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Identification of intermediate species in protein-folding by quantitative analysis of amplitudes in time-domain fluorescence spectroscopy

ANOOP M SAXENA,[1] G KRISHNAMOORTHY,[1,*] JAYANT B UDGAONKAR[2] and N PERIASAMY[1,*]

[1]Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400 005

[2]National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bangalore 565 065

e-mail: {peri, gk}@tifr.res.in

Abstract. In protein-folding studies it is often required to differentiate a system with only two-states, namely the native (N) and unfolded (U) forms of the protein present at any condition of the solvent, from a situation wherein intermediate state(s) could also be present. This differentiation of a two-state from a multi-state structural transition is non-trivial when studied by the several steady-state spectroscopic methods that are popular in protein-folding studies. In contrast to the steady-state methods, time-resolved fluorescence has the capability to reveal the presence of heterogeneity of structural forms due to the 'fingerprint' nature of fluorescence lifetimes of various forms. In this work, we establish this method by quantitative analysis of amplitudes associated with fluorescence lifetimes in multiexponential decays. First, we show that we can estimate, accurately, the relative population of species from two-component mixtures of non-interacting molecules such as fluorescent dyes, peptides and proteins. Subsequently, we demonstrate, by analysing the amplitudes of fluorescence lifetimes which are controlled by fluorescence resonance energy transfer (FRET), that the equilibrium folding–unfolding transition of the small single-domain protein barstar is not a two-step process.

Keywords. Time-resolved fluorescence resonance energy transfer (TR-FRET); fluorescence lifetime; two-state protein-folding; barstar.

1. Introduction

In equilibrium protein-folding studies, the occurrence of a sigmoidal shape of the denaturation titration curve of a protein is interpreted generally in terms of a two-state transition, i.e. a transition leading a protein from its native form to the unfolded form (N $\leftrightarrow$ U) without the involvement of any intermediate structure. The value of a property (parameter) observed at any point on the denaturation titration curve of a protein undergoing two-state transition is given by $P_{obs} = f_1 p_1 + f_2 p_2$, where, p_1 and p_2 are the values of the properties of the two-states respectively, and f_1 and f_2 are the fractions of number of molecules at the point of observation corresponding to p_1 and p_2 respectively, with $(f_1 + f_2) = 1$.

Such studies are generally carried out by observing parameters like absorbance, circular dichroism, fluorescence intensity etc. These spectroscopic properties are ensemble averages of the system under study and hence insensitive to the associated structural heterogeneity. Thus, the observation of sigmoidal transition by monitoring the above mentioned steady-state parameters need not rule out the possibility of the evolution being multi-state (or gradual) at the level of individual structural components. Hence, to determine whether a transition is two-state or multi-state, it is necessary to observe parameters which are sensitive to the structural heterogeneity.

In this regard, experimental methods, based on time-resolved fluorescence (TRF) have proven to be very promising due to their high sensitivity, both in exploring conformational heterogeneity as well as in characterizing the dynamics of various segments of proteins. If the time scale associated with the inter-conversion of various species in a system is longer than their fluorescence lifetimes (τ_i), then TRF-based measurements can provide the fluorescence lifetime (property) associated with each individual species, and also its corresponding fractions (α_i) in the system. TRF measurements have the power to distinguish between two-state and gradual structural transitions

*For correspondence

during a protein-folding reaction. When a protein undergoes a two-state folding transition, the values of lifetimes of native (N) and unfolded (U) species would remain unchanged, while the corresponding fractions would keep varying throughout the transition. In contrast, during a gradual structural transition of folding, the entire system at any point of time would be characterized by only one distinct value of fluorescence lifetime, evolving from the value in the U form to that in the N form. Such distinction between two-state and gradual structural transitions during the protein-folding reaction is not possible by conventional steady-state probes as the property reported by them is ensemble-averaged.

The power of TRF experiments is further enhanced by time-resolved fluorescence resonance energy transfer (TR-FRET)-based measurements of the fluorescence intensity decay of the donor fluorophore in the presence of an acceptor. Efficiency of energy transfer between donor and acceptor is related to the distance between them. Thus, such measurements offer the possibility of monitoring various regions of a protein during the transition from the U to the N form. When coupled with the maximum entropy method (MEM) of analysis of intensity decay curves,[1,2] TR-FRET measurements can resolve heterogeneity in terms of distributions of intramolecular distances. Much has been learnt about conformational heterogeneity in protein-folding,[3] refolding,[4,5] protein-protein association[6] and unfolded proteins[3,7,8] by TR-FRET experiments. Such experimental measurements coupled with MEM analysis have actually demonstrated continuous (gradual) behavior of structure evolution in denaturant-induced equilibrium unfolding of barstar,[9] the equilibrium unfolding curve of which is of sigmoidal shape when determined by monitoring steady-state parameters. However the use of such methods to distinguish between two-state and multi-state behaviour of folding transition is not straightforward due to the non-trivial nature of analysis of fluorescence intensity decays by MEM. Hence, it is desirable to have a simpler and more robust method which can be used for a quick check of whether a protein folds in a two-state or multi-state manner.

