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A stabilized molten globule protein

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Abstract A predominant conformational isomer of non-native α-lactalbumin (α-LA) has been purified by thermal denaturation of the native α-LA using the technique of disulfide scrambling. This unique isomer retains a substantial content of α-helical structure. It is stabilized by two native disulfide bonds within the α-helical domain and two scrambled non-native disulfide bonds at the β-sheet domain. This denatured isomer of α-LA exhibits structural characteristics that are consistent with the well-documented molten globule state. The ability to prepare a stabilized and structurally defined molten globule provides a useful model for studying the folding and unfolding pathways of proteins.

Key words: α-Lactalbumin; Thermal denaturation; Unfolding; Unfolding intermediate; Molten globule; Scrambled α-lactalbumin

1. Introduction

α-Lactalbumin (α-LA) is one of the most extensively investigated models for understanding the mechanism of protein stability, folding and unfolding. Under a variety of mild denaturing conditions, α-LA adopts a partially structured conformation termed as 'molten globule' [1–4]. The structure of α-LA molten globule is characterized by a high degree of native-like secondary structure and a fluctuated tertiary fold [1,2]. Molten globules are thought to be general intermediates in protein folding and unfolding [3,5–9]. However, analysis of their structures has remained to be challenging and results obtained from conflicting studies continue to be a debated subject [7,10–13]. Like most denatured or partially denatured states of proteins [14,15], the structure of molten globule comprises a mixture of conformational isomers that exist in a state of equilibrium. To date, characterization of the molten globule state of α-LA has been achieved by measuring the average property of these collective isomers using a wide range of spectroscopic and physiochemical methods, including circular dichroism [1,2], fluorescence [5,16], nuclear magnetic resonance (NMR) [3,6,7,17,18], disulfide replacement [19,20], limited proteolysis [21], light scattering [22,23] and calorimetric techniques [24]. Further understanding of the molten globule state of α-LA will require fractionation of diverse populations of conformational isomers that constitute the denatured α-LA.

The technique of disulfide scrambling [25] permits isolation of stabilized conformational isomers of a denatured protein. Under denaturing conditions and in the presence of thiol initiator, the native α-LA denatures by shuffling its native disulfide bonds and converts to a mixture of scrambled isomers that are trapped by non-native disulfide bonds. α-LA contains four disulfide bonds and may adopt 104 possible denatured scrambled isomers. They can be fractionated by high performance liquid chromatography (HPLC) and isolated for structural analysis [26,27]. Using the technique of disulfide scrambling and thermal denaturation, we demonstrate the possibility of isolation of a stable conformational isomer of α-LA that exhibits structural characteristics of molten globule.

2. Materials and methods

2.1. Materials

Calcium depleted bovine α-LA (L-6010), acetonitrile, 2-mercaptoethanol and essential chemicals were purchased from Sigma, all with grade purity of greater than 99%.

2.2. Thermal denaturation of the native α-LA

The native α-LA (0.5 mg/ml) was dissolved in the Tris–HCl buffer (0.1 M, pH 8.4, 5 mM EDTA) containing 2-mercaptoethanol (0.25 mM). The samples were incubated at elevated temperature (50–80°C) for up to 60 min. To monitor the kinetics and intermediates of unfolding, aliquots of the sample were removed in a time-course manner, quenched with 4% trifluoroacetic acid and analyzed by HPLC. The denatured sample was subsequently acidified with an equal volume of 4% trifluoroacetic acid and stored at −20°C.

2.3. Structural analysis of scrambled isomers of α-LA

Isolated isomers of α-LA (~10 μg) were treated with 1 μg of thermolysin in 30 μl of *N*-ethylmorpholine/acetate buffer (50 mM, pH 6.4). Digestion was carried out at 37°C for 16 h. Peptides were then isolated by HPLC and analyzed by amino acid sequencing and mass spectrometry in order to identify the disulfide containing peptides.

2.4. Amino acid sequencing and mass spectrometry

Amino acid sequence of disulfide containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (Model 494) equipped with an on-line PTH-amino acid analyzer. The molecular mass of disulfide containing peptides was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Perkin-Elmer Voyager-DE STR).

