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Reduced Fluorescence Lifetime Heterogeneity of 5-Fluorotryptophan in Comparison to Tryptophan in Proteins: Implication for Resonance Energy Transfer Experiments

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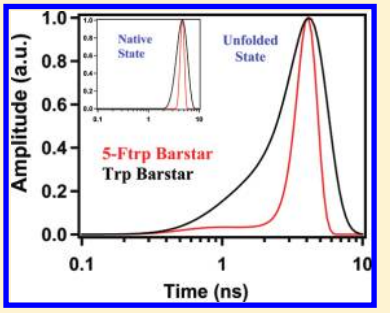
ABSTRACT: Tryptophan (Trp), an intrinsically fluorescent residue of proteins, has been used widely as an energy donor in fluorescence resonance energy transfer (FRET) experiments aimed at measuring intramolecular distances and distance distributions in protein folding−unfolding reactions. However, the high level of heterogeneity associated with the fluorescence lifetime of tryptophan, even in single-tryptophan proteins, imposes restrictions on its use as the energy donor. A search for a tryptophan analogue having reduced lifetime heterogeneity when compared to tryptophan led us to 5-fluorotryptophan (5F-Trp). A single tryptophan-containing mutant form of barstar, a small 89-residue bacterial protein, has multiple lifetime components in its various structural forms including the unfolded state, similar to observations made with several other proteins. Biosynthetic incorporation of 5F-Trp in place of Trp in the mutant barstar resulted in a significant decrease in the level of heterogeneity of fluorescence decay when compared to Trp-barstar, in the native state as well as in the denatured state. Importantly, observation of a major decay component of more than 80% in both the states makes 5F-Trp a significantly better candidate for being the energy donor in FRET experiments, as compared to Trp. This is expected to enable an unambiguous estimation of intramolecular distance distributions during protein folding and unfolding. The sequence insensitivity of the fluorescence decay kinetics of 5F-Trp in proteins was demonstrated by observing the decay kinetics of 5F-Trp incorporated in several synthetic peptides.



1. INTRODUCTION

It is increasingly being recognized that conformational heterogeneity in protein structures may be the rule rather than the exception.[1−3] The diffusive motion of polypeptide chains,[4−7] through a multitude of energy minima separated by barriers of a few kT in magnitude, results in conformational heterogeneity. A quantitative estimation of the conformational heterogeneity displayed by proteins during their folding and unfolding reactions is essential for delineating the mechanisms of these processes. The question whether intermediate states between the native and unfolded states[8−14] are discrete states or continuously evolving structures requires knowledge of the time evolution of conformational heterogeneity during protein folding and unfolding processes. Apart from the unfolded forms and intermediate states that characterize their folding and unfolding pathways, proteins can often adopt alternative structural forms such as molten globules,[15−19] aggregates, and fibrils,[20−22] depending on the external conditions. Such structures are also associated with substantial levels of conformational heterogeneity.[23,24]

NMR spectroscopy is a widely used tool in the study of conformational heterogeneity in proteins[25−30] and in detecting sparsely populated partially folded protein conformations.[31−35] However, NMR spectroscopy will not detect conformational heterogeneity when interconversion between the various conformational states is rapid when compared to the NMR time scale of a few milliseconds, which results in a single value of an observable weighted by the population distribution. Even with a faster spectroscopic technique such as fluorescence, observables such as steady-state intensity cannot reveal the presence of conformational heterogeneity which gets hidden under a population-averaged single value of the observable. On the other hand, the excited state lifetime of a fluorophore, either intrinsic or extrinsically coupled to the protein, has the capability of resolving conformational heterogeneity. The extreme sensitivity of fluorescence lifetime measurements, coupled with the fact that excited state lifetimes of fluorophores (typically a few nanoseconds) are much shorter than the time scales associated with interconversion between various conformational states, endows measurements of fluorescence lifetime distributions[10,13] with the capability of revealing conformational heterogeneity. When fluorescence lifetimes are dominantly controlled by FRET, lifetime distributions obtained from fluorescence decay kinetics can provide, with the use of the Forster equation,[36] intramolecular distance distributions, an observable of immense use. Of the several methods available for estimating lifetime distributions, the method based on Maximum Entropy analysis[37,38] provides a model-free approach.

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Maximum Entropy Method (MEM) analysis does not make any assumption regarding the shape of the distribution, unlike other methods which assume the distribution function (Gaussian or Lorentzian) and the number of peaks prior to analysis.[39] MEM analysis of fluorescence lifetime distributions has, in fact, revealed the presence of multiple populations of conformations in heterogeneous protein structural ensembles.[10,13]

Proper determination of intramolecular distance distributions, from the lifetime distributions obtained from MEM analysis of fluorescence decay curves measured in FRET experiments, requires that the fluorescence lifetime of the energy donor in the absence of the acceptor be homogeneous and single-valued. This is because heterogeneity in the lifetime distributions of the two samples, one with the donor alone and the other with both the donor and acceptor present, may lead to ambiguity in the estimation of intramolecular distances from the Forster equation. In other words, the fluorescence lifetime of the donor, while being sensitive to FRET, should be insensitive to any other environmental perturbation. The advantage of having a single or a narrow distribution of lifetimes for the donor (in the absence of acceptor) is not restricted to the MEM-based method alone. Even for the methods based on predetermined distribution profiles such as Gaussian, having a single lifetime of the donor in the absence of an acceptor would make the estimation of intramolecular distance distributions more robust and reliable.

