Molten globule-like state of human serum albumin at low pH

Salman Muzammil, Yogesh Kumar and Saad Tayyab

Interdisciplinary Biotechnology Unit, Aligarh Muslim University, India

Human serum albumin (HSA), under conditions of low pH, is known to exist in two isomeric forms, the F form at around pH 4.0 and the E form below 3.0. We studied its conformation in the acid-denatured E form using far-UV and near-UV CD, binding of a hydrophobic probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), thermal transition by far-UV and near-UV CD, tryptophan fluorescence, quenching of tryptophan fluorescence using a neutral quencher, acrylamide and viscosity measurements. The results show that HSA at pH 2.0 is characterized by a significant amount of secondary structure, as evident from far-UV CD spectra. The near-UV CD spectra showed a profound loss of tertiary structure. A marked increase in ANS fluorescence signified extensive solvent exposure of non-polar clusters. The temperature-dependence of both near-UV and far-UV CD signals did not exhibit a co-operative thermal transition. The intrinsic fluorescence and acrylamide quenching of the lone tryptophan residue, Trp214, showed that, in the acid-denatured state, it is buried in the interior in a non-polar environment. Intrinsic viscosity measurements showed that the acid-denatured state is relatively compact compared with that of the denatured state in 7 M guanidine hydrochloride. These results suggest that HSA at pH 2.0 represents the molten globule state, which has been shown previously for a number of proteins under mild denaturing conditions.

Keywords: acid denaturation; human serum albumin (HSA); molten globule; pH.

Folding of a protein from a structureless denatured state to an ordered biologically active native state is considered to be a highly complex process because of the lack of information about the folding intermediates formed in the folding pathway. This process is even more complex for multidomain proteins, in which each domain may be capable of refolding independently [1]. Keeping in view information on the formation of the native biologically functional structure in the primary sequence [2-3], previous studies have aimed to increase our understanding of the denatured state of proteins [4] and the role of segment-segment interactions and the interactions between the amino acid side chains with the surrounding medium [5,6] and also to characterize the refolding intermediates [7-9]. The process of protein folding from a denatured state to its native state depends on the type of denatured state, as each method of denaturation is considered to be a distinct process yielding different products [4,10]. It has been shown in several cases that denatured proteins contain some residual structure and therefore are not completely unfolded [11,12]. A comparison of different methods of denaturation showed that the most completely unfolded form can be obtained in either 9 M urea or 6 M guanidine hydrochloride (GdnHCl) [10]. On the other hand, acid denaturation of some proteins results in a denatured state that is often less unfolded than the completely unfolded form obtained in high concentrations of urea and GdnHCl, far from a random coil [4,13,14]. In acid denaturation, the major driving force for unfolding is intramolecular charge repulsion which may fail to overcome interactions that favour folding, such as hydrophobic forces, disulfide bonds, salt bridges and metal ion protein interactions [15]. The exact folding mechanism of a given protein at low pH is a complex interplay between a variety of stabilizing and destabilizing forces. In many cases, the acid-denatured form has been found to represent the ‘molten globule’ state which is assumed to be a major intermediate in protein folding [6,15-18]. The traditional definition of a molten globule state is a compact denatured state with a significant amount of secondary structure but largely disordered tertiary structure [6,12,19]. Characterization of a molten globule state is helpful in identifying the other transition states believed to be located between the molten globule and the native state and the molten globule and the denatured state [20-22].

Serum albumin has been used as a model protein for protein folding and ligand-binding studies over many decades. It contains three structural domains in a single polypeptide chain of 585 amino acids. In acidic medium, it undergoes a conformational transition that is quite different from that of other well-known proteins [23], producing two isomeric forms, the F form at around pH 4.0 and the E form below pH 3.0 [24]. Many studies have been directed towards characterization of the F form [25,26], but limited attempts have been made to characterize the E form. We have here studied the properties of the acid-denatured E form of HSA by near-UV and far-UV CD, tryptophan fluorescence, 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence, viscosity measurements, temperature transition, and acrylamide quenching of the tryptophan fluorescence. We found that the E form of HSA has characteristic properties of a molten globule. In this paper, we present a detailed analysis of the E state of HSA in terms of these spectroscopic and hydrodynamic properties.

