Investigation into pH – dependent conformational isomerisation of Human Serum Albumin using spectroscopic techniques

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By

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CERTIFICATE

This is to certify that dissertation entitled, "**Investigation into the pH-dependent conformational isomerisation of Human Serum Albumin using spectroscopic techniques**", being submitted by **Ms. Jaspreet Kaur** in partial fulfilment of requirements for the award of the degree of **Masters of Science** in **Biochemistry** and being submitted to the School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala is a bonafide work carried out by her under my supervision. The work has reached the standard necessary for submission, and the contents of this dissertation have not been submitted to any other university or institute for the award of any degree or diploma.

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CANDIDATES' DECLARATION

I, hereby, declare that the work being presented in the dissertation entitled **"Investigation into the pH-dependent conformational isomerisation of Human Serum Albumin using spectroscopic techniques"** in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in Biochemistry and being submitted to School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala is my own research work carried out during the period of January to July 2018 under the supervision of **Dr. Mily Bhattacharya**. I have not submitted the contents embodied in this dissertation for the award of any degree elsewhere.

Jaspreet Kaur

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Date: 30/6/18

It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

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ABSTRACT

Human serum albumin (HSA) is a 585-residue, model all α-helical protein comprising three homologous domains (I, II, and III), which is present in the circulatory system and acts as a carrier for various kinds of ligands, metabolites, drugs, etc. Earlier literature reports have indicated that HSA undergoes reversible conformational transitions depending on the solution conditions such as changes in pH, temperature, and also in the presence of denaturants whereby non-covalent interactions such as hydrogen bonding, hydrophobic and van der Waals are disrupted but the disulphides remain intact. In this thesis, we have carried out a systematic study on the conformational isomerisation of HSA as a function of pH using fluorescence and CD spectroscopic techniques. The fluorescence intensity and anisotropy measurements were performed using intrinsic (single tryptophan, Trp-214) and extrinsic (1,8-anilinonaphthalene sulfonate, ANS, hydrophobicity reporter) fluorophores. Results obtained from our spectroscopic measurements suggested that HSA forms a partially-expanded, molten globule-like state at pH 3.5 with a concurrent loss of α-helical content during N↔F transition that appears to be a two-state transition. Additionally, our equilibrium unfolding studies utilizing both thermally- and chemically-induced denaturation of HSA revealed that the unfolding of the molten globule-like state, populated at pH 3.5, occurs in a non-cooperative manner and is less stable than the native HSA at neutral pH.

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LIST OF ABBREVIATIONS

Abbreviation	Name
ANS	8-anilinonaphthalene,1-sulfonic acid
BSA	Bovine Serum Albumin
CD	Circular Dichroism
Cys	Cysteine (Amino acid)
DLS	Dynamic light scattering
DMG	Dry molten globule
DOM	Domain
FRET	Fluorescence resonance energy transfer
GdmCl	Guanidine hydrochloride
HSA	Human Serum Albumin
IAEDANS	5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid
LCP	Left circularly polarized
MRE	Mean residual ellipticity
Phe	Phenylalanine
RCP	Right circularly polarised
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet

Chapter 1 Introduction

Proteins are the most abundant biological functional macromolecules that are present in all cells and carry out a large number of important physiological functions such as transportation, catalysis, recognition, etc [1]. Proteins are biopolymers which are composed of L-α-amino acids that have varying side-chains in terms of size, polarity, electronic structure, etc. The structural hierarchy of a protein shows that the sequence of amino acids, joined together via amide bonds, gives rise to a primary structure. Interplay of various non-covalent interactions such as, van der Waals, hydrophobic interactions and hydrogen bonding, within the primary sequence, leads to the formation of two types of secondary structures i.e. β-strands and α-helices and that further lead to the formation of a tertiary, three-dimensional, folded globular structure mediated by long-range interactions. The diversity in the protein functions is governed by their three-dimensional structures. They act as enzymes, assist in various biochemical reactions by lowering the activation energy of reaction, provide immune protection and mechanical support, transmit nerve impulses, control growth and differentiation, transport and store molecules, etc. However, changes in cellular environments such as pH, temperature and ionic strength, can result in the destabilization of the protein structure that might result in loss-of-function [2]. The loss of structural integrity may cause partial unfolding which may form cross β-sheet-rich, misfolded protein supramolecular aggregates that are involved in a wide range of neurodegenerative diseases e.g. Alzheimer's disease, Parkinson's disease, Huntington's disease, etc [3].

Several studies have suggested that the destabilization of the native conformation of a protein is a crucial step in the formation of amyloid fibril [4]. Perturbation of the native structure leads to the formation of partially-folded intermediates, known as molten-globule state, which can

serve as amyloidogenic precursors. The molten globules are primarily classified into two types namely, wet molten globule and dry molten globule [5]. The wet molten globule retains some secondary structure and a fluctuating tertiary structure whereby side-chain packing interactions are perturbed and it possesses a water-solvated hydrophobic core. Whereas, the dry molten globule is an expanded form of the native state with loose and molten side chain packing and the hydrophobic core is devoid of any water molecule. In case of a multi-domain protein, the native conformation consists of an ensemble of equilibrium intermediate states (I) whose inter-domain region resembles a dry molten globule. The multi-domain proteins are capable of unfolding or refolding independently and various inter-domain interactions may affect the overall folding of proteins. So, the folding process is more complicated in multi-domain proteins such as in Human Serum Albumin (HSA) which has been used as a model protein for several protein folding and ligand – binding studies over the past few decades.

HSA is the most abundant (60% of the total plasma protein content) and a multi-functional protein in the circulatory system with a concentration of 5 g/100 mL in the blood [6]. It is a multi-domain protein which comprises a single polypeptide chain of 585 amino acid residues and has a molecular weight of ~67 kDa. It is an all α-helical protein containing three homologous domains namely, domain I (residues 1 - 195), domain II (residues 196 - 383), and domain III (residues 384 - 585) [7] and each domain is further composed of two sub-domains namely, A and B (Fig. 1.1). Each domain consists of 10 principal helices that are labelled as h1 - h10 [7]. At physiological pH, the arrangement of the domains of HSA resembles a heart-shaped structure and the native form is stabilized by various inter and intra-domain forces such as hydrophobic interactions, salt bridges, disulphide bonds, etc. HSA comprises a total of 17 disulphide bridges, one free cysteine (Cys-34 with a free thiol, -SH group) in domain I and a single tryptophan (Trp-214) in domain II [6]. It is the main carrier of non-

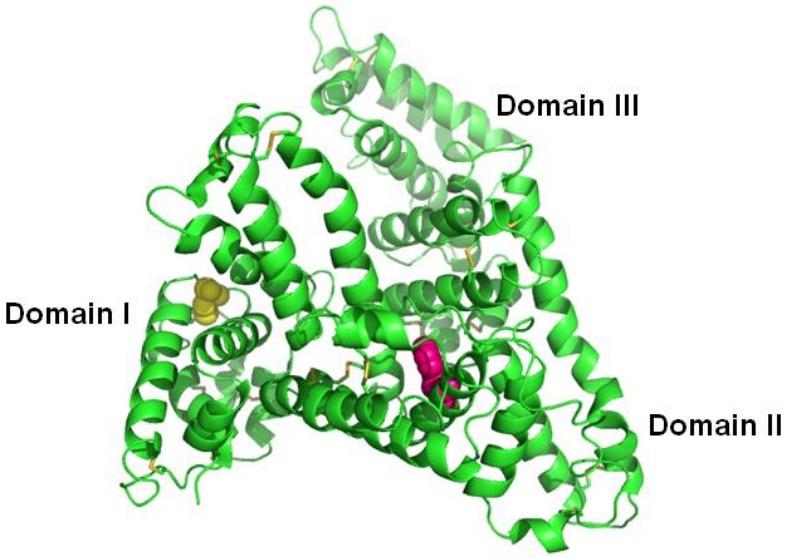

Fig. 1.1 Crystal structure of Human Serum Albumin (HSA); PDB ID: 1UOR, generated using PyMOL (Schrodinger, LLC). The single cysteine (Cys-34 in domain I) and the single tryptophan (Trp-214 in domain II) are shown as yellow and magenta spheres, respectively. The disulphide bonds are shown as yellow sticks.

esterified fatty acids and has a high affinity to a very wide range of substances including hormones, metabolites, nutrients, drugs and metals such as Zn^{2+} and Cu^{2+} [2]. The ligand binding regions are located in the hydrophobic cavities that are present in sub-domains IIA and IIIA [2]. The principal role of HSA is to transport solutes to their respective target organs in addition to the maintenance of the blood pH and colloid osmotic pressure of plasma [8].