In a typical time-resolved fluorescence measurement, a sample containing one or more fluorescent molecules is excited by a short laser pulse at a wavelength where the molecule absorbs, and the emission from the sample is monitored as a function of time. Fluorescence decay is often a sum of exponentials: $I(t) = \Sigma\alpha_i \exp(-t/\tau_i)$, where α_i is the pre-exponent (also called fractional amplitude) and τ_i is the fluorescence lifetime of the ith species. The fluorescence lifetime is characteristic of the species. The pre-exponent, α_i is a measure of the concentration of ith species. Thus, fluorescence decay measurement helps to identify the presence of a distinct species with lifetime τ_i and to obtain an estimate of its concentration. In most applications of TRF, it is always convenient and straightforward to interpret the value of the lifetime of the sample. However, the usefulness of the value of pre-exponents as a quantitative measure of the concentration is generally ignored. There have been reports where the concentration ratio of two fluorescent species in a sample have been determined using pre-exponents,[10,11] using an empirical equation similar to the one derived in this work. However, we should be cautious of the fact that pre-exponents are emission wavelength-dependent. Here, we derive the equation applicable for 'total' fluorescence decay from the sample, including the detector sensitivity, then check the validity of the use of pre-exponent as a quantitative measure of concentration on a series of samples with mixtures of non-interacting fluorophores, and finally test its usefulness by checking whether a protein folds in a two-state or multi-state pathway. This involves a two-component analysis of fluorescence intensity decays at various concentrations of the protein denaturant, and the construction of a plot of goodness of fit (chi square, χ^2) vs the denaturant concentration. This simple analytical plot gives a quick insight into whether a protein folds in a two-state or multi-state manner.

2. Materials and methods

2.1 *Samples and solutions*

Solutions of rhodamine B (Sigma-Aldrich, USA), Fluorescein (SRL, India) were prepared in 20 mM Phosphate (SRL, India) buffer, pH 8. The dye concentrations used were less than 5 μM in order to ensure that intermolecular fluorescence resonance energy transfer between fluorescein and rhodamine B is negligible. Concentration of guanidine hydrochloride (Gibco BRL, USA) solution was checked by refractive index measurements. Trp–Gly, Gly–Trp peptides (purity ~98%) were obtained from Sigma-Aldrich, USA, and used as received.

Wild-type barstar contains three tryptophans (Trp38, Trp44 and Trp53) and two cysteines (Cys40 and Cys82).[12] In our experiments, we used a single tryp-

tophan (Trp53) containing a mutant protein of barstar with mutations W38F/W44F/S12T/C40A obtained by site-directed mutagenesis.[13] The protein was purified as described elsewhere,[14] and the purity was confirmed to be >98% on SDS-PAGE. Human serum albumin (HAS) (purity > 95%) was obtained from Sigma–Aldrich, USA and was used without any further purification. HSA has a single tryptophan at position 214. Concentrations of barstar and HSA solutions were measured using $\varepsilon_{280} = 10{,}000\ M^{-1}\ cm^{-1}$,[15] and $\varepsilon_{280} = 36{,}520\ M^{-1}\ cm^{-1}$,[16] respectively. All the peptide and protein solutions were prepared in a buffer of 20 mM Tris (Sigma-Aldrich, USA) and 250 μM EDTA (Sigma-Aldrich) at pH 8·0.

For TR-FRET measurements, the mutant protein was labeled at the single cysteine site with TNB by reacting with a 100-fold molar excess of 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) in 8 M urea at pH 8·5. After the labelling reaction is complete (~30 min at 25°C), the labelled protein was separated from free dye and urea by passing the reaction mixture through a PD 10 column. The extent of labelling was confirmed to be >98% by DTT assay.[17]

2.2 *Fluorescence measurements and data analysis*

All the steady-state fluorescence measurements were carried out using a SPEX fluorolog (T-format) FL111 spectrofluorimeter. The fraction of unfolded population (f_U) in the equilibrium denaturation titration of barstar by guanidine hydrochloride (GdnHCl) was obtained by exciting Trp53 at 295 nm and measuring the fluorescence signal at 330 nm. The total signal at any denaturant concentration is represented by $y = y_N f_N + y_U f_U$; according to the two-state model[18] where, f_N and f_U are the fractions of molecules that are folded and unfolded, and y_N and y_U are the signals corresponding to the folded and unfolded populations respectively. $f_N + f_U = 1$.

