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Anion-Induced Stabilization of Human Serum Albumin Prevents the Formation of Intermediate During Urea Denaturation

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ABSTRACT **The unfolding of human serum albumin (HSA), a multidomain protein, by urea was followed by far-UV circular dichroism (CD), intrinsic fluorescence, and ANS fluorescence measurements. The urea-induced transition, which otherwise was a two-step process with a stable intermediate at around 4.8 M urea concentration as monitored by far-UV CD and intrinsic fluorescence, underwent a single-step cooperative transition in the presence of 1.0 M KCl. The free energy of stabilization ($\Delta\Delta G_D^{H_2^0}$) in the presence of 1 M KCl was found to be 1,090 and 1,200 cal/mol as determined by CD and fluorescence, respectively.The salt stabilization occurred in the first transition (0–5.0 M urea), which corresponded to the formation of intermediate (I) state from the native (N) state, whereas the second transition, corresponding to the unfolding of I state to denatured (D) state, remained unaffected. Urea denaturation of HSA as monitored by tryptophan fluorescence of the lone tryptophan residue (Trp_{214}) residing in domain II of the protein, followed a single-step transition suggesting that domain(s) I and/or III is (are) involved in the intermediate formation. This was also confirmed by the acrylamide quenching of tryptophan fluorescence at 5 M urea, which exhibited little change in the value of Stern-Volmer constant. ANS fluorescence data also showed single-step transition reflecting the absence of accumulation of hydrophobic patches. The stabilizing potential of various salts studied by far-UV CD and intrinsic fluorescence was found to follow the order: $NaClO_4$ > NaSCN >Na_2SO_4 >KBr >KCl >KF. A comparison of the effects of various potassium salts revealed that anions were chiefly responsible in stabilizing HSA. The above series was found similar to the electroselectivity series of anions towards the anion-exchange resins and reverse of the Hofmeister series, suggesting that preferential binding of anions to HSA rather than hydration, was primarily responsible for stabilization. Further, single-step transition observed with GdnHCl can be ascribed to its ionic character as the free energy change associated with urea denaturation in the presence of 1.0 M KCl (5,980 cal/mol) was similar to that obtained with GdnHCl (5,870 cal/mol). Proteins 2000;40:29–38.** © 2000 Wiley-Liss, Inc.

Key words: human serum albumin; intermediate state; anion-induced stabilization; circular dichroism; ANS fluorescence; protein folding

INTRODUCTION

The mechanism by which proteins fold from a structureless denatured state to their unique biologically active state is an intricate process. However, recent advances in biophysical techniques[1,2] both thermodynamic and kinetic, have shown the presence of stable intermediate conformational states in a number of proteins,[3–5] which helped in the understanding of protein folding phenomenon. The folding process is even more complex in multidomain proteins[6] where each domain may be capable to refold independently[7,8] and interdomain interactions may affect the overall folding process.[9] Salts are known to affect the stability of proteins in a variety of ways.[10] Salts and proteins interact in different ways ranging from preferential hydration to preferential salt binding depending on the type of salt and solvent conditions.[11] Despite development of methodologies (both experimental and theoretical) in the last few decades and several studies on model compounds for the elucidation of thermodynamic principles underlying the effect of neutral salts on protein stability, no consensus has been reached on how salts affect protein stability. Different possible mechanisms have been proposed that include specific and non-specific binding of ions to protein molecules, electrostatic shielding of charges, salts effect on water structure, etc.[12–17] Specific ion binding mechanisms can have important biotechnological implications for the design of thermostable proteins by the construction of ion-binding sites on the surface of globular proteins.[18,19]

Human serum albumin (HSA) is a single chain multidomain protein that aids in the transport, metabolism, and distribution of exogenous and endogenous ligands.[20] X-ray crystallographic studies have shown that serum albumin consists of three domains encompassing the complete

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sequence.[21] Studies on chemically and enzymatically isolated domains have shown that these domains retain native-like conformation and ligand binding properties.[22,23] Recently individual domains (I and III) have been efficiently expressed and secreted in *Saccharomyces cerevisiae,* [24] which further confirms that these domains can form independent structural units capable of folding into stable tertiary structure. Wetlaufer[7] has also described the refolding of BSA fragment 377–582, which is intact domain III. Earlier denaturation studies on serum albumin have shown conflicting results on the presence of an intermediate state. Whereas urea-induced denaturation of serum albumin has been shown to follow a two-step process in earlier studies,[25,26] Wallevik[27] has shown that guanidine hydrochloride-induced denaturation is a single-step process. Absence of intermediate in the GdnHCl-induced transition has been attributed to the positive preferential salt binding for guanidinium ions.[28–30] In view of this, it becomes necessary to study the effect of various salts on the urea-induced denaturation of HSA. In the present work, we studied the effect of salts on the urea-induced denaturation of HSA at pH 7.0, 25°C by far-UV CD, intrinsic fluorescence and ANS fluorescence. We found that salts markedly stabilized HSA towards urea denaturation and anions are chiefly responsible for this stabilization. The order of effectiveness of stabilization followed electroselectivity series of anions towards anion-exchange resins. The results also suggested that stabilization chiefly involved domain III, which is otherwise relatively unstable and is responsible for the formation of intermediate state.

