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Differing structural characteristics of molten globule intermediate of peanut lectin in urea and guanidine-HCl

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ABSTRACT

The structural characteristics of exclusive equilibrium molten globule-like intermediate formed during peanut lectin unfolding in urea and guanidine hydrochloride (GdnHCl) have been investigated by size-exclusion chromatography, circular dichroism, fluorescence, phosphorescence, and chemical modification. The elution behavior and 8-anilino-1-naphthalenesulfonate binding indicate a less compact tertiary structure in urea than in GdnHCl. Further, the urea-induced intermediate reveals perturbed, nonnative typical β-sheet conformation in contrast to native-like atypical β-structure in GdnHCl. N-bromosuccinimide oxidation shows that none of three tryptophan residues is modified for GdnHCl-induced intermediate while one gets oxidized in urea. Such difference in tryptophan environment is supported by acrylamide quenching (Stern–Volmer constant being 3.2 and 5.8 M^{-1} respectively), and phosphorescence studies at 77 K which show a blue-shift of (0, 0) band from 412.4 nm (GdnHCl) to 411.4 nm (urea). These results may provide important insight into the differential effects of GdnHCl and urea on the structural characteristics of intermediate state(s) in protein folding.

1. Introduction

A major difficulty in experimental investigations of protein folding problem is the reliable identification and structural description of the intermediate state(s). The studies of equilibrium unfolding of several globular proteins have elucidated a class of thermodynamically stable intermediates between the compact native and the unfolded random coil state. This class of intermediate states has been christened molten globule intermediates characterized by the presence of a pronounced secondary structure, a high compactness without a rigid packing, and a substantial increase in fluctuations of side chains of the molecule [1,2]. Compared to monomeric proteins, the folding/unfolding problem of oligomeric proteins is more complex as it involves subunit association/dissociation as well as monomer folding/unfolding, and the sequence of the two events is not similar in different systems. However, there is good evidence for the presence of molten globule intermediates whose characterization can help depict folding or unfolding pathway of oligomeric proteins in detail [3].

Lectins are oligomeric carbohydrate-binding proteins that are involved in various biological recognition processes [4]. Of many lectin families, legume lectins have attracted much interest for protein folding studies because they exhibit the same tertiary 'jelly roll' structural fold, a kind of β-sandwich comprising three antiparallel β-sheets [5] but differ considerably in their quaternary structures [6,7]. Peanut agglutinin (PNA) is a galactose-specific legume lectin, and a unique case of tetramer that lacks a 4-fold symmetry. The PNA tetramer can be considered as a dimer of dimers (Fig. 1). Two PNA subunits dimerize through back-to-back association of the flat six-stranded back β-sheets (subunits A and D; B and C; Fig. 1) [8]. Two dimers then form the tetramer with a canonical interface across subunits A and B by side-by-side arrangement of two back β-sheets leading to a contiguous 12-stranded sheet, and the unusual open interface formed across subunits C and D.

Guanidine hydrochloride (GdnHCl) and urea are the most widely used chemical denaturants for protein unfolding reaction. The unfolding pathways of many legume lectins in presence of these denaturants have been shown to be either a simple two-state (monophasic) or a multi-state (multiphasic) involving structured or partially folded intermediates [9–17]. Recently, we have explored the distinguishing structural aspects of soybean agglutinin and concanavalin A in different states that appear in their unfolding/refolding pathways [18,19], and demonstrated a unique molten globule fragment chain of pea lectin in its unfolding reaction in GdnHCl [20]. However, despite their everyday use in protein folding studies, the exact mode of action of GdnHCl and urea on proteins

Abbreviations: PNA, peanut agglutinin; ANS, 8-anilino-1-naphthalenesulfonate; NBS, N-bromosuccinimide; GdnHCl, guanidine hydrochloride; PBS, 20 mM sodium phosphate buffered with 0.15 M NaCl, pH 7.2; CD, circular dichroism; HPLC, high performance liquid chromatography.

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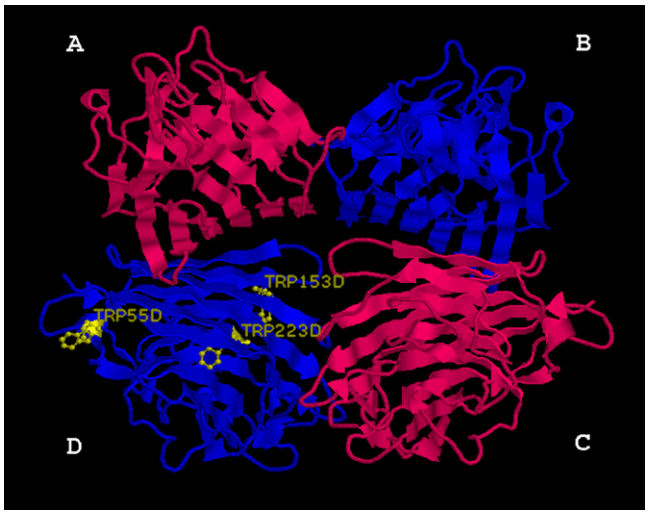

