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Methyl Cyanide Induces α to β Transition and Aggregation at High Concentrations in E-State of Human Serum Albumin

P. Sen, M. A. Iqbal, S. Fatima, and R. H. Khan*

Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India; fax: +91-571-272-1776; E-mail: rizwanhkhan@hotmail.com; rizwanhkhan1@yahoo.com

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Abstract—We have studied the effect of 2,2,2-trifluoroethanol (TFE), an α-helix inducer, versus methyl cyanide (MeCN), a β-sheet inducer, on acid-denatured human serum albumin (HSA) using far-UV circular dichroism, intrinsic fluorescence, 1-anilino-8-naphthalene sulfonate binding, and acrylamide quenching studies. Interestingly, at pH 2.0, where the recovery and resolution of the protein in reverse phase chromatography is high, its secondary structure remains unchanged even in the presence of very high concentration (76% v/v) of MeCN. Gain of 23 and 34% α-helicity was observed in the presence of 20 and 50% TFE, respectively. At pH 7.3, HSA aggregates in the presence of 40% MeCN, but it remains soluble up to 75% MeCN at pH 2.0. The results seem to be important for HSA isolation and purification.

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Key words: acrylamide quenching, aggregation, circular dichroism, E-state, human serum albumin

After the ribonuclease refolding experiment, Anfinsen concluded that all the information necessary to achieve the native conformation of a protein in a given environment is contained in its amino acid sequence [1]. But different schools are still trying to understand how a protein achieves its native conformation in a short span of time. Helices and strands often take their conformational instructions from the solvent [2, 3]. The conformational studies of albumin are important as it is the most multifunctional transport protein known to date. Albumin microspheres are useful carriers of therapeutic agents. Human serum albumin (HSA) is also used in peritoneal dialysis, in combating the harmful effect of antibiotics, and as a scavenger of toxic substances and free radicals [4]. HSA undergoes isomerization forming an "E", or expanded form at around pH 2.0. Further increase in ionic strength leads to a decrease in acid expansion of the molecule, indicating the involvement of ionic forces. Since all carboxyl groups are protonated under these conditions, protonation of basic amino acid side chains leads to mutual repulsion between domains and subdomains of the molecule [5].

Possession of a high content of α-helical structure, high 1-anilino-8-naphthalene sulfonic acid (ANS) binding, loss of cooperativity in the thermal transition with significant loss of tertiary contacts, but retention of compactness suggest that E-state is a molten globule state. In view of previous results showing uncoiling of domain III on acidification, loss of secondary structure can be attributed to the unfolding of domain III, whereas the retention of other properties similar to those of the native state can be ascribed to domains I and II [6]. Binary mixtures of water with alcohols such as methanol, ethanol, or 2,2,2-trifluoroethanol (TFE) denature the tertiary and quaternary structures of proteins while enhancing their helicity [7]. TFE is a protein denaturant that has also been shown to induce the molten globule state in many proteins [8, 9]. Among various alcohols, TFE is often preferred in such studies because of its high potential in stabilizing α-helical structure. The secondary structures stabilized by TFE are assumed to reflect conformations that prevail during early stages of protein folding [9].

Absorption behavior of bovine serum albumin (BSA) and HSA is strongly dependent on their secondary and tertiary structures at different methyl cyanide (MeCN) concentrations, as demonstrated by CD and IR spectroscopy. MeCN is a widely used solvent that assists α-helix to β-sheet transition and β-sheet formation in many

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonic acid; GuHCl, guanidine hydrochloride; HSA, human serum albumin; MeCN, methyl cyanide (or acetonitrile); MRE, mean residue ellipticity; RFI, relative fluorescence intensity; RPC, reverse phase chromatography; TFE, 2,2,2-trifluoroethanol; UV-CD, ultraviolet circular dichroism.

* To whom correspondence should be addressed.

proteins [10]. It is more polar than alcohol, but less polar than water. Among several organic solvents used in reverse phase chromatography (RPC), MeCN is the most widely utilized because of the high resolution attainable, probably due to the low viscosity of water–MeCN mixtures [11]. In this study we have tried to understand whether a protein follows different folding pathways in the presence of different solvent systems or similar pathway irrespective of its environment. The knowledge of the effect of these cosolvents on HSA is important in RPC as well as normal phase liquid chromatography.

MATERIALS AND METHODS

Chemicals. HSA (essentially fatty acid free), TFE, and ANS were supplied by Sigma (USA) and MeCN by Qualigens Fine Chemicals (USA). All other reagents and buffer compounds used were of analytical grade.

A stock solution of 5 mg/ml HSA was prepared in 20 mM glycine-HCl buffer, pH 2.0. Protein concentrations were determined on a Hitachi U-1500 spectrophotometer (Japan) using an extinction coefficient $\varepsilon^{1\%}_{1cm} = 5.3$ at 280 nm [6]. The pH value was measured using an Elico digital pH meter (model LI610) (India).

MeCN and TFE were added to buffer solution to get the desired concentration of the cosolvents. The final volume in each tube was 2 ml. The degree of aggregation induced in the protein was estimated by measuring the absorbance at 350 nm.

