

Formation of a molten globule like state in bovine serum albumin at alkaline pH

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Abstract Little work has been done to understand the folding profiles of multi-domain proteins at alkaline conditions. We have found the formation of a molten globule-like state in bovine serum albumin at pH 11.2 with the help of spectroscopic techniques; like far and near ultra-violet circular dichroism, intrinsic and extrinsic fluorescence spectroscopy. Interestingly, this state has features similar to the acid-denatured state of human serum albumin at pH 2.0 reported by Muzammil et al. (Eur J Biochem 266:26–32, 1999). This state has also shown significant increase in 8-anilino-1-naphthalene-sulfonate (ANS) binding in compare to the native state. At pH 13.0, the protein seems to acquire a state very close to 6 M guanidinium hydrochloride (GuHCl) denatured one. But, reversibility study shows it can regain nearly 40% of its native secondary structure. On the contrary, tertiary contacts have disrupted irreversibly. It seems, withdrawal of electrostatic repulsion leave room for local interactions, but disrupted tertiary contacts fail to regain their original states.

Keywords Aggregation · Alkaline denaturation · Circular dichroism · Molten globule state · Serum albumin

Introduction

Serum albumins undergo a number of pH-dependent conformational transitions. The normal ‘N’ form exists at neutral pH. Between pH 7.0 and 8.0, human serum albumin (HSA) and bovine serum albumin (BSA) undergo

conformation transition to ‘B’ or basic form; known as $N \rightleftharpoons B$ transition, slowly going to ‘A’ or aged form near pH 10.0 (Ahmad et al. 2004). But what happens at higher pH, is not yet studied well. Above pH 10.5, as many workers have observed, proteins get cleaved. Further, in this range of pH tyrosinyl residues ($pK = 10.1$) get deprotonated, resulting in a cooperative transformation of the albumins. At extreme pH the peptidyl residues get positively (for low pH) or negatively (for high pH) charged, which produces local Coulombian force of repulsion that counteracts internal stabilization forces of a protein, and result in unfolded state of a protein (Ahmad et al. 2004).

The mechanism by which protein folds from a structureless denatured state to their unique biologically active state is an intricate process. However, recent advances in biophysical techniques (El Kadi et al. 2006; Plaxco and Dobson 1996), both thermodynamic and kinetic studies, have shown the presence of stable intermediate states in a number of proteins (Horwick 2002; Calamai et al. 2005; Privalov 1996). One such intermediate is called “molten globule state,” characterized by compact secondary structure, but lacking rigid tertiary contacts. Although recent developments support the idea that a molten globule may also possess well-defined tertiary contacts, Goldberg et al. has introduced the term “specific molten globule.. The specific molten globule is a rather compact intermediate with a high content of native secondary structure, but a fluctuating tertiary structure. It contains an accessible hydrophobic surface susceptible to binding a hydrophobic dye, 8-anilino-1-naphthalene-sulfonate (ANS) (Chaffotte et al. 1992).

The folding process is even more complex in multi-domain proteins where each domain may be capable of folding independently and inter-domain interactions may affect the overall folding process (Gelamo et al. 2002;

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Viallet et al. 2000; Privalov 1982). One such example is BSA, a well-known model for protein folding studies. BSA is a single chain polypeptide, devoid of any non-proteinous part, having 583 amino acid residues arranged in three homologous domains, which further have two sub-domains each. In this study, we have tried to understand the unfolding of BSA at extremely high pH and to know whether the structural changes are reversible.

Materials and methods

Essentially fatty acid-free BSA, fraction VI (lot no. 42F-9365) was purchased from Sigma, St Louis, MO, USA, and the purity was checked for monomer by sodium dodecyl sulfate polyacrylamide gel electrophoresis. All other chemicals used in this study were of analytical grade.

All the measurements were carried out at room temperature. Protein stock solutions (5 mg/ml) were prepared in phosphate buffer, pH 7.0 and NaOH solution pH 13.0, respectively. The concentration of native proteins in 0.06 M sodium phosphate buffer was determined spectrophotometrically from the extinction coefficient reported at 280 nm, pH 7. For all studies, protein samples were incubated at room temperature for 1 h before spectroscopic measurements.

