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Elucidation of μs dynamics of domain-III of human serum albumin during the chemical and thermal unfolding: A fluorescence correlation spectroscopic investigation

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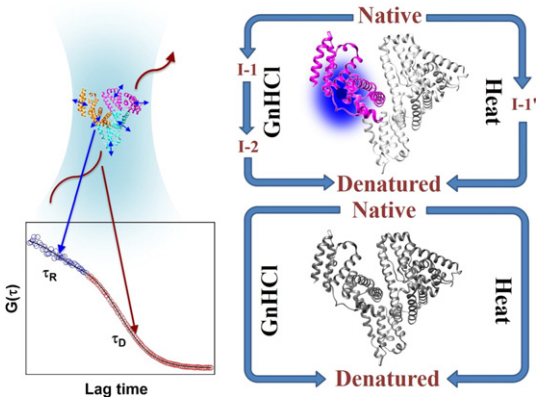
HIGHLIGHTS

- Denaturation of domain-III of HSA is studied by covalent marker for the first time.
- Global and domain-III denaturation were found to follow different pathways.
- Intermediate state/s during the denaturation is detected within the domain-III.
- Effects of GnHCl and heat on domain-III of HSA are found to be different.
- Conformational dynamics during the course of denaturation has been elucidated.

GRAPHICAL ABSTRACT



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ABSTRACT

The local structural dynamics and denaturation profile of domain-III of HSA against guanidine hydrochloride (GnHCl) and temperature has been studied using a coumarin based solvatochromic fluorescent probe p-nitrophenyl coumarin ester (NPCE), covalently tagged to Tyr-411 residue. By the steady state, time-resolved and single molecular level fluorescence studies it has been established that the domain-III of HSA is very sensitive to GnHCl but somewhat resistant to temperature and the domain specific unfolding proceeds in an altered way as compared to the overall unfolding of HSA. While the overall denaturation of HSA is a two-state process for both GnHCl and heat, domain-III adopts two intermediate states for GnHCl induced denaturation and one intermediate state for temperature induced denaturation. Fluorescence correlation spectroscopic investigation divulges the conformational dynamics of domain-III of HSA in the native, intermediates and denatured state.

1. Introduction

Human serum albumin (HSA) is the most abundant transport protein in human body [1–13]. It transports different types of metabolites including hormones, drugs, fatty acids, etc. and has significant esterase

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activity [5–7]. HSA consists of 585 amino acid residues with a molecular weight of 66.5 kDa. Structurally it is divided into three domains, namely domains I, II and III, each of which consists of two subdomains [1,8–9]. The three different domains of HSA serve as binding site for different drugs and metabolites [5–7]. Fluorescence techniques have been employed to understand the structural and functional details of serum albumins [14–25]. It has been established that the three domains of HSA behaves independently both structurally and functionally [1–2,5, 7,14,16–17]. For this reason, the domain specific study of HSA is important to understand the actual structure function relationship.

HSA contains only one tryptophan residue, which is located at the domain-II [26–27]. Thus, domain-II can be studied easily by intrinsic fluorescence of tryptophan, while scrutiny of other two domains by fluorescence methods requires attachment of external fluorophores to the respective domains. Non-covalent probes are less preferred as compared to covalent ones because there is always a chance that the non-covalent probe may slip out of the protein core under structural perturbation. Here it is to note that the covalent fluorescent labeling can provide specific information of the tagging site within the protein. The covalent attachment of thiol specific fluorescent tagging agents to the single free cysteine residue in domain-I of HSA makes the study of domain-I feasible [28–33]. We have already tagged domain-I of HSA with tetramethylrhodamine-5-maleimide (TMR) and N-(7-dimethylamino-4-methylcoumarin-3-yl) iodoacetamide (DACIA) to study the unfolding and conformational dynamics pertaining to domain-I [28–30]. Other thiol specific probes such as 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM), acrylodan are also reported to tag domain-I of HSA [31–33]. However, domain-III of HSA is not studied thoroughly, in spite of holding important role in ligand binding and esterase activity, probably due to the limited scope of site specific labeling of this domain. Recently, we have reported a tyrosine specific probe, *p*-nitrophenyl coumarin ester (NPCE) to selectively label tyrosine-411 of HSA residing in the domain-III [34]. By solvation dynamics, anisotropy and single molecular fluorescence experiments we have characterized the local environment within the domain-III of HSA [34]. In this work we intend to study the denaturation profile of domain-III of HSA.

