



Characterization of a dimeric unfolding intermediate of bovine serum albumin under mildly acidic condition

Amrita Brahma, Chhabinath Mandal, Debasish Bhattacharyya*

Division of Drug Design, Development and Molecular Modeling, Indian Institute of Chemical Biology, 4, Raja S.C. Mallick Road, Jadavpur, Calcutta-700032, India

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Abstract

Protein aggregation is a well known phenomenon related to serious medical implications. Bovine serum albumin (BSA), a structural analogue of human serum albumin, has a natural tendency for aggregation under stress conditions. While following effect of moderately acidic pH on BSA, a state was identified at pH 4.2 having increased light scattering capability at 350 nm. It was essentially a dimer devoid of disulphide linked large aggregates as observed from ‘spin column’ experiments, gel electrophoresis and ultra centrifugations. Its surface hydrophobic character was comparable to the native conformer at pH 7.0 as observed by the extraneous fluorescence probes pyrene and pyrene maleimide but its interactions with 1 anilino 8 naphthelene sulphonic acid was more favorable. Dimerization was irreversible between pH 4.2 and 7.0 even after treatment with DTT. The role of the only cysteine 34 residue was investigated where modification with reagents of arm length bigger than 6 Å prevented dimerization. Molecular modeling of BSA indicated that cys 34 resides in a cleft of 6 Å depth. This indicated that the area surrounding the cleft plays important role in inducing the dimerization.

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1. Introduction

Proteins, which are soluble under normal physiological conditions, sometimes form insoluble aggregates with serious medical implications. Conformation change is an obligatory requirement for initiation of association, no matter whether detectable or not [1]. Aggregation may initiate by a number of ways; properly folded molecules under stress conditions or with aging may acquire conformations susceptible to adhesion. Alternately, a fraction of proteins during its maturation after synthesis may fold

incorrectly leading to meta-stable states prone to aggregation. These type of structural intermediates are considered to be the same or very similar in either case. Further, the general properties of the conformers of all proteins prone to aggregation are believed to be similar. The energy landscapes of such processes have been reviewed [2]. Many deadly consequences like Alzheimer’s disease [3], Parkinson’s disease [4,5], amyloidosis [6,7], etc. are associated with protein aggregation.

Kinetic analysis of protein aggregation includes first-order reversible unfolding followed by association of nonnative species in a higher order process (‘Lumry–Eyring’ model) [8,9]. Propagation of aggregates follows sequential steps starting from association of molecules of low multimericity. Energy analysis from thermodynamic standpoints predicts that elongation of aggregates over small number of nuclei is favorable compared to growth over a large number of nuclei [10]. Thus, initiation of aggregation is regulated by molecules of low multimericity no matter what kind of stress is induced.

Abbreviations: 1-ANS, 1-anilino 8-naphthylene sulfonic acid; BAL, sodium arsenite; BSA, Bovine serum albumin; CD, Circular dichroism; DTNB, dithio bis-trinitrobenzoic acid; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethyl maleimide; MMTS, methyl methane thiosulphonate; PM, pyrene maleimide

* Corresponding author. Tel.: +91 33 2473 3491/3493/0492x164; fax: +91 33 2473 5197/0284.

E-mail address: debasish@iicb.res.in (D. Bhattacharyya).

Since the motive force in biology is derived from gradients created between concentration, density, pH, electromotive force, etc., stress on protein conformation is evident. Variation of pH in intestine within 1–6 depending on requirement is an extreme example of pH driven biological control. The pH of lysosome in cells, which is the major site for protein degradation, remains around 4.8 where proteases function leaving nonfunctional and other proteins denatured. Further, in gastric ulceration and deep wounds, the pH at the site of injury remains as low as 2.0 [11,12]. Thus, pH-induced deformation of proteins is common in biology. Aggregation *in vivo* being a slow continuing process for years, to follow them in the time scale of laboratories, native conformation of proteins is altered under restricted conditions. There the protein should not unfold completely but will attain an unstable structure usually with exposed hydrophobic patches leading to molecular adhesion [13–15].

To have insight into pH-induced aggregation of proteins, BSA has been selected as a model. It has a tendency to aggregate and a wealth of information on its structure and properties are known [16,17]. Secondly, serum albumin remains in circulation at ~50 mg/ml. This leaves a suspicion of its role in the aggregation of accompanying proteins [18]. Thirdly, its easy availability permits physical characterization. Moreover, X-ray crystallographic structure of human serum albumin (HSA), bearing strong sequence homology with BSA, is known at high resolution [19]. It is interesting that though BSA serves as a model in many cases, it has unique properties like binding with a large number of ligands and fatty acids [16,17]. Here, we report characterization of a dimeric state of BSA under mildly acidic condition that appears to play a role in the initiation of aggregation.

2. Materials and methods

2.1. Reagents

BSA (fraction V, 96–99% purity by gel electrophoresis), guanidinium, HCl, (Gdm. HCl, 8 M, sequential grade), pyrene, PM, 1-ANS, MMTS, NEM, iodoacetamide, NBD-Cl and BAL were from Sigma and DTNB was from Pierce chemicals.

2.2. Isolation of monomeric BSA

BSA as procured contained 4–6% of high Mw impurity or multimers which were separated using a Waters Protein Pak 125 SE-HPLC column (fractionation range 20–125 kDa) equilibrated with 20 mM Na-phosphate, pH 7.0 containing 0.2 M NaCl at a flow rate of 0.5 ml/min. Elution was followed at 280 nm. The column was pre-calibrated with the marker proteins: alcohol dehydrogenase (150 kDa), hemoglobin (64 kDa), ovalbumin (43 kDa), lysozyme (14 kDa) and cytochrome *c* (14.3 kDa) where a linear

dependence of log Mw versus elution volume was observed. The major peak corresponding to 65–67 kDa was pooled as monomeric BSA.

