Region-Specific Double Denaturation of Human Serum Albumin: Combined Effects of Temperature and GnHCl on Structural and Dynamical Responses

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ABSTRACT: In this work, we have investigated the effects of denaturing agents, guanidine hydrochloride (GnHCl) and temperature, on the overall structure, domain-I, and domain-III of human serum albumin (HSA) using circular dichroism (CD) spectroscopy and steady-state, time-resolved fluorescence spectroscopy. We have tagged Cys-34 of HSA, located at domain-I, using N-(7-dimethylamino-4-methylcoumarin-3-yl)iodoacetamide and Tyr-411 of HSA, located at domain-III, using p-nitrophenyl coumarin ester, for this purpose. The CD spectroscopy studies reveal the overall denaturation of the protein. The denaturation follows the expected direction in which the protein is denatured with an increase in the concentration of GnHCl or temperature. The $\alpha$-helicity of the native state of HSA was found to be 64.2%, and the minimum value of $\alpha$-helicity was found to be 14.8% in the presence of 6 M GnHCl at room temperature. Steady-state emission studies were carried out on domain-I and domain-III of the protein using site-specific fluorescent tags. The degree of folding of the two domains at different combinations of temperature and GnHCl concentration was calculated and was found to follow a slightly different course of denaturation. Solvation dynamics was found to be quite different for these two domains. The domain-I of HSA has a maximum solvation time of 0.39 ns, and the solvation time tends to decrease with the action of either temperature or GnHCl. On the other hand, the domain-III of HSA showed a much higher solvation time (1.42 ns) and does not show any regular change at higher temperatures or in the presence of GnHCl. This difference could be attributed to the different microenvironment inside the protein cores of the two domains.

1. INTRODUCTION

Human serum albumin (HSA) is the most abundant transport protein present in human blood serum.1−3 Being a huge protein with 585 amino acid residues and 66.5 kDa molecular weight, it has a few hydrophobic pockets which can accommodate macromolecules and transport them to specific locations of the human body.4−10 HSA is responsible for the transport of a variety of molecules including carbohydrates, fatty acids, drugs, hormones, etc.4,9,10 Structurally, HSA is divided into three domains, each of which consists of two subdomains.11−5 It is to be noted that all proteins, including HSA, perform their biological actions in physiological conditions having a specific structure, known as the native state of the protein. In the native state, the protein chain is folded in a unique way to minimize the destabilizing interaction. Any loss in this folded three-dimensional (3D) structure under the effect of any external factor is broadly identified as denaturation of the protein and it eventually leads to the loss in its function.11−18 Understanding the complex mechanism of denaturation has remained a topic of research for several decades.14,15 Besides this, biological and biochemical research has been carried out to monitor the functionality of proteins as a function of external parameters and to relate it with the structural perturbation.20,21

Fluorescence techniques are being vastly applied to study proteins, owing to their sensitivity toward the local environment inside the protein.22−38 Tyrosine and tryptophan are the two main fluorescing amino acid residues, which cause intrinsic fluorescence of proteins. In most proteins, the number of these amino acid residues is more than one, and they are located at different parts of the protein. For this reason, site-specific information is lost, and the signal depicts the overall picture. Fortunately, HSA has a single tryptophan residue located at the 214th position in domain-II, which serves as an efficient marker for monitoring this domain.36,37 However, domain-I and domain-III do not have any site-specific intrinsic fluorophore, and hence, fluorescent labeling is necessary to perform domain-I- and domain-III-specific unfolding studies. Several reports are available on the tagging of HSA with noncovalent and covalent fluorescent markers.32,33,38−40 The covalent markers are always more trusted than the noncovalent.
ones on account of their much stronger attachment with the protein. Domain-I of HSA has a single free cysteine residue at the 34th position, which can be tagged with thiol-specific fluorescence probes.43,33,38,39 In our earlier reports, we have labeled domain-I with tetramethylrhodamine-5-maleimide and N-(7-dimethylamino-4-methylcoumarin-3-yi)iodoacetamide (DACIA).38 Domain-III, on the other hand, has a tyrosine residue at the 411th position, which is much more active than the other tyrosine residues and can be selectively labeled by hydroxyl-specific fluorescent probes.41 However, no suitable fluorescent marker is available commercially to tag the tyrosine residue. In one of our recent reports, we have designed and synthesized a fluorescent marker p-nitrophenyl coumarin ester (NPCE), which successfully tags Tyr-411 and delivers useful domain-specific information.39

