

## Structural changes during the unfolding of Bovine serum albumin in the presence of urea: A small-angle neutron scattering study

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**Abstract.** The native form of serum albumin is the most important soluble protein in the body plasma. In order to investigate the structural changes of Bovine serum albumin (BSA) during its unfolding in the presence of urea, a small-angle neutron scattering (SANS) study was performed. The scattering curves of dilute solutions of BSA with different concentrations of urea in D<sub>2</sub>O at pH  $7.2 \pm 0.2$  were measured at room temperature. The scattering profile was fitted to a prolate ellipsoidal shape ( $a, b, b$ ) of the protein with  $a = 52.2$  Å and  $b = 24.2$  Å. The change in the dimensions of the protein as it unfolds was found to be anisotropic. The radius of gyration of the compact form of the protein in solution decreased as the urea concentration was increased.

**Keywords.** Small-angle neutron scattering; Bovine serum albumin; urea denaturation.

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### 1. Introduction

Serum albumin (SA) is one of the most widely studied proteins. It is the most abundant protein in the circulatory system [1] and is chiefly responsible for the maintenance of blood pH [2]. Bovine serum albumin (BSA) is a single-chain protein and belongs to the class of serum albumins [3]. The folding of BSA is characterized by a unique arrangement of nine disulfide loops created by 17-disulfide bonds [4].

The globular protein BSA in aqueous solution has been extensively studied in the past by small-angle X-ray scattering (SAXS) [5], quasielastic light scattering (QELS) [6] and hydrodynamic techniques [7]. Hydrodynamic measurements of sedimentation coefficients and rotational relaxation time [8] indicate that the defatted BSA molecule has a prolate ellipsoidal shape having a molecular weight of  $66700 \pm 400$  Da [9]. Low-angle X-ray scattering [10] and neutron scattering [11] also indicated serum albumin to be a prolate ellipsoid. However, studies using <sup>1</sup>H NMR indicated that a prolate structure was unlikely; rather a heart-shaped structure was proposed [12]. The shape of albumin reveals a heart-shaped molecule that can be approximated to an equilateral triangle [13]. This was in agreement with X-ray

crystallographic data [13]. In the light of new information of X-ray crystallographic data [1], albumin structure is predominantly alpha-helical (67%).

The change in the conformations of a protein as it folds to its functional form is of great interest [14]. Equilibrium studies, as unfolding of a protein progresses, may give rise to interesting information about the equilibrium folding-states [15,16]. An important question is how many such states are possible, especially for multi-domain proteins like serum albumins. Urea-induced denaturation of proteins has been studied in the past [17]. BSA, during urea denaturation has been shown to follow a two-step, three-state transition [18]. Similar studies on human serum albumin (HSA) show domain III to be primarily responsible for intermediate formation during urea denaturation [19,20]. The unfolding of BSA seems to be a complex process in view of the independent unfolding behavior of all the three domains [21,22].

Present study is aimed to investigate the presence of any folding intermediate(s) during urea denaturation of BSA and to get an idea about the changes in the shape and size during the unfolding of BSA using small-angle neutron scattering (SANS). SANS is used here as a tool for investigating the equilibrium folding states of BSA as unfolding progresses, using urea as denaturant. The experimental curves were compared with theoretical curves of the prolate ellipsoidal model. The radius of gyration ( $R_g$ ) was obtained by means of regularization technique [23–25].

## 2. Materials and methods

### 2.1 Materials and sample preparation

Purified and defatted Bovine serum albumin (BSA) and urea were obtained from Sigma Chemical Co., St. Louis, USA and used as such.  $D_2O$  (99.4 at% of D) was obtained from Heavy Water Division, BARC, India.  $D_2O$  was used as a solvent in all the experiments for better contrast variation. The total scattering length of a dry BSA molecule  $\Sigma b_i = 0.1486 \text{ \AA}$  was calculated from its amino acid sequence [3]. The total number of exchangeable protons are 1018. Sample cells were quartz cuvettes from Hellma, UK with 1 cm path length. The protein samples were equilibrated in  $D_2O$  for 24 h for the complete H/D exchange. After adding urea, about 15 h equilibration time was given in order to attain an equilibrium folding-state. The concentration of BSA used in all the experiments was 1% (w/v). The temperature during the data collection was  $30 \pm 1^\circ\text{C}$ . The pH was measured to be  $7.2 \pm 0.2$  for BSA in pure  $D_2O$  and when urea was added.