Several studies have suggested that HSA undergoes reversible conformational transitions with changes in pH. At pH 7, HSA adopts the native conformation (N-form) which converts into a highly-charged, fast migrating form (F-form) at pH <4.3 that moves "fast" upon gel electrophoresis [12]. Upon further decrease in pH to < 2.7, the F-form changes to a fully extended form (E-form). On the other side, under alkaline conditions, the N-form changes to

a basic form (B-form) at pH >8 and at pH above 10, the structure changes to the aged form (A-form) [9]. Since, it has been reported that pH-induced conformational transitions are preserved across different species, it appears that these pH-dependent isomerization might be implicated in some important physiological functions of HSA. Therefore, it is important to investigate how does the protein structure and size changes simultaneously in a pH-dependent manner. Spectroscopic techniques such as fluorescence and circular dichroism are used to deduce the structural information of proteins during conformational transitions at very low concentration of protein.

In this thesis, we have examined the structural changes occurring during pH-induced conformational isomerization of HSA as a function of pH using both fluorescence spectroscopy and circular dichroism. The fluorescence intensities and anisotropies of both intrinsic (tryptophan) and extrinsic fluorophores allowed us to extract detailed structural information during conformational- and size changes. Additionally, equilibrium unfolding studies using thermal- and chemical denaturation, on pH-induced conformers of HSA, were carried out that provided information about the relative stabilities of the conformers.

Carter and Ho, 1994 reported the single crystal structure of albumins determined by X-ray crystallographic technique. They discussed the three homologous domains in more detail along with their conformational flexibility to undergo transitions with change in pH [8] . He and Carter, 1992 reported the atomic structure of Human Serum Albumin (HSA) to a resolution of 2.8 Å. The single crystal structure of HSA revealed that it consists of three homologous domains (I, II and III) whereby each domain is further divided into 2 subdomains (A and B) and gave information about the molecular configuration. The structure provided evidence about the high affinity of ligand binding to hydrophobic cavities of the subdomains IIA and IIIB [7]. Lee et al., 1992 demonstrated the formation of intermediate I (SH) in HSA by reversible interconversion between denatured reduced form D (SH) and native form N (S-S). The hydrodynamic radii measured by size exclusive chromatography indicated that partially folded intermediate state I (SH) is formed, either by refolding of denatured reduced form D (SH) or by disulphide reduction of native form. The tryptophan fluorescence data of Bovine Serum Albumin (BSA) and HSA showed red-shift in urea-denatured, reduced form whereas, exhibited a blue-shift in the disulphide reduced form, suggesting that out of three, two domains participate during disulphide reduction [10]. Sugio et al., 1999 demonstrated the crystal structure of HSA, extracted from *Pichia pastoris* expression system or human pooled plasma. They determined structure of HSA at 2.5 Å resolution and shed light on the three dimensional structure and their features. They provided evidence that Cys-34 is the only cysteine with a free thiol (-SH) group and does not take part in the formation of disulphide linkage. The pockets of domain-II and domain-III contain hydrophobic and positively charged residues, to which various ligands may be accommodated [6]. Muzammil et al., 1999 studied the conformation of HSA at low pH by using Circular Dichroism (CD) and fluorescence techniques. The great loss of tertiary

structure with a remarkable amount of secondary structure in the low pH was determined by both far- and near-UV CD spectra. The fluorescence experiments with ANS signified the exposure of non-polar patches to aqueous medium. The acrylamide quenching compared with 7 M guanidine hydrochloride denatured state showed that the Trp-214 is buried inside the hydrophobic core. These all results taken together, suggested that the HSA resembles the molten globule state at pH 2 [11]. Dockal et al., 2000 determined pH-dependent conformational transitions of three recombinantly expressed domains of HSA in isolation using fluorescence and CD spectroscopies. Since the entire three-dimensional albumin structure is stabilized by inter- and intra-domain forces, the isolated domains were not able to experience these forces fully as compared to the whole protein. They stated that during the N - F transitions, the HSA-domain III starts loosening, minute fluctuations in secondary structure of domain-I occurs whereas domain-III possesses a molten-globule like state at low pH because it experiences high proportional loss of structure due to its central position [12]. Ahmad et al., 2005 monitored the conformational behaviour of F isomer of HSA in the existence of buffered urea solution by using CD and fluorescence spectroscopic techniques. The N↔F isomerisation occurs by a two-step, three-state transition with the formation of intermediate state at 4.8 – 5.2 M urea concentration whereas unfolding of F isomer of HSA does not form the intermediate state. The ANS-protein complex fluorescence showed that the N↔F transition occured due to unfolding of domain III but the tertiary and secondary structural integrity of both domains I and II is not much damaged. Fluorescence emission of two external fluorescence coupled with binding of chloroform and quenching experiments by acrylamide suggested that the unfolding of F isomer starts at domain II. The shifting of energy maximum towards longer wavelength with a decrease in the Trp fluorescence intensity and chloroform-induced quenching indicate that unfolding of domain II of F-isomer starts at 0.4 M of urea [13]. Vetri et al., 2007 elucidated the thermal aggregation of BSA and

compared it with that of HSA by performing fluorescence kinetics studies and Rayleigh scattering experiments. Their results showed that the intermolecular interactions and lack of electrostatic repulsion affects the aggregation rate and hydrophobic regions are exposed during fluctuations in the tertiary structure. They also demonstrated that pH-induced conformational transitions involve participation from various domains [2]. Kumar et al., 2008 investigated the temperature-dependent interactions of various isoforms of HSA with photosensitizing drug namely, protoporphyrin IX (PPIX) using Dynamic Light Scattering (DLS), CD, and Forster Resonance Energy Transfer (FRET) techniques. The CD spectra showed a loss of secondary structure with an increase in temperature till 75 °C. DLS showed that the temperature-induced unfolding of N-form occurs in a cooperative manner. No such alterations were observed in the fully expanded (E-form) and fully basic (A-form) because they are already expanded. FRET between HSA (donor) and PPIX (acceptor) showed a decrease in the FRET efficiency in the N- and E-forms with an increase in temperature whereas no change in A-form was observed due to its higher stability at a high ionic strength [9]. Bhattacharya et al., 2011 illustrated the presence of large number of partially-folded, molten-globule-like state of BSA at low pH. The decrease in fluorescence intensity of tryptophan and increase in the anisotropy in acidic regime indicates the conformational transitions of BSA molecule into molten-globule-like state. They also monitored the loss of α-helical content by measuring changes in the far UV CD spectra, hence the presence of expanded conformational isoform confirmed at low pH. The binding of ANS and pyrene confirmed the presence of exposed hydrophobic core in molten-globule. The change in the Trp fluorescence as a function of urea concentration suggested the unfolding of the native BSA into expanded form in a non-cooperative manner [14]. Yadav et al., 2014 explained the participation of domain I in the conformational fluctuation through the unfolding of HSA by Guanidine hydrochloride (GdnHCl) and temperature. Fluorescence lifetime data showed a

shallow minimum at 1.25 M GdnHCl and a local maxima at 1.75 M GdnHCl indicating the involvement of intermediate state during unfolding pathway of HSA, whereas such intermediate state was not observed during temperature-induced unfolding of HSA [15]. Acharya et al., 2016 discussed the pH-induced structural perturbation of HSA. As pH decreases to 2.2, an intermediate state having characteristics of dry molten globule is observed. The FRET experiment between labeled Cys-34 – IAEDANS and Trp-214 indicates the expansion of E-form by ~3.5Å due to the disruption in side-chain packing because van der Waals interactions have a steep dependence on interatomic distances. The emission maximum of E-form is blue-shifted whereas it is red-shifted for the unfolded HSA indicating the Trp-214 is near the hydrophobic core of domain II at pH 2.2. The MRE value at 222 nm is -28350 ± 3550 deg cm^2 $dmol^{-1}$, -6500 ± 1000 deg cm^2 $dmol^{-1}$ and -21400 ± 2000 deg cm^2 $dmol^{-1}$ for the N, U and E form. These results suggested that E-form maintains the characteristics of a dry molten globule-like intermediate with 68% of α-helical content in the native protein [16]. Mishra et al., 2017 studied the unfolding of HSA which occurred by the participation of side chain packing by the formation of dry molten globule-like intermediate (I) state populated at pH 5. CD and fluorescence measurements revealed the loosening of side chain packing in the I-state along with the movement of Trp-214 into a hydrophobic environment. The FRET results showed that the inter-domain distance of domain I and domain II increases in I state as compared to the N-state [5].

