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Effect of Physiological Concentration of Urea on the Conformation of Human Serum Albumin

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We report that the presence of very low concentrations (<0.1 M) of urea, a widely used chemical denaturant, induces structure formation in the water-soluble globular protein human serum albumin (HSA) at pH 7. We have presented results suggesting an almost 8% and 5% increase in α-helix in the presence of 10 mM urea (U) and 20 mM monomethylurea (MMU), respectively. Far and near-UV circular dichroism studies along with tryptophan fluorescence and 1-anilino-8-naphthalenesulphonicacid (ANS) binding support our view. We hypothesize that both U and MMU, at such low concentrations, modify the solvent structure, increase the dielectric constant and consequently increase hydrophobic forces resulting in enhanced α-helical content. The implications of these results of the lower urea regime are significant because the physiological blood urea ranges from 2.5 to 7.5 mM.

Key words: ANS binding and human serum albumin, circular dichroism, intrinsic fluorescence, monomethyl urea, urea.

Abbreviations: ANS, 1-anilino-8-naphthalenesulphonicacid; CD, circular dichroism; HSA, human serum albumin; MMU, monomethyl urea; U, urea.

Elucidation of detailed mechanism of protein folding/unfolding remains one of the major challenges in structural biology/protein chemistry (*1–4*). The effect of urea on the structure of biopolymers is not well understood at the molecular level despite the impressive number of works published in this area (*5–7*). Urea is most widely used denaturing agent, being extensively used in biochemistry, not only to denature proteins at high urea concentration, but also to promote controlled folding (*5*). It is the best choice of solute to probe large-scale changes in water accessible surface area (ASA) in protein studies. The role of action of urea as a denaturant has been extensively studied. The denaturing action of urea has been analysed within the framework of two opposite theories, i.e. Direct interaction with the macromolecule or an indirect interaction via rupture of 3D network of water (*8–11*). The traditional explanation for denaturation of globular proteins has been that urea, because of its structural similarity to a peptide group, is able to form stronger hydrogen bonds with peptide groups than water does, and that it is, therefore, able to break interpeptide hydrogen bonding which water cannot (*12*). This explanation is doubtful because: (i) interpeptide and other hydrogen bonds have been shown to be relatively unimportant as a source of free energy stabilizing the native proteins in water solution (*13–18*), and (ii) hydrogen bonds between water and peptide groups of *N*-methyl acetamide have been shown to be considerably stronger than interpeptide bonds between amide molecules (*19*). The indirect mechanism has been widely accepted and many experimental results seem to favour the hypothesis that urea acts as a water structure breaker (*20–22*). The major force stabilizing the globular protein is, thus, believed to be the hydrophobic force which arises from the unfavourable interaction between non-polar parts of a protein molecule and water (*23, 24*). The low concentrations of urea are not expected to perturb the structure of either the initial or final states of protein processes as much as a highly excluded solute (*25*). The transition of proteins from an unfolded state to the native conformation has some resemblance to micelle formation and likewise it is a highly cooperative process (analogous to hydrophobic interactions) (*26*). As preliminary studies (*27*) showed urea behaving oppositely in low and high concentration regimes in micelles, this study was undertaken to understand the general effect of very low concentrations of urea (U) and monomethyl urea (MMU) on proteins. The results presented seem to follow an earlier investigation (*28*) where a comparative study of the effect of very low concentrations of urea and its derivatives on protein (bovine serum albumin)/surfactant was made.

The observations were carried on human serum albumin (HSA), as it is one of the most studied models of globular proteins. HSA is a single polypeptide chain, multi-domain protein of 585 amino acid residues (*29*) that aids in the transport, metabolism and distribution of exogenous and endogenous ligands (*30*). Albumin serves to maintain plasma pH, contributes to colloidal blood pressure, functions as carrier of many metabolites and fatty acids, and serves as a major drug transport protein in plasma (*31, 32*). HSA (MW = 66.5 kDa) consists of

