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Research paper

Pea lectin unfolding reveals a unique molten globule fragment chain

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ABSTRACT

Pea lectin (PSL) is a dimeric protein in which each subunit comprises two intertwined, post-translationally processed polypeptide chains -a long β-fragment and a short α-fragment. Using guanidine hydrochloride-induced denaturation, we have investigated and characterized the species obtained in the unfolding equilibrium of PSL by steady-state and time-resolved fluorescence, phosphorescence, and selective chemical modification. During unfolding, the fragment chains become separated, and the unfolding pattern reveals a β-fragment as intermediate that has the molten globule characteristics. As examined by 8-anilino-1-naphthalenesulfonate (ANS) binding, the fragment intermediate shows ~ 20 fold increase in ANS fluorescence, and a large increase in ANS lifetime (12.8 ns). The tryptophan environment of the molten globule β-fragment has been probed by selective modification with N-bromosuccinimide (NBS), which shows that two tryptophans, possibly Trp 53 and Trp 152 are oxidized while the other Trp 128 remains resistant to oxidation. The different types of tryptophan environment for the intermediate are supported by phosphorescence studies at 77 K, which gives a (0,0) band at 410 nm. These results seem to indicate that the larger fragment chain of PSL can independently behave as a monomeric or single domain protein that undergoes unfolding through intermediate state(s), and may provide important insight into the folding problem of oligomeric proteins in general and lectins in particular.

1. Introduction

Compared to monomeric proteins, folding problem of oligomeric proteins is more complex as their structure formation requires both monomer folding and monomer association. It is believed that protein folding/unfolding does not occur through a specific channel of mandatory intermediate states, and the sequence or hierarchy of the two events, subunit association/dissociation and monomer folding/unfolding, may not be similar even in case of a homologous protein family. Characterization of the intermediate(s) has been considered an important strategy in studying the folding/unfolding of oligomeric proteins [1,2].

Lectins are oligomeric carbohydrate-binding proteins that form a highly diverse group consisting of many protein families [3]. The legume lectins [4,5], and the animal lectins such as the galectins [6], the C-, I- and P-type lectins [7–9] provide classic examples of such families. Legume lectins have attracted much interest because of their easy availability, known three-dimensional structure, and large variation in their quaternary association of the same tertiary structural fold described as 'jelly roll' motif, a kind of a β-sandwich comprising three antiparallel β-sheets [4,10]. Legume lectins exist as different kinds of dimers and tetramers [11,12]. The most frequently observed mode of association is that of the "canonical dimer" [11], where the back β sheets of two subunits interact side-by-side to form a contiguous 12-stranded sheet. Thus legume lectins, which exhibit various kinds of quaternary association, can serve as excellent model systems for investigation of folding and association of oligomeric proteins.

Pea (*Pisum sativum*) lectin (PSL) is a dimeric protein (M_r = 49,000), and is specific for D-mannose/D-glucose [13]. It is a metalloprotein containing Mn^{2+} and Ca^{2+}, and has a single carbohydrate-binding site per monomer. Each subunit is post-translationally cleaved into an α- and a β-chain. The crystal structure of pea lectin shows that the α- and β-chains are closely interdigitated in the structure of one subunit [14]. The interface in the "canonical dimer" is composed almost exclusively of the β-chain.

In the past few years, reports on unfolding/refolding of several legume lectins (including PSL) have appeared in the literature [15–21]. The denaturant-induced unfolding of these proteins have been shown to be either a simple two-state (monophasic) or

Abbreviations: PSL, Pea (*Pisum sativum*) lectin; ANS, 8-anilino-1-naphthalenesulfonate; NBS, N-bromosuccinimide; GdnHCl, guanidine hydrochloride; REES, red edge excitation shift; HPLC, high performance liquid chromatography.

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a multi-state (multiphasic) process involving structured or partially folded intermediate(s). We have previously demonstrated the biphasic equilibrium unfolding of soybean agglutinin and concanavalin A [22,23], and their kinetic pathways of reassociation involving a structured monomeric intermediate [24,25]. Very recently, we have explored the distinguishing structural aspects of SBA in native tetrameric, intermediate monomeric and denatured states that appear in the unfolding/refolding pathway of the protein [26]. Previous studies on the unfolding of PSL in urea reported a thermodynamic analysis based on a two-state model [18]. A recent study by Küster and Seckler [27] shows that PSL reassembles via an intermediate not represented in the structural hierarchy. In this paper, we present a pattern of unfolding of PSL in guanidine hydrochloride that reveals a unique β–fragment as intermediate that has molten globule characteristics of the larger post-translational fragment (β-chain).