Correspondence to S. Tayyab, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202 002, India. Fax: + 91 571 401081, Tel.: + 91 571 401718.

Abbreviations: HSA, human serum albumin; ANS, 1-anilinonaphthalene-8-sulfonic acid; NATA, N-acetyl-l-tryptophanamide.

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MATERIALS AND METHODS

Materials

Essentially fatty acid-free HSA, type A-1887, ANS, type A-3125, N-acetyl-l-tryptophanamide (NATA), type A-6501, and GdnHCl, type G-7153, were obtained from Sigma Chemical Co. HSA was freed from dimers and oligomers by passage through a Sephadex G-100 gel-filtration column. All other chemicals used were of analytical grade.

Protein concentration was determined either spectrophotometrically using $E_{1\text{cm}}^{1%}$ of 5.30 at 280 nm [27] on a Cecil double-beam spectrophotometer, model CE 594, or by the method of Lowry et al. [28]. ANS concentration was also determined spectrophotometrically using a molar absorption coefficient of 5000 $\text{m}^{-1}\cdot\text{cm}^{-1}$ at 350 nm [29].

CD measurements

CD measurements were recorded with a Jasco spectropolarimeter, model J-720, equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All CD measurements were performed at 25 °C with a thermostatically controlled cell holder attached to a NESLAB RTE-110 waterbath (NESLAB Instruments, Inc., Newington, NH, USA) with an accuracy of ±0.1 °C. Spectra were collected with a scan speed of 20 nm min$^{-1}$ and with a response time of 1 s. Each spectrum was the average of four scans. Far-UV CD spectra were recorded at a protein concentration of 1.8 $\mu$M with a 1-mm pathlength cell and the near-UV CD spectra were measured at 18 $\mu$M concentration with a 10-mm pathlength cell. The results are expressed as mean residue ellipticity (MRE) in degrees cm$^2$ dmol$^{-1}$ which is defined as:

$$\text{MRE} = \frac{\theta_{\text{obs}} \cdot (\text{mdeg})}{10 \times n \times l \times C_p}$$

where $\theta_{\text{obs}}$ is the CD in millidegrees, $n$ is the number of amino acid residues (585), $l$ is the pathlength of the cell and $C_p$ is the mole fraction. The $\alpha$-helical content of HSA was calculated from the MRE value at 222 nm using the following equation as described by Chen et al. [30]:

$$\% \text{ helix} = \left( \frac{\text{MRE}_{222} - 2340/30\,300}{} \right) \times 100$$

For temperature-transition studies, a water-jacketed 0.1-mm pathlength cell was used for far-UV CD and a water-jacketed 10-mm pathlength cell was used for near-UV CD attached to the RTE-110 waterbath interfaced with the microcomputer. The same protein solution (18 $\mu$M) was used in both near-UV and far-UV CD measurements. Each reading at the desired temperature was recorded after an incubation time of 20 min at that temperature.

Fluorescence measurements

Fluorescence measurements were performed on a Shimadzu spectrofluorimeter, model RF-540, equipped with a data recorder DR-3, or on an Hitachi spectrofluorimeter, model F-2000. The fluorescence spectra were measured at a protein concentration of 1.8 $\mu$M with a 1-cm pathlength cell. The 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 spectra taken in the range 400–600 nm or at a fixed wavelength of 470 nm. Intrinsic fluorescence was measured by exciting the protein solution at 280 nm, and emission spectra were taken in the range 300–400 nm with a protein concentration of 1.8 $\mu$M.

Acid unfolding of HSA

HSA solutions, of varying pH, were prepared by adding different volumes of HCl/water mixtures of different concentrations (pH values) to a protein stock solution (9 $\mu$M) such that the final volume in each tube was 5.0 mL. These tubes were incubated for 30 min at 25 °C. pH measurements were made on an Elico pH-meter, model L6161, at 25 °C. The pH values of the samples were found to lie within 0.1 pH unit when taken before and after the CD and fluorescence measurements.

For ANS-binding studies, to a protein solution incubated with various concentrations of HCl, a concentrated stock solution of ANS was added to obtain a final concentration of 125 $\mu$M and 1.8 $\mu$M for ANS and protein, respectively.

