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Influence of Fluoro, Chloro and Alkyl Alcohols on the Folding Pathway of Human Serum Albumin

Yogesh Kumar*, Salman Muzammil† and Saad Tayyab‡
Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202 002, India

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Urea-induced equilibrium unfolding of human serum albumin (HSA) when studied by mean residue ellipticity at 222 nm (MRE_{222}) or intrinsic fluorescence measurements showed a two-step, three-state transition with a stable intermediate around 4.6–5.2 M urea. The presence of 2,2,2-trifluoroethanol (TFE) resulted in a single-step, two-state transition with a significant shift towards higher urea concentration, suggesting the stabilizing effect of TFE. The free energy of stabilization ($\Delta\Delta G_D^{H_2O}$) in the presence of 3.0 M TFE was determined to be 2.68 and 2.72 kcal/mol by MRE_{222} and fluorescence measurements, respectively. The stabilizing potential of other alcohols on the refolding behavior of HSA at 5.0 M urea (where the intermediate exists) as studied by MRE_{222} and intrinsic fluorescence measurements showed the following order: 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) > TFE > 2-chloroethanol > tert-butanol > iso-propanol > ethanol > methanol. Further, the extent of refolding at the highest concentration of alcohol was similar in all cases. The stabilizing effect of TFE on guanidine hydrochloride (GdnHCl)–induced unfolding of HSA was nearly equal to that found for urea denaturation, as reflected in the $\Delta\Delta G_D^{H_2O}$ value (2.38 kcal/mol). Taken together, these results suggest that the stabilizing effect of TFE and other alcohols on urea/GdnHCl-induced unfolding of HSA is higher for alcohols that contain bulky groups or fluorine atoms.

Key words: alcohols, circular dichroism, fluorescence, folding pathway, human serum albumin, trifluoroethanol, urea denaturation.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; BSA, bovine serum albumin; CD, circular dichroism; D, denatured; GdnHCl, guanidine hydrochloride; $\Delta\Delta G_D^{H_2O}$, free energy of stabilization; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; HSA, human serum albumin; I, intermediate; MRE, mean residue ellipticity; N, native; TFE, 2,2,2-trifluoroethanol; UV, ultraviolet.

The structural stability of proteins is a reflection of the overall molecular interactions among all amino acid residues. To understand the interactions governing the formation of the three-dimensional structure from the amino acid sequence, studies have focused on the characterization of the denatured state and unfolding/refolding intermediates under several non-native conditions such as high ionic strength, extremes of pH, organic solvents, temperature variation and mild denaturing conditions (*1–5*). Much progress has been made in characterizing these intermediates, which include both transient species detected in kinetic experiments (*6–11*) and stable species that can exist at equilibrium under a variety of conditions where the native state is destabilized (*1*, *12–14*). The folding mechanism of multidomain proteins is more complex because of the autonomous folding of domains and sub-domains and their subsequent interaction to produce the native state (*15–19*). Intermediates of a protein including a molten globule can be stabilized under equilibrium conditions in mild denaturing medium or in organic solvents such as 2,2,2-trifluoroethanol (TFE) (*1*, *20–22*). Alcohols strengthen hydrogen bonding in proteins, leading to increased α-helical content (*23–25*), but destabilize tertiary structure by influencing the hydrophobic interactions within the protein (*23*, *26*). The effect of alcohols, particularly TFE, on the conformation of several intact single-domain proteins has been examined in comparison with the molten globule intermediate obtained under aqueous conditions (*22*, *27*). These studies suggest that TFE stabilizes an open-helical structure, in which the interactions between helical segments are weak and many hydrophobic groups are exposed to the solvent. The open-helical structure is distinct from the compact molten globule state, which is stabilized by the weak but significant inter-helical hydrophobic interaction (*28*, *29*). Whether alcohols affect multidomain proteins in a similar manner to single-domain proteins has not been studied so far.

