Molten Globule-like State of Peanut Lectin Monomer Retains Its Carbohydrate Specificity

IMPLICATIONS IN PROTEIN FOLDING AND LEGUME LECTIN OLIGOMERIZATION

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A central question in biological chemistry is the minimal structural requirement of a protein that would determine its specificity and activity, the underlying basis being the importance of the entire structural element of a protein with regards to its activity vis à vis the overall integrity and stability of the protein. Although there are many reports on the characterization of protein folding/unfolding intermediates, with considerable secondary structural elements but substantial loss of tertiary structure, none of them have been reported to show any activity toward their respective ligands. This may be a result of the conditions under which such intermediates have been isolated or due to the importance of specific structural elements for the activity. In this paper we report such an intermediate in the unfolding of peanut agglutinin that seems to retain, to a considerable degree, its carbohydrate binding specificity and activity. This result has significant implications on the molten globule state during the folding pathway(s) of proteins in general and the quaternary association in legume lectins in particular, where precise subunit topology is required for their biologic activities.

Despite their preponderance in biologic systems, studies on the folding pathways of oligomeric proteins are less common and information regarding the same, meager. The folding process for oligomeric proteins is more complex as the acquisition of the quaternary structure entails both, the intramolecular refolding of the individual polypeptide chains and the simultaneous intermolecular interactions between the various subunits. Hence, elucidation of the hierarchy of events occurring during the denaturation of an oligomeric protein provides important means to delineate such a process.

Legume lectins, a class of highly homologous, carbohydrate-binding proteins exhibit the same jelly roll tertiary structural fold but differ considerably in their ligand specificity and quaternary structures (1, 3). They can hence be considered to be “natural mutants” of quaternary structures. However, apart from their agglutinating activity, the role of oligomerization in lectins is not clearly understood (1–11). Peanut (Arachis hypogaea) agglutinin (PNA),1 a homotetrameric nonglycosylated protein, violates an important principle of quaternary association in globular proteins, a unique case of a tetramer without a structural requirement of a protein that would determine its specificity and activity, the underlying basis being the importance of the entire structural element of a protein with regards to its activity vis à vis the overall integrity and stability of the protein. Existence of such a molten globule-like structure with retention of binding activity is perhaps unprecedented so far. Moreover, the occurrence of such a species for PNA suggests that the monomers of legume lectins are competent to bind sugars and oligomerization appears to impart them stability and necessary spatial disposition of sugar binding sites for manifestation of their respective biologic activities.

EXPERIMENTAL PROCEDURES

Materials—GdnHCl was a product of Amersham. All other chemicals used were of the highest purity available from Sigma. PNA purified as described previously, on SDS-polyacrylamide gel electrophoresis, showed a single band of M, 27,000 (15). The protein concentration was determined by its specific absorbance A280nm = 7.7 (16). Stocks of 8 mM GdnHCl were freshly prepared in appropriate buffers, filtered through 0.45-μm filters, and their concentrations determined by refractive index measurements (17). The buffers used were: 20 mM maleate for pH 3, 50 mM acetate for pH 4 and 5, 50 mM dimethylglyutarate for pH 6, and 50 mM phosphate for pH 7.4.

Unfolding Studies—Protein samples (10 μM) were incubated for 8–10 h at the desired temperature and denaturant solutions before measurements, to ensure equilibrium. Unfolding as a function of GdnHCl concentration was monitored by fluorescence spectroscopy, near- and far-UV circular dichroism. Intrinsic tryptophan fluorescence of protein samples (10 μM) was monitored in a 1-cm quartz cell in the 300–400 nm region, when excited at 280 nm, in a Jasco-FP777 spectrophotofluorimeter connected to a circulating water bath. Excitation and emission band passes of 3 nm were used. Far-UV CD (200–250 nm) and near-UV CD (250–300 nm) was followed in a 1-mm and a 10-mm path length cell, respectively, on a Jasco-J500A spectropolarimeter connected to a circulating water bath. The spectra were collected with a slit width of 1 nm, response time of 8 s, and a scan speed of 10 nm s⁻¹. Each data point was an average of eight accumulations. 8-Anilino-1-naphthalene sulfonate (ANS) emission spectra were recorded in the range of 400–500 nm, of samples excited at 380 nm at the desired temperature using slit widths of 5 nm. The changes in ANS fluorescence induced by the conformational changes in PNA were followed by measuring the intensity at 470 nm at constant concentrations of PNA (10 μM) and ANS (50 μM). The temperature inside spectrometer cells were monitored using a CIE digital thermometer.

Gel Filtration—Samples (10 μM PNA in 200 μl) incubated for 8 h with denaturant were injected onto a Superdex75 HR 10/30 column (1 × 30 cm) connected to a Pharmacia FPLC system preequilibrated and eluted with the 50 mM phosphate buffer (pH 7.4) and the required denaturant concentration at a flow rate of 0.5 ml/min. Samples were monitored by their absorbance at 280 nm.