MATERIALS AND METHODS

Human serum albumin (essentially fatty acid free), type A-1887; 1-anilinonaphthalene-8-sulphonate (ANS), type A-3125; N-acetyl-L-tryptophanamide (NATA), type A-6501, ultrapure urea, type U-0631, and guanidine hydrochloride, type G-7153 were obtained from Sigma Chemical Co. (St. Louis, MO). HSA was freed from dimers and highermers by passing through Sephadex G-100 gel filtration column. All the other chemicals used were of analytical grade.

Protein concentration was determined spectrophotometrically using $E^{1\%}_{1cm}$ of 5.30 at 280 nm[27] on a Cecil double beam spectrophotometer, model CE 594. ANS concentration was also determined spectrophotometrically using molar extinction coefficient of 5,000 M^{-1} at 350 nm.[31] Concentrations of denaturant stock solutions were determined from the data of Warren and Gorden[32] and Nozaki[33] for urea and GdnHCl solutions, respectively, as described by Pace and Scholtz.[34]

Circular Dichroism (CD) Measurements

CD measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulphonic acid. All the CD measurements were made at 25°C with a thermostatically controlled cell holder attached to a Neslab's RTE-110 waterbath with an accuracy of ± 0.1°C. Spectra were collected with a scan speed of 20 nm/min and with a response time of 1 s. Each spectrum was the average of 4 scans. Far-UV CD spectra were taken at a protein concentration of 1.8 μM with a 1 mm path length cell. The results are expressed as mean residue ellipticity (MRE) in deg · cm^2 · $dmol^{-1}$, which is defined as,

$$MRE = \theta_{obs}(mdeg)/10 \times n \times 1 \times C_p \quad (1)$$

where different terms have their usual significance.[35]

Fluorescence Measurements

Fluorescence measurements were performed on a Shimadzu spectrofluorometer, model RF-540 equipped with a data recorder, DR-3. The fluorescence spectra were measured at a protein concentration of 1.8 μM with a 1-cm path-length cell. Excitation and emission slits were set at 10 nm each. For ANS fluorescence in the ANS binding experiments, the excitation wavelength was set at 380 nm and the emission spectrum was recorded in the range of 400–600 nm. Intrinsic fluorescence was measured by exciting the protein solution at either 280 or 295 nm and emission spectra were recorded in the range of 300–400 nm with a protein concentration of 1.8 μM.

Acrylamide Quenching Experiments

In the acrylamide quenching experiments, aliquots of 1 M acrylamide stock solution were added to a protein stock solution to get the desired acrylamide concentration. The final concentration of protein was 1.5 μM. The excitation was set at 295 nm in order to excite tryptophan fluorescence only and the emission spectrum was recorded in the range of 300–400 nm. The slit width was set at 10 nm both for excitation and emission spectra. The decrease in fluorescence intensity at λ_{max} was analyzed according to the Stern-Volmer equation.[36]

Denaturation and Renaturation Experiments

Solutions for the denaturation and renaturation experiments were prepared from stock solutions of protein and denaturants (urea and GdnHCl) prepared in 60 mM sodium phosphate buffer, pH 7.0.

In denaturation experiments, to a stock protein solution, different volumes of the buffer were added first and the denaturant was added last so as to get the desired concentration of denaturant. On the other hand, for renaturation experiments, to a stock protein solution, different volumes of concentrated denaturant solution (10M urea) were added first and the mixture was incubated for 4 hours and finally buffer was added to get desired denaturant concentration. The final solution mixture for both the denaturation and renaturation experiments was incubated for 12 hours at 25°C before CD and fluorescence measurements.

Data Analysis

Unfolding curves were analyzed using either two state or three state mechanisms.

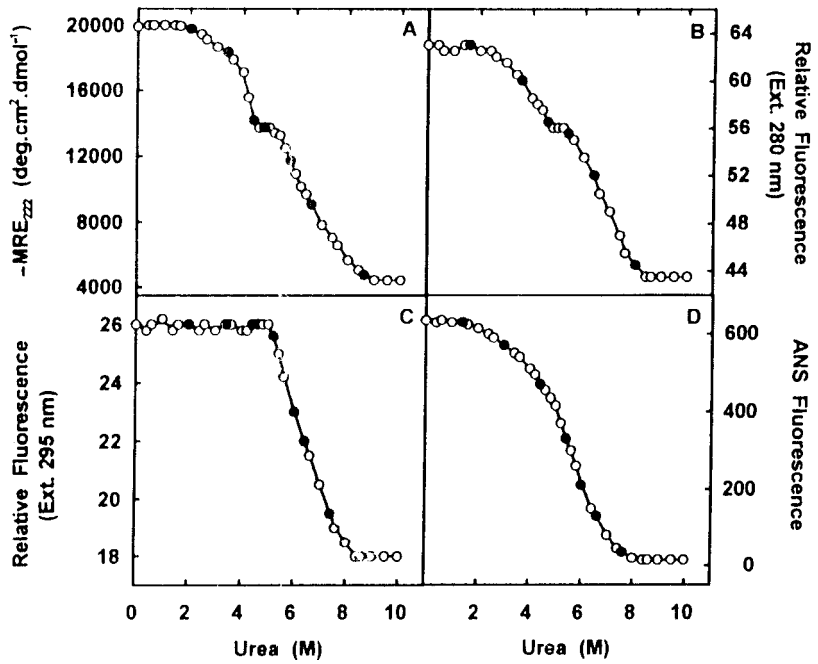

Fig. 1. Urea-induced unfolding (open circles) and refolding (solid circles) of HSA in 60 mM sodium phosphate buffer, pH 7.0, 25°C. The unfolding/refolding were followed by measuring: **A:** MRE at 222 nm. **B:** Fluorescence intensity at 340 nm on excitation at 280 nm. **C:** Fluorescence intensity at 340 nm on excitation at 295 nm. **D:** ANS fluorescence at 480 nm on excitation at 380 nm. The excitation and emission band widths for fluorescence measurement were 10 nm each.

