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Soon-jong Kim
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Fluorescence studies of phosphofructokinase from *Bacillus stearothermophilus*

Kim, Soon-Jong, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991
FLUORESCENCE STUDIES OF PHOSPHOFRUCTOKINASE FROM
BACILLUS STEAROTHERMOPHILUS

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by
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Abstract

Phosphofructokinase catalyzes the phosphorylation of β-fructose 6-phosphate (Fru-6-P) to β-fructose 1,6-bisphosphate. The first part of this dissertation shows that phosphofructokinase from Bacillus stearothermophilus (Bs-PFK) is a tetramer in the region pH 4.5 - 9.5 unlike the enzymes from mammalian sources, which exhibit association-dissociation reactions. Circular dichroism, steady-state fluorescence, and static and dynamic light scattering were used to study the conformational properties of the enzyme. Inactivation of the enzyme below pH 7.0 is not due to dissociation of the tetramer to dimers or monomers.

In the second part, the fluorescence of the lone tryptophan was investigated by steady-state and time-resolved techniques. The decay of Bs-PFK can be best described as a discrete double exponential with lifetimes of ~1.6 and 4.4 ns. The decay-associated emission spectra of the two components are identical. Similar results were obtained in D₂O, suggesting that the heterogeneous emission is not due to excited-state proton transfer. The activation energy for the temperature-dependent nonradiative decay rate was ~ 0.94 Kcal/mol. The emission had a quantum yield of 0.30 ± 0.03 with a
rate constant for acrylamide quenching, $0.62 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ which is 4-fold lower than the value expected for a diffusion controlled reaction. The fluorescence anisotropy is 0.18 suggesting that the tryptophan environment is fairly rigid. From the anisotropy decay measurements, a single exponential decay with rotational correlation time of $\sim 40 \text{ ns}$ with $r(0)$ value of $\sim 0.19$ was obtained.

Finally, the effects of allosteric ligand binding and site-specific mutation on the conformation of Bs-PFK were studied. Addition of adenosine triphosphate (ATP) or Fru-6-P did not cause any change in fluorescence. However, there is about 7% decrease of quantum yield and 2 nm red shift of emission maximum, upon adding the inhibitor PEP. Fru-6-P restores the fluorescence parameters to those of native Bs-PFK indicating the substrate induced allosteric transition from T- to R-conformation. The Arg-252 to Ala-252 mutation caused opposite effects: increase of quantum yield and 2 nm blue shift of emission maximum.
Chapter 1. Introduction

Background

Phosphofructokinase (PFK) (ATP: β-D-fructose 6-phospho-α-D-fructose-1-phosphotransferase, E.C. 2.7.11) is a key enzyme in the control of glycolysis. It catalyzes the transfer of the γ-phosphoryl group of ATP to the C-1 hydroxyl group of β-D-fructose 6-phosphate to produce β-D-fructose 1,6-bisphosphate and ADP as shown in Figure 1.1 (Bloxham & Lardy, 1973; Uyeda, 1979; Younathan et al., 1981). This reaction is an important control point in glycolysis and is extensively regulated by a score of metabolites.

For the PFKs from eukaryotic cells, the minimum fully active form is a tetramer which can further associate to form very large aggregates with apparent molecular weights of several million (Paetkau et al., 1968; Reinhart, G. D. et al., 1980). Also, it reversibly dissociates into subunits upon dilution or mild acidification with concomitant loss of enzyme activity (Goldhammer & Paradies, 1979; Bock & Frieden, 1974; Frieden et al., 1976; Hand & Somer, 1983, 1984; Hand & Carpenter, 1986; Luther et al., 1986) except the PFK from Artemia Embryos (Carpenter & Hand, 1986). For the prokaryotic PFKs, only limited pH-dependent studies on the aggregational property
Figure 1.1. The reaction catalyzed by phosphofructokinase.
of the enzyme have been carried out in this area. PFK from yeast has been characterized and shows dissociation at pH 6.0 below the concentration 1.5 mg/ml (Kopperschlager et al., 1972).

Past studies on the structure-function relationship and mechanism of action of PFKs have relied mostly on enzyme kinetic measurements. Recent determinations of X-ray diffraction structures of Bs-PFK (Evans & Hudson, 1979; Evans et al., 1981; Evans et al., 1986; Schirmer & Evans, 1990) and homologous E. coli PFK (Shirakihara, Y., & Evans, P.R., 1988) have contributed greatly to understanding the structural basis of the reaction mechanism and the allosteric transition of the enzyme. In the last few years, site-specific mutation (Lau et al., 1987; Lau & Fersht, 1987, 1989; Serre & Garel, 1990; Valdez et al., 1988, & 1989; Kundrot & Evans, 1991; Berger & Evans, 1990) has been used also to investigate the structure-function relationships of PFK.

**Classification of PFK**

PFK can be classified either by molecular weight or by allosteric properties. The smallest active form of PFKs from most sources is a tetramer of identical subunits. The PFK from yeast which is composed of two types of nonidentical subunits (Bloxham & Lardy, 1973; Hofmann, 1976) and the minor PFK from E. coli which is a dimer of
identical subunits (Kotlarz & Buc, 1981) are exceptions. Approximate subunit molecular weights of prokaryotic and eukaryotic PFKs are 32 to 38 kDa and 75 to 95 kDa, respectively.

PFKs can also be classified according to their allosteric properties. An allosteric enzyme has one or more regulatory sites besides the catalytic site. PFK from rabbit muscle shows Michaelis-Menten kinetics at pH 8.0 but exhibits sigmoidal kinetics with respect to substrate Fru-6-P at pH 7.2 (Uyeda, 1979). Prokaryotic PFKs show as diverse properties as their mammalian counterparts. For example, the major E. coli PFK, (Atkinson & Walton, 1965; Blangy et al., 1968), Lactobacillus acidophilus PFK (Simon & Hofer, 1977), Streptococcus lactis PFK (Fordable et al., 1982), and Thermus X-1 PFK (Cass & Steelwagen, 1975) follow sigmoidal dependence of enzyme activity on substrate Fru-6-P concentration. However, PFKs from Bacillus stearothermophilus (Valdez et al., 1988), Lactobacillus plantarum (Simon & Hofer, 1977), Streptococcus thermophilus (Simon & Hofer, 1981), Bacillus licheniformis (Marschke & Bernlohr, 1982), Flavobacterium thermophilus (Yoshida, 1972), and the minor PFK from E. coli (Kotlarz & Buc, 1981) show hyperbolic kinetic profiles with respect to Fru-6-P concentration.
Regulation of PFK Activity

The enzyme has been purified from a variety of sources and its activity is regulated by diverse mechanisms, namely by: metabolites, allosteric control, and association-dissociation equilibrium. Unlike the PFKs from higher organisms, the bacterial PFKs are controlled by relatively few modulators. Generally, bacterial PFKs are activated by ADP, and inhibited by PEP (Kolb et al., 1980). The increase in ADP concentration results in the concomitant increase of the glycolytic rate to generate more ATP. In eukaryotic PFKs, the regulation is more complicated. However, in general, eukaryotic PFKs are activated by AMP, cAMP, ADP, P_i, Fru-6-P, Fru-1,6-P_2, Fru-2,6-P, and inhibited by citrate or high levels of ATP (Uyde, 1979; Goldhammer & Paradies, 1979; Kemp & Foe, 1983). Ammonium ions are known to strongly activate both prokaryotic and eukaryotic PFKs (Abrahams & Younathan, 1971; French et al., 1987). Unlike the eukaryotic PFKs, the bacterial enzymes are not inhibited by ATP or citrate, nor activated by AMP.

The reaction mechanisms of PFKs are different depending on their sources. For prokaryotic PFKs, an ordered Bi-Bi sequential mechanism was reported for PFKs from non-allosteric Lactobacillus plantarum (Simon & Hofer, 1977) and the minor PFK from E. coli (Kotlarz & Buc, 1982) in
which Fru-6-P binds to the enzyme followed by release of ADP as the first product. The Bi-Bi sequential mechanism is also observed in the major allosteric PFK from *E. coli* where F-1,6-P₂ is the first product released (Kotlarz & Buc, 1981). In rabbit muscle PFK, the kinetic mechanism was studied by Bar-Tana and Cleland (1974) and found to be sequential random, i.e., the enzyme can bind either one of its two substrates first.

For allosteric enzymes, the regulation of enzyme activity was proposed to obey a two state model belonging to the K-system (Blangy et al., 1968). Based on the X-ray structures of R- and T- states of Bs-PFK (Schirmer & Evans, 1990), the rigid dimers of the enzyme rotate around their common dyad axis which results in the alteration of the binding sites located between dimer pairs for the substrate Fru-6-P. The transition between the two states is mediated by ligands in the effector site. During the R- to T- transition, the eighth helical loop moves in response to activator or inhibitor with a concomitant rearrangement of the sixth helical loop located at the active site of the enzyme. The T state has lower enzymatic activity or lower substrate binding ability, and the R state has higher activity or higher affinity for the substrate. The activity of the enzyme is controlled by switching between the two different conformational states.
Rotation of the dimers is accommodated by removal of the water layer that lies between them in the R state and the low affinity of Fru-6-P for the T state has been found to be due to closing of the binding site of the enzyme. The rigid monomer-monomer subunit interface where the lone tryptophan residue is located does not show any major movement based on the X-ray studies. Unlike the homologous E. coli PFK, which exists in the T state in the absence of Fru-6-P, Bs-PFK is known to exist in the R state in the absence and presence of substrate Fru-6-P, converting to the T state only in the presence of inhibitor, PEP (Valdez et al., 1989).

The allosteric enzyme purified from skeletal muscle appears to be regulated also by association-dissociation equilibrium. It undergoes inactivation at pH below 7.0 (Paeetkau et al., 1968; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Bock & Frieden, 1974; Frieden et al., 1976; Goldhammer & Paradies, 1979; Hand & Somer, 1983, 1984; Hand & Carpenter, 1986; Luther et al., 1986), which coincides with dissociation from the active tetramer to inactive dimer or monomer. The dissociation of PFK is believed to be mediated by the protonation of specific groups of the tetramer, followed by dissociation to the dimers. The association-dissociation process is proposed to be important physiologically in regulation of enzyme
activity (Goldhammer & Paradies, 1979). It is also proposed to be the control mechanism of PFKs from some hibernating animals whose body pH drops by about 0.3-0.4 pH unit in the winter causing an appreciable inhibition of glycolysis (Hand & Somer, 1983). Although the tetramer appears to be the smallest active species of the enzyme, higher aggregation forms may play some role in regulation (Luther et al., 1986). In the case of rabbit muscle PFK, the catalytic activity (Lad et al., 1973) and substrate binding (Hill & Hammes, 1975) are also affected by self-association of the enzyme to bigger aggregates.

**X-ray Structure of Bs-PFK**

X-ray structures of Bs-PFK for both active (R-state) (Evans & Hudson, 1979) and inactive (T-state) forms (Evans et al., 1986; Schirmer & Evans, 1990) are known. The larger subunit interface forms dimers and the dimers pack together with a smaller interface to form the tetramer. The active site is located at the smaller interface and the effector site is located at the larger interface. The tryptophan is located at the same interface as the effector site but not in direct contact with the site.

**Fluorescence Spectroscopy: A tool for structure-function studies of proteins.**
Fluorescence spectroscopy provides a unique method for investigation of basic properties, dynamics, and structure-function relationships of biological macromolecules (Lakowicz, 1983; Beechem & Brand, 1985). The sensitivity, selectivity and rapid time scale of fluorescence can provide real-time observation of very rapid internal motion and excited-state reaction. By fluorescence energy transfer measurements, distance between sites on the molecules of interest can be also determined. Due to the recognition of thermal fluctuation as having potentially important implications for protein structure and function (Karplus & McCammon, 1984), it is now believed to be important to understand the rapid motions of peptides and proteins.

The basic concept of fluorescence spectroscopy is well established (Lakowicz, 1983). Briefly, the fluorescence lifetime $\tau$ is the inverse of the decay rate $k$ from the lowest excited singlet state to the ground state after excitation. The observed lifetime depends on the response of the fluorophore to its environment during the lifetime of the excited state. Figure 1.2 is a Jablonski diagram which explains the radiative and nonradiative decay pathways. The decay rate of fluorescence emission from the excited state can be described as follows:
Figure 1.2. Modified Jablonski diagram $S_0$, $S_1$, and $S_n$ indicate singlet states; $T_1$ is the lowest triplet state. The first order rate constants $k_r$, $k_{ic}$, and $k_{isc}$ refer to radiative emission, internal conversion, and intersystem crossing, respectively.
\[ k = k_r + k_{isc} + k_{ic} = k_r + k_{nr} = 1/\tau \]

where \( k_r \) is the radiative rate, \( k_{isc} \) is the rate of intersystem crossing to the triplet manifold, \( k_{ic} \) is the rate of radiationless decay to the ground state, and \( k_{nr} \) is the total nonradiative decay rate of the \( S_1 \) state. The excited state lifetime varies from a few picoseconds to hundreds of nanoseconds depending on what happens during the lifetime. Relevant events that can occur in proteins on the fluorescence time scale include: 1) interaction among amino acids and interaction between amino acids and solvent molecules, and 2) rotations of the whole protein, segments of the polypeptide chain, and individual amino acid side chains (Beechem & Brand, 1985). Intrinsic and extrinsic probes can be used to measure structural perturbations caused by pH, ionic strength, pressure, and temperature and by ligand binding (Tran et al., 1982; Komiyama & Miwa, 1980; Maliwal & Lakowicz, 1984).

There are three intrinsic fluorophores in proteins: phenylalanine, tyrosine, and tryptophan (Figure 1.3). By exciting at wavelengths longer than 295 nm where almost no absorption from phenylalanine and tyrosine is expected, we can selectively excite tryptophan residues. In the experimental situations, due to energy transfer and lower extinction coefficient and quantum yield of phenylalanine and tyrosine compared to tryptophan, almost 90% of the
Figure 1.3. Extinction coefficients and structures of intrinsic fluorophores in proteins.
fluorescence from proteins is due to tryptophan.

