Molten globule state of human serum albumin in urea

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Preferential interaction and hydration parameters of human serum albumin (HSA) as a function of urea concentration in the entire solubility range are obtained by partial specific volume measurements. At lower concentrations (< 2 M) of urea, HSA exists in a molten globule state with an unusually hydrated structure and increased Stokes radius. Differential mechanism of interaction of urea at low and high concentrations with HSA exists. This is in tune and conformity with the first observation made by Prakash and Timasheff for other proteins.

THE role of classical protein denaturants such as urea and guanidine hydrochloride has always been one transition of the native to denatured state. But in this large concentration of the denaturants, there are many intermediates that exist, which are identified and characterized[1]. One of the main difficulties in experimental investigations of folding and unfolding of proteins is the reliable identification and structural description of the intermediate state/s. But what interests the authors is – at very low concentration of denaturants what kind of intermediates are formed? How stable are they? What is the relevance of such intermediates to molten globule state? Hence it was thought to investigate especially in urea the fate of human serum albumin (HSA) at low concentration of this denaturant as the availability of the data has been scantier and few references have shown that such intermediates are possibly existing.

Urea is a denaturant which can bind to proteins, reversibly in many cases and is exploited to understand the mechanism of protein folding. Although considerable progress has been made in experimental studies of the folding and unfolding, our knowledge is limited by the difficulty in obtaining structural and kinetic data of folding intermediates in solution or through crystallographic studies. Hence more detailed studies are needed to understand the role of solvent and solute with proteins in solution. In this direction, numerous studies have dealt with the behaviour of different proteins in urea solution, but the total mechanism of interaction of urea with proteins is poorly understood.

To understand the forces governing the formation of three-dimensional architecture of proteins from the primary structure, studies were focused on the characterization of denatured structure and unfolding/refolding intermediates under several non-native conditions such as high ionic strength, extremes of pH, organic solvents, temperature/pressure variations and mild denaturant conditions[2]. In this direction, efforts were made to look for the solution behaviour of HSA in urea.

Stabilization of proteins at lower concentration of urea has been of major concern today, with many reports showing the urea-induced stability of a variety of proteins with many different experimental approaches[3,4]. These intermediates in the protein unfolding pathway include *molten globule* state which is characterized at low concentration of urea[5–9]. But none of these reports precisely establish the mechanism of interaction of urea at low concentration with proteins including its relevance in forming *molten globule* structures. There were few evidences where many multimeric seed proteins aggregate at low concentration of urea exhibiting the *molten globule* properties. In this context it was important to obtain quantitative preferential interaction data and structural properties of HSA in the entire solubility range of urea.

However, the interaction of urea at low concentrations[10] has been shown for the first time by Prakash and Timasheff[10] that there are two kinds of interactions, namely initially one that is dominated by preferential hydration at low concentration later, normal preferential interaction overrides[10]. This has been shown in case of RNase and lysozyme[10].

Preferential interaction of urea and volume changes in proteins are the subject matter of study in a number of proteins establishing mechanism of denaturation[11,12]. However, the interaction of urea with HSA at lower concentrations is not clear. Free energy of stabilization of proteins in denaturants is commonly calculated using the two state model $N \rightleftharpoons U$ and the role of Wyman linkage in such a path and also assuming the linear dependency of ΔG_{stab} with denaturant concentrations[13–17]. However, it is observed that the linear extrapolation of ΔG_{stab} to lower concentrations (< 2 M) of denaturants was not fitting into linear equations as it fits in at higher concentrations[18]. There are few evidences[19] to show that the mechanism of interaction of urea at lower concentration seems to be different compared to higher concentration. It was also observed as hydrophobic interaction in the case of multisubunit proteins, where it aggregates at a narrow range of lower concentration of urea[20]. In the case of HSA, the effect of low concentration of urea has been studied to understand the phenomena of intermediate/s, especially from the point of view of hydration and volume changes. Urea–HSA interaction system is studied at lower concentration of urea, using preferential interaction parameters and chromatographic techniques. The data at higher concentration of urea is also derived to explain the structural state of HSA in these concentrations of urea.

Methodology

Materials

Human serum albumin, Cohn fraction V (essentially fatty acid free, lot 42 H9313) and urea (83 F0483) were obtained from Sigma, USA. HSA was passed through Sephadex G-100 and the separated monomer was dialysed against triple glass distilled water at 4°C for 24 h and checked for homogeneity. The urea solutions were prepared according to standard procedures[21,22]. Dialysis membrane was obtained from Spectrum, USA. The buffer salts were of analytical grade obtained from Merck India Ltd, Mumbai, India. Triple glass distilled and deionised water was used in all the experiments.