2.3. Rayleigh's scattering

Rayleigh's scattering at 90° was measured with a Hitachi F 4500 spectrofluorimeter having excitation and emission wavelengths at 350 nm and using slit width of 2.5 nm each. BSA has no absorption at 350 nm (<0.001 for a 5-mg/ml solution). A 3-ml quartz cuvette with teflon cover in a holder attached to a circulating water bath (25±0.5 °C, Polyscience, USA) was used. Samples, after centrifugation was passed through 0.45 µ nylon filter membrane (Millipore). General precautions for washing and dust contaminations were followed [20]. Except kinetic measurements, a 300-s time scan was set for each reading. The lowest *and not the average* scattering was recorded. Minor fluctuation of scattering was unavoidable due to dust contamination during transfer of samples.

For solution of macromolecules or macromolecular assemblies, the basic equation for the angular dependence of light scattering is the Debye–Zimm relation [21]. Considering factors like very low protein concentration, scattering at 90°, irradiation at 350 nm, spherical nature, same partial specific volume of globular proteins and radius of gyration of medium size globular proteins [22], the relation is reduced to $R_\theta \approx K C r_s^3$ where R_θ is the scattering intensity, K is a constant depending on viscosity of solvent, C is concentration of macromolecule and r_s is the Stokes radius. Validity of the relation has been experimentally verified in details ([23], to be communicated elsewhere). A linear dependence of scattering intensity with concentration of BSA was observed (between 0–40 and 0–60 µg/ml at pH 7.0 and 4.2 respectively having $R^2=0.9988$ and 0.9990, where R^2 is regression coefficient). This indicated absence of self-association, etc. under the experimental conditions. The derivation has been explained in Appendix A.

It was ensured that incubation of BSA at low pH was free from out-of-phase precipitation from turbidity measurement at spectral zone where BSA has no absorption. A solution of 0.5–5 mg/ml of BSA at pH 4.2 or 7.0 had A_{350} or $A_{600\text{ nm}}$ of <0.001. Further 5 mg/ml of BSA between pH 2.0 and 7.0 had identical $A_{280\text{ nm}}$ indicating absence of precipitation.

2.4. 'Spin column' centrifugation

Proteins in 100 µl aliquots were loaded onto a pre-spun (2000 rpm for 1 min in a Remi R 8C bench top centrifuge) Sephadex G-100 column (2.9 × 0.7 cm, usually referred as 'spin column') equilibrated with buffers of desired pH, and were eluted by centrifugation under identical conditions. Recovery of BSA at pH 7.0 was 30–35% that was consistent with previous report [24]. Higher recovery of

BSA was related with apparent increase of Stokes radius arising from partial unfolding or multimerization.

2.5. Ultracentrifugation

2.5.1. Sucrose density fractionations

Sucrose density gradient (4–20%) ultracentrifugation was performed in 30 ml tubes using a Beckman L8-80 M ultracentrifuge at 40,000 rpm and at 4 °C for 8 h. Protein samples, (100 μ l, 1 mg/ml) pre-equilibrated with buffer of pH 7.0 or 4.2 for 20 h at 25 °C were placed over sucrose layers. After centrifugation, 1-ml fractions were collected from the bottom of the tubes using a peristaltic pump and distribution of protein was followed by $A_{280\text{ nm}}$ after dilution to 1 ml with water. Cytochrome *c* (12 kDa), ovalbumin (45 kDa), hemoglobin (62 kDa), BSA at pH 7.0 (66 kDa) and alcohol dehydrogenase (150 kDa) as molecular weight marker (100 μ l, 1–5 mg/ml) were also run in parallel [25].

2.5.2. Sedimentation velocity

An analytical ultracentrifuge (Beckman Coulter A-1) was used to determine sedimentation coefficient of BSA at pH 4.2. The protein in three different concentrations in 20 mM Na-acetate, pH 4.2 in a 500- μ l quartz cuvette was centrifuged at 50,000 r.p.m. for 6 h and at 20 °C. Data acquisition and analysis were done by SEDFIT analysis software that yielded $S_{20,W}$ for each set. $S_{20,W}^0$ was derived from the relation:

$$S_{20,W} = S_{20,W}^0(1 - k_S c),$$

where the constant k_S reflects non-ideality effects of the system and c is protein concentration in mg/ml. Once k_S and $S_{20,W}^0$ are known, the molecular weight of the protein was obtained from:

$$M_r = \left(6\pi\eta_{20,w}S_{20,W}^0\right)^{1.5} (3k_S/8\pi)^{0.5}$$

where $\eta_{20,w}$ is the viscosity of the solvent [21].

2.6. Binding of fluorescence probes

The emission spectrum of monomeric pyrene is sensitive towards solvent polarity and is used to estimate polarity of pyrene interacting sites of macromolecules [26,27]. While exciting at 335 nm in apolar solvents, its emission spectrum is often split into five vibronic peaks at 373 nm (I_1), 378 nm (I_2), 384 nm (I_3), 389 nm (I_4) and 394 nm (I_5). In polar and aqueous medium, usually I_1 , I_3 and I_5 are visible. Pyrene maintains a linear relationship between the ratio (I_1/I_3) and apparent dielectric constants of solvents (ϵ). A calibration curve was constructed using 20 nM of pyrene with the following solvents having ϵ and I_1/I_3 as follows; water (78.5, 1.84), 25% methanol (67.0, 1.60), 50% methanol (55.56; 1.59), 75% methanol (44.09; 1.49), methanol (32.63; 1.39), ethanol (24.3; 1.21), diethylether (3.0; 1.03) (Fig. 5a, upper inset) [28]. Excitation and emission bandwidths were 2.5 and 5 nm, respectively. Interaction of

BSA and pyrene was followed between 2.5–100 μ M and 0.2–5 μ M, respectively. The labeling ratio of pyrene to BSA was 2:1 (molar ratio). Association constant of pyrene binding was calculated after [29].

PM reacts specifically with cysteine residues and the covalently attached fluorophore monitors hydrophobicity around the labeled residue [30]. BSA (1 mg/ml) at pH 7.0 was labeled for 60 min at 25 °C by adding 50 fold molar excess of pyrene maleimide (0.07 mM) from a stock in DMSO. Adding excess of 2-ME quenched the reaction. The labeled protein was separated from free PM by dialyzing against low salt buffer, pH 7.0. The concentration of PM-BSA was determined after [31]. Concentration of PM was determined from the absorbance using $\epsilon_{343\text{ nm}} = 42,000\text{ M}^{-1}\text{ cm}^{-1}$ for determining the labeling ratio in PM-BSA [32,33]. The labeling ratio of pyrene to BSA was found to be 1.57.