Circular dichroism measurements

Circular dichroism (CD) was measured with a JASCO J-720 spectropolarimeter calibrated with ammonium d-10 camphorsulfonate. A cell of path length 0.1 and 1 cm was used for scanning between 250–200 and 320–250 nm, respectively. The results were expressed as the mean residue ellipticity (MRE in $\text{deg.cm}^2.\text{dmol}^{-1}$), which is defined as:

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

Where θ_{obs} is the observed ellipticity in degrees, n is the number of peptide bonds per molecule, Cp is the molar fraction, and ' l ' is the length of light path in cm. The α -helical content of proteins was calculated from the MRE value at 222 nm (MRE_{222}) using the following equation:

$$\text{Percent } \alpha - \text{helix} = [(\text{MRE}_{222} - 2340) / 30300] \times 100 \quad (2)$$

Circular dichroism data have also analyzed by online available software, K2d (Andrade et al. 1993).

Fluorescence measurements

Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorophotometer in a 10-mm path length quartz cell. Samples containing different concentrations of

organic solvent were equilibrated at room temperature for 30 min before recording for tryptophan fluorescence measurements. The excitation wavelength was 280 nm and the emission from 300 to 500 nm was recorded.

ANS-fluorescence measurements

8-Anilino-1-naphthalene-sulfonate binding was measured by fluorescence emission with excitation at 380 nm and emission was recorded from 400 to 600 nm. Typically, ANS concentration was 50 times more than protein concentration and protein concentration was 15 μM .

Fractional denaturation studies

The fractional denaturation (f_D) of BSA at different pH was calculated with the help of following equation:

$$f_D = (Y_N - Y) / (Y_N - Y_D) \quad (3)$$

Where Y_N , Y_D and Y are MRE_{222} or relative fluorescence intensities of the native, denatured and at the transition states of the protein, respectively.

Results

pH dependent unfolding of BSA

Bovine serum albumin unfolds as pH increases in alkaline range (Ahmad et al. 2004). We have done the SDS-PAGE of BSA at pH 13.0 with incubation time of 0, 1, 2 and 3 h, respectively, and found that the protein band is visible in first two lanes only (data not shown), which seem to be due to cleavage of the protein after 2 and 3 h of incubations. Cleavage of BSA at higher alkaline pH can be avoided if incubation period is not more than 1 h, which is similar to earlier reports (Aoki et al. 1973). The conformational changes in the secondary structure of BSA have been studied with far UV-CD, in the range of 200–250 nm at pH 7.0, 11.2 and 13.0 (Fig. 1). The spectra at pH 7.0, shows characteristic minima at 208 and 222 nm, a known feature of α -helical proteins (Chen 1974). Moreover, MRE at 222 nm (MRE_{222}) is a widely used probe for α -helical conformation of a protein. Figure 1 (inset) shows decrease in the MRE_{222} of BSA with increase in pH from 7.0 to 13.0, which can be divided into three phases. In the first phase, which extends from pH 7 to 9, there is no significant change in MRE. In the pH range of 9–11.2, there is a little decrease and in the pH range of 11.2–13.0, a sudden fall in the MRE can be observed.

Bovine serum albumin contains two tryptophanyl residues, at 134th position of domain I and 213th position of domain IIA, respectively. Tryptophanyl residue of a