Two state mechanism

Unfolding curves for the N ⇌ D transition were normalized to the apparent fraction of the unfolded form, F_D, using the following equation:[37]

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

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. The difference in free energy between the folded and the unfolded states, ΔG, was calculated by the following equation:

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

where K is the equilibrium constant, R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature.

Three state mechanism

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 obtained from the relation:

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

where $F_I + F_N = 1$.

Similarly, the fraction of denatured state, F_D in the reaction I ⇌ D can be calculated from the relation:

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

where $F_D + F_I = 1$.

The equilibrium constant and the free energy for the above transitions may be calculated from the following relationships:

For N ⇌ I transition,

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

and

$$\Delta G_I=-RT\ \text{In}\ K_I \quad (7)$$

For I ⇌ D transition

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

and

$$\Delta G_D=-RT\ \text{In}\ K_D \quad (9)$$

A least squares analysis of the equations (3), (7), and (9) as a function of denaturant concentration, [D], was used to fit the data to the following equation for the determination of ΔG^{H_2O}, the free energy change in the absence of urea[38]

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

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

RESULTS AND DISCUSSION

Figure 1A–D shows the urea-induced denaturation of HSA as monitored by the measurement of MRE at 222 nm, intrinsic fluorescence at 340 nm by exciting the protein at 280 nm, tryptophan fluorescence at 340 nm after exciting the protein at 295 nm, and ANS fluorescence at 470 nm after exciting the ANS-protein complex at 380 nm, respectively. Urea-induced denaturation of HSA was found to be a two-step process with accumulation of an intermediate state at around 4.6–5.2 M urea concentration when monitored by ellipticity and intrinsic fluorescence measurements (Fig. 1A,B). On the other hand, transition from

native to denatured state was found to be a single-step process with no intermediate state when studied by tryptophan fluorescence and ANS fluorescence measurements (Fig. 1C,D). The denaturation was found to be completely reversible for the transitions studied by different probes under the conditions used. Absence of intermediate in the latter (Fig. 1C,D) simply indicated that neither the environment of Trp residue was significantly affected nor accumulation of hydrophobic patches took place at that urea concentration where intermediate state was detected by MRE and intrinsic fluorescence measurements. Therefore, urea-induced denaturation may be approximated to a two-step, three-state transition and the mechanism for unfolding-refolding of HSA may be represented as:

$$N \rightleftharpoons I \rightleftharpoons D \quad (11)$$

where N, I and D are the native, intermediate and denatured states, respectively. The first transition (Fig. 1A) which corresponded to the transformation of N state to the I state started at around 2 M urea and completed at 4.5 M urea concentration with a midpoint occurring at 3.9 M urea. The intermediate state was stable in the urea concentration range 4.6–5.0 M and characterized by the presence of abundant secondary structure, i.e., ~ 37% α-helix compared to 58% α-helix found in the N state as calculated from the MRE value at 222 nm by the method of Chen et al.[39] The second transition, which corresponded to the unfolding of the I state, started at around 5.2 M urea and finally sloped off to the D state at ~ 8.4 M urea with a midpoint occurring at ~ 6.6 M urea concentration. The transition as monitored by fluorescence measurement by exciting the protein at 280 nm (Fig. 1B) also exhibited the formation of the stable intermediate state around 4.8–5.2 M urea concentration. The midpoint for the first and second transitions occurred at 3.9 M and 6.65 M urea concentrations, respectively. These results were similar to those studied by MRE measurements.

The environment of the tryptophan residue can be selectively investigated by exciting the protein at wavelength 295 nm or greater because at this wavelength, the fluorescence emission is essentially due to tryptophan residue.[40] Figure 1C shows urea-induced denaturation of HSA by measuring the tryptophan fluorescence at 340 nm after exciting the protein at 295 nm. As can be seen from Figure 1C, the transition was single-step with no apparent intermediate state. The transition started at around 5.6 M urea and sloped off at 8.0 M urea with a midpoint occurring at 6.45 M urea concentration. Since HSA contains only one tryptophan (Trp_{214}) residue, which resides in domain II, changes in fluorescence intensity observed after exciting the protein at 295 nm may be ascribed to the conformational changes in domain II. Thus, it can be inferred that no changes occurred in domain II in the urea concentration range 0.0–5.4 M. Further, the changes in fluorescence intensity observed by exciting the protein at 280 nm (Fig. 1B) in the urea concentration range 2.0–4.6 M may be attributed to conformational changes in domain I and/or domain III. Therefore, it appears that the formation of intermediate in the unfolding-refolding transition of serum albumin involves unfolding of domain I and/or domain III. Absence of any spectral shift in the fluorescence spectra of HSA in between 0–4.0 M urea concentration has also been reported earlier, suggesting noninvolvement of domain II in the conformational transition of HSA observed in this region by other probes.[41] An earlier study[25] has shown the involvement of both domains II and III in the formation of intermediate based on their results on urea denaturation of bovine serum albumin (BSA) and its fragment containing domains II and III. It can be concluded from earlier results as well as the present investigation that domain III is primarily responsible for intermediate formation in the unfolding transition of HSA. Involvement of domain II in the intermediate formation as shown in a previous report[25] based on denaturation study of the fragment containing domains II and III is questionable since this fragment contained nicked peptide bond at two positions. Further, it is not certain from the previous study whether an excitation wavelength of 295 nm was used.[25]

