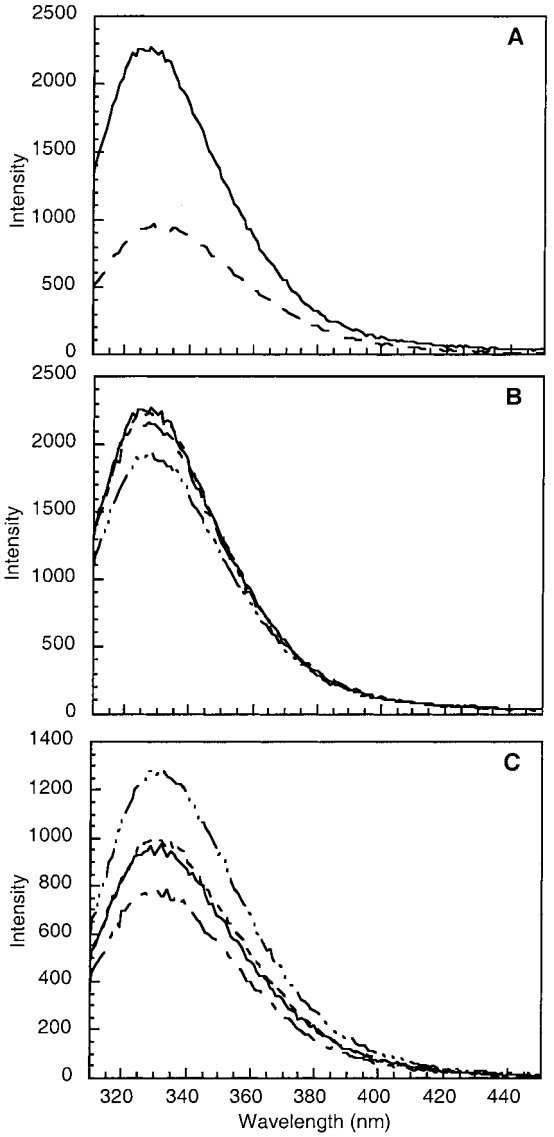

FIGURE 3: (A) Emission spectra of wild type (—) and W179F/F230W (– –) *B. stearothermophilus* PFK. (B) Emission spectra of wild-type *B. stearothermophilus* PFK in the presence of no ligands (—), Fru-6-P (– –), PEP (– ••• –), and MgADP (– • –). (C) Emission spectra of W179F/F230W *B. stearothermophilus* PFK in the presence of no ligands (—), Fru-6-P (– –), PEP (– ••• –), and MgADP (– • –). Spectra were normalized to a protein concentration of 1.0 mg/mL. $\lambda_{ex} = 300$ nm.

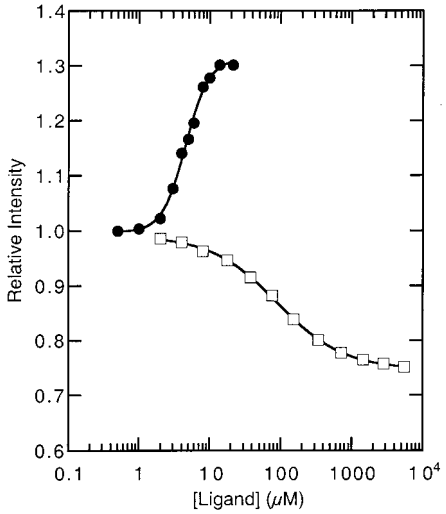

FIGURE 4: Relative fluorescence intensity as a function of [PEP] (●) or [ADP] (□) for W179F/F230W *B. stearothermophilus* PFK. The solid line represents the best nonlinear regression fit to eq 4.

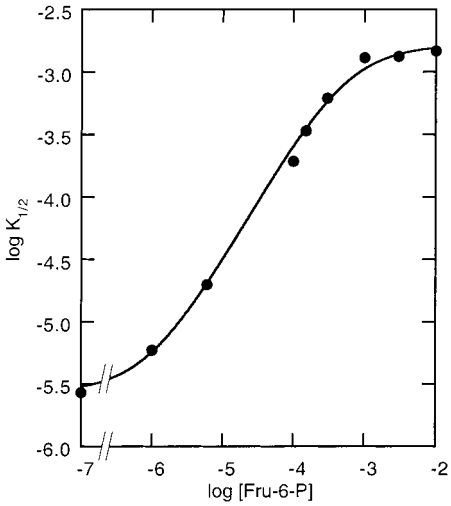

FIGURE 5: Logarithm of the apparent dissociation constant for PEP as a function of [Fru-6-P] in the absence of MgATP. The K_d was determined by titrating W179F/F230W *B. stearothermophilus* PFK with PEP at fixed Fru-6-P concentrations and analyzed as described under Materials and Methods. The solid line represents the best nonlinear regression fit of the data to eq 2. Error bars are shown and appear smaller than the symbols.

fluorescence intensity is observed with the binding of PEP as well as a 25% decrease in the fluorescence intensity with the binding of MgADP as shown in Figure 3C. No changes are observed with the binding of Fru-6-P (Figure 3C) or MgATP (data not shown).