Tryptophan, an intrinsically fluorescent amino acid residue of proteins, has served as the energy donor in TR-FRET experiments designed to obtain intramolecular distance and distance distributions in folding and unfolding reactions.[10,13,40,41] However, Trp very often shows a multiexponential fluorescence decay in different protein conformations, including the native state.[42,43] This imposes a limitation of its use as a universal energy donor in FRET experiments. Several investigators try to get around the problem of heterogeneity in FRET experiments by using mean fluorescence lifetimes, and in doing so, they introduce uncertainty in their measurements. The motivation to determine intramolecular distance distributions unambiguously in proteins led to a search for an environment-insensitive (except by FRET) Trp analogue, differing very little in structure from Trp, to minimize the effect of its incorporation on protein conformation. Such a Trp analogue should ideally display either a single fluorescent lifetime component, or at least close to single lifetime component, in different protein conformations, so that it can usefully take the place of Trp as the energy donor in TR-FRET experiments.

5-Fluorotryptophan (5F-Trp), an analogue of Trp containing a fluorine atom on the fifth carbon atom of the indole ring, has been shown to display a single or close to a single exponential decay in fluorescence, when incorporated in various proteins.[44,45] When incorporated at different positions in mannitol permease, 5F-Trp shows monoexponential fluorescence decay kinetics in the native state of the protein.[44] In α-synuclein, an intrinsically unstructured protein where the fluorophore is solvent exposed,[45] 5F-Trp shows a major lifetime component of 90% at each of many residue positions. In the same proteins, Trp displays at least three fluorescence lifetime components in identical conditions and when incorporated at the same residue positions.[46,47] Such experimental observations on the reduced environment sensitivity of 5F-Trp with respect to Trp are supported by theoretical studies. The value of the ionization potential of 5F-Trp is higher than that of Trp, thereby suppressing electron transfer reactions to the amide group and thus endowing it with environment insensitivity.[48] Nevertheless, significant improvement in the homogeneity of fluorescence lifetimes, of 5F-Trp with respect to Trp, has not been observed in the case of other proteins, suggesting that the fluorescence of 5F-Trp might be sequence-specific or protein conformation-dependent.[49,50] The singularity or close to singularity of the fluorescence lifetime of 5F-Trp in some proteins makes 5F-Trp a suitable replacement for Trp as the energy donor in TR-FRET experiments, to determine distance distributions in these proteins unambiguously.

In barstar, a small 89-residue bacterial protein, Trp53 has close to a monoexponential decay in the native protein, although the unfolded protein shows three fluorescence lifetime components, each with an appreciable amplitude.[10,40] The absence of a major fluorescence lifetime component for the unfolded state imposes a serious limitation in using Trp as the energy donor in TR-FRET enabled determinations of changes in intramolecular distance distributions during folding or unfolding.

In this work, 5F-Trp has been biosynthetically incorporated into barstar at residue position 53. The level of homogeneity of its fluorescence decay kinetics has been compared to that of Trp at the same residue position. Interestingly, 5F-Trp barstar shows a major fluorescence lifetime component of around 80% amplitude in the unfolded state, whereas Trp barstar shows three components of similar amplitudes. The reduction in the level of fluorescence lifetime heterogeneity is observed in both the native and unfolded states. The near-homogeneous fluorescence decay kinetics of 5FTrp-barstar in various structural forms is expected to be great for use in estimating intramolecular distance distributions in TR-FRET experiments. The residual inhomogeneity seen in the fluorescence decay kinetics of 5F-trp in barstar was explored by studying the fluorescence decay kinetics of 5F-Trp in short peptides. Our work on several pentapeptide sequences shows that the residual inhomogeneity associated with the fluorescence decay kinetics of 5F-Trp is an inherent property of 5F-Trp, rather than being caused by near neighbors in the sequence.

2. EXPERIMENTAL METHODS

2.1. Chemicals and Buffers. All the chemicals used were of the highest purity grade available from Sigma Aldrich, Inc. The buffer used in all the experiments was 20 mM Tris (pH 8.0), 250 μM EDTA. Concentrations of urea and GdnHCl stock solutions were determined by refractive index measurements.

2.2. Preparation and Purification of Trp-barstar and 5F-Trp Barstar. Wild-type barstar contains three tryptophans (Trp38, Trp44, and Trp53) and two cysteines (Cys40 and Cys82). The mutant variant of barstar used in this study contains a single tryptophan residue (Trp53) and a single cysteine (Cys82).