Chapter 3 Materials and Methods

3.1. Reagents

Human Serum Albumin (HSA; lyophilized powder, fatty acid and globulin free, ≥ 99% pure) was purchased from Sigma-Aldrich (Catalog number: A3782-1G, EC number: 274-272-6) and used as such. The external fluorophore, 8-Anilino-1-napthalenesulfonic acid (ANS) and denaturing agent, Urea (Catalog number: U5378-1KG) were obtained from Sigma-Aldrich. Various buffer materials such as sodium citrate, sodium phosphate monobasic, sodium phosphate dibasic, glycine, citric acid, Tris were also purchased from Sigma-Aldrich at the highest purity grade and used as such to prepare aqueous solutions of buffers (pH 1.6 – pH 10.5).

3.2. Glassware and Labware used

Micropipettes (Eppendorf Research), micropipette tips, Kim-wipes, centrifuge tubes (15 mL and 50 mL), microcentrifuge tubes (2 mL, 1.5 mL and 0.5 mL). Cuvettes (Fluorescence cuvette 10 × 10 mm, UV-Vis cuvette 10 x 10 mm, Circular Dichroism (CD) cuvette (Far-UV CD: 10 × 1 mm and Near-UV CD : 10 × 10 mm)

3.3. Instruments used in this study

3.3.1. Steady-state Fluorescence Spectrophotometer

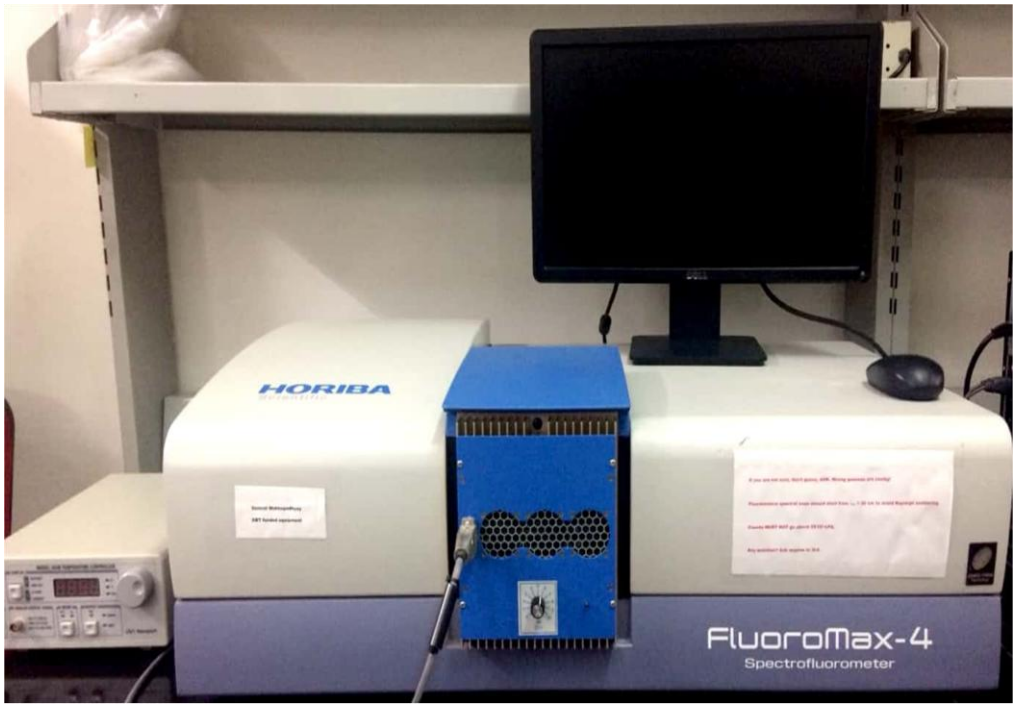

Fig. 3.1 Fluorimeter

Fluorescence spectroscopy is widely used to investigate various processes in chemistry and biology. For my thesis, the steady-state fluorescence intensity, anisotropy and energy transfer efficiency were measured using Fluoromax-4 (Horiba Jobin Yvon, NJ) flourimeter (Fig. 3.1) to monitor the pH-induced conformational changes of HSA. It is an emission phenomenon which occurs in certain molecules called fluorophores or fluorescent dyes. When a fluorophore absorbs photon, an electron is promoted to a higher excited singlet state which exists for a finite time (~10 nanoseconds). During this time, the fluorophore dissipates its energy through the process of internal conversion and relaxes to the lowest vibrational level of the singlet excited state (S_1). The fluorescence emission originates when the fluorophore returns to the ground state ($S_1 \rightarrow S_0$ transition) by emitting photon.

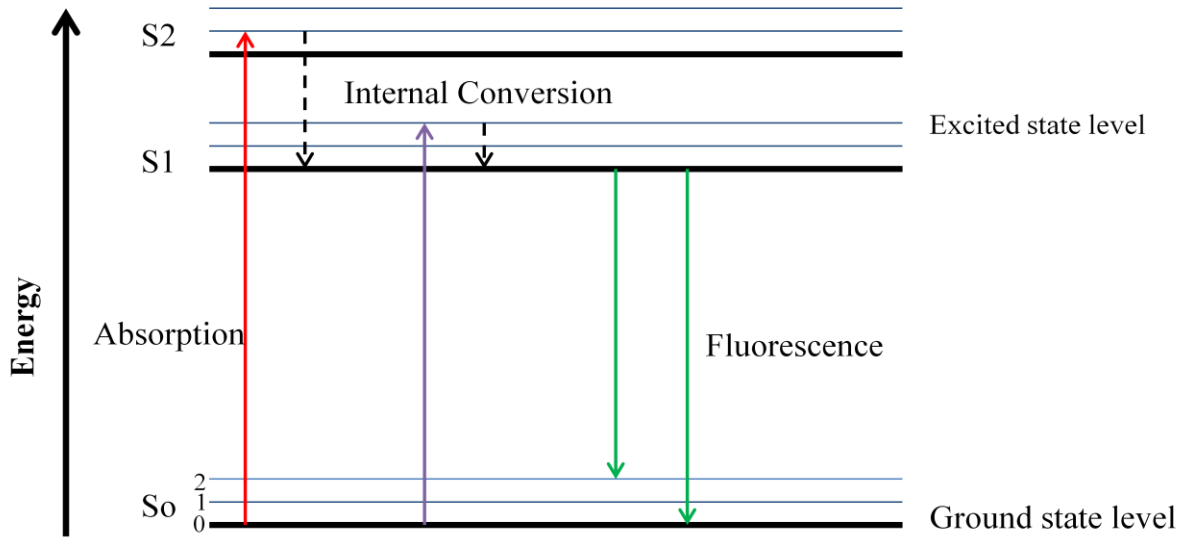

Fig.3.2 Jablonski diagram

In proteins, fluorescence occurs from aromatic amino acids such as tryptophan, tyrosine and phenylalanine. The indole group of tryptophan is the primary source of emission and is highly sensitive to its local environment. The excitation of tryptophan (Trp) occurs at 295 nm and it shows an emission at ~340 nm. In a native protein, when Trp is buried, emission of indole is blue-shifted whereas it is red-shifted (longer wavelength) when Trp is exposed to the aqueous environment with a decrease in the emission intensity e.g. when the protein is unfolded. Therefore, Trp can serve as a useful marker to monitor protein conformational changes. An additional possibility for protein characterization is offered by extrinsic fluorophore such as 1,8-anilinonaphthalene sulfonic acid (ANS) which is weakly- or non-fluorescent in water and shows a very weak emission in the range of 500 – 510 nm, but fluoresces strongly with a significant blue-shift to ~475 nm when it binds to the hydrophobic core of a protein.

