J Fluoresc (2011) 21:1083–1090
DOI 10.1007/s10895-010-0781-3

ORIGINAL PAPER

pH-induced Conformational Isomerization of Bovine Serum Albumin Studied by Extrinsic and Intrinsic Protein Fluorescence

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Received: 29 August 2010 / Accepted: 25 November 2010 / Published online: 3 December 2010

Abstract Serum albumins are multi-domain all α-helical proteins that are present in the circulatory system and aid in the transport of a variety of metabolites, endogenous ligands, drugs etc. Earlier observations have indicated that serum albumins adopt a range of reversible conformational isomers depending on the pH of the solution. Herein, we report the concurrent changes in the protein conformation and size that are inherent to the pH-induced conformational isomers of bovine serum albumin (BSA). We have investigated the fluorescence properties of both intrinsic (tryptophan) and extrinsic (ANS, pyrene) fluorophores to shed light into the structural features of the pH-dependent conformers. We have been able to identify a number of conformational isomers using multiple fluorescence observables as a function of pH titration. Our results indicate that at pH 3, a partially-folded, 'molten-globule-like' state is populated. Moreover, equilibrium unfolding studies indicated that the 'molten-globule-like' state unfolds in a non-cooperative fashion and is thermodynamically less stable than the native state. The fluorescence-based approach described in the present work has implications in the study of pH-induced conformational plasticity of other physiologically relevant proteins.

Keywords Fluorescence spectroscopy · Fluorescence anisotropy · Bovine serum albumin · Conformational isomers · Molten-globule

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Introduction

Serum albumin is the most abundant globular, all α-helical protein (~60% of all plasma proteins) in the circulatory system that is involved in the binding and transport of fatty acids, hormones, metabolites, endogenous ligands and drug delivery in addition to the maintenance of colloid osmotic blood pressure [1, 2]. Human serum albumin (HSA) or its bovine analogue (BSA) is a 585-residue, multi-domain, all α-helical protein that assumes a heart-shaped structure at physiological pH (Fig. 1). The crystal structure of HSA [3, 4] reveals that each of the three domains (I, II and III) is further composed of two sub-domains (A and B) that contain several intra-domain disulfide bonds (17 disulfide bridges in total) and a single tryptophan residue (W214) in domain IIA. The disulfide bonds impart rigidity to the helical, globular structure but allow enough flexibility to the protein to undergo conformational changes in response to changes in pH [5, 6] and upon interaction with fatty acids [1]. BSA shares a 76% sequence homology with HSA, contains one more tryptophan residue in domain IA at the 134th position (in addition to the one in domain IIA) and also undergoes reversible conformational isomerization as a function of pH [7]. Recent reports suggest that the structural organization and compactness of the tertiary structure of BSA vary as a function of pH whereby a few significant modifications in the secondary structures are also observed [8–12]. Five conformational isomers of BSA over different pH ranges have been identified namely, the E-form (Extended; pH<3), the F-form (Fast; pH 3–5), the N-form (Native; pH 5–7), the B-form (Basic; pH 7–8.5) and the A-form (Aged; pH>8.5). It was also suggested that the conformational isomerization of serum albumins is conserved across various species which implies that the pH-dependent isomerization might play a significant physio-