ANS is another hydrophobic surface sensing fluorescence probe for macromolecules whose requirements for anchoring is different from pyrene [34]. BSA (5 μ g/ml) was equilibrated with buffers between pH 2.0 and 8.0 and was treated with 65 μ M of ANS for 15 min. The interaction was followed fluorimetrically (ex: 375 nm; em: 400–600 nm; $\lambda_{\text{em,max}} = 520\text{ nm}$).

2.7. Modification of cysteine residue

BSA (5 mg/ml) in 50 mM Na-phosphate, pH 7.5 was treated with 20 mM DTNB and was followed spectrophotometrically for 30 min using $\epsilon_{412\text{ nm}} = 13,600\text{ M}^{-1}\text{ cm}^{-1}$ for the product [35]. Modification of cysteine reached maximum of 0.72 moles/mole of BSA. BSA (1 mg/ml) in 50 mM Na-phosphate, pH 8.0 was treated with 3-fold molar excess of iodoacetamide for 30 min followed by dialysis against 5 mM Na-phosphate, pH 7.0 [36]. An aqueous solution of iodoacetamide, saturated with nitrogen served as stock and all treatments were done in the dark. Reactions with BAL, NEM, NBD-Cl and MMTS were carried out using protein and reagent concentrations of 1–5 mg/ml and 1–2 mM respectively in presence of 50 mM Na-phosphate, pH 7.0 for 30 min at ambient temperature [37]. Excess reagents were removed by dialysis against low salt buffer at pH 7.0. Estimation of free cysteine residue of these modified proteins with DTNB indicated a value of 0.01 ± 0.01 residues/mole.

2.8. Spectroscopic methods

Fluorescence measurements were done with a Hitachi F 4500 recording spectrofluorimeter having excitation and emission slit widths of 2.5 nm. Conformation change of proteins was monitored from intrinsic fluorescence (ex: 280 or 295 nm; em: 300–400 nm). Optical absorbance was recorded with SICO 200 UV-VIS (India) or Analytik Jena Specord 200 (Germany) recording spectrophotometer. Turbidity of protein solutions was measured at 650 nm with the Specord spectrophotometer. Far UV-CD (190–

250 nm) spectra of the proteins (30 $\mu\text{g/ml}$), incubated in buffers of pH 7.0 and 4.2 at 30 $^{\circ}\text{C}$ for 5 h, were recorded in a 1-mm path length cell, using a J 720 (Jasco) spectropolarimeter. The spectra were recorded with response time of 2 s and scan speed of 20 nm/min. Each data point was an average of five accumulations. FTIR measurements were done with a Boeman IR spectrometer equipped with a dTGS detector. Protein (10 μl , 1 mg/ml) in buffer of pH 7.0 or 4.2 was placed on an ATR attachment with a 6- μm spacer. Air and buffer corrections were done using instrumental programming.

2.9. Atomic distances of modification reagents

Using CS Chem 3D ultra software program, simulation of models of different cysteine modifying reagents was done. The models of adduct after modification of Cys-SH of protein (assumed as $\text{CH}_3\text{-SH}$) were further generated after energy minimization and then distance between the sulfur and the utmost atom was noted.

2.10. Modeling studies

Three-dimensional structure of bovine serum albumin (BSA) was generated by knowledge-based homology modeling using Accelrys, 2000 (San Diego, CA), ABGEN [38], and in UNIX environment. Energy minimization and molecular dynamics were performed with the Accelrys, 2000 (San Diego, CA) package using the cff 91 force field on a Silicon Graphics OCTANE workstation. Energy minimizations were done with a convergence criterion of 0.001 kcal/mol, using a combination of steepest descent and conjugate gradient methods (100 steps each). Molecular dynamics simulations were performed using a time step of 1 for 100 steps of equilibration 1000 steps of dynamics. Repeated steps of molecular dynamics, selection of conformation with least potential energy, and energy minimization were performed until satisfactory conformational parameters were obtained. The electrostatic potential surfaces of the protein models were determined by MOLMOL [39]. Protein BLAST was used for searching the homologous proteins of known structures taking BSA as the query sequence in the Protein Data Bank database [40]. The alignment result of HSA and BSA sequences showed conserved amino acid of 436 out of 576, over all score, 75.7% and homology score=47150.

2.11. Other methods

Protein concentrations at pH 7.5 were determined using $\epsilon_{280\text{ nm}}^{\text{M}}$ [41]. Measurement of pH was done with ELICO pH meter after calibration with standard buffers. The following buffers were used: 20 mM Na-acetate, pH 3.0–6.0, 20 mM Na-phosphate, pH 6.5–8.0 and 20 mM Tris, HCl, pH 8.5–11.0. When a change of buffer was required, the protein was initially dissolved in 5 mM buffer followed by 10-fold

dilution with 50 mM buffer of desired pH. Attainment of pH was ensured from dummy sets. The isoelectric point of BSA being 4.7, its PAGE profiles were generated at pH 4.2 after reversing the polarity of the electrophoresis apparatus.

3. Results

3.1. Light scattering from low pH conformers

Rise of Rayleigh's scattering from a protein solution under non-denaturing condition is an indication of aggregation. Denaturation, on the other hand, helps penetration of solvents to the core structure of proteins, whereby difference of refractive index between the solvent and solute is reduced leading to lowering of scattering intensity. Scattering of BSA between pH 7.0 and 2.0 showed a maximum of 3.5-fold exactly at pH 4.2 followed by gradual fall to one fifth of its intensity at pH 7.0 (Fig. 1 and inset). It indicated that before pH-denaturation, BSA underwent aggregation that was most prominent at pH 4.2. Removal of high molecular weight impurities in BSA did not change the profile suggesting absence of their role in enhancing the scattering.