Circular dichroism measurements. Circular dichroism (CD) was measured with a JASCO J-815 spectropolarimeter (USA) calibrated with ammonium d-10-camphorsulfonate. A cell of 0.1 cm path length was used for scanning between 250-192 nm in the presence of TFE and 250-200 nm in the presence of MeCN, respectively, to avoid low signal to noise ratio. The results were expressed as the mean residue ellipticity (MRE) in deg·cm^2·dmol^{-1}, which is defined as $\theta_{obs}/(10nCl)$, where θ_{obs} is the observed ellipticity in millidegrees, n is the number of peptide bonds per molecule, C is the molar concentration, and l is the length of light path in cm [12]. The CD data were also analyzed using K2d software, an algorithm for the estimation of the percentages of protein secondary structure from UV-CD spectra using a Kohonen neural network with a 2-dimensional output layer. It uses a self-organizing neural network to extract the secondary structure features from a set of circular dichroism spectra ranging from 200 to 240 nm [13].

Fluorescence measurements. Fluorescence measurements were performed on a Shimadzu RFPC 5301spectrofluorometer (Japan) equipped with a Peltier element. The fluorescence spectra were collected at 25°C in a 1 cm pathlength cell. The excitation and emission slits were set at 5 nm. Solutions for the fluorescence experiments were prepared in 20 mM glycine-HCl buffer, pH 2.0. Different volumes of the desired buffer were added to a 0.5 mg/ml HSA stock solution. Intrinsic fluorescence was measured by exciting the protein solution at 280 and 295 nm. For the ANS binding experiments, the excitation was set at 380 nm and the emission spectra were recorded in the range of 400-600 nm. For ANS binding studies, ANS was added to the protein incubated with varying concentration of cosolvents, so that the ANS concentration will be 50 times higher than that of the protein.

Acrylamide quenching. Aliquots of 5 M acrylamide stock solution were added to the protein solution (9.6 μM) to achieve the desired range of quencher concentration (0.1-1.0 M). To excite tryptophan residues only, the excitation wavelength was set at 295 nm and the emission spectra were recorded in the range of 300-400 nm. The data were analyzed according to the Stern–Volmer equation [14]: $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively; Q is the quencher concentration, and K_{SV} is Stern–Volmer quenching constant.

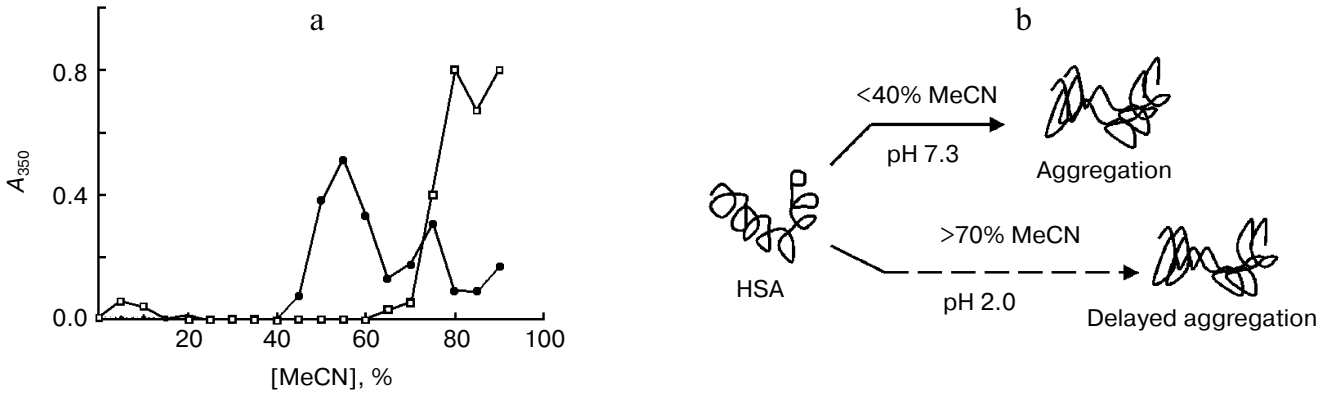

Fig. 1. a) Turbidity measurement of HSA (0.5 mg/ml) at 350 nm in the presence of increasing percentage of MeCN (v/v) at pH 2.0 (open symbols) and pH 7.3 (closed symbols) at 27°C. b) Schematic presentation of aggregation in HSA above 70 and 40% MeCN at pH 2.0 and 7.3, respectively.

RESULTS

Aggregation studies. Organic cosolvents have been shown to promote aggregation of many proteins [15]. Absorbance at 350 nm is used as a probe for aggregation. The degree of aggregation induced in HSA (0.5 mg/ml) by MeCN was observed at 27°C at pH 2.0 (glycine-HCl buffer) and 7.3 (phosphate buffer) (Fig. 1a). At 0-40% MeCN (v/v) at pH 7.3 and 0-70% MeCN (v/v) at pH 2.0, no aggregation was observed. Similar results have been also reported by other workers [16, 17]. Above these concentrations absorbance was increased markedly, showing a zone of aggregation between 40 to 90% and 70 to 90% MeCN at pH 7.3 and 2.0, respectively. In short, at pH 2.0, HSA aggregates in the presence of more than 70% MeCN (v/v), at much higher concentration in comparison to neutral pH (Fig. 1b).

