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# Urea induced unfolding of F isomer of human serum albumin: A case study using multiple probes

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**Abstract**

The human serum albumin is known to undergo N ⇌ F (neutral to fast moving) isomerization between pH 7 and 3.5. The N ⇌ F isomerization involves unfolding and separation of domain III from rest of the molecule. The urea denaturation of N isomer of HSA shows two step three state transition with accumulation of an intermediate state around 4.8–5.2 M urea concentration. While urea induced unfolding transition of F isomer of HSA does not show the intermediate state observed during unfolding of N isomer. Therefore, it provides direct evidence that the formation of intermediate in the unfolding transition of HSA involves unfolding of domain III. Although urea induced unfolding of F isomer of HSA appears to be an one step process, but no coincidence between the equilibrium transitions monitored by tryptophanyl fluorescence, tyrosyl fluorescence, far-UV CD and near-UV CD spectroscopic techniques provides decisive evidence that unfolding of F isomer of HSA is not a two state process. An intermediate state that retained significant amount of secondary structure but no tertiary structure has been identified (around 4.4 M urea) in the unfolding pathway of F isomer. The emission of Trp-214 (located in domain II) and its mode of quenching by acrylamide and binding of chloroform indicate that unfolding of F isomer start from domain II (from 0.4 M urea). But at higher urea concentration (above 1.6 M) both the domain unfold simultaneously and the protein acquire random coil structure around 8.0 M urea. Further much higher $K_{sv}$ of NATA (17.2) than completely denatured F isomer (5.45) of HSA (8.0 M urea) suggests the existence of residual tertiary contacts within local regions in random coil conformation (probably around lone Trp-214).


*Keywords:* N ⇌ F isomerization; Urea denaturation; Chloroform binding; Multidomain protein; Human serum albumin

Over the past four decades, much research has been focused on two central questions: what are the determinants of the protein structures, and how does a polypeptide fold to its unique biologically active state? Folding of a protein in in vitro is likely to differ in many details from that in the cell. However, one feature, which is common in both in vitro and in vivo folding, is the existence of the intermediate conformational state on the folding–unfolding pathway [1,2]. Recent advances in biophysical techniques [3,4] have shown the presence of stable intermediate conformational states in a number of proteins [5–8]. There has been considerable interest in characterizing these partially folded states for gaining an insight into the possible determinants of the protein folds and the mechanism of protein folding [9–14]. The folding process is even more complex in multidomain proteins where each domain may be capable to unfold/refold independently and interdomain interactions may affect the overall folding topology of the proteins [15–17].

Human serum albumin (HSA)[1] is a monomeric, multi-domain protein of 66.5 kDa. It consists of three

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[1] *Abbreviations used:* HSA, human serum albumin; MRE, mean residue ellipticity; I state, intermediate state;

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homologous domains encompassing the complete sequence, each of which displays specific structural and functional characteristics [18]. Its overall native three dimensional conformation is stabilized by various intra- and inter-domain forces such as salt bridges, hydrophobic interactions, and natural boundaries involving helical extensions $h_{10}$ (domain I)–$h_1$ (domain II) and $h_{10}$ (domain II)–$h_1$ (domain III) existing between three domains of albumin [18–20]. It is the principal protein of blood plasma that aids in the transport, metabolism, and distribution of exogenous and endogenous ligands[18,19,21].

HSA is known to undergo different pH dependent conformational transition, the N $\rightleftharpoons$ F transition between pH 5.0 and 3.5, the F $\rightleftharpoons$ E transition between pH 3.5 and 1.2 and N $\rightleftharpoons$ B transition between pH 7.0 and 9.0 [20,21]. Geisow and Beaven [22] proposed that N-F transition involved the unfolding of domain III from the rest of the molecule which was later verified by Khan et al. [23] using proteolytic fragment serum albumin containing residues 377–582. Using three recombinant domains of HSA, Dockal et al. [20] have provided evidence that the loosening of the HSA structure in the N–F transition takes place primarily in domain III.

Urea is known to denature human serum albumin by three state mechanism with accumulation of an intermediate state. While formation of intermediate state in the unfolding pathway of HSA has been widely studied [24–27], but there is little understanding about the conformational state of different domains at intermediate state [8,26]. As discussed above F isomerisation of HSA displays specific structural characteristic. Unfolding studies of this protein (F isomer of HSA) will provide powerful means for understanding important structural and functional characteristic of the native molecule. In view of this, conformational behavior of F isomer of HSA in buffered urea solution (pH 3.5) was monitored using fluorescence and CD spectroscopic measurements and the results were compared with behaviors of native HSA molecules in neutral urea solutions.

## Materials and methods

### *Materials*

Human serum albumin (HSA) essentially fatty acid free, lot no. 90K7604; ultra pure urea, lot no. 42K0133 were obtained from Sigma Chemical, USA. Chloroform was purchased from Sisco Research Laboratories, India.

All the other reagents were of analytical grade. HSA was freed from dimers and highermers by passing through Sephacryl-S100 (HR) (76 × 1.8 cm) gel filtration column.

Protein concentration was determined spectrophotometrically using $E^{1\%}_{1cm}$ of 5.30–280 nm [28] on a Hitachi spectrophotometer, model U- 1500 or alternately by method of Lowry et al. [29].

### *CD measurements*

CD measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulphonic acid. All the CD measurements were made at 25 °C with a thermostatically controlled cell holder attached to Neslab's RTE-110 water bath with an accuracy of ±0.1 °C. Spectra were collected with scan speed of 20 nm $min^{-1}$ and response time of 1 s. Each spectrum was the average of four scans. Far-UV CD and near-UV CD spectra were taken at protein concentrations of 1.8–2.0 and 20 μM with a 1 mm and 1 cm path length cell, respectively. The results were expressed as mean residue ellipticity (MRE) in deg $cm^2$ $dmol^{-1}$ which is defined as $MRE = \theta_{obs}/(10 \times n \times l \times C_p)$, where $\theta_{obs}$ is the CD in millidegree, $n$ is the number of amino acid residues (585), $l$ is the path length of the cell, and $C_p$ is mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al. [30]:

$$\%\alpha\text{-helix} = (MRE_{222nm} - 2340/30300) \times 100. \quad (1)$$

### *Fluorescence measurements*

Fluorescence measurements were performed on Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1 cm pathlength cell. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300–400 nm. A stock solution of ANS was prepared in distilled water and its concentration was determined using an extinction coefficient of $\varepsilon_M = 5000\ M^{-1}\ cm^{-1}$ at 350 nm. For ANS fluorescence in the ANS binding experiments the excitation was set at 380 nm and the emission spectra were taken in the range of 400–600 nm.

### *Denaturation/renaturation experiments*

Stock protein solutions (for N and F isomers) were prepared by exhaustive dialysis of HSA monomer against 60 mM sodium phosphate buffer of pH 7.0 and 10 mM sodium acetate buffer of pH 3.5. To a 0.5 ml stock protein solution (N and/or F isomer), different volumes of the desired buffer were added first; followed by the addition of stock urea solutions prepared in their respective buffer (10 M) to get a desired concentration of denaturant. The final solution mixture (3.0 ml) was