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three structurally similar α-helical domains I–III encompassing the complete sequence (*33*). The charge distribution of HSA at neutral pH for domain I, II, III are −9, −8 and +2, respectively (*29*). Each domain consists of two sub-domains A and B, which are extensively cross-linked by disulphide bridges. There are 10 principal helices in each domain, h1–h6 for sub domain A and h7–h10 for sub-domain B. Domains I–II and II–III in turn are connected through extensions of h10(I)–h1(II) and h10(II)–h1(III), respectively, diminishing the actual number of helices from 30 to 28 (*34*). Its overall native 3D conformation is stabilized by various intra- and inter-domain forces such as salt bridges, hydrophobic interactions and natural boundaries involving helical extensions existing between three domains of albumin. Unfolding of the three domains of bovine serum albumin (BSA), structural analogue of HSA, follows the following order of susceptibility towards alkaline denaturation; domain I > domain III > domain II (*35*). Gd-HCl induced unfolding of HSA proceeds via local unfolding of some stable regions/loops in domain III and at higher concentrations all the three loops start unfolding invariably, the most labile being domain III followed by domain II and domain I (*4*). Domain III is primarily responsible for intermediate formation in the unfolding transition of HSA in case of urea induced unfolding. Here domain I and III unfold before 5 M and domain II unfolds beyond it. Further, the refolding of domain I and III occurs rapidly while the refolding of domain II is slow. The presence of a single cysteine residue in domain I, a single tryptophan residue in domain II, and a highly reactive tyrosine residue in domain III provides a unique opportunity to follow the unfolding and refolding (*31*). This communication details a conformational change in HSA triggered by very low concentrations of U/MMU. CD and spectrofluorometric techniques were used for the said purpose.

MATERIALS AND METHODS

Materials—HSA essentially fatty acid free (lot no.3872A, Sigma Chemicals ltd), U, (Sigma Chemicals Ltd.), MMU (Merck) and ANS (Sigma Chemicals Ltd) were used as received. All other reagents and buffer compounds used were of analytical grade. Double distilled water was used throughout the study. Stock solutions of 5 mg ml^{-1} human serum albumin HSA extensively predialysed, 3 M urea U and 3 M monomethylurea MMU were prepared in 20 mM sodium phosphate buffer, pH 7 and utilized to prepare the samples of desired concentrations.

Protein Concentration Determination—Protein concentrations were determined spectrophotometrically using an extinction coefficient = 5.3 (*36*) on a Hitachi U-1500 spectrophotometer or alternatively by the method of Lowry *et al*. (*37*) pH measurements were carried out on an Elico digital pH meter (model LI610).

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-camphorsulfonic acid. All the CD measurements were carried out at 25°C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1°C. Spectra were collected with a scan speed of 20 nm/min and response time of 1 s. Each spectra was the average of four scans. Far-UV CD spectra were measured at a protein concentration of 5.0 μM and near-UV CD spectra were measured at protein concentration of 10 μM. The path length was 1 mm and 1 cm, respectively. The results were expressed as mean residue ellipticity (MRE) in deg cm^2 dmol^{-1}, defined as

$$\mathrm{MRE} = \frac{\theta_{\mathrm{obs}}}{10 \times n \times Cp \times l}.$$

Where $_{\mathrm{obs}}$ is the CD in millidegree, n is the number of amino acid residues (585), l is the cell pathlength in cm and Cp the molar fraction.

Data Analysis—The secondary structure was estimated from spectra between 200 and 240 nm using K_2d CD secondary structure server, which uses an unsupervised neural network to predict secondary structure (*38*).

Fluorescence Measurements—Fluorescence measurements were performed on Hitachi spectrofluorimeter (model 2500) equipped with a PC. The fluorescence spectra were collected at 25°C with a 1 cm path length cell. The excitation and emission slits were set at 5 nm. The fluorescence spectra were taken with a protein concentration of 5 μM. To the 5 μM protein stock solution, different volumes of the buffer were added followed by the requisite volumes of stock additive solutions to obtain the samples of desired additive concentration. Intrinsic fluorescence was measured by exciting the protein solution at 280 and 295 nm and emission spectra were recorded in the range of 300–400 nm.