Human serum albumin (HSA) is a multidomain protein and the major protein present in the human blood circulation, where it functions mainly to maintain blood osmotic pressure in addition to being the major transporter of free fatty acids as well as other exogenous and endogenous ligands (*30*). In view of the involvement of independent folding of domains and interdomain interactions in the folding mechanism of multidomain proteins, the effect of

*Present address: Basic Science Research Division, University of California at Los Angeles, Los Angeles, CA 90095, USA.
†Present address: Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA.
‡To whom correspondence should be addressed at the present address: Institut Sains Biologi, Universiti Malaya, 50603, Kuala Lumpur, Malaysia. Tel: +603-7967-7118, Fax: +603-7967-4178, E-mail: saadtayyab2004@yahoo.com

alcohols on the folding of these proteins is a matter of interest. Here, we investigated the effect of two fluoro alcohols, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and TFE, a chloro-alcohol, 2-chloroethanol, and four alkyl alcohols, methanol, ethanol, iso-propanol, and tert-butanol, on the urea-induced unfolding of HSA at pH 7.0, 25°C. This paper describes the effect of these alcohols on the structural stability of HSA as studied by far-UV CD, intrinsic fluorescence and ANS fluorescence measurements.

MATERIALS AND METHODS

Materials—Human serum albumin (HSA) (essentially fatty acid free), type A-1887; 1-anilinonaphthalene-8-sulfonate (ANS), type A-3125; ultra-pure urea, type U-0631; guanidine hydrochloride (GdnHCl), type G-7153; 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), type H-8508 and 2,2,2-trifluoroethanol (TFE), type T-3182 were the products of Sigma Chemical Co., St. Louis, MO, USA. HSA monomer was purified by Sephadex G-100 gel chromatography. The homogeneity of the purified preparation was checked by polyacrylamide gel electrophoresis. Methanol, ethanol, iso-propanol, tert-butanol and 2-chloroethanol were of spectroscopic grade. All other reagents used were of analytical grade.

Analytical Procedures—Protein concentration was determined spectrophotometrically on a Cecil double-beam spectrophotometer, model CE-594, by using $E^{1\%}_{1cm} = 5.30$ at 280 nm (*31*).

ANS concentration was determined using an extinction coefficient of 5×10^3 M^{-1} cm^{-1} at 350 nm (*32*).

Concentrations of stock solutions of denaturants were determined from the data of Warren and Gordon (*33*) and Nozaki (*34*) for urea and GdnHCl solutions, respectively, as described by Pace and Scholtz (*35*).

Circular Dichroism (CD) Spectroscopy—CD measurements were carried out at 25°C on a Jasco spectropolarimeter, model J-720, attached to a personal computer. A cuvette with 1-mm path length was used in the far-UV region. The instrument was calibrated with d-10-camphorsulfonic acid. The temperature of the cuvette was controlled (within ±0.1°C) with an attached water bath (Neslab, model RTE-110) by circulating water through a jacket around the cuvette. Each spectrum was the average of four scans. Far-UV CD spectra were taken at a protein concentration of 3.0 μM. The results are expressed as mean residue ellipticity (MRE), which is defined as:

$$\mathrm{MRE} = \theta_{obs}/10 \times n \times Cp \times l \quad (1)$$

where "θ_{obs}" is the observed ellipticity in millidegrees, "*n*" is the total number of amino acid residues (585), "*Cp*" is the mole fraction and "*l*" is the length of light path in centimeters.

Fluorescence Spectroscopy—Fluorescence measurements were carried out on a Shimadzu spectrofluorometer, model RF-540, with an attached data recorder DR-3, using a cell of 1-cm light path. The excitation wavelength was set at 280 nm and the emission spectra were recorded in the wavelength range of 300–400 nm. The bandwidths set for the excitation and emission were 10 nm each. In ANS binding studies, the fluorescence spectra were taken with a protein concentration of 1.8 μM and using an excitation wavelength of 380 nm. The emission was recorded either in the wavelength range of 400–600 nm or at a fixed wavelength of 470 nm. Appropriate blank values were subtracted to obtain the net fluorescence caused by the dye absorption.