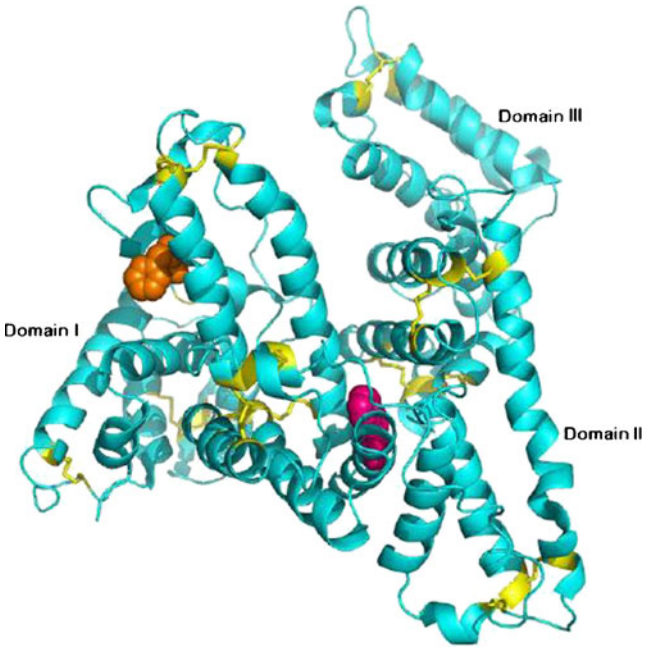

Fig. 1 The crystal structure of human serum albumin (HSA) showing the single tryptophan (W214; magenta), phenylalanine (F134; orange) and disulfides (yellow) generated using PyMol from protein data bank (1UOR.pdb). Bovine serum albumin (BSA), structure of which is not available, shares 76% sequence homology with HSA. BSA contains an additional tryptophan in domain I at residue position 134 instead of the phenylalanine

logical role [1]. The physiological relevance of serum albumins might be linked to pH-dependent binding and release of substrates given the fact that low pH has been measured on the membrane surfaces of several tissues [13]. An interesting and important aspect of serum albumins is to understand how the changes in conformation and size of the protein are coupled with the pH variation.

Fluorescence spectroscopy is a useful and powerful methodology to extract information about the structural features of biomolecules such as proteins [14, 15]. Its exclusivity lies in the fact that the changes in the fluorescence properties of the proteins (either intrinsic fluorophores or extrinsic fluorophores that can be covalently or non-covalently attached to the protein) can be directly correlated to the simultaneous changes in the protein conformation and size. In addition to the structural information, fluorescence is a very sensitive spectroscopic technique which enables one to study the structural aspects at a very low protein concentration and thereby allowing one to study the predominantly monomeric (unaggregated) form. In this work, we have carried out fluorescence-based studies on the structural changes of monomeric BSA by extracting detailed information on conformational changes coupled with the size variation. The fluorescence properties, namely, fluorescence intensity and anisotropy of intrinsic fluorophore, tryptophan and extrinsic fluorophores viz. ANS, pyrene have been utilized in the present work to shed light into the conformational equilibria of BSA as a function of pH.

Materials and Methods

Materials

Bovine Serum Albumin (BSA), glycine, sodium phosphate monobasic, sodium phosphate dibasic, citric acid, potassium chloride, Tris, urea were purchased from Sigma and used without any further purification. The fluorescent probes viz. 8-anilinonaphthalene,1-sulfonic acid (ANS) and pyrene were obtained from Sigma and used as received. Milli-Q water was used for the preparation of solutions and buffers. KCl-HCl (pH 1.6, 2), glycine-HCl (pH 2.5, 3), sodium citrate (pH 3–6), sodium phosphate mono-and dibasic (pH 6.5–8), Tris-HCl (pH 8.5), glycine-NaOH (pH 9–10.5) were used for buffer preparations. All the pH buffers (from pH 1.6–10.5) were always made freshly and adjusted by adding 1N HCl or NaOH prior to every pH titration experiments. The pH of the buffers was checked on Cyberscan pH 510 bench meter from Eutech Instruments. The final pH was in the range of ±0.01 at 24–25 °C.

Preparation of Protein Sample

For pH titration experiments, BSA was dissolved in 50 mM, phosphate buffer of pH 7 to give a stock solution of 1 mM and stored at 4 °C. Accurate protein concentration was determined by measuring the absorbance of tryptophans at 280 nm on Lambda 25, Perkin-Elmer UV-Visible spectrophotometer. The molar extinction coefficient of BSA at 280 nm is 43,824 M^{-1} cm^{-1} [2]. Typically, 1 mM of BSA in phosphate buffer (50 mM, pH 7) was diluted 100-fold by using a required buffer (pH 1.6–10.5, 50 mM) to a final protein concentration of 10 μM and kept for 2 h at 25 °C to ensure complete equilibration to the required isoform. The fluorescence intensity and anisotropy of tryptophans and ANS were measured to study the conformational and size changes in the monomeric protein isomers. For pyrene fluorescence experiments, 1 mM of BSA in aqueous solution was diluted 100-fold into the respective pH buffer (1 mM) and equilibrated for 2 h at 24–25 °C. After 2 h, 1 mM of pyrene (in methanol) was diluted 100-fold into a protein solution and kept in the dark for 5 min each at 24–25 °C prior to the recording of fluorescence spectral scans. All the measurements were carried out at 24–25 °C.

