Molten globule intermediates of human serum albumin in low concentration of urea

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Interaction of non-electrolytes such as urea with proteins especially at lower concentrations is opening-up newer concepts in the understanding of protein stability and folding in proteomics. In this study, the secondary and tertiary structural characteristics and thermal stability of human serum albumin at lower concentrations of urea have been monitored. The protein attains a molten globule like structure at concentration urea below 2 M. This structural state also shows an increase in the α-helical content as compared to the native state. At concentrations of urea above 2 M, human serum albumin starts unfolding, resulting in a three-state transition with two mid points of transitions at around 4 M and 7 M urea concentrations. The characteristics of the partially folded intermediates are discussed with respect to the three component system analyses. Preferential hydration dominates over preferential interaction at lower concentration of urea (up to 2.5 M) and at higher concentration, the preferential interaction overtakes preferential hydration in a competitive manner. Formation of structural intermediates at lower concentration of urea is hypothesized as a general phenomenon in proteins and fits in with the observation with preferential interaction parameters by Timasheff and co-workers in the case of lysozyme and ribonuclease at different pH values.

Human serum albumin (HSA) is a 66 kDa single polypeptide protein composed of three homologous helical domains, and is used as a model protein in this study to understand the interaction of urea at extended lower and higher concentrations. The structural state of several proteins in denaturants has been dealt in depth for a number of proteins from a both energetic point of view, as well as thermodynamic angle. If one looks at the abundant treasure of data, one can see the energy of stabilization and denaturation of proteins is a direct function of concentration of denaturants. Of importance are the observations made by Timasheff, which show for the first time that there are two types of interactions possible with proteins depending on the denaturant concentration; One at low concentration and the other at high concentration of denaturant. The thermodynamics angle of such a phenomena has been looked into both by enthalpic measurements of HSA at low and high concentration of urea through differential scanning calorimetry, as well as apparent thermal transition temperature measurements substantiated by structural analyses. This throws more light on the mechanism of interaction of urea at lower concentration generating the molten globule like structures and would also help understand the mechanism of the structural state of protein at higher concentration of the denaturant. The structural stability of HSA at very low and high concentrations of urea i.e. below 2 M and above 7.5 M, were studied exclusively from the above point of view.

Most of the preferential interaction studies with proteins were performed between 2 M to 8 M of urea and 1 M to 6 M of GdnHCl. The interaction of urea and GdnHCl with RNase A, bovine serum albumin and other proteins showed a decrease in net preferential interaction after reaching the maximum preferential interaction and also completely unfolded state. Anomalous behavior of proteins at low concentration of denaturants has been reported in a number of cases where structural intermediates have been characterized. However, no emphasis is made on the mechanistic angle of denaturant interaction with proteins. Our initial study on such an interaction showed that urea at low concentrations preferentially hydrates HSA and increases the apparent Stokes radius of the molecule. Preferential interaction of urea with HSA was also shown to decrease at very high concentra-
tions (>8 M)\(^9\). The interaction of urea at lower concentrations with proteins in terms of structure and stability are less understood as compared to that at higher concentrations and the general mechanism of interaction of urea at low concentration with proteins is still not clearly understood\(^{10,11}\). The structural intermediates characterized at low denaturant concentrations differ from the kinetic intermediates formed early in the folding pathway\(^{12,13}\). Hence, it is of importance to understand the interaction of urea especially at low concentrations with HSA directly from the calorimetric measurements as well as structural angle of the protein in solution.

Materials and Methods

Materials

Human serum albumin, Cohn fraction V (essentially fatty acid free), ANS and urea were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. The monomeric form of HSA was prepared by passing through Sephadex G-100 gel to remove other polymers and the homogeneity of the eluate was established by SDS-PAGE\(^{13}\). The analytical grade buffer salts, triple quartz distilled water were used in all of the experiments.

Methods

Circular dichroic spectral measurements

Far-ultraviolet circular dichroic studies were performed between 200 nm to 260 nm using Jasco J-20C automatic recording spectropolarimeter at 30°C with 1 nm bandwidth. Near-ultraviolet circular dichroic studies were performed between 250 nm to 350 nm using Jasco J-810 software driven spectropolarimeter at 30°C with 10 nm bandwidth. HSA was equilibrated with varying concentrations of urea by equilibrium dialysis for at least 24 hr with stirring at 30 ± 2°C with the concentration of 2.8×10\(^{-6}\) M and 7.5×10\(^{-6}\) M was used respectively for far-UV and near-UV analyses. The mean residue weight of 113.57 was used for all calculations. The data was analyzed according to the standard procedure\(^{15}\).

