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# Organization and dynamics of tryptophans in the molten globule state of bovine α-lactalbumin utilizing wavelength-selective fluorescence approach: Comparisons with native and denatured states

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ABSTRACT

Bovine α-lactalbumin (BLA) is known to be present in molten globule form in its *apo*-state (*i.e.*, $Ca^{2+}$ depleted state). We explored the organization and dynamics of the functionally important tryptophan residues of BLA in native, molten globule and denatured states utilizing the wavelength-selective fluorescence approach. We observed red edge excitation shift (REES) of 7 nm for the tryptophans in native BLA. Interestingly, we show here that BLA tryptophans exhibit considerable REES (8 nm) in its molten globule state. Taken together, these results indicate that tryptophan residues in BLA in native as well as molten globule states experience motionally restricted environment. We further show that even the denatured form of BLA exhibits a modest REES of 3 nm, indicating that the tryptophans are shielded from bulk solvent, even when denatured, due to the presence of residual structure around tryptophan(s). This is further supported by wavelength-dependent changes in fluorescence anisotropy and lifetime for BLA tryptophans. These novel results constitute one of the first reports of REES in the molten globule state of proteins, and could provide vital insight into the role of tryptophans in the function of BLA in its molten globule state in particular, and other partially ordered proteins in general.



## 1. Introduction

The discovery of intrinsically disordered yet functional proteins has altered the paradigm of structure–function relationship of proteins from the rigid three-dimensional structures, earlier believed to be a mandatory prerequisite of protein function [1–3]. The fact that more than 50% of the total eukaryotic proteins and ~75% of signaling proteins in mammals contain at least one long disordered region (>30 residues) [1,3] has changed the earlier structure–function dogma. In view of this, monitoring the dynamics of proteins, not only in ordered forms but also in disordered forms, such as the molten globule form, assumes relevance. The molten globule state is considered to be an important intermediate in protein folding, and was initially proposed as a partly folded state with stable native-like secondary structure but lacking a specific tertiary structure [4,5]. Molten globule states are now considered to be a milieu of conformations with varying degrees of disorder. Bovine α-lactalbumin (BLA) is a small acidic $Ca^{2+}$-binding protein (mol. wt. 14,200) present in milk and functions as a specificity modifier of galactosyltransferase [6,7]. Interestingly, BLA serves as a useful model for the protein folding problem since it has several partially folded intermediate states and is known to be present in molten globule form under various conditions. For example, it is extensively used to study the molten globule state since it assumes the molten globule state at acidic pH and in the *apo*-state [4,6]. The *apo*-state molten globule is generated by removal of $Ca^{2+}$ at neutral pH and low ionic strength in a narrow range of temperature [6]. BLA has four tryptophans (at positions 26, 60, 104 and 118) out of which Trp-118 belongs to aromatic cluster I, while the other three tryptophans are part of aromatic cluster II [8]. The tryptophans at positions 104 and 118 are conserved in α-lactalbumins among various species since they are involved in the binding of α-lactalbumin to galactosyltransferase and the stimulation of its lactose synthase activity [9,10]. Importantly, tryptophan residues have been reported to be crucial for the global stability of α-lactalbumin [11].

In this paper, we have monitored the organization and dynamics of the functionally important tryptophan residues of BLA in native, denatured and *apo*-state molten globule conditions utilizing the wavelength-selective fluorescence approach. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy that can be used to directly monitor the environment and dynamics around a fluorophore in a complex system [12–15]. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths,

*Abbreviations:* BLA, bovine α-lactalbumin; FRET, fluorescence resonance energy transfer; REES, red edge excitation shift

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caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). REES arises due to slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which depends on the motional restriction imposed on the solvent molecules (or the dipolar environment, as in green fluorescent protein [16]) in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. We have previously shown that REES serves as a sensitive tool to monitor the organization and dynamics of peptides and proteins in solution [17–19], and when bound to membranes [20]. Since the dynamics of hydration is directly associated with the function of proteins, REES has proved to be a useful tool to explore the organization and dynamics of soluble and membrane proteins under varying degrees of hydration [21,22]. This makes the use of REES in particular, and the wavelength-selective fluorescence approach in general, extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events including protein folding [23].

