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Research paper

Novel unfolding sequence of banana lectin: Folded, unfolded and natively unfolded-like monomeric states in guanidine hydrochloride

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ABSTRACT

The sequence of unfolding events of dimeric banana lectin (Banlec), as induced by guanidine hydrochloride (GdnHCl), has been investigated by size-exclusion HPLC, fluorescence, far-UV CD, low temperature phosphorescence and selective chemical modification. 8-Anilino-1-naphthalenesulfonate (ANS) binding indicates a structured unfolding intermediate which has been characterized as dissociated monomer by size-exclusion chromatography. Interestingly, the unfolding elution pattern reveals two distinct unfolded states. One is a usual random coil. The other represents a novel species having elution behavior and structural compactness (Stokes radius) similar to dissociated monomer but showing no regular secondary structure as determined by far-UV CD, thus resembling a natively unfolded state. *N*-Bromosuccinimide (NBS) oxidation shows that single tryptophan residue remains unmodified in dissociated monomer intermediate while the same is oxidized in natively unfolded-like species. Such difference in tryptophan environment in these species is supported by acrylamide quenching studies, and phosphorescence results at 77 K which show a blue-shift of (0,0) band from 414.8 nm to 409.2 nm. The present results reveal subtlety of structural characteristics of unfolded states of Banlec in GdnHCl, which provide important insight in protein unfolding reaction.

1. Introduction

Guanidine hydrochloride (GdnHCl) is believed to be an ideal chemical denaturant for protein unfolding reaction. Notwithstanding its widespread use, how the structural and energetic properties of proteins change with varying concentration of GdnHCl is not well understood. It is held that binding or interaction of GdnHCl occurs to both folded and unfolded states of proteins and the number of binding sites is more in the unfolded state than in the native state [1,2]. The results of denaturation studies of several proteins in the presence of high concentration (4–6 M) of GdnHCl indicate that the unfolded proteins could be described as random coils [3]. It was thereafter proposed that the unfolded states may also be distinct from unstructured random coils [4,5]. The question then arises: how unfolded are these states? Apart from random coils, if the unfolded states possess some structural features, how should they be classified? Dunker et al. [6] have formulated that the native intracellular proteins can exist in, not just the ordered state, but any of the three thermodynamic states: ordered, molten globule and disordered. Such intrinsically disordered proteins are known as natively unfolded proteins [7,8]. They lack ordered secondary structure and possess no specific or tightly packed tertiary structures. They are very flexible but may adopt relatively rigid conformation upon binding of ligands or changes in the environment.

Experimental investigation of protein unfolding reaction involves reliable identification and structural description of the species in unfolding pathways. Compared to monomeric proteins, the unfolding problem of oligomeric proteins is more complex as it involves subunit dissociation as well as monomer unfolding, and the sequence of the two events may vary in different systems. An important class of oligomeric proteins comprises lectins that bind carbohydrates specifically, and are involved in various biological recognition processes [9]. The denaturant-induced unfolding of many legume lectins is shown to be either a simple two-state process with no intermediate or a multi-state process involving structured or partially folded intermediates [10–15]. We have reported recently the distinguishing features of soybean agglutinin and concanavalin A in different structural states in their unfolding pathways [16,17], and demonstrated a unique molten globule

Abbreviations: Banlec, banana lectin; GdnHCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalenesulfonate; NBS, *N*-bromosuccinimide; PBS, 10 mM sodium phosphate buffered with 0.15 M NaCl, pH 7.2; CD, circular dichroism; HPLC, high performance liquid chromatography.

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fragment chain of pea lectin in GdnHCl-induced unfolding [18]. Very recently, we have explored the differential structural characteristics of the molten globule intermediate formed in the unfolding reaction of peanut agglutinin in urea and GdnHCl [19].

Banana lectin (Banlec) is a non-glycosylated homodimeric protein with specificity for mannose and high mannose oligosaccharides [20]. It is a member of the jacalin-related superfamily of lectins [21]. The structure of Banlec monomer comprises a single polypeptide chain that forms twelve β-strands in a β-prism-I fold (Fig. 1A). In this fold, three greek keys, each with four-stranded antiparallel β-sheets, are arranged like the triangular face of a prism with pseudo three-fold symmetry. The dimer is formed with the interface constituted primarily from the first face of the β-prism of each monomer (Fig. 1B). Banlec is unique among the members of the family that it possesses two distinct sugar binding sites per subunit [22]. Recently, it has been reported that Banlec is a potent inhibitor of HIV infection by binding to the mannose rich viral envelope and blocking cellular entry [23].

In this article, we have explored the GdnHCl-induced equilibrium unfolding sequence of Banlec using several techniques including intrinsic and ANS fluorescence, size-exclusion HPLC, far-UV CD, low temperature phosphorescence and selective chemical modification. Previously a two-state mechanism for GdnHCl-induced unfolding of Banlec was proposed [24]. In contrast, the present results demonstrate an unfolding sequence that involves, apart from a structured monomeric intermediate, two distinct unfolded states comprising unstructured random coil and a novel unfolded species resembling natively unfolded state. This appears to be the first report, to our knowledge, of an unfolded species resembling a natively unfolded state in the GdnHCl-induced unfolding sequence for oligomeric lectins.