Effect of MeCN and TFE on far-UV-CD of E-state. Far-UV-CD spectra were studied to observe changes in the secondary structure of the protein [13, 16]. Figures 2a and 2b show the far-UV-CD spectra of HSA at pH 2.0 in the presence of different concentrations of MeCN and TFE, respectively. In Fig. 2a, wavelengths below 200 nm were not included in the result to avoid lower signal to noise ratio. At pH 2.0, the CD spectrum of HSA showed minima near 208 and 222 nm, the characteristics of α-helical proteins [6]. With the increase in MeCN concentration from 0 to 70% (v/v), MRE decreased, showing increase in α-helical structure. But further increase in MeCN concentration (78 and 80% v/v) led to the formation of a typical β-sheet structure with a characteristic minimum near 213 nm [18].

The MRE of the protein decreased with increase in the concentration of TFE up to 90% (v/v), without any change in its characteristic minima at 208 and 222 nm (Fig. 2b). Figure 2c and Table 1 summarize the changes in the percentage of secondary structures (α-helix and β-sheet) in the protein against increasing concentration of cosolvents as calculated by K2d (see "Materials and Methods"). The secondary structure did not show much change until 76% MeCN (v/v), but then an abrupt loss of apparent α-helical structure was observed with a concomitant instant increase in apparent β-sheet structure. The curve of percent helicity of the protein against TFE concentrations can be divided into three parts. In the first

Table 1. Calculated percentage of α- and β-structures of HSA (pH 2.0) in the presence of different concentrations of MeCN and TFE using K2d software [13]

Cosolvent	Concentration, % v/v	α-Helix, %	β-Structure, %
–	–	45	23
MeCN	76	44	21
TFE	20	68	4
TFE	50	79	0

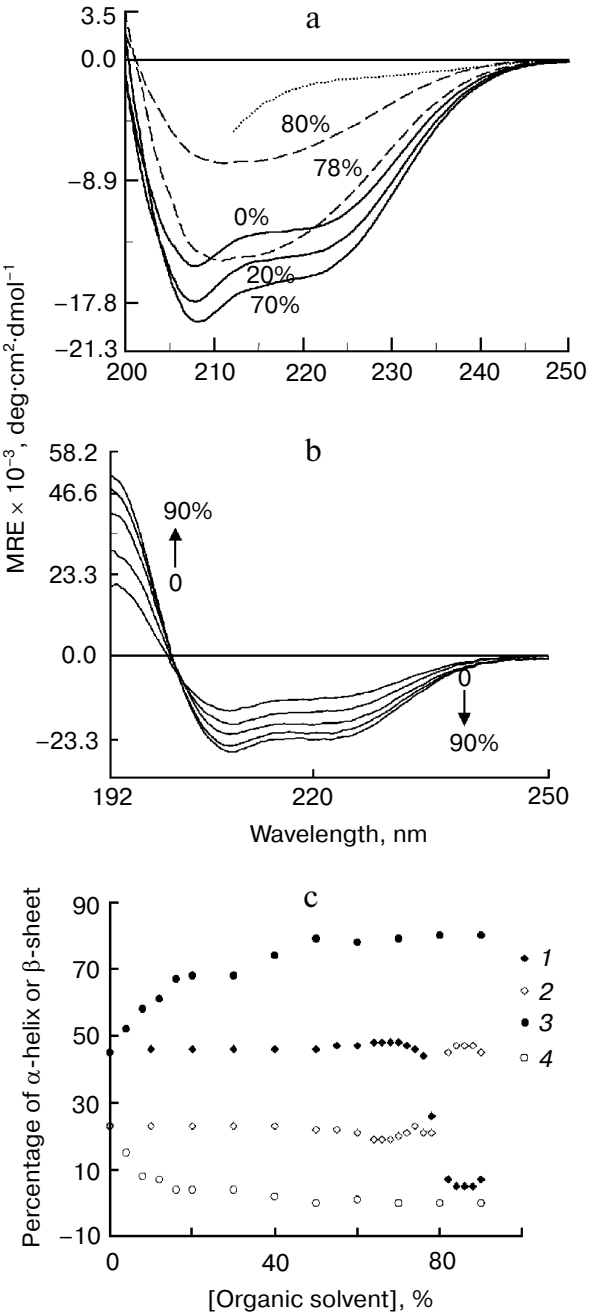

Fig. 2. Far-UV-CD spectra of HSA (0.5 mg/ml) at pH 2.0 in the presence of 0, 20, 70, 78, and 80% MeCN and 6 M GuHCl-denatured HSA (dotted line) (a) or in the presence of 0, 4, 30, 60, and 90% TFE (b). c) Change in percentage of α-helix (*1*) and β-sheet (*2*) in the presence of MeCN and the same of α-helix (*3*) and β-sheet (*4*) in the presence of TFE, as calculated by the K2d software.

part, i.e. from 0 to 20% (v/v), the increase in helicity is 23%, while in the second part, i.e. from 20 to 50% (v/v), the increase in helicity is only 11%. In the third part (50 to 90% v/v) the change is negligible (~1%). Low concentration of TFE (20%) seems to stabilize the secondary structure of the acid denatured state, but much higher (>76%) concentration of MeCN is needed to induce any change in it.