Preferential interaction of urea

Preferential interaction parameters were obtained from densimetry method using Anton Paar DMA-58 ultra-precision densitymeter at 20 ± 0.01°C according to standard procedures[23,24]. The data was analysed for thermodynamical parameters using a three-component system: water, protein and denaturant as components 1, 2 and 3 respectively, following the standard notations[25,26].

Size-exclusion HPLC studies

HPLC experiments were carried out using a Shimadzu LC-RA series chromatographic system with a SPD-6AV ultraviolet detector. A Progel-TSK, G2000 SWXL (300×7.8 mm) (Supelco) column was pre-equilibrated with buffer containing specific concentrations of urea before loading the protein. HSA dissolved in buffer containing specific concentrations of urea and dialysed against the same for 24 h at 10°C. The dialysed samples were centrifuged at 10,000 rpm for 1 h at 10°C before loading on to the column and the exact protein concentration in urea was determined with appropriate corrections for molar absorption coefficient of HSA. In all the experiments, 50 µl of HSA of concentration 20 mg/ml and a flow rate of 1.5 ml/min was used. The Stokes radius of HSA was calculated using standard procedure[27].

In order to quantitate the urea interaction and see the hydrodynamic changes in the HSA molecule, partial specific volume measurements were made both at constant molal and constant chemical potential conditions. The density of different HSA concentrations ranging from 5 to 25 mg/ml in single urea concentration was measured and converted to partial specific volume using the standard method described under materials and methods. The isomolal value remained fairly constant in all the urea concentrations below 8 M. But the isopotential values changed significantly. The values of HSA for isomolal and isopotential conditions, respectively, for different concentrations given are 1 M (0.735 ml/g, 0.739 ml/g), 6 M (0.733 ml/g, 0.722 ml/g) and 8 M (0.734 ml/g, 0.730 ml/g) respectively. These are the average values of four identical experiments with an error bar of ± 0.001 ml/g. It is well documented in the literature that normally increased partial specific volume of protein under isopotential conditions in a three-component system indicates the preferential hydration of the macromolecule[28].

Table 1 shows the changes in preferential interaction parameters (ξ_3) with increasing urea concentration having more data points below 3 M. There was a negative preferential interaction of HSA till 2 M concentration of urea where it reaches a maximum with ξ_3 value of -0.0215 ± 0.0063 g/g. Above 2 M concentration, preferential interaction of urea predominates as observed by the positive values of ξ_3. The isomolal value of HSA remained fairly constant but isopotential values increased below 2 M concentrations of urea. There is a steep increase in the preferential interaction of urea between 2 M and 6 M, reaching a maximum at 6 M with the ξ_3 values of 0.0646 g/g. Preferential interaction parameter and related parameters with error bars at low and high concentrations of urea at 20°C are shown in Tables 2 and 3 respectively.

At low concentration of urea up to 2 M, there is decrease in ΔV values, reaching a maximum value of -233 ± 16 ml/mol and the transition passes through to the positive side at 3 M urea with a value of 199 ± 18 ml/mol. This transition is possibly due to changes in the transition from hydrated molecule at lower concentration of urea to that of the unfolded molecule at higher concentration of urea and an equilibrium existing between the two. And the equilibrium shifts toward the unfolding process as the concentration of urea increases. The ΔV reaches a maximum value at around 5.6–6 M, after which it decreases with a value at 6 M being 997 ± 60 ml/mol. This decrease in the ΔV value after reaching a maximum is possibly due to the unfolding of protein molecule and exposure of a number of both hydrophobic and hydrophilic sites to bulk solvent, leading to the net change in the preferential interaction, which is lower. At higher concentration, the data follows a direct relation between ΔV and urea concentration as observed for other proteins also[11].

The size of the protein was determined by a direct method of size exclusion HPLC and the Stokes radius of HSA was calculated. Figure 1 shows the Stokes radius of HSA at different concentrations of urea. HSA at lower concentrations of urea eluted much earlier with lower distribution coefficients than the HSA in native buffer. At 0.5 and 1.6 M of urea there was 9 and 6% increase, respectively, in the Stokes radius of HSA molecule compared to native HSA, whereas at 4 M about 15% increase was seen. Urea at these concentrations did not affect the permeation capabilities of the stationary phase of the column.

