
Dynamic light scattering study of peanut agglutinin: Size, shape and urea denaturation

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Peanut agglutinin (PNA) is a homotetrameric protein with a unique open quaternary structure. PNA shows non-two state profile in chaotrope induced denaturation. It passes through a monomeric molten globule like state before complete denaturation (Reddy *et al* 1999). This denaturation profile is associated with the change in hydrodynamic radius of the native protein. Though the molten globule-like state is monomeric in nature it expands in size due to partial denaturation. The size and shape of the native PNA as well as the change in hydrodynamic radius of the protein during denaturation has been studied by dynamic light scattering (DLS). The generation of two species is evident from the profile of hydrodynamic radii. This study also reveals the extent of compactness of the intermediate state.

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1. Introduction

The knowledge of the dimensions of proteins in different state is always essential to get a complete understanding of protein conformation and its role in protein folding. A number of studies have enhanced our understanding of the protein secondary structure in non-native state but less attention has been paid to their dimension during unfolding process (Dev *et al* 2006b; Mitra *et al* 2005). Proteins can fold back to their native state by a two-state cooperative process (Sinha *et al* 2005; Mitra *et al* 2002; Jackson and Fersht 1991) or in multiple steps populating one or more intermediates (Louzada *et al* 2003; Hobart *et al* 2002). However, the intermediates are often transient and difficult to trap for characterizing their thermodynamic and kinetic properties. Often an intermediate called “molten globule” is observed during the folding pathway of many proteins. “Molten globule” state generally possess native like secondary structure but lacks specific tertiary interactions. It is also considerably more compact as compared to the unfolded state (Dolgikh *et al* 1981; Ohgushi and Wada 1983; Kuwajima 1989; Baum *et al* 1989; Christensen and Pain 1991; Dobson 1992; Redfield 2004). Direct measurement of any particular quantity in the

denatured state is more difficult compared to the native state as the unfolded structure is an ensemble average of several possible conformations. X-ray and neutron scattering techniques (Gast *et al* 1994) provide precise information on the distance distribution function of the molecule. The dimension of the protein in the different unfolded states gives insight about the protein structural changes that occur during denaturation. As compared to X-ray and neutron scattering the hydrodynamic radius (R_h) measurement is a less direct but an easily measurable physical dimension. R_h can be measured by dynamic light scattering (DLS). DLS measures the instantaneous intensity fluctuation arising from the Brownian motion of particles in a small volume. This instantaneous fluctuation can be analysed to get the diffusion coefficients of the particles. R_h can be obtained from diffusion coefficient by Stoke's-Einstein relationship. R_h obtained this way is the size of a spherical particle that would have a diffusion coefficient equal to the protein in solution. Most proteins are certainly not spherical. Their apparent hydrodynamic radii may strongly vary depending upon their shape and size. Besides, water molecules bound to the proteins may affect the diffusion affecting their hydrodynamic radii which can differ in some situations from the actual size of the molecule. Protein molecules