An approximate calculation relating Stoke's radius, scattering intensity and multimericity of BSA conformers were done; for example, a BSA solution of concentration C and $r_s=33.9\text{ \AA}$ [42], scattering intensity at pH 7.0 will be $R_{\theta[1]}=38.96 \times 10^3\text{ K}$. C (in arbitrary units) where the subscript $[n]$ stands for multimericity. In case, the monomers under identical experimental conditions form a dimer, trimer, tetramer or pentamer by point contact on surface without unfolding, concentration and Stokes radius of the particles will be $C/2$, $C/3$, $C/4$, $C/5$ and 67.8, 74.6, 101.7 and 101.7 $\text{\AA}$, respectively (approximately as derived from solid sphere model). Thus, the scattering ratios will be $R_{\theta[2]}/R_{\theta[1]}=3.99$; $R_{\theta[3]}/R_{\theta[1]}=3.54$; $R_{\theta[4]}/R_{\theta[1]}=6.74$ and $R_{\theta[5]}/R_{\theta[1]}=5.41$, respectively. Observed $R_{\theta[\text{pH } 4.2]}/R_{\theta[\text{pH } 7.0]}$ for

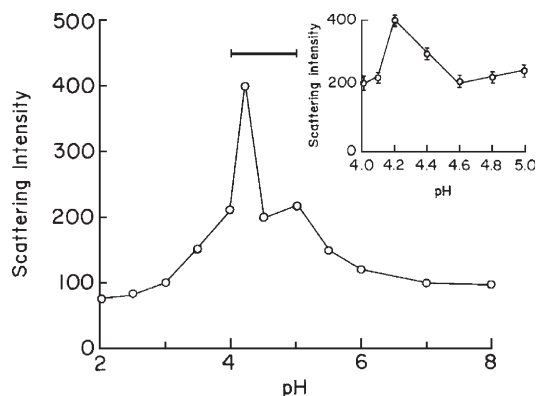


Fig. 1. Change in scattering intensity of BSA (0.5 mg/ml) between pH 2.0 and 8.0. Data were presented after correction of background intensity from buffers. (Inset) The portion between pH 4.0 and 5.0 as marked by the bar in the original figure has been enlarged.

BSA being 3.55, this was suggestive of average multimericity of the conformer to be in the order of 2–3.

3.2. Molecular state

A Sephadex G-100 ‘spin column’ was conveniently applied to know the possible range of molecular weight of the conformer at pH 4.2. Recovery of BSA between pH 7.0 and 8.0 was $31 \pm 5\%$, consistent with its calibration profile [24]. Recovery increased monotonously up to 80% as pH was lowered to 2.0. However, compared to pH 4.1 and 4.3, there was a rise of around 10% recovery at pH 4.2 (Fig. 2). This profile was reproducible and suggestive of formation of small aggregates of multimericity 2–3 on an average. In case BSA trimers were formed accompanied by partial unfolding, expected recovery was $>95\%$.

SDS-PAGE profiles of BSA exposed at pH 4.2 between 1 and 3 h in presence and absence of 2-ME were almost identical with that of native protein at pH 7.0, indicating that the conformer at pH 4.2 was devoid of disulphide links. PAGE at pH 4.2 revealed a fast migrating major band accompanied by two other slower migrating minor components but no high molecular weight aggregate denying entry into the gel. PAGE profiles at pH 7.0 with or without preincubation at pH 4.2 showed that exposure at low pH allowed aggregate formation to a low extent (results not shown). Thus, the aggregate formed was unstable in electrophoresis medium.

A 4–20% sucrose density ultracentrifugation at pH 4.2 determined the molecular state of BSA from its migration with respect to a calibration curve (Fig. 3a and inset). It showed that at pH 4.2, BSA acquired essentially a dimeric structure without accumulation of higher multimers consistent with previous results.

Monomeric and dimeric BSA at pH 7.0 have been subjected to extensive analytical ultracentrifugal studies yielding $S_{20,w}^0$ of 4.5 and 6.7, respectively [16]. BSA at pH 4.2 showed concentration dependence of $S_{20,w}$ as follows; 0.45 mg/ml, 6.46; 0.86 mg/ml, 6.67 and 1.29 mg/ml, 6.88. Linear relation between $S_{20,w}$ versus concentration yielded

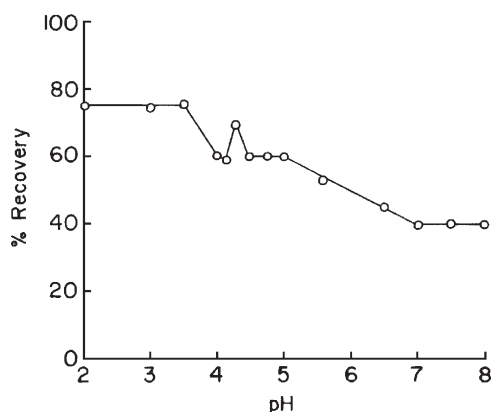


Fig. 2. Recovery of BSA (1 mg/ml) from Sephadex G-50 ‘spin columns’ equilibrated with buffers of pH 2.0–8.0.

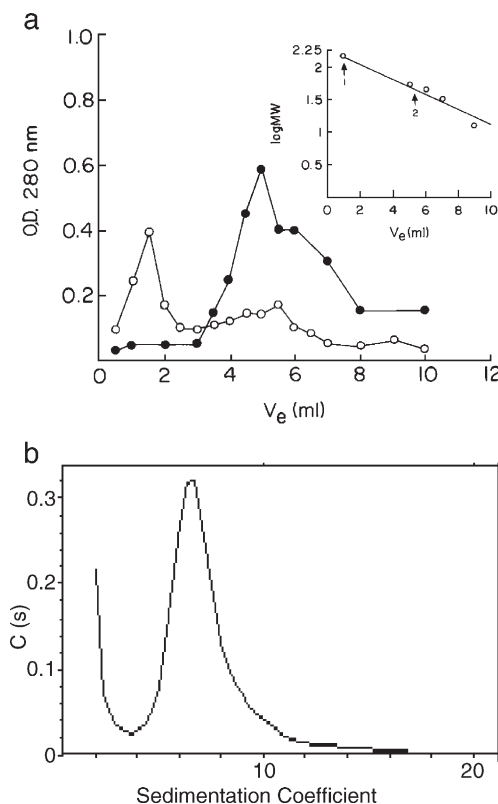


Fig. 3. (a) Ultracentrifugal patterns of BSA at pH 7.0 (solid circles) and 4.2 (open circles). A volume of 100 μ l containing 1 mg of BSA was applied in each case. Experimental conditions have been described in the text. (Inset) Calibration curve of the ultracentrifugal run showing dependence of log Mw versus elution volume (V_e) from the bottom of the centrifuge tubes. The open circles, 1 and 2 represent positions of marker proteins, BSA at pH 4.2 and pH 7.0, respectively. (b) Distribution of sedimentation coefficients of BSA (0.86 mg/ml) at pH 4.2. The peak top corresponds to $S_{20,w}$ of 6.67.