There are several reports on the effect of external parameters on the structure and dynamics of proteins, but there are only a few reports on the effect of more than one denaturant simultaneously.42 Such a situation of multiple denaturing effects is important as similar situations may be occurring in biological systems. Moreover, the effect of such multiple denaturation may not be uniform throughout the protein structure, and thus, a domain-specific study is warranted. Similar studies on the effect of more than one denaturant were carried out by Shaw and Pal, where they observed that the donor–acceptor distance of the alkali-induced conformer of HSA does not change with temperature, whereas in the case of acid-induced and native conformer, the distance was found to decrease with an increase in temperature. The donor and acceptor in this case were Trp-214 and a photosensitizing drug, decahydroindigo, respectively.43 In another study carried out by Ahmad et al., domain-specific ligands have been used to study the unfolding of different domains of HSA under the action of guanidine hydrochloride (GnHCl), and they reveal that in the presence of GnHCl, domain-III is more liable to unfolding compared to the two other domains.44

In the present study, we aimed to map the unfolding behavior of domain-I and domain-III of HSA under a double denaturing condition with GnHCl (0–6 M) and temperature (283–353 K). We have used steady-state as well as time-resolved fluorescence measurements to understand the nature of unfolding.

2. RESULTS

2.1. Effect of Double Denaturation on the Overall Structure of HSA. The overall structural features of unfolding of HSA were monitored by measuring the α-helicity content by circular dichroism (CD) spectroscopy using the following equation:

\[
\% \alpha \text{ helicity} = \frac{\text{MRE}_{222} - 3000}{-36,000 - 3000} \times 100
\]

where the mean molar residual ellipticity at 222 nm (MRE\(_{222}\)) is defined as

\[
\text{MRE}_{222} = \frac{\theta_{222} \times M}{n \cdot c \cdot l}
\]

In the above equation, \(\theta_{222}\) is the intensity of CD signal at 222 nm, \(M\) is the molecular weight of HSA, \(n\) is the number of amino acid residues, \(c\) is the concentration in g/L, and \(l\) is the path length of the cuvette. The helicity of the native state of HSA has been calculated as 64.2%, which is in good agreement with the reported value.38,39

Upon denaturation, the α-helicity has been found to decrease both with an increase in temperature and with an increase in GnHCl concentration. At high concentrations of GnHCl, CD spectra could not be recorded below 210 nm because of significant interference from GnHCl. Figure 1 shows the CD spectra of HSA at four different combinations of GnHCl concentration and temperature. The concentration of HSA in each case is 1.85 μM, and the path length in each case is 2 mm.

\[
f_i = \frac{\alpha_i - \alpha_U}{\alpha_N - \alpha_U}
\]

where \(f_i\) is a measure of the degree of folding (varies between 1 and 0), \(\alpha_i\) is the α-helicity at a particular denaturing condition (i.e., either at some specific temperature or at some specific GnHCl concentration or both together), and \(\alpha_N\) and \(\alpha_U\) are the α-helocities of HSA in its unfolded (i.e., in the presence of 6 M GnHCl at 298 K) and native (i.e., in the absence of GnHCl at 298 K) states, respectively. Figure 2 shows the contour diagram of the calculated extent of folding as a function of GnHCl concentrations and temperatures. From the contour diagram, it is evident that the effect of temperature is less pronounced than the effect of GnHCl.