Keywords. Chaotropic denaturation; DLS; hydrodynamic radius; PNA

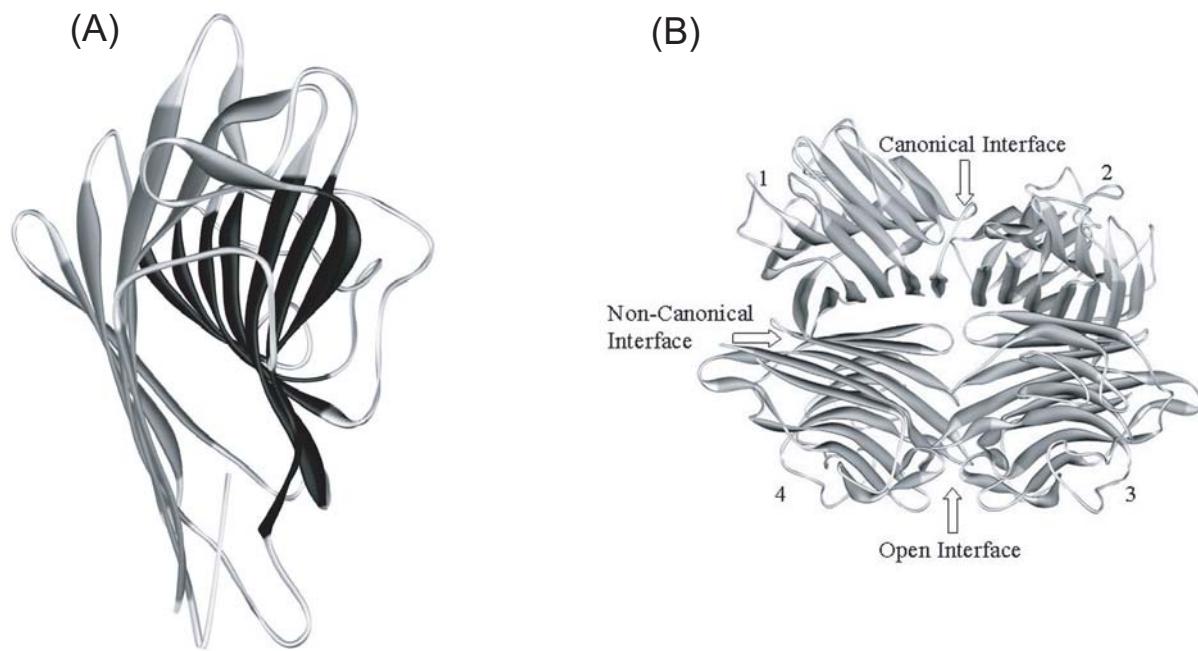


Figure 1. (A) Three-dimensional structural fold of PNA. Six stranded flat back beta sheet (dark gray), seven stranded curved front beta sheet (black) and small five stranded top beta sheet (light gray). (B) Tetrameric PNA with all the three different interfaces marked.

undergo changes in hydrodynamic radii during unfolding. Observation of change in R_h may be a good indication of protein unfolding pattern. It has been attempted for a homotetrameric protein peanut agglutinin (PNA) in this study.

PNA is a nonglycosylated homotetrameric protein (Banerjee *et al* 1996). Mainly three different beta sheets form the monomeric subunit of the tetramer (figure 1A). A flat six stranded back β sheet and a seven stranded curved β sheet at the front of the molecule are joined by a five stranded short top β sheet and several loops. The structure of the monomer is such that three different loops (91-116, 125-135 and 144-148) interact among them and with the front β sheet to form a secondary hydrophobic core besides the primary hydrophobic core present between the front and curved β sheet. The monomers assemble by three different types of interfaces (figure 1B). Two pairs of monomers (subunit 1-4 and 2-3) associate in a back-to-back fashion with an inclination with respect to each other of 86° (non-canonical interface, GSIV type). Monomers 1 and 2 involve side by side anti-parallel alignment of the back β sheet (canonical). The other interface consists of the monomers 3 and 4 which constitute a unique open interface. PNA shows a non-two state unfolding pattern involving the formation of a molten globule like intermediate (Reddy *et al* 1999) in thermal and chaotrope induced denaturation. This study is an attempt to follow a chaotrope induced denaturation of PNA by change in R_h .

2. Materials and methods

2.1 Materials

Ultra pure urea was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of the highest purity available. Urea solution was prepared in 20 mM PBS (phosphate buffer saline buffer) with 150 mM NaCl. Each urea solution was prepared fresh and concentration was determined by refractive index measurement as described by Pace (1990).

2.2 Protein purification

PNA was extracted from groundnut seeds and purified by lactosyl Sepharose affinity column. Sample purity was checked by SDS-PAGE. The concentration of protein was determined by specific extinction coefficient $A^{1\%}_{280} = 7.7$ (Lotan *et al* 1975).

2.3 DLS Measurement

DLS measurements were done using a DynaPro MS800 equipped with temperature control (Proterion, Protein Solutions, Wyatt Technology, Santa Barbara, CA, USA) operating at wavelength 830 nm and 256-channel multi tau correlator. The protein solutions were spun at 10 K

for 10 min and filtered through 100 nm pore size filter membrane (Whatman) into 12 μ l micro-cell. The translation diffusion coefficient was calculated by Dynamics V6 software, provided by the supplier. Hydrodynamic radius was calculated from translation diffusion coefficient by Stoke's-Einstein relationship. Experiments could not be done at very high urea concentration due to the solvent irregularity. Experiments at the other concentrations of urea did not consider any change in refractive index or viscosity of the solvent. The refractive index and viscosity values were taken for the phosphate buffer solution as provided by the software.