$S_{20,w}^0$ and k_s of 6.28 and 0.063 ml/mg. This led to approximate molecular weight of the conformer to be 112 kDa. This value, though lower for the dimer, matched well to 117 kDa reported for the dimer from [43]. Distribution profile of BSA (0.86 mg/ml) at pH 4.2 against $S_{20,w}$ was symmetrical (Fig. 3b). Lower concentration of the protein under identical centrifugal condition yielded an asymmetric profile indicating presence of low proportion of particles of $S_{20,w}$ 4.5 likely to be the monomers. Good correlation between $S_{20,w}$ of the dimer at pH 4.2 and 7.0 appeared to originate from its shape at neutral pH where a substantial part of the monomers remain non-overlapping. This leaves adequate provision for partial unfolding of the molecule to attend similar $S_{20,w}$ [43]. Movement of the boundary in the sedimentation velocity experiments was symmetric and thus nearly complete conversion to the dimeric conformer was evident.

It is worthwhile to note that application of Sephadex G-200 gel filtration chromatography and Waters Protein Pak 300 SE-HPLC were unsuccessful in determining the molecular mass at pH 4.2. In either case, the protein adhered to the column leading to abnormal migration. Generation of unusual properties of partially unfolded proteins has been reported earlier [44].

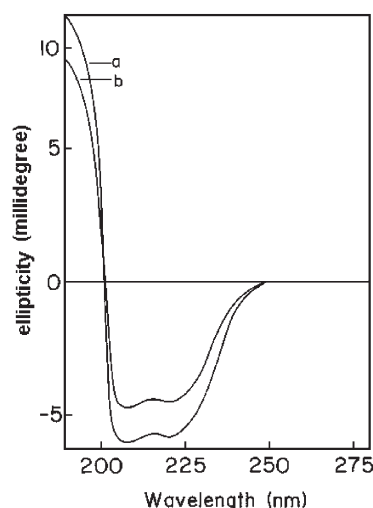


Fig. 4. Far-UV CD spectra of BSA (30 $\mu\text{g/ml}$) at pH 7.0 (a) and pH 4.2 (b) between 190 and 280 nm. Data were presented after solvent correction and averaging each set ($n = 5$). $[\theta]_{\text{MRE}}$ at 225 nm observed for BSA at pH 7.0 and pH 4.2 were 20,280° and 14,550° $\text{cm}^2 \text{dmol}^{-1}$ respectively. Corresponding $[\theta]_{\text{MRE}}$ at pH 7.0 was reported earlier to be approximately 20,000° $\text{cm}^2 \text{dmol}^{-1}$ [54].

3.3. Physical properties of pH 4.2 conformer

The CD spectra of BSA at pH 4.2 between 190 and 250 nm showed maximum molar ellipticity at 208 nm typical of a protein containing significant amount of α -helical structure (Fig. 4). Analysis after Chow and Fasman [45], yielded 43% helicity of this conformer. This is comparable to 55, 45 and 33% helicity of BSA conformers at pH 7.0, 4.3 and 2.9, respectively [16].

Alteration of surface hydrophobicity of proteins under mildly denaturing conditions is an important parameter that generates new properties like molecular adhesion, impairment of solubility etc [13–15]. Emission patterns of pyrene, interacting with BSA at pH 7.0 or 4.2 was found to be exactly overlapping (Fig. 5a). The I_1/I_3 ratio of these spectra at the respective pH was 1.44 and 1.42, which indicated a dielectric constant of 35 of the pyrene binding site. Polarity of the binding site was derived from the linear dependence of I_1/I_3 of pyrene emission versus dielectric constants of different solvents (Fig. 5a, upper inset). Calculated association constant (K_a) for pyrene binding at pH 7.0 and 4.2 were $24.0 \pm 1.2 \times 10^4 \text{ M}^{-1}$ and $19.5 \pm 1.5 \times 10^4 \text{ M}^{-1}$ respectively. This suggested higher affinity for pyrene at pH 7.0 (Fig. 5a, lower inset). Contrasting results were obtained after interaction with another fluorescence probe 1-ANS. It also senses hydrophobic patches on protein surfaces with enhancement of emission intensity and blue shift of emission maxima (ex: 375 nm; em: 520 $\rightarrow$ 480 nm). Emission intensity of ANS interacting with BSA showed a sharp increase of around 20-fold exactly at pH 4.2 indicating anchoring of the probe specifically with the conformer (Fig. 5b).

FT-IR spectroscopy is amenable to estimate the overall secondary structure of large proteins under equilibrium and

non-equilibrium conditions [46]. The mode by far best characterized is the so-called amide I band (1600–1700 cm^{-1}) together with less characterized amide II and amide III bands [47]. Overlapping FT-IR spectra of BSA at pH 7.0 and 4.2 show that the peak at 3306 for pH 4.2 conformer, responsible for C–H, O–H and N–H stretching, was absent at pH 7.0. This is due to conformational change of BSA at low pH, where the molecule was partially unfolded and the bonds got stretched (result not shown).