The gene for the mutant barstar was expressed under the control of a tac promoter, using the barstar expression plasmid pMT316[51] as described previously.[10,17]For incorporation of 5F-Trp into barstar, the expression plasmid was transformed into cells of an auxotrophic strain of *E. coli* (CY15602). Transformed colonies were inoculated into 500 mL of rich medium containing 500 μL of ampicillin (100 mg/mL stock) and grown at 37 °C. The rich medium used contained 6 g of bactotryptone, 12 g of yeast extract, 1.2 g of KH_2PO_4, 6.25 g of K_2HPO_4, and 2.5 mL of glycerol. After 6.5 h, when the OD_{600} had reached a value of ~1.5, the cells were pelleted down and washed with M9 medium containing 1% casamino acid, 0.25 mM 5-F-Trp, and 100 μg/mL of ampicillin and subsequently pelleted down three times.

Table 1. Parameters Obtained from Discrete Analysis of Fluorescence Intensity Decays at 25 °C for 5F-Trp-barstar and Trp-barstar[a]

	lifetimes (ns)			amplitudes			mean lifetime	
protein	τ_1	τ_2	τ_3	α_1	α_2	α_3	(τ_m) (ns)	χ_{red}^2
5F-trp barstar in native state								
λ_{em} = 340 nm	4.78			1.00			4.78	1.04
Trp barstar in native state								
λ_{em} = 340 nm	4.92	1.29		0.93	0.07		4.66	0.96
5F-trp barstar in 7 M urea								
λ_{em} = 350 nm	3.94	1.39		0.76	0.24		3.33	1.05
λ_{em} = 370 nm	4.00	1.24		0.78	0.22		3.39	0.97
λ_{em} = 390 nm	4.10	1.49		0.83	0.17		3.66	1.07
5F-trp barstar in 5 M GdHCl								
λ_{em} = 350 nm	3.14	1.28		0.69	0.31		2.56	1.05
λ_{em} = 370 nm	3.19	1.11		0.74	0.26		2.65	1.02
λ_{em} = 390 nm	3.27	1.33		0.76	0.24		2.80	1.08
Trp barstar in 7 M urea								
λ_{em} = 350 nm	4.50	1.84	0.35	0.50	0.35	0.15	2.95	1.05
λ_{em} = 370 nm	4.60	1.86	0.26	0.50	0.36	0.14	3.01	0.99
λ_{em} = 390 nm	4.78	2.16	0.50	0.48	0.38	0.14	3.18	1.01
Trp barstar in 5 M GdHCl								
λ_{em} = 350 nm	3.94	1.70	0.38	0.47	0.41	0.12	2.59	1.12
λ_{em} = 370 nm	4.10	2.04	0.55	0.38	0.43	0.19	2.54	1.15
λ_{em} = 390 nm	3.76	1.35	0.17	0.44	0.37	0.19	2.19	1.24

[a] Errors associated with the estimation of parameters are ~15% for τ_3 and α_3 and ~10% for others. Individual errors are not given to minimize congestion.

The M9 medium contains M9 salts (12.8 g/L of Na_2HPO_4, 3.0 g/L of KH_2PO_4, 0.5 g/L of NaCl, 1 g/L of NH_4Cl) along with 2 mL of 2 M $MgSO_4$, 20 mL of 20% glucose, 0.1 mL of 1 M $CaCl_2$, and 1 mL of 10% thiamine in 1 L of the medium. Then the cells were resuspended in 500 mL of expression medium (M9 medium containing 1% casamino acid, 0.25 mM 5-fluorotryptophan, and 100 μg/mL of ampicillin). IPTG was added after 30 min to the culture to make the final concentration 10 mg/L. After 6 h of shaking, cells were harvested by centrifugation. The purification procedure was the same as that for Trp-barstar.[17] The mass and purity of 5F-Trp barstar were checked by ESI mass spectroscopy. ESI mass spectra showed 85% incorporation of 5F-Trp barstar.

2.3. Synthesis of Peptides. The details of peptide synthesis are described elsewhere.[52] The terminal group of all the peptides was kept in amide form. Fmoc-Rink amide MBHA resin was used for the amination of the carboxy group at the C-terminus. Acetylation of the N-terminal amine was done using acetic anhydride in the final step of peptide synthesis. The peptides were purified using reverse-phase chromatography; in each case, the peptide eluted out as a single peak. To make sure that the peptide preparation did not contain any fluorescent impurity, fluorescence emission spectra were obtained by excitation at two different wavelengths, 280 and 300 nm: the emission spectra were found to be independent of excitation wavelength.

2.4. Fluorescence Measurements. All the steady-state fluorescence measurements on Trp barstar and 5F-Trp barstar were carried out using a SPEX Fluorolog-3 (T-format) FL3-11 spectrofluorimeter.

Time-resolved fluorescence intensity decay measurements were carried out using a time-correlated single-photon-counting setup. Pulses (1 ps) of 885 nm radiation from a Ti-sapphire femto/picosecond (Spectra Physics, Mountain View, CA) laser, pumped by an Nd:YAG laser (Millenia X, Spectra Physics), were frequency tripled to 295 nm by using a frequency doubler/tripler (GWU, Spectra physics). Fluorescence decay curves were obtained at the laser repetition rate of 4 MHz, by use of a microchannel plate photomultiplier (model R2809u; Hamamatsu Corp.) coupled to a time-correlated single-photon-counting setup. The instrument response functions (IRF) at 295, 300, and 308 nm were obtained using a dilute colloidal suspension of dried nondairy coffee whitener. The width (fwhm) of the IRF was 40 ps. A 320 nm cutoff filter was used for the fluorescence measurements. In fluorescence lifetime measurements, the emission was monitored at the magic angle (54.7°) to eliminate the contribution from the decay of anisotropy.

