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ORIGINAL ARTICLE

Sensing Tryptophan Microenvironment of Amyloid Protein Utilizing Wavelength-Selective Fluorescence Approach

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Abstract Structural transition among various forms of proteins involves subtle interplay between structure and dynamics and is crucial in human diseases. Red edge excitation shift (REES) represents a suitable approach to explore the environmental organization and dynamics surrounding tryptophan residues in proteins. Although REES from tryptophan residues has been reported for native, molten globule and denatured states of proteins, such data on the amyloid form of proteins is lacking. κ-casein is one of the most important constituents of casein micelles in milk and has a tendency to form amyloid fibril. We report here REES of the sole tryptophan residue for native, acid-denatured and urea-denatured forms of κ-casein. More importantly, we show that the amyloid form of κ-casein displays REES of 4 nm. We analyze these results in terms of tryptophan microenvironment in various forms of κ-casein, particularly the amyloid form. We conclude that REES is a sensitive tool to monitor structural plasticity in proteins.

Keywords Intrinsically disordered protein · Amyloid fibril · κ-casein · REES · Thioflavin T fluorescence

Introduction

Intrinsically disordered proteins (IDPs) are unique due to their intrinsic structural flexibility. They do not obey the conventional sequence-structure-function paradigm [1–4]. Despite being unstructured, IDPs perform several cellular functions [3, 5–7]. Sequence analysis has shown the prevalence of flexible, disorder promoting amino acids and high ratio of charged to hydrophobic amino acids in IDPs relative to globular proteins [2]. The structural flexibility induces conformational heterogeneity (plasticity) and therefore the structural characterization utilizing x-ray crystallography is far more complicated and challenging.

The excessive conformational heterogeneity of IDPs makes them prone to assume a misfolded state in an altered environmental condition. The inter-chain interactions become stronger than the chain-solvent interaction resulting in the formation of aggregated structures. Protein misfolding, followed by amyloid formation and fibrillation, plays a critical role in the pathogenesis of a number of diseases involving the deposition of aggregates of different proteins or peptides in various organs and tissues. Alzheimer's disease, Parkinson's disease, type II diabetes mellitus, and several other age-related neurodegenerative and systemic disorders are protein-misfolding diseases that are characterized by the accumulation of insoluble protein and peptide deposits [7–9].

Several spectroscopic and microscopic techniques have been exploited to detect the amyloid formation and fibrillation of IDPs [10–12]. However, solvent dynamics around a given amino acid in amyloid forms is relatively less explored area [13]. The interplay between structure and dynamics is crucial for protein organization and function, particularly in case of IDPs. Water has a seminal role in the folding, structure, dynamics and therefore the function of proteins and peptides [14–20]. There is a requirement of minimum level of hydration to activate the dynamics and function of proteins and therefore hydration acts as a catalyst for hydrogen bond exchange in protein folding [15, 19]. Hydration has even been termed as 'foldase' due to its important role in protein folding

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[15]. Water molecules are generally confined on the surfaces of proteins. Such interfacial confinement destroys the water-water hydrogen bond network and couples the motion of water molecules with the slow moving protein molecules [21, 22], thereby impeding the dynamics of water molecules. In such a situation, red edge excitation shift (REES) represents a suitable approach to explore the dynamic organization of proteins [23]. REES is opearationally defined as the shift in the wavelength of maximum fluorescence emission toward higher wavelengths, induced by a shift in the excitation wavelength toward the red edge of absorption spectrum. The origin of REES lies in slow solvent relaxation (relative to fluorescence lifetime) in the excited state and this assumes relevance in organized molecular assemblies such as proteins and membranes.