For extrinsic fluorescence measurements in the ANS binding studies, the excitation was set at 380 nm and the emission spectra were taken in the range of 400–600 nm or at a fixed wavelength of 470 nm. U or MMU did not give any significant CD or fluorescence spectra in the absence of protein.

RESULTS

Far-UV CD—The changes in the secondary structure of HSA were monitored by far-UVCD in the range 200–250 nm. Figure 1A and B shows spectra of HSA in the absence and presence of 10, 20 and 2000 mM U and MMU, respectively. The spectrum of untreated HSA at pH 7 shows negative minima nearly at 208 and 222 nm, characteristic of α-helical structure (*39*). Alterations of ellipticity at 222 nm are useful probe for visualizing varying-helical contents (*40*). The untreated HSA contained about 67% α-helical structure as estimated by K_2d (*38*). This is in agreement with literature value (*41*) and also agrees fairly well with the values obtained by employing CONTINLL; an algorithm uses ridge regression to fit a linear combination of spectra of known composition to match a spectrum of unkown composition (*42, 43*). As shown in the Fig. 1A and B HSA shows a decrease in CD values as the concentration of U reaches 10 mM and the decrease in MMU is observed till 20 mM, the CD values at 2 M U/MMU are found to be higher than that of untreated HSA for both U and MMU. The graph of −MRE at 222 nm versus the concentration

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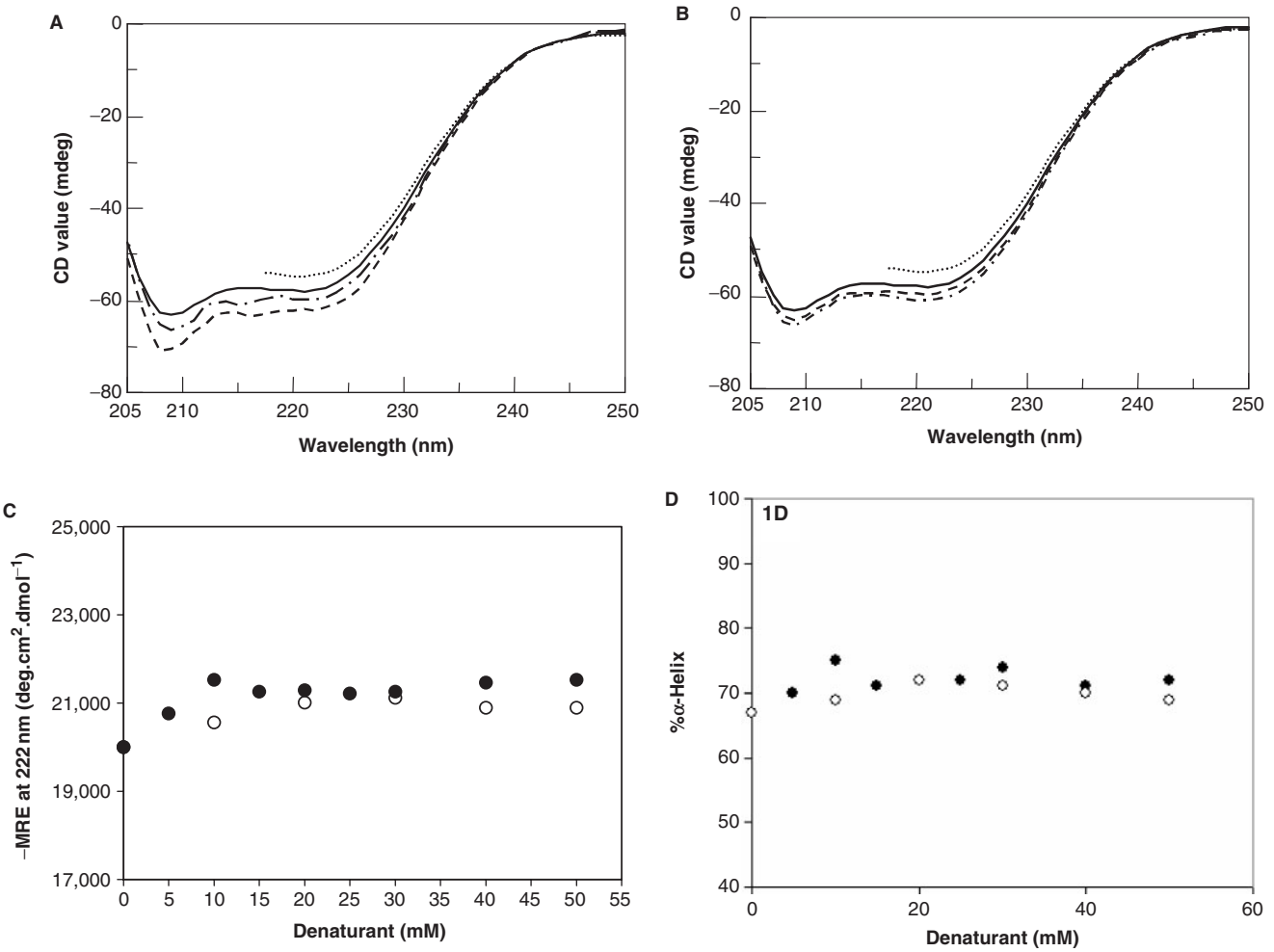