3. Results and discussion

PNA unlike several lectins like ECORL, SBA etc; exhibits a non-two state unfolding behaviour. A study on the three-state fitting of the non-two state unfolding induced by urea of PNA to extract all the relevant thermodynamic data has been reported recently (Dev *et al* 2006a). The study involved fitting of the non-two state unfolding process considering deoligomerisation from the tetramer to the monomeric molten globule initially and then its dissociation and unfolding to completely denatured state. An earlier study from this lab was concerned on the analysis of the sites that become accessible to a protease in the molten globule like intermediate generated during denaturation (Mitra *et al* 2005). Far and near UV circular dichroism studies in the presence of different concentrations of urea were also presented there.

Generally proteins fold into compact globular domains and are tightly packed. No voids or water molecules are accommodated in the interior though the surface of the protein is rather bumpy. We first determined the size and shape of the native PNA. Volume occupied by a single protein molecule can be found out from equation 1.

$$V = 1.212(\text{nm}^3/\text{Da}) * 10^{-3} * M(\text{Da}). \quad (1)$$

Here M is the molecular weight in Dalton and V , the volume of the protein in nm^3 . The molecular weight of PNA tetramer was taken as 110,000 Da (Natchiar *et al* 2004). The volume of the protein thus obtained was 133.32 nm^3 . The radius of the smallest globular protein that occupies a volume of 133.32 nm^3 was 3.16 nm. This is the theoretically determined value of the radius of the compact PNA molecule.

The hydrodynamic radius is highly affected by the shape of a protein molecule. Generally sedimentation coefficient or frictional coefficient is used to obtain information whether the protein is globular or elongated. Perrin equation, though widely used does not really work for proteins as their surface contours are rather uneven. Therefore, they experience more hydrodynamic drag compared to a smooth ellipsoid. We can have a broad estimation of the shape of the protein by

calculating

$$\frac{f}{f_{\min}} = \frac{S_{\max}}{S}. \quad (2)$$

f_{\min} or S_{\max} are the frictional coefficient or sedimentation coefficient of an unhydrated sphere corresponding to given mass of a protein. Sedimentation coefficient is related to frictional coefficient by the equation

$$S = \frac{M(1 - v\rho)}{N_0 f}. \quad (3)$$

M = molecular weight (Da); v = partial specific volume of the protein (typical value is 0.73 cm^3/g); ρ = density of solvent (1.0 g/cm^3 for H_2O); N_0 is the Avogadro's number (6.023×10^{23}); f is the frictional coefficient which depends on the shape of the protein. A more elongated shape creates more hydrodynamic drag and a larger value of f . Also, proteins are hydrated – the layer of 'frozen' water held by hydrogen or ionic bonds, or frozen by the interaction at the hydrophobic surface, increases the effective diameter and hydrodynamic drag of protein. Thus the factor f reflects both the shape and hydration.

Again, f is inversely related to diffusion coefficient, which in turn relates to hydrodynamic radius to Stoke's-Einstein relation

$$D = \frac{kT}{6\pi\eta R_h}, \quad (4)$$

η is the viscosity of the solvent (0.01 $\text{g}/\text{cm.s}$ for H_2O). By substituting all the relevant parameters solution for S_{\max} is obtained as in eq. (5).

$$S_{\max} = 0.00361 \times M^{2/3}, \quad (5)$$

where, S_{\max} is expressed in Svedberg's unit. This gives us the S_{\max} value to be 8.28 S. The experimentally determined value of S for PNA is 6.4 S (Fish *et al* 1978). Thus S_{\max}/S value is found out to be 1.29; which is consistent with a number of globular proteins (Tanfod 1961). The Perrin factor when multiplied by the theoretical radius of PNA gives a value of 4.08 nm. The R_h measured by DLS is 3.9 nm (figure 2). Hence, the theoretical value matches quite well with the experimentally observed value.

DLS has been used to study the hydrodynamic properties of PNA during chaotrope induced denaturation. Several data points have been taken for each urea concentration to calculate the hydrodynamic radii. The data points are marked manually. Data points show a fairly close distribution. The software fits these values to extract out the value of hydrodynamic radius. The fit yields the hydrodynamic radii of each of the species along with their percent Pd and percent intensity in the regularization view. The values of percent intensity of each species have been plotted in figure 4. Figure 5 shows a representative regularization fit at 4.3 M urea concentration. The observed hydrodynamic