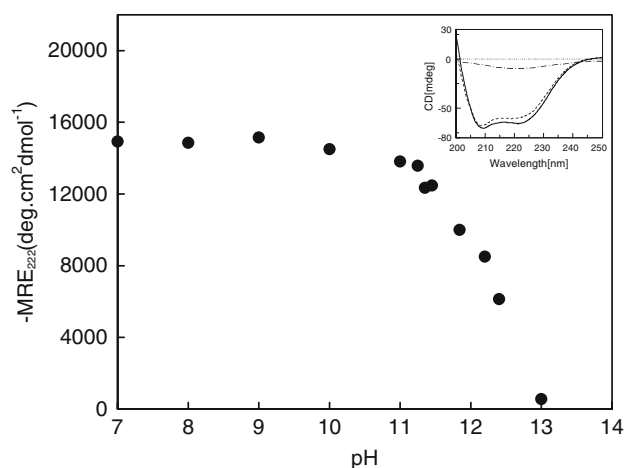


Fig. 1 Effect of pH on the mean residue ellipticity (MRE) of BSA was monitored at 222 nm by far UV-CD. Protein concentration used was 7.5 μ M. (Inset) Far UV-CD spectra of BSA at pH 7.0 (solid line), 11.2 (dashed line) and 13.0 (dotted-dashed line), respectively

protein exclusively excites at 295 nm, but at 280 nm it excites along with tyrosinyl residues. Figure 2 depicts the changes in maximum emission wavelength after exciting at 280 and 295 nm against increasing pH from 7.0 to 13.0. Maximum emission wavelengths were found to be 340 and 346 nm after exciting at 295 and 280 nm, respectively, at pH 7.0. They remained unaltered till pH 9.0, but then decreased to 333 and 338 nm, respectively, at pH 11.2. On the contrary, above pH 11.5, it shows a red shift of 12 and 9 nm, respectively.

The protein does not show any significant change in its secondary (Fig. 1) or tertiary structures (Fig. 2) in the pH range of 7.0–9.0. But significant decrease in tertiary constraints may be observed in the pH range of 9.0–11.2 (Figs. 2, 3), without any significant change in secondary structure (Fig. 1). It may have happened due to formation of a state where the secondary conformation of the protein

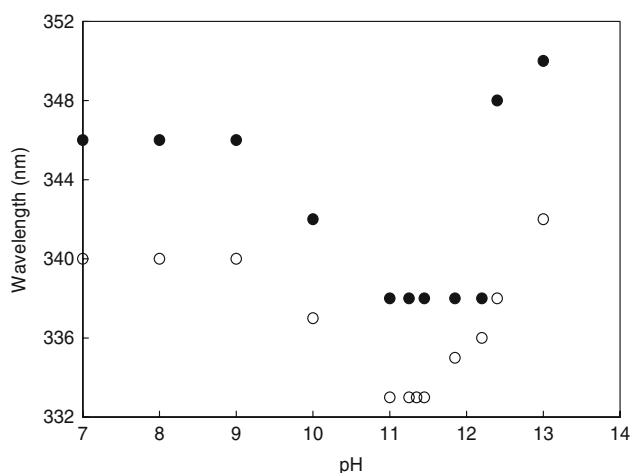


Fig. 2 Wavelength maxima of BSA, after exciting at 280 nm (dark filled circle) and 295 nm (circle) against increasing pH

almost remains unaltered with total disruption of tertiary contacts, called ‘molten globule’ state.

Figure 2 shows clear blue shift in the maximum emission wavelength in the chromophoric amino acid residues in this pH range. Blue shift in emission wavelength shows that the tryptophan has shifted to a more apolar environment. It may have happened due to internalization of tryptophan around pH 11.2. It also supports stabilization of an intermediate state with abrupt decrease in the non-local and increase in the local contacts.

To make the picture clearer, we have plotted a graph of fractional denaturation (f_D) of the secondary (MRE₂₂₂) and tertiary (relative fluorescence intensities after exciting at 280 and 295 nm, and as emitted at 340 nm) structures against increase in pH from 7.0 to 13.0 (Fig. 3a). It clearly shows there is almost 40% loss of tertiary structure as pH increases from 7.0 to 11.2, but no change in secondary structure can be observed. Almost 70% loss of both