## 2. Materials and methods

### 2.1. Materials

Calcium depleted BLA, ultrapure grade urea, $CaCl_2$ and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

### 2.2. Sample preparation

Native BLA solution was prepared by dissolving calcium depleted BLA (*apo*-form) in 10 mM Tris, 1 mM $CaCl_2$, pH 7.4 buffer. The molten globule form of BLA was generated by dissolving calcium depleted BLA (*apo*-form) in 10 mM Tris, pH 7.4 buffer at ~23 °C. Native BLA was denatured by incubating in 8 M urea for 2 h. Concentration of pure BLA in buffer was estimated using its molar extinction coefficient (28,540 $M^{-1}$ $cm^{-1}$) at 280 nm [24]. The final protein concentration in all cases was 32 μM. Experiments were carried out at ~23 °C.

### 2.3. Fluorescence measurements

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes as described previously [18]. Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using IBH 5000F NanoLED equipment (Horiba Jobin Yvon, Edison, NJ) with DataStation software in the time-correlated single photon counting mode as described previously [25]. A pulsed light emitting diode (LED) (NanoLED-17) was used as an excitation source. This LED generates optical pulse at 294 nm of pulse duration less than 750 ps, and is run at 1 MHz repetition rate. Intensity-averaged mean lifetimes $\langle\tau\rangle$ for triexponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the following equation [26]:

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2 + \alpha_3\tau_3^2}{\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3} \quad (1)$$

### 2.4. Circular dichroism measurements

CD measurements were carried out at room temperature (~23 °C) on a JASCO J-815 spectropolarimeter as described previously [18].

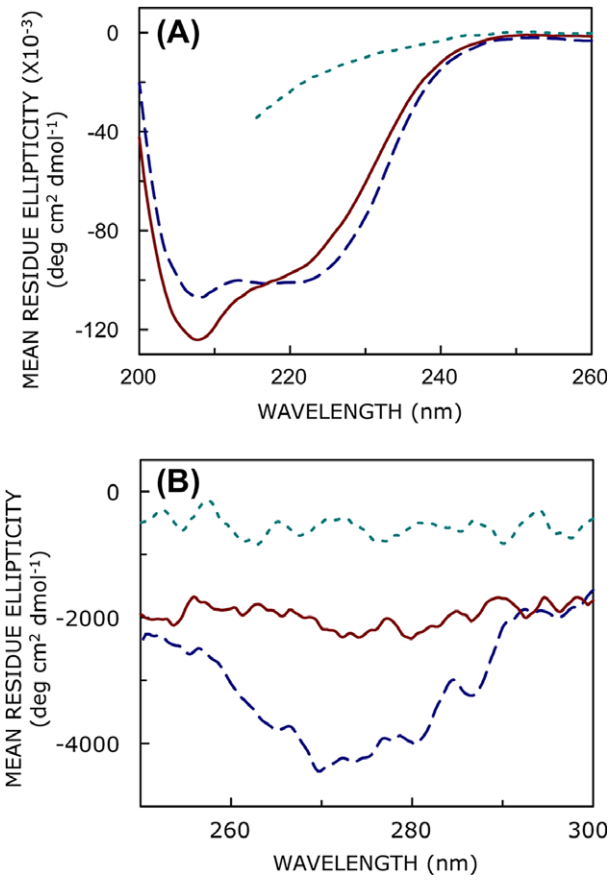


**Fig. 1.** Representative (A) far-UV and (B) near-UV CD spectra of BLA in native (blue, – –), molten globule (red, —) and urea-denatured (cyan, - - - -) states. The concentration of BLA was 32 μM in all cases. See Section 2 for other details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 3. Results

Circular dichroism (CD) spectroscopy represents a convenient approach to monitor molten globule conformations [4]. The far- and near-UV CD spectra of BLA in various conditions are shown in Fig. 1. The calcium depleted *apo*-form BLA in low ionic strength buffer shows lack of appreciable tertiary structure in near-UV CD (panel B) and representative secondary structure in the far-UV region (panel A), thereby confirming its molten globule character. The far- and near-UV CD spectra of BLA in presence of 1 mM $CaCl_2$ shows representative native structure. The urea-denatured BLA, on the other hand, shows concomitant loss of secondary and tertiary structures (but see later).