Equilibrium Unfolding Studies on Protein Using Urea

Typically, 1 mM BSA in phosphate buffer (50 mM, pH 7) was prepared in 2 sets for the unfolding experiments at pH 7 and pH 3 and stored at 4 °C. A stock solution of urea (8 M) in the respective pH buffers (phosphate buffer: pH 7, 50 mM and Gly-HCl buffer: pH 3, 50 mM) was prepared separately and the pH of the urea solutions were checked and adjusted

accordingly at 24–25 °C. The stock solution of BSA was then diluted 100-fold in varying concentrations (0–8 M) of urea and the respective pH buffer to yield a final protein concentration of 10 μM. The solutions were incubated at 25 °C for 2 h to ensure partial/complete denaturation at that particular concentration of urea. The unfolding of BSA was monitored by tryptophan fluorescence intensity and anisotropy.

Fluorescence Measurements

All the steady-state fluorescence measurements were performed on an LS 55 luminescence spectrometer from Perkin Elmer. In general, the final protein concentration used for fluorescence measurements was 10 μM. Different concentrations of ANS viz. 4, 10 and 40 μM were obtained by suitable dilution of a stock solution (10 mM) of ANS (prepared in Milli-Q water and stored at 4 °C) into protein solution. The excitation bandpass was fixed at 2.5 nm for recording fluorescence data of all the probes used in this study. The fluorescence intensity and anisotropy were collected at constant wavelength with an integration time of 5 and 30 s, respectively. The steady-state anisotropy is given by: $r = (I_{II} - GI_{\perp})/(I_{II} + 2GI_{\perp})$, where $I_{II,}$ $I_{\perp}$ are fluorescence intensities collected using parallel and perpendicular geometry of the polarizers, respectively and the perpendicular components were corrected using respective G-factors. The error associated with the fluorescence anisotropy measurements was below 0.01. All the emission spectra were scanned at a rate of 10 nm/min and averaged over 5 scans. The following parameters were adjusted for monitoring tryptophan fluorescence intensity and anisotropy during pH titration: λ_{ex}=300 nm, λ_{em}=350 nm, excitation bandpass=2.5 nm, emission bandpass=4 nm (for intensity) and 5–7 nm (for anisotropy). For recording ANS fluorescence intensity and anisotropy: λ_{ex}=350 nm, λ_{em}=475 nm, emission bandpass of 2.5 nm (for both intensity and anisotropy at [ANS]=4 μM and 10 μM) and 0.5 nm (for both intensity and anisotropy at [ANS]=40 μM). For monitoring pyrene fluorescence intensity, λ_{ex}=335 nm, excitation bandpass=2 nm and emission bandpass=2.5 nm. The FRET efficiency between the tryptophans and ANS was estimated using $E = (1-I_{DA}/I_D)$ where I_D is the fluorescence intensity of the donor alone and I_{DA} is the fluorescence intensity of the donor in presence of the acceptor. Several data points at a given condition were collected to get an estimate of the standard deviation associated with the measurement. The graphs were plotted using OriginPro Version 8.0 software.

Circular Dichroism (CD) Spectroscopy

The far-UV CD spectra of the protein samples were recorded on a J-810 Jasco CD spectrometer at room temperature. Typically, the protein sample solution at a given pH was diluted 30-fold to a final protein concentration of 3 μM, which was taken in a quartz cuvette of 1 mm pathlength and the secondary structural changes were recorded in the range of 195-250 nm. The scan rate was 100 nm/min and the final spectrum was averaged over 5 scans.

