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ORIGINAL PAPER

Exploring tryptophan dynamics in acid-induced molten globule state of bovine α-lactalbumin: a wavelength-selective fluorescence approach

Devaki A. Kelkar · Arunima Chaudhuri · Sourav Haldar · Amitabha Chattopadhyay

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Abstract The relevance of partially ordered states of proteins (such as the molten globule state) in cellular processes is beginning to be understood. Bovine α-lactalbumin (BLA) assumes the molten globule state at acidic pH. We monitored the organization and dynamics of the functionally important tryptophan residues of BLA in native and molten globule states utilizing the wavelength-selective fluorescence approach and fluorescence quenching. Quenching of BLA tryptophan fluorescence using quenchers of varying polarity (acrylamide and trichloroethanol) reveals varying degrees of accessibility of tryptophan residues, characteristic of native and molten globule states. We observed red edge excitation shift (REES) of 6 nm for the tryptophans in native BLA. Interestingly, we show here that BLA tryptophans exhibit REES (3 nm) in the molten globule state. These results constitute one of the early reports of REES in the molten globule state of proteins. Taken together, our results indicate that tryptophan residues in BLA in native as well as molten globule states 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 water dipoles around the excited-state tryptophans. These results are supported by wavelength-dependent changes in fluorescence anisotropy and lifetime for BLA tryptophans. These results 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.

Keywords Molten globule · α-Lactalbumin · Red edge excitation shift · Fluorescence quenching · Fluorescence anisotropy · Fluorescence lifetime

Abbreviations

BLA	Bovine α-lactalbumin
GLA	Goat α-lactalbumin
CD	Circular dichroism
MG	Molten globule
REES	Red edge excitation shift
TCE	Trichloroethanol

Introduction

The molten globule state is considered to be an important intermediate in protein folding, and was initially proposed as a partially folded state with stable native-like secondary structure but lacking a specific tertiary structure (Dolgikh et al. 1981; Ohgushi and Wada 1983). Molten globule states are now considered to be a milieu of conformations with varying degrees of disorder (De Laureto et al. 2002). Over the years, 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

D. A. Kelkar · A. Chaudhuri · S. Haldar · A. Chattopadhyay (✉)
Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Uppal Road, Hyderabad 500 007, India
e-mail: amit@ccmb.res.in

Present Address:
D. A. Kelkar
Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR 97239, USA

(Flynn et al. 1993; Booth et al. 1997; Ren et al. 1999; Wright and Dyson 1999). All these processes require partially unfolded proteins.

Bovine α-lactalbumin (BLA) is a small acidic Ca^{2+}-binding protein (mol. wt. 14.2 kDa) present in milk; it functions as a specificity modifier of galactosyltransferase. 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 (Kuwajima 1996; Permyakov and Berliner 2000). 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 (Dolgikh et al. 1981; Kuwajima 1996). α-Lactalbumins undergo conformational transition induced by low pH, giving rise to molten globule intermediates (referred to as A states), which are slightly expanded compared with the native state but retain a compact shape (Kuwajima 1989). Low-pH-induced molten globules are regarded as prototypes of classical molten globules. BLA is intrinsically fluorescent due to the presence of four tryptophans (at positions 26, 60, 104, and 118), of which Trp-118 belongs to aromatic cluster I while the other three tryptophan residues are part of aromatic cluster II (Chrysina et al. 2000). 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 (Vanhooren et al. 2006; Grobler et al. 1994). Importantly, tryptophan residues have been reported to be crucial for the global stability of α-lactalbumin (Vanhooren et al. 2005).

In this work, we monitored the organization and dynamics of the functionally important tryptophan residues of BLA in native, and acid-induced molten globule conditions by application of the wavelength-selective fluorescence approach. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex system (Mukherjee and Chattopadhyay 1995; Demchenko 2002, 2008; Chattopadhyay 2003; Raghuraman et al. 2005). A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime (Mukherjee and Chattopadhyay 1995; Demchenko 2002, 2008; Chattopadhyay 2003; Raghuraman et al. 2005). 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 (Haldar and Chattopadhyay 2007)] 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. The unique feature of REES is that, while all other fluorescence techniques (such as fluorescence quenching, energy transfer, and anisotropy measurements) yield information about the fluorophore itself, REES provides information about the relative rates of solvent relaxation dynamics, which cannot be obtained using other techniques. We have previously shown that REES serves as a sensitive tool to monitor the organization and dynamics of peptides and proteins in solution (Guha et al. 1996; Chattopadhyay et al. 2003; Kelkar et al. 2005; Raghuraman and Chattopadhyay 2006), and when bound to membranes and membrane-mimetic systems (Raghuraman and Chattopadhyay 2003; Kelkar and Chattopadhyay 2005; Rawat et al. 2004). Since the dynamics of hydration is directly associated with the function of proteins, REES has proved to be a valuable tool to explore the organization and dynamics of soluble and membrane proteins under varying degrees of hydration (Raghuraman and Chattopadhyay 2003; Kelkar and Chattopadhyay 2005). 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 (Wyttenbach and Bowers 2009).