Tryptophan in proteins can be used as a sensitive probe to study the conformational dynamics of the microenvironment around individual residues (Steiner, 1983; Lakowicz, 1983; Beechem & Brand, 1985). Despite the wide use of tryptophan as a probe for structure and dynamics of proteins and polypeptides due to its sensitivity to the local environment (Longworth, 1971; Beechem & Brand, 1985), the photophysical properties of tryptophan, and its behavior toward environmental perturbations are not clear. To understand the complexity, the photophysics of indole and tryptophan has been studied extensively (Creed, 1984). A complex fluorescence decay is also observed for single tryptophans in polypeptides and proteins presumably due to: 1) different ground-state conformations of the indole side chain, or 2) excited-state reactions of the tryptophan (Beechem & Brand, 1985). A mixture of fluorophores will give rise to multiexponential decay, with the number of decay constants equal to the number of components in the ground state. Chemical forms, aggregation species, or solvent can cause a heterogeneous ground state environment. However, it is also possible that a single tryptophan residue in a protein which exists in two conformations can show biexponential decay kinetics.
Multi- or non-exponential decay behavior may also arise from a pure homogeneous fluorophore undergoing an excited-state reaction. These excited-state reactions include solvent or protein reorientation around the excited fluorophore and the formation of exciplexes. Nevertheless, the existence of a single tryptophan in a protein is attractive due to the simpler assignment of steady-state fluorescence signal compared to the other proteins containing multiple tryptophans.

**Objective of Studies**

The purpose of this research project is to study the properties of the lone tryptophan in *Bacillus stearothermophilus* PFK (Bs-PFK) by fluorescence measurements. The gene for this enzyme was previously cloned, sequenced, and expressed in *E. coli* by Professor Chang's lab in the Department of Biochemistry, Louisiana State University. The high-level of recombinant gene expression enables interested investigators to purify adequate amounts of Bs-PFK for biophysical studies to: (1) characterize the photophysical properties of the lone Trp-179 residue, and (2) determine the effects of mutation and ligand binding on the tryptophan fluorescence.

The possible existence of multiple aggregation states: tetramer, dimer, or monomer could lead to complex decay of
tryptophan fluorescence in Bs-PFK due to ground-state heterogeneity. Therefore, this dissertation begins with the effects of pH on Bs-PFK activity and conformation. CD, steady-state fluorescence, and static and dynamic light scattering measurements were used to monitor the protein conformation and its states of aggregation.

The second part of this dissertation involves fluorescence studies of the lone tryptophan residue in Bs-PFK. Steady-state and time-resolved techniques were used to measure fluorescence quantum yield and lifetime, solute quenching, and emission anisotropy as functions of pH and temperature.

Finally, the effects of allosteric ligands and site-specific mutation on the conformation of Bs-PFK were studied. The two different conformational states: R and T were compared using steady-state fluorescence spectroscopy.
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Chapter 2. Effects of pH on Activity and Conformation of *Bacillus stearothermophilus* Phosphofructokinase.

**Summary.** The dependence of enzyme activity and aggregation state of PFK from *Bacillus stearothermophilus* has been studied as a function of pH at 25°C. Unlike the mammalian PFK, the bacterial enzyme shows no indication of either reversible association-dissociation or multiple aggregation states. The enzyme activity had a broad optimum at pH 7.0-9.0, but dropped to half-maximal activity at pH 6.5. Protein conformational changes were monitored by fluorescence and CD spectroscopy. Molecular weight and shape were determined by static and dynamic laser light scattering. No changes in molecular weight or hydrodynamic radius were detected over the pH range 5.0 to 9.0. These results show that the loss of enzyme activity below pH 7.0 which follows similar activity profile as that of mammalian PFK is not due to dissociation of an active tetramer to inactive dimer or monomer. The finding can be attributed to three possibilities: 1) changes in the state of ionization of Asp-127 residue shown by X-ray studies to be located at the active site of this enzyme; 2) pH-dependent conformational changes detected by CD, and 3) substrate titration.
The X-ray structures of Bs-PFK and *E. coli* PFK (Evans & Hudson, 1979; Evans et al., 1981, 1986; Shirakihara & Evans; 1988; Schirmer & Evans, 1990) are known. The bacterial enzyme has 4 identical subunits of 34 kDa with a single tryptophan per subunit. Each subunit consists of two domains; the larger one binds the substrate ATP, and the smaller one binds Fru-6-P (Evans & Hudson, 1979). There are two interfaces per subunit; the large monomer interface forms the dimer and the small interface forms the tetramer. The effects of certain single site mutations on the kinetics and allosteric characteristics of Bs-PFK and *E. coli* PFK have been also studied (Lau et al., 1987; Lau & Fersht, 1987, 1989; Serre et al., 1990; Valdez et al., 1988 & 1989; Berger & Evans, 1990; Kundrot & Evans, 1991).

The enzyme purified from skeletal muscle undergoes inactivation at pH values below 7.0 (Goldhammer & Paradies, 1979; Bock & Frieden, 1974; Frieden et al., 1976; Hand & Somer, 1983, 1984; Hand & Carpenter, 1986; Hofer & Krystek, 1975; Luther et al., 1986). The inactivation is accompanied by dissociation of the active tetramer enzyme to inactive dimer or monomer (Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Hand & Somer, 1983). Unlike the mammalian PFK which shows complicated association-dissociation reactions or
multiple aggregation states depending on concentration or pH, most prokaryotic PFK's exist as a tetramer in solution around physiological pH (Uyeda & Kurooka, 1970; Glodhammer & Paradies, 1979). The pH dependence of enzyme activity of *Flavobacterium thermophilum* (Yoshida, 1972), *Bacillus licheniformis* (Marschke & Bernlohr, 1973), *Clostridium pasteurianum* (Uyeda & Kurooka, 1975), *Lactobacillus acidophilus* (Simon & Hofer, 1977), and *Streptococcus thermophilus* (Simon & Hofer, 1981) and structural studies of yeast PFK, which dissociates at pH 6.0 with concentration below 1.5 mg/ml (Kopperschlager et al., 1972), have been reported. Low temperature induced dissociation of *Bacillus licheniformis* PFK (Marschke & Bernlohr, 1973) and small-angle X-ray scattering study of homologous *E. coli* PFK (Paradies et al., 1977; Goldhammer & Paradies, 1979) showing a hydrodynamic radius of ~ 44 Å has been also reported. However, no studies have been made to correlate these pH-induced changes in activity with changes in protein structure. In this chapter, enzymatic activity, fluorescence, CD, and static and dynamic light scattering of the enzyme are measured in the range pH 3.0-10.0 in order to gain more information about the structure-activity relationship of Bs-PFK. The pH dependence of the structure and the implications for activity of this bacterial enzyme are discussed.
Material and Methods

Gel Electrophoresis. The homogeneity and isoelectric point of the enzyme were determined by using a PhastGel System (Pharmacia). PhastGel 10-15% gradient sodium dodecyl sulfate gel was used for the purity check and PhastGel IEF3-9 gel was used for the isoelectric focusing. The gel was stained with Coomassie Brilliant Blue. Gel electrophoresis exhibited a single band with no observable contamination.

Absorbance. Absorption spectra were recorded on an Aviv Model 118DS UV-VIS spectrophotometer. The extinction coefficient of Bs-PFK was determined to be $\epsilon_{278} = 16690 \text{ M}^{-1} \text{ cm}^{-1}$ based on the monomer $M_r = 33,900$ (Evans & Hudson, 1979). Protein concentration determination for the extinction coefficient was made by a BioRad protein assay kit. The absorbance shift of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm upon binding with protein was followed to determine the amount of protein.

Fluorescence. Emission spectra and intensity measurements were made by using an SLM Model 8000 photon counting spectrofluorometer with single excitation (4 nm band-pass) and emission (8 nm band-pass) monochromators. Bs-PFK samples were excited at 296 nm to avoid excitation of tyrosine. Anisotropic effects were eliminated by using magic angle polarizers which were set to 55° on the
excitation side and 0° on the emission side to avoid the Wood's anomaly of the emission grating. Sample absorbance was < 0.1 at 296 nm with a 0.4-cm path length. Fluorescence emission was measured in the ratio mode and background fluorescence from a solvent blank was subtracted. Emission spectra were corrected for the wavelength dependence of the instrument response using correction factors determined with a standard lamp from Optronics, Inc. The temperature was controlled at 25 ± 0.05 °C by a Lauda water bath. The center of gravity $v_{cg,i}$ (in nm$^{-1}$) of the emission spectrum was calculated from (Lakowicz & Hogan, 1981)

$$v_{cg,i} = \frac{\sum_{j} I_i(\lambda_j)\lambda_j^{-3}}{\sum_{j} I_i(\lambda_j)\lambda_j^{-2}} \quad \text{Eqn. 2.1}$$

where the wavelength $\lambda_j$ goes from 320 to 360 nm in 1-nm intervals.

Fluorescence quantum yields were measured at 296 nm excitation wavelength by comparison to tryptophan (Sigma, recrystallized four times from 70% ethanol) in glass-distilled water. Sample quantum yields were calculated using a value of 0.14 for tryptophan (Chen, 1967).

Fluorescence emission anisotropy was measured at 340 nm emission wavelength. Each data point was acquired for five 10-s time intervals and the values were averaged. Steady-state anisotropy $\langle r \rangle$ was calculated by using the equation:
\[ \langle r \rangle = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + 2G I_{VH})} \quad \text{Eqn. 2.2} \]

where the first and second subscripts refer to the orientation of the excitation and emission polarizers, respectively (V = 0°; H = 90°), and G = \( I_{HV}/I_{HH} \) is an instrumental correction factor.

**Enzyme Assays.** Bs-PFK activity was determined from the rate of Fru-1,6-P₂ production at 25 °C as monitored by NADH oxidation. Absorbance of NADH was monitored at 340 nm in a Gilford 240 recording spectrophotometer. The linear phase of the reaction was used to calculate initial rates. Single point for the sequential assay and time points for the coupled method were used. For the sequential assay, a single point at 2 min after the reaction start was selected. The selection of the time point was based on separate experiments showing linear enzyme kinetics within 2 min for all pH studied.

**Coupled Assay.** A coupled assay (Koltlarz & Buc, 1982) was used in the pH range 6.0 to 10.0 where the auxiliary enzymes (aldolase, triose-phosphate isomerase, and glycerol 3-phosphate dehydrogenase) are 40-100% active. The standard assay mixture contained 50 mM buffer at the desired pH, 10 mM MgCl₂, 0.2 mM NADH, 1 mM Fru-6-P, 1 mM ATP, 0.02 µg Bs-PFK, 112 µg aldolase, 18 µg triose phosphate isomerase, and 2.4 µg glycerol 3-phosphate dehydrogenase in a final volume of 1 ml.
Buffers used were: acetate, pH 3.0-6.0; Bis-Tris, pH 6.5-7.0; Tris, pH 7.5-9.0; and CHES, pH 9.5-10.0. The auxiliary enzymes were mixed and were dialyzed for 24 hr against three changes of 50 mM Tris buffer, pH 8.0 to remove the ammonium sulfate prior to use. The pH profile of the auxiliary enzyme activity was determined separately in an assay mixture containing 50 mM buffer, 0.2 mM NADH, 0.1 mM Fru-1,6-P2, 112 μg aldolase, 18 μg triose phosphate isomerase, and 2.4 μg glycerol 3-phosphate dehydrogenase in a final volume of 1 ml.

Sequential Assay. The sequential assay method (Yoshida, 1972) with minor modification was used in two separate steps to avoid the possibility that auxiliary enzymes become rate limiting in the pH region 3.0 to 6.0 where their activity was diminished more than 40%. The assay mixture in the first step contained 50 mM buffer at the desired pH, 10 mM MgCl2, 1 mM Fru-6-P, 1 mM ATP, and 0.02 μg of PFK in a final volume of 1 ml. The reaction was initiated by adding the ATP. The reaction was terminated by filtering about 1 ml of the mixture through a Millipore 0.1 μm nitrocellulose filter to remove the PFK. The pH of the filtrate was adjusted to 8.2. The amount of Fru-1,6-P2 formed in the first step was determined by adding 0.2 mM NADH and the auxiliary enzymes: 112 μg aldolase, 18 μg triose phosphate
isomerase, and 2.4 µg glycerol 3-phosphate dehydrogenase and continuing the reaction to completion. The final volume of assay mixture in the second step was 1.0 ~ 1.1 ml. The amount of Fru-1,6-P₂ formed in the first step was determined from the amount of NADH oxidized in the second step, taking into consideration that two mols of NAD oxidized correspond to one mol of Fru-1,6-P₂.

Light Scattering Measurements. A Lexel Model 95 argon ion laser with maximum output of approximately 1 W at 514.5 nm was used as a light source. The apparatus has undergone only minor modifications since last described (Russo et al., 1986). The temperature was controlled at 25 ± 0.05 °C by a Lauda water bath.

Scattering cells were 12-mm outer diameter silanated cells. Each cell was individually tested for cleanliness by filling it with clean water gravity fed (approx 1 m pressure head) through a Gelman 0.2 µm cartridge filter, resulting in a flow rate of about 400 ml/min, which is sufficiently slow to prevent filter shedding. The water appeared completely free of dust: none could be seen when a focused laser beam was observed traversing the water at 100x magnification. The cells were checked for cleanliness by observing the laser beam traversing the cell at about 100x 30° scattering angle.
The rinsing process was repeated until less than one
"dust-event" per 30 s occurred.

About 0.2 mg/ml sample for DLS and 0.03 mg/ml - 0.2
mg/ml samples for SLS were prepared in buffer solutions
and transferred into the clean cells via 0.2 μm Sargent-
Welch "Anotop" prefabricated inorganic membrane filters,
which exhibited very low protein binding as confirmed by
UV and fluorescence measurements. Some variability in
the performance of these filters was evident, so the
ability of each to deliver clean filtrate was judged by
the laser observation test before use. Cells were
capped with Teflon tape and light scattering measurements
were carried out immediately. Occasionally, it was
necessary to centrifuge the samples, directly in the
measurement cells. Typical centrifugation conditions
were at 9000 xg for 1 hr.

SLS data were obtained at 45° scattering angle. The
data were converted to Rayleigh factor using toluene as a
reference standard. The Rayleigh factor for toluene at
90° scattering is 1.402 x 10⁻⁵ cm⁻¹ at 632.8 nm (Leite et
al., 1965). The value at 514.8 nm was calculated from
R_{toluene,514.5} = R_{toluene,632.8} (632.8/514.5)⁴. The
relationship below shows the equation represented by the
Zimm plot:

\[
\frac{Kc}{R_\theta} = \frac{M_w}{(1 + (16\pi^2 R_g^2/3\lambda^2)\sin^2\theta/2) + 2A_2c} \quad \text{Eqn 2.3}
\]
Assuming a point scatterer and no angular dependence,

\[ \frac{Kc}{R_\theta} = M_w^{-1} + 2A_2c \quad \text{Eqn. 2.4} \]

where \( K \) is an optical constant containing the specific refractive index increment, \( \frac{dn}{dc} \), \( c \) is the concentration (mg/ml), and \( R_\theta \) is the Rayleigh ratio (Utiyama, 1972). By plotting \( \frac{Kc}{R_\theta} \) vs \( c \), and extrapolating to \( c = 0 \), \( M_w^{-1} \) is obtained from the intercept of the line.