Results and Discussion

pH-dependent Conformational Changes of BSA

Steady-state fluorescence spectroscopy was employed to investigate the conformational and size changes of monomeric BSA as a function of pH. The fluorescence intensity, which is a measure of the fluorophore's quantum yield, is sensitive to its environment [15]. This implies that any change in the protein conformation that affects the surroundings of the intrinsic tryptophan residues will be reflected in the change in tryptophan fluorescence intensity. Moreover, a shift in the tryptophan emission spectrum is observed depending on whether the tryptophan residues are exposed or buried due to the protein conformational changes. The fluorescence anisotropy measurements offer information about the overall size and the rigidity of the probe attached to biomolecules. The steady-state fluorescence anisotropy is related to the overall size of the protein assuming that the overall size changes is predominant compared to the changes in internal (local) dynamics. In this set of experiments, the fluorescence intensities and anisotropies of intrinsic tryptophans (present in domains I and II at positions 134 and 214, respectively [1]) and non-covalently-bound ANS (at different concentrations) were measured to extract information about the changes in conformation and size simultaneously. Additionally, the vibronic bands in the fluorescence spectra of pyrene were utilized as the polarity reporters of the various pH-dependent conformational isomers of BSA. Far-UV CD was employed to monitor the changes in secondary structural content of the α-helical protein during pH titration. In order to determine the protein concentration range at which it remains monomeric, tryptophan fluorescence anisotropy was measured over a wide range of protein concentration (2–100 μM). No change in tryptophan fluorescence anisotropy from 2–50 μM was observed (data not shown). This indicated that BSA remains predominantly monomeric in the aforementioned concentration range at various pH. Hence, the following studies were carried out at a low protein concentration (10 μM) where it exists largely as monomers (for details, see "Materials and Methods").

Tryptophan Fluorescence of pH-induced Conformational Isomers

The tryptophans of the native, monomeric protein at neutral pH exhibited maximum fluorescence intensity at ~350 nm. During the course of pH titration, the tryptophans exhibited an emission maximum at ~340 nm in the acidic pH range implying that the conformational transition brings about a change in the location of tryptophans from solvent exposed to hydrophobic region. As the pH is increased towards the neutral regime, ~10 nm red-shift in the tryptophan emission maximum (340 nm→350 nm) occurs which indicates that the tryptophans are exposed to the aqueous environment upon transition from low to neutral pH (Fig. 2a). As the pH is increased further beyond neutrality, ~5 nm blue-shift in the tryptophan emission spectrum (350 nm→345 nm) is observed in the basic pH regime. Figure 2b represents the tryptophan fluorescence intensity profile at 350 nm as a function of pH which corroborates the previous findings for HSA [16] and clearly indicates a change in the tryptophan environment as a consequence of pH-induced conformational isomerization of BSA. In addition to fluorescence intensity, the steady-state fluorescence anisotropy of the tryptophans was measured as pH of the medium was varied from acidic to alkaline as shown in Fig. 2c. The tryptophans in the native form at pH 7 had an average anisotropy of ~0.20 [16]. As the pH was lowered, the fluorescence anisotropy of the low pH form showed a peak at pH 3 (~0.23), which may indicate that upon lowering the pH, BSA molecule undergoes a conformational expansion. The acid-expanded form shares structural similarities with the 'molten-globule-like' conformers available in the literature [8, 16, 17]. A further lowering of the pH resulted in a further drop in the tryptophan fluorescence anisotropy ~0.18 indicating that the 'molten-globule-like' conformer converts into a more extended conformer. In the basic pH regime, the tryptophan fluorescence anisotropy of the conformational isomers remains unaltered when compared to that of the native protein.

In order to shed light into the secondary structural changes during pH-dependent conformational isomerization, far-UV CD spectra of the isomers were collected. The spectra of the monomeric conformational isomers showed distinct changes in helicity upon change in pH (Fig. 2d) that is in accordance with earlier reports [16]. A significant loss in helical content of the conformational isomers was observed in the low pH regime. The present set of experiments indicated the presence of expanded conformational isomers at low pH whereby an expanded, 'molten-globule-like' state is populated at pH 3.

ANS Fluorescence of pH-induced Conformational Isomers

In the previous set of experiments, a blue-shift in tryptophan emission spectrum was observed at low pH, suggestive of a non-polar environment in the acid-induced conformational isomers. In order to ascertain the changes