Table 1. Preferential interaction parameter of human serum albumin in aqueous urea at selected lower and higher concentrations

Molarity of urea	0.5	0.8	1.0	1.6	2.0	3.0	4.5	6.0	7.0	8.0	9.0	10.0
$(\delta g_3/\delta g_2)T$, μ_1, μ_3 (g/g)	0.009 ± 0.002	0.012 ± 0.008	0.015 ± 0.008	0.017 ± 0.004	0.022 ± 0.006	−0.009 ± 0.003	−0.026 ± 0.007	−0.065 ± 0.014	−0.056 ± 0.013	−0.028 ± 0.009	−0.012 ± 0.004	0.046 ± 0.028

Table 2. Partial specific volume, preferential interaction parameter and related parameters of HSA interaction at specific lower concentration of urea

	Molarity of urea					
Interaction parameter	0.5	0.8	1.0	1.6	2.0	3.0
ϕ_2^0 (ml/g)	0.735 ± 0.001	0.735 ± 0.001	0.735 ± 0.002	0.734 ± 0.001	0.735 ± 0.001	0.734 ± 0.001
$\phi_2'^0$ (ml/g)	0.735 ± 0.001	0.738 ± 0.001	0.739 ± 0.001	0.738 ± 0.001	0.740 ± 0.001	0.732 ± 0.001
g_3 (g/g)	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.10 ± 0.03	0.13 ± 0.03	0.21 ± 0.04
m_3 (mol of solvent per 1000 g H_2O)	0.52 ± 0.08	0.83 ± 0.16	1.06 ± 0.28	1.72 ± 0.32	2.19 ± 0.40	3.43 ± 0.60
$(\delta g_1/\delta g_2)T$, μ_1, μ_3 (g/g)	0.274 ± 0.033	0.242 ± 0.036	0.241 ± 0.068	0.161 ± 0.049	0.163 ± 0.060	0.045 ± 0.17
$(\delta m_3/\delta m_2)T$, μ_1, μ_3 (mol/mol)	9.37 ± 2.02	13.28 ± 3.02	17.04 ± 4.32	18.34 ± 5.02	23.73 ± 5.95	−10.33 ± 2.90
ΔV (ml/mol)	−126 ± 10	− 199 ± 13	− 233 ± 16	− 199 ± 13	− 332 ± 19	+ 199 ± 18

Table 3. Partial specific volume, preferential interaction parameter and related parameters of HSA interaction at specific higher concentration of urea

	Molarity of urea					
Interaction parameter	4.5	6.0	7.0	8.0	9.0	10.0
ϕ_2^0 (ml/g)	0.734 ± 0.001	0.733 ± 0.001	0.734 ± 0.001	0.734 ± 0.001	0.734 ± 0.001	0.730 ± 0.001
$\phi_2'^0$ (ml/g)	0.729 ± 0.001	0.722 ± 0.001	0.727 ± 0.001	0.730 ± 0.001	0.733 ± 0.001	0.736 ± 0.001
g_3 (g/g)	0.34 ± 0.06	0.49 ± 0.08	0.61 ± 0.10	0.75 ± 0.20	0.91 ± 0.22	1.09 ± 0.26
m_3 (mol of solvent per 1000 g H_2O)	5.62 ± 0.60	8.17 ± 1.20	10.15 ± 1.60	12.41 ± 1.80	15.16 ± 2.00	18.08 ± 2.60
$(\delta g_1/\delta g_2)T$, μ_1, μ_3 (g/g)	0.078 ± 0.032	0.132 ± 0.059	0.091 ± 0.040	0.041 ± 0.019	0.013 ± 0.020	0.042 ± 0.016
$(\delta m_3/\delta m_2)T$, μ_1, μ_3 (mol/mol)	− 29.20 ± 4.10	− 71.42 ± 6.57	− 61.44 ± 7.10	− 30.44 ± 8.50	− 13.10 ± 8.00	50.69 ± 9.05
ΔV (ml/mol)	+ 399 ± 20	+ 997 ± 60	+ 598 ± 35	+ 365 ± 25	+ 166 ± 10	− 102 ± 8