Materials and methods

Materials

Calcium-depleted BLA, $CaCl_2$, trichloroethanol (TCE), and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Ultrapure-grade acrylamide was from Invitrogen Life Technologies (Carlsbad, CA). The purity of acrylamide was checked from its absorbance using its molar extinction coefficient (ε) of 0.23 $M^{-1}cm^{-1}$ at 295 nm and optical transparency beyond 310 nm (Eftink 1991a). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Sample preparation

Native BLA solution was prepared by dissolving calcium-depleted BLA in 10 mM Tris, 1 mM $CaCl_2$, pH 7.4 buffer. Acid-induced molten globule form of BLA was generated

by treatment of native BLA solution with 5 mM HCl at room temperature (~23°C). The concentration of pure BLA in buffer was estimated using the molar extinction coefficient (ε) of 28,540 $M^{-1}cm^{-1}$ at 280 nm (Engel et al. 2002). All experiments were carried out at ~23°C.

Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed by using a Hitachi F-4010 spectrofluorometer using 1-cm-path-length quartz cuvettes. Excitation and emission slits with nominal bandpass of 5 nm were used. All spectra were recorded using the correct spectrum mode. Background intensities of samples in which BLA was omitted were negligible in most cases and were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak. The spectral shifts obtained with different sets of samples were identical in most cases, or were within ±1 nm of the ones reported. Fluorescence anisotropy measurements were performed at room temperature (~23°C) using a Hitachi polarization accessory. Anisotropy values were calculated from the equation (Lakowicz 2006):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor that corrects for wavelength-dependent distortion of the polarizers and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}. All experiments were performed with multiple sets of samples; average values of anisotropy are shown in Fig. 5.

Fluorescence quenching measurements

Acrylamide and TCE quenching experiments of tryptophan fluorescence were carried out by measurement of fluorescence intensity after serial addition of small aliquots of either a freshly prepared stock solution of 2 M acrylamide in water or neat TCE (10.42 M) to a stirred sample, followed by incubation for 3 min in the sample compartment in the dark (shutter closed). The excitation wavelength used was 295 nm, and emission was monitored at 335 nm. The fluorescence intensities obtained were corrected for dilution. Corrections for inner filter effect for acrylamide quenching were made using the following equation (Chattopadhyay et al. 2003):

$$F = F_{obs}\ \text{antilog}\ [(A_{ex} + A_{em})/2], \quad (2)$$

where F is the corrected fluorescence intensity and F_{obs} is the background-subtracted fluorescence intensity of the sample (also corrected for dilution). A_{ex} and A_{em} are the measured absorbance at the excitation and emission wavelengths. The absorbances of the samples were measured using a Hitachi U-2000 ultraviolet (UV)–visible absorption spectrophotometer. Inner filter effects in case of TCE quenching were negligible. Quenching data were analyzed according to the Stern–Volmer equation (Chattopadhyay et al. 2003):

$$F_0/F = 1 + K_{SV}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (acrylamide or TCE), respectively, $[Q]$ is the molar quencher (acrylamide or TCE) concentration, and K_{SV} is the Stern–Volmer quenching constant.

Time-resolved fluorescence measurements

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. A pulsed light-emitting diode (LED) (NanoLED-17) was used as an excitation source. This LED generates optical pulses at 294 nm with pulse duration less than 750 ps, and was run at 1 MHz repetition rate. The LED profile (instrument response function) was measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. In order to optimize the signal-to-noise ratio, 10,000 photon counts were collected in the peak channel. All experiments were performed using emission slits with bandpass of 6 nm or less. The sample and the scatterer were alternated after every 5% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam, thereby avoiding any possible photodamage to the fluorophore. Data were stored and analyzed using DAS 6.2 software (Horiba Jobin Yvon, Edison, NJ). Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (4)$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\sum_i \alpha_i = 1$. The program also includes statistical and plotting subroutine packages (O'Connor and Phillips 1984). The goodness of fit of a given set of observed data and the chosen function was evaluated by the χ^2 ratio, the weighted residuals (Lampert

et al. 1983), and the autocorrelation function of the weighted residuals (Grinvald and Steinberg 1974). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with minimum χ^2 of not more than 1.4. 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 (Lakowicz 2006):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2 + \alpha_3\tau_3^2}{\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3} \quad (5)$$

Circular dichroism measurements

CD measurements were carried out at room temperature (~23°C) on a JASCO J-815 spectropolarimeter which was calibrated with (+)-10-camphorsulfonic acid. Spectra were scanned in a quartz optical cell with path length of 0.1 cm, and recorded in 0.5 nm wavelength increments with 2 s response and bandwidth of 2 nm. For monitoring changes in secondary structure and tertiary structures, spectra were scanned from 200 to 260 nm in the far-UV range, and from 250 to 310 nm in the near-UV range. The scan rate was 50 nm/min, and each spectrum is the average of four scans with full-scale sensitivity of 100 mdeg. Spectra were corrected for background by subtraction of appropriate blanks and were smoothed, ensuring that the overall shape of the spectrum remained unaltered. Data are represented as mean residue ellipticities and were calculated using the equation:

$$[\theta] = \theta_{obs}/(10Cl) \quad (6)$$

where θ_{obs} is the observed ellipticity in mdeg, l is the path length in cm, and C is the concentration of peptide bonds in BLA in mol/L.