Fig. 1. **Far-UV CD of HSA at pH 7, in the native state (—) and in the presence of 10 mM (- - -), 20 mM(–·–·) and 2 M (·····) U (A) and MMU (B).** MRE values at 222 nm (C) and %α-helix (D) against increasing concentration of U (●) and MMU (○).

shows an increase in −MRE in the concentration range of 0–10 mM U and 0–20 mM MMU (Fig. 1C) and it remains constant till 100 mM U and 150 mM MMU (data not shown). The variation of % α-helical content with U/MMU concentration as determined by K_2d (Fig. 1D), is found to follow the same pattern as that of −MRE at 222 nm (Fig. 1C). The helical content is found to rise from 67% for native protein to 72% as the MMU concentration reaches 10 mM. In case of Urea a significant rise of 8% (i.e. 67–75%) in presence of 10 mM U, which is very near to the physiological urea concentration, is detected (Table 1).

Near-UV CD—Near-UV CD spectra were used to probe the asymmetry of the protein's aromatic amino acid environment (*44*). Figure 2A shows the near UV CD spectra of HSA in the range of 250–300 nm for the native (pH 7) and in the presence of 10 mM, 20 mM and 2 M MMU. The near-UV CD spectra for the native state showed two minima at 262 and 268 nm and shoulder at 283 and 293 nm, characteristics of disulphide and aromatic chromophores, which is in accordance with previous studies (*45*). Figure 2B shows the near-UV CD ellipticity data of HSA in the presence of varying concentrations of MMU at 262 and 268 nm, respectively, and their respective MRE values are given in Table 2. It can be seen from the figure that the CD values

Table 1. **Percentage of Secondary structural elements of HSA estimated byK_2d: %α, Percentage of α-helix; %β, Percentage of β-sheet; %R, Percentage of remainder structural elements.**

(U/MMU) mM	U			MMU		
	%α	%β	%R	%α	%β	%R
0	67	4	29	67	4	28
10	75	2	24	69	4	27
20	72	3	25	72	3	25
30	74	2	24	71	3	26
40	71	3	26	70	3	26
50	72	2	26	69	4	27