The DLS were measured at 60° using the same experimental conditions as in the SLS measurements. Each autocorrelation function was obtained as the sum of several "short" runs, sometimes collected under manual supervision while observing a rate meter to guard against dust artifacts.

Selected short runs were summed after testing each for consistency of intensity, average decay rate, degree of nonexponentiality, optical coherence, and agreement of computed (Russo et al., 1986) and measured baselines. Measurements of average intensity were accomplished by repeated photon counting trials. Each measurement was accepted only after reproducibility was established. High intensity trials could be eliminated from consideration, as a dust-discriminating tool. The data were fit using CORAN (Koppel, 1972), MARLIN (developed by Professor Russo's group at LSU), and CONTIN (Provencher, 1979). The standard deviation in the hydrodynamic
radius was obtained from the deviation calculated from
CORAN and MARLIN. The hydrodynamic radius, $R_h$, was
calculated by using the Stokes-Einstein relation:

$$D = \frac{kT}{6\pi \eta_0 R_h} \quad \text{Eqn. 2.5}$$

where $D$ is the translational diffusion coefficient, $k$ is
the Boltzmann constant, $T$ is the absolute temperature,
and $\eta_0$ is the viscosity of the solvent assuming
$\eta_{\text{buffer}} = \eta_{\text{water}}$.

CD spectra were recorded with an Aviv CDS M62DS
spectropolarimeter at 25 °C. Spectra were measured on
about 0.2 mg/ml sample solutions by scanning from 260 to
180 nm at 25°C using a 1.0-cm path length cell.
Solvent blank spectra were subtracted from the sample
spectra. Mean molar residue ellipticity, $[\theta]_M$, was
calculated from the observed ellipticity, $\theta$ (deg):

$$[\theta]_M = 100 \frac{\theta}{c \lambda} \quad \text{Eqn. 2.6}$$

where, $c$ is concentration (ML$^{-1}$), and $\lambda$ is the path
length (cm).

Results

Sample Purity. Bs-PFK samples eluted from an ATP-
agrose column were analyzed on SDS-polyacrylamide gel to
assess purity. A typical gel shown in Figure 2.1
indicates no impurity in the sample. Absorption and
Figure 2.1. Coomassie-blue-stained SDS/polyacrylamide gel electrophoresis of Bs-PFK eluted from ATP-agrose column. Lanes 1,2: Bs-PFK; lane 3, molecular weight markers: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa, α-lactalbumin, 14 kDa.
fluorescence spectra of Bs-PFK. Absorption and corrected steady-state emission spectra of Bs-PFK at pH 8.0 are presented in Figures 2.2 and 2.3, respectively. The absorption maximum is located at 278 nm and the emission maximum is at 328 nm with a center of gravity at 330 nm. The emission maximum of Bs-PFK is blue shifted compared to indole compounds in water which generally display emission maxima around 350 nm (Longworth, 1971). The emission maximum and anisotropy of 0.175 ± 0.03 suggest that the residue is in a rigid hydrophobic environment. The lone tryptophan has a high fluorescence quantum yield of 0.30 ± 0.003 at 25 °C.

The pH profile of enzyme activity measured at saturating concentration of substrate is presented in Figure 2.4. The activity profile is similar to that of the major PFK from E. coli (Evans, private communication), other bacterial PFKs (Uyeda & Kurooka, 1975; Yoshida, M., 1972; Simon & Hofer, 1977), and the mammalian PFK (Bock & Frieden, 1974; Frieden et al., 1976; Kitajima et al., 1983). To separate the effects of pH on the auxiliary enzyme activity, the measurement was carried out using both coupled and sequential methods described in the Material and Method section. The results presented in Figure 2.1 show that the two methods are in good agreement. The activity is maximal at pH
Figure 2.2  Absorption spectrum of Bs-PFK in 50 mM Tris, pH 8.0 at 25 °C. Sample concentration was ~ 1.1 x 10^{-5} M.
Figure 2.3. Fluorescence emission spectrum of Bs-PFK in 50 mM Tris, pH 8.0 at 25 °C. Sample concentration was ~ 6.0 x 10^{-6}M. Excitation at 296 nm.
Figure 2.4. pH Profile of Bs-PFK activity at 25°C. Enzyme activity was determined in (•) coupled or (□) sequential assay as described in Material and Method. PFK concentration was 0.02 µg/ml.
7.0 - 9.0 and drops sharply between pH 6.0 - 7.0. The pH profile of the curve below the maximum agrees with the titration curve of an ionizable group with a pK$_a$ = 6.5 ± 0.1.

In order to determine the quaternary state of the enzyme, the molecular weight was measured by static light scattering at pH 6.0 and 8.0. As the hydrodynamic radii were quite small compared to the laser wavelength, at least in the absence of aggregation, it was impossible to measure static radii of gyration. On the positive side, molecular weights could be determined reliably by scattering measurements at just one scattering angle. Figure 2.5 shows intensity data plotted as suggested by Eqn. 2.4, which governs the scattering of vertically polarized incident light from a small, optically isotropic particle, where dn/dc was assumed to be 0.195 ml/g (Utiyama, 1972), a reasonable value for proteins. From the extrapolation, we can get the molecular weight from the inverse of the intercept. That the data do not fall onto a perfect line reflects the very low concentrations and small excess scattering. However, there is no difficulty in extracting the intercepts to within about ±15%, yielding molecular weights of 116 kDa and 122 kDa for pH 8.0 and pH 6.0, respectively. These values are within error of the molecular weight of 136 kDa considering the difficulties of measurements with
Figure 2.5. Static light scattering as a function of concentration for Bs-PFK at (o) pH 6.0 and (□) pH 8.0. \( \lambda_o = 514.5 \text{ nm} \), scattering angle at 45°. \( M_w \) determined from the reciprocal of the intercept at zero concentration from linear regression.
very low concentration of samples which were prepared in the concentration range used for fluorescence and CD measurements.

Dynamic light scattering of Bs-PFK was measured from pH 3.5 to 9.5. At the low concentrations required for accurate comparison with the fluorescence results, the scattering signal was relatively weak—in some cases, only a few percent above that of buffer alone. Therefore, apparent optical coherence (Ford, 1983) was reduced compared to strongly scattering solutions. Still, acceptably quite correlation function, Figure 2.6, could be acquired within a reasonable time, and it was not difficult to determine the average decay rate. The z-average (Pecora, 1983) diffusion coefficient, \( D_z \), and hydrodynamic radius \( R_h \) (Table 2.1), can be obtained. The hydrodynamic radius was constant at about 40 Å between pH 6.5 and 8.5, in good agreement with the 44 Å hydrodynamic radius determined for the homologous E. coli enzyme by X-ray scattering (Goldhammer & Paradies, 1979) or 38 Å estimated from the crystal structure. At higher and lower pH, the Stokes' radius increased dramatically, presumably due to aggregation (Table 2.1). The enzyme has more tendency to aggregate in alkaline than in acidic condition. The hydrodynamic radius of the sample at pH 9.5 is \( \sim 9600 \pm 800 \) Å which is about 80 times bigger than that of the sample measured at pH 4.5.
Figure 2.6. Correlation function for Bs-PFK sample at pH 8.5. Measured at $\theta = 60^\circ$, 0.2 mg/ml. (upper left) $f(A)$ represents the amount of usable signal above the baseline scattering. The closer $f(A)$ is to 1, the larger the usable signal. (upper right) Raw correlation function $G^{(2)}(t)$; (lower left) semilog representation, showing lack of curvature indicating that the sample is monodisperse; (lower right) error analyses by cumulants. The distance from the center of bar to line of zero error represents the error of fit. The abscissa is the same time axis as the upper right and lower left panels.
Table 2.1. Static* and Dynamic Light Scattering

<table>
<thead>
<tr>
<th>pH</th>
<th>$M_w$(kDa)*</th>
<th>$R_h$(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>---</td>
<td>153 ± 23</td>
</tr>
<tr>
<td>4.5</td>
<td>---</td>
<td>115 ± 15</td>
</tr>
<tr>
<td>5.5</td>
<td>---</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>6.0*</td>
<td>122</td>
<td>---</td>
</tr>
<tr>
<td>6.5</td>
<td>---</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>7.5</td>
<td>---</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>8.0*</td>
<td>116</td>
<td>---</td>
</tr>
<tr>
<td>8.5</td>
<td>---</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>9.5</td>
<td>---</td>
<td>9600 ± 800</td>
</tr>
</tbody>
</table>
The isoelectric point of the enzyme was measured to check the possibility that the slight decrease and erratic behavior of the hydrodynamic radius at pH 6.5 may come from the loss of charge at the isoelectric point. The pI value of \(~5.5\) was determined on an isoelectrofocusing gel (Figure 2.7), which indicates that the decrease of the radius may not be coming from the loss of charge.

The lone Trp-179 residue of Bs-PFK is located at the monomer subunit interface (Evans et al., 1979). The intrinsic tryptophan fluorescence is a sensitive indicator of dissociation of dimer to monomer (Garel, 1990). The fluorescence quantum yield (Figure 2.8) was constant at \(0.30 \pm 0.03\) between pH 4.5 and 9.0. This result is consistent with the light scattering data showing no subunit dissociation in this pH range. Fluorescence quenching by acrylamide also showed no difference in the Stern-Volmer constant, \(K_{SV}\), at pHs 6.0 (\(K_{SV} = 2.1\)) and 8.0 (\(K_{SV} = 2.2\)) indicating no detectable change in solute accessibility of Trp-179. However, in the pH range below pH 4.5 and above pH 9.5, a marked decrease of fluorescence quantum yield (Figure 2.8) is observed. For example, the quantum yield drops sharply from about 0.30 above pH 4.5 to 0.15 at pH 4.0.

Steady-state anisotropy (Figure 2.9) was also measured over the same pH range. The fluorescence
Figure 2.7. Isoelectric focusing gel. Lanes 1, 2; Bs-PFK, Lane 3; pH standards.
Figure 2.8. pH Dependence of fluorescence quantum yield. $6.0 \times 10^{-6}$ M Bs-PFK at 25 °C.
Figure 2.9. pH Dependence of steady-state anisotropy. Emission at 340 nm, 6.0 x 10^{-6} M Bs-PFK at 25 °C.
anisotropy was about 0.175 ± 0.003 between pH 5.0-9.5, indicating a rigid tryptophan environment (Lakowicz, 1983). It dropped slightly to about 0.16 at pH 4.5. As the quantum yield also dropped around this pH, the decrease in anisotropy is probably due to increased mobility of the tryptophan below pH 5.0. In Figure 2.10, the pH dependence of the emission maximum of Bs-PFK is presented. Below pH 5.5 and above pH 9.0 there is some red shift of the emission maximum from 327 nm up to 333 nm. In order to compare these results with the enzyme in the completely dissociated and unfolded state, the effect of guanidinium chloride on fluorescence anisotropy and emission maximum was measured. In Figure 2.11, only a single transition is evident by either measurement and the effective transition concentrations of the two curves are near 4.0 M guanidinium chloride. The transition point shows a sharp contrast compared to homologous E. coli PFK which has an effective transition concentration of ~ 0.6M guanidinium chloride (Teschner & Garel, 1989). Because the quantum yield dropped from 0.3 to below 0.15 at higher guanidinium chloride concentrations, the anisotropy drop to 0.14 most likely indicates increased flexibility of the tryptophan in the unfolded protein. The 350 nm limiting value of the emission maximum in 5.5 M guanidinium chloride is similar to the value expected
Figure 2.10. pH Dependence of fluorescence maximum.  
6.0 x 10^{-6} M Bs-PFK at 25 °C.
Figure 2.11. The effect of guanidinium chloride on anisotropy (●) and fluorescence maximum (Δ). Emission monitored at 340 nm for anisotropy measurements, 6.0 x 10⁻⁶ M Bs-PFK in 50 mM Tris buffer, pH 8.0 at 25 °C.
for a fully solvent exposed tryptophan (Lakowicz, 1983). Because of the smaller red shift and anisotropy drop at low pH compared to the corresponding values for the denatured protein, it appears that the tryptophan at lower pH has a more structured environment.

To follow possible secondary structural changes, circular dichroism spectra of Bs-PFK were measured. Figure 2.12 shows the CD spectrum of Bs-PFK with two negative bands located around 222 nm and 208 nm. The negative ellipticity at 222 nm is greatest at pH 7.5-8.5, dropping off gradually at lower and higher pH (Figure 2.13). The molar ellipticity appears to have dropped 10% at pH 6.5 and 50% at pH 4.0. The decreased CD at 222 nm indicates gradual loss of secondary structure.

**Discussion**

We can reach the following conclusions: 1) The loss of Bs-PFK activity below neutral pH does not appear to be due to changes in the protein quaternary structure as judged by static and dynamic light scattering. 2) The fluorescence quantum yield and emission anisotropy of the single tryptophan located at the monomer subunit interface do not change in the pH region of the activity loss (pH 6.0-7.0). This is consistent with no changes
Figure 2.12. Circular dichroism spectrum of Bs-PFK in 10 mM phosphate buffer, pH 8.0 at 25 °C. Sample concentration was ~ $3.0 \times 10^{-6}$ M.
Figure 2.13. pH Dependence of the molar ellipticity of Bs-PFK at 222 nm.
in quaternary structure. 3) The CD decreases about 50% in the pH region 4.0-7.5. This is consistent with changes in the secondary structure of the protein. 4) Comparing the fluorescence parameters induced by pH change or by presence of guanidinium chloride, it is likely that at lower pH the tryptophan environment retains part of its native structure. Pertinently, the observation that the effective transition concentration of guanidinium chloride is about 0.6 M for *E. coli* PFK and 4.0 M for *Bs-PFK* reflects the stronger forces that maintain the secondary structure of the latter enzyme.

There are three possible explanations for the pH dependent loss in bacterial PFK activity at acidic pH:

i) Changes in protein secondary structure,

ii) Protonation of Asp-127, proposed (Hellinga & Evans, 1987) to be a key residue in catalysis.

iii) Protonation of the substrate Fru-6-P below its pK$_a$ value (pK$_a$ =6.1).