Thermal denaturation studies

(i) Spectroscopic method

HSA solution concentration of 2.8×10\(^{-6}\) M in different urea concentration was dialyzed against large amount of the respective concentration of urea for at least 24 hr with stirring at 30°C. The respective dialyzates were used as blanks to monitor the thermal denaturation profile of equilibrated protein using Gilford Response-II spectrophotometer. The absorbance at 287 nm was monitored over a temperature range of 25°C to 95°C, with the temperature increment programmed for 0.6°C/min. Apparent thermal transition temperature (T\(_{m,(app)}\)) was calculated according to standard procedure using also the software of Gilford Response-II spectrophotometer\(^{16}\).

(ii) Differential scanning calorimetric method

Differential scanning calorimetric measurements were made using MC-2 ultrasensitive microcalorimeter (Microcal Inc., USA) at a scan rate of 1.5°C/min. Instrumental baseline was determined with both the cells filled with degassed dialyze before scanning of each sample. HSA equilibrated either with buffer or with urea was degassed and exact concentration was determined with appropriate corrections for molar extinction coefficients of HSA. A concentration of 6 mg/ml of HSA was used in all of the experiments. The data was analyzed using Origin software (2.9 Ver.) and was best fit to non-two-state model of denaturation. The thermodynamic parameters were calculated according to the procedure described elsewhere\(^{17}\).

Fluorescence studies

(i) HSA intrinsic fluorescence

A protein of concentration of 2.8×10\(^{-6}\) M was equilibrated to required concentration of urea by dialysis. The fluorescence emission properties of the equilibrated protein were monitored in the range of 300-400 nm at 30°C using Shimadzu RF-5000 spectrofluorimeter\(^{18}\).

(ii) ANS induced fluorescence studies

HSA at concentration of 2.8×10\(^{-6}\) M was equilibrated with different urea concentrations and was mixed with freshly prepared ANS. The mixture was incubated with constant shaking at 100 rpm at 30°C for 3 hr in Queue orbital shaker. Fluorescence emission was monitored between 450-550 nm keeping the excitation at 395 nm. Only ANS with different urea concentrations and ANS with HSA in buffer were used as proper blanks. ANS:HSA mole ratio of 300:1 was used with varying equilibrium concentration of urea.
Results and Discussion

Secondary structural analysis is used to monitor the subtle conformational changes in proteins derived either from specific and/or non-specific ligand/solvent interactions with different structural motifs and/or domains of proteins. Circular dichroic measurements of HSA equilibrated to different urea concentrations were performed and the rotations at 222 nm are plotted as a function of urea concentration, as shown in Fig. 1A. At lower urea concentration, the α-helical content of HSA increased and reached a maximum value of about 10% at 1.2 M urea concentration where the rotations at 222 nm reaches a maximum value. Above this concentration, the rotations gradually decreased with the decrease in α-helix. But the near-ultraviolet studies showed only a subtle decrease in the tertiary structure up to 2 M of urea concentration (data not shown).

Effect of secondary structural change induced by urea at low concentrations on the thermal stability of HSA was monitored using both spectroscopic and differential scanning calorimetric methods. In Fig. 1B is shown the effect of different concentrations of urea on the T_m(app) of HSA. A single thermal transition was observed at low concentration of urea (<2 M) which was monitored for the cumulative effect of tyrosine and tryptophan at 287 nm. HSA in phosphate buffer showed a T_m(app) of 73°C and at low concentration of urea it increased up to 81°C at 1.8 M of urea. Above 2 M urea concentrations no thermal transition was observed (Fig. 1B).

Differential scanning calorimetric measurements were made as an additional probe of thermal denaturation spectroscopy and also to evaluate the heat capacity changes of denaturation. In Fig. 2 is shown the representative differential scanning thermogram of HSA in 0.8 M urea (open circles). The profile was best-fit to a non-two-state denaturation model, which is de-convoluted into two clear transitions (dotted lines), first at 61°C and the second at 72°C. In Fig. 3 is shown the effect of urea on the ΔC_p changes of the two transitions of HSA at different lower concentrations of urea. The ΔC_p increases and reaches a maximum value at 1.5 M urea concentration after which it decreases gradually with increase in urea concentration. Above 3 M urea concentration there was only one transition observed, the midpoint of which decreased with the increase in denaturant concentration following typical denaturation process.