Results and discussion

Molten globule conformations can be conveniently characterized using CD spectroscopy (Dolgikh et al. 1981). The far-UV and near-UV CD spectra of native and acid-induced molten globule forms of BLA are shown in Fig. 1. The molten globule conformation exhibits lack of appreciable tertiary structure in near-UV CD (panel b) and representative secondary structure in the far-UV region (panel a). The far-UV and near-UV CD spectra of native BLA show representative native structure.

The fluorescence emission spectra of BLA in native and molten globule forms are shown in Fig. 2. Tryptophans in native BLA exhibit an emission maximum at 331 nm, in agreement with previous literature (Kronman et al. 1981). The fluorescence emission spectrum of molten globule

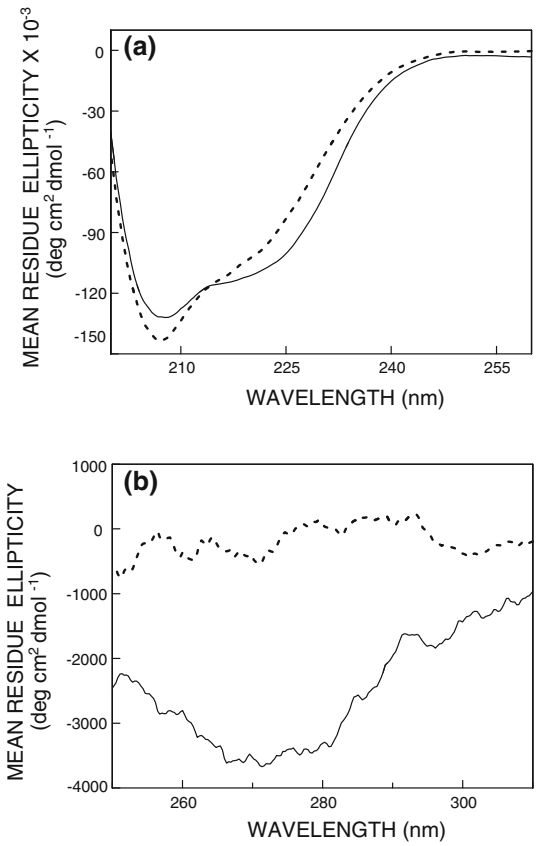

Fig. 1 Representative **a** far-UV and **b** near-UV CD spectra of BLA in native (*solid line*) and molten globule (*broken line*) states. The concentration of BLA was 32 μM in both cases. See "Materials and methods" for further details

BLA displays a red shift and the emission maximum[1] is shifted to 342 nm (i.e., a red shift of 11 nm with respect to native BLA). This indicates a partially disordered conformation in the molten globule state due to loss of tertiary structure, allowing more water penetration near some of the tryptophan residues, which results in a red shift in emission maximum.

The inset to Fig. 2 shows the relative fluorescence intensities of BLA in the molten globule state with respect to the native form at their respective emission maxima. The molten globule form of BLA exhibits an increase (~30%) in fluorescence intensity relative to the native form. This indicates the possible release of intramolecular quenching of tryptophan fluorescence in the molten globule state,

[1] We use the term "maximum of fluorescence emission" in a somewhat broader sense here. In every case, we 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 of these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission is reported as the fluorescence maximum.

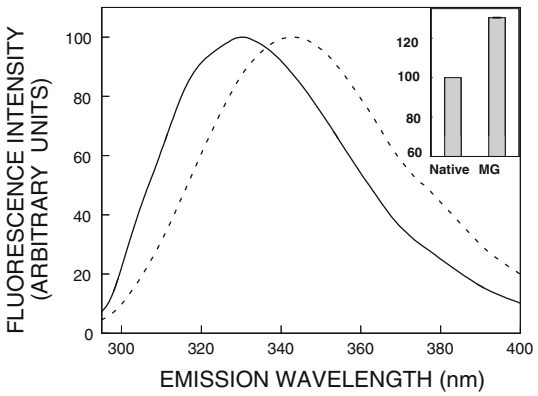

Fig. 2 Fluorescence emission spectra of BLA in native (*solid line*) and molten globule (*broken line*) states. The excitation wavelength was 280 nm in both cases. Spectra are intensity-normalized to the respective emission maximum. The *inset* shows relative fluorescence intensities of BLA in native and molten globule conformations at their respective emission maximum. The excitation wavelength was 280 nm in both cases. All other conditions are as in Fig. 1. See "Materials and methods" for further details