3. Results and discussion

The native α-LA was allowed to denature and unfold in the Tris–HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.25 mM) under elevated temperatures (50–80°C) for up to 60 min. Denatured samples were trapped by acidification and ana-

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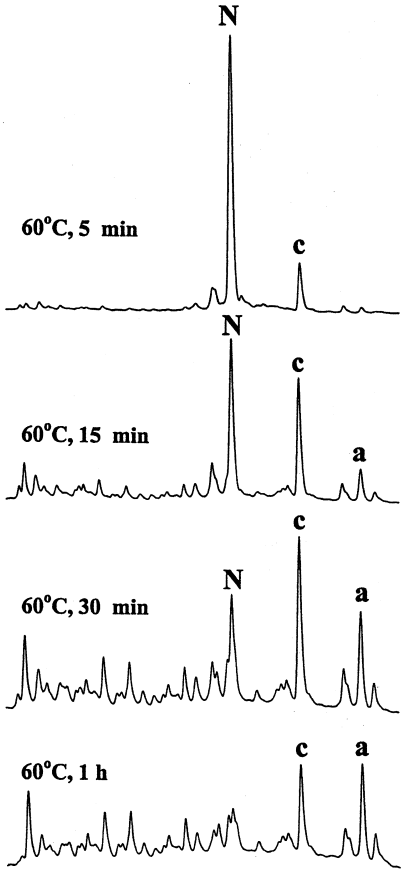

Fig. 1. Thermal denaturation of α-LA. Denaturation was carried out at 60°C in the Tris–HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.25 mM). Unfolding intermediates were trapped by acidification (4% trifluoroacetic acid) and analyzed by HPLC. Analysis confirms that aside from the native α-LA (N), all other fractions are scrambled 4-disulfide species of denatured α-LA. The two predominant isomers (**a** and **c**) of denatured α-LA are marked. Solvent A for HPLC was water containing 0.05% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.042% trifluoroacetic acid. The gradient was 22%B to 37%B in 15 min, 37%B to 56%B from 15 to 45 min. The flow rate was 0.5 ml/min. The column was Zorbax C-18 for peptides and proteins, 4.6 mm, 5 μm. Column temperature was 23°C.

lyzed by reversed phase HPLC. Chromatograms obtained from samples denatured at 60°C are presented in Fig. 1. The structure of denatured α-LA is highly heterogeneous. It comprises a mixture of fully oxidized scrambled isomers. The presence of fully oxidized (4-disulfides) isomers is supported by the following observations: (1) the HPLC profiles of the denatured α-LA remain indistinguishable regardless of whether the sample is quenched by acidification (4% trifluoroacetic acid) or by alkylation (iodoacetic acid); (2) denatured samples quenched by alkylation were further analyzed for their content of free cysteine and disulfide bond by the dabsyl chloride method [28] and MALDI mass spectrometry. Both methods confirm that the denatured α-LA contains 4-disulfide bonds.

Kinetic analysis reveals the presence of a predominant isomer (isomer **c**) during the early stage of thermal unfolding of α-LA (Fig. 1). Under selected conditions (e.g. 60°C, 15–20 min), the yield of isomer **c** may account for as much as 36% of the total content of the denatured α-LA. A more extensively denatured α-LA consists of 40 fractions of well-populated isomers, including two prevalent isomers **c** and **a** (1 h sample of Fig. 1). These two major isomers were isolated for structural analysis. They were digested with thermolysin. Peptides were then separated by HPLC and analyzed by Edman amino acid sequencing and MALDI mass spectrometry in order to identify peptides that contain disulfide bonds. Their structures are summarized in Fig. 2. Isomer **a** is crosslinked by four pairs of consecutive neighboring cysteines (also known as *beads-form*). This isomer adopts the smallest disulfide loops and presumably represents the most extensively unfolded structure among the 104 possible isomers. This conclusion is consistent with its increasing yield during the time-course unfolding (Fig. 1) [25–27]. Isomer **c** was found to contain two native disulfide bonds (Cys^{6}–Cys^{120}, and Cys^{28}–Cys^{111}) within the α-helical domain and two non-native disulfide bonds (Cys^{61}–Cys^{73}, and Cys^{77}–Cys^{91}) within the β-sheet (calcium binding) domain (Fig. 2). The disulfide structure alone clearly implies that isomer **c** is a partially unfolded α-LA with a largely intact α-helical domain and an unstructured, disordered β-sheet region. Measurement of circular dichroism further shows that isomer **c** retains a substantial amount of α-helical structure (Fig. 3). The circular dichroism spectrum of the *N*-isomer (four native disulfide bonds) reveals the pres-