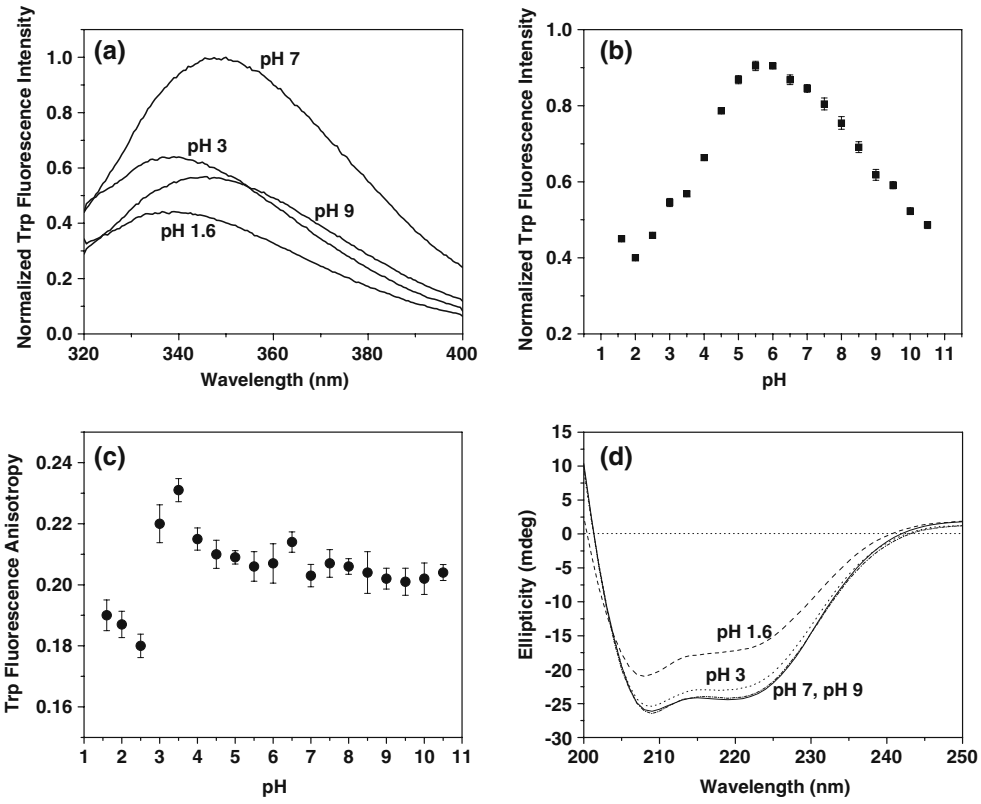

Fig. 2 Conformational isomerization of BSA as a function of pH. Variation in **a** tryptophan emission spectra, **b** tryptophan intensity, **c** tryptophan anisotropy and **d** far-UV CD spectra as observed during pH titration. The error bars in the intensity and anisotropy are included in the symbols

in the hydrophobicity of the core of BSA across a wide pH range, the steady-state fluorescence intensity and anisotropy of different concentrations of 8-anilinonaphthalene-1-sulfonic acid (ANS) were measured. ANS is an extrinsic fluorophore which is weakly fluorescent in aqueous environment but fluoresces strongly (with a concurrent blue-shift in its emission maximum from ~515 nm to ~475 nm) when located in a hydrophobic environment [18]. It has been reported that though ANS binds to the hydrophobic regions of both domains II and III of BSA, it exhibits a preferential binding in IIIA at low concentrations of ANS (when [ANS]: [protein]=0.4) [19]. From the crystal structure of HSA, it has been suggested that the binding domains IIA and IIIA share a common interface and hence, binding in III affects conformational changes and binding affinities in II [3, 4]. Therefore, during the course of pH titration, any change in the conformation of domain III would most likely influence the structural arrangement in domain II. Such an event, in turn, would affect the binding of ANS and hence, its fluorescence properties. Control experiments showed that the fluorescence properties of free, aqueous ANS remained unchanged with a change in pH. Therefore, the observed changes in the ANS fluorescence intensity and anisotropy could be solely attributed to the pH-induced structural changes in domains II and III of BSA. Figure 3a depicts the changes in fluorescence intensity of ANS at [ANS]= 4 μM (predominantly occupying domain III [19]; top panel), 10 μM (both domains II and III are occupied; middle panel) and 40 μM (ANS saturated; bottom panel), respectively as a function of pH ([BSA]=10 μM). The fluorescence intensity profile is more resolved at an ANS concentration of 10 μM whereas the change in fluorescence intensity at either low or high ANS concentrations is very subtle. At 10 μM of ANS, three distinct peaks at ~ pH 3, 7 and 9.5 were observed. Therefore, three different conformational isomers of the protein with varying extent of hydrophobicity in the acidic, neutral and alkaline pH regime could clearly be identified from the ANS intensity profile. This observation reveals that the structural variations of the domains in the pH-induced conformational isomers affect the fluorescence quantum yield of the bound ANS. Figure 3b shows the concentration-dependent variation in ANS fluorescence anisotropy as a function of pH. A critical look at the anisotropy data ([ANS]=10 μM) reveals that at low pH, the fluorescence anisotropy of the acid-expanded isomer is larger than that of the native-form, which corroborates the tryptophan anisotropy data indicating an expanded conformational state at low pH. This set of experiments suggested the presence of exposed hydrophobic regions in the expanded conformational populations at ~ pH 3. Upon lowering the pH from 3 to 1.6, there is a further loss in the protein structure leading to acid-induced unfolding.