implying thereby that the tryptophan residues in the native conformation are surrounded by amino acids that may quench their fluorescence. Earlier work from a number of laboratories using homeodomain proteins and immunophilins has shown that the intramolecular quenching of tryptophan fluorescence could be due to mechanisms such as excited-state electron transfer (with neighboring residues such as glutamine, asparagine, glutamic acid, aspartic acid, cysteine, and histidine), excited-state proton transfer (lysine and tyrosine), or through self energy transfer (homo-FRET) between tryptophan residues (Silva and Prendergast 1996; Rouviere et al. 1997; Nanda and Brand 2000). In addition, phenylalanine and tyrosine residues are known to quench tryptophan fluorescence by NH$\cdots\pi$ interaction (Nanda and Brand 2000). It is evident from the crystal structure of BLA (Chrysina et al. 2000) that some of the tryptophan residues are in close proximity to amino acids that could induce quenching. For example, Trp-26 is in the vicinity of Lys-16 (within 5.2 Å) and 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 (Moens et al. 2004). Trp-60 is also in close proximity to Cys-73 (4.03 Å) and Cys-61 (6.91 Å), and Trp-118 is in close proximity to Cys-28 (3.30 Å), His-32 (4.07 Å), and Glu-117 (3.91 Å). 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), while 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, some of these intramolecular quenching effects are released due to the changes in BLA conformation, as shown from the increase in steady-state fluorescence intensity (Fig. 2) and fluorescence lifetime (see later, Fig. 6). Interestingly, the increase in intensity is not proportional to the increase in lifetime, suggesting that the mechanism of quenching could be a combination of static and dynamic quenching (Nanda and Brand 2000).

Based on nuclear magnetic resonance (NMR) studies, it has been reported that, out of the four tryptophans, only Trp-26 is solvent inaccessible in the molten globule conformation of BLA. In contrast, in the native state of BLA, all four tryptophans are largely solvent excluded (Mok et al. 2005). Studies with tryptophan mutants of GLA [which contain four tryptophans at positions similar to BLA and have 71% sequence similarity with BLA (Pettersson et al. 2006)] have shown that the fluorescence of Trp-60, Trp-104, and Trp-118 are strongly quenched. This accounts for partial indirect quenching of Trp-26 in the native form. The fluorescence signal of native GLA is dominated by Trp-26 (Vanhooren et al. 2005, 2006). Upon comparison of these earlier reports with our results, it is likely that Trp-26 is the major emitter in native BLA.

The red shifted fluorescence emission maximum of molten globule BLA is indicative of higher solvent exposure of BLA tryptophans in the molten globule state. Acrylamide quenching of tryptophan fluorescence is widely used to monitor tryptophan accessibilities in proteins and peptides (Eftink 1991b). Figure 3a shows representative Stern–Volmer plots of acrylamide quenching of BLA tryptophans. The slope (K_{SV}) of such a plot is related to the accessibility (degree of exposure) of the tryptophans to the aqueous quencher. The linearity of the plot with no apparent downward curvature indicates minimal heterogeneity in tryptophan population, as assessed by acrylamide quenching in this concentration range. The quenching parameters obtained by analysis of Stern–Volmer plots are presented in Table 1. The Stern–Volmer constant (K_{SV}) for acrylamide quenching of native BLA was found to be 1.88 M^{-1}. The corresponding value of K_{SV} for the molten globule state was higher (4.87 M^{-1}), indicating increased exposure of the tryptophans in the molten globule state. The K_{SV} for acrylamide quenching of BLA tryptophans therefore increases by $\sim$159% with the conformational transition to the molten globule state.

As mentioned earlier, BLA has four tryptophans (at positions 26, 60, 104, and 118) and this gives rise to heterogeneity in tryptophan environments in terms of accessibility. In fact, such heterogeneity has been reported for the related human (Chakraborty et al. 2001) and goat (Vanhooren et al. 2006) α-lactalbumins. Acrylamide quenching usually provides information on the environment of surface-accessible tryptophans. On the other hand, TCE is a hydrophobic neutral quencher of tryptophan