The observed changes associated with PEP and MgADP binding were examined more closely by titrating the enzyme with each ligand. By monitoring the change in intensity as a function of PEP concentration (Figure 4), an apparent dissociation constant ($K_{1/2}$) for PEP of 2.71 ± 0.04 μM was obtained by fitting the data to eq 4. The binding of PEP was also found to be cooperative with a Hill coefficient of 2.9 ± 0.3. Similarly, a dissociation constant for MgADP of 90.4 ± 3.5 μM was measured using the observed decrease in fluorescence intensity (Figure 4). The Hill coefficient is equal to 1.

The coupling parameter for the allosteric interaction between Fru-6-P and PEP under equilibrium conditions in the absence of MgATP was determined by monitoring the apparent dissociation constant for PEP, determined from fluorescence titrations such as shown in Figure 4, as a function of Fru-6-P resulting in the data presented in Figure 5. The dissociation constants for Fru-6-P and PEP and the coupling constant determined in this manner for the allosteric interaction are given in Table 2. The dissociation constant for Fru-6-P determined from these fluorescence titrations is one-thirtieth as large as that obtained from steady-state kinetics. This discrepancy most likely is due to antagonism between Fru-6-P and MgATP in the active site as observed previously for *B. stearothermophilus* PFK (*18*) and for *E. coli* PFK (*19*). The value of Q_{ay} determined by both techniques is quite similar, however, indicating that even though MgATP disrupts Fru-6-P binding, MgATP binding

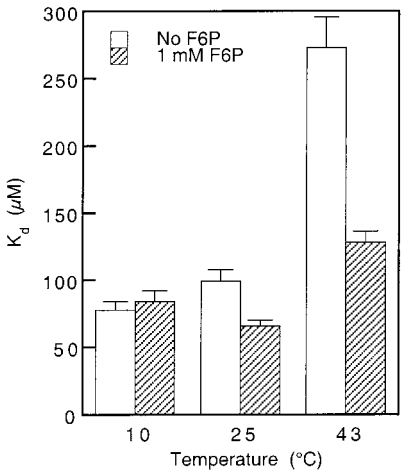

FIGURE 6: Apparent dissociation constant for MgADP in the absence (white bars) and presence (hatched bars) of Fru-6-P at multiple temperatures. Error bars indicate ± the standard error of each determination.

does not affect the allosteric interaction between Fru-6-P and PEP.

The temperature dependence of Q_{ay} was then examined, and the data are presented in the form of a van't Hoff plot in Figure 2. The thermodynamic components ΔH_{ay} and ΔS_{ay}, determined from van't Hoff analysis of these data, are presented in Table 3 for comparison to the values obtained for wild-type and W179F/F230W from steady-state kinetics. The sign and magnitude of ΔG_{ay} at 25 °C are unchanged from the values determined using kinetic assays as would be predicted by the relationship of ΔG_{ay} and Q_{ay} (eq 5). The absolute values of ΔH_{ay} and ΔS_{ay} are each increased slightly compared to the values obtained with kinetic assays of W179F/F230W in the presence of MgATP. However, the signs of ΔH_{ay} and ΔS_{ay} are both negative regardless of technique used, showing that the coupling free energy is associated with a high degree of enthalpy−entropy compensation and that entropy establishes the positive coupling free energy (and hence the nature of the allosteric effect) even under true thermodynamic equilibrium conditions.

The dissociation constant for MgADP from W179F/F230W in the absence and saturating presence of Fru-6-P at various temperatures was determined in the absence of MgATP using fluorescence titrations, and the results are presented in Figure 6. The data indicate that at low temperature Fru-6-P has little or no effect on MgADP binding while at higher temperatures Fru-6-P substantially enhances MgADP binding. The trend suggests that at yet lower temperatures Fru-6-P will inhibit the binding of MgADP, consistent with previous observations made of the steady-state kinetic behavior of wild-type *B. stearothermophilus* PFK (*17*). Consequently, this crossover of the nature of the MgADP influence is independent of MgATP and instead is attributable to the high degree of entropy−enthalpy compensation as previously suspected (*6*, *17*).