Fig. 1. Ribbon representation of three-dimensional structure of PNA tetramer (PDB entry 2DV9). Subunits A and D form one dimer while B and C form the other, through back-to-back association. In the tetramer, subunits A and B form the canonical interface and the interface between subunits C and D is the unusual 'open' type. The three tryptophan residues in subunit D are indicated in white ball-and-stick. Tryptophan residues for other subunit are not shown for clarity.

is not understood. Similar unfolding pathways are observed for the two denaturants in some protein systems while the pathways become different in others [21]. Further, under subdenaturing concentrations, both denaturants may stabilize the protein, though to different extent [22]. The unfolding pathway of peanut agglutinin (PNA) has been reported to be similar and biphasic for the two denaturants [23,24]. In this paper, we have addressed the possible effects of GdnHCl and urea on the intermediate species formed under equilibrium condition in order to characterize and compare the unfolding mechanism, and presented the differential structural characteristics of the molten globule intermediate formed in these denaturants. The unfolding reaction has been ensured to reach equilibrium whose composition has been examined by size-exclusion chromatography. Far-UV CD has been used to monitor the secondary structural characteristics of the intermediate. PNA has three tryptophan residues per monomer. Utilizing these tryptophans as intrinsic probe, we have investigated the tertiary structure with respect to tryptophan environment by fluorescence, phosphorescence at 77 K, and selective chemical modification of tryptophan(s) that might be accessible in the molten globule intermediate formed in GdnHCl and urea.

2. Materials and methods

2.1. Materials

Peanut seeds (*Arachis hypogaea*) were purchased from a local store. 8-Anilino-1-naphthalenesulfonate (ANS), guanidine hydrochloride (GdnHCl), acrylamide and guargum were obtained from Sigma. Cross-linked guar gum matrix was prepared as described [17]. The concentration of ANS was determined spectrophotometrically [25] using its molar extinction coefficient, $\varepsilon = 5000\ M^{-1}\ cm^{-1}$ at 350 nm. Urea (AR, E.Merck) was further crystallized from hot ethanol to remove possible contamination by cyanate ions. Urea and GdnHCl concentrations were determined by refractive index measurements [26]. The purity of acrylamide was checked from its absorbance at 295 nm using $\varepsilon = 0.23\ M^{-1}\ cm^{-1}$, and optical transparency beyond 310 nm [27]. All other reagents used were of analytical grade. Double distilled water was used in all experiments.

2.2. Protein purification

PNA was purified from the crude extract of peanut seeds by affinity chromatography as described [28] except that cross-linked guar gum was used as an affinity matrix. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [29]. The concentration of PNA was determined spectrophotometrically at 280 nm from its specific extinction coefficient, $A^{1\%,\,1\,cm} = 7.7$ [28], and molar concentration was expressed in terms of monomer ($M_r = 27{,}500$).

2.3. Protein unfolding

The equilibrium unfolding experiments in GdnHCl were carried out in PBS, pH 7.2 by treating PNA with varying concentrations of GdnHCl at 37 °C overnight. For unfolding in urea, the protein samples in different concentrations of urea in PBS were incubated at 37 °C for ≥40 h to ensure equilibrium. The protein concentration was 100 μg/mL.

2.4. Size-exclusion chromatography

The experiments on size-exclusion chromatography were performed using a Superdex-75 10/300 GL column attached to a Waters HPLC system. An aliquot of 200 μL of a protein sample (1 mg/mL) prepared by incubation with required concentration of urea or GdnHCl at pH 7.2 as described above was injected into the column. The column was preequilibrated with the same buffer in which the protein sample was prepared. The flow rate was 0.5 mL/min, and eluent was detected on-line by Waters 2489 UV–vis detector at 280 nm. The fractions corresponding to the separated peaks for urea-induced unfolding were collected and concentrated by membrane ultrafiltration units (GE Healthcare).

2.5. Steady-state absorption and fluorescence measurements

Ultraviolet absorption was measured in a Hitachi U-4100 double-beam spectrophotometer using Sigma cuvette (volume: 2 mL; path length: 1 cm).

Steady-state fluorescence measurements were performed with a Hitachi F-7000 spectrofluorometer (equipped with a 150 W xenon lamp) using Sigma cuvette (volume: 2 mL; path length: 1 cm). The excitation and emission band pass was 5 nm each, and scan speed was 60 nm/min. All spectra were corrected by subtraction of appropriate blanks without PNA.

ANS binding experiments were carried out at pH 7.2 with native PNA and different protein samples in urea or GdnHCl. In a typical experiment, PNA sample (4 μM) was incubated with 200 μM ANS for 5 min at 25 °C. Excitation wavelength was fixed at 370 nm, and the emission scanned from 400 to 600 nm.

2.6. CD measurements

CD experiments were performed on a J-815 spectropolarimeter (Jasco, Japan) equipped with a Peltier type temperature controller. CD measurements in the far-UV region were carried out at 25 °C in the wavelength range of 260–190 nm using path length of 1 mm, scan speed 50 nm/min and a response time of 2 s. The protein concentration was 0.3 mg/mL. The spectra were averaged over at least five scans to eliminate signal noise. The experiments were carried out in 20 mM sodium phosphate buffer at pH 7.2 in absence or presence of requisite quantities of denaturant.