Based on the CD spectra (Figure 2.13), there is a gradual loss of the protein secondary structure with decreasing pH. Therefore, it is possible that the changes in secondary structure may play a role in the observed decrease in activity. From the site-specific mutation studies of *Bs-PFK* (Hellinga & Evans, 1987), it has been shown that changing Asp-127 to Ser-127 reduced
the turnover number by a factor of 18,000. The authors postulated that this residue can probably act as a base in the reaction mechanism. This residue must be deprotonated in order to carry out the acid-base catalysis involved in the phosphoryl group transfer. Therefore, the protonation of negatively charged oxygen of Asp-127 at low pH will hinder the deprotonation of the C-1 hydroxyl of the Fru-6-P (Figure 2.14). This will result in blocking the phosphoryl transfer reaction. The pKᵢ value of aspartic acid is ~ 4.5. However, there are many cases showing abnormal pKᵢ values due to perturbation of the electrostatic environment caused by nearby amino acid residues (Matthew et al., 1979; Ohe & Kajita, 1980; Chau et al., 1990). Other candidates for acid-base catalysis at the active site are Asp-129, Glu-222, His-249, Arg-252, and Arg-243. However, judging from the X-ray diffraction studies, none of these residues is as close to the C-1 hydroxyl of Fru-6-P as Asp-127. The pKᵢ value of the phosphate group of Fru-6-P is ~ 6.1. Therefore, it is also plausible that protonation of this group on Fru-6-P causes a decrease of the affinity of the enzyme for Fru-6-P due to the alteration of the charged interaction between the substrate and positively charged residues: Agr-252, Arg-243 and Arg-162 (Figure 2.14).
Figure 2.14. Mechanism of action of phosphofructokinase from *B. stearothermophilus* (Adapted from Hellinga & Evans, 1987).
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Chapter 3. Time-Resolved Fluorescence of Phosphofructokinase.

Summary. We have characterized the fluorescence properties of the lone tryptophan by steady-state and time-resolved techniques. The fluorescence emission had a maximum at 328 nm with a bandwidth of ~45 nm at $\Delta \lambda_{1/2}$ and a quantum yield of 0.30 ± 0.03. Fluorescence decay curves acquired at 296 nm excitation and various emission wavelengths were deconvolved in global analysis assuming a sum of exponentials and a continuous lifetime distribution of Gaussian shape. The reduced chi-square ($X_r^2$) values for a discrete double exponential are slightly better than those of bimodal Gaussian fits. Moreover, the discrete double exponential fits were favored due to very narrow widths of the bimodal distributions (about 200 ps). The fluorescence decay of Bs-PFK was found to be a biexponential with 1.6 and 4.4 ns lifetimes having amplitudes of 40% and 60%, respectively, and almost invariant with emission wavelength. The decay-associated emission spectra of the two components are identical. Similar results were obtained in D$_2$O, suggesting that the heterogeneous emission is not due to excited-state proton transfer reactions involving solvent accessible protons. The Stern-Volmer rate constants determined from steady-
state fluorescence measurements for acrylamide and KI quenching, 5.4 x 10^8 M^-1s^-1 and 5.0 x 10^7 M^-1s^-1 respectively, indicate almost no accessibility of the fluorophore to iodide. Rate constants of 9.0 x 10^8 M^-1s^-1 and 1.8 x 10^8 M^-1s^-1 for shorter and longer lifetime components, respectively were obtained from the lifetime quenching of tryptophan fluorescence by acrylamide suggesting a 5-fold difference in accessibility of the two components to the solute quencher. The steady-state anisotropy at 296 nm excitation wavelength was 0.175 ± 0.003. From anisotropy decay measurements, a single exponential decay with rotational correlational time of roughly 40 ns with r(0) value of ~0.19 were obtained. From the temperature dependent measurements, activation energies of ~ 1.5 kcal/mol from quantum yield and ~1.2 kcal/mol from lifetime were obtained, which are in reasonable agreement.
Tryptophan in water has a double exponential decay at neutral pH. The major component has a lifetime of 3.1 ns with emission maximum at 350 nm, and the minor component has a lifetime of 0.5 ns with emission maximum at 335 nm (Szabo & Rayner, 1980). There are various models to interpret the existence of the dual lifetimes. Dual emission from overlapping excited states of indole ring (Rayner & Szabo, 1978) faded out due to the observation of a monoexponential decay of indole, 3-methylindole, and N-acetyl-C-tryptophanamide (NATA). Tryptophan in proteins is used as a sensitive probe to study the conformational dynamics of the microenvironment around the residues. However, it is well known that single tryptophan containing proteins show complex behavior of fluorescence decay due to: different conformers or excited state-reactions (Grinvald & Steinberg, 1976; Beechem & Brand, 1985). Nonexponential decays, which often means not single exponential, have been reported for a number of single tryptophan containing proteins (Ross et al., 1981; Szabo et al., 1983; Ludescher et al., 1985; Lakowicz et al., 1986; Chen et al., 1987; Eftink & Ghiron, 1987; Eftink et al., 1991; Petrich et al., 1987). Nevertheless, the existence of single tryptophans in proteins is attractive due to the simpler assignment of steady-state fluorescence signal than in proteins containing multiple tryptophans. In the past
ten years, the dynamic aspects of protein structure have been extensively studied (Careri et al., 1979; Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; McCammon & Karplus, 1983; Frauenfelder & Debrunner, 1983). It is now generally accepted that a protein can fluctuate among a number of possible substates (Austin et al., 1975; Frauenfelder, 1985; Frauenfelder & Gratton, 1985; Ansari et al., 1987). With these facts in mind, it is relevant to consider the distribution of protein conformations in the analysis of protein dynamics. Traditionally, in the analysis of fluorescence lifetime data, discrete analyses which describe the decay in terms of a minimum number of exponential decay terms were used. Recently, Alcala et al. (1987a-c) and other groups (Albery et al., 1985; James & Ware, 1985; James et al., 1985) showed that in some cases nonexponential decays can be better described by a continuous distribution of lifetimes rather than a sum of exponentials. Continuous distributions of decay times were subsequently tested on proteins containing a single tryptophan (Alcala et al., 1987c; Lakowicz et al., 1987; Eftink & Ghiron, 1987; Eftink & Wasylewski, 1989).

Here we present steady-state and time domain fluorescence measurements of the single tryptophan containing Bs-PFK. The comparison of decay data analyzed in terms of distributed and discrete lifetimes was carried
out to test the models for the fluorescence decay of the enzyme. The solute quenching, temperature-dependence studies, and anisotropy decay measurements were carried out to understand the photophysical properties of the lone tryptophan in Bs-PFK.

**Material and Methods**

Materials. All chemicals were of analytical grade or better. Acrylamide (ultrapure) was purchased from Schwarz/Mann Biotech. p-Terphenyl was obtained from Aldrich (99+) and N-acetyltryptophanamide and tryptophan were purchased from Sigma. Bs-PFK purification is described in Appendix. For a solvent isotope effect experiment, the Bs-PFK sample in 50 mM Tris, pH 8.0 was frozen at -70°C and freeze dried. Then, the sample was dissolved in unbuffered D2O solution to measure solvent accessibility of the tryptophan environment. The Bs-PFK showed no activity loss or changes in circular dichroism and steady-state emission spectrum after lyophilization.

Absorbance, fluorescence quantum yield, and steady-state emission anisotropy measurements are described in Chapter 2.

Fluorescence Decay. Fluorescence lifetimes were measured by time-correlated single photon counting technique on a Photochemical Research Associates pulse
fluorometer interfaced to a Machintosh IIcx. A diagram of the instrument is shown in Figure 3.1. Samples were excited at 296 nm with the frequency-doubled output of a Rhodamine 6G dye laser (Coherent Model 701-3) cavity-dumped at 760 kHz, synchronously-pumped by a mode-locked Nd-YAG laser (Quantronics Model 416). The dye laser output was passed through a BBO crystal to frequency double into the UV, a half-wave retarder to rotate the polarization, a Newport 9335 attenuator to adjust the intensity, and a Pellin-Broca crystal to remove the fundamental. The stop timing signal was provided by the dye laser output, which was detected by an Antel Optronics AR-S2 fast photodiode, amplified by a PRA 1763 fast preamplifier, discriminated in one channel of a Tennelec 454 quad constant fraction discriminator, and delayed by a Tennnelec 412A delay. The start timing signal was provided by the fluorescence emission, which was detected by a cooled Hamamatsu R955 photomultiplier, amplified by a PRA 1763 fast preamplifier, and discriminated by an Ortec 583 constant fraction discriminator. The Ortec 457 time-to-amplitude converter was operated in reverse mode. The R955 photomultiplier was run at 1380 V. The width of the instrumental response was 350-400 ps FWHM. Fluorescence decays were acquired in 512 channels of 0.054 ns/channels. The data acquisition was controlled by a program using the LABVIEW software
Figure 3.1. Schematic diagram of time-resolved single photon counting laser fluorometer. PMT; photomultiplier tube, CFD; constant fraction discriminator, rf; radio frequency, ML; mode-locker, FPD; fast photodiode, CD; cavity dumper, SHG; second harmonic generator, MCA; multichannel analyzer.
package (Stryjewski, 1991). Lifetime data were collected at the magic angle (54.7°) relative to the vertically polarized laser beam. Probe and sample decays were collected by alternation to have peak counts of ~30,000 in the sample decay in the L-format. A solution of p-terphenyl in aqueous ethanol and 0.8 M KI (containing a trace of sodium thiosulfate to retard I$_3^-$ formation) was used as reference fluorophore. Lifetimes of 0.23 ± 0.01 ns at 15 °C to 0.15 ± 0.01 ns at 50 °C for the quenched terphenyl were determined in separate experiments using N-acetyltryptophanamide in 50 mM Tris, pH 8.0 as the monoexponential standard. Temperature was regulated with a Lauda circulating bath and OMEGA Model HH-72TH 2252 Ohm Thermistor.

Analysis of Fluorescence Data. Fluorescence decay data were fitted by reference, deconvolution (Kolber & Barkley, 1986) according to discrete and distribution functions in both single and multiple curve analyses (Knutson et al., 1983; Beechem et al., 1983). Programs TFIT, DFIT, and AFIT were obtained from Dr. J. Knutson and the program GLOBAL was kindly provided by Dr. J. Beechem. For global analysis, simultaneous nonlinear least-squares fits on multiple, linked data sets acquired from 315 to 360 nm (5-nm intervals) were done. The global analysis assumes that the lifetimes but not the preexponential factors are
independent of wavelength. For the discrete analysis, the
decay data were fitted to a sum of exponentials;
\[ I(t) = \sum \alpha_i \exp \left( -\frac{t}{\tau_i} \right) \] Eqn. 3.1
where \( \alpha_i \) is amplitude and \( \tau_i \) is lifetime. For the
distribution analysis using DFIT and GLOBAL, the decay data
were fitted to a continuous Gaussian distribution;
\[ I(t) = \int f(\tau) \tau^{-1}e^{-\tau/\tau_d}dt \] Eqn 3.2
\[ f(\tau) = A e^{(\tau-C)^2\ln2/4W^2} \]
where \( f(\tau) \) is a Gaussian function, \( C \) is the lifetime center,
\( W \) is the FWHM, and \( A \) is a constant determined by the
normalization. In distribution global analysis, lifetime
center and FWHM were linked with 0.1 ns as the chosen mesh
size. The integration in Eqn. 3.2 was approximated by
mesh size of \( \tau_i = i\Delta t \). Goodness of fit was judged by the
magnitude of reduced chi-square \( \chi^2_r \) and the shape of the
autocorrelation function of the weighted residuals
(Grinvald & Steinberg, 1974).

Decay-Associated Spectra (DAS). Decay associated
emission spectra \( I_i(\lambda) \) were derived by combining time-
resolved and steady-state data from the equation:
\[ I_i(\lambda) = I(\lambda) \left[ \sum \alpha_i(\lambda) \tau_i / \sum \alpha_i(\lambda) \tau_i \right] \] Eqn. 3.3
where \( I(\lambda) \) is the corrected steady-state intensity and
\( \alpha_i(\lambda) \) is the amplitude of component \( i \) at wavelength \( \lambda \)
determined from lifetime measurements. The center of
gravity $\psi_{cg,i}$ (in nm$^{-1}$) was calculated from (Lakowicz & Hogan, 1981)

$$\psi_{cg,i} = \sum_j I_j(\lambda_j) \lambda_j^{-3} / \sum_j I_j(\lambda_j) \lambda_j^{-2} \quad \text{Eqn. 3.4}$$

where the wavelength $\lambda_j$ goes from 315 to 360 nm in 5-nm increments.

Solute Quenching. Quenching experiments were carried out by using iodide and acrylamide. Stock solutions of 1 M KI in water, and 1, 2, and 8 M acrylamide in water were prepared. The ionic strength was kept constant for iodide quenching experiments by diluting Bs-PFK sample into Tris containing 1 M KCl. For the steady-state quenching, intensities were acquired for five 10-s time intervals at 340-nm emission wavelength, and the values were averaged. The data were analyzed by using the Stern-Volmer equation:

$$I_0/I = 1 + \tau_0 k_q [Q] = 1 + K_{SV}[Q] \quad \text{Eqn. 3.5}$$

where $I_0$ is the fluorescence intensity in the absence of quencher, $I$ is the intensity at quencher concentration $[Q]$, $\tau_0$ is the fluorescence lifetime in the absence of quencher, $k_q$ is the bimolecular Stern-Volmer rate constant for collisional quenching, and $K_{SV}$ is the Stern-Volmer constant. The Stern-Volmer rate constant, $k_q$ was also obtained from lifetime quenching experiments. In the analysis by GLOBAL, multiple decay data sets collected in different quencher concentrations were linked by the Stern-Volmer relationship to have $k_q$ invariant.
Temperature Effects. The effects of temperature on the quantum yield and lifetime of sample were studied. The temperature dependence of the fluorescence quantum yield in case only one deactivation process is significant can be expressed as follows (Kirby & Steiner, 1970):

\[
(\phi^{-1} - 1) = \frac{A}{k_f} \exp\left[-\frac{E^*}{RT}\right] \quad \text{Eqn. 3.6}
\]

where \( \phi \) is the fluorescence quantum yield at temperature \( T \), \( k_f \) is radiative rate constant, \( A \) is the frequency factor of the temperature-dependent deactivation process, \( R \) is the gas constant, \( E^* \) is the activation energy of the deactivation process, and \( T \) is absolute temperature. Therefore, from a semilogarithmic plot of \( \ln(Q^{-1} - 1) \) vs \( 1/RT \), we can get \( E^* \) from the slope. Likewise, a similar equation can be applied to temperature-dependent lifetime measurements:

\[
\tau^{-1} = k_0 + A \exp\left[-\frac{E^*}{RT}\right] \quad \text{Eqn. 3.7}
\]

where \( \tau \) is the lifetime of fluorophore, \( k_0 \) is the temperature-independent rate constant taken as \( 9.9 \times 10^7 \text{ s}^{-1} \) (Colucci et al., 1990), \( A \) is the frequency factor, and \( E^* \) is the activation energy. In the temperature-dependent lifetime analysis by GLOBAL, multiple decay data sets collected at different temperatures were linked by the Arrhenius equation (Eqn. 3.7) to have \( E^* \) and \( A \) invariant.