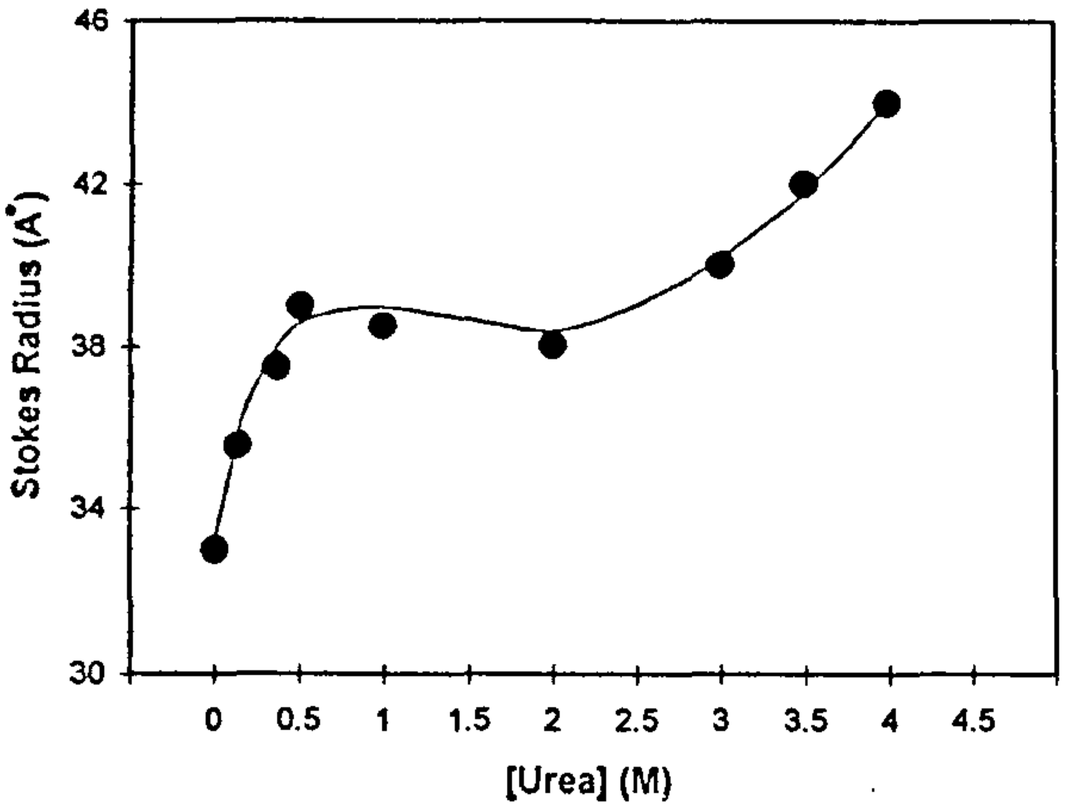

Figure 1. Stokes radius of human serum albumin as a function of urea concentration calculated from size exclusion–HPLC experiments. Urea did not alter the permeation properties of the gel matrix.

These results show a transition in the size of the protein which initially increases by about 6 Å units up to a concentration of 1 M urea, above which it plateaus and above 1.5 M urea there is a constant increase in the average size of the protein. It is significant to note that the initial increase in the size of the molecule is due to the hydration status of the molecule, possibly in the *molten globule* state. At higher urea concentrations, the HSA size increases in a manner similar to that observed for a large number of proteins.

Preferential exclusion overrides the preferential interaction of urea, resulting in the net decrease of preferential interaction above 8 M concentration of urea when compared to binding at the plateau region. Therefore, for any meaningful evaluation of preferential interaction or preferential hydration data, one has to evaluate the ξ_3 value over the entire concentration range of denaturant and not at single point measurement, indicating the nature of subtle changes that can occur in the protein as reflected in the ξ_3 results in the present study. Here there is a fine balance between binding and exclusion. These observations are similar to the interaction of $MgCl_2$ (ref. 28) and DMSO[29] with different proteins. The thermodynamics and the molecular meaning of binding are explained clearly in a three-component system[30].

The sedimentation velocity data was collected for HSA both at native state and at 1.8 M urea concentration where maximum changes were observed in terms of preferential interaction/preferential hydration and Stokes radius. The sedimentation coefficient decreased from a native $S_{20,w}$ value of 4.2 S to 3.8 S. This can only be possible if the molecule has an increased buoyant density, $(1 - \bar{V}\rho)$, of the solution. The increased hydration shell of the protein must be the driving force to increase the buoyant factor, thus decreasing the sedimentation coefficient of the protein molecule.