2.5. Analysis of the Fluorescence Decay for Discrete Lifetimes. The fluorescence decay curves collected at the magic angle were analyzed by deconvoluting with the IRF to obtain the intensity decay which was subsequently represented as a sum of 1−3 exponentials

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \quad i = 1-3$$

where $I(t)$ is the fluorescence intensity at time t and α_i is the amplitude of the ith lifetime τ_i such that $\Sigma_i \alpha_i = 1$. Mean lifetime was calculated using the equation: $\Sigma \tau_m = \alpha_i \tau_i$.

2.6. Analysis of Fluorescence Decay by the Maximum Entropy Method (MEM). The fluorescence lifetime distributions of 5F-Trp and Trp, in the proteins as well as in the peptides, were obtained from MEM analysis of the fluorescence intensity decays. The analysis does not require any prior assumption regarding the mathematical function fitting the distribution. A range of lifetime values from 10 ps to 10 ns were assumed to cover the expected lifetime values of the fluorophore in the

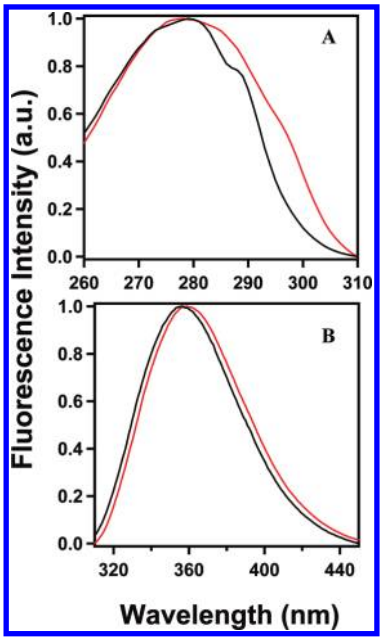

Figure 1. (A) Fluorescence excitation spectra of 5F-Trp-barstar (red line) and Trp-barstar (black line) both unfolded in 7 M urea in pH 8 buffer containing 20 mM Tris and 250 μM EDTA at 25 °C. The emission wavelength was 360 nm. The spectra were peak-normalized. (B) Fluorescence emission spectra of 5F-Trp-barstar (red line) and Trp-barstar (black line) both unfolded in 7 M urea in pH 8 buffer containing 20 mM Tris and 250 μM EDTA at 25 °C. The excitation wavelength was 300 nm. The spectra were peak normalized.

distribution; the lower value was limited by the instrument resolution, whereas the upper value can be obtained from the literature[42] as the maximum lifetime component of the fluorophore. At the beginning of analysis, the range of lifetimes is divided into a large number (150) of lifetime components of equal amplitude. Each successive iteration looks for a distribution to minimize χ^2 as well as to maximize the Shannon−Jaynes entropy function defined as $S = -\Sigma p_i \log p_i$ where $p_i = \alpha_i/\Sigma\alpha_i$ is the probability of the ith lifetime. If several distributions have similar χ^2 values of around 1.0, then the distribution having the maximum entropy value is accepted.

3. RESULTS AND DISCUSSION

3.1. Comparison of Fluorescence Properties of Trp and 5F-Trp in Barstar. The fluorescence intensity decay kinetics of Trp53 in barstar is well-studied.[10,40] It follows nearly homogeneous kinetics with a major (>90%) lifetime component of 4.8 ns in the native state, and highly heterogeneous decay kinetics in the unfolded state showing three lifetimes having significant amplitudes (Table 1). Observation of multiple lifetime components for single Trp proteins in different protein conformations is commonly seen.[43] As mentioned earlier, such heterogeneous fluorescence decay in the absence of FRET causes uncertainties in the estimation of an intramolecular distance distribution by FRET. The main aim of this work is to evaluate the suitability of 5F-Trp as the energy donor in FRET experiments designed to estimate intramolecular distance distributions in different protein conformations in an unambiguous way. 5F-Trp was incorporated in place of the single Trp at position 53 in barstar.

Figure 1 shows the fluorescence excitation and emission spectra of Trp-barstar and 5F-Trp-barstar unfolded in 7 M urea. It can be seen that the excitation spectrum of 5F-trp-barstar is slightly red-shifted when compared to that of Trp-barstar, as also observed for other proteins.[44,45] This feature is useful for selective excitation of 5F-Trp (say at ∼300 nm) in situations where both Trp and 5F-Trp are present, either in the same protein or when the level of incorporation of 5F-Trp is less than 100%, resulting in contamination of the fluorescence signal of 5F-Trp by that of Trp. For similar reasons, 295 nm is generally used to excite Trp in proteins, to avoid the excitation of Tyr or Phe, even though the absorption maximum of Trp is at 280 nm. The emission spectra (Figure 1B) of both the proteins are very similar to each other except for the slight red-shift of 5F-Trp-barstar. The observation of fluorescence peak maxima of 5F-Trp at 359 nm is an indication of solvent exposure of the fluorophore in the denatured state as the free amino acid has the peak at 355 nm in water.[45] The emission spectra of both the proteins in their native states have a peak around 330 nm (data not shown) indicating that the environments of 5F-Trp and Trp are very similar to each other and incorporation of the Trp analogue does not alter the structure significantly.