Fluorescence anisotropy measurement give information about the overall size of a protein [17]. The technique is based on the principle of photoselection of fluorophores by an incident polarized light. Only those fluorophores absorb photons whose transition moments are

aligned parallel to the electric vectors of the incident polarized light. The steady state anisotropy is given by:

$$r = (I_{||}-GI_{\perp}) / (I_{||}+2GI_{\perp})$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities collected using parallel and perpendicular geometry of the polarizers.

Another phenomenon used in the application of fluorescence is Fluorescence Resonance Energy Transfer (FRET). It occurs by energy transfer between a donor molecule (D) in the excited state and an acceptor molecule (A) in the ground state. The efficiency (E) of energy transfer is measured by:

$$E = 1 - F_{DA}/F_{D}$$

where F_D and F_{DA} are the donor fluorescence intensities in the absence and presence of the acceptor.

3.3.2. Circular Dichroism (CD) Spectrophotometer

Circular dichroism (CD) is a valuable spectroscopic technique which is used to analyse the secondary and tertiary structures of proteins in solution. For my thesis, the protein stock concentration was estimated and secondary as well as tertiary structural changes in HSA were monitored using Chirascan CD Spectrophotometer (Applied Photophysics, UK) (Fig. 3.3).

Fig. 3.3 Circular Dichroism Spectrophotometer

A plane polarised light is a sum of two circularly polarized light that have equal magnitude, one rotating counter clockwise (left-circularly polarised light) and the other clockwise (right-circularly polarised light). When light is passed through an optically active compound, the absorbance of LCP (A_L) is different from the absorbance of RCP (A_R) and this difference in absorbance is the basis of circular dichroism. The radii of electric field vector for one of the components is different after passing through the sample, and combination of these two components results in an elliptically polarized light. Ellipticity is defined as the arc tangent of the ratio of the minor axis to the major axis of the ellipse, i.e. , $\Theta = \tan^{-1}$ (b/a).

$$Circular\ dichroism = \Delta A(\lambda) = A(\lambda)LCPL - A(\lambda)RCPL$$

To study the structural changes in more details, CD measurements in the Far-UV CD and Near-UV CD were carried out that provided information on the secondary and tertiary structural changes, respectively of HSA.

3.4. Methodologies

3.4.1. Preparation of Buffers

Appropriate buffers of various pH were selected depending on the pH range (pH 1.6-10.5, at 0.5 intervals) used in this study. Following are the buffer materials and their pH range: 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) and glycine-NaOH (pH 9 - 10.5). At first, a stock solution of these buffers were prepared in milli-Q water at a concentration of 500 mM. For our spectroscopic studies, a sub-stock of 50 mM buffer solutions were prepared by 10-fold dilution and 5 mM buffer solutions were prepared by further 10-fold dilution of the respective 50 mM buffers. All the buffers and solutions was prepared in milli-Q water and their pH were adjusted by adding either HCl or NaOH and checked using Metrohm 827 lab pH meter. The final pH of each buffer was kept in the range of ±0.01 at 24 – 25 ^{o}C. All the buffers were stored in a refrigerator at 4 °C.

3.4.2. Preparation of HSA Stock Solution

The HSA stock solution of 1 mM was prepared by dissolving HSA in phosphate buffer of 5 mM, pH 7 and stored at 4 ^{o}C. The concentration of the stock solution was determined by measuring the absorbance at 280 nm with the help of Chirascan CD Spectrophotometer. The extinction coefficient of HSA is 35219 $M^{-1}cm^{-1}$ at 280 nm. The final spectra were averaged over 3 scans and buffer-subtracted.

3.4.3. Preparation of ANS Stock Solution

A stock solution of ANS of concentration 10 mM was prepared in milli Q water followed by the preparation of sub stock of 1 mM. For our spectroscopic studies, working concentration of ANS was 10 µM which was achieved by taking an aliquot of 20 µL of 1 mM sub stock which was then added into 2 mL of protein solution.

3.4.4. Steady State – Fluorescence measurements

The final working concentration of HSA for fluorescence measurements was 10 µM. The fluorescence cuvette used for experiments was 10 × 10 mm with a sample volume of 2 mL. All the experiments were performed at least thrice and the data were plotted using Origin software.

3.4.4.1. Tryptophan (Trp) Fluorescence

The pH-induced conformational transitions in HSA were monitored by measuring the Trp fluorescence intensity (in the absence and presence of 1,8-Anilinonaphthalene sulfonate (ANS) and anisotropy. Following parameters were adjusted for monitoring tryptophan fluorescence intensity: λ_{ex} = 295 nm, λ_{em} = 320 – 500 nm with excitation and emission band pass of 1 and 2 nm, respectively. Integration time = 0.5 sec, No. of accumulations = 2. For collecting tryptophan fluorescence anisotropy: λ_{ex} = 295 nm, λ_{em} = 350 nm, excitation band pass = 2.5 nm and emission band pass = 3 nm. Integration time = 2 sec, No. of accumulation of anisotropy values = 3.

3.4.4.2. 1,8-Anilinonaphthalene sulfonate (ANS) Fluorescence

The pH-induced conformational transitions in HSA were monitored by an external fluorophore by measuring the ANS fluorescence intensity and anisotropy. Following parameters were adjusted for monitoring ANS fluorescence intensity: λ_{ex} = 350 nm, λ_{em} = 400 - 600 nm with excitation and emission band pass of 0.5 and 1.5 nm, respectively. Integration time = 0.5 sec, No. of accumulations = 2. ANS fluorescence anisotropy was recorded at: λ_{ex} = 350 nm, λ_{em} = 475 nm, excitation and emission slits of 1 nm and 2.5 nm, Integration time = 2 sec, No. of accumulation of anisotropy values = 3.

3.4.5. Urea-induced denaturation studies using Trp fluorescence

The fluorescence intensity and anisotropy of the intrinsic Trp were measured to monitor the protein structural changes during urea-induced chemical denaturation of HSA. Two different urea stock solutions of 10 M each were prepared in pH 7 and pH 3.5. Typically, at first, different volumes of the desired buffer were added to a 40 µL of stock protein solution (500 µM). Following this, an appropriate volume of urea stock solution was added into the protein solution. The final urea concentration was varied from 0 M – 8 M at an interval of 0.5 M. HSA was incubated in the respective urea solution for at least 3 hours at room temperature to ensure complete equilibration of a particular conformer at that urea concentration prior to collecting the fluorescence data. Following parameters were used for monitoring tryptophan fluorescence intensity: λ_{ex} = 295 nm, λ_{em} = 320 – 500 nm with excitation and emission band pass of 1 nm and 2 nm, respectively. Integration time = 0.5 sec, No. of accumulation = 2. For collecting tryptophan fluorescence anisotropy: λ_{ex} = 295 nm, λ_{em} = 350 nm, excitation band pass = 2.5 nm and emission band pass = 3 nm. Integration time = 2 sec, No. of values accumulated = 3.

3.4.6. Circular Dichroism (CD) Experiments

To study the HSA secondary and tertiary structural changes as a function of pH, CD experiments in the far-UV CD and near-UV CD were performed at room temperature. The protein solution at a various pH (5 mM for far-UV CD and 50 mM for near-UV CD) was diluted to final concentration of protein i.e. 3 µM and 10 µM for far and near-UV CD measurements, respectively. Each spectrum was averaged over three scans and were corrected with buffer baseline subtraction. All the experiments were performed at least thrice and the data were plotted using Origin software. The far-UV CD spectra was recorded within

the range of 195 – 250 nm with a scan speed of 2 nm/sec, using a 10 × 1 mm quartz cell of volume 200 µL. For collecting the near-UV CD spectra using a 10 × 10 mm quartz cuvette with a total volume of 2 mL, following parameters were adjusted: scan range = 250 – 350 nm, steps = 1 nm, time per point = 0.5 sec.