The results suggest that, as indicated in the case of RNase and lysozyme[10] and in HSA also there are two major structural features in urea. One, *molten globule* state of the protein at lower concentration of urea and second, the unfolding process of the protein leading to the denatured state at higher concentration of urea. There is a remarkable transition between the two structures as evidenced by the above data, which clearly indicates the role played by water at lower concentration of urea in increasing the hydrodynamic volume of the protein and secondary structural alterations typical to *molten globule* state. At higher concentration of urea the profile indicates the typical denaturation process as seen in large number of proteins. These data have substantial thermodynamic implications in the behaviour of proteins at lower concentration of non-electrolyte denaturant like urea and can only be explained by the existence of *molten globule* state of HSA in these concentrations of denaturant.

1. Ptitsyn, O. B., *Adv. Protein Chem.*, 1995, **47**, 83–229.
2. Kuwajima, K., *Protein: Struct. Funct. Genet.*, 1989, **6**, 87–103.
3. Mayr, L. M. and Schmid, F. X., *Biochemistry*, 1993, **32**, 7994–7998.
4. Johnson, C. M. and Fersht, A. R., *Biochemistry*, 1995, **34**, 6795–6808.
5. Yu, X-C., Shen, S. and Strobel, H. W., *Biochemistry*, 1995, **34**, 5511–5517.
6. Ptitsyn, O. B., *Curr. Biol.*, 1995, **5**, 74–78.
7. Guha, S. and Bhattacharya, B., *Biochemistry*, 1995, **34**, 6925–6931.
8. Filippis, D. E., Vassiliev, V. B., Beltramin, M., Fontana, A., Salvato, B. and Gaitskhoki, V. S., *Biochem. Biophys. Acta*, 1996, **1297**, 119–123.
9. Mendoza-Hernandz, G. and Rendon, T. L., *Biochem. Biophys. Acta*, 1996, **1297**, 219–227.
10. Prakash, V. and Timasheff, S. N., manuscript in preparation.
11. Skerjanc, J. and Lapanje, S., *Eur. J. Biochem.*, 1970, **25**, 49–54.
12. Prakash, V., Loucheux, C., Scheuffle, S., Gorbunoff, M. J. and Timasheff, S. N., *Arch. Biochem. Biophys.*, 1981, **210**, 455–464.
13. Wyman, J., *Adv. Protein Chem.*, 1964, **19**, 223–286.
14. Schellman, J. A., *Biopolymers*, 1978, **17**, 1305–1322.
15. Pace, C. N. and Vanderburg, K. E., *Biochemistry*, 1979, **18**, 228–292.
16. Ahmad, F. and Bigelow, C. C., *Biopolymers*, 1986, **25**, 1623–1633.
17. Schellman, J. A., *Biophys. Chem.*, 1993, **45**, 273–279.
18. Santoro, M. M. and Bolen, D. W., *Biochemistry*, 1992, **31**, 4901–4907.
19. Noelkn, M. E. and Timasheff, S. N., *J. Biol. Chem.*, 1967, **242**, 5080–5085.
20. Prakash, V. and Nandi, P. K., *Int. J. Pept. Protein Res.*, 1977, **9**, 97–106.
21. Arakawa, T. and Timasheff, S. N., *Biochemistry*, 1984, **23**, 5924–5929.
22. Noazki, Y., *Methods Enzymol.*, 1972, **26**, 43–54.
23. Lee, J. C. and Timasheff, S. N., *Biochemistry*, 1974, **13**, 257–265.
24. Scatchard, G., *J. Am. Chem. Soc.*, 1946, **68**, 2315–2319.
25. Prakash, V., *J. Biosci.*, 1982, **4**, 347–359.
26. Prakash, V. and Timasheff, S. N., *Methods Enzymol.*, 1985, **117**, 53–60.
27. Ackers, G. K., *J. Biol. Chem.*, 1967, **242**, 3237–3242.
28. Arakawa, T., Bhat, R. and Timasheff, S. N., *Biochemistry*, 1990, **29**, 1914–1923.
29. Kita, Y., Arakawa, T., Lin, T. Y. and Timasheff, S. N., *Biochemistry*, 1994, **33**, 15178–15189.
30. Timasheff, S. N., *Biochemistry*, 1992, **31**, 9857–9864.