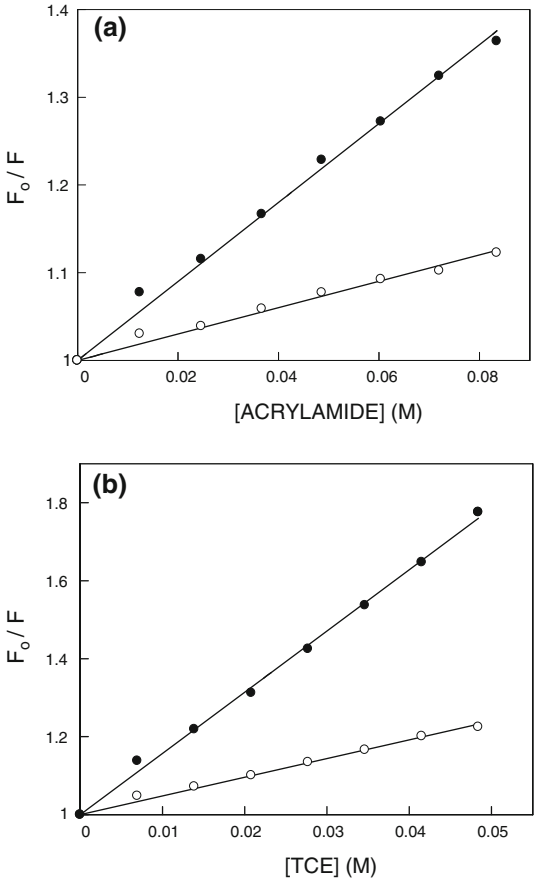

Fig. 3 Quenching of BLA tryptophan fluorescence using quenchers of varying degrees of accessibility. Representative data for Stern–Volmer analysis of **a** acrylamide and **b** TCE quenching of native (○) and molten globule (●) BLA fluorescence. F_0 is the fluorescence in the absence of quencher, F is the corrected fluorescence in the presence of quencher. The excitation wavelength was fixed at 295 nm, and emission was monitored at 335 nm in both cases. The concentration of BLA used was 1 μM. See "Materials and methods" for further details

Table 1 Quenching of tryptophan fluorescence of BLA using acrylamide and TCE

Condition	Quencher used	K_{SV}[a] (M^{-1})
Native	Acrylamide	1.88 ± 0.20
Molten globule	Acrylamide	4.87 ± 0.27
Native	TCE	5.37 ± 0.47
Molten globule	TCE	12.39 ± 1.67

The concentration of BLA was 1 μM. The excitation wavelength was 295 nm; emission was monitored at 335 nm. See "Materials and methods" for further details

[a] Calculated using Eq. (3). The quenching parameters shown represent mean ± standard error (SE) of at least three independent measurements, while quenching data shown in Fig. 3 are from representative experiments

fluorescence which is less polar than acrylamide and has been earlier shown to be a more effective quencher of tryptophan fluorescence in hydrophobic pockets than acrylamide (Kelkar et al. 2005; Eftink et al. 1977; Raja et al. 1999). The advantage of TCE as a quencher lies in the fact that it can penetrate into the interior of the protein matrix due to its relatively nonpolar nature. Representative Stern–Volmer plots of TCE quenching of BLA tryptophans are shown in Fig. 3b. The Stern–Volmer plots obtained are linear, and the quenching parameters (K_{SV}) obtained by analysis of Stern–Volmer plots are presented in Table 1. The increased K_{SV} for TCE quenching ($\sim$5.37 M^{-1}) of native BLA tryptophans as compared with quenching by acrylamide indicates the presence of hydrophobic patches in the vicinity of tryptophan residues (Giovanni and Berliner 1985), since TCE is known to preferentially quench tryptophans localized in such areas (Eftink et al. 1977). The value of K_{SV} for TCE quenching in case of the molten globule state was found to be higher (12.39 M^{-1}), i.e., an increase of $\sim$131%. This indicates a change in the organization of tryptophan residues in the hydrophobic region of BLA in the molten globule state.

Our quenching results show a higher K_{SV} with TCE quenching compared with quenching by acrylamide, thereby reflecting that hydrophobic pockets harboring the major emitting tryptophan(s) are present in both native and molten globule conformations. As reported earlier, two distinct hydrophobic cores are present in native BLA that contain solvent-inaccessible tryptophan residues (Chrysina et al. 2000; Mok et al. 2005). These are aromatic cluster I (containing Trp-118) and aromatic cluster II (Trp-26, Trp-60, and Trp-104), sometimes referred to as the "hydrophobic box." In the molten globule state also, residues belonging to aromatic cluster II (Trp-26) remain in the solvent-inaccessible hydrophobic patches. A relatively high K_{SV} value of 12.39 M^{-1} with TCE as the quencher in the molten globule state probably reflects significant quenching of Trp-26.

The shifts in the maxima of fluorescence emission of BLA as a function of excitation wavelength are shown in Fig. 4. Upon excitation at 280 nm, tryptophans in native BLA exhibit an emission maximum at 331 nm. As the excitation wavelength is changed from 280 to 307 nm, the emission maximum of native BLA is shifted from 331 to 337 nm, which corresponds to REES of 6 nm (see inset to Fig. 4). It is possible that there could be further red shift when native BLA is excited beyond 307 nm. We found it difficult to work in this wavelength range because of very low signal-to-noise ratio and artifacts due to the solvent Raman peak that sometimes remained even after background subtraction. 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