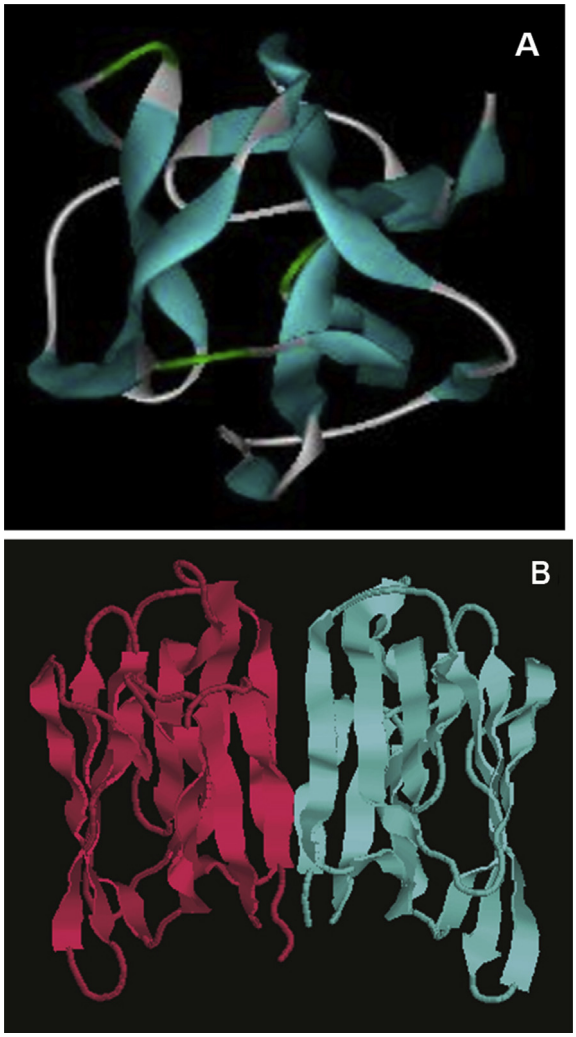

Fig. 1. (A) Ribbon representation of β-prism-I fold of Banlec monomer (PDB entry 1X1V). In this fold, three greek keys are arranged like the triangular face of a prism with pseudo three-fold symmetry. (B) The structure of Banlec dimer with the interface constituted primarily from the first face of the β-prism of each monomer.

2. Materials and methods

2.1. Materials

Ripe bananas (*Musa paradisiaca*) were purchased from a local store. 8-Anilino-1-naphthalenesulfonate (ANS), ultrapure guanidine hydrochloride (GdnHCl), acrylamide, D-mannose and Sepharose 4B were obtained from Sigma. The concentration of ANS was measured spectrophotometrically [25] from its absorbance at 350 nm using its molar extinction coefficient, $\varepsilon = 5000\ M^{-1}\ cm^{-1}$. GdnHCl concentrations were determined by refractive index measurements [26]. The purity of acrylamide was checked using its $\varepsilon = 0.23\ M^{-1}\ cm^{-1}$ at 295 nm, and optical transparency beyond 310 nm [27]. All other reagents were of analytical grade. Double distilled water was used throughout.

2.2. Protein purification

Banlec was purified from the crude extract of bananas by affinity chromatography on mannose-Sepharose 4B matrix as described [24] with slight modification. In the final step, the protein was eluted with 1.5 M D-mannose, and the eluent was concentrated using 10-kDa cut-off membrane ultrafiltration units (GE Healthcare) and stored at 4 °C. The protein solution was dialyzed extensively against appropriate buffer before each experiment. The integrity of dimeric native structure of Banlec was confirmed by size-exclusion HPLC on Superose-12 10/300 column and the purity of the sample was also checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [28]. The protein content was assayed by the method of Lowry et al. [29]. Based on this assay, the absorbance of Banlec, $A^{1\ mg/mL,\ 1\ cm}$ at 280 nm has been estimated to be 0.9.

2.3. Protein unfolding

The unfolding experiments of Banlec were carried out in varying concentrations of GdnHCl in PBS (10 mM sodium phosphate buffer containing 0.15 M NaCL, pH 7.2). The protein was incubated at 37 °C for 18 h to ensure equilibrium. The protein concentration was 100–250 μg/mL.

2.4. Size-exclusion chromatography

The size-exclusion chromatography experiments were performed on a Superose-12 10/300 GL column attached to a Waters HPLC system. An aliquot of 200 μL of protein samples (1 mg/mL) prepared by incubation with varying concentrations of GdnHCl in PBS, pH 7.2 was injected into the column. The column was pre-equilibrated with the appropriate buffer containing requisite concentration of GdnHCl. The flow rate was adjusted to 0.5 mL/min, and eluent was detected on-line by Waters 2489 UV–visible detector at 280 nm. To determine the size of the intermediate in 2.5 M GdnHCl, the column was calibrated with the following marker proteins under the same concentration of GdnHCl in PBS: carbonic