We further extended our ANS-binding studies to record conformational changes using fluorescence resonance energy transfer (FRET) since tryptophan and ANS are known to be a FRET-pair [15]. Figure 3c depicts the stack plots of FRET-efficiency from tryptophan (located in domains I and II) to ANS which clearly shows that the FRET efficiency increases with increasing concentration of ANS. However, at low concentrations of ANS ([ANS] ≤10 μM), the FRET efficiency is lower at acidic conditions compared to that at neutral and alkaline pH which could be ascribed to the loss in intra-domain helicity (corroborating the far-UV CD data; Fig. 2d), that affects the energy transfer efficiency between tryptophan (located in domains I and II) and ANS. Thus, the tryptophan and ANS fluorescence observables together with the CD data indicated that BSA

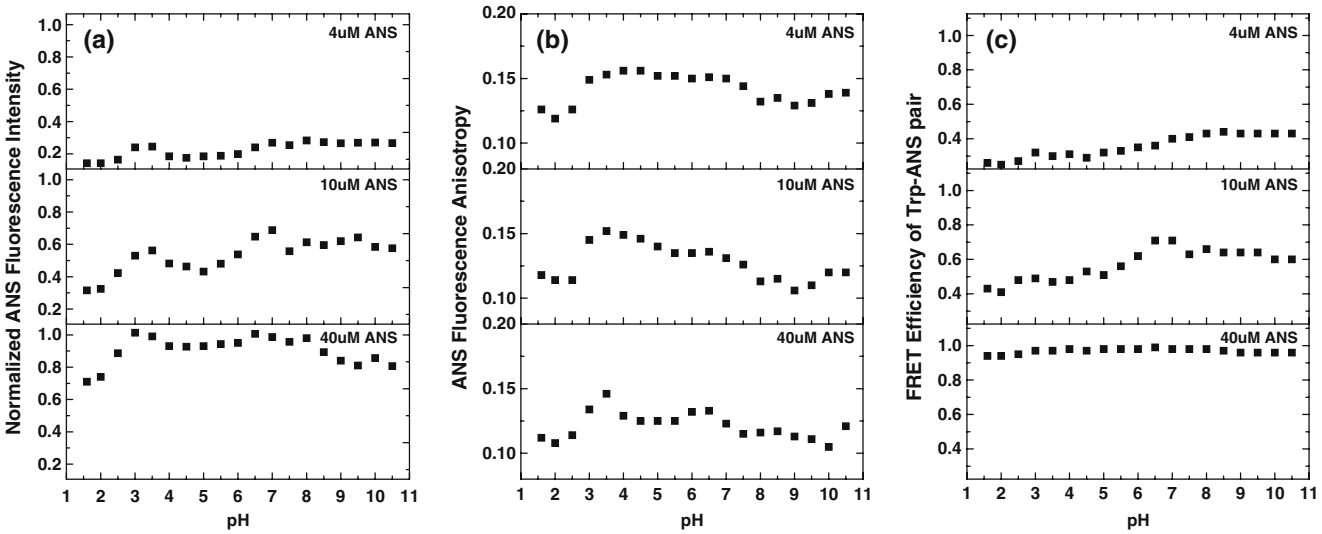

Fig. 3 Conformational isomerization of BSA as a function of pH at various ANS concentrations viz. 4, 10 and 40 μM. Stack plots showing the variation in **a** ANS fluorescence intensity, **b** ANS fluorescence anisotropy and **c** FRET-efficiency between tryptophan and ANS as observed during pH titration. The error bars in the intensity and anisotropy are included in the respective symbols used for the plots