Excited state decay kinetics of 5F-Trp-barstar is significantly different from that of Trp-barstar (Table 1). The difference is prominent in the unfolded state when compared to the situation in the native state. While the Trp-barstar shows three lifetime components of ∼0.4, ∼2, and ∼4.5 ns with amplitudes in the range of 0.15−0.5 in the unfolded state, as observed in earlier studies,[40] 5F-Trp-barstar shows only two components, viz., ∼4s and ∼1.5 ns, with the ∼4 ns component having ∼80% amplitude. Thus, unfolded 5F-Trp-barstar shows a significantly reduced level of heterogeneity in its fluorescence decay kinetics, in comparison to Trp-barstar, as also observed in the cases of mannnitol permease[44] and synuclein.[45] The fluorescence decay pattern of 5F-Trp is independent of the denaturant used (Table 1) indicating that the observed second lifetime (∼1.5 ns) is not due to any interaction with the denaturant used. Furthermore, the decay parameters did not show any appreciable dependence on the emission wavelength, probably ruling out solvent relaxation and any other excited state process as the source of multiple lifetimes. In their native states, both 5F-Trp-barstar and Trp-barstar show nearly homogeneous decay kinetics. While Trp-barstar is associated with two lifetime components of 4.9 ns (93%) and 1.3 ns (7%) in the native state, 5F-Trp-barstar shows a single exponential of 4.8 ns (100%). A similar observation has been made in the case of apoflavodoxin.[53] Thus, the difference observed in the decay patterns of the two proteins in their native states is not as dramatic as that observed in their unfolded states (however, see later).

To check whether the observed smaller (∼1.5 ns) component for unfolded 5F-Trp-barstar is due to the presence of Trp-barstar as a contamination (the level of incorporation of 5F-Trp is ∼85%), the dependence of the decay parameters on the excitation wavelength was checked. This was motivated by the observation that the excitation spectra of 5F-Trp-barstar show a pronounced shoulder on the red side (Figure 1A). The decay parameters obtained by exciting at 295, 300, and 308 nm are 4.06 ns (82%) and 1.48 ns (18%); 4.10 ns (83%) and 1.49 ns (17%); and 4.14 ns (79%) and 1.42 ns (21%), respectively. The near independence of the observed decay parameters on the excitation wavelength indicates that the ∼1.5 ns component

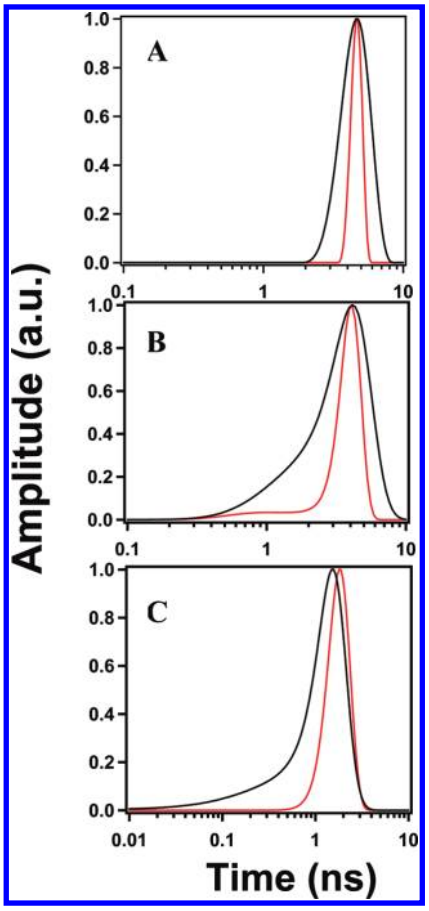

Figure 2. (A) Fluorescence lifetime distributions of 5F-Trp-barstar (red line) and Trp-barstar (black line) in their native states. The observations were made in 20 mM Tris, 250 μM EDTA, pH = 8.0 at 25 °C. The samples were excited at 300 nm, and decays were collected at 340 nm. The χ_{red}^2 values are 1.25 and 1.15 for 5F-Trp-barstar and Trp-barstar, respectively. The time axis is in a logarithmic scale. Each distribution is peak-normalized separately. (B) Fluorescence lifetime distributions of 5F-Trp-barstar (red line) and Trp-barstar (black line) in their unfolded states. The observations were made in 20 mM Tris, 250 μM EDTA, 7 M urea, pH = 8.0 at 25 °C. The samples were excited at 300 nm, and decays were collected at 390 nm. The χ_{red}^2 values are 1.02 and 1.15 for 5F-Trp-barstar and Trp-barstar, respectively. The time axis is in a logarithmic scale. Each distribution is peak-normalized separately. (C) Fluorescence lifetime distributions of 5F-Trp-barstar (red line) and Trp-barstar (black line) in their unfolded states at 70 °C. The observations were made in 20 mM Tris, 250 μM EDTA, 7 M urea, pH = 8.0. The samples were excited at 300 nm, and decays were collected at 390 nm. The χ_{red}^2 values are 1.09 and 1.06 for 5F-Trp-barstar and Trp-barstar, respectively. The time axis is in a log scale. Each distribution is peak normalized separately.

arises from 5F-Trp-barstar itself rather than from the low level of contamination by Trp-barstar.