Time-resolved fluorescence intensity measurements were carried out using a time-correlated single photon counting set-up. For the excitation of tryptophan in proteins, 1 ps pulses at 887 nm from the Ti-sapphire femto/pico second (Spectra Physics, Mountain View, CA) laser, pumped by an Nd : YLF laser (Millenia X, Spectra Physics), were frequency tripled to 295 nm by using a frequency doubler/tripler (GWU, Spectra physics). Excitation wavelength of 290 nm, obtained by frequency tripling of 1 ps pulses at 870 nm, was used as the excitation source for tryptophan in peptides. Similarly, for excitation of fluorescein and rhodamine B dye solutions, 1 ps pulses at 830 nm were frequency doubled to 415 nm. Excitation pulses of 308 nm (4 ps width) radiation, were derived from a cavity-dumped rhodamine 6G dye laser pumped by CW-mode locked Nd : YAG laser for measurements on fluorescein and rhodamine B. The repetition rate of the excitation pulses was 4 MHz and fluorescence decay was monitored by a micro-channel plate photomultiplier (Model R2809U; Hamamatsu Corp.) coupled to a time-correlated single-photon counting setup.[8,19] The time per channel of the instrument was 40 ps. The instrument response function (IRF) at the excitation wavelength was obtained by using a dilute colloidal suspension of dried non-dairy coffee whitener. The full width at half maximum of the IRF was ~40 ps.

The analysis described in this paper requires that the total fluorescence decay from the sample be acquired. The total fluorescence decay, integrated over the entire emission spectrum, was obtained as follows. The photon count rate at the peak of the emission spectrum was adjusted to be about 8×10^3 counts per second. The decay was recorded while scanning the emission monochromator between the lower and upper limits of the spectrum using a suitable cut-off filter to exclude scattered photons at the excitation wavelength. The fluorescence emission range for the fluorescein solution, rhodamine B solution, and the solutions of a mixture of fluorescein and rhodamine B dyes was 475–625 nm, 525–675 nm, and 475–675 nm respectively. Similar measurements on protein and peptide (Trp–Gly, Gly–Trp, and mixtures of Trp–Gly + Gly–Trp) solutions were done in their total emission range of 310–410 nm and 305–450 nm, respectively. In fluorescence-lifetime measurements, the emission was monitored at the magic angle (54·7°) to eliminate the contribution from the decay of anisotropy.

The fluorescence decay curve was analysed by deconvoluting the observed decay with the IRF to obtain the intensity decay function represented as a sum of three or four exponentials:

$$I(t) = \sum \alpha_i \exp(-t/\tau_i)\ \ i = 1\text{–}4, \qquad (1)$$

where $I(t)$ is the fluorescence intensity at time t and α_i is the amplitude of the ith lifetime τ_i such that $\sum_i \alpha_i = 1$.[19]

3. Theory

In a solution containing a mixture of fluorophores, let C_i be the concentration of the ith species and A_i be

its absorbance at an excitation wavelength, λ_{ex}. The passage of a short δ-function like a laser pulse, with an integrated intensity I_0 (in terms of number of photons), through the sample produces the excited states. The number of such excited states, produced at time $t = 0$ in the excitation volume, defined by the sample thickness and the cross section of the beam, will be

$$N_0^* = I_{abs} = I_0(1 - e^{-2.303Ai}). \quad (2)$$

The decay of these excited states at any time t is given by,

$$\frac{dN_t^*}{dt} = -(k_r + \sum k_{nr})N_t^* = -\frac{1}{\tau}N_t^*, \quad (3)$$

where, k_r and k_{nr} are rate constants associated with the radiative and non-radiative processes, and τ is the fluorescence lifetime.

Solving the above equation with the boundary condition that at $t = 0$, $N_t^* = N_0^*$,

$$N_t^* = N_0^* e^{-t/\tau}, \quad (4)$$

Actually in fluorescence spectroscopy, we do not observe the number of excited states, rather we detect the fluorescence intensity (I), defined in terms of number of photons emitted per unit time (s). The radiative decay of the excited population of species A can be presented as,

$$A^* \xrightarrow{k_r} A + \text{photon}.$$

The fluorescence intensity at time t would be,

$$I(t) = k_r N_t^* = k_r N_0^* e^{-t/\tau} \text{ photons/s}. \quad (5)$$

Substituting the value of N_0^* from (2) in (5),

$$I(t) = k_r I_0[1 - e^{-2.303A}]e^{-t/\tau}. \quad (6)$$

Therefore for ith species in solution, the time-dependent emission intensity (integrated over the emission spectrum) is