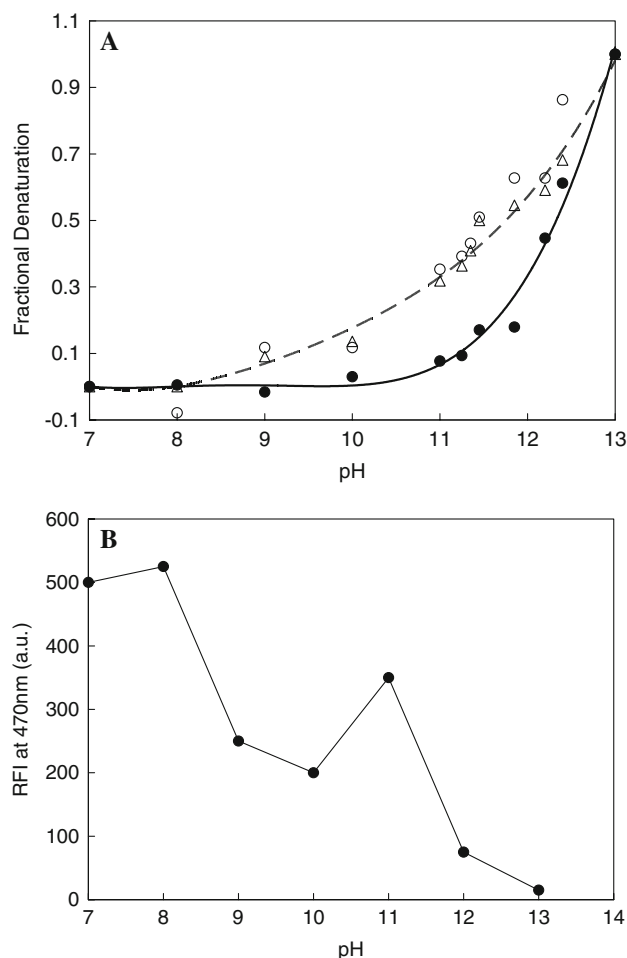


Fig. 3 a Fractional denaturation (f_D) of BSA when the protein was excited at 280 nm (circle) and 295 nm (triangle) and MRE₂₂₂ (dark filled circle) against increasing pH. b Relative fluorescence intensity at 470 nm of ANS–BSA complex with increasing pH after exciting at 380 nm

secondary and tertiary structures can be seen at pH 12.2. Tertiary structure of BSA starts decreasing at pH 9.0, while secondary structure at pH 11.2 and both disrupt totally by pH 13.0. Not only that, the rate of unfolding of the secondary structure is higher than that of the tertiary structure as pH increases from 11.2 to 13.0.

Protein ANS complex is excellently used as a probe for molten globule states. ANS is a hydrophobic dye that binds at the hydrophobic sites of a protein. Figure 3b shows the change in relative fluorescence intensities of BSA–ANS complex at 470 nm with increase in pH (Matulis and Lovrien 1998). With increase in pH the fluorescence intensities of BSA–ANS complex have decreased, except at pH 11. It again supports the possibility of formation of molten globule state in BSA near pH 11.

Reversibility studies of alkaline unfolded BSA

The reversibility properties of the alkaline unfolded protein have thoroughly checked by far and near UV-CD and absorption spectroscopy. Figure 4a depicts the far UV-CD spectra of BSA at pH 7.0, 13.0, the alkaline unfolded (at pH 13.0) BSA redissolved in the buffer at pH 7 and the protein denatured with 6 M guanidinium hydrochloride (GuHCl). At pH 7, the far UV-CD spectrum shows the characteristics of α -helical protein with the negative minima at 208 and 222 nm. At pH 13.0, the far UV-CD spectrum of BSA is closer to the baseline than that of the spectrum of 6 M GuHCl denatured protein. It seems that the alkaline-unfolded BSA at pH 13.0 acquires an unfolded state that is highly devoid of any secondary structure as compared to 6 M GuHCl denatured BSA. Interestingly, the alkaline-unfolded protein regains $\sim 40\%$ structure as it has redissolved in the refolding buffer of pH 7.0. But the tertiary structure of the unfolded protein has not changed in a similar way, as seen with the help of the change in near UV-CD spectrum of BSA (Fig. 4b). Near UV-CD of BSA at pH 7.0, 13.0, the alkaline-unfolded (at pH 13.0) BSA redissolved in pH 7 buffer and of the protein denatured with 6 M GuHCl have done in the range of 250–320 nm. Spectra in the region 260–320 nm arise from the aromatic amino acids. Each of the amino acid has characteristic wavelength range. Tryptophan shows fine structures between 290 and 305 nm; tyrosine between 275 and 282 nm and phenylalanine shows weaker bands around 255 and 270 nm. BSA at pH 7.0 shows a characteristic spectrum similar to earlier report (Lee and Hirose 1992) with shoulders near 262, 268 and 280 nm (Fig. 4b), but at pH 13.0, it shows a distinctly different spectrum with a positive peak near 255 nm, which is again quite different from the spectrum of 6 M GuHCl denatured protein. Interestingly, the reversibility point shows spectrum very similar to the alkaline-unfolded one. From these observations, we