Fluorescence lifetime distributions determined by the MEM is an ideal way to compare the level of heterogeneity of fluorescence decay kinetics.[10,13,37,38,43] Figure 2 shows the lifetime distribution obtained from MEM analysis of decay kinetics. The striking observation is the significant reduction in the overall spread of the lifetime distribution observed for 5F-Trp-barstar when compared to that of Trp-barstar, in the native state as well as in the denatured state. Both 5F-Trp and Trp show nearly symmetrical lifetime distributions, although the width is much narrower in the case of 5F-Trp. The lifetime distributions obtained from MEM analysis clearly distinguish their lifetime heterogeneities, which were not evident from the decay parameters obtained from discrete analysis. In the unfolded state, the enhanced level of homogeneity in the fluorescence decay of 5F-Trp-barstar is seen as a near symmetrical single Gaussian distribution as opposed to the prominent tail in the rising part of the broader distribution obtained for Trp-barstar. Furthermore, a comparison of the distributions of lifetimes obtained from MEM analysis with the decay parameters obtained from discrete analysis (to a sum of 2 or 3 exponentials) shows that the discrete analysis (which is computationally faster) is only an approximation to the description of the decay kinetics arising from a continuous distribution of conformers.

Table 2. Fluorescence Decay Parameters Obtained at 7 M Urea and 70 °C for 5-Fluoro-Trp-barstar and Trp-barstar[a]

	lifetimes (ns)			amplitudes			mean lifetime	
protein	τ_1	τ_2	τ_3	α_1	α_2	α_3	(τ_m) (ns)	χ_{red}^2
5F-trp barstar in 7 M urea								
λ_{em} = 350 nm	1.96	0.85		0.69	0.31		1.62	1.17
λ_{em} = 370 nm	1.99	0.91		0.72	0.28		1.69	1.00
λ_{em} = 390 nm	2.04	1.02		0.70	0.30		1.73	0.96
Trp barstar in 7 M urea								
λ_{em} = 350 nm	2.18	1.16	0.31	0.22	0.55	0.23	1.19	0.90
λ_{em} = 370 nm	2.19	1.27	0.45	0.22	0.55	0.23	1.28	0.89
λ_{em} = 390 nm	2.24	1.17	0.33	0.25	0.59	0.16	1.30	1.06

[a] Errors associated with the estimation of parameters are ~15% for τ_3 and α_3 and ~10% for others. Individual errors are not given to minimize congestion.

It is necessary to check whether the observed 1.5 ns component seen for 5F-Trp-barstar upon discrete analysis, or the broad peak around 1 ns seen in the MEM distribution (Figure 2B), is due to any residual structure of the protein in the presence of denaturants. The presence of residual structures in such conditions has been reported for several proteins.[41] Temperatures around 70 °C are expected to melt away any residual structure.[43] Table 2 and Figure 2C show the decay parameters for the denatured proteins at 70 °C, obtained from discrete analysis and MEM, respectively. The persistence of the shorter (~1 ns) lifetime component seen in the discrete analysis (Table 2) can be taken as an indication that the origin of this lifetime component is from the 5F-Trp fluorophore itself, rather than any residual structure in the unfolded state of the protein. The apparently contradictory observation of a near-symmetrical single Gaussian distribution by MEM (Figure 2C) and two lifetimes (~2 and ~1 ns, Table 2) by discrete analysis for 5F-Trp-barstar at 70 °C shows once again the inadequacy of discrete fits in representing the heterogeneity associated with protein structures in general.

3.2. Effect of Near-Neighbor Sequence on the Decay Profile of 5F-Trp in Small Peptides. 5F-Trp shows monoexponential fluorescence decay kinetics at different residue positions of native mannitol permease, indicating that the fluorophore is environment-insensitive.[44] However, deviation from such homogeneous decay kinetics is observed when 5F-Trp is next to a Cys residue.[44] A low-temperature phosphorescence study has suggested that the thiol group of the Cys residue is involved in electron transfer from the excited fluorophore resulting in an additional lifetime component leading to biexponential decay kinetics.[44] Previous reports also suggest that Cys and some other