DISCUSSION

In our initial search for potential residues to mutate to tryptophan, we had two basic criteria. (1) We looked only at phenylalanine residues that we considered to be a conservative mutation (*20*). (2) We looked for phenylalanine residues that were far removed from the active and allosteric sites so as not to disturb binding at these sites. W179F/F230W was the first mutant to fit these criteria. The double mutant has been successfully constructed, expressed, and purified.

This mutant shows kinetic properties very similar to wild-type *B. stearothermophilus* PFK with respect to activity and allosteric inhibition by PEP. This consistency is important when relating the fluorescence characteristics of the mutant directly to wild-type *B. stearothermophilus* PFK. According to the data presented here, the mutant is very similar to wild-type PFK with the following exceptions: the mutant is inhibited to a slightly greater extent by PEP, and the tryptophan fluorescence of the mutant is more responsive to conformational changes induced by ligand binding in the allosteric site. Consequently, we feel that the properties uniquely revealed by this latter property should pertain to native enzyme.

By comparing the steady-state kinetic behavior with the ligand binding data determined by monitoring the fluorescence changes under true equilibrium conditions, several conclusions can be made. The dissociation constant for PEP using both techniques is similar. Since kinetic assays were done in the saturating presence of MgATP and fluorescence assays were done the absence of MgATP, we can say that MgATP has little effect on the binding of PEP. It is obvious, however, that MgATP does inhibit the binding of Fru-6-P. This inhibition has been previously reported to result from interactions within each active site (*18*). The coupling constant quantitatively describing the nature and magnitude of the allosteric antagonism between Fru-6-P and PEP, Q_{ay}, is also the same using both experimental techniques. This result indicates that MgATP has no effect on the allosteric interaction between Fru-6-P and PEP. Similar results were obtained from *E. coli* PFK (*5*).

The ability to monitor binding of PEP directly has also revealed a pronounced positive cooperativity in the absence of other ligands. This cooperativity diminishes at very high concentrations of Fru-6-P. Such changes in cooperativity in response to saturation of the coupled ligand can contribute to the magnitude of the apparent heterotropic coupling if there is no compensating change in cooperativity in the binding of the coupled ligand, in this case Fru-6-P (*21*).

However, possibly the most noteworthy conclusion to draw from the ability to monitor ligand binding using the fluorescence changes of W179F/F230W involves an analysis of the van't Hoff behavior of the coupling between Fru-6-P and PEP. For the first time, we have demonstrated an example of an enzyme that has an apparent entropy dominated coupling free energy using a purely thermodynamic approach.[4] Previously we have observed such cases with steady-state kinetics (*6*, *22*, *23*) that required that the rapid-equilibrium assumption be invoked in order to justify the thermodynamic interpretation. With these results, no such ambiguity exists. The allosteric antagonism between the binding of Fru-6-P and PEP to PFK from *B. stearothermo-*

[4] As explicitly shown for an allosteric dimer (*21*), energetic coupling terms in an oligomer actually represent the arithmetic combination of multiple individual heterotropic and homotropic coupling terms, a fact that must be acknowledged when considering the possible mechanistic origins of these terms.

philus, in contrast to PFK from *E. coli*, derives from contributions of ΔH_{ay} and ΔS_{ay} that are both opposite in sign to the coupling free energy. Consequently, ΔS_{ay} is larger than ΔH_{ay} in absolute value and thereby establishes both the sign of the coupling free energy and the nature of the allosteric effect. This entropy domination pertains to both the PEP inhibition and the MgADP activation of Fru-6-P binding, the latter being sufficiently weak at 25 °C that an inversion in the nature of the allosteric effect by MgADP is suggested at low temperature as had been noted for the wild-type enzyme using steady-state kinetics (*6*, *17*).

The importance of entropy in ligand binding has recently been discussed in regard to inhibitor design for HIV-1 protease (*24*). In most drugs designed to inhibit HIV-1 protease, the binding of the inhibitor to the enzyme is tight due to overwhelming favorable entropic interactions that compensate for the unfavorable enthalpy of binding. However, by designing inhibitors that bind tightly due to less favorable entropy that is compensated by favorable enthalpy, more effective inhibitors might be produced because they would have more flexible structures that would be less affected by mutations in the binding site. In the case of *B. stearothermophilus* PFK, entropy dominates the allosteric influence associated with the coupling interactions. However, in *E. coli* PFK, enthalpy dominates (*4*, *5*). The thermodynamic differences in these two enzymes, which otherwise seem to be so similar structurally, might suggest a fundamentally different mechanism, perhaps involving the dynamics of the protein structure, is responsible for the allosteric response.

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