3.4. Role of Cys 34

Different sulphydryl modification reagents were employed to ascertain the role of Cys-34 in the dimerization process after confirming no significant oxidation of this residue. Pyrene maleimide (PM), an effective thiol modifier, contains a fluorescence reporter group that monitored the hydrophobicity of the cysteine group modified. BSA was

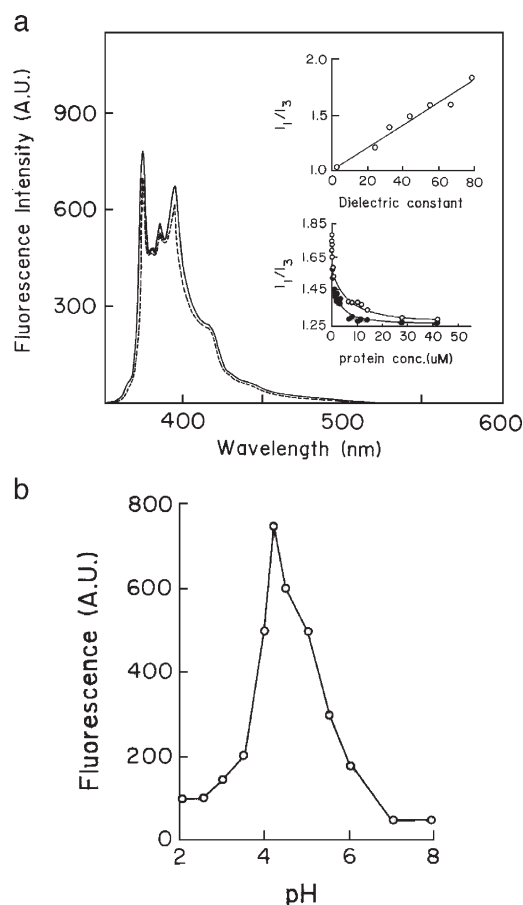
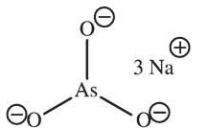
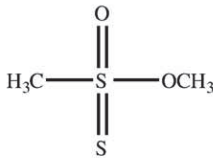
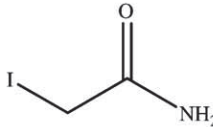
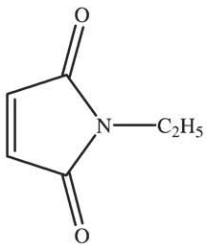
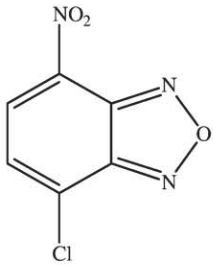
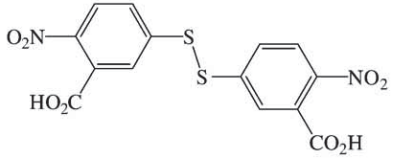
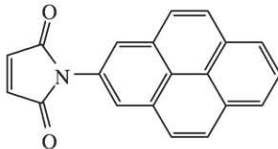


Fig. 5. (a) Fluorescence spectra of 0.2 μM of pyrene in presence of 5 $\mu\text{g/ml}$ of BSA at 25 °C showing five vibronic peaks at 373, 378, 384, 389 and 394 nm. Spectrum of BSA at pH 7.0 (solid line) and pH 4.2 (dotted line) were indistinguishable. (Inset upper panel) Calibration curve showing linear dependence of the ratio, I_1/I_3 , and apparent dielectric constant, ϵ of various solvents. (Inset lower panel) Plot of the ratio I_1/I_3 of pyrene fluorescence as a function of increasing BSA concentrations at pH 7.0 ($\bullet$) and 4.2 ($\circ$). Ex: 335 nm. (b) Interaction of ANS (65 μM) with BSA (20 $\mu\text{g/ml}$) between pH 2.0 and 8.0. Ex: 375 nm; Em: 480 nm.

Table 1
Modification of Cys-34 residue with thiol modifying reagents

Thiol modifiers	Structure	Arm length (Å)	Raise of scattering (%) ^a
Sodium arsenite-BAL		3.320	130
Methyl methane thiosulphonate		4.125	120
Iodoacetamide		4.804	100
N-Ethyl maleimide		6.170	5
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole		6.902	2
5, 5'-Dithiobis-(2-nitrobenzoic acid)		8.023	0
N-(1-Pyrenyl) maleimide		12.195	0

^a An increase of 100% corresponds to rise of scattering intensity of unmodified BSA while changing its pH from 7.0 to 4.2.

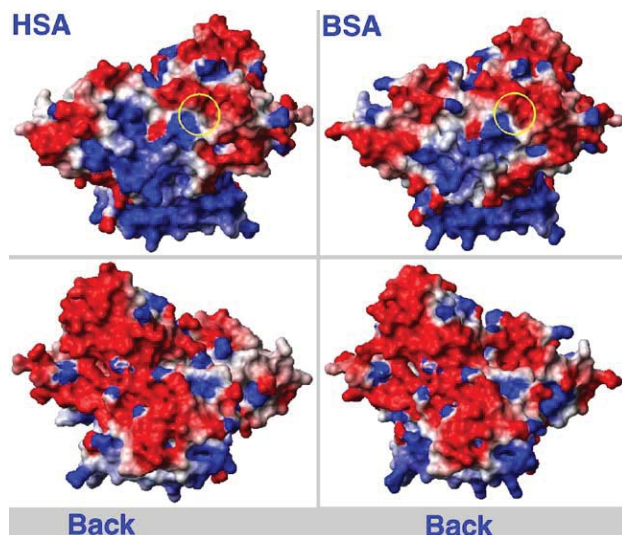


Fig. 6. Homology modeling of BSA (right panel) as compared to HSA (left panel). Negatively and positively charged residues have been presented as red and blue, respectively, while white represents both hydrophobic and neutral polar residues. Position of Cys-34 has marked with a yellow circle.