Table 3. Parameters Obtained from Discrete Analysis of Fluorescence Intensity Decays at 25 °C for 5F-Trp (W*) and Trp (W) in Peptides[a]

	lifetimes (ns)			amplitudes			mean lifetime	
peptides	τ_1	τ_2	τ_3	α_1	α_2	α_3	(τ_m) (ns)	χ_{red}^2
sequence LEW*RQ:NH_2−CO-Leu-Glu-5Ftrp-Arg-Gln-CO−NH_2								
λ_{em} = 350 nm	3.26	0.80		0.89	0.11		2.99	1.09
λ_{em} = 370 nm	3.33	0.85		0.91	0.09		3.11	0.96
λ_{em} = 390 nm	3.38	1.19		0.89	0.11		3.14	0.96
sequence LEW*SQ: NH_2−CO-Leu-Glu-5Ftrp-Ser-Gln-CO−NH_2								
λ_{em} = 350 nm	3.23	1.21		0.82	0.18		2.87	1.05
λ_{em} = 370 nm	3.38	1.46		0.81	0.19		3.02	0.98
λ_{em} = 390 nm	3.44	1.63		0.79	0.21		3.06	1.04
sequence LSW*RQ: NH_2−CO-Leu-Ser-5Ftrp-Arg-Gln-CO−NH_2								
λ_{em} = 350 nm	3.08	1.21		0.81	0.19		2.72	1.00
λ_{em} = 370 nm	3.20	1.40		0.82	0.18		2.88	1.14
λ_{em} = 390 nm	3.17	1.30		0.86	0.14		2.91	0.99
sequence LEWRQ: NH_2−CO-Leu-Glu-trp-Arg-Gln-CO−NH_2								
λ_{em} = 350 nm	2.56	1.12	0.32	0.40	0.39	0.21	1.53	1.08
λ_{em} = 370 nm	2.55	1.08	0.29	0.38	0.42	0.20	1.48	1.02
λ_{em} = 390 nm	2.98	1.51	0.42	0.23	0.49	0.28	1.53	1.06
sequence LGW*GQ:NH_2−CO-Leu-Gly- 5Ftrp-Gly-Gln-CO−NH_2								
λ_{em} = 350 nm	2.78	0.65		0.82	0.18		2.40	1.06
λ_{em} = 370 nm	3.00	1.16		0.81	0.19		2.65	0.98
λ_{em} = 390 nm	3.10	1.08		0.77	0.23		2.64	0.99

[a] Errors associated with the estimation of parameters are ∼15% for τ_3 and α_3 and ∼10% for others. Individual errors are not given to minimize congestion.

amino acids can participate in such nonradiative processes with the excited indole ring.[54] Thus, the decay kinetics of 5F-Trp can be expected to be sequence-dependent. In barstar, Glu and Arg are the near neighbors of Trp 53. Although these side-chains have not been implicated in quenching of tryptophan fluorescence,[54] their electrostatic effect could assist quenching by the backbone amide.[55] Close contact of either the negative Glu with the Trp or the positive Arg with the amide could lead to quenching of fluorescence by modulating the energy gap associated with electron transfer.[55]

Exploration of the effect of nearby residues on the fluorescence of 5F-Trp can be done by mutating the residue to an amino acid residue having a nonpolar side-chain and comparing the fluorescence decay patterns in the unfolded state. However, an alternative and easier way to address the issue is by incorporating 5F-Trp in a small peptide with varying sequence, arguably unstructured and containing solvent-exposed fluorophore. Pentapeptides in which 5F-Trp is placed at the third position were chosen to serve this purpose. The peptide sequence was taken from barstar, as a starting point. Arg and Glu residues next to 5F-Trp were changed to Ser or Gly, one at a time as well as together. All the peptides contain amide groups at their two terminals, to avoid the effects of the charged ammonium and carboxyl groups on the fluorescence decay kinetics of 5F-Trp.

The fluorescence lifetimes of 5F-Trp in various peptides are listed in Table 3, and their lifetime distributions are shown in Figure 3. 5F-Trp in the barstar sequence shows a major component of ∼3.3 ns with the amplitude of ∼90%. Trp shows three lifetime components of ∼2.6, ∼1.1, and ∼0.3 ns having amplitudes in the range from 0.2 to 0.5 in similar sequences. The similarity of the

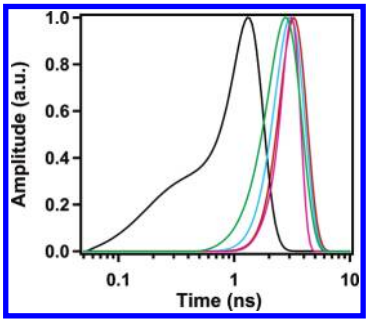

Figure 3. Fluorescence lifetime distributions of peptide sequences NH_2−CO-Leu-Glu-5Ftrp-Arg-Gln-CO−NH_2 (pink), NH_2−CO-Leu-Glu-5Ftrp-Ser-Gln-CO−NH_2 (cyan), NH_2−CO-Leu-Ser-5Ftrp-Arg-Gln-CO−NH_2 (brown), NH_2−CO-Leu-Glu-Trp-Arg-Gln-CO−NH_2 (black), and NH_2−CO-Leu-Gly-5Ftrp-Gly-Gln-CO_-NH_2 (green) with χ_{red}^2 values 1.11, 1.39, 1.25, 1.03, and 1.23, respectively. The observations were made in 20 mM Tris, 250 μM EDTA, pH = 8.0 at 25 °C. The samples were excited at 300 nm, and decays were collected at 390 nm. The time axis is in a log scale. Each distribution is peak normalized separately.

lifetime components of 5F-Trp and Trp in the peptides in comparison to the unfolded state of barstar indicates that the construction of such small peptides can be a good model system to explore the effect of the sequence on the 5F-Trp fluorescence decay pattern.