may conclude that although BSA loses all its structure at extremely high pH and acquires a state similar to 6 M GuHCl denatured one, it can regain its secondary structure to an extent. On the contrary, the tertiary contacts fail to reestablish.

Discussion

Alkaline denaturation of BSA: formation of a state with secondary structure lesser than 6 M GuHCl denatured one

Proteins are assumed to be featureless (random coils) under sufficiently denaturing conditions; like in the presence of 6 M GuHCl (Tanford 1968) random coils are freely joined chains in which there is no correlation between the

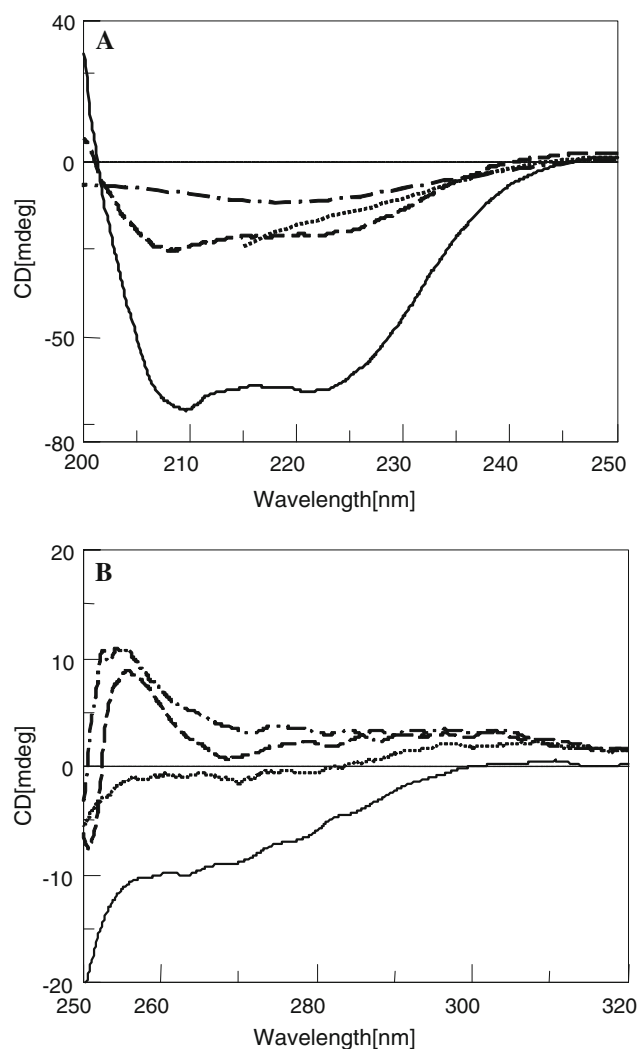


Fig. 4 **a** Far UV-CD, and **b** near UV-CD spectra of BSA at pH 7.0 (solid line), 13.0 (dotted-dashed line), reversibility (dashed line) and GndHCl denatured (dotted line), respectively. Protein concentration used was 7.5 μ M