Anisotropy Decay. Time-dependent fluorescence anisotropy was measured using the same instrument and
method described in Fluorescence Decay with the following differences: T-format sample collections and vertical and horizontal detection of sample signals. Anisotropy decay, \( A(t) \), is given by:

\[
A(t) = \frac{(I_{VV}(t) - I_{VH}(t))}{(I_{VV}(t) + 2GI_{VH}(t))} = \sum \beta_i \exp(-t/\phi_i) \quad \text{Eqn. 3.8}
\]

where \( I_{VV}(t) \) is the emission intensity monitored through a polarizer oriented parallel to the excitation polarization, \( I_{VH}(t) \) is the emission intensity monitored through a perpendicular polarizer, \( G \) is a factor used to correct for the polarization sensitivity of the detection system, \( \beta_i \) is the amplitude of \( i \)th rotational mode, \( r(o) = \sum \beta_i \) is the fluorescence anisotropy of the initially prepared excited state, and \( \phi_i \) is the rotational correlation time of the molecule. Fluorescence decay curves collected for anisotropy measurements contained at least 35,000 counts in the peak channel for the curve with fewest counts. The anisotropy decays were analyzed by the program AFIT. For the association of decay time with a particular rotational correlation time, the linkage scheme described by Brand et al. (1984) was used. The fitting functions for \( I_{VV}(t) \) and \( I_{VH}(t) \) are:

\[
I_{VV}(t) = \frac{1}{3} \sum_{i=1,m} \alpha_i e^{-t/\tau_i} [1 + 2\sum_{j=1,n} \beta_j I_{ij} e^{-t/\phi_j}] \quad \text{Eqn. 3.9}
\]
\[ I_{VH}(t) = \frac{1}{3} \sum_{i=1,m} \alpha_i e^{-t/\tau_i} \left[ 1 - \sum_{j=1,n} \beta_j L_{ij} e^{-t/\phi_j} \right] \] Eqn. 3.10

where \( m \) is the number of total intensity lifetimes, and \( n \) represent the total number of rotational correlation times.

The matrix \( L \) is defined as:

\[ L_{ij} = 1 \text{ if lifetime } i \text{ is associated with rotation } j, \]
\[ L_{ij} = 0 \text{ otherwise.} \]

The G-factor (see Appendix) was determined from the relative intensities of left and right PMT observed from melatonin (Sigma) in water.

**RESULTS**

Fluorescence Lifetime. Decay curves of Bs-PFK were measured at several emission wavelengths from 315 to 360 nm at 25 °C. In Figures 3.2 and 3.3, the fluorescence intensity decay of Bs-PFK fitted to single and double exponentials is presented. Tables 3.1 and 3.2 summarize the results of the double and triple exponential analysis, respectively. In general, it was found that two exponential terms are adequate to fit the data. The \( \chi_r^2 \) values for the double exponential fit are in the range 1.30 - 1.60. The trials to fit the data to a triple exponential give slightly lower \( \chi_r^2 \) values in the range 1.26 - 1.57. However, the double exponential fit was selected due to the small decrease of the \( \chi_r^2 \) values and based on the
Figure 3.2. Fluorescence decay of Bs-PFK at pH 8.0, 25 °C. Emission wavelength 340 nm. Left curve is reference decay. Points are sample decay; smooth curve through points is best fit to single exponential function, $\tau = 3.91$ ns; $\chi^2 = 24.88$. 
Figure 3.3. Fluorescence decay of Bs-PFK at pH 8.0, 25 °C. Emission wavelength 340 nm. Left curve is reference decay. Points are sample decay; smooth curve through points is best fit to double-exponential function, $\alpha_1 = 0.39$, $\tau_1 = 1.63$, $\alpha_2 = 0.61$, $\tau_2 = 4.42$; $\chi^2 = 1.51$. 

TIME (0.054 ns/channel)
Table 3.1. Double-Exponential Lifetime Analysis of Bs-PFK Fluorescence Decay at pH 8.0, 25 °C.\(^a\)

<table>
<thead>
<tr>
<th>WL, nm</th>
<th>(\alpha_1)</th>
<th>(\tau_1,\text{ns})</th>
<th>(\tau_2,\text{ns})</th>
<th>(\chi_r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>0.40 (0.42)</td>
<td>1.53</td>
<td>4.35</td>
<td>1.60 (1.61)</td>
</tr>
<tr>
<td>320</td>
<td>0.38 (0.38)</td>
<td>1.71</td>
<td>4.45</td>
<td>1.35 (1.36)</td>
</tr>
<tr>
<td>325</td>
<td>0.38 (0.37)</td>
<td>1.71</td>
<td>4.46</td>
<td>1.46 (1.48)</td>
</tr>
<tr>
<td>330</td>
<td>0.39 (0.38)</td>
<td>1.73</td>
<td>4.46</td>
<td>1.38 (1.41)</td>
</tr>
<tr>
<td>335</td>
<td>0.39 (0.40)</td>
<td>1.46</td>
<td>4.37</td>
<td>1.53 (1.65)</td>
</tr>
<tr>
<td>340</td>
<td>0.39 (0.40)</td>
<td>1.63</td>
<td>4.42</td>
<td>1.51 (1.51)</td>
</tr>
<tr>
<td>345</td>
<td>0.40 (0.40)</td>
<td>1.64</td>
<td>4.44</td>
<td>1.45 (1.47)</td>
</tr>
<tr>
<td>350</td>
<td>0.40 (0.40)</td>
<td>1.65</td>
<td>4.44</td>
<td>1.34 (1.35)</td>
</tr>
<tr>
<td>355</td>
<td>0.41 (0.39)</td>
<td>1.70</td>
<td>4.48</td>
<td>1.31 (1.36)</td>
</tr>
<tr>
<td>360</td>
<td>0.41 (0.40)</td>
<td>1.64</td>
<td>4.45</td>
<td>1.30 (1.32)</td>
</tr>
<tr>
<td>Average</td>
<td>0.39</td>
<td>1.64</td>
<td>4.4</td>
<td>±0.02</td>
</tr>
<tr>
<td></td>
<td>±0.02</td>
<td>±0.07</td>
<td>±0.1</td>
<td></td>
</tr>
</tbody>
</table>

Global (0.40) (1.63) (4.43) (1.45)

\(\pm 0.02\)

\(^a\) Linked global analysis results in parentheses.

\(^b\) Partial \(\chi_r^2\) from global analysis.
Table 3.2. Triple-Exponential Lifetime Analysis of Bs-PFK Fluorescence Decay at pH 8.0, 25°C a) with and b) without linking the lifetimes.

<table>
<thead>
<tr>
<th>WL, nm</th>
<th>$\alpha_1$</th>
<th>$\tau_1$, ns</th>
<th>$\alpha_2^b$</th>
<th>$\tau_2$, ns</th>
<th>$\alpha_3^b$</th>
<th>$\tau_3$, ns</th>
<th>$\chi^2_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>-0.35</td>
<td>0.43</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td>1.57</td>
</tr>
<tr>
<td>320</td>
<td>-0.16</td>
<td>0.37</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
<td>1.35</td>
</tr>
<tr>
<td>325</td>
<td>-0.25</td>
<td>0.36</td>
<td>0.64</td>
<td></td>
<td></td>
<td></td>
<td>1.42</td>
</tr>
<tr>
<td>330</td>
<td>-0.24</td>
<td>0.38</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
<td>1.36</td>
</tr>
<tr>
<td>335</td>
<td>0.59</td>
<td>0.16</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>1.29</td>
</tr>
<tr>
<td>340</td>
<td>-0.32</td>
<td>0.41</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td>1.36</td>
</tr>
<tr>
<td>345</td>
<td>-0.23</td>
<td>0.40</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
<td>1.44</td>
</tr>
<tr>
<td>350</td>
<td>-0.23</td>
<td>0.39</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
<td>1.31</td>
</tr>
<tr>
<td>355</td>
<td>-0.31</td>
<td>0.40</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
<td>1.26</td>
</tr>
<tr>
<td>360</td>
<td>-0.02</td>
<td>0.39</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
<td>1.32</td>
</tr>
<tr>
<td>Global$^a$</td>
<td>0.03</td>
<td>1.62</td>
<td>4.43</td>
<td></td>
<td></td>
<td></td>
<td>1.36</td>
</tr>
</tbody>
</table>

$^a$ Global result with three lifetimes linked without fixing lifetimes. $^b$ Amplitudes of component in percentage scale calculated assuming the negative amplitude component contribution as 0%.
b).

<table>
<thead>
<tr>
<th>WL, nm</th>
<th>$\alpha_1$</th>
<th>$\tau_1$, ns</th>
<th>$\alpha_2$</th>
<th>$\tau_2$, ns</th>
<th>$\alpha_3$</th>
<th>$\tau_3$, ns</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>0.29</td>
<td>1.76</td>
<td>0.12</td>
<td>1.13</td>
<td>0.59</td>
<td>4.38</td>
<td>1.60</td>
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<tr>
<td>320</td>
<td>0.26</td>
<td>2.41</td>
<td>0.19</td>
<td>1.25</td>
<td>0.55</td>
<td>4.56</td>
<td>1.31</td>
</tr>
<tr>
<td>325</td>
<td>0.26</td>
<td>2.33</td>
<td>0.18</td>
<td>1.24</td>
<td>0.56</td>
<td>4.55</td>
<td>1.43</td>
</tr>
<tr>
<td>330</td>
<td>0.24</td>
<td>2.40</td>
<td>0.21</td>
<td>1.34</td>
<td>0.55</td>
<td>4.55</td>
<td>1.36</td>
</tr>
<tr>
<td>335</td>
<td>0.31</td>
<td>2.27</td>
<td>0.19</td>
<td>0.75</td>
<td>0.50</td>
<td>4.55</td>
<td>1.21</td>
</tr>
<tr>
<td>340</td>
<td>0.15</td>
<td>1.94</td>
<td>0.25</td>
<td>1.49</td>
<td>0.60</td>
<td>4.43</td>
<td>1.51</td>
</tr>
<tr>
<td>345</td>
<td>0.26</td>
<td>2.33</td>
<td>0.20</td>
<td>1.20</td>
<td>0.54</td>
<td>4.55</td>
<td>1.43</td>
</tr>
<tr>
<td>350</td>
<td>0.22</td>
<td>2.29</td>
<td>0.23</td>
<td>1.32</td>
<td>0.55</td>
<td>4.52</td>
<td>1.32</td>
</tr>
<tr>
<td>355</td>
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<td>2.23</td>
<td>0.24</td>
<td>1.43</td>
<td>0.56</td>
<td>4.53</td>
<td>1.30</td>
</tr>
<tr>
<td>360</td>
<td>0.33</td>
<td>2.17</td>
<td>0.15</td>
<td>0.93</td>
<td>0.52</td>
<td>4.58</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Average $0.2$ 2.2 0.20 1.2 0.52 4.5

$\pm 0.1$  $\pm 0.4$  $\pm 0.08$  $\pm 0.3$  $\pm 0.08$  $\pm 0.1$
observation that the triple exponential fit resulted in negative amplitude of the third component in the linked global analysis (Table 3.2). The four-exponential analysis always resulted in no further improvement in the $\chi_r^2$ values. In the lifetime linked double-exponential analysis (Table 3.1), the major component (~60%) has a lifetime of about 4.4 ns and the minor component about 1.6 ns with no wavelength dependence of decay parameters. In Tables 3.3 and 3.4, the data analyses in terms of continuous Gaussian distribution are also presented. Unimodal Gaussian distribution analysis (Table 3.3) resulted in unacceptably high $\chi_r^2$. In general, the bimodal distribution analysis results gave about the same lifetime with slightly higher $\chi_r^2$ values than the double exponential. Also, the full widths at half maximum (FWHM) of the two components (Figure 3.4) in the bimodal Gaussian distribution are very narrow (about 200 ps).

Decay Associated Spectra. Figure 3.5 presents the steady-state spectrum of Bs-PFK and the decay-associated spectra of the two components constructed according to Eqn. 3.3 from the decay parameters in Table 3.2. The solid curve is the total intensity spectrum with center of gravity, $\nu_{cg}^{-1} = 329$ nm. Component 2 with lifetime $\tau_2 = 4.4$ ns has $\nu_{cg}^{-1}$ at about 329 nm and contributes about 80%
Table 3.3. Unimodal Gaussian Distribution Analysis of Bs-PFK Fluorescence Decay at pH 8.0, 25°C.

<table>
<thead>
<tr>
<th>WL, nm</th>
<th>$\tau$, ns$^a$</th>
<th>FWHM, ns</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>3.77</td>
<td>0.39</td>
<td>28.97</td>
</tr>
<tr>
<td>320</td>
<td>3.85</td>
<td>0.31</td>
<td>21.41</td>
</tr>
<tr>
<td>325</td>
<td>3.87</td>
<td>0.32</td>
<td>22.55</td>
</tr>
<tr>
<td>330</td>
<td>3.85</td>
<td>0.30</td>
<td>21.50</td>
</tr>
<tr>
<td>335</td>
<td>3.81</td>
<td>0.35</td>
<td>31.54</td>
</tr>
<tr>
<td>340</td>
<td>3.81</td>
<td>0.32</td>
<td>36.87</td>
</tr>
<tr>
<td>345</td>
<td>3.82</td>
<td>0.41</td>
<td>34.02</td>
</tr>
<tr>
<td>350</td>
<td>3.82</td>
<td>0.35</td>
<td>29.17</td>
</tr>
<tr>
<td>355</td>
<td>3.83</td>
<td>0.34</td>
<td>29.84</td>
</tr>
<tr>
<td>360</td>
<td>3.81</td>
<td>0.35</td>
<td>29.17</td>
</tr>
<tr>
<td>Average</td>
<td>3.82±0.05</td>
<td>0.38±0.07</td>
<td></td>
</tr>
<tr>
<td>Global$^b$</td>
<td>3.83</td>
<td>0.37</td>
<td>28.48</td>
</tr>
</tbody>
</table>

$^a$Lifetime centers. $^b$Lifetime center and FWHM linked global result.
Table 3.4. Bimodal Gaussian Distribution Analysis of Bs-PFK Fluorescence Decay at pH 8.0, 25°C.