1 The abbreviations used are: PNA, peanut agglutinin; GdnHCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalene sulfonate; FPLC, fast protein liquid chromatography; ConA, concanavalin A; mPNA, native PNA; mPNA, monomeric PNA; ITA, isothermal titration calorimeter.
Isothermal Titration Calorimetry— Isothermal titration calorimetric experiments were performed as described previously, on an Omega titration calorimeter (Microcal Inc.) (12, 13, 18). Aliquots of the ligand solution at 10–20 μl were added via a 250-μl rotating stirrer-syringe to the solution cell containing 1.5 ml of the 0.6–1.2 mM protein.

RESULTS

Chaotrope-induced Unfolding Profiles of PNA Display Biphasic Nature—The simple two-state assumption for denaturant-induced unfolding of protein may not apply for many oligomeric proteins as, in addition to the interactions within the same polypeptide chain, those between the subunits may make distinct contributions to its overall conformational stability. Consequently, although unfolded oligomers are unlikely to exist, the occurrence of native-like compact monomeric states is not excluded (19). In such instances, the overall unfolding reaction may be more appropriately described as

\[ A_n \leftrightarrow nA \leftrightarrow nU \]

where the unfolding may start with the folded oligomers \( A_n \), end with unfolded monomers \( U \), but have a significantly populated monomeric state. Such a non-two-state transition may display biphasic denaturation curves and/or nonsuperimposable transitions when the reaction is followed by different spectroscopic probes (19, 20). The GdnHCl-induced unfolding of PNA followed by fluorescence (at 321 nm) and near-UV CD and shows a very distinct biphasic nature (Fig. 1A). These unfolding transitions were found to be completely reversible. In the fluorescence and the near-UV CD transition, this intermediate species is populated predominantly in the 1.0–2.0 M GdnHCl range. In the far-UV CD transition, the intermediate appears in 1.4–2.2 M GdnHCl range. Additionally, the melting curves obtained by the different spectroscopic probes do not superimpose (Fig. 1A, inset). Thus, additional intermediates during the unfolding pathway of PNA may indeed occur, but we were unable to observe them as they are not populated significantly.

The Chaotrope Intermediate Is Monomeric in Nature—That the first transition could well be associated with the dissociation of tetramer to monomers was examined by size exclusion chromatography (Fig. 1B). Whereas the native PNA and that in the presence of 0.6 M GdnHCl (which correspond to the pre-transition base-line region of the denaturation profile) eluted as a tetramer (retention time: 18.4 min), the completely denatured form of the protein (in 5.2 M GdnHCl) eluted earlier (at 13 min), which is consistent with the retention time expected for a completely unfolded random polypeptide of a PNA monomer. However, at 1.8 M GdnHCl (the intermediate region of the melting curve) it elutes at 38.5 min, the position where a monomer of 25 kDa is expected to emerge. Also, under no circumstances was a dimer, in the entire region of the denaturation curve of the protein, observed. Taken together, these data show that the intermediate species is monomeric and that the unfolding of PNA proceeds through a compact monomeric form to a completely unfolded polypeptide chain. At pH 3.0 PNA is known to exist mostly as a dimeric species (21), consistent with its elution (28 min) in FPLC (not shown). The unfolding of the dimer also involves a monomer as the intermediate. Thus PNA dimer also unfolds through the monomeric form (Fig. 1A).

The Intermediate Has “Molten Globule-like” Characteristics—The nature of the intermediate was further examined by comparing its near- and far-UV CD spectra with the native and the fully denatured forms and by its ability to bind to the hydrophobic indicator ANS. As can be seen from Fig. 2, A and B, while its tertiary structure is lost dramatically (70%), the secondary structure is retained significantly. Thus the unfolding of PNA by chaotropes leads to the formation of a stable intermediate species, with most of its secondary structure intact but substantial loss of tertiary structure. Such intermediate species with similar spectral losses have been observed for a number of proteins, such as α-lactalbumin, cytochrome c, apomyoglobin, etc. (20–29).

Binding of the hydrophobic fluorescent probe, ANS, to proteins occurs upon the exposure of hydrophobic clusters during the unfolding process. We observed that ANS binds neither to the native nor the fully denatured states of PNA, but it does so very strikingly to the intermediate (Fig. 3A). Obviously, there are large clusters of solvent exposed hydrophobic regions that are unveiled in the intermediate and that are impinged by molecules of ANS, with a resultant increase in its emission intensity and a blue shift of its emission maximum. The intermediate is thus a molten globule-like state (26–30). Moreover, as shown in Fig. 3B, the maximum of fluorescence intensity of ANS occurs at progressively lower temperatures as the pH is lowered. Thus the intermediate occurs with greater propensity at more acidic pH which is not surprising as these conditions.
would promote deoligomerization of PNA (30).