### 2.2 Neutron scattering experiments and data analysis

Small-angle scattering data were collected using standard procedures on the SANS diffractometer at Dhruva reactor, Trombay, India. The scattering curves were measured at a mean neutron wavelength,  $\lambda = 5.2 \text{ \AA}$  covering the momentum transfer ( $Q$ ) range  $0.018 \text{ \AA}^{-1} < Q < 0.20 \text{ \AA}^{-1}$  ( $Q = 4\pi \sin \theta / \lambda$ , where  $2\theta$  is the scattering

angle). Scattering intensities were corrected for background, solvent and transmission and normalized to the cross-sectional unit using standard procedures. The normalized experimental data were fitted to the theoretically calculated intensity [26]. The fitting parameters were varied until satisfactory agreement is achieved by visually monitoring the fit.

Assuming the protein molecules to be a monodisperse system, the scattering probability per unit solid angle per unit path length is

$$d\Sigma/d\Omega(Q)(\text{in cm}^{-1}) = I(Q) = n \left[ 1 + \frac{\langle F \rangle^2}{\langle F^2 \rangle} \{S(Q) - 1\} \right] P(Q), \quad (1)$$

where  $\langle F \rangle^2 = P(Q) = \int_0^1 d\mu |F(Q, \mu)|^2$  is the intra-particle structure factor and  $S(Q)$  is the inter-particle structure factor.

$$\langle F \rangle^2 = \left| \int_0^1 d\mu F(Q, \mu) \right|^2 \quad \text{and} \quad F(Q, \mu) = (\rho - \rho_0) V 3j_1(u)/u$$

$$u = Q[a^2\mu^2 + b^2(1 - \mu^2)]^{1/2} \quad \text{and} \quad \mu = \cos \omega.$$

$n$  is the number density of the particles in solution;  $\omega$  is the angle between  $a$  and  $Q$ ;  $j_1(u)$  is the spherical Bessel function;  $\rho$  and  $\rho_0$  are the mean neutron scattering densities of hydrated BSA molecules and of the solvent, respectively. For weakly or non-interacting systems where  $S(Q) \sim 1$ , eq. (1) reduces to the familiar form

$$I(Q) = nP(Q). \quad (2)$$

This assumption is true as the BSA solutions used in the experiments were sufficiently dilute and  $S(Q) \sim 1$  in the  $Q$  range used [11].

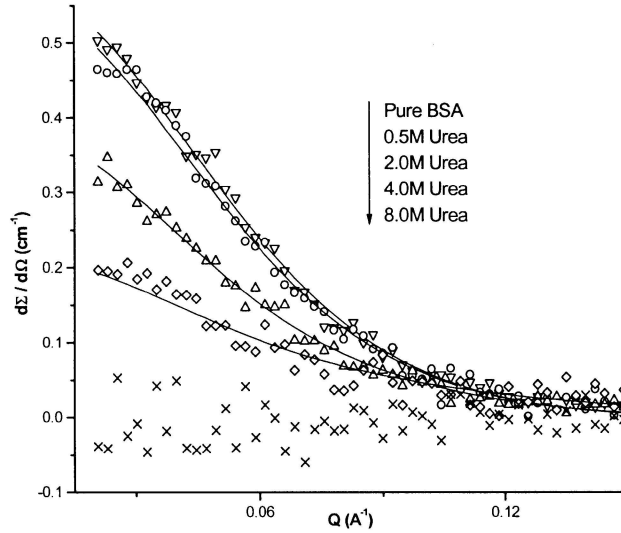
The experimental distance distribution functions  $P(r)$  were obtained by numerical calculation of the Fourier transformation

$$P(R) = \frac{1}{2\pi^2} \int_0^\infty I(Q) \cdot QR \sin(QR) dQ, \quad (3)$$

where  $I(Q)$  is the scattering curve extrapolated to zero angle using Guinier approximation [24]. The radii of gyration  $R_g$ , assuming monodisperse system of spheres, were evaluated by the indirect Fourier transform program GNOM [25].