Effect of MeCN and TFE on tertiary structure of E-state. At 280 nm, tryptophan, tyrosine, and phenylalanine fluorophores are excited, but tryptophan and tyrosine fluorescence dominates [16, 18]. Figure 3a shows the emission spectra of HSA at pH 2.0 in the absence and presence of MeCN and TFE. The peak of the spectrum of E-state was observed near 325 nm, which is similar to an earlier report [6]. In the presence of 10% cosolvents, the fluorescence intensity increased without any wavelength shift. In the presence of 80% cosolvents, the wavelength maximum shifted towards 306 nm, although in the presence of MeCN the spectrum appeared with distinguishably higher intensity. To analyze these effects, we plotted normalized relative fluorescence intensity (RFI) at 325 nm versus MeCN and TFE concentrations (Fig. 3b). The graph can be divided into two parts. First, there was a sharp but small increase in fluorescence intensity in the presence of 0-10% cosolvents, followed by a decrease in fluorescence in the presence of 10-20% cosolvents. The sharp rise in the fluorescence intensities might be attributed to the apolar nature of the cosolvents. In the second part, further increase in cosolvent concentration showed very different spectral changes in the presence of MeCN and TFE. In the presence of TFE, only baseline changes were observed, but almost 60% increase in fluorescence intensity was observed in the range of 20-65% MeCN, followed by a plateau until 80% MeCN.

At pH 2.0 HSA acquires a "molten globule state" with a secondary structure similar to native state with the least amount of tertiary contact [6, 19]. From these results, it seems that the unfolded protein perhaps is stabilized in the presence of TFE. In contrast, the increased fluorescence intensity is an indicator of further exposure of the fluorophores in the presence of MeCN [11].

Effect of TFE and MeCN on tryptophan fluorescence of E-state. Tryptophan residues of a protein are exclusively excited nearly 295 nm, and they emit in the range of 300 to 400 nm [20, 21]. HSA possesses only one tryptophan residue in the domain IIA, at position 214. It is located almost centrally of the domain II of the protein and is further buried in the protein core at pH 2.0. Therefore, the changes in its fluorescence report on a local conformational change at the core of the protein [6]. Figure 4a shows the emission spectra of HSA at pH 2.0 in the absence and presence of MeCN and TFE. At pH 2.0, the peak of HSA fluorescence was observed at 330 nm, which later on shifted towards 340 nm with increasing concentration of cosolvents. To analyze the structural changes of HSA in the presence of TFE and MeCN, the normalized RFI at 340 nm was plotted versus concentration of cosolvents (Fig. 4b). Little change was observed in the range of 0-10% TFE and 0-20% MeCN. In the range of 10 to 20% TFE, a 40% decrease in fluorescence intensity was observed. On the other hand, 90% increase in fluorescence intensity was observed in the range of 20-60% MeCN. To show the changes in wavelength maxima of HSA, the ratio of RFI at 330 to 340 nm (with excitation at 295 nm) versus concentration of cosolvents was plotted in Fig. 4c. In the presence of 0 to 20% TFE, the fluorescence intensity decreased with a red shift. With further increase in TFE concentration (20-80%), a marginal blue shift was observed. But in the case of MeCN there was a continuous red shift starting from 0 to 80%.

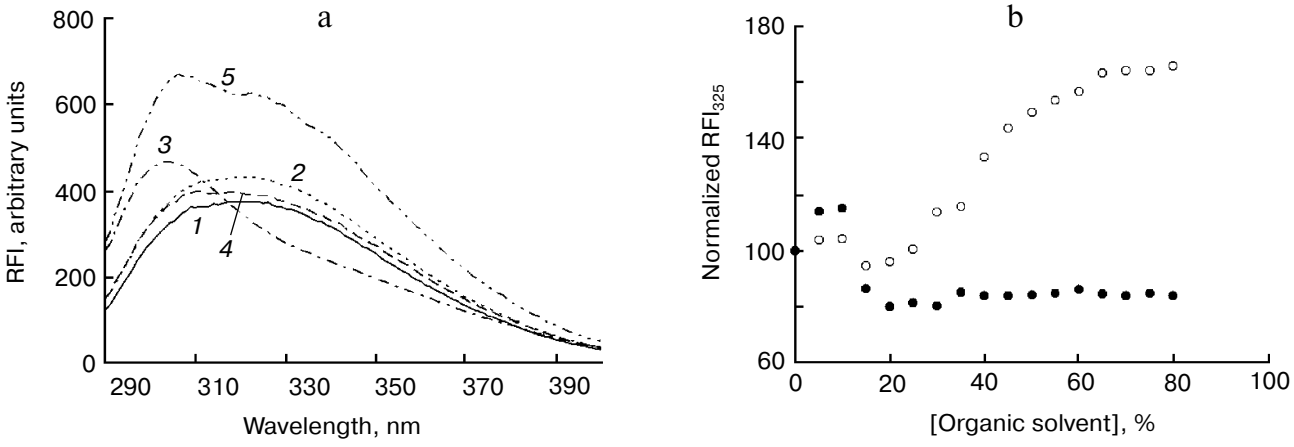

Fig. 3. a) Emission spectra of HSA (0.5 mg/ml) with excitation at 280 nm at pH 2.0 in the absence (*1*, bold solid line) and presence of 10% (*2*, dotted line) and 80% TFE (*3*, dash-dot line) and 10% (*4*, dashed line) and 80% MeCN (*5*, dash-dot-dot line). b) Normalized RFI of HSA at pH 2.0 at 325 nm with excitation at 280 nm against increasing concentration of TFE (closed symbols) and MeCN (open symbols).