Two different peptide sequences were designed with the near neighbors Arg and Glu replaced by Ser, but one at a time. Both the sequences show the major component of ∼3.0−3.3 ns with amplitude ∼80% in their biexponential fluorescence intensity

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decays. This result indicates that the side chains of Glu and Arg do not have any significant effect on the 5F-Trp decay pattern. Subsequently, two Gly residues were inserted into the sequence in place of Glu and Arg. This sequence also shows the major component of ~3.0 ns with the amplitude of ~80%. The similarity in the fluorescence intensity decay patterns of 5F-Trp across different sequences indicates that the neighboring group has no effect on the lifetime property 5F-Trp, even when the residue is in the extended conformation of the peptide chain. Interestingly, the presence of the smaller lifetime component of ~1.0−1.5 ns in different peptides supports the assertion that the similar component observed in denatured proteins is not due to incomplete (<100%) incorporation of 5F-Trp or due to the presence of any residual structure.

The lifetime distributions of the peptides are shown in Figure 3. In all the peptides, 5F-Trp shows narrow unimodal distributions with the peak ~3.0 ns. The similarity in the lifetime distributions of 5F-Trp across different sequences is an indication that the decay kinetics of 5F-Trp is near-neighbor insensitive. In contrast, Trp shows broad lifetime distributions when placed in the peptide sequences, with considerable amplitude around hundreds of picoseconds, unlike 5F-Trp.

3.3. Ground-State Heterogeneity and Lifetime Distribution. A broad distribution of lifetimes or three lifetime components of Trp are generally observed in different denatured proteins having a single tryptophan.[43] The presence of three lifetime components has been explained as due to the presence of three rotamers that interconvert on a time scale slower than their fluorescence lifetime.[42] The three rotamers of tryptophan (around the $C_\alpha-C_\beta$ bond) differ from each other in the distance between the indole ring and the nearby quencher group, the carbonyl group in the case of proteins, giving rise to differences in the rate constant of charge transfer and, hence, in their lifetimes. A study on a cyclic hexapeptide as a model compound showed that the presence of three rotamers gives rise to three lifetimes of Trp with their relative amplitudes similar to the relative populations as observed in an NMR experiment.[56] The occurrence of two lifetime components of 5F-Trp with the major being 80% in the denatured state of the protein, as well as in the peptides, indicates the reduction in the sensitivity of the 5-fluoroindole ring to the nearby quencher groups in different rotamers, although the rotamer populations are not hindered due to steric effects unlike in the native state. The narrow lifetime distribution as well as the absence of lifetime components around hundreds of picoseconds in the case of 5F-Trp in comparison to Trp is again an indication of the fact that the presence of the fluorine atom in the indole ring suppresses the extent of electron transfer reactions. This supports the theoretical study that the increase of the ionization potential of 5-fluoroindole in comparison to indole decreases the extent of electron transfer reactions and reduces the number of lifetime components as well as the lifetime heterogeneity as observed in their lifetime distributions.[48] The origin of the minor component of ~1.5 ns in all the samples having 5F-Trp studied here (except native barstar) could lie in the presence of a rotamer whose fluorescence lifetime is altered by the energy gap associated with electron transfer quenching.[48] Introduction of fluorine does not seem to abolish totally electron transfer to the amide as indicated by quantum yield measurements of 3-methylindole, NATA, and their 5F-analogues.[44] Also, the observation of similar values of quantum yields for free tryptophan and 5F-trp[49] indicates that proton transfer quenching is also not totally suppressed by fluorine substitution. An additional mechanism for the origin of the ~1.5 ns component could be the presence of a reversible equilibrium between the excited indole and a charge transfer dark state[57] leading to biexponential decay. Finally, the results from the present work establish 5F-Trp as an effective substitute for Trp in FRET experiments designed for generating robust intramolecular distance distributions for addressing various important issues involved in protein folding and unfolding reactions.

4. CONCLUSIONS

The main conclusions that can be derived from the present work are the following: (i) The fluorescence decay kinetics of 5F-Trp-barstar is significantly more homogeneous when compared to that of Trp-barstar in the native state, as well as in the unfolded states in 7 M urea, in 5 M GdnHCl, and in 7 M urea at 70 °C, as shown clearly by their lifetime distributions (Figure 2). (ii) The environment sensitivity shown by Trp when the protein is in various structural forms is largely reduced on replacement of Trp by 5F-Trp. (iii) The fluorescence decay kinetics of 5F-Trp in unfolded barstar is very similar to those observed in pentapeptide sequences having barstar-like sequences; the observation of near-neighbor insensitivity on the decay kinetics of the peptides shows that the observed biexponential decay kinetics is intrinsic to the photophysics of 5F-Trp. (iv) The high level of homogeneity and environment insensitivity of the fluorescence decay kinetics of 5F-Trp makes it an ideal fluorescence donor in estimating intramolecular distance distributions in proteins, by the combined use of time-resolved FRET and MEM.

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