TABLE I. Stern-Volmer Constants of Acrylamide Quenching for HSA and NATA

Subject	K_{sv} [a]
Native HSA	6.0
HSA in 5 M urea	6.6
HSA in 7 M urea	8.8
HSA in 10 M urea	10.7
NATA	20.3

[a] K_{sv} values were calculated by linear least-squares fitting of Stern-Volmer plots.

The exposure of the lone tryptophan residue Trp_{214} (present in domain II) was examined by fluorescence quenching using neutral quencher, acrylamide.[36] Tryptophan analogue, NATA, was also used as a standard for complete accessibility to quencher. Values of Stern-Volmer constant (K_{sv}) fitted to the linear early part of Stern-Volmer plots (figure omitted for brevity) are shown in Table I. As can be seen from Table I, K_{sv} value of HSA did not change significantly up to 5 M urea concentration, suggesting little or no conformational change in domain II of the protein. However, at higher urea concentrations (>5.0 M), a significant increase in K_{sv} value was noticed indicating marked conformational change in domain II. These results also suggested that the conformational changes in HSA occurring at <5.0 M urea concentration may be attributed to domain I and/or domain III whereas the changes at concentrations >5.0 M urea may be assigned to domain II. It must be noted that HSA in 10 M urea concentration wherein protein was considered to exist in a random coil conformation contained some local regions probably around Trp_{214} retaining residual structure as evident from the much higher K_{sv} of NATA than HSA in 10 M urea (see Table I). This was in agreement with previous reports suggesting retention of native-like secondary and/or tertiary structures within local regions in a random coil conformation.[42–44] ANS fluorescence data (Fig. 1D) showed no apparent intermediate state in the