The Molten Globule-like Intermediate Retains Its Carbohydrate Binding Activity—Finally, the FPLC-purified monomeric intermediate in 2 M GdnHCl, which was confirmed to retain its molten globule-like characteristics at the concentrations used for titrations, bound to the sugar ligands almost akin to its native counterpart (Fig. 4 and Table I). Their binding constant was nearly 80% that of native PNA. The stoichiometry \( n \) of interaction of the intermediate was comparable with that of the native PNA, both being close to one. It thus seems that the molten globule-like intermediate retains, to a significant extent, the sugar binding capability of the protein.

DISCUSSION

The unfolding process of peanut agglutinin studied by GdnHCl-induced denaturation was found to be completely reversible. The profiles were biphasic in nature when monitored by all the three spectroscopic probes, suggesting a non-two-state unfolding process. The intermediate in the unfolding of PNA was further characterized spectroscopically (fluorescence, near- and far-UV-CD), by gel filtration and by titration calorimetry, to determine the extent of its activity. The intermediate has about 80% of its secondary structural elements intact, yet has considerably decreased tertiary structure (nearly 70% loss of tertiary structure). It seems to be fairly compact in nature (its gel filtration elution volume corresponds to that of the monomer of PNA), and yet highly solvent exposed, as observed by its strong ability to bind ANS. This species may hence be described as a molten globule-like intermediate, as similar properties have been observed for the molten globule states of a number of proteins. For example, in the case acid isolated molten globule of \( \alpha \)-lactalbumin, there is nearly a 75% loss of tertiary structure, while the secondary structure is almost intact in addition to being highly compact in nature. Likewise, in cytochrome \( c \) this state is accompanied by a loss of approximately 23–30% of the secondary structure (20, 21, 23–26). Yet, unusually, the PNA intermediate, despite the reduced tertiary structure, retains its carbohydrate binding activity to a considerable degree. It thus seems that, at least for PNA, the architecture of the binding site in the intermediate seems sufficiently intact, necessary for its ligand recognition. These data, hence, not only provide information on the folding pathway of peanut agglutinin tetramer, which may be correlated with its three-dimensional structure, but also provide novel insights on the architecture of a functional monomer of a legume lectin. It is thus pertinent, at this point, to describe briefly the unusual features of its quaternary structure. Unlike any other legume lectin, PNA is best described as a dimer of two back to back dimers each 2-fold symmetric (10, 11). Although the two dimers, \( \text{viz.} \) subunits 1 and 4 and 2 and 3 in the tetramer, are also related by a dyad, the different dyads neither intersect nor are they mutually perpendicular. Consequently, the molecule has an open quaternary arrangement. The interactions between the two dimers involve two distinct kinds of interfaces.
Carbohydrate Binding Activity of a Molten Globe-like Structure of PNA

One is a side by side antiparallel alignment of the flat six-stranded back β-sheets, between subunits 1 and 2, like the highly extended "canonical" dimeric interface in legume lectins such as concanavalin A (ConA), pea lectins, etc. However, the two sheets at this interface do not come close enough as in ConA, pea lectin, etc. Instead the association between the two back β-sheets from subunits 1 and 2 are stabilized by six water bridges. The other interface, viz. between subunits 3 and 4, an incidental consequence of the presence of the two back to back (1-4 and 2-3) and a side by side (1-2) interfaces, has not been observed in any other tetrameric protein so far. The back to back association between the two flat back β-sheets across the subunits 1-4 and subunits 2-3 of PNA tetramer appears to be intrinsically less stable as compared with the canonical dimeric interfaces of the legume lectins. The 1-2 interface in peanut agglutinin, which is common to 1-4 and 2-3 dimers, does not link up to give a contiguous 12-strand sheet, as it does so in ConA, pea lectins, etc., hence it is unable to confer stability to the extent observed in the latter class of lectins and with a slight chaotrope perturbation, dissociates into monomers readily. The resultant exposure of the flat six-stranded back β-sheet may lead to the display of extensive hydrophobic patches that is recognized by ANS, although the exposure of a recently described additional hydrophobic region in PNA is not ruled out altogether (11). This monomeric state has considerably reduced tertiary structure and yet binds the ligand, indicating that the regions of the two sheets supporting the four carbohydrate binding loops as well as the loops themselves retain the geometric features of the combining site like that in the native protein.

Legume lectins exist as oligomers, the nature of which has important functional implications (1, 2). Studies by Brewer and co-workers (3, 6), for example, have shown that various lectins form well defined and characteristic lattices when complexed with multivalent oligosaccharides that may underlie their effects on cells such as mitogenesis. From our studies, it appears that lectin monomers may have all the necessary features compatible with carbohydrate binding and that oligomerization essentially appears to endow them with additional stability and the requisite topology of binding sites for manifesting their biologic activity.

REFERENCES

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