2. Materials and methods

2.1. Materials

Seeds of pea (*Pisum sativum*) were purchased from a local store. 8-Anilino-1-naphthalenesulfonate (ANS), guanidine hydrochloride (GdnHCl), acrylamide and methyl α-D-mannopyranoside were obtained from Sigma. Sephadex G100 was obtained from Pharmacia. The concentration of ANS was determined spectrophotometrically [28] using its molar extinction coefficient, $\varepsilon = 5000\ M^{-1}cm^{-1}$ at 350 nm. GdnHCl concentration was determined by refractive index measurements [29]. All other reagents used were of analytical grade. Double distilled water was used throughout.

2.2. Protein purification

PSL was purified from pea seeds by affinity chromatography on Sephadex G 100 as described [13]. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [30]. The concentration of PSL was determined spectrophotometrically at 280 nm from its specific extinction coefficient, $A^{1\ \%,\ 1\ cm} = 15$ [31], and expressed in terms of monomeric protein concentration using monomer mass of 24.5 kDa.

2.3. Protein unfolding

The equilibrium unfolding experiments were carried out in 20 mM sodium acetate buffer, pH 5 containing 0.15 M NaCl in presence of varying concentrations of GdnHCl at room temperature for ≥72 h or at 37 °C for ≥24 h. The monomeric protein concentration was 4–8 μM. In some experiments, the denaturation was carried out in presence of binding sugar, methyl α-D-mannopyranoside (10 mM). The unfolding reactions were monitored by intrinsic (tryptophan) as well as extrinsic (ANS) fluorescence.

2.4. Size-exclusion chromatography

The experiments on size-exclusion chromatography were performed using a Superose-12 10/300 GL column attached to a Waters HPLC system. An aliquot of 200 μL of a protein sample (20 μM) prepared by incubation with required concentration of GdnHCl at pH 5 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–Visible detector at 280 nm. The fractions corresponding to the separated peaks were collected and concentrated by membrane ultrafiltration units (Sigma). The column was calibrated with the following marker proteins: bovine serum albumin (66 kDa), soybean trypsin inhibitor (20.1 kDa), chicken egg ovalbumin (45 kDa) and cabonic anhydrase (29 kDa).

The extinction coefficient at 280 nm of the separated species as β-chain and α-chain was calculated using the method of Pace et al. [32] taking the molar mass of 17 kDa and 7 kDa, respectively [13]. The concentration of the species was determined by measuring absorbance at 280 nm, and expressed in terms of monomeric chains.

2.5. Steady-state absorption and fluorescence measurements

Ultraviolet absorption was measured in a Hitachi U 3210 double-beam spectrophotometer using Sigma cuvette (volume: 2 mL; path length: 1 cm).

Steady-state fluorescence measurements were performed with a Hitachi F-4010 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 PSL.

ANS binding experiments were carried out at pH 5 with native PSL, different protein samples during PSL unfolding in GdnHCl as well as the separated species obtained from size-exclusion chromatography of unfolding mixture. In a typical experiment, protein sample (8 μM) was incubated with 100 μM ANS for 7 min at 25 °C. Excitation wavelength was fixed at 370 nm, and the emission scanned from 400 to 600 nm.

2.6. Time-resolved fluorescence measurements

Fluorescence lifetime was measured by the Time Master Fluorometer from Photon Technology International (PTI), using PTI's patented strobe technique and gated detection. The software Felix 32 controls all acquisition modes and data analysis. For ANS fluorescence, excitation was done at 378 nm using a thyroton-gated nitrogen flash lamp. Lamp profiles were measured at 378 nm using slits with a band pass of 3 nm and Ludox as the scatterer. Intensity decay curves were fitted as a sum of exponential terms:

$$F(t) = \sum \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i represents the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered using a nonlinear iterative fitting procedure based on the Marquardt algorithm. The quality of fit has been assessed over the entire decay including the rising edge, and tested with a plot of weighted residuals and other statistical parameters, e.g., the reduced χ^2 ratio and the Durbin–Watson (DW) parameters. Mean lifetime $\langle\tau\rangle$ for biexponential decays were calculated using the equation [33]:

$$\langle\tau\rangle = \left(\alpha_1\tau_1^2 + \alpha_2\tau_2^2\right) \Big/ (\alpha_1\tau_1 + \alpha_2\tau_2) \quad (2)$$

2.7. Fluorescence quenching measurements

Acrylamide quenching of tryptophan fluorescence was measured from the decrease of fluorescence intensity obtained after serial addition of small aliquots of freshly prepared acrylamide solution (2 M) to a sample of PSL taken in a cuvette followed by mixing and incubation for 10 min in the sample compartment in the dark. With excitation wavelength of 295 nm, emission intensity was monitored at the respective emission wavelength maximum for each sample. The fluorescence intensities obtained were