At 2 M and 10 M urea concentrations the difference in the partial molar volume and other spectral parameters of HSA may be same but the extent of interaction are different as shown in our previous report. Thermal denaturation studies also showed that the stability of HSA was increased at lower concentrations of urea as reflected in the increase in T_m(app) of HSA. Differential scanning calorimetric data showed

Fig. 1—A: Changes in the circular dichroic spectral rotations of HSA at 222 nm as a function of low concentrations of urea at 30°C [Circular dichroic studies were performed between 200 nm to 260 nm using Jasco J 20-C automatic recording spectropolarimeter at 30°C with 1 nm bandwidth. Rotations at 222 nm were evaluated. HSA solution concentration of 2.8×10⁻⁵ M was used for spectral analysis. The mean residue weight of 113.57 was used for all calculations. [θ] is in deg cm² dmol⁻¹]; B: Changes in the apparent thermal transition temperature (T_m(app)) of HSA as a function of low concentrations of urea [The thermal transitions were measured to an accuracy of ± 0.5°C using the absorbance at 287 nm on a Gilford Response—II thermal programming spectrophotometer]
the increased stability of HSA as reflected from the molar enthalpic values at lower concentration of urea. Temperature induced transitions of HSA from native to denatured state at lower concentrations of urea are broader compared to native protein indicating less co-operativity of the folding intermediates, which is a general observation made in case of many proteins.

Role of such differential effect of urea at low and high concentrations on the surface properties of HSA was interesting to probe. The hydrophobic clusters on the surface of proteins are very sensitive to tertiary structural changes derived from specific and non-specific protein interactions. This is especially true with a third component such as ANS and has been very successful as a hydrophobic fluorophore to monitor such changes. In Fig. 4 is shown the interaction of ANS with HSA as monitored by the increased fluorescence emission intensity of ANS at 485 nm upon binding to protein. ANS also binds to native HSA in buffer. Changes in the ANS fluorescence only in urea with the increase in concentration are not very significant. In the case of HSA equilibrated with urea, the ANS fluorescence intensity increased significantly with the increase in denaturant concentration reaching a maximum at 1.5 M of urea after which it started declining.

**Fig. 2**—Representative differential scanning thermogram of HSA at 0.8 M of urea concentration [The data were obtained with the scan rate of 1.5°C/min and curve-fitted to non-two-state denaturation mechanism. The first (61°C) and second (72°C) transitions are represented by 'a' and 'b' respectively].

**Fig. 3**—Molar enthalpic changes of the two thermal transitions of HSA (a' and 'b) obtained from the differential scanning calorimetric experiments are shown as a function of low concentrations of urea [The data were best deconvoluted to give two transitions using non-two state of denaturation analysis].

**Fig. 4**—Fluorescence emission intensity of ANS in presence of HSA at different concentrations of urea (squares) [The intensity at zero denaturant concentration represents the ANS binding to HSA in the native state. ANS:HSA mol/mol ratio of 300:1 was used in all the experiments with freshly prepared ANS in buffer. ANS fluorescence in buffer containing different concentration of only urea is shown as control (circles)].
state is positively hydrated as compared to native state giving more stability to the protein as indicated by $T_{m(app)}$ values and also partial molar volume of HSA. ANS, a hydrophobic fluorescent probe has been used in many protein systems to characterize the molten globule state either in lower concentration of denaturant or with different pH. Here, we show that ANS binding to HSA reaches a maximum at 2 M concentration of urea where hydration also reaches maximum. Since ANS is in aqueous media it can reach the exposed hydrophobic groups and bind strongly to them. Once the preferential binding of urea starts, the ANS binding decreases, overriding all the characteristics of the molten globule. HSA has 55% $\alpha$-helix that can be easily perturbed by the change in the solvent environment (Prakash and Muralidhara, unpublished results) and weak reversible binding of ligands like chlorogenic acid and caffeic acid to HSA$^{19}$. Increase of rotations up to 1500 at 222 nm resulting in 10% increase in the $\alpha$-helix in the maximum hydration region is shown in Fig. 1A. Fluorescence studies also showed that there is no perturbation of the single tryptophan (Trp-214) located in domain II of HSA at lower concentrations of urea as there was no change in the emission maximum. However, the changes in the tertiary structure were subtle since the HSA structure is mainly dominated by secondary structural elements. The molten globule state of proteins are mainly characterized at lower concentration of denaturants and there are many reports in the literature till recently, showing the molten globule properties like decreased protein volume in case of ribonuclease$^{20}$, increased thermal stability in case of barnase$^{21}$ and possibility of hydration of exposed hydrophobic groups in case of tubulin$^{22}$.