Unfolding/Refolding Studies—Solutions for the unfolding and refolding studies were prepared from stock solutions of protein and denaturants (urea/GdnHCl) in 0.06 M sodium phosphate buffer, pH 7.0. In unfolding experiments, different volumes of buffer were first added to a fixed volume of stock protein solution, then denaturant was added to the desired concentration. A similar procedure was employed in preparing the solutions for refolding experiments, except that the protein was first denatured by adding denaturant solution, and then diluted with buffer. The final mixture for both unfolding and refolding experiments was incubated for 12 h at 25°C before CD and fluorescence measurements.

Analysis of Data—The pre- and post-transition baselines were treated by linear extrapolation (*36*, *37*), and the unfolding curves for the N ⇌ D transition were normalized to the apparent fraction of the unfolded form, F_D, using Eq. 2

$$F_D = (Y - Y_N)/(Y_D - Y_N) \quad (2)$$

where Y is the observed variable parameter, and Y_N and Y_D are the values of the variable characteristic of the folded and unfolded conformations, respectively. The difference in free energy between the folded and unfolded states, ΔG, is given by Eq. 3

$$\Delta G = -RT \ln K_D = -RT \ln [F_D/(1-F_D)] \quad (3)$$

where different terms have their usual significance (*38*).

For the unfolding transition, N ⇌ I ⇌ D, where I is an intermediate state, each step may be assumed to follow a two-state mechanism. The fraction of the intermediate state, F_I in the reaction N ⇌ I, can be given as:

$$F_I = (Y - Y_N)/(Y_I - Y_N) \quad (4)$$

where $F_I + F_N = 1$

Similarly, the fraction of the denatured state, F_D in the reaction I ⇌ D, can be given as:

$$F_D = (Y - Y_I)/(Y_D - Y_I) \quad (5)$$

where $F_D + F_I = 1$

Values of equilibrium constant and free energy for the above transitions can be obtained in the following way.

For the N ⇌ I transition,

$$K_I = F_I/(1 - F_I) \quad (6)$$

and

$$\Delta G_I = -RT \ln K_I \quad (7)$$

For the I ⇌ D transition

$$K_D = F_D/(1 - F_D) \quad (8)$$

and

$$\Delta G_D = -RT \ln K_D \quad (9)$$

A least-squares analysis of the data of ΔG as a function of denaturant concentration [D], was used to fit the data to

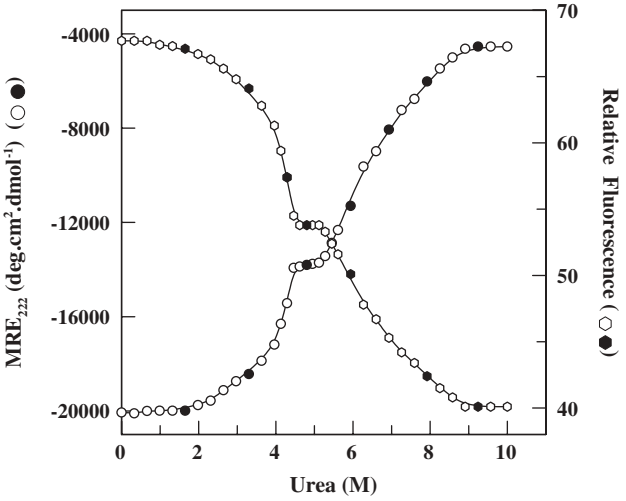

Fig. 1. **Urea-induced unfolding (open circles and hexagons) and refolding (filled circles and hexagons) of HSA in 0.06 M sodium phosphate buffer, pH 7.0, 25°C.** The unfolding/refolding was followed by measuring MRE at 222 nm (circles) or fluorescence at 340 nm (hexagons) on excitation at 280 nm.