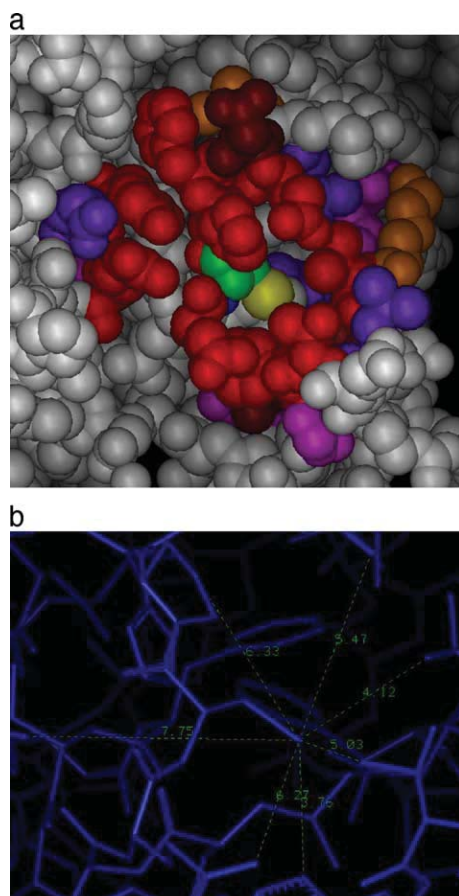


Fig. 7. (a) A close up view of space filling model of BSA around Cys-34 residue marked as yellow and green. The red, brown, violet, orange and pink residues are located within 6, 7, 8, 9 and 10 Å from Cys-34, respectively. (b) Stick representation showing distances (green) of different residues (blue) around Cys-34.

modified with PM at pH 7.0 followed by dialysis against buffers of pH 7.0 and 4.2. The dialyzed samples generated indistinguishable emission spectra after excitation at 335 nm after normalization. The I_1/I_3 ratio, vibronic tones of the emission spectra of the protein conjugate related with hydrophobic environment of the fluorophore, was 0.818 and 0.820 at pH 7.0 and 4.2, respectively. No excimer formation of the PM-derivatives, related with enhancement of emission intensity centered at 450 nm was observed. This indicated absence of molecular adhesion of the derivatives. It was interesting to note that when the pH of the protein conjugate was readjusted from 7.0 to 4.2, no enhancement of scattering intensity was observed suggesting prevention of dimer formation.

To investigate this further, a series of modification reagents were selected having variable arm lengths. They were classified into two groups, one having arm length between 3.0 and 5.0 Å comprising of BAL, MMTS and IA; while the other having arm length between 6.0 and 12.0 Å comprising of NEM, NBD-Cl, DTNB and PM. BSA was modified at pH 7.0 by these reagents. The rise of scattering of modified BSA by the former class of reagents after exposure to pH 4.2 was at par with the unmodified protein. This indicated formation of the dimer at pH 4.2. In contrast, under similar treatment with the later class of reagents, the rise of scattering was completely prevented. The results have been presented in Table 1. This indicated that the protruding end of the reagents that went off the cleft played a role in preventing dimerization.

3.5. Modeling studies

Based on 78% amino acid sequence homology between HSA and BSA and reported X-ray crystallographic structure of HSA [19], structure of BSA has been derived by molecular simulation. Similar derived structure of BSA has been used in comparison to HSA for ionic surfactant studies [48]. Front and back views of the structures of BSA and HSA show similar overall spherical patterns (Fig. 6). A remarkable feature of these structures is most of the surface residues were polar supporting their high solubility

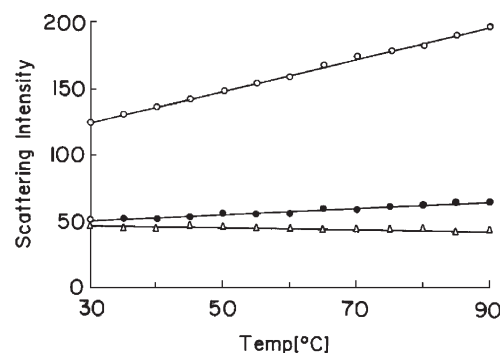


Fig. 8. Increase of scattering intensity of BSA (1 mg/ml) with rise of temperature at pH 7.0 (Δ), 3.0 (●) and 4.2 (○). Heating rate was 2 °C / min.

in aqueous solvents. Location of Cys-34 has been marked. The structure of BSA has also been represented where position of Cys-34 is visible on the surface of the molecule but in a cleft (Fig. 7a). When this site was magnified, Cys-34 in the cleft was clearly visible. This structure further allowed identification of the surface residues constituting the cleft region and measurement of distances from Cys-34. These are Tyr-84 (distance in Å 3.76); Val-77 (4.12); Thr-83 (5.03); Glu-38 (5.47); Gln-33 (6.27); Pro-35 (6.33) and Arg-144 (7.75) (Fig. 7b). Thus, it has been observed that most of the residues constituting the surface of the cleft were within 6 Å from Cys34, thus measuring an approximate depth of the cleft. This matches exactly with the chemical modification data (Table 1).

3.6. Irreversibility

Assuming that the rise of scattering at pH 4.2 originated from dimerization, BSA (1.00 mg/ml) was initially incubated in 10 mM Na-acetate, pH 4.2 for 10 min followed by 10-fold dilution with 250 mM Na-acetate, pH 4.2 or Na-phosphate, pH 7.0. In either case, the scattering intensities were almost similar and remained constant for an hour. Thus, the quaternary structure of the conformer at pH 4.2 remained stable at pH 7.0. This irreversible character was also indicated after interaction with the fluorophore ANS. Also, the enhanced emission intensity of the conformer at pH 4.2 was retained by 95% once the pH of the solution was readjusted to 7.0 by dilution with buffer and was stable for an hour.

3.7. Thermal stability

Stability of BSA is reflected against its thermal aggregation. Scattering intensity of BSA (1.00 mg/ml) at pH 7.0 remained constant once the temperature was raised from 30 to 90 °C. In contrast, the conformer at pH 4.2 under identical conditions showed continuous rise of scattering by about 2-fold. Presence and absence of high molecular weight impurities in BSA could not alter this scattering profile. Thus, the aggregate prone character of the pH 4.2 conformer was not induced by the multimers present. Further the conformer of pH 3.0, where dimerization was apparently prevented, was similarly treated assuming that unfolding might expose additional hydrophobic patches to help multimer formation. But the tendency was low. Relative rates of aggregate formation at pH 7.0, 4.2 and 3.0 were 0.0, 1.3 and 0.3, respectively (in arbitrary units of change of scattering intensity/°C) (Fig. 8).