$$I(t)_i = k_{r,i} I_0[1 - e^{-2.303A_i}]e^{-t/\tau_i}. \quad (7)$$

For a diluted solution, $A_i \ll 1$, and (7) reduces as,

$$I(t)_i = k_{r,i} I_0[2.303A_i]e^{-t/\tau_i}. \quad (8)$$

Also, incorporating the geometry factor, G, of the detection system and the detector sensitivity ψ_i for the ith species in the sample,

$$I(t)_i = (2.303GI_0)(k_{r,i}\psi_i A_i e^{-t/\tau_i}). \quad (9)$$

The value of ψ_i is species-specific. This value is obtained from the emission spectrum of the species and the wavelength-specific detector sensitivity. Specifically, ψ_i (≤ 1) is the ratio of the areas of the emission spectrum uncorrected and corrected for detector sensitivity.

For more than one species (let us say, two species) in the solution,

$$I(t) = 2.303GI_0 \sum_i k_{r,i}\psi_i A_i e^{-t/\tau_i}. \quad (10)$$

The above equation is generally written as multi-exponential decay,

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}. \quad (11)$$

Comparing (10) with (11), we get,

$$\alpha_i = (2.303GI_0)k_{r,i}\psi_i A_i, \quad (12)$$

where, $A_i = \varepsilon_i C_i L$; ε_i is molar extinction coefficient of ith species ($M^{-1}\,cm^{-1}$) and L is sample thickness (cm).

Because of the unknown value for geometry factor, the absolute value of α_i is not useful; however, the ratio,

$$\frac{\alpha_i}{\alpha_j} = \frac{C_i\varepsilon_i k_{r,i}\psi_i}{C_j\varepsilon_j k_{r,j}\psi_j}, \quad (13)$$

is a useful one for quantitative measurement of the ratio of concentrations, radiative rate constants, or extinction coefficients. Since the above equation is derived for total fluorescence of the sample, the ratio, (α_i/α_j) is dependent only on the excitation wavelength, λ_{ex} through ε. It is important to note that this method is valid only when individual species in a mixture differ significantly in terms of their fluorescence decays.

It must be emphasized that the above equation is derived for a mixture of fluorophores with each

fluorophore having a single lifetime. There are examples where the sample is a mixture of molecules and one or more molecules may show multi-exponential fluorescence decay. A mixture of proteins or a protein in two or more conformations (e.g. protein-folding/unfolding) is such an example. Similarly, a single fluorophore in multiple environments displaying multi-exponential decay is common. In such cases it is necessary to modify (13) suitably, so that the concentrations of molecules and associated lifetimes are explicitly stated.

Thus, for a mixture of two species 1 and 2, each with multi-exponential fluorescence decays,

$$I(t)_1 = \sum \alpha_i e^{-t/\tau_i};\ I(t)_2 = \sum \alpha_j e^{-t/\tau_j}. \tag{14}$$

The observed total fluorescence decay from the mixture is,

$$I(t) = \gamma_1 \Sigma_1 \alpha_i e^{-t/\tau_i} + \gamma_2 \Sigma_2 \alpha_j e^{-t/\tau_j}, \tag{15}$$

where, Σ_1 and Σ_2 represent the sum of exponentials, representing the multi-exponential fluorescence decay of the individual species. γ_1 and γ_2 are the amplitude parameters for the analytical fitting of the fluorescence decay; where,

$$\frac{\gamma_1}{\gamma_2} = \frac{C_1 \varepsilon_1 \psi_1}{C_2 \varepsilon_2 \psi_2}. \tag{16}$$

In the above equation, radiative rate constants k_{r1} and k_{r2} do not appear as they have been assumed to have similar values for the fluorophore in both the species.

4. Results and discussion

The validities of (13) and (16) were tested directly on mixtures of various fluorescent molecules.

4.1 *Mixture of fluorescein and rhodamine B dyes*

The validity of (13), showing the relation between the ratios α_i/α_j and C_i/C_j, was directly tested on an equimolar solution mixture of fluorescein and rhodamine B dyes. Time-resolved fluorescence measurements on fluorescein, rhodamine B, and an equimolar mixture of fluorescein and rhodamine B were done at two different excitation wavelengths of 308 nm and 415 nm in the total emission spectral range (figure 1). Fluorescence lifetime measurements on fluorescein and rhodamine B solutions in water give single lifetime values of 4·15 ns and 1·6 ns, respectively. The equimolar solution mixture of fluorescein and rhodamine B gives lifetime values of $\tau(\alpha)$ ns = 4·15 (0·42), 1·6 (0·58); (at λ_{ex} = 308 nm) and $\tau(\alpha)$ *ns* = 4·15 (0·60), 1·6 (0·40); (at λ_{ex} = 415 nm). These measurements gave the experimental values of α_{Rh}/α_{Fl} at 308 and 415 nm.