2.2. Effect of Double Denaturation on Domain-I of HSA. 2.2.1. Steady-State Measurement. As mentioned earlier, the domain-I of HSA was tagged with DACIA to study the unfolding behavior of domain-I in the presence of GnHCl and temperature. However, first, we have to be sure that the tagging of DACIA does not alter the secondary structure of HSA, which was verified by measuring the CD spectra. The same study was also performed with NPCE-tagged HSA (note: NPCE binds to the domain-III of HSA). Figure 3 shows the CD spectra of the native HSA, HSA tagged with DACIA and of HSA tagged with NPCE at four different combinations of GnHCl concentration and temperature. From the contour diagram, it is evident that the tagging of DACIA does not alter the secondary structure of HSA.
with DACIA, and HSA tagged with NPCE. The calculated values of \( \alpha \)-helicity and the spectral signatures are similar for all the three cases, indicating that the attachment of the fluorescent label (either DACIA or NPCE) does not affect the secondary structure of HSA. Further, to investigate the effect of tagging on the tertiary structure of HSA, we have recorded the near-UV CD spectra (250–320 nm) using a higher HSA concentration (15 \( \mu \)M) and a longer path length (10 mm). The resulting spectra of tagged and untagged HSA possess similar features, indicating that the tertiary structure is also retained upon tagging with either DACIA or NPCE.

The fluorescent tag DACIA, which has been used to tag domain-I, is a solvatochromic probe, which shows strong solvent polarity dependence on emission spectra. Upon being tagged to domain-I of HSA, DACIA shows a 20 nm blue shift (\( \lambda_{\text{em max}} = 457 \) nm) in emission maximum from 477 nm in buffer, owing to the more hydrophobic environment inside the protein core. As HSA unfolds, the emission maximum shows a bathochromic shift as shown in Figure 4 for four different GnHCl–temperature combinations. In order to verify if the tags undergo any structural changes at elevated temperatures, we have recorded the excitation spectra of DACIA and NPCE at higher temperatures and observed that the spectrum at 348 K is almost overlapping with that at 298 K. This confirms that both these dyes are stable within our experimental range of temperature. The amount of shift normalized by the maximum amount of shift has been used to denote the degree of folding as per the following equation.

\[
f_i = \frac{\lambda_U - \lambda_i}{\lambda_U - \lambda_N}
\]

where \( \lambda_N \) and \( \lambda_U \) are the emission maxima in the native and unfolded states, respectively, and \( \lambda_i \) is the emission maxima for the intermediate temperatures and GnHCl concentrations. The value of \( \lambda_i \) at 298 K in the absence of GnHCl has been taken as \( \lambda_N \), and the value of \( \lambda_i \) at 298 K in the presence of 6 M GnHCl has been taken as \( \lambda_U \). The contour plot (Figure 5) shows the variation of the degree of folding at different GnHCl concentrations and temperatures. Expectedly, as per the definition, the extent of folding is unity at 298 K in the absence of GnHCl. Upon increasing the temperature and GnHCl concentration, the extent of folding decreases. As evident, the effect of temperature is similar at all GnHCl concentrations.
concentrations, and the denaturation effect of GnHCl is much more pronounced as compared to that of the temperature. Apart from this regular observation, we observe two striking features. The first one is that at 323 K, the extent of folding is more than unity. Second, at 283 K, in the absence of GnHCl, the extent of folding is less than unity.

2.2.2. Lifetime Measurement. To gather further information on the nature of heat and GnHCl-induced denaturation, the fluorescence lifetime measurements were performed for a few samples. The excited-state lifetime of the free DACIA molecule was measured at its emission maximum, and the fluorescent transients were fitted using a triexponential function. The decay components were found to be 0.28 ns (3%), 1.85 ns (85%), and 3.31 ns (12%) with an average lifetime of 1.98 ns. Once tagged to domain-I of HSA, the average lifetime was found to increase to 3.64 ns with three decay components 0.37 ns (3%), 1.81 ns (22%), and 4.28 ns (76%).