4. Discussion

BSA at pH 4.2 irreversibly forms a partially unfolded dimeric intermediate with alteration of surface hydrophobicity. BSA is known to undergo pH induced conformational

isomerization, one having transition at pH 4.3 (N ↔ F) and the other at pH 2.7 (F ↔ E, where N, F and E represent native, fast and expanded states) accompanied by loss of helix content from 55 to 35% [16,17]. This report presents that the conformer at pH 4.2, presumably close to F state, could attain a dimeric configuration.

The contact point of the dimeric molecule has been assigned to the region around the sole Cys-34 residue by modification reagents. A decisive factor of 6 Å arm length of reagents that prevented dimerization is comparable to the depth of the cleft where Cys-34 resides (Fig. 7a). This cysteine plays crucial role in achieving various structural alterations in the molecule. Blocking of this residue prevented formation of mixed disulphides in aged albumin, as well as the occurrence of the albumin dimer [16]. Previous studies suggested that as temperature was raised, ‘some molecular regions’ become accessible to new intramolecular interactions, producing soluble aggregates through disulphide and noncovalent bonds [49,50]. Involvement of Cys-34 in this process was later indicated [51,52]. It is rather difficult to conceive that this region is so important in molecular adhesion because hydrophobic residues do not prevail this area as per molecular modeling (Fig. 6).

The significance of this study rests on direct correlation between prevention of dimer formation and thermal aggregation. Our preliminary studies indicate that only those reagents, which prevented dimer formation, also protected BSA from thermal aggregation (Fig. 7 and Table 1). Formation of aggregates follows sequential steps starting from molecular associates of very low multimetricity [1,53]. ‘In following a kinetic process, modeling the initiation of the process poses the greatest challenges. This is because the later stages are usually more amenable to direct observation, whereas the initial phases are more likely to be controlled by intermediates that are difficult to observe directly’ [10]. Whether a similar dimeric structure plays vital roles in initiation of aggregation of BSA under other stress conditions remains speculative at this stage.

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Appendix A

For solutions of macromolecules like proteins, the basic equation for light scattering in the uv-vis zone is the Debye–Zimm relation [21]:

$$Kc/R_\theta = \left\{ 1 + \left(16\pi^2 R_g^2 / 3\lambda^2 \right) \sin^2(\theta/2) \right\} (1/M_r) + 2Bc, \quad (1)$$

where, R_θ =Rayleigh scattering, c =concentration of protein, K =an experimental constant dependant on solvent refractive index, R_g =radius of gyration, M_r =weight average molecular weight of the protein, λ =wavelength of scattering light, θ =angle of scattering and B =second virial coefficient.

In case of a spectrofluorimeter, $\theta=90^\circ$ and for very low concentration of protein, where $2Bc \rightarrow 0$; Eq. (1). is reduced to:

$$Kc/R_\theta = \left\{ 1 + \left(8\pi^2 R_g^2 / 3\lambda^2 \right) \right\} (1/M_r) \text{ or}$$

$$R_\theta/Kc = M_r \left\{ 1 + \left(8\pi^2 R_g^2 / 3\lambda^2 \right) \right\}^{-1} \approx M_r \left\{ 1 - 8\pi^2 R_g^2 / 3\lambda^2 \right\},$$

neglecting higher terms of the binomial by expression... (2)

For globular proteins up to around 100 kDa and irradiation at 350 nm, the composite term $(8\pi^2 R_g^2 / 3\lambda^2)$ attains negligible numerical value as has been illustrated for some standard proteins in Table 1.

For very large proteins this approximation is not valid, e.g., for myosin (493 kDa, $R_g=46.8$ nm), the composite term is 0.470. Thus with certain approximations Eq. (2) is reduced to,

$$R_\theta = K \cdot c \cdot M_r. \quad (3)$$

Further assuming that all globular proteins are spheres, Rayleigh's scattering will ultimately depend on Stoke's radius of the molecule as:

$$R_\theta = K \cdot c \cdot (4/3)\pi r_s^3 \rho \quad (4)$$

where r_s =Stokes radius and ρ =partial specific volume of proteins. The assumption that Mw is linearly related with r_s^3 , has been verified separately in cases of monomeric, dimeric and tetrameric proteins [42]. Eq. (4) may be further simplified to

$$R_\theta = K' \cdot c \cdot r_s^3 \text{ or } R_\theta \propto r_s^3 \quad (5)$$

where K' is a modified form of K . This constant term is related to the difference of refractive index between solvent and solute. Eq. (5), therefore indicates that R_θ should maintain a linear relation with K' , c and r_s^3 when any two of them remain invariable. These have been experimentally verified with a number of proteins under different experimental conditions [23].

Table 1

(Appendix A): Different proteins and their physical terms

Proteins	Mol. Wt. (kDa)	R_g (nm)	$(8\pi^2 R_g^2 / 3\lambda^2)$
Ribonuclease	12.7	1.48	0.00047
α -lactalbumin	13.5	1.45	0.00045
lysozyme	13.6	1.43	0.00044
β -lactoglobulin	36.7	2.17	0.0010
Bovine serum albumin	67.0	2.98	0.0019

When protein molecules come in contact with each other without denaturation, the association is assumed to be between solid spheres without fusion. Thus, for monomeric BSA of concentration C and $r_s=33.9$ Å, its scattering intensity will be, $R_{\theta[N]}=K'C[3.99]^3=38.96 \times 10^3 K'$. C (in arbitrary units), where $[N]$ stands for native conformer.

In case, monomeric BSA under identical experimental conditions form dimers, corresponding concentration and Stokes radius will be $C/2$ and $(33.9+33.9)=67.8$ Å, respectively.

Therefore, $R_{\theta[2]}=K' \cdot C/2 [67.8]^3$ (in same arbitrary units)= $155.83 \times 10^3 K'C$, where $[2]$ stands for dimer. So, $R_{\theta[2]}/R_{\theta[N]}=155.83/38.96=3.99$.

Similarly, using simple geometrical models, the ratios for trimeric, tetrameric and pentameric assemblies will be 3.54, 6.74 and 5.4 respectively. In case of BSA, $R_{\theta[pH 4.2]}/R_{\theta[pH 7.0]}=3.55$. Though it matches very closely to trimer formation, in reality it may not be so because a degree of unfolding prohibits assumption of the spherical model. However, it suggests that the BSA conformer at pH 4.2 is a small assembly of monomers.

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