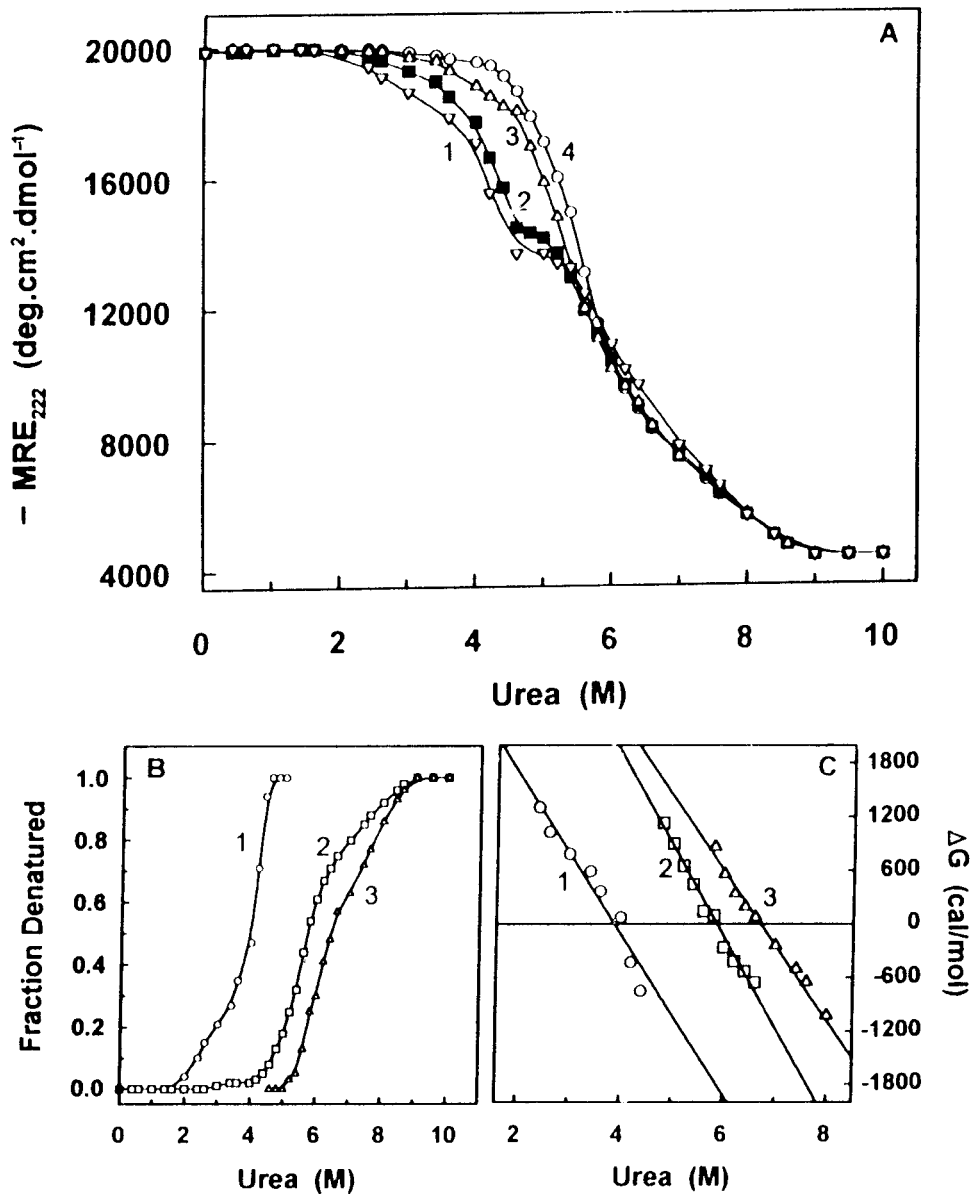

Fig. 2. Urea-induced unfolding of HSA as followed by MRE measurements at 222 nm at pH 7.0, 25°C in the absence as well as presence of different concentrations of KCl. **A:** Transition curves in the presence of (1) 0 M KCl, (2) 0.2 M KCl, (3) 0.75 M KCl, and (4) 1 M KCl. **B:**Normalized curves for the transitions: (1) N ⇌ I; (3) I ⇌ D and (2)N ⇌ D in the presence of 1 M KCl. **C:** Dependence of free energy change on urea concentration for the transitions shown in B.

urea-induced denaturation reflecting the absence of accumulation of hydrophobic patches. This seems to be understandable in view of the more or less even distribution of nonpolar residues in different domains of the protein.[45]

In order to examine the role of salts in the conformational stability of HSA, we studied urea-induced denaturation of HSA in the presence of different concentrations of KCl. Figure 2A shows the effect of increasing KCl concentrations on the urea-induced transition of HSA at pH 7.0, 25°C as followed by MRE measurements at 222 nm. As can be seen from Figure 2A, HSA had undergone single-step transition with no intermediate in the presence of 1.0 M KCl (Fig. 2A, curve 4) against two-step transition observed in its absence (Fig. 2A, curve 1). The presence of increasing concentrations of KCl shifted the initial zone of denaturation curve towards higher urea concentration thus abolishing the formation of intermediate at highest KCl concentration used (Fig. 2A, curves 1–4). In other words, salt was found to stabilize transition I that corresponded to the formation of intermediate state whereas the II transition that corresponded to the unfolding of the intermediate state exhibited no change. In view of the involvement of domain III in the I transition, it can be inferred that salt ions stabilized domain III that unfolded in a cooperative manner with rest of the molecule. The urea denaturation curve shown in Figure 2A (curve 1) was normalized assuming two-step transition by plotting F_I and F_D values obtained by using eqs. (4) and (5), respectively, against urea concentration whereas curve 4 of Figure 2A was normalized assuming single-step transition into F_D values obtained by using eq. (2) against urea concentration (see Fig. 2B, curves 1–3). Assuming both the transitions shown in Figure 2B (curves 1 and 3) to follow two-state mechanism, we calculated the free energy of unfolding, ΔG_I and

TABLE II. Values of Conformational Free Energy of HSA Using CD and Fluorescence

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)	$\Delta G_D^{H_2O}$ [a] (N⇌D) (cal/mol)	$\Delta\Delta G_D^{H_2O}$ (cal/mol)
MRE_{222}	3,490	1,400	4,890	5,980	1,090
Fluorescence[b]	3,400	1,400	4,800	6,000	1,200

[a]Determined in the presence of 1 M KCl.
[b]Excitation and emission wavelengths were 280 and 340 nm, respectively.

ΔG_D, respectively, as a function of urea concentration as described in equations (7) and (9). Similarly, for the transition curve (Fig. 2B, curve 2) in the presence of 1.0 M KCl, values of ΔG_D were calculated using equation (3). A least squares analysis was used to fit the data to eq. (10) to determine $\Delta G_I^{H_2O}$, ΔG_D^*, and $\Delta G_D^{H_2O}$ (in the presence of KCl), the free energy of unfolding in the absence of urea. Value of ΔG_D^* represents the value obtained from extrapolation of ΔG_D values up to the starting of the process, I ⇌ D. Figure 2C shows the variation of ΔG as a function of urea concentration and Table II 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 3,490 cal/mol and 900 cal · mol^{-1} · M^{-1} of urea concentration, respectively, whereas ΔG_D^*, the free energy change associated with I ⇌ D transition was found to be 1,400 cal/mol and m value 840 cal.mol^{-1}.M^{-1}. Free energy change associated with N ⇌ D transition in the absence of salt can be obtained by summing the free energy change of the individual steps, i.e., $\Delta G_I^{H_2O}$ and ΔG_D^*[46] since ΔG^{H_2O} being a thermodynamic property does not depend on the path. The $\Delta G_{total}^{H_2O}$ i.e. the free energy change associated with the transformation of N state to I state and finally to D state, was calculated to be 4,890 cal/mol. The $\Delta G_D^{H_2O}$ and m values for N ⇌ D transition in the presence of 1.0 M KCl were calculated to be 5,980 cal/mol and 1,020 cal · mol^{-1} · M^{-1}, respectively. The free energy of stabilization $\Delta\ \Delta G_D^{H_2O}$, i.e., the free energy change of unfolding of HSA in the presence of KCl than in its absence was found to be 1,090 cal/mol. These results suggested that KCl markedly stabilized HSA conformation which seems to result mainly from the relative weak binding of anions and/or cations by the native folded conformation of the protein especially in the III domain. Halle and Lindman[47] studying the chloride ion binding to HSA suggested that the high-affinity chloride binding sites in HSA are doubly cationic amino acid pairs. Of the nine such pairs in the HSA sequence, six are found in domain III (at residues 336, 413, 444, 484, 524 and 573). Hence these six sites might be the loci at which binding of chloride, or other anions, stabilizes domain III against denaturation.