3.4.7. Temperature-induced denaturation studies using far-UV CD measurements

The far-UV CD spectra (195 - 250 nm) were also recorded to monitor temperature-induced denaturation of four distinct conformational isomers of HSA namely, E-form (pH 1.6), F-form (pH 3.5), N-form (pH 7), and B-form (pH 10.5). All the spectra were averaged over 3 scans with a scan speed of 2 nm/sec. The temperature inside the sample chamber was varied from 25 ^{o}C – 90 ^{o}C with an interval of 5 ^{o}C and 0.20 tolerance. The protein sample was allowed to attain a thermal equilibrium at each temperature for 5 minutes before the spectra were collected.

The intrinsic fluorescence of proteins is due to the presence of three aromatic amino acids residues namely, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). The dominant intrinsic fluorophore is tryptophan, so that the energy absorbed by phenylalanine and tyrosine is transferred to the tryptophan in the same molecule. It is well-known that the protein fluorescence is highly sensitive to its local environment. Hence, in order to determine the conformational as well as size (overall flexibility) changes of monomeric HSA as a function

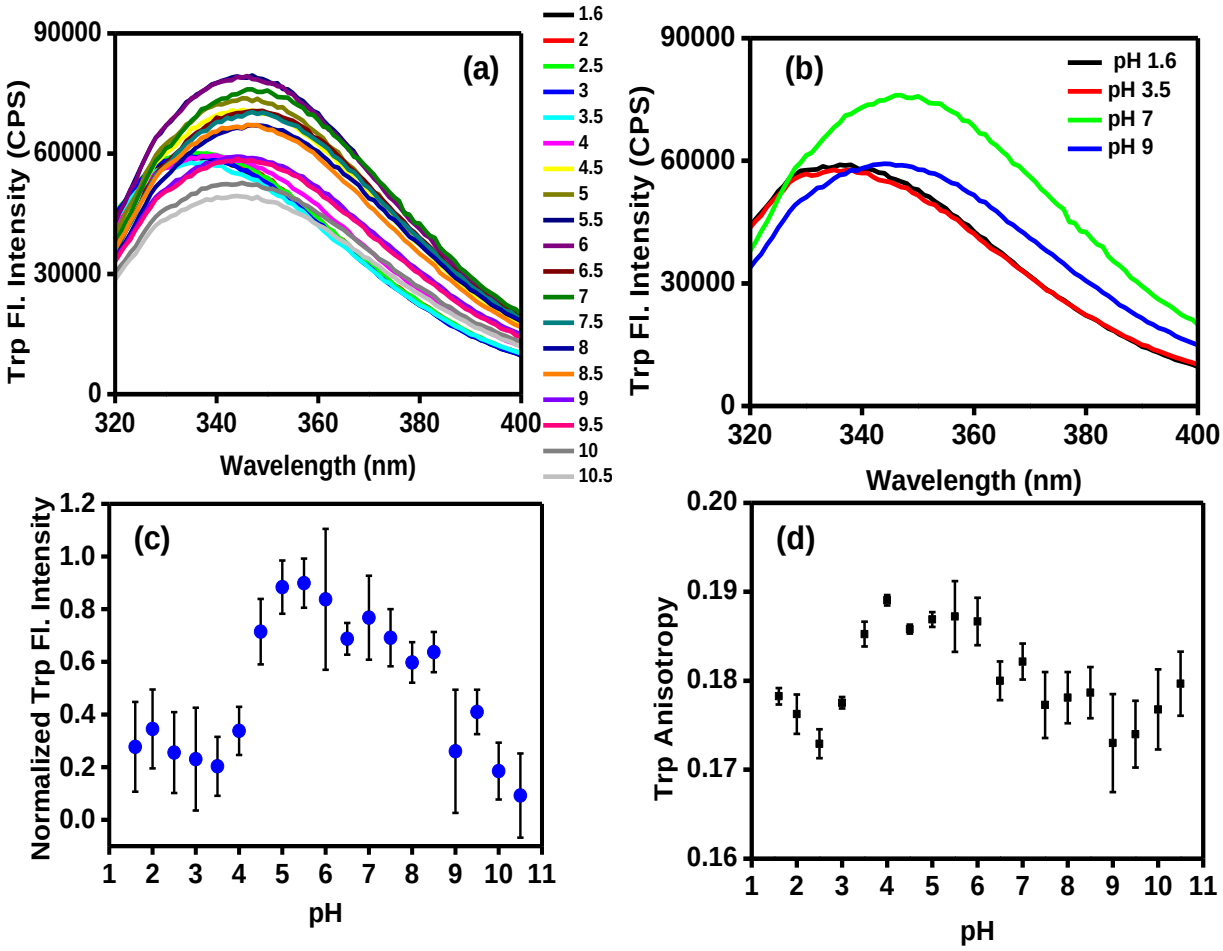

Fig.4.1 Conformational isomerisation of HSA as a function of pH. **(a)** Tryptophan emission spectra, **(b)** Representative tryptophan emission spectra of HSA at pH 1.6 (E-form), 3.5 (F-form), 7 (N-form) and 9 (B-form) for better clarity, **(c)** Normalised tryptophan intensity at 350 nm and **(d)** Tryptophan anisotropy. All of the experiments were repeated at least thrice.

of pH, the fluorescence emission of intrinsic Trp (single Trp located in domain IIA of HSA) was collected (Fig. 4.1). In addition to Trp fluorescence, an extrinsic fluorophore was also used to monitor the pH-induced conformational isomerization of HSA (Fig. 4.2). A brief discussion is as follows.

4.1 Tryptophan emission in various pH-induced conformational isomers of HSA

The samples were incubated overnight in an appropriate buffer solution from pH 1.6 to 10.5 (See Materials and methods) at 25 ^{o}C. The intrinsic tryptophan fluorescence spectra of all the samples were recorded. Figure 4.1a shows the tryptophan emission spectra as a function of pH. The tryptophan emission in native state of protein at pH 7 (N-form) exhibits an emission maximum at ~350 nm which determined that the lone tryptophan is exposed to the aqueous environment (Fig. 4.1b). As the pH is decreased towards acidic range, the emission maximum is blue-shifted to an extent of ~10 nm and occurs at ~340 nm at both pH 1.6 (E-form) and at pH 3.5 (F-form) (Fig. 4.1b). This indicated that as the pH is lowered, the tryptophan is surrounded by hydrophobic region in the acidic pH. However, if the pH is increased towards the basic range, the tryptophan emission shows a maximum at ~345 nm at pH 9 (B-form). Taken together, the variation in tryptophan emission profile as a function of pH (Fig. 4.1c) indicates that the conformational isomerization shows an apparent two-state transition which involves a change in the tryptophan environment from an exposed (N- and B-forms) to a buried (E- and F-forms). Additionally, the steady-state tryptophan fluorescence anisotropy (Fig. 4.1d) was measured, which tells us about the overall size of the conformational isomers of HSA. At pH 7, the average anisotropy of tryptophan is ~0.18 which remains almost constant from pH 7 (N-form) to pH 9 (B-form) (Fig. 4.1d). When pH is lowered towards acidic region, the fluorescence anisotropy shows a peak at pH 4 (F-form), and upon lowering

the pH further, a decrease in the anisotropy is observed for the E-form (Fig. 4.1d). The fluorescence anisotropy data indicate that the HSA undergoes conformational alterations whereby the F-form (pH >3-5) exhibits the characteristics of a molten-globule-like state. These results suggest that the acid-induced structural transition causes alterations in the tertiary as well as in the secondary structure, whereas base-induced transitions cause changes in the tertiary structure only. In the following section, we discuss our results obtained using ANS steady-state fluorescence experiments.