Acrylamide quenching

In the acrylamide-quenching experiments, aliquots of 5 $\mu$M acrylamide stock solution were added to a protein stock solution (15 $\mu$M) to achieve the desired acrylamide concentration. Excitation was set at 295 nm in order to excite tryptophan fluorescence only, and the emission spectrum was recorded in the range 300–400 nm. The slit width was set at 10 nm for both excitation and emission. The decrease in fluorescence intensity at $\lambda_{\text{max}}$ was analysed according to the Stern–Volmer equation [31]:

$$F_0/F = 1 + K_{sv}[Q]$$

where $F_0$ and $F$ are the fluorescence intensities at an appropriate wavelength in the absence and presence of a quencher (acrylamide), respectively, $K_{sv}$ is the Stern–Volmer constant for the collisional quenching process, and [Q] is the concentration of the quencher.

Viscosity measurements

A calibrated Cannon–Fenske viscometer (Cannon Instrument Co., State College, PA, USA) with a flow time of about 400 s for distilled water at 25 °C was used. The time of fall was recorded with a stop watch reading to 0.1 s. The temperature of the viscometer was maintained by dipping it in a glass tank filled with distilled water and attached to a water bath which maintained the temperature within ±0.1 °C. The reduced viscosity of a protein is defined as [23]:

$$\eta_r = \frac{(t - t_0)}{t_0} + \frac{(1 - P \psi)P_0}{P_0}$$

where $t_0$ is the time of fall of the solvent, $t$ is the time of fall of the protein solution, $c$ is the concentration in g mL$^{-1}$, $\psi$ is partial specific volume of protein, and $P_0$ is the density of the solvent. $\psi$ was taken to be 0.734 [32]. Intrinsic viscosity was obtained by linear extrapolation, by the method of least squares, of reduced viscosity to zero protein concentration. The viscosity measurements were made at four different concentrations ranging from 2 to 8 mg mL$^{-1}$.

RESULTS AND DISCUSSION

Figure 1 shows the acid-induced denaturation of HSA as monitored by ellipticity measurements in the far-UV region at 222 nm and by ANS fluorescence. The CD spectrum of a protein in the far-UV region was used to assay the conformation of the polypeptide backbone, whereas ANS, a hydrophobic dye that binds to exposed hydrophobic regions of partially folded proteins, was used to monitor these hydrophobic regions as
described previously [33,34]. The ellipticity measurements at 222 nm showed no apparent change between pH 6.5 and 4.0, but when the pH was decreased below 4.0, the ellipticity decreased markedly to a minimum value at around pH 2.1 and remained unchanged down to pH 1.8. A further decrease in the pH below 1.8 resulted in a second transition, corresponding to the formation of secondary structure, which in turn tailed off at pH 1.3. In the first transition, only 30% loss of secondary structure took place, calculated on the basis of the difference in ellipticity at 222 nm between the native and the denatured state in 7 M GdnHCl taken to be 100%. However, in the second transition, 7% of the secondary structure re-formed. When the effect of pH was assessed by ANS fluorescence, a gradual increase in fluorescence intensity was observed on decreasing the pH below 5.0, reaching a maximum at around pH 2.5, whereas no change in ANS fluorescence was observed in the pH range 6.8–5.0. A further decrease to below 2.0 led to a gradual decrease in the ANS fluorescence which tailed off at pH 1.1. In addition to this, emission maxima showed a red shift from 470 nm to 480 nm on decreasing the pH from 4.5 to 2.5, which remained constant up to pH 2.0 and decreased to 474 nm on decreasing the pH below 2.0. The observed red shift in emission maxima and increase in ANS fluorescence within the pH ranges 4.5–2.5 and 5.0–2.5, respectively, were indicative of exposure of hydrophobic regions to the solvent. A decrease in ellipticity at 222 nm within the pH range 4.0–2.1 suggested the loss of some secondary structure, making some hydrophobic regions available for ANS binding. Interestingly, below pH 2.0, another transition, characterized by the formation of secondary structure indicated by the increase in ellipticity and decrease in ANS binding as well as the blue shift, was observed which was complete at pH 1.1. The transition curves produced both by ellipticity measurements at 222 nm and ANS fluorescence showed a co-operative transition with no apparent intermediate state. These results are similar to those reported previously [35], which also indicated no intermediate state. These results also suggest that the protein’s tertiary structure was more sensitive to pH change than the secondary structure, as the transition monitored by ANS fluorescence started around pH 5.0 whereas that assessed by ellipticity measurement at 222 nm started around pH 4.0. These data are in accordance with previous results on acid-induced unfolding of HSA [35] in which more unfolding was observed below pH 4.0 reaching a maximum at pH 2.1 and which was partially restored at pH values below 2.0. A shift in the point of transition observed in this study (∼4.0) from the native to the denatured state compared with the value (pH 5.0) observed previously [27] can be explained by salt effect [15], because, in the earlier study, transition was seen in the presence of 0.2 M KCl [27]. Partial restoration of secondary structure at pH 1.1, as observed by the increase in ellipticity, can be ascribed to the anion-induced folding [16], which decreased the number of hydrophobic patches resulting in less binding of ANS.