Similar effect of salt (KCl) was also noticed when the urea-induced transition of HSA was monitored by fluorescence measurements. Data were treated in the same way as described above. $\Delta G_I^{H_2O}$ and ΔG_D^* values for the first and second transitions were determined to be 3,400 cal/mol and 1,400 cal/mol, respectively, whereas the m values for the two transitions were 920 and 900 cal · mol^{-1} · M^{-1},

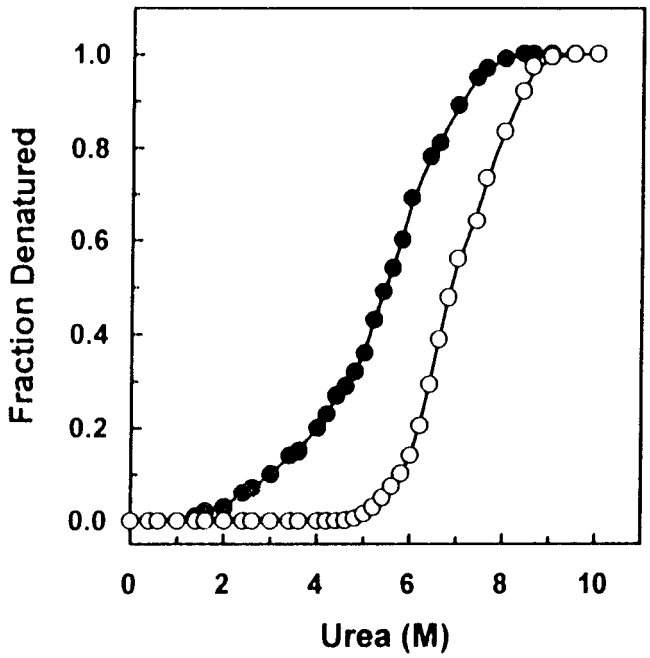

Fig. 3. Normalized transition curves for urea-induced unfolding of HSA as followed by ANS fluorescence measurements at pH 7.0, 25°C in the absence (solid circle) as well as presence of 1 M KCl (open circle). Concentrations of protein and ANS were 1.5 μM and 75 μM, respectively.

respectively. $\Delta G_{total}^{H_2O}$ was calculated to be 4,800 cal/mol. The free energy change, $\Delta G_D^{H_2O}$ for the transition (N ⇌ D) in the presence of 1 M KCl was calculated to be 6,000 cal/mol and m value, 970 cal · mol^{-1} · M^{-1}. Therefore, $\Delta\Delta G_D^{H_2O}$ by fluorescence measurements was calculated to be 1,200 cal/mol. These results also suggested that KCl increased the conformational stability of HSA to the same extent as determined by MRE measurements.

Figure 3 shows the normalized transition curves of urea denaturation of HSA both in the absence as well as presence of 1 M KCl, obtained by plotting F_D against urea concentration, when studied by ANS binding. Although both transition curves showed a single-step transition, treatment of data for the calculation of free energy change as employed above seems to be erroneous in view of the binding of all three ligands (urea, KCl, and ANS) to the protein and their competition with each other for binding.[48] But it is very much clear from the data that the presence of KCl stabilized the protein against urea denaturation.