Fig. 2 shows the fluorescence emission spectra of BLA in various conformations. As shown in the figure, tryptophans in native BLA exhibit an emission maximum at 330 nm[1] in agreement with previous literature [27]. The emission spectrum of BLA in molten globule state exhibits a red shift and the maximum is shifted to 338 nm (*i.e.*, a red shift of 8 nm with respect to the native state). This indicates a partially disordered conformation due to the loss of tertiary

[1] We have used the term maximum of fluorescence emission in a somewhat broader sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass of the fluorescence emission, in the symmetric part of the spectrum. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.

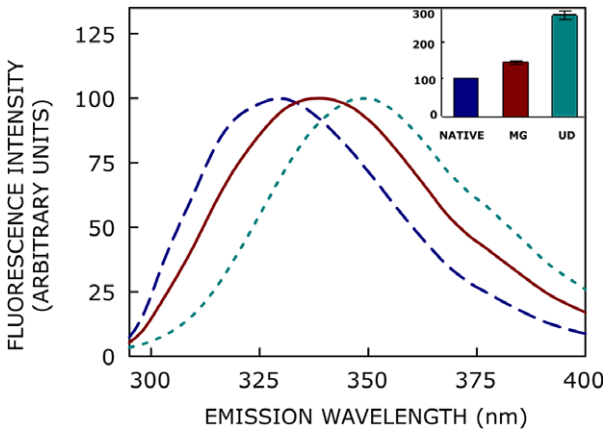


**Fig. 2.** Representative fluorescence emission spectra of BLA in native (blue, – –), molten globule (red, —) and urea-denatured (cyan, - - - -) states. The inset shows relative fluorescence intensities of BLA in native, molten globule (MG) and urea-denatured (UD) conformations at their respective emission maximum. The excitation wavelength was 280 nm in all cases. Spectra are intensity-normalized at the respective emission maximum. Data shown are means ± SE of three independent measurements. All other conditions are as in Fig. 1. See Section 2 for other details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structure, allowing more water penetration near some of the tryptophan residues. Fig. 2 shows that the emission spectrum of urea-denatured BLA displays a further red shift and the emission maximum is at 348 nm. This red shift can be attributed to increased exposure of BLA tryptophans to water upon denaturation. Interestingly, tryptophan in water exhibits an emission maximum of 355 nm [28]. The emission maximum of 348 nm for denatured BLA therefore indicates that the tryptophans are not completely exposed to water, even when denatured.

Fig. 2 (inset) shows the relative fluorescence intensities of BLA in various conformations. The denatured form exhibits a pronounced increase (~172%) in fluorescence intensity relative to the native form. The increase in fluorescence intensity in case of the molten globule form is more modest (~43%). This indicates the possible release of intramolecular quenching of tryptophan fluorescence in these states. This implies that the tryptophan residues in the native conformation are surrounded by amino acids that may quench its fluorescence. Previous work using homeodomain proteins have shown that the intramolecular quenching of tryptophan fluorescence could be due to mechanisms such as excited state electron transfer (with neighboring residues), excited state proton transfer or through self energy transfer (homo-FRET) between tryptophan residues [29]. In addition, phenylalanine and tyrosine residues are known to quench tryptophan fluorescence by NH…π interaction [29]. It is evident from the crystal structure of BLA [8] that some of the tryptophan residues are in close proximity of amino acids that could induce quenching. For example, Trp-26 is in the vicinity of Lys-16 (within 5.2 Å), His-107 (7.8 Å), residues that could act as potential quenchers of tryptophan fluorescence. Trp-26 is also in close proximity to Trp-104 (4.05 Å), Trp-60 (11.36 Å) and Trp-118 (11.3 Å). These distances are well suited for homo-FRET among tryptophans [30]. Interestingly, some of the tryptophan residues are also close to either phenylalanine or tyrosine residues. For example, Trp-104 is close to Tyr-103 (3.51 Å) and Phe-53 (4.15 Å), Trp-60 is in the vicinity of Tyr-103 (3.59 Å), and Trp-118 is in close proximity to Phe-31 (3.59 Å). Upon molten globule formation or denaturation, some of these intramolecular quenchings are released due to conformational change, as shown from the increase in fluorescence intensity and lifetime (see Fig. 4A). Interestingly, the increase in intensity is not proportional to the increase in lifetime (particularly, in case of denatured BLA), suggesting that the mechanism of quenching could be a combination of static and dynamic quenching [29].