Effect of cosolvents on protein–ANS binding. Protein–ANS complex formation is an excellent probe for "molten globule" state. ANS is a hydrophobic dye, which binds at the hydrophobic sites of a protein. Only a few ANS molecules bind to a native protein, due to scarcity of solvent accessible hydrophobic patches. In a molten globule state, where the protein core becomes more accessible to solvent, more dye molecules bind to protein. In contrast, an unfolded state of a protein generally loses most of the ANS binding sites [19]. Figure 5a shows changes in emission spectra of ANS–HSA complex with increasing cosolvent concentrations when excited at 380 nm. As organic solvents are hydrophobic in nature, baseline corrections were done with appropriate controls. At pH 2.0, a fine spectrum of HSA–ANS complex was observed with a wavelength maximum at 477 nm (Fig. 5a). It increased further in the presence of 0-5% cosolvents. With the increasing concentrations of MeCN and TFE, the fluorescence intensity decreased in the range of 5-30% cosolvents. With further increase in MeCN, the ANS binding increased, but in the presence of TFE no further change was noted (Fig. 5b).

The emission maxima of the protein shifted from 477 to 490 nm as the concentrations of the cosolvents was increased (Fig. 5a). To give a clear picture of the wavelength shift, we have taken the ratio of the fluorescence intensities at 477 and 490 nm (Fig. 5c). In the presence of MeCN and TFE, a decrease in the ratio was observed as the cosolvent concentration was increased from 0 to 40%. Above this concentration, a continuous decrease (red shift) in the ratio was observed in the presence of TFE, whereas an increase in the ratio (blue shift) was observed in the range of 40 to 70% MeCN. A red shift with decrease in fluorescence in the range of 0 to 40% MeCN was indicative of a decrease in protein–ANS binding, but on further increase in MeCN concentration, increase in fluorescence with blue shift was observed. Aggregates are prone to bind ANS. Although visible aggregates seems to be formed above 70% MeCN as shown by Rayleigh scattering (Fig. 1a), invisible aggregates seems to arise above 40% MeCN, resulting in blue shift and increase in protein–ANS complex fluorescence. In the presence of TFE, protein–ANS binding decreased, as shown by decreasing fluorescence with a red shift.

Effect of cosolvents on HSA detected by quenching of tryptophan fluorescence. Tryptophan residues of a protein are exclusively excited near 295 nm, and they emit in the range of 300 to 400 nm [20, 21]. HSA contains only one tryptophan residue and at pH 2.0 shows fluorescence emission at 330 nm when excited at 295 nm (Fig. 4a). The fluorescence intensity of HSA gradually decreased on addition of acrylamide, showing dynamic quenching [20, 22]. Figure 6 shows the Stern–Volmer plot of quenching of fluorescence by acrylamide in HSA at pH 2.0 in the absence and presence of MeCN and TFE. The observed Stern–Volmer constant is given in Table 2. The K_{sv} value

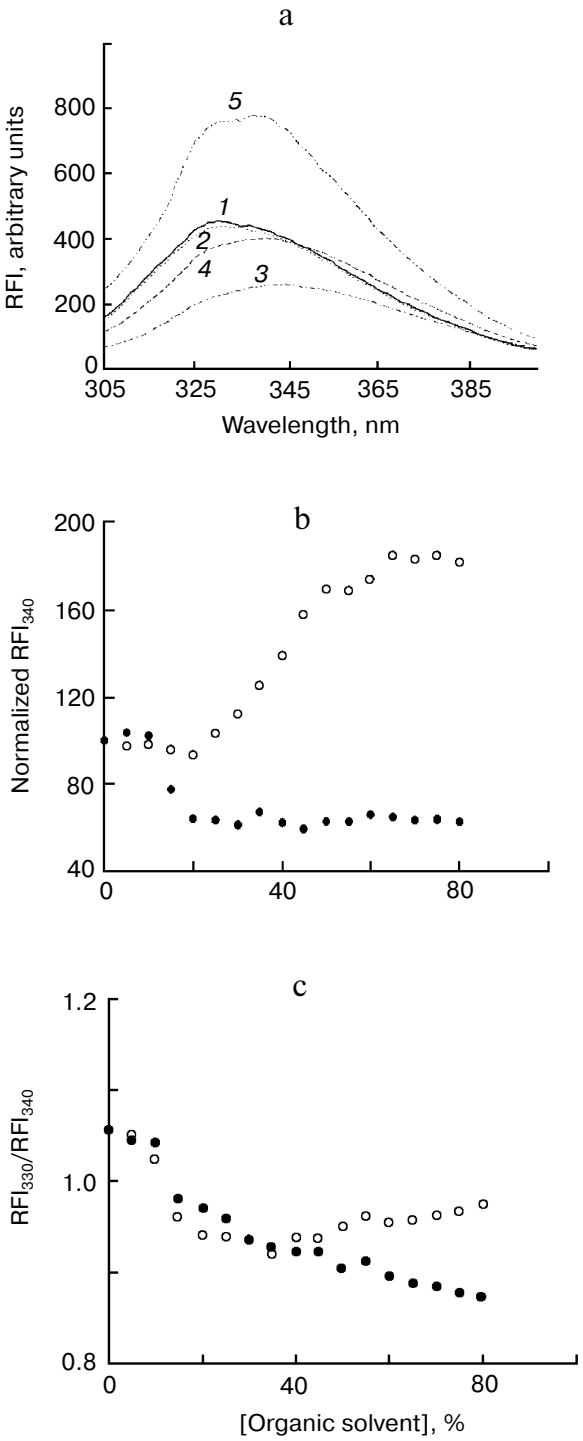

Fig. 4. a) Emission spectra of HSA (0.5 mg/ml) with excitation at 295 nm at pH 2.0 in the absence (*1*, bold solid line) and presence of 10% (*2*, dotted line) and 80% TFE (*3*, dash-dot line) and 20% (*4*, dashed line) and 80% MeCN (*5*, dash-dot-dot line). b) Normalized RFI of HSA at pH 2.0 at 340 nm with excitation at 295 nm against increasing concentration of TFE (closed symbols) and MeCN (open symbols). c) Ratio of RFI of HSA at pH 2.0 at 330 and 340 nm with excitation at 295 nm against the concentration of TFE (closed symbols) and MeCN (open symbols).