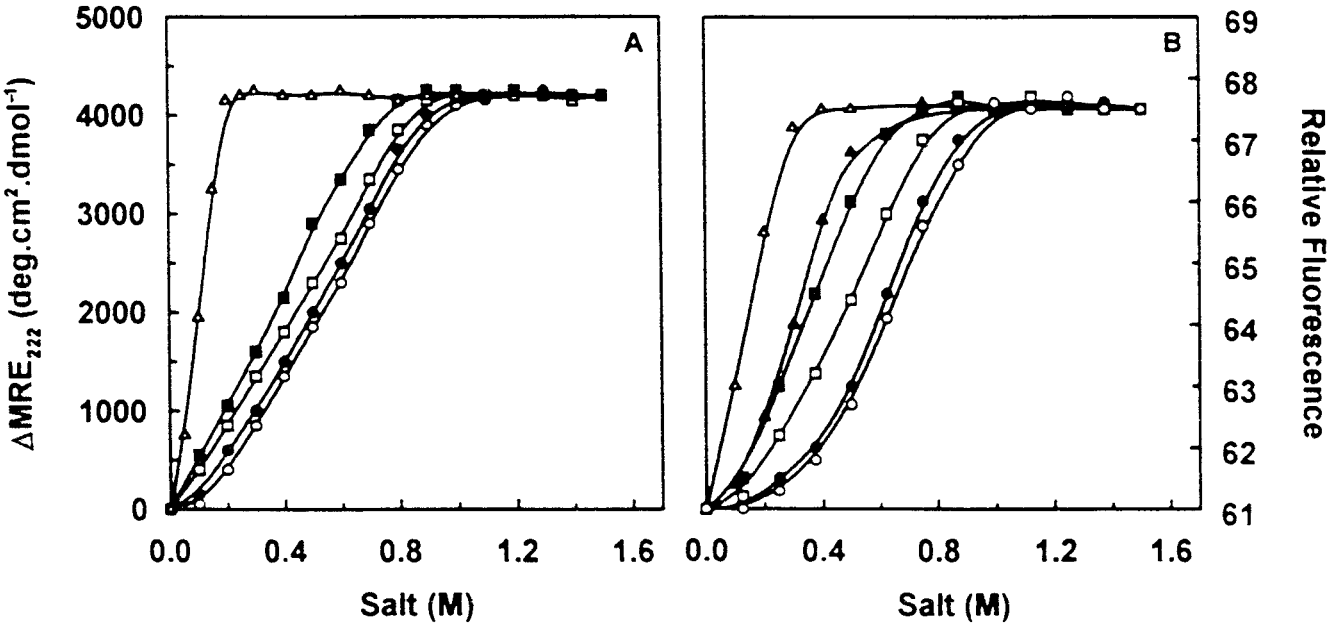

Fig. 4. Effect of various salts on the refolding of HSA at 4.8 M urea concentration at pH 7.0, 25°C as measured by MRE_{222} and intrinsic fluorescence. **A:** Dependence of MRE (the difference in the MRE_{222} value at 4.8 M urea concentration in the absence and presence of various salts) on molar salt concentration. **B:** Dependence of fluorescence intensity on molar salt concentration. Different salts used were: $NaClO_4$ (open triangle); NaSCN (closed triangle); Na_2SO_4 (solid square); KBr (open square); KCl (closed circle) and KF (open circle). The protein concentration used was 1.8 μM both for MRE and fluorescence measurements.

In order to compare the stabilizing effect of different neutral salts, we studied the refolding behavior of HSA at 4.8 M urea, the concentration at which the intermediate (I) state existed, both by MRE measurements at 222 nm and intrinsic fluorescence measurements at 340 nm. Figure 4A shows the dependence of ΔMRE_{222}, i.e., the difference in the MRE_{222} values at 4.8 M urea in the absence and presence of different salts (Na_2SO_4, $NaClO_4$, KF, KBr, and KCl) on molar salt concentration. As can be seen from Figure 4A, all the salts used induced refolding. The extent of refolding by all the salts studied was found to be similar at higher salt concentrations (>1.0 M). However, the value of MRE_{222} obtained at this salt concentration (17,900 deg · cm^2 · dmol^{-1}) was 10% less than the MRE_{222} value obtained with native HSA. On the other hand, at lower salt concentration (< 0.7 M), the extent of refolding was found to be different with different salts. C_m values as determined from Figure 4A for different salts are given in Table III.As can be seen from Figure 4A and Table III, the relative effectiveness of various salts in inducing refolding of HSA followed the series:

$$NaClO_4 > Na_2SO_4 > KBr > KCl > KF$$

Comparing the effect of various potassium salts (KBr, KCl, and KF) on the refolding process of HSA, it may be concluded that the extent of refolding of the protein was markedly influenced by the nature of anions present in the medium. At 0.2 M salt concentration, ΔMRE_{222} values obtained were 400, 600, and 800 deg · cm^2 · dmol^{-1} with KF, KCl, and KBr, respectively. On the other hand, both KCl and NaCl induced the refolding of HSA in a similar manner (data not shown). This suggested that anions play an important role in the stabilization of HSA. Figure 4B shows the effect of different salts, namely, Na_2SO_4, NaSCN, $NaClO_4$, KF, KBr, and KCl on the refolding behavior of HSA at 4.8 M urea as monitored by fluorescence at 340 nm. All the salts used were found to induce refolding as revealed from the increase in relative fluorescence intensity at 340 nm on increasing the salt concentration. At higher salt concentration (>1.0 M), the extent of refolding by all salts was similar. However, at lower salt concentration (< 0.5 M), the extent of refolding varied among different salts (see Fig. 4B). Comparing the C_m values (Table III), the relative effectiveness of various salts in inducing refolding followed the series:

$$NaClO_4 > NaSCN > Na_2SO_4 > KBr > KCl > KF$$

This series was similar to the one obtained with MRE measurements at 222 nm. NaSCN, which could not be studied by MRE measurements due to high noise in the far-UV region, occupied the position in between $NaClO_4$ and Na_2SO_4.

TABLE III. Anion-Induced Refolding of HSA

Salt	C_m value (mM)[a]	
	MRE_{222}	Fluorescence
$NaClO_4$	100	62.5
NaSCN	N.D.	155
Na_2SO_4	390	360
KBr	460	490
KCl	520	610
KF	560	640

[a]Calculated from the data of Figure 4A and B.