The shifts in the maxima of fluorescence emission of BLA as a function of excitation wavelength are shown in Fig. 3A. Upon excitation at 280 nm, tryptophans in native BLA exhibit an emission maximum at 330 nm. As the excitation wavelength is changed from 280 to 307 nm, the emission maximum of native BLA is shifted from 330 to 337 nm, which corresponds to a REES of 7 nm (see inset in Fig. 3A). Such a shift in the wavelength of emission maximum with change in excitation wavelength is characteristic of the red edge effect and indicates that the tryptophans in native BLA experience motionally restricted environment. Since BLA is a multitryptophan protein, REES could be indicative of the average environment experienced by the tryptophans. Nevertheless, such a result would directly imply that the regions surrounding at least some of the BLA tryptophans offer considerable restriction to the reorientational motion of the solvent (water) dipoles around the excited state tryptophans.

The fluorescence emission spectrum of BLA in molten globule state displays a red shift and the emission maximum is shifted to 338 nm (see Fig. 2). Analysis of REES effect in the molten globule

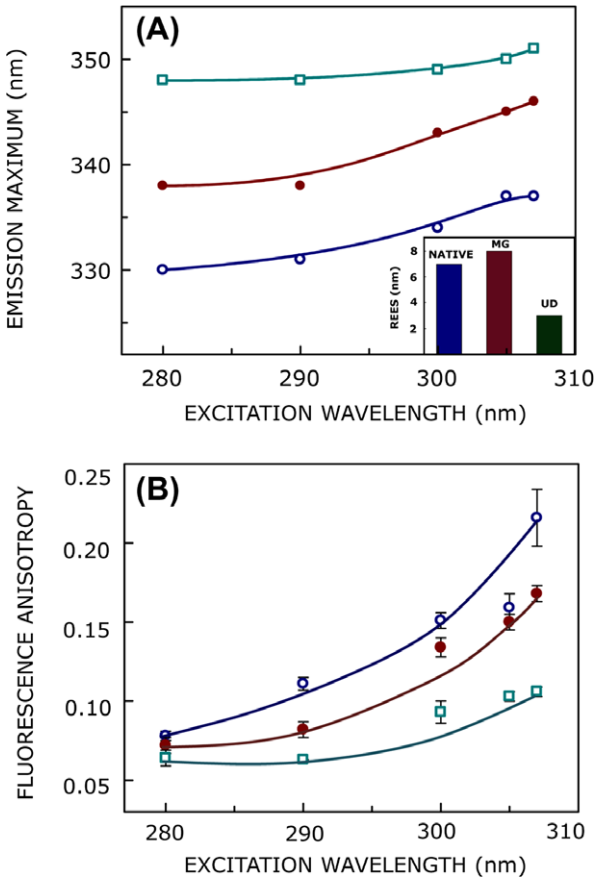


**Fig. 3.** (A) Effect of changing excitation wavelength on the wavelength of maximum emission of BLA in native (blue, ○), molten globule (red, ●) and urea-denatured (cyan, □) states. The inset shows the magnitude of REES obtained for BLA in various conformations. The magnitude of REES corresponds to the total shift in emission maximum when the excitation wavelength is changed from 280 to 307 nm. (B) Fluorescence anisotropy of BLA as a function of excitation wavelength in native (blue, ○), molten globule (red, ●) and urea-denatured (cyan, □) states. The emission wavelength was fixed at 330, 340 and 350 nm for native, molten globule and urea-denatured states, respectively. Data shown are means ± SE of three independent measurements. All other conditions are as in Fig. 1. The lines joining the data points are provided merely as viewing guides. See Section 2 for other details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