The lifetime measurements of DACIA-tagged HSA were carried out in the absence of GnHCl and in the presence of 2 and 5 M GnHCl at three different temperatures: 298, 328, and 348 K at their respective emission maxima. In all cases, the fluorescence transients were fitted using triexponential functions. In the presence of 2 M GnHCl at 298 K, the average lifetime was found to decrease to 3.29 ns, whereas in the absence of GnHCl at 328 K, the average lifetime became 3.41 ns. The emission maxima and the average lifetime of each of the cases are given in Table 1. It can be seen that the lifetime of DACIA tagged to HSA decreases with an increase in the concentration of GnHCl and with an increase in temperature, which indicate an increase in the nonradiative rate constant of the excited DACIA molecule at higher temperatures and higher GnHCl concentration. However, this study is not much informative, and we proceed with solvation dynamics study to have a better picture of the dynamics of associated water molecules within the domain.

2.2.3. Solvation Dynamics Measurement. We have studied the dynamics of solvation within the domain-I of HSA using the solvatochromic property of DACIA. For this purpose, the fluorescence transients at 20 different wavelengths spread over the steady-state emission spectrum (from 405 to 550 nm) were recorded at the magic angle condition. The fluorescence transients collected at longer wavelengths showed a distinct growth component, which was absent at shorter wavelengths. All transients were fitted using a triexponential function. At 298 K and in the absence of GnHCl, the average lifetime at 405 nm was found to be 1.44 ns with the three lifetime components 0.13 ns (41%), 1.04 ns (30%), and 3.70 ns (29%). At 550 nm, the average lifetime was calculated to be 4.08 ns with components 0.37 ns (−17%), 1.26 ns (43%), and 4.86 ns (74%). Some of the transients are shown in Figure 6. The emission maxima and average lifetimes of DACIA-tagged HSA in the presence of different concentrations of GnHCl at different temperatures are given in Table 1.

Figure 6. Fluorescence transients of DACIA tagged to domain-I of HSA in phosphate buffer solution of pH 7.4 at wavelengths 405, 440, and 550 nm upon exciting at 375 nm. Transients in a short time window are shown in the inset.

Figure 7. TRES of DACIA tagged to domain-I of HSA in phosphate buffer solution of pH 7.4. The steady-state emission spectrum is also shown for comparison.

While constructing the solvent response function from TRES, we observed that the convergence of data was not very good, which may be arising due to the complex environment and constant fluctuations of the protein core. In such cases, choosing an appropriate \( \nu(\infty) \) value (eq 2) could be challenging, and any change in the chosen \( \nu(\infty) \) value usually changes the value of the solvation time drastically. For our experiments, the time spectrum whose peak frequency closely matches to that of the steady-state spectrum has been chosen as the \( \nu(\infty) \), which is the spectrum at 2 ns for the present case. The solvent response function, thus calculated, is shown in
From Table 2, it can be seen that either an increasing temperature or a high concentration of GnHCl results in a decrease in solvation time. However, the effect of temperature in the presence of GnHCl (2 or 5 M) is quite strange. In these two cases, we observed that the solvation time is unaffected by raising the temperature from 298 to 328 K; however, further increase in the temperature to 348 K leads to slower solvation. Another important observation is that at 348 K, the solvation time hardly depends on the GnHCl concentration. The dynamic Stokes shift was found to decrease with an increase in temperature or with an increase in the concentration of GnHCl in each of the cases we monitored. Certainly, a significant portion of the total Stokes shift has not been observed in our study unlike the slowest part of the solvation dynamics. The missing components of dynamic Stokes shift in our picosecond setup were calculated using the method demonstrated by Fee and Maroncelli.\(^{48}\) The emission frequency at time zero, \(\nu_{\text{em}}^p(0)\), was calculated using the equation

\[
\nu_{\text{em}}^p(0) = \nu_{\text{abs}}^p - [\nu_{\text{abs}}^p - \nu_{\text{em}}^p(\infty)]
\]

where \(\nu_{\text{abs}}^p\) is the absorption frequency in a polar medium and \(\nu_{\text{em}}^p\) and \(\nu_{\text{em}}^p(\infty)\) denote the steady-state absorption and emission frequencies of DACIA in a nonpolar medium (in our case, cyclohexane). The missing components of Stokes shift for DACIA-tagged HSA under different denaturing conditions are listed in Table 2.