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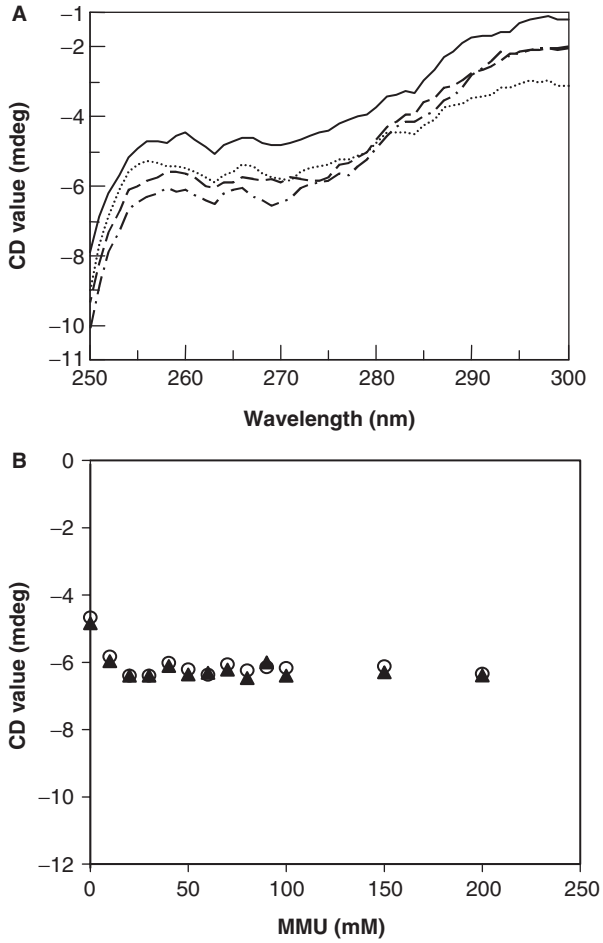

Fig. 2. **Near-UV CD (A) of HSA in the native (—) and in the presence of 10 mM(- - -), 20 mM(—·—·) and 2 M(·····) MMU at pH 7. CD values at 262 nm (▲) and 268 nm (○) against increasing concentration of MMU (B).**

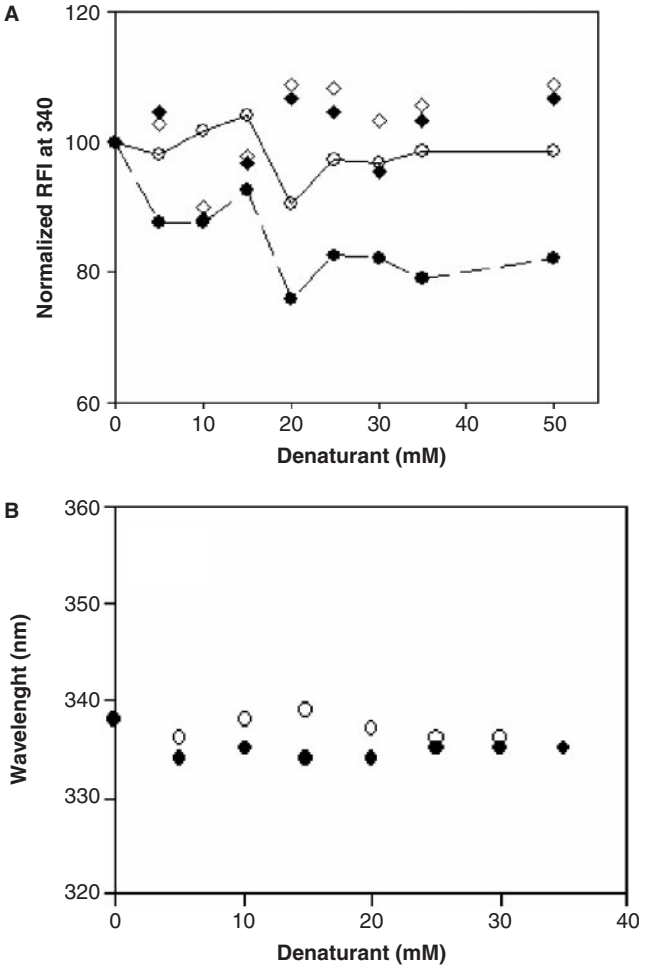

Fig. 3. **Normalized RFI of HSA in the presence of U (●), (○) and MMU (◆), (◇) on the basis of fluorescence intensity at 340 nm by exciting at 295 nm (●,◆) and 280 nm (○,◇)respectively (A).** Change in maximum emission wavelength of U (●) and MMU (○) when excited at 280 nm (B).

Table 2. **–MRE (Mean Residue Ellipticity) at 262 and 268 nm of HSA at different concentrations of U/MMU at pH 7.**

	–MRE262	–MRE268
0	1,654	1,627
10	2,041	1,993
20	2,188	2,188
2,000	1,959	1,918

have simultaneously decreased from −4.8 (262 nm) and −4.6 (268 nm) to −6.4 in the presence of 20 and 30 mM MMU, respectively, and remains consistent up to 200 mM as shown in Fig. 2B. This decrease may be attributed to the increase in tertiary contacts due to the increase in-helix. A decrease in –MRE values have been registered at 2 M MMU (Table 2). Near UV-CD experiments for U couldn't be performed because of very high dynode voltage in the entire wavelength range of 250–300.