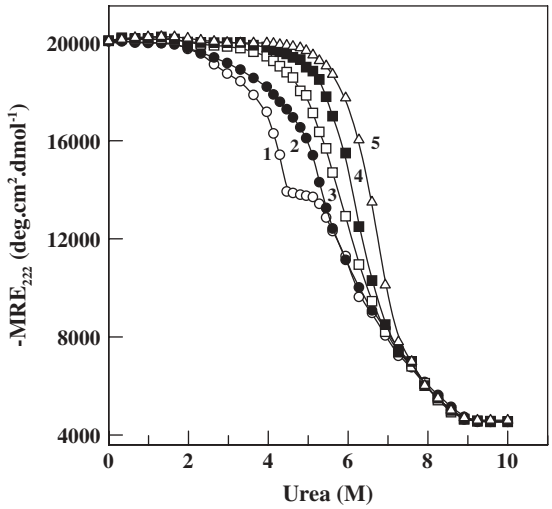

Fig. 2. **Urea-induced unfolding of HSA as followed by MRE measurements at 222 nm at pH 7.0, 25°C in the absence (1) and presence of 0.5 M TFE (2), 1.0 M TFE (3), 2.0 M TFE (4) and 3.0 M TFE (5).**

the following equation for the determination of ΔG^{H_2O}, the free energy change in the absence of urea.

$$\Delta G = \Delta G^{H_2O} - m[D] \quad (10)$$

where m is a measure of the dependence of ΔG on denaturant concentration in cal mol^{-1} M^{-1}.

RESULTS

Urea-Induced Transition of HSA—Figure 1 shows the urea-induced equilibrium unfolding of HSA as studied by MRE and intrinsic fluorescence measurements at 222 and 340 nm, respectively. Urea-induced unfolding of HSA showed a two-step transition from native (N) state to denatured (D) state with a stable intermediate (I) state around 4.6–5.2 M urea. The first transition, showing the transformation of N state to I state, started at 2.0 M urea and was complete at 4.5 M urea with a mid-point at 3.9 M urea. The second transition (I ⇌ D) started at 5.2 M urea and finally sloped off at ~8.8 M urea with a mid-point at ~6.6 M urea. Similar values were obtained from intrinsic fluorescence measurements.

Effect of TFE on the Conformational Stability of HSA—The effect of TFE on the conformational stability of HSA was examined by studying urea-induced denaturation of HSA in the presence of different concentrations of TFE. Figure 2 shows the urea-induced transition of HSA at pH 7.0, 25°C as followed by MRE measurements at 222 nm in the absence (curve 1) and presence of increasing concentrations of TFE (curves 2–5). The transition curves (curves 2–5) obtained in the presence of different concentrations of TFE showed a single-step transition (N ⇌ D) without an intermediate state, as compared with a two-step transition (curve 1) in the absence of TFE. The denaturation curve of HSA in the presence of increasing concentrations (0.5–3.0 M) of TFE was shifted towards higher urea concentration, as the mid-point of transition was observed at 6.7 M urea in the presence of 3.0 M TFE as compared with 5.6 M urea in the presence of 0.5 M TFE.

Urea denaturation curves of HSA (in the absence as well as presence of 3.0 M TFE) were normalized assuming a two-step transition for the former and a single-step transition for the latter, as described in Materials and Methods. Figure 3A shows the normalized curves thus obtained. By assuming that the transitions shown in curves 1 and 2 of Fig. 3A both follow a two-state mechanism, the free energy of unfolding, ΔG_I and ΔG_D, respectively, was calculated as a function of urea concentration as described in Eqs. 7 and 9. Similarly, for transition curve 3 in the presence of 3.0 M TFE, values of ΔG_D were calculated using Eq. 3. Figure 3B shows the variation of ΔG as a function of urea concentration. A least-squares analysis was used to fit the data to Eq. 10 to calculate $\Delta G_I^{H_2O}$, ΔG_D^* and $\Delta G_D^{H_2O}$ (in the presence of TFE). The value of ΔG_D^* represents the value obtained from the extrapolation of ΔG_D values to the start of the process, I ⇌ D. Table 1 summarizes the ΔG^{H_2O} values under different conditions. The $\Delta G_I^{H_2O}$ and m values obtained for the first transition (N ⇌ I) were 3,510 cal/mol and 910 cal mol^{-1} M^{-1} of urea concentration, respectively, whereas ΔG_D^*, the free energy change associated with the I ⇌ D transition was found to be 1,850 cal/mol and the m value to be 810 cal mol^{-1} M^{-1}. Free energy change associated with the N ⇌ D transition in the absence of TFE can be obtained by summing the free energy change of the individual steps, *i.e.*, $\Delta G_I^{H_2O}$ and ΔG_D^*. Therefore, $\Delta G_{total}^{H_2O}$, *i.e.*, the free energy change associated with the transformation from N state to I state and finally to D state, was calculated to be 5,360 cal/mol. The $\Delta G_D^{H_2O}$ and m values for the N ⇌ D transition in the presence of 3.0 M TFE were calculated to be 8,040 cal/mol and 1,190 cal mol^{-1} M^{-1}, respectively. The free energy of stabilization $\Delta\Delta G_D^{H_2O}$, *i.e.*, the difference between the free energy change of unfolding of HSA in the presence and absence of TFE was found to be 2,680 cal/mol.