### 2.3. Effect of Double Denaturation on Domain-III of HSA

#### 2.3.1. Steady-State Measurement

The fluorescent label NPCE, which is used to tag domain-III of HSA, shows the emission maximum at 493 nm in buffer. After being covalently attached to HSA, the emission maximum shifts to 478 nm. The solvatochromic behavior of NPCE is utilized to study the double denaturation effect of this specific domain. The fluorescence maximum of NPCE-tagged HSA gets red-shifted monotonously with an increase in temperature almost at all GnHCl concentrations. Figure 10 shows the emission spectra of NPCE-tagged HSA at two different GnHCl concentrations and at two different temperatures. Using eq 6, the change in degree of folding of this domain has been calculated from the shifts in the emission maxima of NPCE. Here also, the same nomenclature of \(\lambda_N\) and \(\lambda_U\) has been used. The variation of extent of folding upon thermal and chemical denaturation is shown in the contour plot in Figure 11.

#### 2.3.2. Lifetime Measurement

The excited-state lifetime of NPCE in phosphate buffer was measured at its emission maximum, the transients were best fitted using a single

![Figure 8](image-url)

**Figure 8.** Decay of solvent response function of DACIA tagged to domain-I of HSA in phosphate buffer of pH 7.4.

0.68 ns (45%) with an average solvation time of 0.39 ns. The Stokes shift was observed to be 480 cm\(^{-1}\). For the purpose of comparing our data with a previously reported data\(^{32}\) of the same system where the \(\nu(\infty)\) value was chosen at 10 ns, we also performed the same line of analysis choosing \(\nu(\infty)\) value at 10 ns. The solvent correlation function thus obtained gave an average solvation time of 2.56 ns, which is very close to the value reported in the literature (2.50 ns).\(^{32}\)

In a similar fashion, the solvation dynamics of domain-I of HSA has been studied for nine different combinations of temperatures and GnHCl concentrations. At 328 K, in the absence of GnHCl, the lifetime components at 405 nm were found to be 0.14 ns (43%), 1.00 ns (33%), and 3.60 ns (24%) with an average lifetime of 1.25 ns. At 550 nm, the three components are 0.29 ns (−13%), 1.45 ns (68%), and 4.90 ns (45%) with an average lifetime of 3.15 ns. TRES were constructed from the decay components, and a dynamic Stokes shift was observed to be 480 cm\(^{-1}\). The two components obtained from the fitting of solvent response function using a biexponential function were found to be 0.25 ns (96%) and 1.29 ns (4%) with an average solvation time of 0.29 ns. The biexponential function were found to be 0.14 ns (55%) and 1.00 ns (33%), and 3.60 ns (24%) with an average lifetime of 1.25 ns. At 550 nm, the three components are 0.29 ns (−13%), 1.45 ns (68%), and 4.90 ns (45%) with an average lifetime of 3.15 ns. TRES were constructed from the decay components, and a dynamic Stokes shift was observed to be 480 cm\(^{-1}\). The two components obtained from the fitting of solvent response function using a biexponential function were found to be 0.25 ns (96%) and 1.29 ns (4%) with an average solvation time of 0.29 ns. The biexponential function were found to be 0.14 ns (55%) and 1.00 ns (33%), and 3.60 ns (24%) with an average lifetime of 1.25 ns. At 550 nm, the three components are 0.29 ns (−13%), 1.45 ns (68%), and 4.90 ns (45%) with an average lifetime of 3.15 ns. TRES were constructed from the decay components, and a dynamic Stokes shift was observed to be 480 cm\(^{-1}\). The two components obtained from the fitting of solvent response function using a biexponential function were found to be 0.25 ns (96%) and 1.29 ns (4%) with an average solvation time of 0.29 ns. The components of solvent response functions and the observed dynamic Stokes shifts of DACIA tagged to domain-I of HSA at different temperatures and at different concentrations of GnHCl are compiled in Table 2, and the solvent correlation functions along with their fittings are shown in Figure 9.