To ascertain whether the protein state observed at pH 2.0 represented the extended state (E state) as reported previously [24,25,36], we compared the near-UV and far-UV CD spectra of HSA at pH 2.0 with those obtained at pH 6.0 (native state) and in the GdnHCl-denatured state. Figure 2A shows the
far-UV CD spectra of HSA in the native state (pH 6.0), the acid-denatured state (pH 2.0) and the GdnHCl-denatured state (7 M) in which the protein is considered to exist in a random coil conformation [4]. The curve for the native state has two minima, one at 208 nm and the other at 222 nm, characteristic of α-helical structure. HSA in the native state contained around 57% α-helical structure (Table 1) as determined from the ellipticity value at 222 nm by the method of Chen et al. [30], which is in agreement with the values reported by other workers [37]. The curve for the acid-denatured state retained all the elements of secondary structure, although there was a decrease in the ellipticity value, indicating the loss of ≈15% α-helical structure from the native state. HSA in the presence of 7 M GdnHCl lost all the elements of secondary structure, as can be seen in Fig. 2A (see also Table 1). Figure 2B shows the near-UV CD spectra in the 250–300 nm range for the native state (pH 6.0), the acid-denatured state (pH 2.0) and in 7 M GdnHCl. These spectra were used to probe the asymmetry of the protein’s aromatic amino acid’s environment. The near-UV CD spectra for the native state showed two minima at 262 nm and 268 nm and shoulders at 275 nm and 292 nm, characteristic of disulfide and aromatic chromophores [35]. In the acid-denatured state, there was a loss of signal and fine structure at 275 nm and 292 nm; nonetheless the protein retained the two minima at 262 and 268 nm whereas in 7 M GdnHCl there was complete disappearance of all minima. A comparison of near-UV and far-UV CD spectra of acid-denatured HSA with that of native and GdnHCl-denatured HSA (Table 1) shows that the acid-denatured form retained quite a lot of secondary structure as well as a significant proportion of tertiary structure.

Exposure of the hydrophobic surface to solvent in the acid-denatured state was studied by ANS binding. Binding of ANS to the hydrophobic regions of proteins results in an increase in fluorescence intensity [34], which has been widely used to detect the molten globule states of different proteins [38]. Figure 3 shows the fluorescence spectra of ANS in the 400–600 nm wavelength range in the presence of native and acid-denatured protein. As can be seen, binding of ANS to the acid-denatured state at pH 2.0 produced a large increase (sixfold, compared to the native HSA) in fluorescence intensity (Table 1) accompanied by a shift in spectral maximum from 470 to 480 nm, indicating exposure of hydrophobic regions of the protein molecule on acidification. Thus it appears that the acid-denatured state, although it retained a significant amount of secondary and tertiary structure, had sizeable amounts of exposed hydrophobic region.

Figure 4A,B shows the temperature-induced unfolding of the native HSA at pH 6.0 as well as acid-denatured HSA at pH 2.0 by ellipticity measurements in the far-UV region at 222 nm and in the near-UV region at 262 nm, respectively. The thermal unfolding of the native protein as seen in the far-UV region at 222 nm and in the near-UV region at 262 nm is a co-operative process (Table 1). In the far-UV region, the transition started at 40 °C whereas in the near-UV region it began at 60 °C, suggesting that secondary-structural changes are more sensitive to temperature than tertiary structural changes. On the other hand, thermal transition of the

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* Taken from Tanford [10]. b Calculated by the method of Chen et al. [30].