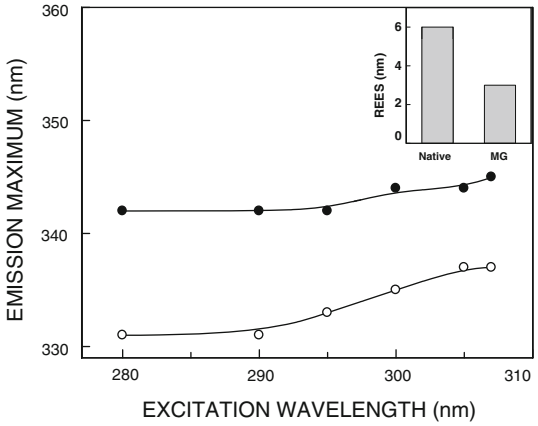

Fig. 4 Effect of changing excitation wavelength on the wavelength of maximum emission of BLA in native (○) and molten globule (●) states. The concentration of BLA used was 24 μM (in case of native) or 12 μM (for molten globule). The *lines* joining the data points are provided merely as viewing guides. The *inset* shows the magnitude of REES obtained in both conformations. The magnitude of REES corresponds to the total shift in emission maximum when the excitation wavelength is changed from 280 to 307 nm. All other conditions are as in Fig. 1. See "Materials and methods" for further details

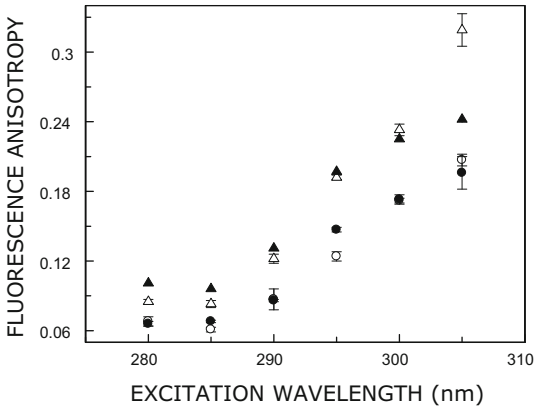

Fig. 5 Fluorescence anisotropy of BLA as a function of excitation wavelengths in native (○ buffer, Δ glycerol) and molten globule (● buffer, ▲ glycerol) states. The emission wavelength was fixed at 330 and 340 nm for native and molten globule states, respectively. The concentration of BLA was 12 μM in buffer and 90 μM in glycerol. Data shown are mean ± SE of three independent measurements. All other conditions are as in Fig. 1. See "Materials and methods" for further details

would directly imply that the regions surrounding at least some of the BLA tryptophans offer considerable restriction to reorientational motion of the solvent (water) dipoles around the excited-state tryptophans. Heterogeneity in tryptophan environment in terms of both solvent accessibility and quantum yield in α-lactalbumins has previously been reported (Chakraborty et al. 2001; Vanhooren et al. 2006). For example, some of the functionally important tryptophans in α-lactalbumins (e.g., Trp-104) are believed to display low solvent accessibility in the native state (Chakraborty et al. 2001).

The fluorescence emission spectrum of BLA in molten globule state displays a red shift, and the emission maximum is shifted to 342 nm (Fig. 2). Analysis of the REES effect in the molten globule state provides interesting results. Figure 4 shows that, as the excitation wavelength is changed from 280 to 307 nm, the emission maximum of the tryptophans is shifted from 342 to 345 nm, which corresponds to a REES of 3 nm (see inset to Fig. 4). This indicates that the motional restriction around the tryptophans, on average, is reduced in the molten globule state compared with in the native state, possibly because of change in the organization of tryptophan residues state due to increased dynamics. For example, it has been earlier reported that Trp-118 is buried in a hydrophobic core with low solvent accessibility in the molten globule state (as opposed to Trp-104, which is characterized by low solvent accessibility in the native state; see above) (Chakraborty et al. 2001). The analysis of fluorescence from multitryptophan proteins is often complicated because of the complexity of fluorescence processes in such systems, and the heterogeneity in fluorescence parameters (such as quantum yield and lifetime) due to environmental sensitivity of individual tryptophans (Eftink 1991c; Chattopadhyay and McNamee 1991). Analysis of REES of individual tryptophans in BLA could therefore provide useful information on the organization and dynamics of individual tryptophans contributing to the experimentally measured REES. We have recently performed such analysis for the ion-channel peptide gramicidin (Chattopadhyay et al. 2008).

In addition to the shift in emission maximum upon red edge excitation, fluorescence anisotropy is known to be dependent on excitation wavelength in motionally restricted media (Mukherjee and Chattopadhyay 1995). Due to strong dipolar interactions with the surrounding solvent molecules, there is a decreased rotational rate of the fluorophore in the solvent-relaxed state. Upon red edge excitation, selective excitation of this subclass of fluorophore occurs. Because of strong interactions with the polar solvent molecules in the excited state, one may expect these "solvent-relaxed" fluorophores to rotate more slowly, thereby increasing anisotropy. The excitation anisotropy spectra (i.e., plots of steady-state anisotropy versus excitation wavelength) of BLA in native and molten globule states are shown in Fig. 5. The figure shows that the anisotropy of BLA tryptophans undergoes considerable change upon altering the excitation wavelength from 280 to 305 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, especially in restricted environments, has been previously reported (Guha et al. 1996). Another possible reason for the