Radiative rate constants for fluorescein and rhodamine were calculated according to the Strickler-Berg equation,[20]

$$k_r = 2{\cdot}88 \times 10^{-9} n^2 \frac{\int I(\bar{\nu}) \mathrm{d}\bar{\nu}}{\int I(\bar{\nu}) \bar{\nu}^{-3} \mathrm{d}\bar{\nu}} \int \frac{\varepsilon(\bar{\nu})}{\bar{\nu}} \mathrm{d}\bar{\nu}, \tag{17}$$

where, n is the refractive index of the medium, which is 1·333 for water; $I(\bar{\nu})$; is the fluorescence intensity at wave number $\bar{\nu}$. $\varepsilon(\bar{\nu})$ is the extinction coefficient at wave-number $\bar{\nu}$. Using the fluorescence emission spectrum and the extinction coefficient spectrum of the dyes, k_r of fluorescein and rhodamine dyes were calculated and their values were found to be k_r (fluorescein) = $2{\cdot}1 \times 10^8$ s^{-1} and k_r (rhodamine B) = $2{\cdot}52 \times 10^8$ s^{-1}. The values of $\psi_{(Fl)}$ and $\psi_{(Rh)}$ were determined from the areas of the corrected and uncorrected spectra. The values of α_i/α_j at 308 and 415 nm were calculated using (13) and known values of other parameters. Both the calculated and experimental values of ratio α_{Rh}/α_{Fl} were found to agree well with each other (table 1), within error limits.

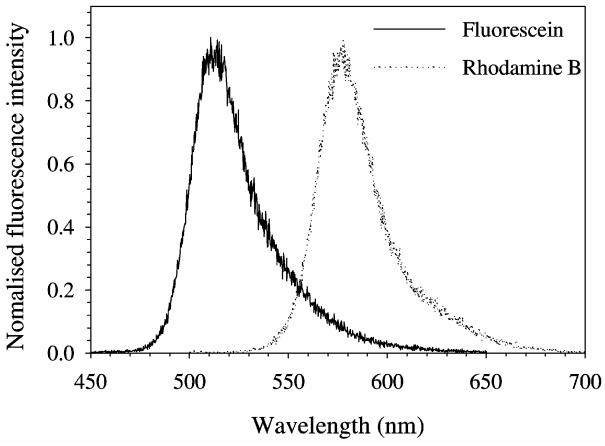

Figure 1. Fluorescence emission spectra of fluorescein and rhodamine B dyes in 20 mM phosphate, pH 8.

Table 1. Calculated and experimental values of ratio, α_{Rh}/α_{Fl} in an equimolar mixture of fluorescein and rhodamine B.

λ_{ex} (nm)	Fluorophore	ε (M^{-1} cm^{-1})	k_r (s^{-1})	ψ	α_{Rh}/α_{Fl} (calculated)	α_{Rh}/α_{Fl} (experimental)
415	Fluorescein	2800	$2{\cdot}10 \times 10^8$	0·085 ± 0·002	0·72 ± 0·07	0·67 ± 0·01
	Rhodamine B	3000	$2{\cdot}52 \times 10^8$	0·048 ± 0·001		
308	Fluorescein	5800	$2{\cdot}10 \times 10^8$	0·085 ± 0·002	1·57 ± 0·15	1·40 ± 0·04
	Rhodamine B	13600	$2{\cdot}52 \times 10^8$	0·048 ± 0·001		

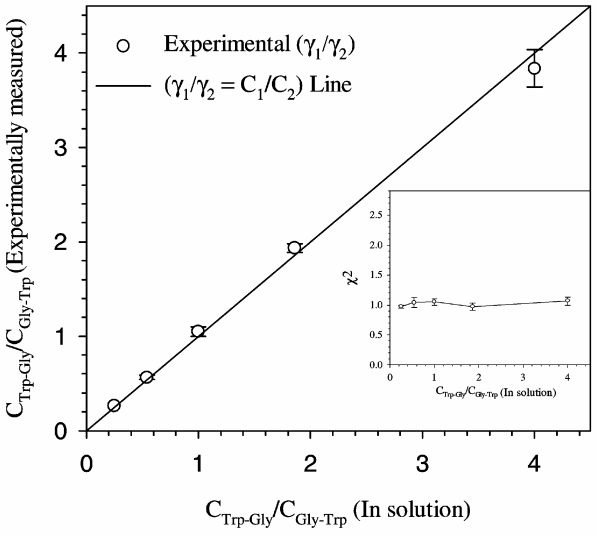

Figure 2. Ratio of concentrations of Trp–Gly and Gly–Trp peptides, $C_{Trp-Gly}/C_{Gly-Trp}$ determined experimentally by time-resolved measurements vs the actual ratio of concentrations in the solution. The data are shown by open circles with error bars. The inset represents the variation in the chi square (χ^2) value of the two component analytical fitting of total fluorescence decays.