state provides interesting results. Fig. 3A shows that as the excitation wavelength is changed from 280 to 307 nm, the emission maximum of the tryptophans is shifted from 338 to 346 nm, which corresponds to a REES of 8 nm (see inset in Fig. 3A). We have previously reported REES of tryptophan(s) in case of native (ordered) [17,19] and denatured [18] proteins. Our present results show that tryptophan(s) in the molten globule state of BLA exhibit REES. These results constitute one of the first reports of REES in the molten globule state of proteins. Although the magnitude of REES is slightly increased in the molten globule state compared to REES displayed in the native state, the organization of tryptophan residues appear to be altered in the molten globule state. For example, all four tryptophans are largely solvent-excluded in the native conformation, while only Trp-26 is buried in a hydrophobic core with low solvent accessibility in the molten globule state [31].

Fig. 3A shows that the fluorescence emission maximum of urea-denatured BLA exhibits a further red shift and is at 348 nm. As the excitation wavelength is changed from 280 to 307 nm, the emission maximum of the tryptophans is shifted from 348 to 351 nm corresponding to a relatively modest REES of 3 nm (see inset in Fig. 3A). Although tryptophans in denatured proteins generally do not exhibit REES due to fast solvent relaxation in the denatured state [17], we have previously reported that denatured erythroid spectrin exhibits REES due to residual structures that remain even after denaturation in 8 M urea [18]. The presence of REES in urea-denatured BLA, along with the emission maximum of 348 nm, indicate that some amount of structure is maintained (residual structure) around the tryptophan(s), even when denatured. This is further supported by previous NMR results showing that BLA does not unfold fully in presence of 8 M urea [32].

In addition to the shift in emission maximum on red edge excitation, fluorescence anisotropy is known to be dependent on excitation wavelength in motionally restricted media [12]. The excitation anisotropy spectra (*i.e.*, a plot of steady state anisotropy *vs*. excitation wavelength) of BLA in native, molten globule and urea denatured states are shown in Fig. 3B. The figure shows that the anisotropy of BLA tryptophans undergoes considerable change upon altering the excitation wavelength from 280 to 307 nm, with a sharp increase toward the red edge of the absorption band. Such a characteristic increase in anisotropy upon red edge excitation for peptides and proteins containing tryptophans in media of reduced mobility has been previously reported [17]. Another possible reason for the increase in anisotropy at the red edge of excitation could be the reduced efficiency of self energy transfer (homo-FRET) among tryptophan residues, sometimes referred to as Weber's red edge effect [33]. This reinforces our earlier conclusion that at least some of the tryptophans in BLA are in a motionally restricted region (due to the presence of slow reorienting solvent shell) not only in its native state, but also in molten globule state and even in denatured state.

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is localized. All fluorescence decays could be fitted well with a triexponential function. We chose to use the mean fluorescence lifetime as an important parameter since it is independent of the method of analysis and the number of exponentials used to fit the decay. The mean fluorescence lifetimes of BLA tryptophans were calculated using Eq. (1) and are shown in Fig. 4A. The figure shows that while the mean fluorescence lifetime of BLA tryptophans in the native state is ~2.4 ns, it is increased considerably in both the molten globule state (~3.5 ns) and the urea denatured state (~3.9 ns). In general, tryptophan lifetimes are known to be reduced when exposed to polar environments [34]. Since molten globule state is less ordered than the native state, more water penetration is expected resulting in a reduction in fluorescence lifetime. However, there are other factors that need to be considered while interpreting changes in fluorescence lifetime. The increase in mean fluorescence lifetime