Caseins are tremendously flexible, small proteins that lack specific secondary structure and have been classified as IDPs [3, 11, 24]. Among this family, κ-casein is an important member since it is believed that κ-casein is responsible for the steric stability of casein micelles through coating of the micellar structure [25, 26]. κ-casein is a 169 residue, 18.9 kDa protein containing a single tryptophan (residue 76) and two cysteine (residues 11 and 88) residues. By generating the κ-casein null mutation in mice, it has been shown that κ-casein is essential for lactation [25]. κ-casein shows a very high tendency to form amyloid fibrils in vitro [11]. The solvation dynamics of tryptophan residue is different in its native, amyloid and denatured forms. In this paper, we have utilized REES to differentiate the native, urea-denatured, acid-denatured and amyloid forms of κ-casein. REES represents a unique approach that utilizes slow solvent reorientation in the excited state of the fluorophore and can be conveniently used to examine the environment and dynamics around a fluorophore in an organized molecular environment such as biological membranes and proteins [23, 27–31]. Measurements of REES of tryptophan fluorescence in various forms of κ-casein, such as native, acid-denatured (AD), urea-denatured (UD) and amyloid, clearly demonstrate that the solvent environment and dynamics around the tryptophan residue are different among various forms of κ-casein. These results are indicative of folding state-dependent solvent dynamics in proteins.

Experimental

Materials

κ-casein, urea, DTT, Tris, GdmCl, iodoacetic acid, thioflavin T and phosphotungstic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Solvents used were of spectroscopic grade, and water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Monomerization of κ-Casein

Reduced and carboxymethylated (RCM) monomeric κ-casein was prepared using a previously published protocol [10]. Briefly, 1 mM κ-casein solution was prepared in 6 M GdmCl (pH 8, 100 mM Tris), to which 10 mM DTT was added. To ensure complete reduction (cleavage of intra- and inter-strand disulfide linkages) and denaturation, the reaction mixture was stirred for 1 h at 37 °C. Subsequently, 50 mM iodoacetic acid (freshly prepared in DMSO) was added into the reaction mixture to react with the thiol groups of the resulting free cysteines, and the mixture was further stirred for 30 min at 37 °C. After the reaction was complete, traces of free iodoacetic acid were removed and the protein was concentrated using AMICON YM-10 (10 kDa cut-off; Millipore) by washing it extensively with 6 M GdmCl (50 mM phosphate buffer, pH 7). Concentration of RCM κ-casein was determined by measuring the tryptophan absorbance at 280 nm by UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA) using a molar extinction coefficient of 11,500 M^{-1} cm^{-1} [10]. RCM κ-casein was freshly prepared prior to spectroscopic measurements.

Urea- and Acid-Denatured κ-Casein Preparation

Freshly prepared RCM κ-casein was incubated in 8 M urea prepared in 5 mM phosphate buffer at pH 7 to prepare the urea-denatured κ-casein. For the preparation of acid-denatured κ-casein, we incubated κ-casein in 5 mM KCl-HCl solution at pH 1.6 [32].

Steady State Fluorescence Measurements

Steady state fluorescence measurements were performed with Fluorolog-3 Model FL3–22 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal band pass of 3 nm were used for all measurements involving tryptophan fluorescence. The excitation wavelength used for thioflavin T (ThT) fluorescence was 450 nm. Background (without protein) intensities of samples were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts.

Transmission Electron Microscopy

Transmission electron microscopy was carried out with JEM2100 (Jeol, Japan) transmission electron microscope. The samples were spread over a copper grid coated with carbon. Phosphotungstic acid was used as a negative stain for the visualization of amyloids under transmission electron microscope.

Results and Discussion

In order to explore the environment of tryptophan in different folded states (native, denatured and amyloid) of proteins we utilized REES, as it monitors the solvation dynamics around the fluorophore [23, 27–31]. We used κ-casein for this study since κ-casein belongs to the IDP family and forms amyloid in certain conditions [11]. Naturally occurring κ-casein generally exists in micellar form due to inter- and intra-molecular disulfide bond formation. We therefore prepared RCM κ-casein to generate the monomeric form of κ-casein by reducing the disulfide bonds followed by carboxymethylation in denatured condition. Carboxymethylation of the –SH group followed by the reduction of the disulfide bonds between two cysteines prevents the possibility of further disulfide bond formation so that κ-casein remains monomeric in solution until amyloid formation is initiated by altering the condition.