4.2 *Mixture of Trp–Gly and Gly–Trp peptides*

Quantitative analysis of total fluorescence decay obtained from a mixture of two peptides or proteins, where each species exhibits a multi-exponential fluorescence decay, requires a different approach. In this procedure, the analysis is constrained so that only the unknown values (ratio γ_1/γ_2) are obtained from the analysis. The validity of this method was checked by using mixture of the dipeptides Trp–Gly and Gly–Trp, the fluorescence properties of which are different from each other. The total fluorescence decay of the dipeptide is multi-exponential. The fluorescence lifetime (amplitude) parameters for Trp–Gly are, $\tau(\alpha)$ *ns* = 6·8 (0·01), 1·85 (0·84), 0·45 (0·15) and those for Gly–Trp are, $\tau(\alpha)$ ns = 1·26 (0·44), 0·58 (0·26), 0·14 (0·30). The multi-exponential behaviour of the tryptophan fluorescence decay in these peptides was due to various χ1 rotamers of the Trp residue, originating from rotation about the C^{α}–C^{β} bond.[19,21] The total fluorescence decay of the mixture is also multi-exponential and in principle is expected to be a six-exponential decay. The experimentally measured decay for the mixture (1 : 1 and other ratios) shows a best fit to three or four exponentials. These lifetime and amplitude values do not have any relationship to the lifetimes and amplitudes of the pure dipeptides. This reduction in the number of exponentials and the observation of new lifetimes in the mixtures are not due to the formation of new species, but due to the lack of precision (signal to noise ratio) of the numerical data in the decay for reliable estimation of six-exponentials. The procedure of constrained analysis assumes that the lifetime and amplitude parameters are unchanged for each dipeptide in the mixture. The total fluorescence decay of the mixture is then expressed by (15).

According to (16), the experimentally determined ratio (γ_1/γ_2) should be equal to the concentration ratio (C_1/C_2) of the dipeptides if the difference in absorption spectra and/or emission spectra of the two dipeptides is negligible. Figure 2 shows the good correlation of the concentration ratios determined experimentally with the actual values for various mixture solutions of the two peptides validating the relation in (16).

4.3 *Mixture of proteins*

The validity of the method was also checked by determining the ratio of concentrations in a mixture of two proteins, each having multi-exponential fluorescence decay, from the total fluorescence decay measurements. Samples containing two different proteins, each with a single tryptophan as the flourophore, were chosen for this study. The proteins used were HSA (Human serum albumin) with a size of 66·4 kDa and barstar, an intracellular inhibitor of extracellular RNase barnase in *Bacillus amyloliquefaciens*, with a size of 10 kDa. Both HSA and the mutant variant of barstar used here had a single tryptophan at locations

214 and 53, respectively, which served as the natural fluorophore in the proteins. Their fluorescence decays were collected in the total emission range of 310–410 nm. Trp53 of barstar showed values for the lifetime of $\tau(\alpha)$ *ns* = 4·93 (0·88), 1·84 (0·12) and Trp214 of HSA showed $\tau(\alpha)$ *ns* = 7·04 (0·50), 2·98 (0·33), 0·46 (0·17).

Figure 3 shows the correlation between the concentration ratio determined experimentally, with its actual value for various mixtures of the two proteins, validating the method described in this work. The inset of figure 3 is the representation of the goodness of fit (chi square, χ^2) of fluorescence intensity decays for two components at various concentration ratios of the two proteins.

4.4 *Folding/unfolding of barstar*

Barstar is a well-studied model system for protein-folding/unfolding studies. Its denaturant-induced equilibrium folding/unfolding transition has been modelled as a two-state transition (N ↔ U).[13,14] This means the protein at any denaturant concentration exists as a mixture of native and unfolded populations. Wild-type barstar contains three tryptophans (Trp38, Trp44 and Trp53) and two cysteines (Cys40 and Cys82). In our experiments, we used a mutant variant of barstar with a single tryptophan (Trp53) and a single cysteine (Cys82).