A similar stabilizing effect of TFE was also found when the urea-induced transition of HSA was monitored by fluorescence measurements. Data were treated in the same way as described above (see Table 1). Values of

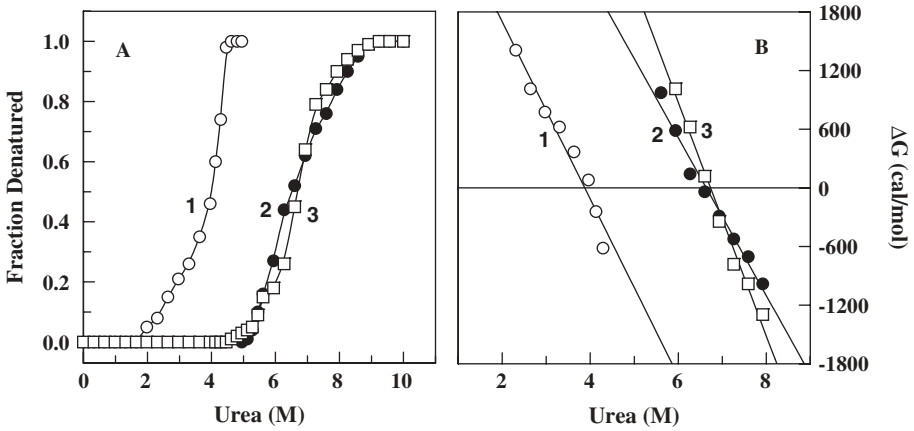

Fig. 3. **(A) Normalized curves for the transitions: (1) N ⇌ I, (2) I ⇌ D and (3) N ⇌ D in the presence of 3.0 M TFE. (B) Dependence of free energy change on urea concentration for the transitions shown in (A).**

Table 1. **Values of conformational free energy of HSA obtained from analysis of CD and fluorescence data.**

Probe	$\Delta G_I^{H_2O}$ (N ⇌ I) (cal/mol)	ΔG_D^* (I ⇌ D) (cal/mol)	$\Delta G_{total}^{H_2O}$ (N ⇌ I ⇌ D) (cal/mol)	$\Delta G_D^{H_2Oa}$ (N ⇌ D) (cal/mol)	$\Delta\Delta G_D^{H_2O}$ (cal/mol)
MRE_{222}	3,510	1,850	5,360	8,040	2,680
Fluorescence[b]	3,490	1,850	5,340	8,060	2,720

[a]Determined in the presence of 3.0 M TFE. [b]Excitation and emission wavelengths were 280 and 340 nm, respectively.

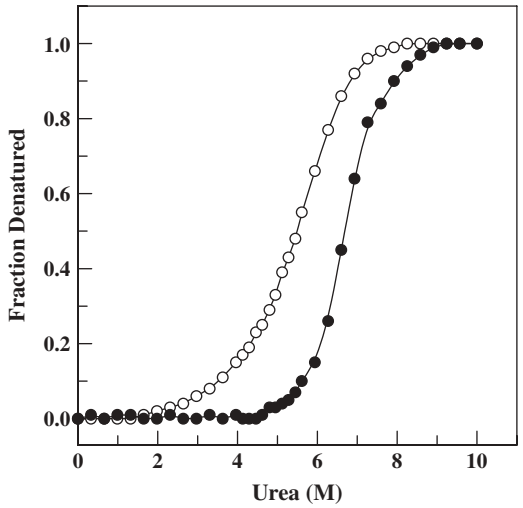

Fig. 4. **Normalized transition curves for urea-induced unfolding of HSA as monitored by ANS fluorescence measurements at pH 7.0, 25°C in the absence (open circles) and presence of 3.0 M TFE (filled circles).** Concentrations of protein and ANS were 1.8 μM and 90 μM, respectively.