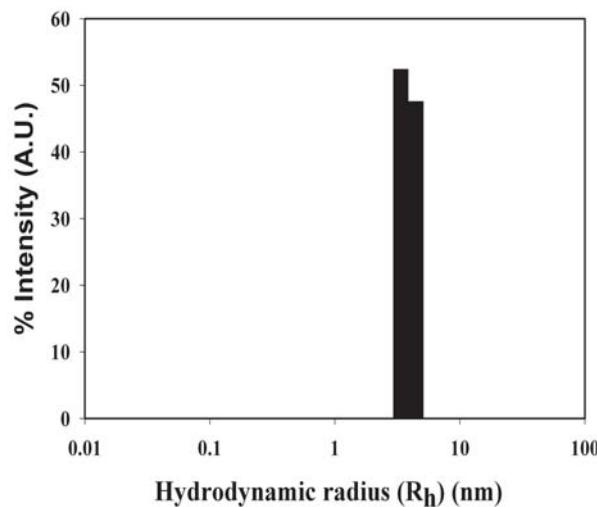


Figure 2. Hydrodynamic radius of native PNA. The protein concentration was 1 mg/ml in 20 mM PBS with 150 mM NaCl.

Table 1. Hydrodynamic radii and polydispersity of the protein at different concentration of urea.

Urea Conc.(M)	$R_h(1)$ (nm)	$Pd(1)$ (%)	$R_h(2)$ (nm)	$Pd(2)$ (%)
0	3.9	18.4		
1.07	4.1	12.8		
2.15	4.2	13.8	11.7	15.9
2.69	5	22.1	14.7	26.3
3.2	6.2	19.8	18.1	24
3.76	8.7	22.6	22.3	23.1
4.3	10.7	24.6	23.7	13.9
4.84	8	13.2	21.6	11.5
5.3	7.6	14	20	7.5
6.4	8.3	11.3	20.9	7.5
7.5	6.7	0.0	20.7	14.8
8.6	6	13.2	20.5	0.0

radii are shown in table 1. The hydrodynamic radii can not be followed at very high urea concentration due to solvent irregularity. A perusal of the profile of the hydrodynamic radii (figure 3) shows generation of intermediate species along with denatured state from 2 M urea onwards and the protein radius becomes almost constant at 5 M urea.

The intensity profile reported here (figure 4) shows the generation of the two species more precisely. The percent intensity does not correspond to the concentration of each species present; rather it is an indicator of relative population of the species. Initially at very low urea concentration only native form is present. With increase in concentration both the species start forming and as denatured species has higher

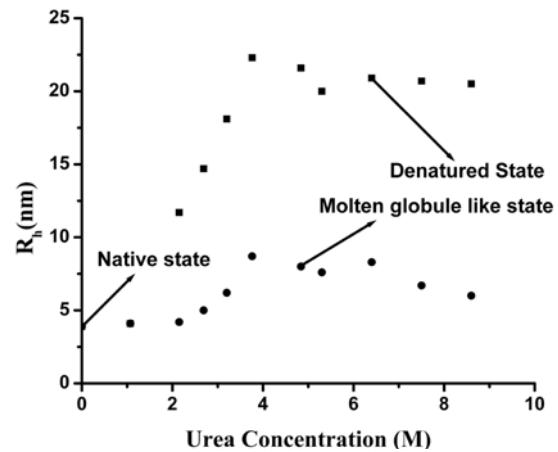


Figure 3. Change in hydrodynamic radius of the native PNA with increasing concentration of urea. Generation of the molten globule like state along with the denatured state can be identified. The value of hydrodynamic radius has been taken by deleting the odd data points and taking the value from the regularization view of the software.

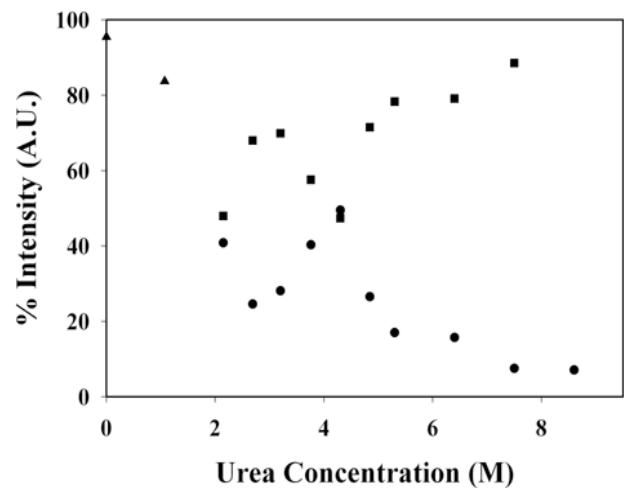


Figure 4. Intensity profile of all the species generated at different concentration of urea. The value of percent intensity is shown along with the hydrodynamic radius obtained by the regularization fit of the DLS data according to the software provided by the manufacturer. Those values have been taken and plotted. (▲), the native tetramer, (■), denatured species and (●), molten globule like state.