4.1.1 ANS emission in various pH-induced conformational isomers of HSA

Following the tryptophan fluorescence experiments, the conformational changes in HSA as a

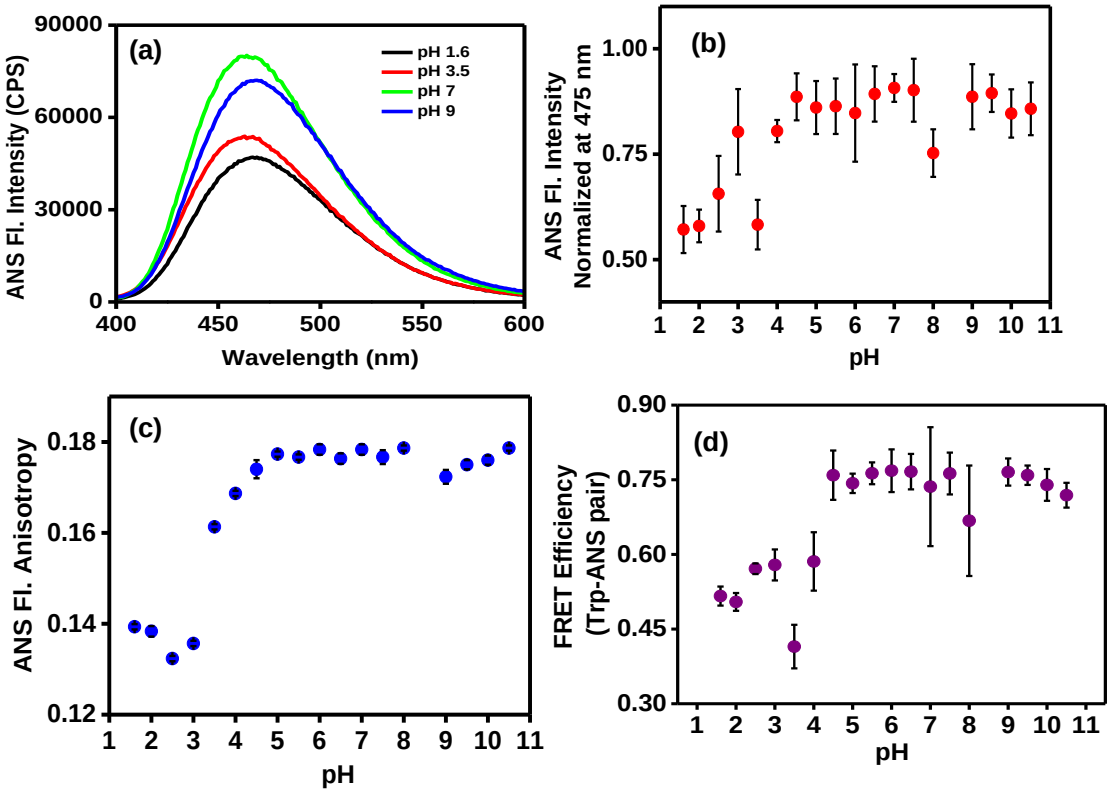

Fig. 4.2 Conformational isomerisation of HSA as a function of pH monitored by ANS fluorescence. **(a)** Representative ANS emission spectra of HSA at pH 1.6 (E-form), 3.5 (F-form), 7 (N-form) and 9 (B-form) for better clarity, **(b)** Normalised ANS intensity at 475 nm, **(c)** ANS fluorescence anisotropy and **(d)** FRET efficiency profile of Trp-ANS. All of the experiments were repeated at least thrice.

function of pH were investigated further by using an external fluorophore namely, 1,8-anilinonaphthalene sulfonate (ANS). ANS is non-fluorescent or weakly-fluorescent in water, but when it is bound to proteins, it fluoresces strongly. It is known that ANS binds to HSA at two sites. For instance, ANS binds to subdomain IIIA strongly and shows a higher binding affinity whereas it binds weakly to subdomain IIA with a lower binding affinity. For our experiments, 20 μL of 1 mM ANS solution was added to our HSA protein samples at various pH so that the final concentration of ANS was 10 μM (see Materials and Methods). The ANS fluorescence spectra of all samples was recorded and a few representative spectra at pH 1.6 (E-form), pH 3.5 (F-form), pH 7 (N-form) and pH 9 (B-form) are shown in Fig. 4.2a. In the N- and B-forms, ANS shows an emission maximum at ~475 nm which shows a blue-shift to ~460 nm in the E- and F-forms. Overall, it appears that the subdomains IIA and IIIA of HSA of the N- and the B-forms are more hydrophobic than that in the E- and F-forms. In order to gain a better clarity on how the hydrophobicity extent of HSA varies with pH, the fluorescence intensity profile of ANS, bound to HSA, at 475 nm was plotted as a function of pH (Fig. 4.2b). The plot indicates an apparent two-state transition again, similar to the tryptophan emission profile, wherein the low-pH forms (E- and F-isomers) show a slight drop in the hydrophobicity compared to that of the neutral and alkaline isomers (N- and B-forms). This could be due to the following: In the acidic regime, HSA adopts an expanded structure whereby the helices in the subdomains IIA and IIIA (ANS binding sites) unwind due to which the binding sites are exposed to the aqueous environment and hence, results in a lowering of the ANS emission intensity. This is further supported by a drop in the ANS fluorescence anisotropy indicating a higher flexibility of ANS in the E- and F-forms compared to that of the N- and B-forms (Fig. 4.2c). In addition to these experiments, the conformational changes of HSA were further probed by fluorescence resonance energy transfer (FRET) measurements since Trp-ANS is a well-known FRET pair. In case of HSA,

Trp is located in domain II and ANS is located in domain II/III. It is observed that the FRET efficiency of the low pH-induced forms is lower than that of the native and alkaline isomers which suggest that a structural loss in the intra-domain helicity occurs as the pH is lowered (Fig. 4.2d).

4.2 Investigations on HSA secondary structural changes as a function of pH using Circular Dichroism (CD) spectroscopy

In order to investigate the secondary structural changes during pH-induced conformational

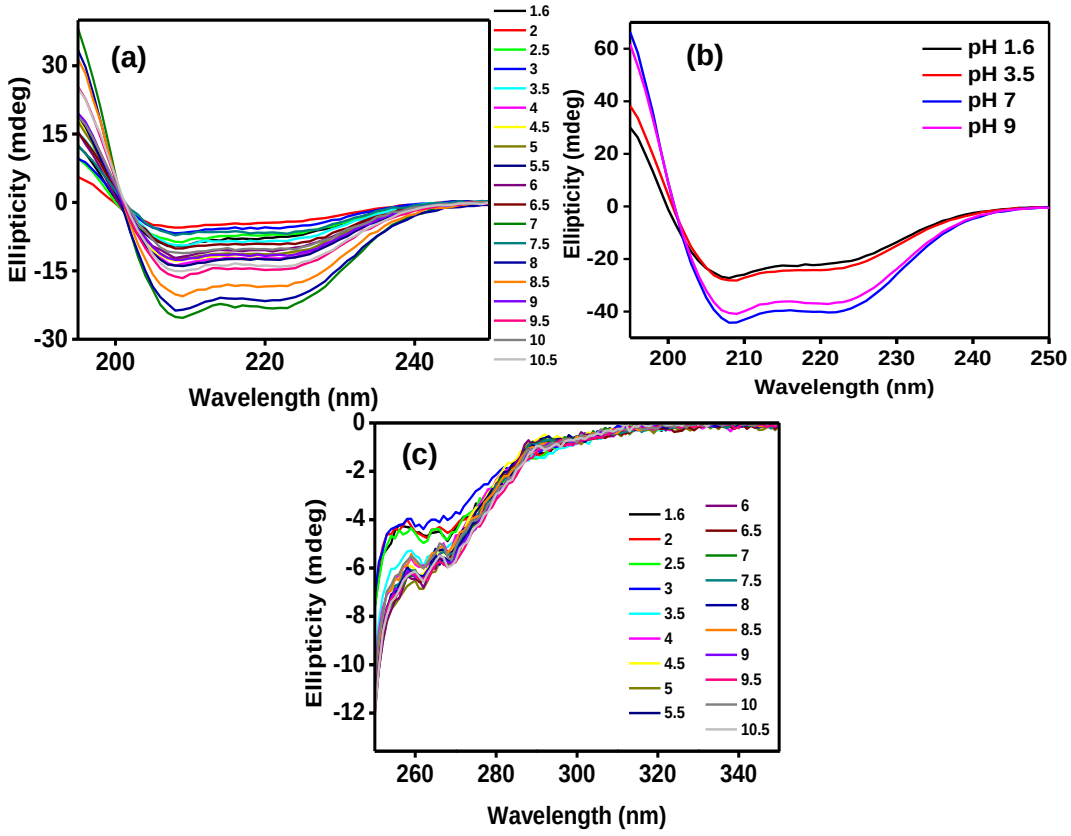

Fig. 4.3. Secondary structural changes of HSA conformational isomers formed at various pH **(a)** Far-UV CD spectra**, (b)** Far-UV CD spectra at pH 1.6 (E-form), 3.5 (F-form), 7 (N-form) and 9 (B-form) for better clarity, and **(c)** Near-UV CD spectra. All of the experiments were repeated at least thrice.