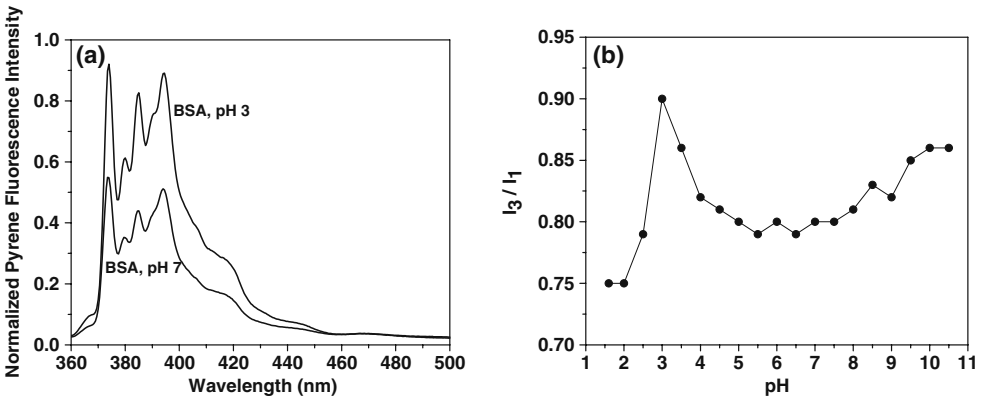

Fig. 4 **a** Fluorescence emission spectra of pyrene in the presence of BSA at pH 7 and pH 3 at 25 °C. **b** The ratio of the third (I_3; 384 nm) and first (I_1; 373 nm) vibronic peak intensities of pyrene in BSA as a function of pH obtained from calculation of the emission spectra. All the experiments were repeated separately three times to get an estimate of the error associated with the measurements which was <0.01

forms an expanded, 'molten-globule-like' state at pH 3 which is accompanied by a partial loss in helicity.

Pyrene Fluorescence of pH-induced Conformational Isomers

In order to ascertain whether the pH-induced conformational isomers of BSA can also be distinguished based on their polarity index, pyrene fluorescence experiments were undertaken. Pyrene is a rigid, polycyclic aromatic hydrocarbon fluorophore whose fluorescence emission spectrum is dependent on the polarity of its microenvironment and is frequently employed to estimate the polarity of micelles and model lipid membranes [20–22]. In a non-polar medium, the fluorescence emission spectrum of pyrene exhibits five vibronic bands, namely, I_1, I_2, I_3, I_4 and I_5 at 373 nm, 378 nm, 384 nm, 389 nm and 394 nm, respectively which reduces to three bands in a polar medium (I_1, I_3 and I_5) [21, 23, 24]. A qualitative estimate of the polarity of the pyrene microenvironment can be obtained from the ratio of the fluorescence intensities at I_3 and I_1. Higher I_3/I_1 ratio indicates that the environment surrounding the pyrene molecules is more hydrophobic. Figure 4a depicts representative fluorescence emission spectra of pyrene (normalized at 373 nm) bound to native BSA (pH 7) and one of the conformational isomers of BSA at acidic pH regime (pH 3). The data clearly shows the split of the emission spectra into five vibrational peaks which clearly indicate that pyrene is bound to the hydrophobic regions of BSA, given that the serum albumins have two distinct binding sites [1, 2]. The I_3/I_1 ratio of pyrene was also estimated to obtain an idea of the relative polarity of the pH-induced conformational isomers. The ratio was higher in native BSA (~0.78) than that in water (~0.65) which is suggestive of the fact that the pyrene molecules are localized in the hydrophobic binding sites of BSA. It is noteworthy to state that the I_3/I_1 ratio in BSA at pH 3 (~0.90) was considerably higher than that of the native protein and all other pH-induced isomers (I_3/I_1 ratio ~0.78), emphasizing the existence of an expanded conformational state of BSA comprising exposed hydrophobic pockets at pH 3. Upon lowering pH further down to 1.6, BSA undergoes acid-induced unfolding that disrupts the hydrophobic binding pockets. Such observation also corroborates the ANS-fluorescence data obtained for the pH-dependent conformational isomers of BSA.