hydrodynamic radius compared to the intermediate species, denatured species contributes more in total intensity than the molten globule like state. With increase in concentration of urea as more and more molten globule like state starts forming, percent intensity of the denatured state starts decreasing and reaches the minima at 4.3 M urea. At the

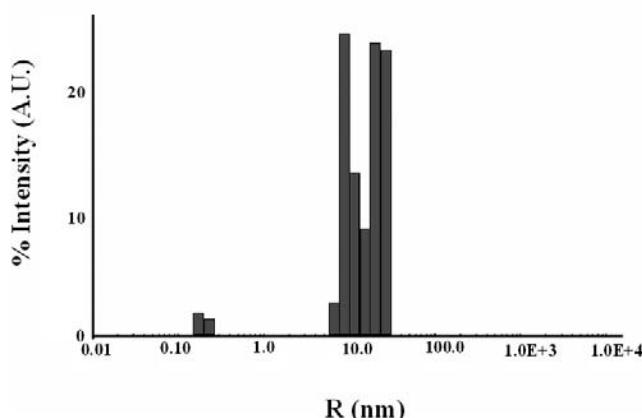


Figure 5. A representative regularization fit at 4.3 M urea concentration. The fit is obtained by the regularization fit, obtained as described in § 3, of the experimental correlation function. The fit shows the presence of three species. The first peak is from the solvent. Any species having radius less than 1 nm is considered to be emanating from the solvent. The hydrodynamic radii for the other two species are 10.7 nm and 23.7 nm. These values have been taken for comparison at different urea concentrations. .

same time the contribution to the total intensity count by the intermediate species increases. After 4.3 M of urea concentration, molten globule, like intermediate transforms to denatured species, hence its percent intensity starts decreasing. Analysis of the hydrodynamic radius (R_h), as presented here, is a good way to follow the simultaneous generation of two species. Fluorescence and circular dichroism in contrast provide information about the species present in the solution as a whole and constitute an average measurement of their physical properties in an ensemble but R_h on the other hand deals with the individual property. The compactness factor of the molten globule like state can be described as:

$$\text{Compactness Factor} = \frac{R_D - R_I}{R_D - R_N}, \quad (6)$$

R_D , R_I and R_N are the hydrodynamic radius of denatured, intermediate and native state respectively. If R_D , R_I and R_N are taken to be 20.5 nm, 10.7 nm and 3.9 nm respectively then compactness factor comes out to be 59.03%. Almost 40% unfolding of molten globule like state tallies very well with the amount present in the isothermal melting study with urea (Dev S, Nirmala Devi K, Sinha S and Surolia A, unpublished result). The homogeneity of the solution is reflected in the parameter percent polydispersity (Pd). Polydispersity is the measurement of standard deviation of the size of the particle. Native PNA shows polydispersity around 15–20%, corresponding to a monomodal species. With increase in concentration of

urea polydispersity keeps on increasing to more than 25% (table 1) along with the generation of the molten globule like state and the denatured state. Polydispersity shows a maximum value at 4.3 M urea concentration. With further increase in concentration of urea the concentration of the molten globule like state decreases in solution and the denatured species predominates. Polydispersity also decreases reflecting increase in homogeneity of the solution. Native PNA always exists with a small percentage of aggregate but there is no evidence of increase in percentage of aggregate compared to the native state during denaturation.

4. Refolding

Hydrodynamic radii during refolding of PNA at 7 M urea concentration was also followed by DLS. Dilution of the denatured protein from 7 M urea causes aggregation. Refolding experiments were not successful due to fluctuation in intensity due to aggregation. A preliminary study on refolding of PNA by tryptophan fluorescence shows that it passes through an intermediate state. The first step, folding from denatured to the intermediate step is quite fast while the second step viz. the folding from the intermediate state to the native state is very slow and requires several hours of dialysis (Dev *et al.* 2006a).

5. Conclusion

DLS has been used to monitor change in dimension of the protein during denaturation and renaturation (Nicholi and Benedek 1976; Gast *et al.* 1986). This has been extended here for studying chemical denaturation of PNA. PNA, the tetrameric protein exhibits scattering behaviour which is consistent with a globular protein as supported from the DLS studies. The change in R_h during chemical denaturation is a good measure of protein radii during unfolding. It provides a direct evidence for the generation of an intermediate during the unfolding process of PNA as exemplified here.

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