isomerization of HSA, far-UV CD spectra were recorded (Fig. 4.3a, b). The CD spectra show two minima at 208 nm and 222 nm, which signifies the α-helical content in HSA structure. In the native state at pH 7, the more negative value of ellipticity with two minima indicated the presence of helical structure. As the pH was lowered, a significant decrease in the ellipticity value at both 208 nm and 222 nm indicates the loss of the α-helical conformation (Fig. 4.3b). These results are similar to those reported previously [13] which indicate the existence of expanded conformational isomers at low pH and the presence of a 'molten – globule – like' state at pH 3.5 during N↔F transition. Moreover, the near-UV CD spectra also show a drop in the tertiary structure (Fig. 4.3c). All of these data are in accordance with the tryptophan and ANS fluorescence data described in earlier sections.

4.3 Thermal denaturation of HSA conformational isomers

Next, in order to ascertain the thermal stabilities of various conformational isomers of HSA, temperature-induced unfolding transitions of the N, B, F and E isoforms of HSA at pH 7, 9, 3.5 and 1.6, respectively were monitored using far-UV CD in the range of 195 – 250 nm (Fig. 4.4). All of the protein samples were prepared in different buffers and incubated overnight at room temperature to carry out the measurements (See Materials and Methods). As expected, the CD spectra of all the states show two minima, one at 208 nm and another at 222 nm with varying extent of ellipticity. As the temperature rises, the protein undergoes a transition from the native to the denatured state with a loss in the α-helical content of the protein due to thermally-induced disruptions in the hydrogen bonding and helical packing.

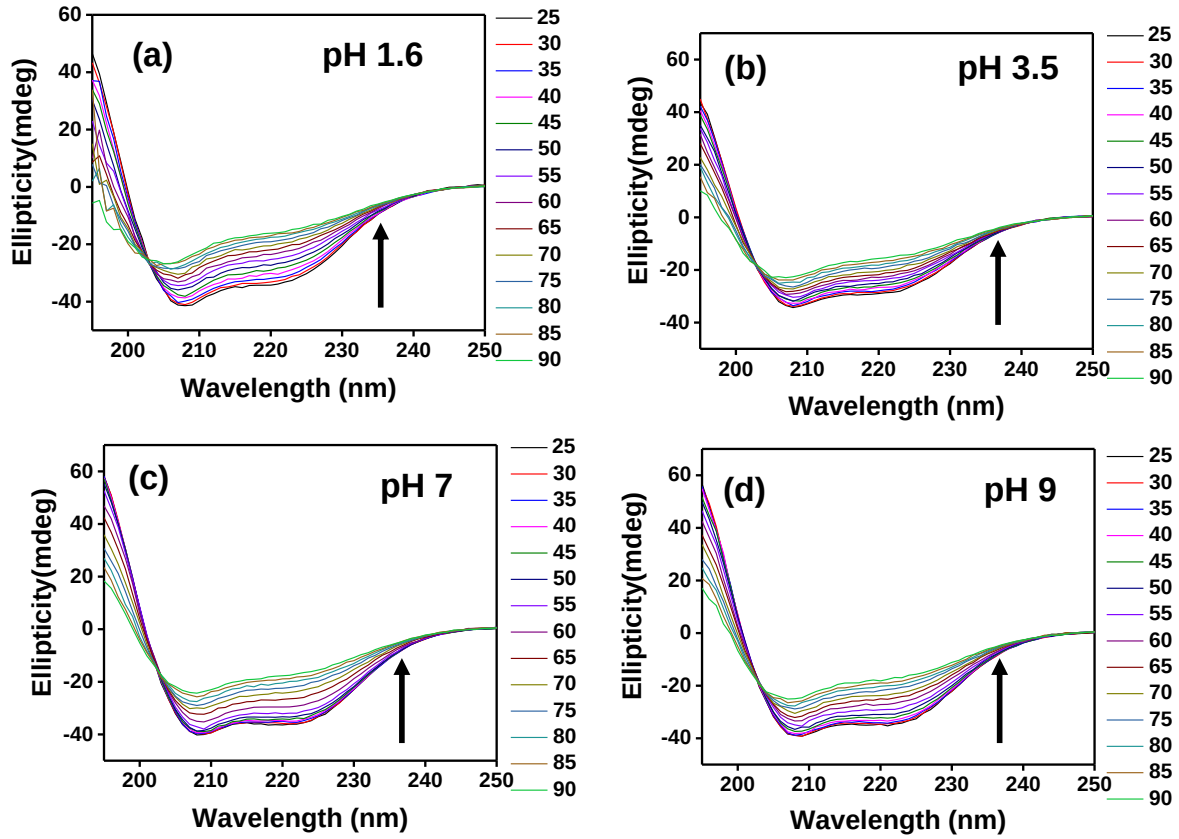

Fig. 4.4 Thermal denaturation of conformational isomers of HSA monitored using far-UV CD at **(a)** pH 1.6 (E-form), **(b)** pH 3.5 (F-form), **(c)** pH 7 (N-form) and **(d)** pH 9 (B-form). The temperature range was 25 – 90 °C. The upward black arrow represents a loss in the helical content. All of the experiments were repeated at least twice.

In order to assess the mid-point of melting transitions (T_m), the observed ellipticity at 222 nm were plotted as a function of temperature for each isomer (Fig. 4.5) since the changes in the ellipticity value at 222 nm is a useful tool to analyse the α-helical content. The plots reveal that the T_m of both N- and B-isomers is ~65 °C and unfolding transitions for both isomers are found to be cooperative. Whereas, the T_m of both E- and F-isomers is found to be lower and unfolding occurs gradually in a non-cooperative fashion. Hence, E- and F-forms appear to be thermally less stable than the N- and B-isomers. This is expected because in both the low pH-induced E- and F-forms, a significant loss of α-helical structure occurs due to the disruption

of intra- and inter-domain packing of the constituent side-chains. Consequently, increasing the temperature results in a faster denaturation of these isomers formed in the acidic regime.