<table>
<thead>
<tr>
<th>WL, nm</th>
<th>$\alpha_1$</th>
<th>$\tau_1, \text{ns}^a$</th>
<th>$\tau_2, \text{ns}^a$</th>
<th>FWHM$_1, \text{ns}$</th>
<th>FWHM$_2, \text{ns}$</th>
<th>$\chi^2_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>0.31</td>
<td>1.58</td>
<td>4.34</td>
<td>0.22</td>
<td>0.15</td>
<td>1.79</td>
</tr>
<tr>
<td>320</td>
<td>0.28</td>
<td>1.44</td>
<td>4.27</td>
<td>0.19</td>
<td>0.16</td>
<td>1.86</td>
</tr>
<tr>
<td>325</td>
<td>0.28</td>
<td>1.54</td>
<td>4.33</td>
<td>0.20</td>
<td>0.16</td>
<td>1.77</td>
</tr>
<tr>
<td>330</td>
<td>0.28</td>
<td>1.47</td>
<td>4.27</td>
<td>0.19</td>
<td>0.16</td>
<td>2.02</td>
</tr>
<tr>
<td>335</td>
<td>0.31</td>
<td>1.50</td>
<td>4.37</td>
<td>0.23</td>
<td>0.17</td>
<td>1.57</td>
</tr>
<tr>
<td>340</td>
<td>0.29</td>
<td>1.46</td>
<td>4.28</td>
<td>0.20</td>
<td>0.16</td>
<td>2.08</td>
</tr>
<tr>
<td>345</td>
<td>0.32</td>
<td>1.66</td>
<td>4.42</td>
<td>0.25</td>
<td>0.19</td>
<td>1.56</td>
</tr>
<tr>
<td>350</td>
<td>0.31</td>
<td>1.56</td>
<td>4.37</td>
<td>0.22</td>
<td>0.17</td>
<td>1.51</td>
</tr>
<tr>
<td>355</td>
<td>0.30</td>
<td>1.55</td>
<td>4.37</td>
<td>0.21</td>
<td>0.17</td>
<td>1.57</td>
</tr>
<tr>
<td>360</td>
<td>0.31</td>
<td>1.54</td>
<td>4.36</td>
<td>0.22</td>
<td>0.17</td>
<td>1.51</td>
</tr>
<tr>
<td>Avg</td>
<td>0.30</td>
<td>1.53</td>
<td>4.34</td>
<td>0.21</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.02</td>
<td>±0.09</td>
<td>±0.07</td>
<td>±0.04</td>
<td>±0.02</td>
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</tr>
<tr>
<td>Global$^b$</td>
<td>b1.59</td>
<td>4.38</td>
<td>0.21</td>
<td>0.16</td>
<td>1.56</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Lifetime centers.  $^b$Lifetime center and FWHM linked global result.
Figure 3.4. Fluorescence decay data of Bs-PFK at pH 8.0, 25 °C, emission wavelength 340 nm fit by bimodal Gaussian distribution.
Figure 3.5. Decay-associated emission spectra (DAS) of Bs-PFK. (solid line) steady-state spectrum of Bs-PFK, $v_{cg}^{-1} = 329$ nm, (▲) steady-state contribution from 1.6-ns component, $v_{cg}^{-1} = 330$ nm, and (●) steady-state contribution from 4.4-ns component, $v_{cg}^{-1} = 329$ nm.
of intensity. Component 1 with lifetime $\tau_1 = 1.6$ ns has $\nu_{cg}^{-1} = 330$ nm and contributes about 20% of the intensity.

Temperature Dependence. The temperature dependence of the lifetime of Bs-PFK was measured as a function of temperature from 15 to 50 °C. In Table 3.5, the single curve fits to both discrete and Gaussian distribution of decay are presented. For the discrete analysis, the lifetime of each component decreased with increasing temperature, but the relative amplitudes fluctuated around $\alpha_1 = 38\% - 45\%$ suggesting that the amplitudes are independent of temperature. About 10% decrease in mean lifetime was obtained upon raising the sample temperature from 15 to 50°C. This compares to tryptophan in aqueous solution which shows a decrease of quantum yield about five fold over this temperature range (Robbins et al., 1980). Figure 3.6 shows an Arrhenius plot of the fluorescence lifetime data. The shorter lifetime component has more scatter than the longer lifetime component and has an activation energy, $E^*$, of 1.1 kcal/mol with $A = 34.1 \times 10^8$. The longer lifetime component has the same activation energy of 1.1 kcal/mol with $A = 8.0 \times 10^8$. The mean lifetime, $<\tau>$, which is calculated by using a relationship,

$$<\tau> = \Sigma \alpha_i \tau_i^2 / \Sigma \alpha_i \tau_i$$

for these two components gives an activation energy of 1.2 kcal/mol and $A = 11.9 \times 10^8$, which is in reasonably good
Table 3.5. Single Curve Analysis of Fluorescence Decay as a Function of Temperature.

a) discrete

<table>
<thead>
<tr>
<th>T, °C</th>
<th>$\alpha_1$</th>
<th>$\tau_1$,ns</th>
<th>$\tau_2$,ns</th>
<th>$\chi_r^2$</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>0.40</td>
<td>1.66</td>
<td>4.51</td>
<td>2.33</td>
</tr>
<tr>
<td>20</td>
<td>0.40</td>
<td>1.69</td>
<td>4.34</td>
<td>1.60</td>
</tr>
<tr>
<td>25</td>
<td>0.38</td>
<td>1.74</td>
<td>4.34</td>
<td>1.51</td>
</tr>
<tr>
<td>30</td>
<td>0.45</td>
<td>1.63</td>
<td>4.23</td>
<td>1.57</td>
</tr>
<tr>
<td>35</td>
<td>0.45</td>
<td>1.55</td>
<td>4.16</td>
<td>1.46</td>
</tr>
<tr>
<td>40</td>
<td>0.44</td>
<td>1.48</td>
<td>4.10</td>
<td>1.55</td>
</tr>
<tr>
<td>45</td>
<td>0.45</td>
<td>1.48</td>
<td>4.03</td>
<td>1.53</td>
</tr>
<tr>
<td>50</td>
<td>0.44</td>
<td>1.39</td>
<td>3.96</td>
<td>1.62</td>
</tr>
</tbody>
</table>

b) Gaussian Distribution

<table>
<thead>
<tr>
<th>T, °C</th>
<th>$\alpha_1$</th>
<th>$\tau_1$,ns</th>
<th>$\tau_2$,ns</th>
<th>FWHM$_1$,ns</th>
<th>FWHM$_2$,ns</th>
<th>$\chi_r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.35</td>
<td>1.39</td>
<td>4.38</td>
<td>0.23</td>
<td>0.22</td>
<td>2.43</td>
</tr>
<tr>
<td>20</td>
<td>0.35</td>
<td>1.65</td>
<td>4.28</td>
<td>0.23</td>
<td>0.21</td>
<td>1.76</td>
</tr>
<tr>
<td>25</td>
<td>0.34</td>
<td>1.65</td>
<td>4.29</td>
<td>0.22</td>
<td>0.20</td>
<td>1.55</td>
</tr>
<tr>
<td>30</td>
<td>0.38</td>
<td>1.60</td>
<td>4.19</td>
<td>0.24</td>
<td>0.20</td>
<td>1.64</td>
</tr>
<tr>
<td>35</td>
<td>0.38</td>
<td>1.51</td>
<td>4.09</td>
<td>0.24</td>
<td>0.20</td>
<td>1.63</td>
</tr>
<tr>
<td>40</td>
<td>0.37</td>
<td>1.47</td>
<td>4.06</td>
<td>0.23</td>
<td>0.19</td>
<td>1.73</td>
</tr>
<tr>
<td>45</td>
<td>0.38</td>
<td>1.47</td>
<td>3.99</td>
<td>0.24</td>
<td>0.20</td>
<td>1.77</td>
</tr>
<tr>
<td>50</td>
<td>0.37</td>
<td>1.45</td>
<td>3.97</td>
<td>0.23</td>
<td>0.18</td>
<td>1.83</td>
</tr>
</tbody>
</table>
Figure 3.6. Arrhenius plot for Bs-PFK. Linear least-squares fit of temperature dependence of fluorescence lifetimes of Bs-PFK at pH 8.0 to Arrhenius equation, \( \ln(\tau^{-1} - k_0) = \ln A - E^*/RT \), assuming \( k_0 = 9.9 \times 10^7 \text{ s}^{-1} \) (Colucci et al., 1990). (▲)\( \tau_1 \), (●)\( \tau_2 \), and (■)mean lifetime, \( \langle \tau \rangle = \Sigma \alpha_i \tau_i^2 / \Sigma \alpha_i \tau_i \).
agreement with the value determined from steady-state quantum yield measurements (1.45 kcal/mol). A summary of the results from Arrhenius plots of quantum yield and lifetime data is presented in Table 3.6. In Figure 3.7, an Arrhenius plot of the fluorescence quantum yield data is shown. For proteins, activation energy values between ~2.5 and 4 kcal have been reported (Weinryb & Steiner, 1970; Wasylewski et al., 1987). However, in some proteins, low activation energy (Szabo et al., 1983; Eftink et al., 1987; Eftink & Wasylewski, 1989) values similar to Bs-PFK are observed.

Solute Quenching. To probe the accessibility of Bs-PFK tryptophan to small solute quenchers, acrylamide and iodide were used. Acrylamide is a neutral polar quencher that quenches indole fluorescence with an efficiency of unity, as defined by the ratio of the apparent quenching rate constant to the diffusion-limited rate constant calculated from the time-independent portion of the von Somoluchowski equation (Eftink & Ghiron, 1981).

The mechanism of acrylamide quenching has been proposed to be electron transfer rather than electronic energy transfer (Evans et al., 1978) due to the small spectral overlap between protein fluorescence and acrylamide and the low extinction coefficient of the quencher. In Figure 3.8, a Stern-Volmer plot for acrylamide and iodide quenching
Table 3.6. Summary of Data for Arrhenius Plots of Bs-PFK in 50 mM Tris, pH 8.0.a

<table>
<thead>
<tr>
<th></th>
<th>$E^*$, kcal/mol</th>
<th>$A$, s$^{-1}$</th>
<th>$k_0$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>quantum yield</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lifetime</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>short</td>
<td>1.1</td>
<td>34.1 x 10⁸</td>
<td></td>
</tr>
<tr>
<td>(0.24)</td>
<td></td>
<td>(3.7 x 10⁸) (8.0 x 10⁷)</td>
<td></td>
</tr>
<tr>
<td>long</td>
<td>1.1</td>
<td>8.0 x 10⁸</td>
<td></td>
</tr>
<tr>
<td>(0.53)</td>
<td></td>
<td>(8.0 x 10⁷) (5.0 x 10⁸)</td>
<td></td>
</tr>
<tr>
<td>$&lt;T&gt;$</td>
<td>1.2</td>
<td>11.9 x 10⁸</td>
<td></td>
</tr>
</tbody>
</table>

aData analyzed by GLOBAL in parentheses. We are currently testing the GLOBAL program with the probe deconvolution and the results are presented as a comparative purpose.
Figure 3.7. Arrhenius plot for Bs-PFK. Linear least-square fit of temperature dependence of fluorescence quantum yield of Bs-PFK at pH 8.0 to Arrhenius equation, \( \ln(\phi^{-1} - 1) = \ln A/k_\ell - E^*/RT \), where \( \phi \) is the quantum yield, \( A \) is the frequency factor, and \( k_\ell \) is the radiative rate constant.
Figure 3.8: Stern-Volmer plot for Bs-PFK quenched by (▲) KI and (●) acrylamide at pH 8.0, 25 °C.
data is presented. Bimolecular rate constants were calculated from the Stern-Volmer constants and lifetimes measured separately. The acrylamide quenching rate constant (Table 3.7) of $0.54 \times 10^9$ M$^{-1}$ s$^{-1}$ is roughly a quarter of the value expected for a diffusion controlled reaction. Based on the X-ray structure, the acrylamide can approach as near as about 10 A to the tryptophan through the cylindrical water hole along the q molecular dyad axis of the Bs-PFK molecule (Figure 3.9). Therefore, it is possible to have an electron transfer process between the acrylamide and tryptophan. The quenching of tryptophan by iodide ion is believed to result from enhanced intersystem crossing to an excited triplet state, which is promoted by spin-orbit coupling of the excited singlet fluorophore and the iodide (Lakowicz, 1983). Therefore, it is unlikely that the iodide can influence tryptophan fluorescence without a direct collisional encounter. In Figure 3.10, Stern-Volmer plots for the two lifetime components obtained from lifetime quenching experiment are presented. The bimolecular rate constants calculated from the lifetime drops were about $0.9 \times 10^9$ M$^{-1}$s$^{-1}$ for the shorter lifetime component and $0.18 \times 10^9$ M$^{-1}$s$^{-1}$ for the longer lifetime component indicating about 5 times more efficient quenching of the short-lifetime component by acrylamide. From the mean lifetime drop, a rate constant of $\sim 0.33 \times 10^9$ M$^{-1}$s$^{-1}$
Table 3.7. Summary of Bimolecular Quenching Rate Constants Obtained for Quenching of Bs-PFK Fluorescence at pH 8.0, 25 °C.\textsuperscript{a}

<table>
<thead>
<tr>
<th>method</th>
<th>quencher</th>
<th>$k_q \times 10^9$ M$^{-1}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>intensity</td>
<td>acrylamide</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>I$^-$</td>
<td>0.05</td>
</tr>
<tr>
<td>lifetime</td>
<td>acrylamide</td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td></td>
<td>0.90 (0.40)</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td></td>
<td>0.18 (0.14)</td>
</tr>
<tr>
<td>$&lt;\tau&gt;$\textsuperscript{b}</td>
<td></td>
<td>0.33</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data analyzed by GLOBAL results in parentheses. We are currently testing the GLOBAL program with the probe deconvolution and the results are presented as a comparative purpose.\textsuperscript{b}Mean lifetimes were calculated using the formula; $<\tau> = \Sigma a_i \tau_i^2 / \Sigma a_i \tau_i$. 
Figure 3.9. X-ray structure of tetramer Bs-PFK. At the center of the molecule, there is a water hole of ~7Å in diameter.
Figure 3.10. Stern-Volmer plot for Bs-PFK quenched by acrylamide, pH 8.0, 25 °C. (▲) short-lifetime component and (●) long-lifetime component.
was obtained, which is in reasonable agreement with the rate constant of \( \approx 0.54 \times 10^9 \text{M}^{-1}\text{s}^{-1} \) obtained from steady-state measurement indicating no static quenching process.