The order of effectiveness by which different salts stabilize the protein molecule generally follows the Hofmeister series[10], which is believed to occur through the modification of water structure.[49] The Hofmeister series is as follows:

sulfate > phosphate > fluoride > chloride > bromide > iodide > perchlorate > thiocyanate

Kosmotropic anions, i.e., anions to the left of chloride in the above series, are believed to stabilize the native structure of the protein molecule by causing preferential hydration of proteins, i.e., preferential exclusion of kosmotropes from the protein surface[50] whereas the chaotropic or lyotropic anions, i.e., anions to the right of chloride destabilize the protein structure by preferentially interacting with the protein.[51] The above series for HSA as determined in this study did not obey this general rule and followed the opposite order of the Hofmeister series. Thus, it may be inferred that the effect of salts on water structure is not responsible for the stabilization of HSA molecule. The above series for HSA was found similar to the electroselectivity series of anions towards anion exchange resins which is as follows:[52]

perchlorate > iodide > trichloroacetate > thiocyanate > nitrate > bromide > trifluoroacetate > chloride > acetate > fluoride

The relative effectiveness of various sodium salts in increasing the thermostability of BSA was also found to follow the same order as observed in this study.[53] Since chaotropic anions interact more preferentially with protein molecules and less favourably with the surrounding water in comparison to kosmotropic anions, which interact less favourably with the protein molecules and more favourably with the surrounding water molecules, it seems likely that former stabilizes unfolded conformation whereas later favours folding of protein. If anion-induced refolding observed in this study resulted from the changes in water structure, it should follow the Hofmeister series. However, the electroselectivity series was found to be followed by different anions used in this study, which is based on valency, charge, and effective size of anions rather than on hydration. It is, therefore, concluded that the electrostatic binding of anions to the positively charged sites of HSA is the major factor responsible for the anion-induced conformational stabilization.

The unfolding of HSA was also studied using GdnHCl as denaturant. The transition was found to follow a single-step two-state transition as monitored by MRE measurements at 222 nm. Figure 5 shows the normalized transition curve of HSA as a function of GdnHCl concentration. The free energy of unfolding, $\Delta G_D^{H_2O}$ and m value were calculated to be 5,870 cal/mol and 1,995 cal.mol^{-1}.M^{-1}, respectively. This value of free energy of unfolding (5,870 cal/mol) was found be similar to the value obtained with urea denaturation curve in the presence of 1.0 M KCl (5,980 cal/mol) and higher than the value (4,890 cal/mol) obtained in its absence. GdnHCl is considered to be a much stronger denaturant than urea[38] although the relative

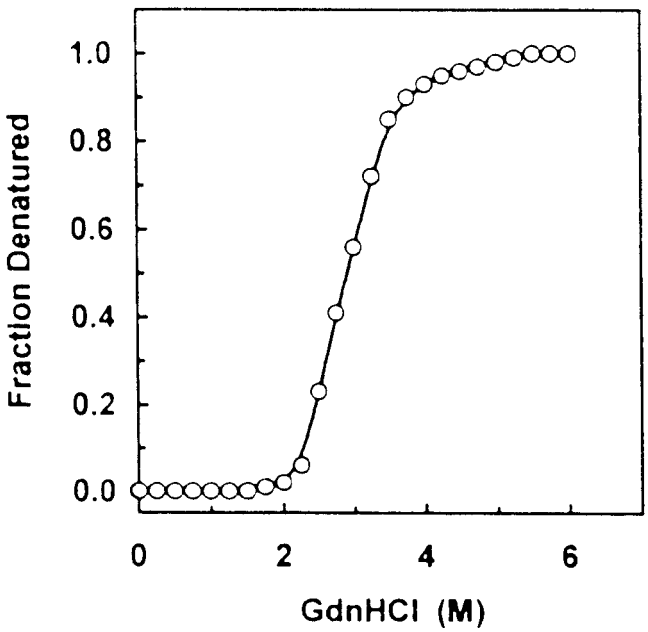

Fig. 5. Normalized transition curve for GdnHCl-induced unfolding of HSA as followed by MRE measurements at 222 nm at pH 7.0, 25°C.

effectiveness of the two denaturants depends on the nature of protein.[54] Both urea and GdnHCl are presumed to bind to peptide bonds.[55,56] Since there are a greater number of non-interacting binding sites on the unfolded conformation than folded conformation, the protein unfolds and more sites are exposed to the denaturant molecules.[57,58] In comparison to urea, GdnHCl gave a higher estimate of free energy change, $\Delta G_D^{H_2O}$ as found in this study. The difference in the estimate of free energy change may be attributed to the ionic property of GdnHCl. Earlier studies on coiled coils[59] suggested that GdnHCl cannot distinguish the contribution of electrostatic interactions to the proteins which were otherwise effectively monitored by urea. GdnHCl, being a salt, ionizes in aqueous solution to Gdn^+ and Cl^- ions, which then mask the positively and negatively charged amino acid side chains of the protein, hence reducing or completely eliminating any stabilizing or destabilizing electrostatic interactions. Therefore, it may be suggested that the difference in the estimates of free energy of unfolding by urea and GdnHCl depends on the nature of the electrostatic interactions stabilizing the protein. This also explains that urea-induced transition is a two-step process whereas GdnHCl-induced denaturation is single step as GdnHCl plays a dual role both as a stabilizer and a denaturant.[28] Similar types of denaturation curves in urea and GdnHCl have been observed earlier with other proteins.[60,61]

HSA, owing to its colloidal property, is widely used as a therapeutic agent. In 1992, its market value touched $1.1 billion.[62] The overall distribution, metabolism, and efficacy of many drugs can be altered based on their affinity to serum albumin.[20] Our results suggested that construction of anion binding sites on the surface of native HSA through recombinant DNA technology would provide the means for

a protein with a higher conformational stability and this, together with a complete understanding of albumin structure, would open avenues for drug design and therapy.

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