We probed the environment of the sole tryptophan residue using REES. REES depends on slow solvent reorientation in the excited state of a fluorophore, and a particular excitation wavelength selectively excites a particular sub-population of fluorophores [31]. Figure 1a shows the effect of changing excitation wavelength (280 to 307 nm) on the wavelength of maximum emission for native, urea- and acid-denatured κ-casein. It should be noted that the position of the emission maximum of κ-casein exhibited difference in various folding states. The emission maximum for native κ-casein was 341 nm (when excited at 280 nm), while the emision maximum for acid-denatured and urea-denatured states were 338 and 346 nm, respectively. Interestingly, the acid-denatured state displayed a blue shifted emission maximum (338 nm) when compared to the emission maximum (341 nm) observed in native κ-casein. This could be due to the generation of local hydrophobic patches in κ-casein in acidic environment. At low pH, the net negative charges of κ-casein would be neutralized and this could induce local compactness as a result of the attenuation of charge-charge repulsion. When the excitation wavelength was progressively increased from 280 to 307 nm, the emission maximum exhibited a concomitant shift from 341 to 348 nm, giving rise to REES of 7 nm for native κ-casein. The corresponding REES for acid-denatured and urea-denatured κ-casein was found to be 7 nm (338 to 345 nm) and 5 nm (346 to 351 nm), respectively. The magnitude of REES for native, urea- and acid-denatured states of κ-casein is shown in Fig. 1b. These results clearly indicate that the tryptophan microenvironment of the two differently denatured states (urea- and acid-denatured) varies in terms of polarity (extent of water) and dynamics. We have previously reported such difference in tryptophan microenvironment for the molten globule state [33, 34].

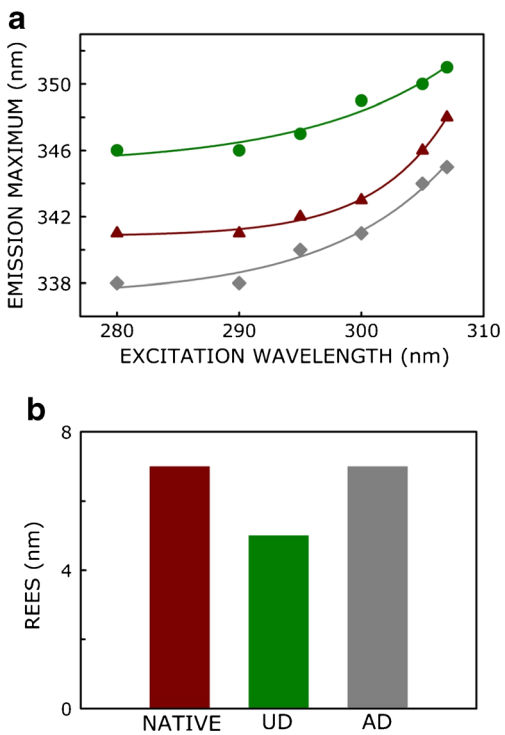

Fig. 1 **a** Effect of changing excitation wavelength on the wavelength of maximum emission of native (▲), urea-denatured (●) and acid-denatured (♦) forms of κ-casein. Lines joining the data points are provided merely as viewing guides. **b** The magnitude of REES of native, urea-denatured (UD) and acid-denatured (AD) forms of κ-casein. The magnitude of REES corresponds to the total shift in the emission maximum when the excitation wavelength is changed from 280 to 307 nm. The concentration of κ-casein in all cases was 5 μM. See Experimental for further details

