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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].

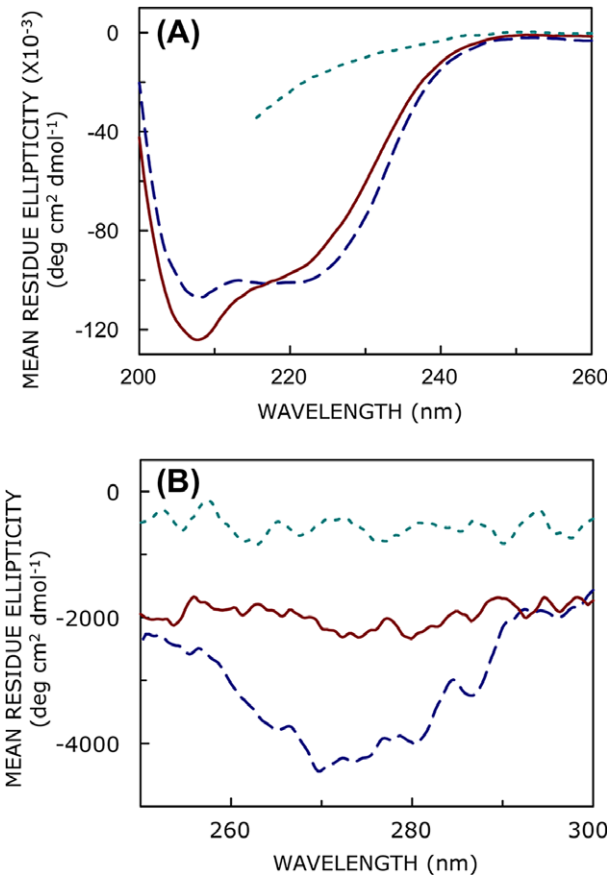

Fig. 1. Representative (A) far-UV and (B) near-UV CD spectra of BLA in native (blue, – –), molten globule (red, —) and urea-denatured (cyan, - - - -) states. The concentration of BLA was 32 μM in all cases. See Section 2 for other details. (For interpretation of the references to color in this figure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