orientations of two chain monomers at any length scale. Flory's treatment of unfolded chains stems from his 'isolated-pair hypothesis', that the φ , Φ angles that specify the backbone conformation of each residue are independent of one another (Flory 1969). However, failure of the Flory isolated-pair hypothesis (Pappu et al. 2000), existence of systematic local steric restrictions beyond the dipeptide (Fitzkee and Rose 2004b) and demonstration of random coil statistics for partially organized peptide chains (Fitzkee and Rose 2004a) suggest that the random coil state achieved by 6 M GuHCl need not to be an unfolded state. Alkaline denaturation of BSA at pH 13.0 is showing lesser secondary structure than that of 6 M GuHCl. Even the near-UV-CD data clearly show the similarity of the spectra of the native and the 6 M GuHCl denatured BSA in comparison to that of alkaline denatured one. GuHCl being a salt ionizes in aqueous solution to Gu^+ 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 (Robles-Vasquez et al. 1994). On other hand, increase in pH gradually anionizes first the uncharged and then the positively charged amino acid side chains. First the tertiary structure is disrupted, and then the secondary structure, as we can see from the far UV-CD and the tryptophanyl and tyrosinyl fluorescence studies. Tertiary contacts like electrostatic interactions, non-local hydrogen bonding and hydrophobic interactions may have cleaved first. Contrarily, the secondary structure seems to be very rigid to disruption before pH 11 due to the presence of high percentage of α -helix, and then it collapses immediately with increase in pH. It can be explained by the phenomenon that, hydrogen bonding together with steric considerations limits backbone segments to a small repertoire of accessible conformers. Characteristic all or none folding behavior is triggered by the shift to or from conditions that favor or disfavor intramolecular hydrogen bond formation. Furthermore, an extended state devoid of any well-defined secondary or tertiary contact seems to arise by the intra-molecular and inter-molecular electrostatic repulsion.

There are clear indications that the alkaline denaturation of BSA is reversible, but the tertiary contacts remain similar to the unfolded state. It can regain as much as 40% of its α -helical structure to form a native-like state. The withdrawal of the electrostatic repulsion may have left room for local intra-molecular hydrogen bonding.

The alkaline denaturation of BSA can be divided into three phases. In the first phase, in the range of pH 7.0–9.0, there is almost no significant change in its conformation. It is followed by a phase (\sim pH 9.0–11.0) of decrease in tertiary contacts without much visible changes in secondary conformations and with imminent blue shift in tryptophanyl fluorescence. It seems that disruption of

tertiary contacts has favored some local hydrogen bonding formations, so the tryptophan residue gets buried further. Interestingly, the pK values of α and ϵ amino groups, sulfhydryl and hydroxyl groups lie in this pH range. In the third phase (pH range of 11.0 to 13.0), the rest of the tertiary contacts collapse along with the total unfolding of secondary structure and red shift in tryptophanyl fluorescence. Increase in solvent accessibility of tryptophanyl residues again supports unfolding of the protein molecule.

High pH induced molten globule state in BSA

The formation of secondary structure in the early steps of protein folding has been observed for many proteins (Ballery et al. 1993; Ptitsyn 1995). Such early species with high content of secondary structures were named "molten globule state" (Ohgushi and Wada 1983) and suggested that it was a general intermediate in the folding pathway of proteins (Ptitsyn 1995). The formation of molten globule as an early intermediate has been reported for several proteins, among them α -lactalbumin, carbonic anhydrase, β -lactamase, the α and β_2 subunits of tryptophan synthase, bovine growth hormone, phosphoglycerate kinase, stem bromelain, HSA etc. (Chaffotte et al. 1992; Ballery et al. 1993; Ptitsyn 1995). Early disruption of tertiary contacts before the melting of secondary structure results in the formation of molten globule-like state in BSA around pH 11. Similar result has been found with the acid denaturation of HSA at pH 2.0. The acid-denatured state has shown sixfold increases in ANS binding from the native state (Muzammil et al. 1999). ANS binding decreases in protein with increase in pH. But at pH 11.0, an increase in ANS binding has been observed, due to the formation of molten globule state.

In short, BSA unfolded at pH 13.0 with structural contents lesser than that of 6 M GuHCl denatured BSA. It can also regain nearly 40% of the native secondary structure in the presence of 0.06 M sodium phosphate buffer of pH 7.0. At pH 11.2, a molten globule-like state seems to form with native-like secondary structure and 40% loss in tertiary structure. We can further characterize that the states arises due to alkaline denaturation of BSA with various stabilizing cosolvents.

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