It has been shown that the time-dependent amyloid formation of RCM κ-casein takes place at pH 6 at 37 °C [10]. In order to explore the tryptophan microenvironment upon amyloid formation, we incubated κ-casein in 10 mM phosphate buffer, pH 6 at 37 °C for amyloid formation. Amyloid formation was monitored by measuring the emission intensity of thioflavin T at 480 nm [35, 36]. ThT selectively binds to antiparallel β-sheets, the major structural element of amyloid fibrils. The binding of ThT to the antiparallel β-sheets enhances the fluorescence intensity of ThT. The kinetics of amyloid formation could therefore be monitored by measuring the fluorescence intensity of ThT as a function of time. Figure 2a shows the time course of ThT fluorescence for κ-casein incubated in 10 mM phosphate buffer, pH 6 and 37 °C. The time-dependent increase in ThT fluorescence intensity is a clear indication of κ-casein amyloid formation. Amyloid formation was confirmed from transmission electron microscopic (TEM) images of amyloid κ-casein. Figure 2b shows the TEM image of κ-casein fibrils using phosphotungstic acid as a negative stain.

The dynamics of the tryptophan environment in native and amyloid fibril of κ-casein was monitored utilizing REES. The amyloid formed after 27 h of incubation of κ-casein in 10 mM phosphate buffer, pH 6 and 37 °C (last data point of Fig. 2a) was used for REES measurements. Figure 3a shows the effect of changing excitation wavelength on the wavelength of

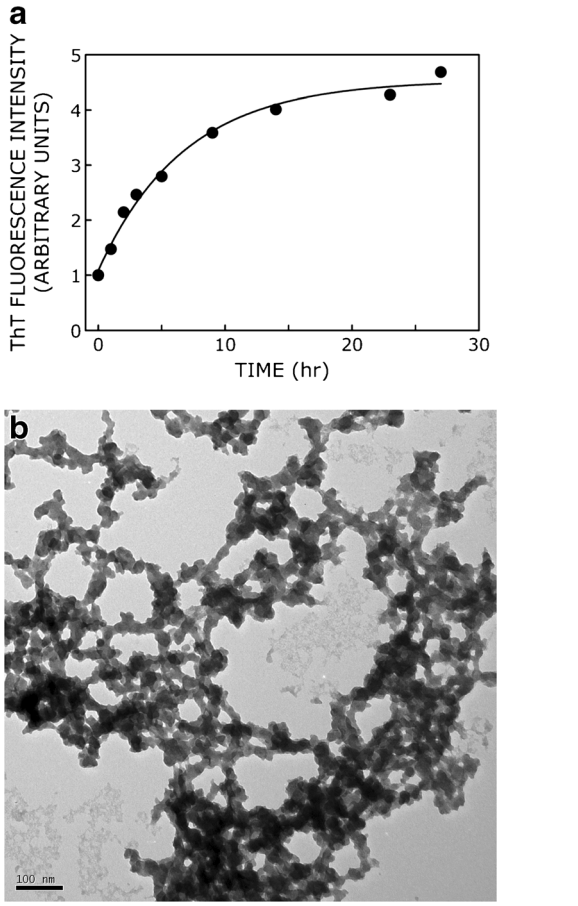

Fig. 2 **a** Increase in thioflavin T fluorescence intensity as a function of time due to the formation of the amyloid form of κ-casein. The concentration of κ-casein was 20 μM. The line joining the data points is provided as a mere viewing guide. **b** Transmission electron micrograph of negatively stained amyloid κ-casein. Phosphotungstic acid was used for negative staining. The scale bar denotes 100 nm. See Experimental for further details