Intrinsic Fluorescence—Fluorescence spectroscopy is widely employed to study proteins and peptides. The aromatic amino acids tryptophan, tyrosine and phenylalanine offer intrinsic fluorescence probes of protein conformation, dynamics and intermolecular interactions. Of the three, tryptophan is the most popular probe (*46*). The fluorescence of the indole chromophore is highly sensitive to the environment making it an ideal choice for reporting protein conformation changes and interactions with other molecules. The changes in the fluorescence intensities at 340 nm by exciting at 295 and 280 nm can be used as a probe for folding/unfolding of proteins (*47*). A single tryptophanyl residue is present almost at the middle of domain II of HSA. The changes in the fluorescence spectra observed by exciting HSA at 295 nm, at which only tryptophan get excited, can be used as a probe for changes in its vicinity. On the other hand, at 280 nm both tryptophan and tyrosine residues get excited and this can be used to probe changes in their respective microenvironments. In Fig. 3A, changes in the normalized fluorescence

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intensities with respect to the native protein have been plotted against the increasing concentration of the U/MMU. Normalized fluorescence intensities have been calculated by taking the intensity of the untreated protein as 100. Figure 3A shows a clearcut decrease in tryptophanyl fluorescence ($\lambda_{ex} = 295$ nm) with as low as 5 mM U, which is physiologically significant. A two-step decrease in fluorescence intensity has been detected, first from 0 to 5 mM and then from 15 to 20 mM U. The change is very distinct for tryptophanyl fluorescence; the intensity has been found to vary similarly when the protein is excited at 280 nm but the decrease is found to be noticeably less. The decrease in intensity may be associated to the increase in compactness due to the increase in α-helical content that may result in the internalization of the aromatic fluorophores decreasing their exposure to the solvent medium and thus leading to decreased emission. The less noticeable decrease at 280 nm may be due to the wide distribution of the tyrosine residues in the protein.

In the presence of MMU, the change in quantum yield is not significant. From far and near UV CD data it is very clear there is increase in structure in the presence of both U and MMU. But MMU is much apolar than urea. In the presence of apolar solvents fluorophores usually show increased emission. Probably the decrease in emission by the increased compactness of the protein has been compensated due to the increased emission owing to a more apolar environment.

The environment sensitivity of wavelength of the emission maximum (λ_{max}) is well understood. It is an excellent parameter to monitor the polarity of tryptophan environment in the protein, and is sensitive to the protein conformation (*48*). Figure 3B depicts the variation of (λ_{max}) plotted against increasing concentrations of U/MMU at an excitation wavelength of 280 nm. (λ_{max}) has been found to decrease from 338 to 334 nm in the presence of only 5 mM and remains unchanged up to 35 mM MMU. In the presence of 5 mM U the decrease in wavelength is only 2 nm. After a short increase in wavelength at 15 mM U, the wavelength has decreased ultimately as the concentration reaches 25 mM and remains consistent thereafter. The results clearly indicate that the tryptophan, along with other aromatic residues, has shifted towards the apolar inner core of the protein as the helical structure increases. The significant decrease in the intensity also suggests internalization of aromatic residues hindering their emission and lending support to our viewpoint that proteins become more compact in presence of low U/MMU concentrations.