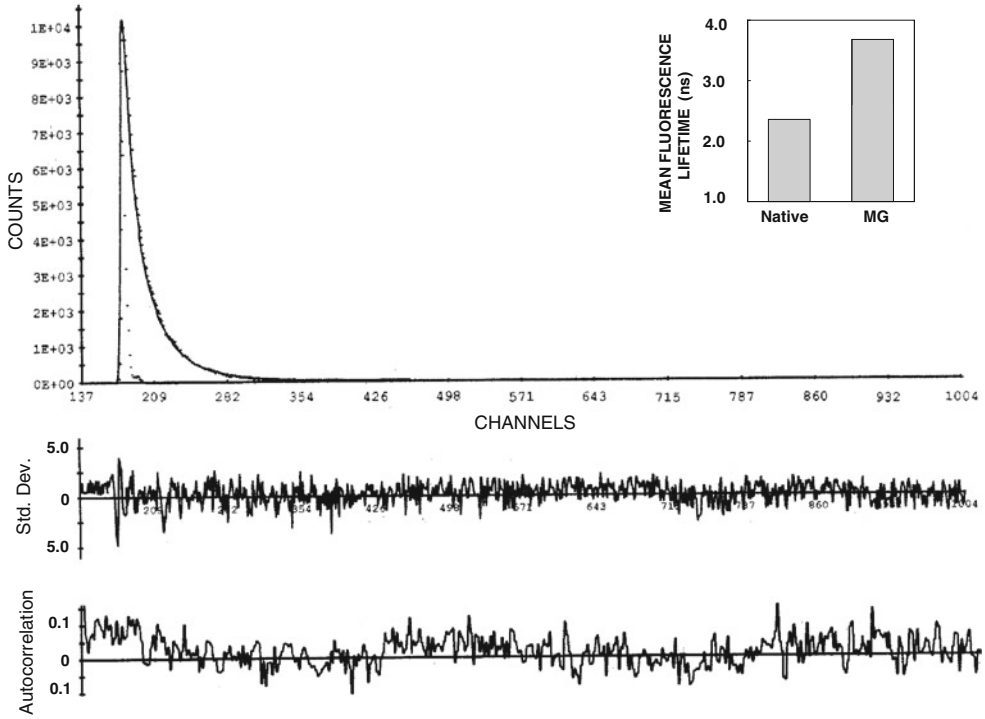

Fig. 6 Representative time-resolved fluorescence intensity decay profile of BLA in molten globule state. Excitation wavelength was 294 nm, corresponding to pulsed diode light source, and emission was monitored at 340 nm. The *sharp peak* on the *left* corresponds to the profile of the pulsed light-emitting diode (LED). The relatively *broad peak* on the *right* is the decay profile, fitted to a triexponential function. The two *lower plots* show the weighted residuals and the autocorrelation function of the weighted residuals. The *inset* shows the mean fluorescence lifetime of BLA in native and molten globule conformations. The excitation wavelength used was 294 nm, while the emission wavelength was fixed at 330 and 340 nm for native and molten globule states, respectively. The concentration of BLA was 32 μM. See "Materials and methods" for further details

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 (Weber and Shinitzky 1970). This reinforces our earlier conclusion that tryptophans in BLA are in a motionally restricted region in both states.

In order to damp the effect of rotational motion (present in buffer) on the homotransfer, if any, we compared the steady-state anisotropy excitation spectra of native and molten globule forms of BLA in highly viscous medium (90% glycerol, v/v) (Vincent et al. 1992). Under such condition in multitryptophan proteins, excitation at the red edge may predominantly reflect the release of homo-FRET and an enhancement of anisotropy. Figure 5 shows that the release of homo-FRET at the red edge of excitation is greater in native form as compared with in the molten globule form, particularly in viscous medium. This could imply that the appreciable homo-FRET between the tryptophans (prominently Trp-26 and Trp-104 in the "hydrophobic box") in the native state may not be maintained in the molten globule state. This is supported by NMR results which indicate that only Trp-26 is solvent inaccessible in the molten globule state (Mok et al. 2005).

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is localized (Prendergast 1991). A typical decay profile of BLA tryptophans with its triexponential fitting and the statistical parameters used to check the goodness of fit are shown in Fig. 6. Table 2 presents the tryptophan lifetimes for BLA in native and molten globule states. All fluorescence decays for BLA tryptophans could be fitted well using 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 time-resolved fluorescence decay. The mean fluorescence lifetimes of BLA tryptophans were calculated using Eq. (5) and are shown in the inset to Fig. 6. The inset shows that, while the mean fluorescence lifetime of BLA tryptophans in the native state is $\sim$2.4 ns, it is increased considerably in the molten globule state to $\sim$3.7 ns. In general, tryptophan lifetimes are known to be reduced when exposed to polar environments (De Lauder and Wahl 1971). Since the molten globule state is less ordered than the native state, more water penetration is expected, resulting in reduction in fluorescence lifetime. However, there are other factors that need to be considered when

Table 2 Representative fluorescence lifetimes of BLA in native and molten globule states as a function of emission wavelength