However, the data from Timasheff$^2$ for the first time indicate that at low concentration of urea, RNase and lysozyme have a characteristic preferential hydration very similar to the data observed in HSA$^9$, suggesting the predominance of preferential hydration of proteins at these lower concentrations of urea. Supporting data are evident from the interaction of model peptides with urea suggesting favourable interaction of model peptides at lower concentration of urea$^{23}$. This certainly establishes the distinguishing feature of the mechanism of interaction of the low and high concentration of urea with HSA. If a molecule exists in two forms, namely native and unfolded, then the effect of the added co-solvents will be the stabilization of the native protein if the cosolvent is preferentially
excluded, of course relative to water from the protein surface. But in a solvent like urea, in this study, it will preferentially interact with the unfolded protein and further increase in urea concentration decreases the preferential interaction due to steric repulsion and also affects the structural features of the unfolded protein.

Conclusion

Interaction of urea at high concentrations with several proteins was monitored by partial specific volume measurements and calculations of various preferential interaction parameters\(^2\). Many proteins acquire molten globule state at specific lower concentrations of urea and recently it has been shown in the case of RNase and lysozyme by Timasheff at different pH values\(^2\). HSA is preferentially hydrated to different extents as the urea concentration increased up to 2 M with the hydration maximum at 1.8 M. The preferential binding of urea starts with the binding minimum after sliding through the stage of maximum preferential hydration as shown in our earlier report\(^2\). The preferential binding and unfolding region of HSA in urea, as our results show, is not very sharp when compared to RNase\(^2\) and wheat germ lipase\(^26\). The reason may be due to the more compact and stable structure of HSA with 17 disulfide bridges. The binding maximum reaches plateau before the much standard 8 M urea concentration, i.e. at 6.5 M. This has been shown in case of HSA interactions with aqueous urea and alkylurea by densimetry method\(^27\) and also with the interaction of anions in presence of urea\(^2\).

The structural stability data from this study correlates well with the preferential hydration phenomena of HSA\(^3\) and RNase\(^3\) at low urea concentrations. This explains one of the possible mechanism for the anomalous behavior of proteins at low denaturant concentrations whose structure is quite different from the kinetic intermediates formed in the early folding stages\(^29,30\). The prime difference between the stabilization and destabilization of HSA by urea arises from the fact that preferential hydration overrides preferential interaction up to 2.5 M of urea after which the unfolding process would result in preferential interaction dominating the preferential hydration to bring about the negative values of partial molar volume. As the protein opens up completely at higher concentration of urea exposing more sites, then the differential of the preferential hydration and preferential interaction reduces considerably, thus one sees the fall in preferential interaction values. This emphasizes the significance of molten globule state of proteins at low denaturant concentrations which has uniquely different properties and apparently more stable than the native protein itself. The formation of stable structural intermediates especially at low concentrations of denaturants may possibly be a general phenomenon in several globular proteins.

When we look at preferential binding of urea to human serum albumin, there is complexity in terms of both positive and negative \(\xi\), where one cannot directly attribute it just for unfolding of the protein molecule alone. Such variation in the transfer of free energy with varying urea concentration is due to the unfavourable thermodynamic interaction of urea up to 1.5 M and the measured free energy of interaction in the transition region after 1.5 M which can be a function of the fraction of the production in the native and denatured proteins in terms of chemical potentials as being indicated by Timasheff\(^2\) for RNase. Lapanje and co-workers\(^31-33\) in their examination of preferential interaction of alkyl urea with \(\beta\)-lactoglobulin\(^31\) and myoglobin\(^32\) have shown that alkyl ureas are preferentially excluded at low concentrations. The transfer enthalpies measured calorimetrically were negative for urea\(^33\) and positive for alkyl urea and has been interpreted as due to the domination of hydrophobic nature of the alkyl groups. An excellent interpretation has been given by Timasheff\(^2\) for such a generic observation that the exchangeable sites are characterized by identical contact enthalpies and have identical exchange affinities and these results certainly would represent the site occupancy of the protein molecule. This is evident according to Timasheff\(^2\) that the \((\delta\mu_1)/(\delta\mu_2)\) are much smaller than the number of surface sites on the protein molecule as well as number of sites that the RNase can make intact for their studies. This is true of human serum albumin also and \((\delta\mu_3)/(\delta\mu_4)\) we have observed can have a similar interpretation. This rightly indicates that the site occupancy and the thermodynamic binding stoichiometry carry opposite signs\(^2\). However, one should keep in mind the large number of disulfide linkages (seventeen) that stabilise the HSA molecule in vivo, probably in different solution conditions to carry on its functions.

References

4 Pace C N & Vanderburg K E (1979) Biochemistry 18, 288-292
20 Tamura Y & Gekko K (1995) Biochemistry 34, 1878-1884