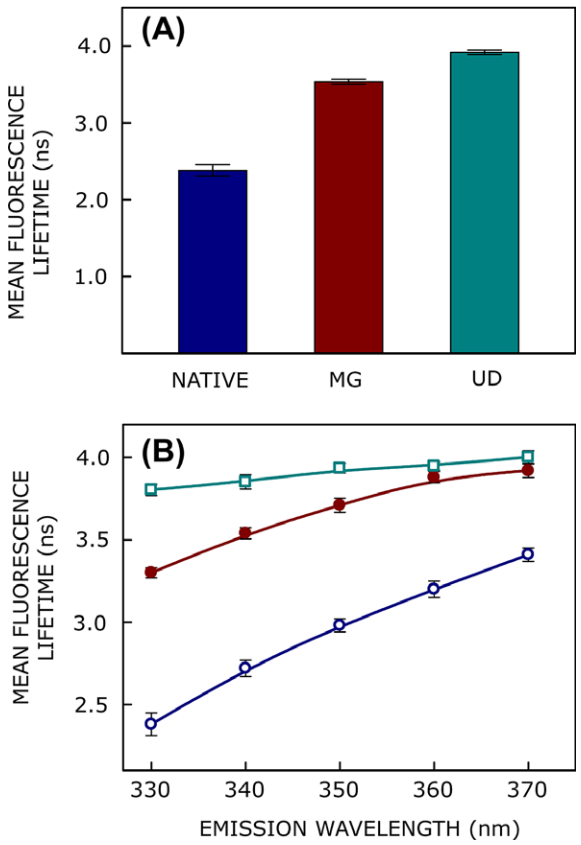


**Fig. 4.** (A) Mean fluorescence lifetime of BLA in native, molten globule (MG) and urea-denatured (UD) states. The emission wavelength was fixed at 330, 340 and 350 nm for native, molten globule and urea-denatured states, respectively. (B) Mean fluorescence lifetime of BLA as a function of emission wavelength in native (blue, ○), molten globule (red, ●) and urea-denatured (cyan, □) states. The excitation wavelength was 294 nm in all cases. Mean fluorescence lifetimes were calculated using Eq. (1). Data shown are means ± SE of three independent measurements. All other conditions are as in Fig. 1. See Section 2 for other details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of BLA tryptophans in the molten globule state could be due to the reorganization of the tryptophan residues in the molten globule conformation, thereby altering the solvent accessibility of some of the tryptophan residues. This increase in mean fluorescence lifetime could be possibly due to release of quenching by neighboring amino acids (see above) as a result of reorganization of the structure in these states. Urea denaturation results in further increase in fluorescence lifetime (see Fig. 4A). The increase in lifetime, however, could be partly attributed to release of quenching upon denaturation (as discussed above). Another important factor, relevant in case of the urea denatured state, is the viscosity of the medium that increases considerably. An increase in medium viscosity generally brings about an increase in fluorescence lifetime [35]. This would result in an increase in mean fluorescence lifetime upon denaturation by urea.

The change in mean fluorescence lifetime of BLA tryptophans in various states as a function of increasing emission wavelength are shown in Fig. 4B. Interestingly, the mean fluorescence lifetime displays a considerable increase in all cases with increasing emission wavelength from 330 to 370 nm. Similar observation of increasing lifetime with increasing emission wavelength has previously been reported for tryptophans in environments of restricted mobilities [17]. Such increasing lifetimes across the emission spectrum may be interpreted in terms of solvent reorientation around the excited state fluorophore [12].

## 4. Discussion

In this paper, we have utilized the wavelength-selective fluorescence approach to monitor the organization and dynamics of the functionally important tryptophan residues of BLA in native, molten globule and denatured states. We observed REES of 7 nm for the tryptophans in native BLA. This indicates that the tryptophans in native BLA experience motionally restricted environment and that the regions surrounding at least some of the BLA tryptophans offer considerable restriction to the reorientational motion of the solvent (water) dipoles around the excited state tryptophans. Interestingly, our results show that BLA tryptophan residues exhibit considerable REES (8 nm) in its molten globule state. This is a novel observation since solvent relaxation studies of partially disordered yet functional proteins is only beginning to be addressed [36]. Our results therefore constitute one of the first reports of REES in the molten globule state of proteins. We also show that even the denatured form of BLA exhibits a modest REES of 3 nm, similar to our earlier results with erythroid spectrin [18].

The molten globule conformation has acquired much relevance in cellular processes since it has been shown that molten globules are involved in interaction with molecular chaperones, translocation across biological membranes, amyloid formation and in gene regulation. All these processes require partially unfolded proteins. Understanding the dynamic environment of tryptophan residues in molten globule-like conformations in proteins could therefore lead to a better understanding of their function.

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