$\Delta G_I^{H_2O}$ and ΔG_D^* for the first and second transitions were determined to be 3,490 and 1,850 cal/mol, respectively, whereas the *m* values for the two transitions were 900 and 805 cal mol^{-1} M^{-1}, respectively. $\Delta G_{total}^{H_2O}$ was calculated to be 5,340 cal/mol. The free energy change, $\Delta G_D^{H_2O}$ for the transition (N ⇌ D) in the presence of 3.0 M TFE was calculated to be 8,060 cal/mol and the m value to be 1,200 cal mol^{-1} M^{-1}. Therefore, $\Delta\Delta G_D^{H_2O}$ determined by fluorescence measurements was 2,720 cal/mol. These results were in close agreement with those obtained by MRE measurements.

Figure 4 shows the normalized transition curves of urea denaturation of HSA in the absence and presence of 3.0 M TFE, obtained by plotting F_D against urea concentration, when studied by ANS binding. Both transition curves showed a single-step transition. However, the mid-point of transition was shifted towards higher urea concentration (6.7 M) in the presence of 3.0 M TFE as against 5.5 M in its absence.

Effect of Different Alcohols on the Conformational Stability of HSA—In view of the stabilizing effect of TFE, the stabilizing potential of other alcohols was analyzed by studying the refolding behavior of HSA at 5.0 M urea, where an intermediate (I) state exists, both by MRE and intrinsic fluorescence measurements at 222 and 340 nm, respectively. Figure 5A shows the dependence of MRE_{222} value at 5.0 M urea in the absence and presence of different alcohols (HFIP, TFE, 2-chloroethanol, tert-butanol, iso-propanol, ethanol and methanol) on molar alcohol concentration. The extent of refolding induced by all the alcohols was found to be similar at higher alcohol concentrations, but to differ for different alcohols at lower concentrations (< 1.0 M). The C_m values determined from Fig. 5A for different alcohols are given in Table 2. As can be seen from Fig. 5A and Table 2, the relative effectiveness of various alcohols in refolding of HSA followed the series: HFIP > TFE > 2-chloroethanol > tert-butanol > iso-propanol > ethanol > methanol. Intrinsic fluorescence measurements gave similar results (see Fig. 5B and Table 2).

Effect of TFE on GdnHCl-Induced Transition of HSA—The unfolding of HSA was also studied using GdnHCl as a denaturant. The transition was found to follow a single-step, two-state transition as monitored by measuring MRE at 222 nm. Figure 6 shows the normalized transition curves of HSA obtained in the absence and presence of 3.0 M TFE as a function of GdnHCl concentration. The free energy of unfolding, $\Delta G_D^{H_2O}$, and m values were calculated to be 5,860 cal/mol and 2,020 cal mol^{-1} M^{-1}, respectively in the absence of TFE, as compared with 8,245 cal/mol and 2,100 cal mol^{-1} M^{-1}, respectively, in the presence of 3.0 M