The denaturation of HSA has been studied by various groups worldwide [36–39]. Being a multi-domain protein, HSA shows different denaturation profiles for different domains [17]. In a recent review by Anand et al., the overall unfolding and refolding pathways of serum albumins are summarized [19]. Bright and co-workers have studied the dynamics of acrylodan labeled HSA in the course of thermal and chemical denaturation and reported the presence of intermediate states in the unfolding process [31]. Ahmad et al. reported that chemical denaturation of HSA initiates by a local unfolding of stable loops in domain-III [15]. In another work, Ahmad et al. reported that urea induced unfolding of fast moving isomer (F-isomer) of HSA does not proceed through any intermediate state, where as N-isomer does [35]. Vlasova et al. confirmed the existence of two stages in the SDS induced denaturation of HSA. First one being the disintegration of globules, the second one involves the complete unfolding of the amino acid chain of HSA [21]. Analysing the intrinsic tryptophan fluorescence of HSA they commented that both stages of denaturation is operational at a pH below the isoelectric point (pI = 4.7) of HSA, but when the pH is above pI, denaturation stops in the first stage only. Picó adopted a model *native* ↔ *unfolded reversible* → *unfolded irreversible* to represent the thermal unfolding behaviour of HSA [40]. According to him, the irreversible change happens only at above 347 K. Previously we have shown that in the presence of guanidine hydrochloride (GnHCl), domain-III unfolds first followed by domain-I and domain-II [17]. We have also elucidated the domain specific unfolding pathway of domain-I in the course of chemical and thermal denaturation using bulk and single molecular level spectroscopic studies [28].

Here we report the unfolding characteristics of domain-III of HSA using NPCE as a site specific covalent tag. Steady state, time resolved and single molecular level fluorescence experiments have been performed to elucidate the conformational dynamics during the course of denaturation.

2. Experimental section

2.1. Materials

Human serum albumin (HSA, fatty acid free), coumarin-343, guanidine hydrochloride (GnHCl), 4-dimethylamino pyridine (DMAP), N,N-dicyclohexylcarbodiimide (DCC) and 4-nitrophenol were purchased from Sigma-Aldrich and used as received. Analytical grade di-sodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Merck, India and used to prepare 50 mM buffer (pH 7.4). Dialysis membrane tubing (14 kDa cut-off) was purchased from Sigma-Aldrich. Before using the dialysis tubes, glycerol and sulfur compounds were removed according to the procedure given by Sigma-Aldrich. Centrifugal filter units (Amicon Ultra, 10 kDa cutoff) have been purchased from Merck Millipore, Germany. HPLC grade dimethyl sulfoxide (DMSO) and dichloromethane (DCM) were purchased from S. D. Fine Chemicals Limited and used after distillation. Deuterieted chloroform ($CDCl_3$) was purchased from Sigma-Aldrich.

2.2. Instrumentation

The steady-state absorption and emission spectra were recorded in a commercial UV–visible spectrophotometer (UV-2450, Schimadzu, Japan) and spectrofluorimeter (FluoroMax4, JobinYvon, USA), respectively, using a quartz cuvette having the path length of 10 mm. Circular dichroism spectra were recorded in a commercial CD spectrometer (J-815, Jasco, Japan) in 1 mm quartz cuvette. Centrifugation was done in Eppendorf centrifuge 5810R, at 5000 rpm. Lyophilisation was done in Heto VR-1 set up. For emission measurements, samples were excited at 446 nm. Time-resolved fluorescence were collected using a commercial TCSPC setup (Life Spec II, Edinburgh Instruments, UK). For lifetime measurements, samples were excited with a 442 nm diode laser (EPL-445, Edinburgh Instruments, UK). Peak counts of 10,000 were collected with the emission polarizer oriented at magic-angle polarization compared to the polarization of the excitation light. Dilute ludox solution was used for the measurement of instrument response function (IRF), which is measured to be 110 ps and used for de-convolution of the fluorescence transients. All the experiments were done at 298 K unless stated otherwise.

2.3. Synthesis of NPCE

NPCE (Scheme 1) was synthesized following the already reported procedure [34]. Briefly, coumarin-343 (0.38 mmol), para-nitrophenol (0.38 mmol) and 4-dimethylamino pyridine (DMAP) (0.38 mmol) were taken in 5 mL dichloromethane and stirred in ice bath for 10 min. 0.38 mmol of N,N-dicyclohexylcarbodiimide (DCC) was then added to the reaction mixture under nitrogen atmosphere with stirring. The reaction mixture was stirred at 0 °C for 20 min and then at 20 °C for 24 h. The organic layer was first washed with 35 mL of 1.2 M HCl and then with 35 mL saturated $NaHCO_3$ aqueous solution and was dried over $MgSO_4$. The residue was suspended in methanol and the precipitate was washed with 100 mL of methanol to remove the unreacted coumarin-343. The precipitate was collected in dichloromethane, dried under vacuum and characterized by ^1H NMR, IR and mass spectrometric studies.

2.4. Protein labeling and sample preparation

NPCE tagged HSA (Scheme 1) was prepared following the procedure described by Wang et al. [41]. 40 mg of HSA was dissolved in 9 mL of 50 mM phosphate buffer (pH = 8.0) and NPCE solution (in 1 mL DMSO) was added to it slowly such that the molar ratio of protein and