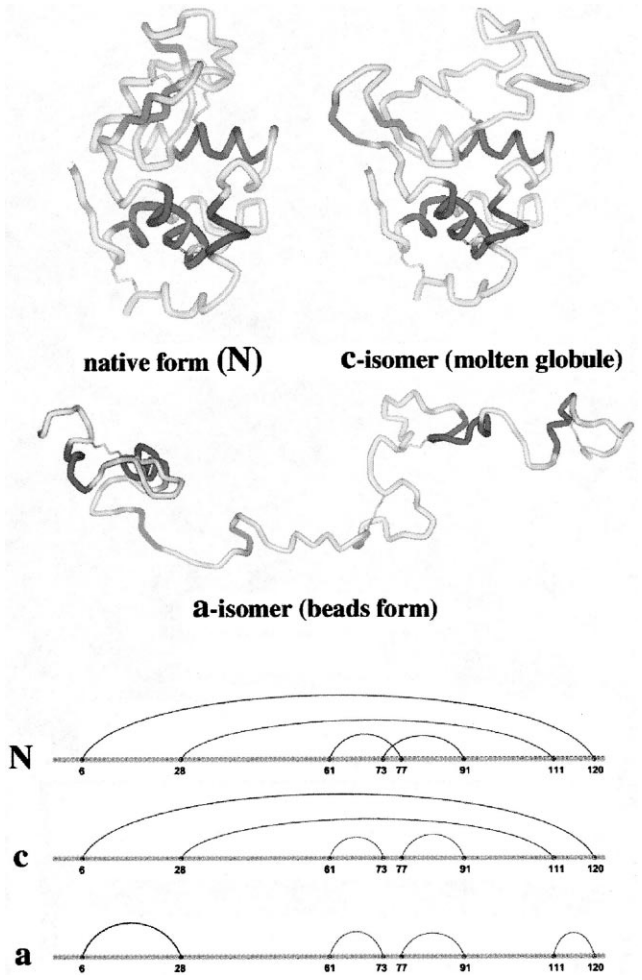

Fig. 2. The structures of the native and denatured α-LA. Top: 3D structure of the native α-LA and schematic presentation of two denatured scrambled isomers (**a** and **c**). Bottom: Disulfide structures of the native α-LA and isomers **a** and **c**.

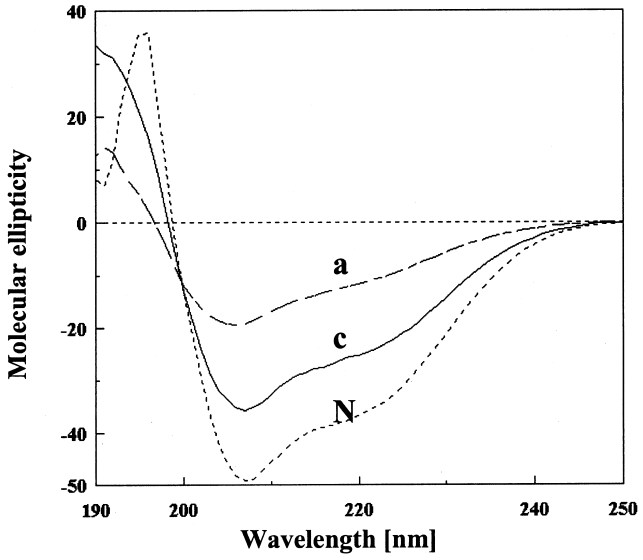

Fig. 3. The circular dichroism spectra of the native and denatured isomers (**a** and **c**) of α-LA. Samples were measured at the protein concentration of 0.5 mg/ml in the phosphate buffer (10 mM, pH 7.0) in a 100 μl cuvette (1 mm light pass) with the Jasco J-715 spectropolarimeter. The protein concentration was determined according to the Bradford method (BioRad kit). The individual isomers were purified by HPLC, as described.

ence of both β-sheet (peak at 197 nm) and α-helical structures, as expected from its known structure. Interestingly, the β-structure characteristics are absent in the **c** isomer. The only secondary structure observed for this isomer is α-helical, which is consistent with its disulfide structure. What is to some extent unexpected is that isomer **a** (beads-form) also retains a residual α-helical structure.

The unique structural properties of isomer **c** bear striking resemblance to those of the well characterized molten globule of α-LA. Structural elements that stabilize and specify the structure of molten globule of α-LA have been investigated by many different laboratories [7–13,16,17,20]. Recent data has shown that it is not a non-specific, collapsed polypeptide, rather, it has a native-like tertiary fold [10,11,12,29]. A systematic analysis of point mutations in the helical domain of α-LA demonstrates that native-like packing of core amino acids helps stabilize the structure of molten globule [12,30]. Hydrogen exchange NMR analysis also indicates that the most persistent structure in the α-LA molten globule is localized in the helical domain [31]. More specifically, Kim et al. have shown that the molten globule properties of α-LA are mainly confined to one of its two domains. The α-helical domain forms a helical structure with a native-like tertiary fold, whereas the β-sheet domain is essentially disordered [11,29]. These new findings differ somehow from the common definition of molten globule in which a fluctuated tertiary fold is believed to encompass the entire polypeptide chain [1,2]. Our data support those recent new findings and confirm that the molten globule of α-LA is composed of a structured and an unstructured domain. More significantly, our study demonstrates a simple method for preparation of a stabilized and structurally (disulfide) defined molten globule. Isomer **c** should be a useful model for understanding the folding/unfolding intermediates of proteins.

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