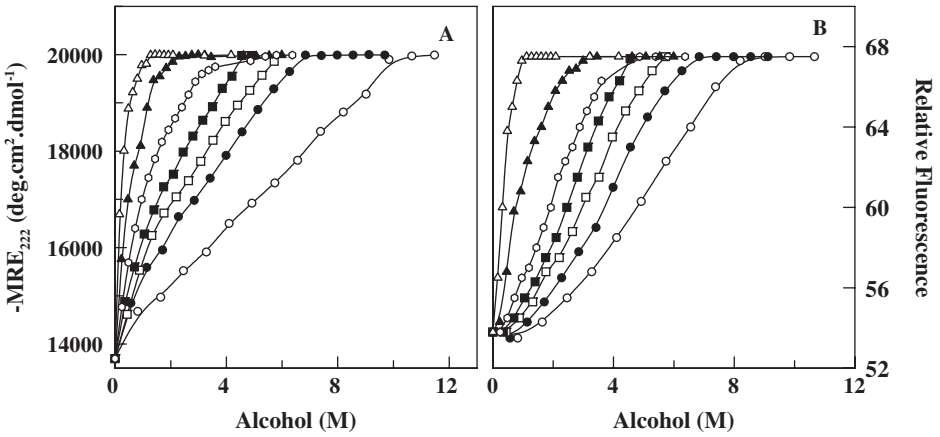

Fig. 5. **Effect of various alcohols on the refolding of HSA at 5.0 M urea and at pH 7.0, 25°C as measured by MRE_{222} and intrinsic fluorescence.** (A) Dependence of MRE on molar alcohol concentration. (B) Dependence of fluorescence intensity on molar alcohol concentration. Alcohols used were: HFIP (open triangles); TFE (filled triangles); 2-chloroethanol (open hexagons); tert-butanol (filled squares); isopropanol (open squares); ethanol (filled circles) and methanol (open circles). The protein concentrations were 3 μM and 1.8 μM for MRE and fluorescence measurements, respectively.

Table 2. **Alcohol-induced refolding of HSA.**

Alcohol	C_m value (M)[a]	
	MRE_{222}	Fluorescence
Methanol	4.90	4.90
Ethanol	2.40	3.90
Iso-propanol	2.20	3.00
tert-Butanol	1.40	2.45
2-Chloroethanol	0.96	2.02
TFE	0.46	0.92
HFIP	0.16	0.40

[a]Calculated from the data of Fig. 5, A and B.

TFE. A comparison of these values gave the $\Delta\Delta G_D^{H_2O}$ value in the presence of 3.0 M TFE as 2,385 cal/mol, which was similar to the value obtained for the urea transition of HSA.

DISCUSSION

The equilibrium denaturation of HSA by urea was found to be completely reversible, and changes in the fluorescence and CD signal may be described by a three-state, two-step denaturation/renaturation model with a stable intermediate populated around 4.6–5.2 M urea. These results were in accordance with the earlier results reported for HSA and BSA (*38–41*). This intermediate retained a significant amount of secondary structure (37%) compared to 58% found in native protein. However, the urea-induced transition of HSA in the presence of TFE followed a two-state mechanism without any detectable intermediate. These data suggested the stabilization of the native state of HSA at low TFE concentration. Earlier studies have also shown an increase in the structural stability of many globular proteins in the presence of low concentrations of organic solvents (*42–44*). A hydrophobic environment may stabilize or even induce native-like structure in a peptide chain, as is evident from the maintenance of many native-like characteristics by lysozyme, ubiquitin and peptide fragment of α-lactalbumin in methanol or TFE-containing solution (*22*, *45*, *46*). Our results on TFE-induced stabilization of HSA seem to be consistent with earlier findings showing that a transition state of kinetic folding is stabilized by the addition of a small amount of TFE due to the stabilization of local hydrogen bonds if the

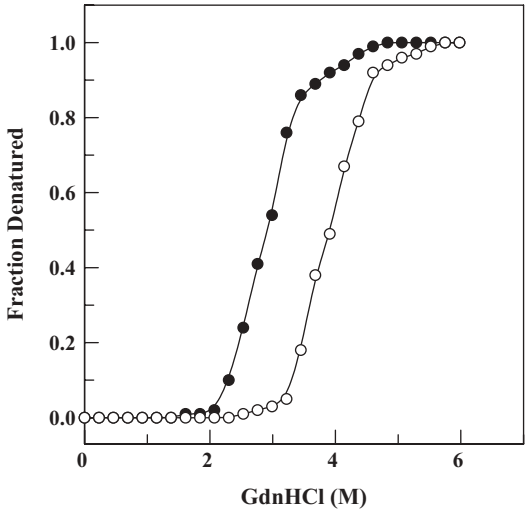

Fig. 6. **Normalized transition curves for GdnHCl-induced unfolding of HSA in the absence (filled circles) and presence (open circles) of 3.0 M TFE as monitored by measuring MRE at 222 nm at pH 7.0, 25°C.**