Extrinsic Fluorescence—In water-soluble proteins about 25–30% of the amino acid side chains are generally significantly hydrophobic, and 45–50% are typically ionic or contain uncharged hydrophilic side chains. The complete removal of hydrophobic side chains from contact with water is generally not possible. In most native proteins some hydrophobic groups remains exposed at the molecular surface or in crevices (*49*). If sufficiently large hydrophobic patches are formed, they may constitute binding sites for hydrocarbon or amphiphile molecules. ANS is a widely used fluorescent probe known to bind to the hydrophobic patches of the protein (*50*). There are two different apparent ranges over which ANS binds to the proteins like serum albumins (*51*). One of these is a broad range involving as many as 100 binding sites at pHs <5, where most of the bound ANS is not fluorescent. In a much narrower range of binding, where ANS is assumed to act as a hydrophobic probe, ANS may become fluorescent. Five hydrophobic sites have been detected on native serum albumins in the pH range 5–7 (*52, 53*). The fluorescence exhibited by the native HSA at pH 7 in our case may be attributed to the presence of these hydrophobic patches and the variation of the fluorescence intensity can be effectively used to monitor the accessibility of the hydrophobic patches. Generally, increase in ANS protein binding indicates opening of hydrophobic sites and thus unfolding of protein and vice versa. Bound ANS excites at 380 nm and emits maximally at 480 nm. Figure 4 depicts the change in fluorescence intensities at 480 nm with increasing concentration of U/MMU. In the presence of just 5 mM U/MMU, ANS binding has shown significant decrease of fluorescence intensity, i.e. 71.4 at native to 50 (U) and ~45 (MMU), and remains more or less unaltered up to 35 mM of both U as well as MMU. Decrease in ANS binding may be a reflection of decrease in hydrophobic patches accessible to the dye probably due to the internalization of the hydrophobic patches because of structure induction. The internalization along with the structure induction may make the protein more compact resulting in decreased exposure of the fluorophores to the solvent as already suggested. Thus these results faithfully follow those of CD and intrinsic fluorescence studies presented in the earlier part of this article.

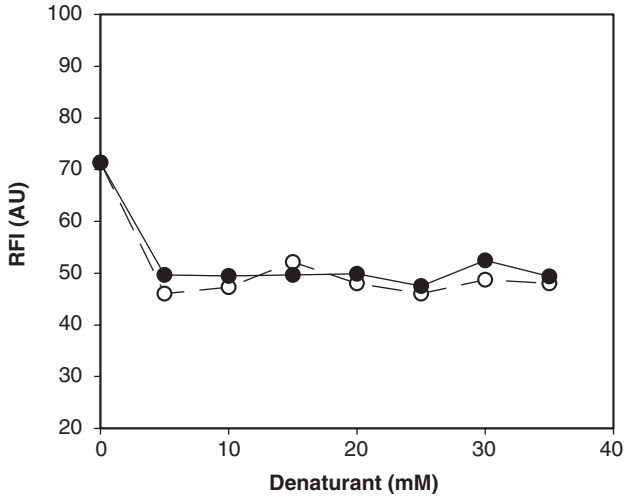

Fig. 4. **Fluorescence intensity at 480 nm of ANS bound HSA in the presence of U (●) and MMU (○) when excited at 380 nm.**

DISCUSSION

Biomolecules evolved in water and therefore their properties are intrinsic related to water properties. In fact, it is difficult to find a single phenomenon of

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biological relevance, that is not related some how with water (*54*). Water is considered to have two populations, strongly hydrogen bonded or intact population where water molecules are in an ice like environment and weakly hydrogen bonded or broken population (*55*). Structure forming agents or kosmotropes increase water structure by increasing the intact water population while as structure breaking agents or chaotropes decrease the water structure by increasing the broken water population (*56*). 'Kosmotropes' (order-maker) and 'Chaotropes' (disorder-maker) originally denotes solutes that stabilized or destabilized, respectively, proteins and membranes. Kosmotropes may lead to extensive hydrogen bonding that enhances hydrophobic forces and thus stabilizes the structure of macromolecules in solution while chaotrope decreases hydrophobic interactions, increases the structural freedom of the macromolecule and thus facilitates protein denaturation. It is generally believed that proteins are stabilized by hydrophobic effect, hydrogen bonding and packing (Van Der Waals) interactions. Urea and its derivatives are well known denaturants of proteins, because of their ability to weaken hydrophobic interactions in aqueous solutions. Since the hydrophobic force seems to be the main factor stabilizing the globular proteins, therefore, any factor enhancing this force might be expected to stabilize a protein (*23, 24*). Urea, at low concentrations might be able to play this role was suggested sometimes ago by observing a decrease in the cmc of SDS in presence of low urea (*27*). Earlier the cmc decrease was reported in presence of low butylurea (*57*). The stabilization of methane–methane contact pair by using molecular dynamic simulations (*58*) and the renaturation of urea in the presence of low urea (*59*) concentrations also reinforces this fact. Notable concentration dependent intermolecular rearrangements of aqueous urea solutions were also reported while studying the physical properties of these solutions (*60*). The refractive index was found to decrease below 0.1 M U from its value in pure aqueous medium, showed fluctuating behaviour till 2.5 M U and exhibited a linear increase with higher urea concentrations. A compact state was also observed in Chainia Xylanase in the lower urea range while studying the urea effect in the concentration range 0–2 M (*61*). Despite these reports it is surprising that no systematic attempt was made to study the effect of urea and its derivatives on protein conformation.