GdnHCl-induced equilibrium unfolding of the protein was carried out by measuring fluorescence from the single Trp53 at 330 nm. Figure 4 represents the fractions of unfolded protein at various concentrations of GdnHCl modelled from Trp53 fluorescence intensity measurements at 330 nm. This curve represents the equilibrium populations of the native and unfolded protein molecules at various concentrations of the denaturants according to the two-state model. The data in figure 4, shown by open triangles, represent the fraction of unfolded protein molecules at various concentrations of GdnHCl, obtained by the analysis of fluorescence decays of Trp53 for two components. The goodness of fit of these decays for two components is shown in terms of the chi square, χ^2, value (~1) by open circles in figure 5. The data obtained both from the steady-state fluorescence and the two component analysis of fluorescence intensity decays at various GdnHCl concentrations coincide with each other. Thus two independent measurements of fraction of unfolded protein molecules at various denaturant concentrations show that equilibrium unfolding of barstar appears to be a two-state transition

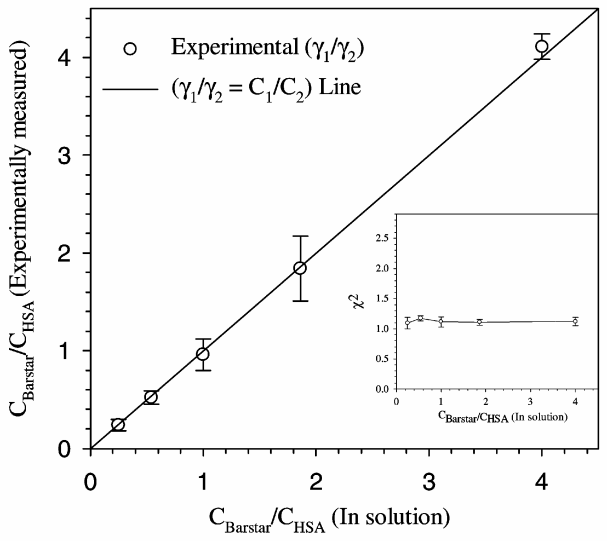

Figure 3. Ratio of concentrations of proteins, barstar and HSA, $C_{Barstar}/C_{HSA}$ determined experimentally by time-resolved measurements vs the actual ratio of concentrations in the solution. The data are shown by open circles with error bars. The inset represents the variation in the chi square (χ^2) value of the two-component analytical fitting of total fluorescence decays of mixtures of barstar and HAS proteins at the various concentration ratios.

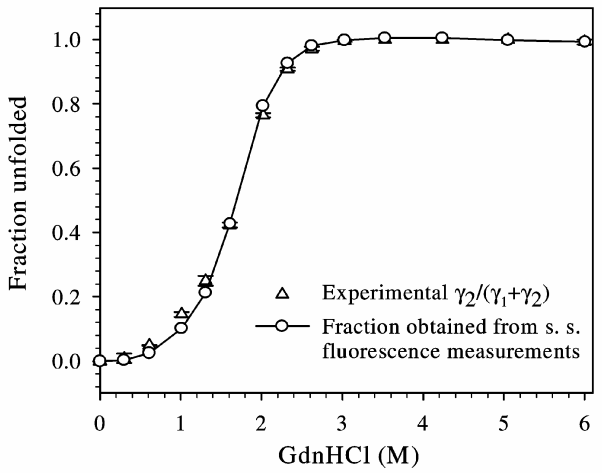

Figure 4. Fraction of unfolded barstar protein as a function of the denaturant (GdnHCl) concentration obtained by steady-state Trp53 fluorescence intensity measurements (open circles and the solid line through them). The fractions obtained from the concentration ratio estimation by time-resolved fluorescence measurements are shown by open triangles with error bars.