D\(_2\)O Effects. The fluorescence quantum yield and lifetime of indole derivatives are increased when the solvent is changed from H\(_2\)O to D\(_2\)O (Ricci, 1970; Gudgin et al., 1981, 1983). This solvent isotope effect indicates excited state proton transfer (Stryer, 1966). Thus, the effect of D\(_2\)O on the tryptophan fluorescence can be used as a measure of degree of exposure of the tryptophan residue to solvent and a way of checking for excited state proton transfer reactions. Fluorescence intensity and lifetime were measured after 2, 6 and 30 days in deuterated solvent and summarized in Table 3.8. No changes in the fluorescence were observed. Thus, the tryptophan residue in Bs-PFK is not located at a solvent-exposed position as expected from the crystal structure.

**Discussion**

It is known that the fluorescence maxima for proteins containing "exposed" tryptophan residues are red-shifted (Sun & Song, 1977), while the fluorescence maxima of many proteins containing "buried" tryptophan residues occur between 325 and 335 nm (Burnstein et al., 1973). Therefore, based on the emission maximum (328 nm), solute
Table 3.8. Bs-PFK Fluorescence Decay in H₂O and D₂O, pH(D) = 8.0, 25 °C, 340 nm emission wavelength.

<table>
<thead>
<tr>
<th>Days/D₂O</th>
<th>( \alpha_1 )</th>
<th>( \tau_1, \text{ns} )</th>
<th>( \tau_2, \text{ns} )</th>
<th>( I_{D\text{2O}}/I_{H\text{2O}} )</th>
<th>( \chi^2_r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.39</td>
<td>1.63</td>
<td>4.42</td>
<td>1.0</td>
<td>1.51</td>
</tr>
<tr>
<td>2</td>
<td>0.43</td>
<td>1.50</td>
<td>4.60</td>
<td>1.0</td>
<td>1.45</td>
</tr>
<tr>
<td>6</td>
<td>0.45</td>
<td>1.81</td>
<td>4.61</td>
<td>1.0</td>
<td>1.27</td>
</tr>
<tr>
<td>30</td>
<td>0.46</td>
<td>1.58</td>
<td>4.64</td>
<td>1.0</td>
<td>1.48</td>
</tr>
</tbody>
</table>
quenching and solvent isotope results, the single tryptophan residue is in a hydrophobic environment inaccessible to solvent. This is in good agreement with the X-ray structure around the tryptophan (Figure 3.11) showing mainly hydrophobic amino acid residues. Also, the 8 Å around the tryptophan shows no amino acid residues with a strong quenching efficiency based on the electron scavenging efficiency except one methionine, which can be considered as a moderate quencher of tryptophan fluorescence (Steiner & Kirby, 1969). Therefore, it is not a surprise that Bs-PFK has a relatively high quantum yield.

From the results of discrete and distribution analysis of the time-resolved fluorescence data, we conclude the following: 1) the unimodal Gaussian distribution fit is unacceptable due to high $\chi_r^2$ values and 2) the bimodal Gaussian distribution is also less favored due to comparable $\chi_r^2$ values for discrete and continuous lifetime distributions. Besides, the very narrow FWHM of about 200 ps resembles a discrete double rather than a real distribution. Considering the chosen mesh size of 100 ps in the distribution analysis, the recorded FWHMs are reliable within the resolution of the analysis window. If
Figure 3.11. X-ray structure of Bs-PFK 8A around Trp-179 residue.
the decay follows a real distribution, adding the extra lifetime components around the lifetime center should improve the result in discrete analysis without showing redundant exponential components. However, when the data were analyzed in higher orders: 3-exponential and 4-exponential, no such trend was observed. Single tryptophan containing proteins show complex decay behaviors (Beechen and Brand, 1985; Eftink, 1991). In the Bs-PFK case, possible origins of the double-exponential decay are: 1) multiple aggregation species or dissociation of tetramer into dimers or monomers, 2) R- and T- allosteric conformers, and 3) internal motion of tryptophanyl residue. In the preceding chapter, we have characterized the aggregation states of Bs-PFK. Unlike the mammalian PFK from muscle, Bs-PFK exists as a homogeneous tetramer and does not undergo dissociation or aggregation into higher molecular weight forms in the concentration range we are studying (Chapter 3). Therefore the possibility of double exponential from different quaternary structures can be eliminated. The possibility of two lifetimes from R- and T-conformers is unlikely. If the longer lifetime component and the shorter one are coming from the R and T conformational states, it is expected that induction of the R-T transition by inhibitor PEP should influence the amplitudes of the two lifetime components. However, the decay parameters measured in the presence of inhibitor...
showed no significant changes (Chapter 4). Based on the comparative X-ray studies of R- and T-states of Bs-PFK (Evans, 1990), it has been shown that there are no significant changes in the tryptophan environment during the R-T transition. Also, the kinetic studies of the homologous E. coli PFK (Blangy & Monod, 1968) show that E. coli PFK exists in one state (T-state) with less than $10^{-6}$ of the protein in the other state (R-state). Therefore the preexponential factors obtained from the decay of Bs-PFK of ~60% for the longer lifetime component and ~40% for the shorter lifetime component are not likely to be coming from these two states. Internal motion of the residue in the protein matrix is also implausible because of the rigid environment of tryptophan with a relatively high steady-state anisotropy value of ~0.18 (Lakowicz et al., 1983). Also, from the analysis of the anisotropy decay of Bs-PFK at 25 °C (Table 3.9), single-exponential analysis gives the result of $\beta = 0.19$ and $\phi = 36$ ns with $\chi^2_r = 3.97$. The double exponential fit gives a slightly higher $\chi^2_r$ value with $\phi_1 \sim 7$ ns and $\phi_2 \sim 118$ ns. The internal motion of the residue in the protein matrix is also implausible from the recovered $r(0)$ value of ~0.19 at 296 nm excitation. The value is close to the expected $r(0)$ for an immobile tryptophan in a protein (Lakowicz et al., 1983).
Table 3.9. Tryptophan Emission Anisotropy Decay of Bs-PFK in 50 mM Tris, pH 8.0 at 25 °C.

<table>
<thead>
<tr>
<th></th>
<th>$\beta_1$</th>
<th>$\phi_1,\text{ns}$</th>
<th>$\beta_2$</th>
<th>$\phi_2,\text{ns}$</th>
<th>$\chi^2_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>single</td>
<td>0.19</td>
<td>36.0</td>
<td></td>
<td></td>
<td>3.97</td>
</tr>
<tr>
<td>double</td>
<td>0.05</td>
<td>7.1</td>
<td>0.14</td>
<td>117.8</td>
<td>4.29</td>
</tr>
</tbody>
</table>

Emission at 340 nm.
It is possible to estimate the value of $\phi$ for a rigid hydrated sphere according to an empirical relationship

$$\phi_{\text{calc}} = \frac{M(v + h)\eta}{RT} \quad \text{Eqn 3.11}$$

in which $M$ is the molar mass, $v$ the partial specific volume (0.735 cm$^3$/g), $h$ the degree of hydration (0.2 cm$^3$/g), $\eta$ the viscosity (~ 0.009 cP at 25 °C), $R$ the gas constant (8.314 x $10^7$ erg M$^{-1}$ K$^{-1}$), and $T$ the absolute temperature (298 K).

For Bs-PFK at 25 °C Eqn. 3.11 predicts a $\phi$ value of ~50 ns, which is larger than the experimentally obtained value of ~36 ns. The discrepancy can be explained due to the difficult analysis of the data which has too long rotational correlation time compared to its lifetime. The expected rotational correlation time and lifetime ratio of 60 ns/4 ns, or a factor of fifteen makes it difficult to recover the correct parameters. Based on the X-ray structure (Figure 3.11), Trp-179 is mostly surrounded by hydrophobic residues with very low electron scavenging efficiency except Met-311, which is within 8Å from Trp-179.

After examination of the other amino acids around the Trp-179 by distances (Table 3.10), the Met-311 seems to be the only possible candidate to influence tryptophan fluorescence. Even though the indole ring in tryptophan is immobilized, it is possible that the fluctuation of a near-by amino acid can create two distinctive,
Table 3.10. Distribution of amino acids 8 Å around the tryptophan.

<table>
<thead>
<tr>
<th>distance</th>
<th>R-form</th>
<th>T-form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Lue-178</td>
<td>same as R-form</td>
</tr>
<tr>
<td></td>
<td>Ser-180</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leu-182</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala-182</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Asp-175</td>
<td>same as R-form</td>
</tr>
<tr>
<td></td>
<td>Ile-176</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala-177</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly-181</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Phe-135</td>
<td>same as R-form</td>
</tr>
<tr>
<td></td>
<td>Asp-140</td>
<td>+ Asp-134</td>
</tr>
<tr>
<td></td>
<td>Leu-143</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leu-147</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly-174</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ile-182</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala-183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly-184</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly-185</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ile-307</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asp-308</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met-311</td>
<td></td>
</tr>
</tbody>
</table>

*aDistance from tryptophan in Å.*
energetically stable microconformations around the tryptophan. Because we have eliminated other possibilities: multiple aggregation states, allostERIC forms, and internal mobility of tryptophan, the two lifetime components in Bs-PFK fluorescence decay probably represent two microenvironments created by proximity difference between Trp-179 and Met-311. The observed short lifetime component can be explained in terms of more efficient electron transfer rate from excited singlet state of Trp-179 to Met-311 during closer contact between the Trp and one of two possible Met-311 conformers. The absence of a solvent isotope effect on the lifetime parameters further suggests electron transfer as the possible mechanism for shortening one or both lifetimes in the two microstates.
References


Wasyewski, Z., Sucharski, P., Wolak, A., & Eftink,
Chapter 4. Binding of Allosteric Ligands and Site-specific Mutation of Bs-PFK

Summary. Effects of ligand binding and site-specific mutation on the conformation of Bacillus stearothermophilus phosphofructokinase were followed by intrinsic tryptophan fluorescence. Substrates ATP and Fru-6-P and activator ADP had no effects on the fluorescence of Bs-PFK. However, upon binding of inhibitor PEP, the quantum yield decreased about 7% with a concomitant 2-nm red-shift of the emission maximum. Solute quenching experiments by acrylamide showed very slight differences in accessibility to the quencher for the sample investigated. Arg-252 to Ala-252 mutant and the T-conformation induced by PEP binding have slightly better accessibility than wild-type Bs-PFK toward neutral quencher acrylamide. KI has almost negligible accessibility to the tryptophan environment for R- and T-forms but slightly better accessibility to Arg-252 to Ala-252 mutant. The red-shift of emission maximum and decrease of quantum yield induced by inhibitor binding were reversed by adding the substrate Fru-6-P, presumably due to the R-T conformational transition.
In allosteric regulation, the activity of an enzyme is controlled by switching between two conformationally different states (Blangy et al., 1968). The T-state has either lower enzymatic activity or lower substrate binding affinity, and the R-state has higher activity or higher affinity for substrate. Switching between the two states is mediated by ligands that bind at remote sites. Effecting ligands may be either activators or inhibitors.

Unlike the homologous *E. coli* PFK which exists in the T-state in the absence of Fru-6-P, wild-type Bs-PFK is in the R-state in the absence and presence of substrate Fru-6-P, converting to the T-state only in the presence of inhibitor, PEP (Valdez et al., 1989). While the enzymatic properties and crystal structures of both R- and T-states have been determined, the effects of ligand binding and site-specific mutation on the solution conformation of this enzyme have not been studied. Dr. Simon Chang's group made and characterized an Arg-252 to Ala-252 mutant (R252A), which is proposed to have altered allosteric properties (Valdez et al., 1989). X-ray study (Evans et al., 1981) shows the direct involvement of the Arg-252 residue in binding of Fru-6-P in the active site (Figure 4.1) through a charge interaction. The substitution of the positively charged Arg-252 residue by uncharged Ala-252 was proposed to induce the
Figure 4.1. X-ray structure of Bs-PFK around active site. Arg-252 is involved with Fru-6-P through charge interaction.
T-conformation. R252A showed increased $K_m$ value for Fru-6-P from 0.029 to 41 mM with lower Hill coefficient of 1.7 compared to 2.2 for wild-type Bs-PFK (Valdez et al., 1989) indicating reduced corporativity in the mutant. The increase of $K_m$ value was explained as an indication of possible conformational change from R-form to T-form. However, the kinetic data showed a hyperbolic kinetics profile of R252A, which is similar to the wild-type Bs-PFK. The wild-type Bs-PFK follows sigmoidal kinetics only in the presence of PEP. Sigmoidal kinetics is expected if R252A were locked in the T-form. In this study, we compare the fluorescence properties of three samples, R252A and wild-type Bs-PFK in the absence (R-form) and presence (T-form) of PEP, to understand the differences using fluorescence spectroscopy.

**Material and Methods**

Materials. PEP and phosphoglycolate (PGC) were purchased from Sigma. Other chemicals are described in the Appendix. Bs-PFK was prepared as described in the Appendix. Purified R252A was a generous gift of Professor S. Chang.

UV absorption, circular dichroism, emission spectra, center of gravity, quantum yield, steady-state
anisotropy, solute quenching, time-resolved measurement and analysis of lifetime data were performed as described in Chapters 2 and 3. The concentration of PFK used was \(~ 6.0 \times 10^{-6}\) M, and the concentration of Fru-6-P and PEP (or PGC) used were \(~0.1\) M, which is more than 100 fold above the \(K_m\) (0.4 mM) and \(K_i\) (0.8 mM) values of wild-type PFK (Kundrot & Evans, 1991).

**Results**

**Steady-State Fluorescence.** In Figure 4.2, corrected steady-state fluorescence spectra of wild-type Bs-PFK (R-form), R252A, and wild-type PFK in the presence of PEP (T-form) are presented. The emission maximum of wild-type Bs-PFK (R-form) occurs at around 328 nm. Interaction of wild-type enzyme with inhibitor PEP (T-form) causes a red shift of emission spectrum from 328 to 330 nm (inverse center of gravity from 325 nm to 327 nm) and about 7% decrease in the fluorescence quantum yield without change in half width \(\Delta \lambda_{1/2}\). The steady-state anisotropy of wild-type Bs-PFK was 0.18 in the absence and 0.17 in the presence of PEP, which is the same within experimental error. R252A showed small changes in the opposite direction (Table 4.1): blue shift of emission maximum from 328 to 326 nm (center of gravity from 325 nm to 323 nm) and about 10% increase of quantum yield with no changes in steady-state anisotropy. The effect of
Figure 4.2. Fluorescence emission spectra of Bs-PFK at 296 nm excitation wavelength, 25 °C; (---) Bs-PFK, (---) Bs-PFK+PEP, and (-----) R252A. Spectra are normalized at their peak.
Table 4.1. Steady-state Fluorescence of PFK at pH 8.0, 25 °C. Anisotropy was determined at 340 nm emission wavelength.