Urea has great ability to undergo hydrogen bonding with water because of three potential centers on each molecule (*62*). It increases the dielectric constant of water (*62, 63*). In the light of the aforementioned studies, we suggest induction of α-helical structure in HSA by low urea concentrations for the following reasons:

(i) Urea, at very low concentrations may enhance the hydrogen bonding in the solvent system (urea + water) resulting in a more structured solvent medium that may lead to the predominance of hydrophobic interactions. This may increase the intramolecular hydrogen bonding in the protein consequently increasing the α-helical content. The decrease of the hydrophobic interactions at higher concentrations is presumably the major factor explaining denaturation of the native conformation or the stabilization of the unfolded state of the protein at higher urea concentrations. Urea has been reported to behave as a kosmotrope in the presence of a macromolecule and a chaotrope at higher urea concentrations. Here we want to suggest that urea may behave as a kosmotrope at low U resulting in an increase in α-helical content of HSA.

(ii) The presence of urea + water (instead of pure water) causes an increase in the dielectric constant of the solvent system. According to Coulomb's law, the electrostatic force between two charges is inversely proportional to the dielectric constant of the medium. Due to the increased dielectric constant, the hydrogen bonding between water and the exposed parts of the protein may have weakened compared with those in pure water protein system. This may also weaken the bonding between trp π cloud and hydroxyl groups. These factors may enhance hydrophobic interactions leading to an increase in intramolecular hydrogen bonding which in turn may enhance helical structure.

The opposite effect exhibited by the same solvent depending upon its concentration is not very unusual. Many organic solvents at low concentrations enhance hydrophobic interactions (*64, 65*) while at higher concentrations the same solvents destabilize these interactions (*66*). According to a recent study some salts, which are well known kosmotropes behave as chaotropes at low concentrations (~50 mM) (*67*). Keeping in view the earlier studies and going by the data of the present study, it is not very unlikely that urea, at low concentrations, may make the solvent more structured, enhance hydrophobic interactions and lead to an increase in α-helical content. At higher urea concentrations the destabilized hydrophobic interactions outweigh the effect of the stabilization caused by the lower concentrations and thus result in protein denaturation.

CONCLUSION

In conclusion, we can say that the protein conformation is significantly influenced by urea addition and that its effect depends upon the concentration. In the very low U regime (<0.1 M), the protein conformation is influenced by an increase in solvent structure and dielectric constant, which enhances the hydrophobic forces and thus results in increased α-helical structure while higher U decreases the hydrophobic forces and thus leads to protein denaturation. This effect of very low concentration of U claims significance because the physiological U lies in the range 2.5–7.5 mM. It is not, therefore, unlikely that the blood urea influences the globular conformation of HSA, the most abundant plasma protein. Since HSA plays a major role in some of the important biological functions, the physiological concentration of urea might be vital to these functions, as well. Besides these experiments offer an explanation for observations, which suggest that the usual assumption of toxicologists,

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i.e. the effect of a drug increases with an increase in its concentration, need not be valid. Indeed a harmful effect at low concentration may convert to a beneficial effect at extremely low concentrations and vice versa. This may go someway to validate the claims of homeopathy. These observations suggest that the effect of low urea on HSA is an important problem that demands additional investigation from biophysical as well as therapeutic point of view.

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