Emission wavelength (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)
(a) Native						
330	0.19	0.12	0.65	0.95	0.16	3.86
340	0.20	0.23	0.60	1.05	0.20	4.07
350	0.19	0.26	0.57	1.10	0.24	4.26
360	0.55	1.13	0.17	0.23	0.28	4.33
370	0.21	0.34	0.47	1.31	0.32	4.46
(b) Molten globule						
330	0.49	1.97	0.20	0.40	0.31	4.81
340	0.49	2.02	0.17	0.39	0.34	4.81
350	0.16	0.38	0.52	2.14	0.32	5.10
360	0.54	2.27	0.16	0.45	0.30	5.25
370	0.53	2.31	0.14	0.42	0.33	5.31

The excitation wavelength was 294 nm. The number of photons collected at the peak channel was 10,000. All other conditions are as in Fig. 6. See "Materials and methods" for further details

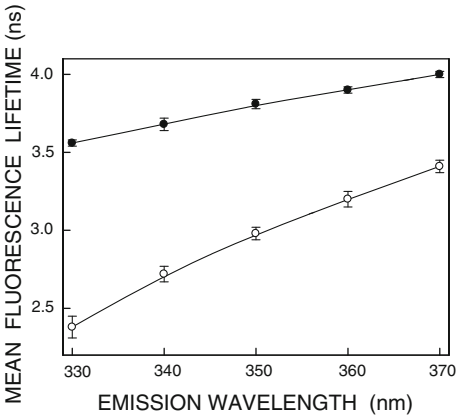

Fig. 7 Mean fluorescence lifetime of BLA as a function of emission wavelengths in native (○) and molten globule (●) states. The excitation wavelength used was 294 nm. Mean fluorescence lifetimes were calculated from Table 1 using Eq. (5). Data shown are mean ± SE of three independent measurements. The concentration of BLA was 32 μM. See "Materials and methods" for further details

interpreting changes in fluorescence lifetime. The increase in mean fluorescence lifetime 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. An essential feature of the molten globule conformation in α-lactalbumins is that the α-helical domain (containing Trp-26, Trp-104, and Trp-118) is highly structured, whereas the *β*-sheet domain (which contains Trp-60) is partially ordered (more unfolded) (Kuwajima 1996; Vanhooren et al. 2006). This results in Trp-60 having the highest solvent accessibility in the molten globule state (Chakraborty et al. 2001). The increase in mean fluorescence lifetime in the molten globule state of BLA could possibly be due to release of quenching by neighboring amino acids [such as histidine residues and disulfide bridge between cysteine residues (Vanhooren et al. 2006)] as a result of reorganization of the structure in these states.

The changes in mean fluorescence lifetime of BLA tryptophans in native and molten globule states as a function of increasing emission wavelength are shown in Fig. 7. Interestingly, the mean fluorescence lifetime displays a considerable increase in both 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 (Guha et al. 1996). The extent of increase in mean fluorescence lifetime was ∼43% for native BLA and ∼12% for the molten globule state. The difference in the extent of increase in mean fluorescence lifetime is indicative of the difference in their environments in the respective states. Such increasing lifetimes across the emission spectrum may be interpreted in terms of solvent reorientation around the excited-state fluorophore as follows. Observation of emission spectra at shorter wavelengths selects for predominantly unrelaxed fluorophores. Their lifetimes are shorter because this population is decaying both at the rate of fluorescence emission at the given excitation wavelength and by decay to longer (unobserved) wavelengths. In contrast, observation at the longer emission wavelength (red edge) selects for the more relaxed fluorophores, which have spent enough time in the excited state to allow increasingly larger extents of solvent relaxation.

In this work, we utilized the wavelength-selective fluorescence approach to monitor the organization and dynamics of the functionally important tryptophan residues of BLA in native and molten globule states. We observe REES of 6 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 3 nm REES in acid-induced molten globule state. This is a novel observation since REES of partially disordered yet functional proteins (such as the molten globule state) is only beginning to be addressed (Leal and Gomes 2007). We have previously reported REES of tryptophan(s) in case of native (ordered) (Guha et al. 1996; Raghuraman and Chattopadhyay 2006) and denatured (Chattopadhyay et al. 2003) proteins. Our present results show that tryptophan(s) in the molten globule state of BLA exhibit REES. To the best of our knowledge, these results

constitute one of the early reports of REES in the molten globule state of proteins.

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 for protein function (Tompa 2005; Dyson and Wright 2005; Dunker et al. 2008; Xie et al. 2007). The fact that more than 50% of total eukaryotic proteins and ~75% of signaling proteins in mammals contain at least one long disordered region (>30 residues) (Dunker et al. 2008; Xie et al. 2007) has changed the earlier structure–function dogma. In this context, monitoring the dynamics of proteins, not only in ordered forms but also in disordered forms, such as the molten globule form, assumes significance. Our present results could provide crucial 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. Understanding the dynamic environment of tryptophan residues in molten globule-like conformations in proteins could therefore lead to better understanding of their function.

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