<table>
<thead>
<tr>
<th></th>
<th>Bs-PFK</th>
<th>Bs-PFK + PEP</th>
<th>Bs-PFK+PEP + Fru-6-P</th>
<th>R252A Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} ) (nm)</td>
<td>328</td>
<td>330</td>
<td>328</td>
<td>326</td>
</tr>
<tr>
<td>Quantum Yield</td>
<td>0.30</td>
<td>0.28</td>
<td>0.30</td>
<td>0.33</td>
</tr>
<tr>
<td>Anisotropy</td>
<td>0.18</td>
<td>0.17</td>
<td>0.18</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Concentration of Bs-PFK was \( 10^{-6} \) M; concentrations of PEP and Fru-6-P were ~ 0.1 M.
substrate Fru-6-P on the fluorescence of wild-type Bs-PFK was also studied. Unlike PEP, binding of Fru-6-P caused no intensity change or emission spectral shift. Moreover, adding Fru-6-P to Bs-PFK in the presence of PEP (T-form) restored the fluorescence properties of Bs-PFK (Table 4.1).

Solute Quenching. In order to study differences in solute quenching of the tryptophan emission, Stern-Volmer quenching experiments were carried out and the quenching parameters are summarized in Table 4.2. The bimolecular quenching rate constants were calculated from the Stern-Volmer constant and the lifetime values determined in a separate experiment (Table 4.3). The iodide rate constant increased three-fold from $5.0 \times 10^7$ M$^{-1}$s$^{-1}$ to $1.5 \times 10^8$ M$^{-1}$s$^{-1}$ for R252A. Inhibitor binding (T-form) caused a two-fold decrease of the iodide rate constant to $2.0 \times 10^7$ M$^{-1}$s$^{-1}$. The slight changes of iodide bimolecular rate constant in T-form and R252A reflect minor changes in the tryptophan environment. The Stern-Volmer plot for KI quenching is presented in Figure 4.3. For acrylamide quenching, T-form (PEP bound Bs-PFK) and R252A have almost the same bimolecular rate constant reflecting similar accessibility to neutral quencher acrylamide. There is a slight increase of $K_{sv}$ value from $5.4 \times 10^8$ M$^{-1}$s$^{-1}$ for the R-form to
Table 4.2. Acrylamide and KI Quenching Parameters for Bs-PFK, PEP Complex, and R252A Mutant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quencher</th>
<th>$K_{sv}$, M$^{-1}$</th>
<th>$k_q$ ($x$ 10$^{-8}$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bs-PFK</td>
<td>acrylamide</td>
<td>2.2 ± 0.3</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.19</td>
<td>0.5</td>
</tr>
<tr>
<td>+ PEP</td>
<td>acrylamide</td>
<td>2.4 ± 0.2</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.08</td>
<td>0.2</td>
</tr>
<tr>
<td>R252A</td>
<td>acrylamide</td>
<td>2.7 ± 0.3</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.71</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Samples in Tris buffer, pH 8.0, 25 °C. $k_q$ values calculated from $K_{sv}$ and mean lifetime, $<\tau>$, given in Table 4.3.
Table 4.3. Lifetimes of Bs-PFK in the Absence and Presence of Inhibitor PEP and in R252A Mutant.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_1$</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\chi^2_{r}$</th>
<th>$\langle \tau \rangle$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bs-PFK</td>
<td>0.37</td>
<td>1.65</td>
<td>4.52</td>
<td>1.33</td>
<td>4.01</td>
</tr>
<tr>
<td>+ PEP</td>
<td>0.40</td>
<td>1.64</td>
<td>4.43</td>
<td>1.51</td>
<td>3.87</td>
</tr>
<tr>
<td>R252A$^a$</td>
<td>0.14</td>
<td>1.84</td>
<td>4.80</td>
<td>1.13</td>
<td>4.62</td>
</tr>
</tbody>
</table>

$\alpha_i$ and $\tau_i$ are the amplitudes and lifetimes of the biexponential fluorescence decay, respectively. Emission at 340 nm in 50 mM Tris, pH 8.0, 25 °C. The concentration of Bs-PFK was $10^{-6}$M and the concentration of PEP was 0.1 M. $^a$flash lamp excitation result. The different amplitudes of R252A mutant may come from different excitation source. Wild-type Bs-PFK measured with flash lamp excitation also has similar amplitudes observed in R252A mutant.
Figure 4.3. Stern-Volmer plots of KI quenching data for (■) Bs-PFK, (○) Bs-PFK+PEP complex and, (▲) R252A mutant.
6.3 x 10^8 M^-1S^-1 for the T-form, which is not significantly different within the experimental errors. This is consistent with the X-ray structure, which indicates that the tryptophan is located remote from the active and effector sites (Schirmer & Evans, 1991). Site-specific mutation studies show that the tryptophan is not located in the communication line between the two sites studied using homologous E. coli PFK (Kundrot & Evans, 1991). In Figure 4.4, the Stern-Volmer plot for acrylamide quenching is shown.

In Figure 4.5, circular dichroism spectra of the three samples are presented. Due to the high extinction coefficient of inhibitor PEP around 260 nm, a non physiological allosteric inhibitor (Evans, 1986) PGC (phosphoglycolate) was used to measure the effect of allosteric inhibition on Bs-PFK. PGC is known to bind to the same site and shows the same kinetic properties as PEP. As seen in the figure, PGC binding did not cause any changes in the CD spectrum of Bs-PFK. R252A showed the same CD peak (222 nm) as wild-type PFK, indicating that the secondary structures of the three protein samples are similar.
Figure 4.4. Stern-Volmer plots of acrylamide quenching data for (■) Bs-PFK, (●) Bs-PFK+PEP complex and, (▲) R252A.
Figure 4.5. CD spectra of (---) R252A mutant.
Discussion

In examining the effects of site-specific mutation and inhibitor binding, we have found the following: 1) the Arg-252 to Ala-252 mutation and inhibitor binding caused slight changes in the tryptophan fluorescence of the enzyme. However, the emission maxima and quantum yield data show that changes due to inhibitor binding and R252A are not in the same direction. The inhibitor binding caused a red shift of the emission maximum with decreased quantum yield and the Arg-252 to Ala-252 mutation caused a blue shift with increased quantum yield; 2) the solution conformations of wild-type and R252A mutant enzymes are the same based on steady-state fluorescence and CD data. However, the mutation has little effect on the tryptophan environment in Bs-PFK; and 3) the small differences in fluorescence upon binding the inhibitor can be ascribed to conformational differences in the R- and T-forms. These findings are consistent with the X-ray results (Evans et al., 1986, 1990) that the R-T transition is accompanied by rotation at the smaller subunit interface. The larger subunit interface where the inhibitor binding site and the tryptophan are located does not rotate in theallosteric transition. The reversal of fluorescence changes upon binding of substrate Fru-6-P is consistent with an
allosteric transition from inactive T-form to active R-form shown in enzyme activity measurements (Valdez et al. 1989). Therefore, the slight spectral changes of the inhibitor bound Bs-PFK can be ascribed to the T-conformation. The opposite trends of T-form and R252A indicate minor differences in tryptophan environment compared to T-form. The results show that Arg-252 does not appear to be the key amino acid residue to induce the conformationally locked enzyme in the T-form, which supports the enzyme kinetic data (Valdez et al., 1989) showing that the allosteric transition still occurs in the mutated protein.
References


Appendix

Materials

Aldolase (Type IV), mixture of triose-phosphate isomerase, glycerol 3-phosphate dehydrogenase (Type III), ethylenediamintetraacetic acid (EDTA), magnesium chloride, nicotinamide adenine dinucleotide (NADH), sodium chloride, sodium hydroxide, phenylmethysulfonyl fluoride, fructose 6-phosphate (sodium salt), adenosine triphosphate (sodium salt), adenosine diphosphate (sodium salt), phosphoenolpyruvate, ampicillin, N-acetyltryptophanamide (NATA), melatonin, PEP, and tryptophan were purchased from Sigma. Bacto-tryptone, bacto-yeast extract, and bacto-agar were obtained from Difco Laboratory. Protein assay kit and dithiothreitol were came from BIO-RAD. Tris (hydroxymethyl)aminomethane (Tris) Ultra Pure and KI were purchased from Fisher Scientific. Nitrocellulose filters were purchased from Millipore; and Anatop filters were obtained from Sergent Welch. ATP-agarose (Type II) was obtained from Pharmacia. Acrylamide (ultrapure) was purchased from Schwarz/Mann Biotech. p-Terphenyl was obtained from Aldrich (99+%).
YT Medium: Adjust pH to 7.5

10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
1 L water

Buffer: pH 7.5

50 mM Tris
1 mM DTT
Purification of Cloned Bs-PFK

Bs-PFK was isolated from *E. coli* strain DF1020/pBR322/Bs-PFK provided by Dr. Simon Chang using the procedure of Kotlarz and Buc (1982) and modified by French et al (1987). All the purification steps were carried out at 0-5 °C except the heat treatment step. Enzymatic activity was measured routinely throughout purification as outlined in Figure A.1.

Cell Culture. A colony of transformed DF1020 was used to inoculate 5 ml 2X-YT broth containing 100 μg/ml ampicillin. After overnight culture at 37 °C on a roller shaker, about 1.5 ml medium was mixed with 500 ml 2X-YT medium containing 100 μg/ml ampicillin. After about 14 hrs of incubation on a rotary shaker set to 250 rpm, 1.5 ml aliquots were taken every 2 hrs from the medium to check the activity. Cells were spun down by centrifugation at 5000 xg for 10 min at 4 °C. The bacterial pellet was stored at -20 °C.

(step 1) Preparation of crude extract. Packed cells of *E. coli* DF1020 carrying pBR322 were suspended in buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for protease inhibition to give 20% cell suspension (1.5 g wet weight in 7.5 ml of buffer) and disrupted by sonication at 0 °C in ice chest for 5 min (one min cooling after each
Figure A.1. Phosphofructokinase assay. Enzyme activity was monitored by absorbance of NADH at 340 nm. TPI, triose phosphate isomerase; GPD, glyceraldehyde phosphate dehydrogenase.
one min sonication to prevent overheating) using a Heat System Ultrasonic W-225R sonicator (level 6, pulsed, horn tip, 50%). Intact cells and debris were removed by centrifugation at 12,000 xg for 30 min at 4 °C and the supernatant was saved. Above steps (sonication on down) were repeated two more times using the collected cells from centrifugation.

(step 2) Heat treatment. The supernatant solution was kept at 70 °C for 30 min and was centrifuged at 12,000 xg for 2 hrs after chilling on ice. The precipitate was discarded and the supernatant was dialyzed into buffer containing 0.1 M NaCl. Before dialysis, NaCl concentration of the supernatant was adjusted to 0.1 M by adding 5.0 M NaCl solution while stirring the sample.

(step 3) Affinity chromatography. The dialyzed solution was applied to an ATP-agarose column equilibrated with buffer containing 0.1 M NaCl. The ATP-agarose column was washed with buffer containing 0.1 M NaCl until the absorbance of the eluate at 280 nm was less than 0.02. Bound Bs-PFK was eluted with buffer containing 50 μM Fru-6-P and 0.1 M NaCl at a flow rate of 50-60 ml/hr. The elution profile from the ATP-agarose column is shown in Figure A.2. The active fractions were combined and concentrated with pressure-dialysis in buffer containing 50% glycerol or were freeze dried.

(step 4) Freeze Drying of Bs-PFK. Unlike the mammalian
Figure A.2. Elution profile of Bs-PFK from ATP-agarose column. The sample was eluted with 50 μM Fru-6-P. The volume of each fraction was ~ 8 ml.
PFK, freezing at -70 °C or freeze drying did not cause any activity loss or secondary structure changes measured by CD.
Anisotropy Measurement using Probe Method.

The use of a reference for the analysis of pulse lifetime data has been discussed by several authors (Wahl et al., 1974; Libertini and Small, 1984; Kolber & Barkley, 1986) as a method to account more accurately for photomultiplier "color effects", in which the transit time of photoelectrons through the photomultiplier can vary depending on the wavelength of light impinging on the photocathode (Lewis et al., 1973). However, the analysis of data using a reference is less commonly used compared to the well-known procedures for analysis using a scatterer (O'Connor, & Phillips, 1984). Even though the probe method for lifetime analysis has been used extensively, no method has been developed to analyze the anisotropy decay using the probe method. However, the extension of the deconvolution procedures using the reference to the anisotropy decays seems to be straightforward. Likewise the lifetime analysis using the probe method (Kolber & Barkley, 1986), probe term instead of the scatterer term was used in deconvolution. The following steps are involved in G-factor determination.

1) For the L-format measurement, G factor was measured from the relationship, \( G = \frac{I_{HV}(t)}{I_{HH}(t)} \). \( I_{HV} \) and \( I_{HH} \) were determined from the intensity of melatonin using one PMT.
2) For the T-format measurement, \( I_{HV} \) and \( I_{HH} \) were determined separately using left and right PMTs respectively. Unlike L-format, signals in T-format also reflect the sensitivity differences of two PMTs.

3) A constant \( k \) determined from the following relationship was multiplied by one of the decay files before the decay file is put into the analysis program.

\[
<r> = \frac{ (I_{VV}/I_{VH}) - G(I_{VH}/I_{VH}) }{ (I_{VV}/I_{VH}) + 2G(I_{VH}/I_{VH}) } \]
\[
= \frac{ I_{VV} - G(L_{VV}/L_{VH})I_{VH} }{ I_{VV} + 2G(L_{VV}/L_{VH}) I_{VH} } \]
\[
= \frac{ I_{VV} - kI_{VH} }{ I_{VV} + 2kI_{VH} } \]

Here, \( <r> \) is the steady-state anisotropy value, \( I \) and \( L \) represent the sample and probe decays. The subscripts \( VV \) and \( VH \) represent the orientation of the emission polarizer vertical and horizontal to the excitation (vertically polarized).
Abbreviations

AA acrylamide
ADP Adenosine diphosphate
AMP adenosine monophosphate
cAMP cyclic adenosine monophosphate
ATP Adenosine triphosphate
Bs-PFK *Bacillus stearothermophilus* Phosphofructokinase
CD circular dichroism
DTT Dithiothreitol
*E. coli* *Escherichia coli*
Fru-1,6-P₂ Fructose 1,6-bisphosphate
Fru-6-P Fructose 6-phosphate
NAD Nicotinamide adenine dinucleotide
NADH Nicotinamide adenine dinucleotide (reduced)
Pᵢ inorganic phosphate
PEP phosphoenolpyruvate
PFK 6-phosphofructo-1-kinase
PGC phosphoglycolate
PMSF phenylmethylsulfonyl fluoride
UV ultraviolet
References

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