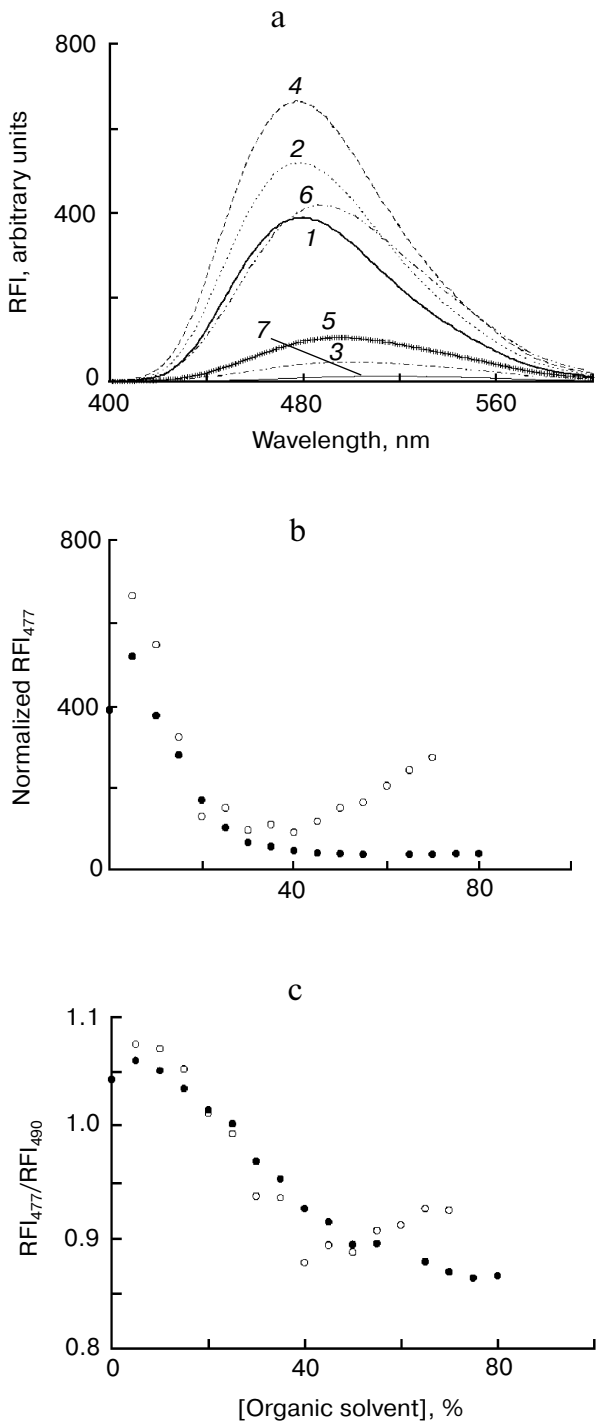

Fig. 5. a) Emission spectra of HSA–ANS complex with excitation at 380 nm at pH 2.0 in the absence (*1*, bold solid line) and presence of 5% (*2*, dotted line) and 80% TFE (*3*, dash-dot line), 5% (*4*, dashed line), 40% (*5*, plus line), and 80% MeCN (*6*, dash-dot-dot line) along with 6 M GuHCl-denatured protein (*7*, solid line). b) Normalized RFI of HSA–ANS complex at pH 2.0 at 477 nm with excitation at 380 nm against concentration of TFE (closed symbols) and MeCN (open symbols). c) Ratio of RFI of HSA–ANS complex at pH 2.0 at 477 and 490 nm with excitation at 380 nm against the concentration of TFE (closed symbols) and MeCN (open symbols).

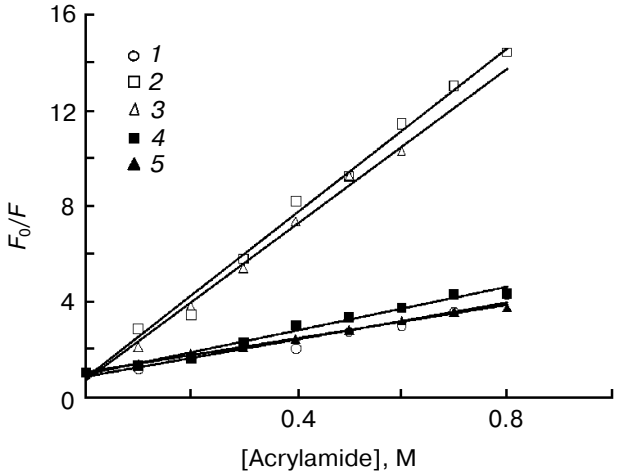

Fig. 6. Stern–Volmer plot of quenching induced by acrylamide in HSA (0.5 mg/ml) at pH 2.0 in the absence (*1*) and presence of 40 (*2*) and 70% MeCN (*3*) and 10 (*4*) and 30% TFE (*5*) with excitation at 295 nm (27°C).

for the E-state was found to be lower (3.6 M^{-1}) than that in the presence of 40 and 70% MeCN (16.9 and 15.6 M^{-1}, respectively). In the presence of 10 and 30% TFE, K_{sv} was 5.0 and 3.3 M^{-1}, respectively. These results indicate that the tryptophan residue in the presence of MeCN was more accessible for quenching by acrylamide than in the E-state, whereas in the presence of TFE the accessibility of the quencher was more or less equal to that of the E-state. This further supports exposure of the tryptophan residue in the presence of MeCN.