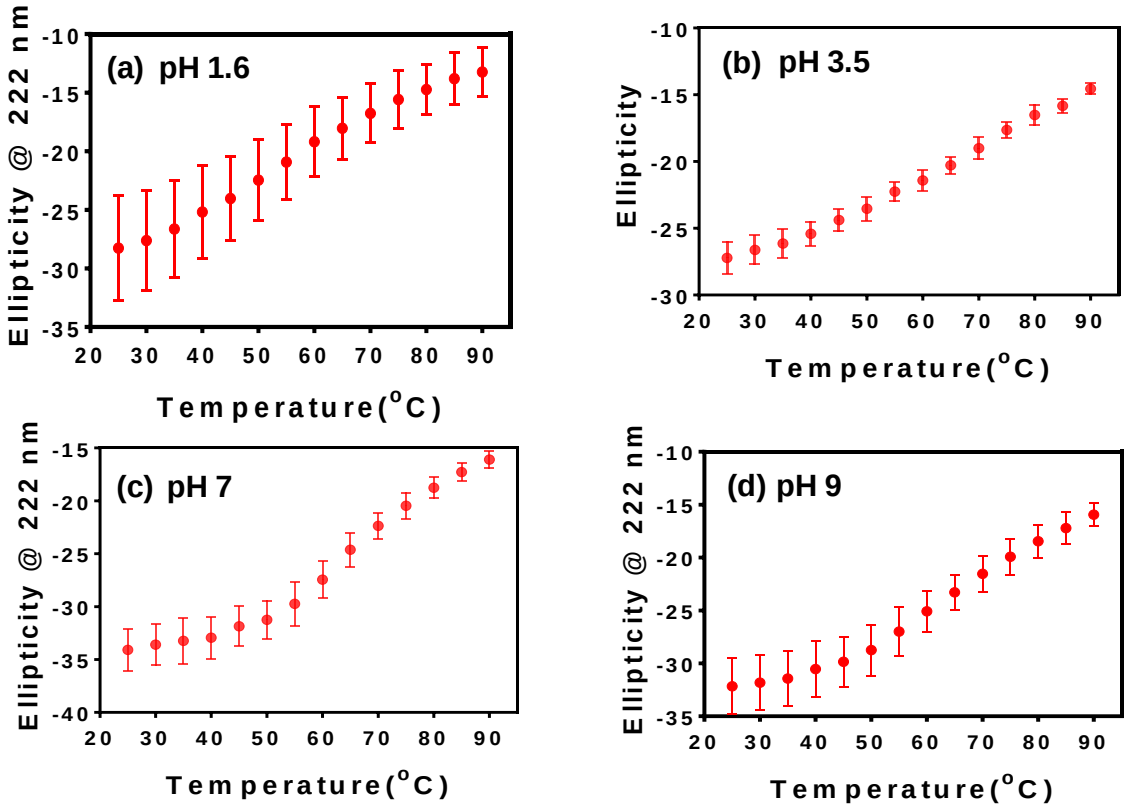

Fig. 4.5 Variation in the ellipticity at 222 nm as a function of temperature for various conformational isomers of HSA at (a) pH 1.6, (b) pH 3.5, (c) pH 7 and (d) pH 9. All of the experiments were repeated at least twice. The error bars at each ellipticity value indicate the standard deviations calculated from all measurements.

4.4 Chemical denaturation of HSA conformational isomers

From the above discussion on fluorescence and CD measurements, it is evident that HSA exists as a partially-unfolded, molten globule-like state at pH 3.5. Thermal denaturation measurements revealed that the thermal stability of the F-form at pH 3.5 is lesser than that of the N-form at pH 7. In order to probe their stabilities further, chemical denaturation

measurements were carried out using various concentration of urea (from 0 M to 8 M). The protein samples were incubated in varying urea concentrations at room temperature at least for three hours to ensure complete equilibration of the particular conformation attained under that condition. The urea-induced unfolding transitions curves of the N- and F-isomers of HSA were monitored by steady-state fluorescence intensity and anisotropy of the intrinsic tryptophan (Fig. 4.6). Figure 4.6a and 4.6b show the changes in tryptophan fluorescence

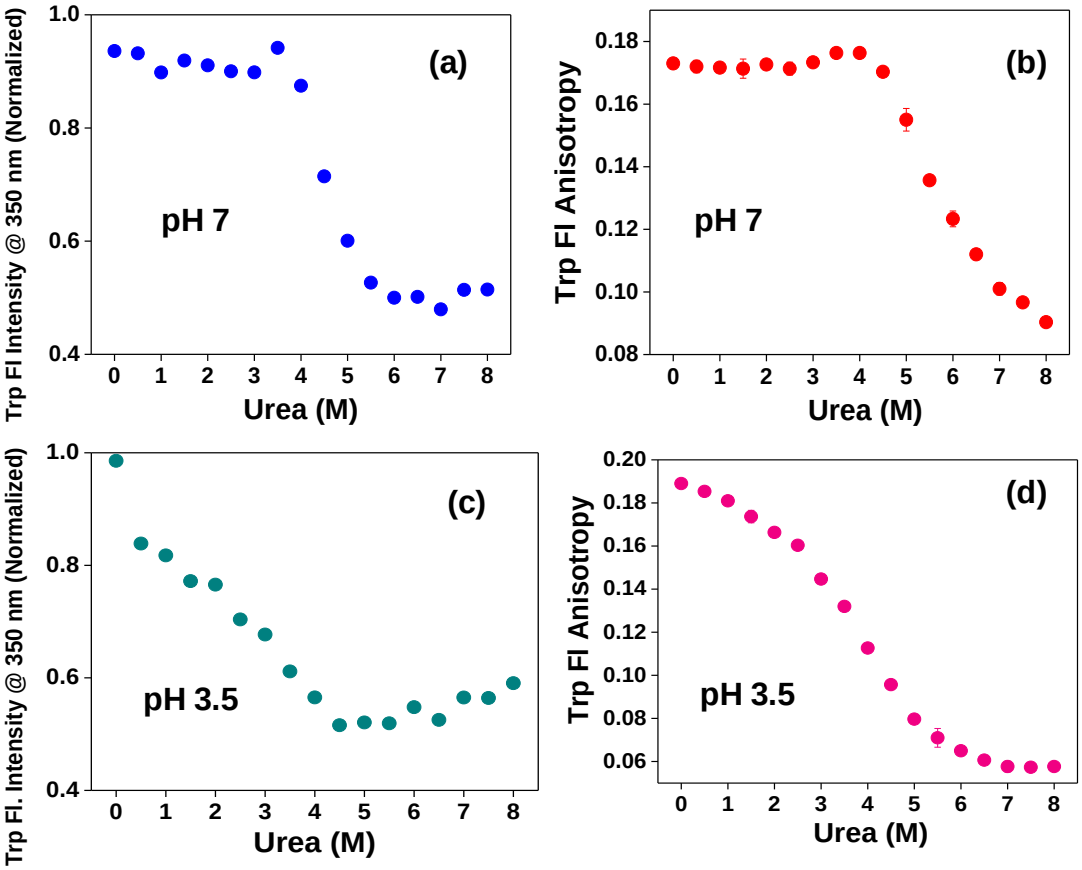

Fig. 4.6 Chemical denaturation of conformational isomers of HSA monitored using intrinsic tryptophan fluorescence. Changes in **(a)** tryptophan fluorescence intensity at 350 nm and **(b)** tryptophan fluorescence anisotropy as a function of urea concentration at pH 7 (N-state). Changes in **(c)** tryptophan fluorescence intensity at 350 nm and **(b)** tryptophan fluorescence anisotropy as a function of urea concentration at pH 3.5 (F-state). All of the experiments were repeated at least twice. The error bars in each plot are included within the symbols.

intensity and anisotropy, respectively of the N-form (pH 7) as a function of urea concentration which show a cooperative unfolding. Both the intensity and the anisotropy plots (Fig. 4.6a, b) show two-state unfolding of the N-state of HSA with a mid-point of transition ~4.5 - 5 M. On the other hand, a gradual decrease in tryptophan fluorescence intensity and anisotropy in a continuous manner is observed for the partially-expanded F-state of HSA formed at pH 3.5 which becomes almost constant. These results are similar to those obtained during thermal denaturation experiments.

Chapter 5 Conclusions

The present study describes an extensive characterization of pH-induced conformational isomers of monomeric HSA using steady-state fluorescence and circular dichroism (CD) spectroscopy. Our studies indicate that the all α-helical, heart-shaped native HSA forms a partially-expanded, molten-globule-like structure at pH 3.5 that is characterized by a significant loss in the α-helical content in the domains II and III due to disruptions in the hydrogen bonding and side-chain packing interactions. The unwinding of helices also leads to the exposure of the hydrophobic pockets towards the aqueous environment. Additionally, our thermal- and chemical denaturation studies support that the molten-globule state is less stable than the native state. All of these observations are relevant in the context that the partially-expanded, molten globule-like state with exposed hydrophobic regions might be aggregation-competent which might have implications in protein conformational diseases.

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