### 3. Results and discussion

The scattering profiles were fitted to prolate ellipsoidal shape ( $a, b, b$ ) (figure 1) of the BSA molecule. Native protein has  $R_g = 26.61$  Å with semimajor axis,  $a = 52.2$  Å and semiminor axis,  $b = 24.2$  Å (figure 2). The  $R_g$  values (table 1) were found to be decreasing with increasing concentration of urea. This is due to the unfolding of the polypeptide chain and hence the size of the compact part of the protein decreases. The variation in the parameters  $a$  and  $b$  are shown in table 1. The two distance distribution functions shown in figure 3 for native BSA and BSA in 2 M urea showing the change in  $R_g$ , were obtained by GNOM [25]. As the protein

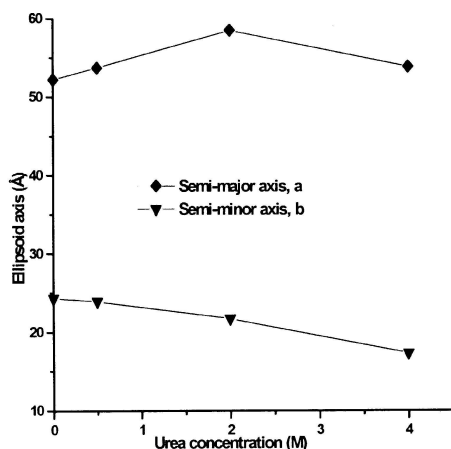


**Figure 1.** SANS profile showing the plot of scattering cross-section ( $d\Sigma/d\Omega$ ) vs. wave vector transfer ( $Q$ ) of 1% (w/v) BSA in the presence of urea. Solid line is the fitted profile.

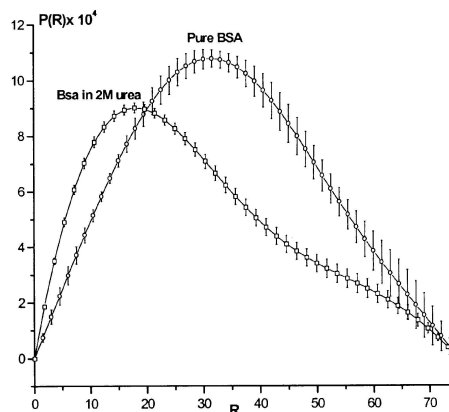
**Table 1.** Variation in the dimensions of the fitted ellipsoid with urea concentration.

Pure BSA	Semimajor axis, $a$ (Å)	Semiminor axis, $b$ (Å)	$R_g$ (Å)
In 0.0 M urea	52.2	24.2	26.61
In 0.5 M urea	53.7	23.9	26.45
In 2.0 M urea	58.5	21.7	23.56
In 4.0 M urea	53.9	17.3	19.40

unfolds, unfolded portions being random coils, are not seen by the neutrons. Thus scattering intensity decreased with increase in the urea concentration (figure 1) indicating that the protein is unfolding. There was an increase in the semimajor axis  $a$  up to 2 M urea then there was a decrease, indicating that the protein initially swells, and then seems to unfold along this axis (figure 2 and table 1). The initial swelling may be due to the destruction of compact secondary structure of BSA by urea and subsequent loosening and separation of the domains along the  $a$ -axis. The semiminor axis  $b$  decreased continuously up to 4 M urea (figure 2 and table 1). This shows that protein starts to unfold along the  $b$ -axis even at low concentrations of urea. The intensity decreased significantly at urea concentrations above 4 M. At 8 M urea concentration, data had no observable signal signifying that the protein has fully denatured (figure 1).



**Figure 2.** Changes in  $a$  and  $b$  with urea concentration.



**Figure 3.** Distance distribution functions of native BSA and BSA in 2 M urea evaluated by GNOM.

#### 4. Conclusions

It is a common belief that the unfolding of a polypeptide chain depends on many aspects of the tertiary and secondary structures, and the weak, non-covalent interactions involved therein [14,15]. As the unfolding of the polypeptide chain progresses, the tertiary structure may loosen up as a result of the breaking of non-covalent interactions at various locations in it. This further results in the reorientation and relaxation of the secondary structures, and their eventual disappearance. Urea is known to destroy the structure of water [27–29], which in turn, disrupts the secondary and the tertiary structures of proteins. Modeling of these events in their correct and chronological sequence is an interesting, but very difficult problem to be solved on the basis of SANS data alone. The results indicated that the unfolding of BSA below 4 M urea is not isotropic along the two ellipsoidal axes. Along  $b$ -axis, unfolding starts at low concentration of urea whereas along  $a$ -axis, the protein first seems to be swelling before the onset of unfolding. The protein was fully denatured at 8 M urea concentration with no compact structure in solution. As the protein unfolds at each equilibrium state there is a compact structure present. The size of this compact structure decreased with the increase in the urea concentration indicated by the decrease in  $R_g$ . There was no observable folding intermediate formation up to 4 M urea concentration.

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