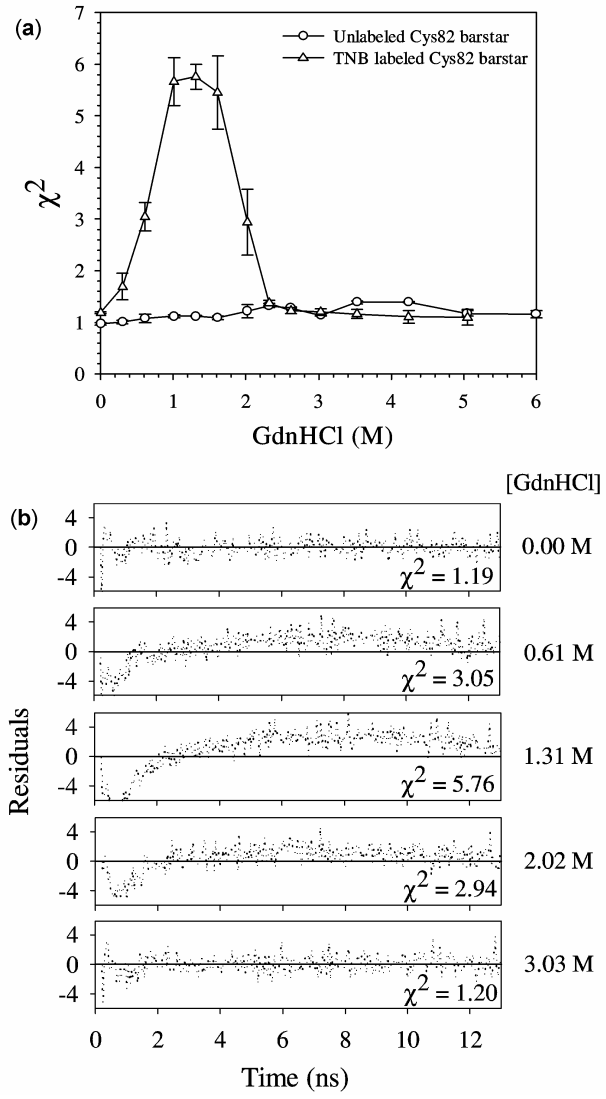

Figure 5. (**a**) Variation in chi square (χ^2) value of the analytical fitting of total fluorescence decay, of donor (Trp53) in the absence of acceptor (shown by open circles) and in the presence of acceptor, TNB, attached to Cys82 (when the fluorescence lifetimes are controlled by FRET and shown by open triangles) of barstar at various GdnHCl concentrations, for a two-component model. (**b**) Residuals (dotted lines) of the analytical fitting of total fluorescence decay, of donor (Trp53) in the presence of acceptor, TNB, attached to Cys82 (FRET controlled lifetimes) of barstar at various GdnHCl concentrations, for a two component model. The misfit of the data ($\chi^2 > 1$ in panel a and non-random distribution of residuals in panel b) is seen prominently in the unfolding transition zone (0·5–2·0 M GdnHCl) for FRET-controlled lifetimes.

when monitored by either steady-state or time-resolved fluorescence of Trp53.

However, when the equilibrium folding of barstar was studied by TR-FRET measurements, the total fluorescence decay of Trp53 in the presence of the acceptor, thionitrobenzoic acid (TNB) attached at single Cys82 site of the protein could not be analysed by a two-component (native and unfolded species) model. This suggests that changes in the intramolecular distance between Trp53 and Cys82 do not follow a two-state model even though steady-state fluorescence intensity data can be fitted to a two-state model. This is represented in figure 5 in terms of the variation in the chi square (χ^2) value from the analytical fitting for two components, of fluorescence decay of the donor Trp53 in the absence of acceptor, TNB (shown by open circles) and in the presence of acceptor, TNB, attached to Cys82 (shown by open triangles) during titration with GdnHCl. Although the fluorescence decays of the donor, Trp53, alone could be well fitted ($\chi^2 \sim 1$), the decays of Trp53 in the presence of acceptor, TNB (which induces FRET) could not be fitted satisfactorily ($\chi^2 > 1{\cdot}5$) to only two components in the whole concentration range of GdnHCl. The non-random distribution of residuals (figure 5b) obtained from two-state fitting of TR-FRET data confirms further that folding-unfolding transition cannot be modelled by a two-state process.

Although the equilibrium unfolding structural transition of barstar, when monitored by either CD or fluorescence intensity measurements can be fitted satisfactorily by a two-state model, steady-state and time-resolved fluorescence anisotropy measurements revealed the presence of partially structured forms.[22] This suggested that the structural transition during the equilibrium unfolding of barstar can not be a two-state process. In the absence of FRET, the fluorescence lifetime of single Trp53 which is buried in the core of folded barstar is sensitive only to the environment in its immediate vicinity. Hence, if the intermediates present on the equilibrium folding path have the fluorescence lifetime of single Trp53 almost similar to that in either the folded or unfolded protein, then fluorescence lifetime measurements would be blind to the intermediates and the folding process would seem to be a two-state one. This is confirmed in the present work, where the fluorescence decay of Trp53, in the absence of FRET, at various concentrations of GdnHCl can be fitted satisfactorily to a two-component (folded and unfolded) model (chi-square, $\chi^2 \sim 1$; open circles in figure 5). In TR-FRET experiments, the fluorescence lifetime of the donor

fluorophore in the presence of an acceptor is highly sensitive to the distance between donor and acceptor on the protein. In this context, TR-FRET-based experimental parameters become much more sensitive to changes in structure in various regions of a protein. In fact, TR-FRET measurements between Trp53 and the thionitrobenzoic acid moiety attached to Cys82 in barstar, coupled with MEM analysis have shown the equilibrium unfolding transition to be multi-state with continuous expansion of folded structure to the unfolded form.[9] The present study also suggests that the equilibrium unfolding of barstar is not a two-state process but it may be occurring through intermediate states, which could have TR-FRET parameters different from those in either the folded or unfolded forms. Thus, the analytical method described in the present work is useful to check whether the folding of a protein follows two-state or multi-state structural transition.

5. Conclusions

The present work demonstrates the application of two-component analysis of total fluorescence decay of a fluorophore in a protein, for easy and robust discrimination between 'two-state' and 'multi-state' models for the structural transition in folding.

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