DISCUSSION

Alcohols are commonly used as a structure inducing cosolvents. Addition of alcohols enhances the helicity of peptides or induces helicity in peptides that are essentially disordered [20]. Alcohols act on proteins in two ways. First, they modify water structure in such a way that hydrogen bonding between water and protein molecules decreases, hence encouraging formation of intramolecular hydrogen bonding and increase in secondary structure. Second, alcohols are nonpolar solvents, so they bind with hydrophobic core of proteins [23].

MeCN is less polar than water but more polar than ethanol [24]. Nonpolar side chains of proteins show preference towards hydrophobic interactions with the nonpolar moiety (CH_3 group) of the MeCN molecule from water. The hydrophobic bonding capacity of these nonpolar side chains is weakened by addition of MeCN leading to destabilization of the tertiary structure of the proteins. Free tryptophan also shows increase in solubility with increase in MeCN concentration in water–MeCN mixtures. On the other hand, the backbone peptide groups

Table 2. Stern–Volmer constant of HSA in the absence and presence of different concentration of cosolvents at pH 2.0

Cosolvent	Concentration, % v/v	K_{sv}, M^{-1}
–	–	3.6
MeCN	40	16.9
MeCN	70	15.6
TFE	10	5.0
TFE	30	3.3

show decrease in solubility of the peptide group upon addition of MeCN, leading to the enhancement of the peptide–peptide hydrogen bonding [11]. The stability and solubility of protein are directly related to the separation of proteins and peptides in RPC. The knowledge gained from our observations is vital because MeCN as well as other solvents are used in acidic condition to increase both recovery and resolution of many proteins in RPC. The results clearly show that even high concentration of MeCN does not perturb the secondary structure of HSA and the alterations in the tertiary structure are probably recoverable [10, 11]. The acidity of the mobile phase contributes to retention by altering (i) charge distribution on proteins, (ii) ionization of surface silanols of the support, and (iii) the denaturation of the proteins. Nonpolar MeCN molecules are excluded from the charged surfaces of both protein and support through "local salting out" mechanism that it diminished at acidic conditions due to relative increase in the solubilizing effect of MeCN for nonpolar groups, leading to good recovery and resolution of proteins [11].

The α-helix to β-sheet transition of proteins is a key issue for understanding the folding and biological function of a number of proteins. For example, α to β transitions have been suggested in various conformational diseases, such as prion or Alzheimer's diseases, where normal protein turns from α-helix to β-sheet to be amyloidogenic. In several pathological disorders and in various *in vitro* experiments, proteins turn into "amorphous aggregate", without local order. Aggregation is thought to be an irreversible process of self-association of several identical protein molecules driven by stereospecific intermolecular contacts. A substantial body of information supports the idea that protein aggregation arises from partially folded intermediates through hydrophobic interaction [25-27]. Although at pH 7.0 albumins show α-helix to β-sheet transition at very low MeCN concentration (20%) [10], apparent α-helix to β-sheet transition takes place only at very high concentration of MeCN (80%) at pH 2.0, perhaps due to high ionic strength of solvent. This phenomenon might have an important role in delay of aggregation. But the formation of β structures might be a consequence rather than a cause of aggregation, as β structure is considered as a sign of already aggregated proteins in misfolding diseases.

In the presence of TFE (a well known α-helix inducer) and MeCN (a β-sheet inducer of some proteins) HSA showed very different trends of conformational transitions [27]. HSA loses 9% helical structure as treated with buffer of pH 2.0, with a blue shift (from 340 to 328 nm) in tryptophan fluorescence and a six-fold increase in ANS binding with a red shift of 10 nm (from 470 to 480 nm) [6]. This means that HSA loses a small amount of secondary structure, tryptophan residue shifts to apolar inner core of the protein from the place in between the IA and IIA subdomains and hydrophobic patches are more exposed in the E-state [22].

In the current study, the results of far-UV-CD studies showed that although low concentration of TFE induced α-helicity in the E-state of the protein, no change in secondary structure was observed in the presence of up to 70% MeCN. In the presence of 20% TFE, the protein acquired 68% α-helicity, which is similar to that of the native state of the protein [6], with red shift and decrease in fluorescence intensities and decrease in ANS binding. All these observations indicate that the E-state can have native-like spectral features in the presence of 20% TFE. Further increase in TFE (50%) induced higher α-helical structure (~80%), with a further red shift in tryptophan fluorescence. In the presence of 0-70% MeCN, very little change was observed in the secondary structure with exposure of the lone tryptophan residue in the polar environment, shown by increase in fluorescence with a red shift and significant increase in Stern–Volmer constant (K_{SV}). This observation is very important in relation to chromatography (especially RPC), because at pH 7.0 albumins turn turbid in the presence of only 40% MeCN [10]. Further addition of MeCN resulted in an apparent loss of 40% α-helix against 25% gain of β-sheet (Table 1), without any further change in the probes of tertiary structure.