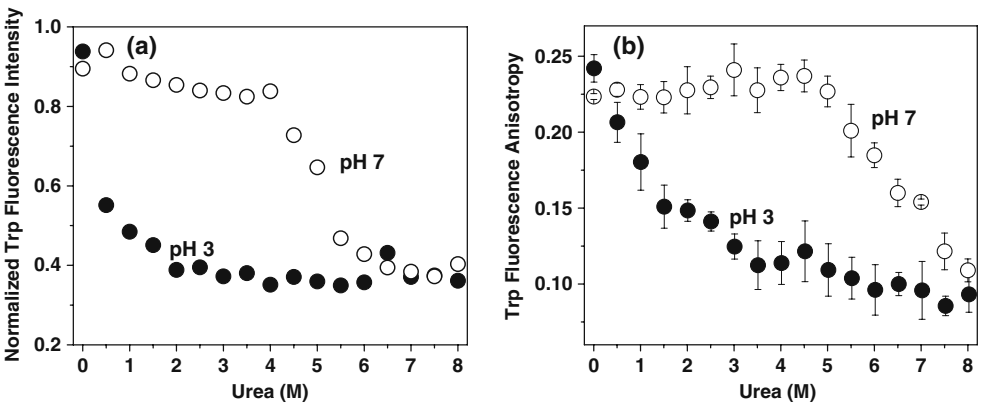

Fig. 5 Chemical denaturation of the native BSA (pH 7: open circle) and the partially-folded conformational isomer of BSA (pH 3: filled circle) as a function of urea concentration at 25 °C. Changes in the **a** tryptophan fluorescence intensity and **b** tryptophan fluorescence anisotropy as observed during unfolding of BSA

Equilibrium Unfolding Studies of BSA at Acidic and Physiological Solution Conditions

All the fluorescence observables have indicated that the native BSA isomerizes to a partially-extended, 'molten-globule-like' state predominantly populated at pH 3. In order to compare the thermodynamic stabilities between the native and the expanded isomer of BSA, the unfolding of BSA, induced by urea, was investigated by steady-state fluorescence spectroscopy under physiological and acidic pH (pH 7 and pH 3, respectively) conditions. Changes in the fluorescence intensity and anisotropy of the tryptophans were monitored as a function of urea concentration. The fluorescence emission maximum and steady-state anisotropy value of the tryptophan residues in the native BSA at pH 7 were found to be 350 nm and ~0.21 respectively. At pH 7, a cooperative transition from the native to unfolded state of BSA was evident from the sigmoidal curves for both in tryptophan fluorescence intensity and anisotropy as a function of denaturant concentration (Fig. 5). The mid-point of transition was centered around 5 M urea concentration. The chemical denaturation studies were also carried out at pH 3 where the partially-extended conformers were populated. Both tryptophan fluorescence intensity and anisotropy did not demonstrate a cooperative transition; rather they showed a continuous change as a function of denaturant concentration. No significant changes were observed after 3 M of urea (Fig. 5). The overall fluorescence intensity plot of BSA at pH 3 suggested that, as expected, the partially-extended conformational population unfolds in non-cooperative fashion.

Conclusion

The present work describes the identification and characterization of multiple pH-dependent conformational isomers of BSA using fluorescence measurements of extrinsic and intrinsic probes. We have utilized tryptophan, ANS and pyrene as fluorescent reporters of conformational- and size changes. Our findings also indicated that BSA forms a partially-folded, 'molten-globule-like' form in the acidic pH regime. We suggest that the domain II might play the key role in the formation of the 'molten-globule-like' conformer since it has been shown earlier that recombinantly expressed domain II of HSA adopts a 'molten-globule' state at low pH [16]. Additionally, our chemical denaturation studies for both native- and low pH-forms support that the conformational isomer formed at pH 3 is less thermodynamically stable than the native protein. Our finding is relevant in the context of the observation that partially-destabilized proteins comprising exposed hydrophobic regions are prone to aggregation that might have important implications in protein misfolding and aggregation related disorders. We believe that the fluorescence-based approach described in this work will find broader applications in the study of pH-induced conformational change of many physiologically important proteins.

Acknowledgements We thank IISER Mohali for financial support during the course of the present investigation and the Guptasarma Lab at IMTECH Chandigarh for CD measurements.

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