structural property of the transition state is closely similar to that of the native state (*47*, *48*). At the same time, alcohols at higher concentrations have been reported to denature proteins by weakening hydrophobic interactions, but stabilize the peptide helices in proteins (*49*). It is difficult to say whether high concentration of TFE stabilizes the open helical structure of HSA in the absence of denaturants like urea or GdnHCl, as attempts to study urea denaturation of HSA in the presence of higher concentrations of TFE (> 3.0 M) failed due to the precipitation of native HSA. However, in an earlier study, we have shown the stabilization of the open-helical structure and loss in the asymmetry of protein's tertiary structure in HSA at pH 2.0 in presence of 8.4 M TFE, suggesting the denaturation effect of TFE at high concentration (*50*). Many organic solvents at low concentrations increase the structure of solvent water and thus enhance hydrophobic interactions (*51*, *52*), which may be responsible for the stabilization of native protein structure. Further, the low dielectric constant of TFE (compared to that of pure water) favors intramolecular hydrogen bonding and electrostatic interactions and may also enhance the stabilization of the folded state in TFE.

At higher concentrations, organic solvents destabilized hydrophobic interactions (*53*), and this destabilization may be far higher than the stabilization of hydrogen bonds and electrostatic interactions and thus account for the denaturation of proteins. Further, the greater effectiveness of alkyl alcohols with bulky alkyl groups compared to those with small alkyl groups in denaturing proteins suggests that hydrophobic interactions are involved in the denaturation process, as reported earlier (*54*, *55*). Alcohols used in this study were found to promote refolding of the intermediate state, which may be ascribed to the complex folding mechanism of serum albumin involving independent folding of domains and their subsequent interactions to give rise to the native structure. Crystal structure has shown that HSA is comprised of three homologous domains, I, II and III, that assemble to form a heart-shaped molecule. These domains are not only topologically identical but also very similar in three-dimensional structure. Each domain can be further divided into sub-domains "a" and "b", which are composed of six (a-h1 to a-h6) and four (b-h1 to b-h4) α-helices respectively, and thus each domain contributes 10 principal helices joined by stretches of extended polypeptides (*56*, *57*). Domains I and II are almost perpendicular to each other to form a T-shaped assembly, whereas domain III protrudes from sub-domain II b at an angle of about 45° to form a Y-shaped assembly. Domain III interacts with only sub-domain II b. Each domain retains its conformation during complex formation with the ligand, except that two C-terminal helices in domain III move towards the outside of the molecule due to their few interactions with other parts of the molecule (*57*). This probably makes domain III more labile towards urea denaturation. Our results with TFE suggest that association of domain III with sub-domain II b is stronger in presence of alcohol. HSA contains an unusually large number (seventeen) of disulfide bonds and one free sulfhydryl group. Though the effect of TFE and other solvents on reduced HSA is to induce helical content, none of the solvent conditions restored the helicity of reduced HSA to the native form (*58*). In view of the primary involvement of domain III in the intermediate formation (*38–41*), it appears that TFE mainly stabilizes domain III against urea denaturation.

GdnHCl is considered to be a much stronger denaturant than urea (*59*), although the relative effectiveness of two denaturants depends on the nature of the protein involved (*60*). The mechanism by which urea and guanidinium ion denature proteins is not clear. Available literature suggests that these molecules may denature proteins either directly by binding to peptide groups and therefore weakening internal hydrogen bonds or indirectly through changing the structure of water's hydrogen bond network around hydrophobic groups and thus weakening the hydrophobic effect in the protein molecule (*61–63*). Since there are more non-interacting binding sites on the unfolded conformation than the folded conformation, the protein unfolds and more sites are exposed to the denaturant molecules (*64*, *65*). GdnHCl-induced denaturation of HSA was also stabilized in the presence of TFE as transition was shifted to higher GdnHCl concentration in the presence of TFE. Thus, it appears that TFE protects the protein structure to some extent from undergoing denaturation through an indirect solvent effect.

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