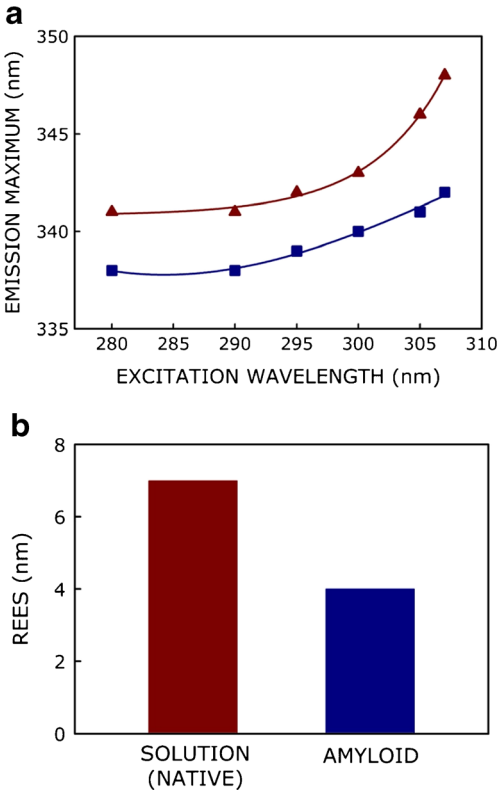

Fig. 3 **a** Effect of changing excitation wavelength on the wavelength of maximum emission of native (▲) and the amyloid form (■) forms of κ-casein. Lines joining the data points are provided merely as viewing guides. **b** The magnitude of REES of native and amyloid forms of κ-casein. The magnitude of REES corresponds to the total shift in the emission maximum when the excitation wavelength is changed from 280 to 307 nm. The concentration of κ-casein was 5 and 20 μM for native and amyloid forms, respectively. See Experimental for further details

maximum emission for amyloid κ-casein (the data for native κ-casein is shown as a control). The amyloid form of κ-casein exhibits an emission maximum of 338 nm, and REES of 4 nm. This indicates that water is a poor solvent for the amyloid form and that results in dehydration of the intermolecular spaces between proteins and making the protein-protein aggregation interface less hydrated. When the excitation wavelength was progressively shifted from 280 to 307 nm, the emission maximum of the amyloid form of κ-casein displayed a shift from 338 to 342 nm, giving rise to REES of 4 nm. The magnitude of REES is shown in Fig. 3b. Although REES of native, molten globule [33, 34] and denatured [37] proteins has been reported by us and others, there are very few reports on REES of proteins in the amyloid state (see Fig. 4). Very recently, REES of the amyloid form of α-synuclein has been reported [13]. Our present results, therefore, add strength to this very small number of literature reports available on REES of the amyloid form of proteins, which could be potentially useful, specially keeping in mind the physiological relevance of the amyloid form in human health and disease.

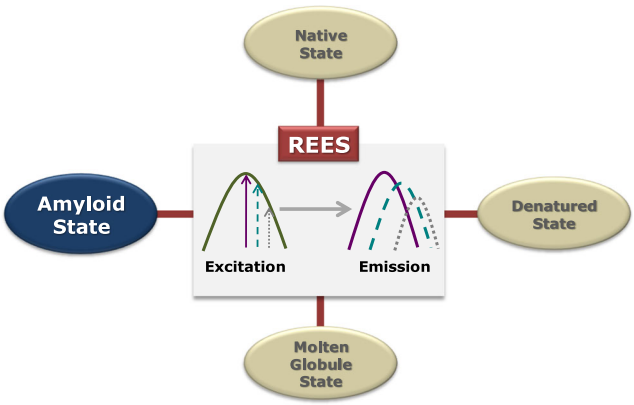

Fig. 4 A schematic representation showing that REES is sensitive to different forms (native, denatured, molten globule and amyloid) of proteins. Although REES of native, molten globule and denatured proteins has previously been reported, *we report REES of the amyloid form (shown in a different color) in this work.* Adapted and modified with permission from ref. 23. Copyright (2014) American Chemical Society

Structural modulation among different forms of proteins involves subtle interplay between structure and dynamics in biophysical terms. When considered from human disease perspective, such structural variation could be the key determinant in healthy and diseased states. It is in this overall context, we envision that REES could be effectively used to differentiate various forms of proteins and could be a handy tool with increasing advancements in instrumentation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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