In the presence of MeCN, fluorescence intensities show similar changes as induced by TFE, but only up to 10-15%. Interestingly, this similarity can be seen up to 40% in ANS binding. The pH denaturation of HSA results in blue shift of the tryptophan fluorescence with increase in RFI. On the other hand, Fig. 4 shows that the RFI decreased with a red shift of ~10 nm as the TFE concentration was increased. This might result from reestablishment of the tryptophan residue in its native position from the inner apolar core of the protein [6]. ANS binding studies also indicate that the state formed might have native-like topology.

The addition of MeCN up to 10% shows a similar trend in tryptophan fluorescence and up to 40% in ANS

binding. As MeCN concentration increased above 10%, an exponential enhancement in RFI was observed with a small blue shift, indicating that the tryptophan residue shifted to a nonpolar microenvironment. Above 60% MeCN, no further increase in fluorescence was observed, indicating maximum solvent accessibility of the tryptophan residue.

In conclusion, when the E-state of HSA is treated with TFE, an α-helix inducer, and MeCN, a β-structure inducer, they first stabilize the protein and then turn it into quite different types of conformations. In the light of tertiary structure studies done here it seems that the E-state of HSA acquires a native-like state in the presence of ~20% TFE and a hyper-helical state in the presence of 50% TFE. In the presence of MeCN, at first, it follows the trends of TFE up to 10-20%, which might have happened due to stabilization of the structure. At higher TEF content, HSA shows a sign of denaturation of tertiary structure although secondary structure remains intact up to 70% MeCN. On a further increase in MeCN concentration, HSA shows apparent β-sheet formation along with aggregation. But more interesting is the fact that up to ~20% TFE and MeCN follow similar pathways. The effect of MeCN, a chief solvent for RPC of HSA (a clinically very important protein), is important for HSA recovery and resolution. At pH 2.0, where HSA recovery and resolution are higher, it retains secondary structure even in the presence of high concentration of MeCN.

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REFERENCES

1. Anfinsen, C. B. (1973) *Science*, **181**, 223-230.
2. Haq, S. K., Rasheedi, S., Sharma, P., Ahmad, B., and Khan, R. H. (2005) *Int. J. Biochem. Cell Biol.*, **37**, 361-374.
3. Bohidar, H. B., and Mohanty, B. (2004) *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.*, **69**, 021902.
4. Peters, T., Jr. (1996) in *All About Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, N. Y., pp. 251-284.
5. Peters, T., Jr. (1996) in *All About Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, N. Y., pp. 9-54.
6. Muzammil, S., Kumar, Y., and Tayyab, S. (1999) *Eur. J. Biochem.*, **266**, 26-32.
7. Khan, F., Khan, R. H., and Muzammil, S. (2000) *Biochim. Biophys. Acta*, **1481**, 229-236.
8. Buck, M., Radford, S. E., and Dobson, C. M. (1993) *Biochemistry*, **32**, 669-678.
9. Fan, P., Bracken, C., and Baum, J. (1993) *Biochemistry*, **32**, 1573-1582.
10. Kandori, K., Uoya, Y., and Ishikawa, T. (2002) *J. Colloid Interface Sci.*, **252**, 269-275.
11. Gekko, K., Ohmae, E., Kameyama, K., and Takagi, T. (1998) *Biochim. Biophys. Acta*, **1387**, 195-205.
12. Kumar, Y., Muzammil, S., and Tayyab, S. (2005) *J. Biochem.*, **138**, 335-341.
13. Andrade, M. A., Chacon, P., Merelo, J. J., and Moran, F. (1993) *Protein Eng.*, **6**, 383-390.
14. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry*, **15**, 672-680.
15. Naeem, A., Khan, K. A., and Khan, R. H. (2004) *Arch. Biochem. Biophys.*, **432**, 79-87.
16. Ahmad, B., Khan, M. K. A., Haq, S. K., and Khan, R. H. (2004) *Biochem. Biophys. Res. Commun.*, **314**, 166-173.
17. Rasheed, Z., Khan, M. W., and Ali, R. (2006) *Autoimmunity*, **39**, 479-488.
18. Kundu, S., Sundd, M., and Jagannadham, M. V. (2002) *J. Biochem. Mol. Biol.*, **35**, 155-164.
19. Matulis, D., Baumann, C. G., Bloomfield, V. A., and Lovrien, R. E. (1999) *Biopolymers*, **49**, 451-458.
20. Ahmad, B., Ankita, and Khan, R. H. (2005) *Arch. Biochem. Biophys.*, **437**, 159-167.
21. Bhakuni, V. (1998) *Arch. Biochem. Biophys.*, **357**, 274-284.
22. He, X. M., and Carter, D. C. (1992) *Nature*, **358**, 209-215.
23. Manavalan, P., and Johnson, W. C. (1983) *Nature*, **305**, 831-832.
24. Pittz, E. P., and Timasheff, S. N. (1978) *Biochemistry*, **17**, 615-623.
25. Dobson, C. M. (2003) *Nature*, **426**, 884-890.
26. Ohnishi, S., and Takano, K. (2004) *Cell Mol. Life Sci.*, **61**, 511-524.
27. Srisailam, S., Kumar, T. K., Srimathi, T., and Yu, C. (2002) *J. Am. Chem. Soc.*, **124**, 1884-1888.