| [GnHCl] (M) | temperature (K) | \(r_1\) (ns) | \(r_2\) (ns) | \(r_3\) (ns) | observed Stokes shift (cm\(^{-1}\)) | missing component (%) | degree of folding calculated from emission spectra |
|---|---|---|---|---|---|---|---|
| 0 | 298 | 0.15 (0.55) | 0.68 (0.45) | 0.39 | 480 | 64 | 1.00 |
| 0 | 328 | 0.25 (0.96) | 1.29 (0.04) | 0.29 | 430 | 67 | 1.09 |
| 0 | 348 | 0.20 (0.96) | 1.80 (0.04) | 0.26 | 360 | 79 | 0.86 |
| 2 | 298 | 0.10 (0.60) | 0.33 (0.40) | 0.19 | 400 | 73 | 0.59 |
| 2 | 328 | 0.17 (1.00) | 0.17 | 360 | 80 | 0.44 |
| 2 | 348 | 0.24 (1.00) | 0.24 | 200 | 93 | 0.33 |
| 5 | 298 | 0.07 (0.80) | 0.53 (0.20) | 0.16 | 250 | 85 | 0.03 |
| 5 | 328 | 0.08 (0.45) | 0.21 (0.55) | 0.15 | 130 | 93 | 0.02 |
| 5 | 348 | 0.09 (0.13) | 0.28 (0.87) | 0.26 | 110 | 94 | 0.09 |

From Table 2, it can be seen that either an increasing temperature or a high concentration of GnHCl results in a decrease in solvation time. However, the effect of temperature in the presence of GnHCl (2 or 5 M) is quite strange. In these two cases, we observed that the solvation time is unaffected by raising the temperature from 298 to 328 K; however, further increase in the temperature to 348 K leads to slower solvation. Another important observation is that at 348 K, the solvation time hardly depends on the GnHCl concentration. The dynamic Stokes shift was found to decrease with an increase in temperature or with an increase in the concentration of GnHCl in each of the cases we monitored. Certainly, a significant portion of the total Stokes shift has not been observed in our study unlike the slowest part of the solvation dynamics. The missing components of dynamic Stokes shift in our picosecond setup were calculated using the method demonstrated by Fee and Maroncelli.\(^{48}\) The emission frequency at time zero, \(\nu_{\text{em}}^p(0)\), was calculated using the equation

\[
\nu_{\text{em}}^p(0) = \nu_{\text{abs}}^p - [\nu_{\text{abs}}^p - \nu_{\text{em}}^p(\infty)]
\]
exponential function, and the lifetime was found to be 4.1 ns. After tagging to domain-III of HSA, the fluorescence transient becomes triexponential with an average lifetime of 3.7 ns. Similar to the experiments carried out with DACIA-tagged domain-I of HSA, the lifetime of NPCE-tagged HSA was also measured in the presence of denaturing agents—GnHCl and temperature, and in all cases, the fluorescence transients were fitted with a triexponential function. In the presence of 2 M GnHCl at 298 K, the average lifetime reduced to 3.05 ns. In the absence of GnHCl at 328 K, a decrease in the lifetime was also observed. The average lifetimes of NPCE tagged to domain-III of HSA in the presence of different concentrations of GnHCl at various temperatures are given in Table 3. It could be seen that there is only a small variation in the lifetime of NPCE tagged to domain-III of HSA with an increase in temperature and with an increase in GnHCl concentration.

2.3.3. Solvation Dynamics Measurement. Solvation dynamics within the domain-III of HSA was measured using the tagged NPCE. The fluorescence transients at different wavelengths along the steady-state emission spectrum of the molecule were measured, and using the wavelength-dependent lifetime data, TRES were constructed. Following this, the solvent correlation function has been constructed as described in section 2.2.3. The observed solvation time, dynamic Stokes shift, and the missing components of Stokes shift within the domain-III of HSA for nine different combinations of GnHCl and temperatures are tabulated in Table 4, which are also depicted in Figure 12.