**Table 1. Summary of some physical properties of HSA.**

**Fig. 3. Fluorescence emission spectra of ANS bound to native HSA at pH 6.0 (--) and acid-denatured HSA (--) at pH 2.0. The excitation wavelength was 380 nm.**
acid-denatured protein at pH 2.0, as seen in the near-UV and far-UV regions, is a continuous non-co-operative process (Table 1). Maximum ellipticity of acid-denatured HSA was found at 0 °C in both the near-UV and far-UV regions, which decreased continuously up to 90 °C and then tailed off. In view of the gradual transition associated with the conformation containing only loosely ordered secondary structure [18], and lack of a co-operative thermal unfolding transition associated with the molten globule state [39], it appears that acid-denatured HSA at pH 2.0 represents the molten globule state of the protein.

Figure 5 shows the effect of pH in the 6.5–2.0 range on tryptophan fluorescence maximum, $\lambda_{\text{max}}$. The inset shows the tryptophan fluorescence emission spectra for HSA in the native state at pH 6.0 and acid-denatured state at pH 2.0 in the 300–400 nm range after exciting the protein at 280 nm. The emission spectra was characterized by the emission maximum at 340 nm. Lowering the pH to 2.0 caused a blue shift of 12 nm (Table 1) and increase in fluorescence intensity, indicating internalization of tryptophan in a non-polar environment which was otherwise located between the IIA and IIIA interface in the native state [36] near the surface of the protein molecule, as judged by its sensitivity to photo-oxidation [40]. To confirm the environment of the tryptophan residue, we compared the
exposure of the single tryptophan residue, Trp214, in the native state with that in the acid-denatured state and GdnHCl-denatured state by a fluorescence-quenching experiment, using uncharged molecules of acrylamide as described by Eftink & Ghiron [31]. Figure 6 shows a Stern–Volmer plot of quenching of fluorescence by acrylamide in a native, acid-denatured and GdnHCl-denatured HSA. Results for the tryptophan analogue NATA are also included as a standard for complete accessibility to quencher. Table 2 shows the Stern–Volmer constants fitted to the early linear parts of the curves in Fig. 6. $K_{sv}$ for the native state was found to be higher (5.9) than that for the acid-denatured state (4.0) and was accompanied by a blue shift in $\lambda_{max}$ from 342 to 334 nm. These results indicate that the tryptophan residue in the native state was more accessible to quenching by acrylamide than in the acid-denatured state. The blue shift in the emission maximum of tryptophan indicated that the residue became more inaccessible to the solvent in the acid-denatured state. That the $K_{sv}$ values for the GdnHCl-denatured state and NATA were significantly higher than those for the native and acid-denatured states suggests that, even in the native state, the tryptophan residue was not fully accessible, which is in agreement with previous results [41].

Intrinsic viscosity measurements were also used for the detection of conformational changes in native HSA. The intrinsic viscosity values, $[\eta]$, for the native, acid-denatured and GdnHCl-denatured states are listed in Table 1. The value measured for the acid-denatured state (8.4 cm$^3$·g$^{-1}$) was a little more than twice that measured for the native state (3.9 cm$^3$·g$^{-1}$), but was considerably lower than that of the GdnHCl-denatured state (22.9 cm$^3$·g$^{-1}$). In the light of the molten globule theory, these data indicate that the compactness of HSA at acidic pH was similar to the molten globule state of other proteins [42].

Taken together, these results, i.e. the presence of a high content of secondary structure ($\approx 42\%$ $\alpha$-helix), higher magnitude of ANS binding, loss of co-operativity in the thermal transition and significant loss of tertiary structure but retention of compactness, suggest that the acid-denatured state of HSA at pH 2.0 resembles the molten globule state as defined for other proteins. However, it should be remembered that HSA contains three domains which are assumed to fold independently [43]. In view of previous results showing uncoiling of domain III on acidification, loss of secondary structure may be attributed to unfolding of domain III, whereas retention of other properties similar to those of the native state can be ascribed to domains I and II.

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### References