At 298 K and in the absence of GnHCl, the average solvation time is found to be 0.76 ns with its components 0.05 ns (23%) and 0.97 ns (77%). The observed Stokes shift in this case is 225 cm⁻¹. Surprisingly, for domain-III, unlike domain-I, we have not observed a definite trend in solvation time either on changing the temperature or on changing the GnHCl concentration.

Table 3. Emission Maxima and Average Lifetimes of NPCE-Tagged HSA in the Presence of Different Concentrations of GnHCl at Different Temperatures

| GnHCl concentration (M) | temperature (K) | emission maximum (nm) | average τ (ns) |
|--------------------------|-----------------|-----------------------|----------------|
| 0                        | 298             | 479.0                 | 3.70           |
| 0                        | 328             | 478.5                 | 3.69           |
| 0                        | 348             | 480.0                 | 3.79           |
| 2                        | 298             | 485.0                 | 3.68           |
| 2                        | 328             | 482.5                 | 3.32           |
| 2                        | 348             | 489.5                 | 3.32           |
| 5                        | 298             | 494.0                 | 3.81           |
| 5                        | 328             | 494.0                 | 3.67           |
| 5                        | 348             | 495.0                 | 3.76           |
for the formation of the tertiary structure of the proteins.49 Electrostatic interactions and hydrophobicity are responsible for hydrogen bonding, whereas other noncovalent interactions such as secondary structures of proteins are stabilized by hydrogen bonds. Under the action of denaturants, these interactions are broken. Understanding the complex mechanism of denaturation has remained a topic of considerable research for several decades. Temperature-, pH-, and chemical-induced denaturation pathways of various proteins have been extensively studied by several groups.12–19,52,53 In the case of GnHCl, this unfolding is driven by the attractive interaction of the protein surface with the GnHCl molecule. When this interaction overcomes the hydrogen bonding network of the protein, which is responsible for the stabilization of the native state, the protein gets denatured.54 To understand the thermal unfolding of the protein in aqueous solution, a proper understanding of the role of water molecules is necessary. Inside an aqueous solution, the hydrophobic protein molecules tend to disrupt the intermolecular hydrogen bond network between water molecules. In order to retain the network, the water molecules tend to form cagelike ordered structures around the hydrophobic core at the cost of high entropic loss. In order to minimize the effect of unfavorable entropy change, the protein molecules tend to fold so as to reduce the surface area of contact with the water molecules. An interesting fact is that this entropy change associated with the hydrophobic effect depends on the temperature. With an increase in temperature, the ordered cagelike structure tends to break down, thus leading to a decrease in a negative entropy effect. This leads to the gradual unfolding of the protein with increasing temperature.55 The course of denaturing action of GnHCl is related with their capacity to break down the salt bridges that are responsible to stabilize the folded structure of the protein.

In our experiments where we have monitored the denaturation of HSA at a temperature range 283–353 K and in the presence of 0–6 M GnHCl, we could find that the temperature has a less effect to cause the denaturation of the two domains of the protein as compared to GnHCl. In the absence of GnHCl, the degree of folding of both domain-I and domain-III was found to decrease from 1.0 at 298 K to ∼0.7 at 353 K (Figure 5). However, the overall unfolding of the protein was found to be more sensitive to temperature as the degree of folding was found to vary from 1.0 at 298 K to ∼0.4 at 353 K (Figure 2). Also, at any given concentration of GnHCl, the degree of folding due to the change in temperature was not found to vary to a great extent. On the contrary, the degree of folding of both the domains as well as the overall protein decreased from 1 to 0 with an increase in the concentration of GnHCl from 0 to 6 M (by definition, the degree of folding is 1 at 0 M GnHCl, 298 K, and is 0 at 6 M GnHCl, 298 K).

The two domains were also found to show some differences in their nature of unfolding because of GnHCl. In the case of domain-I, the extent of unfolding due to GnHCl was more pronounced at lower temperature (283 K) than at higher temperature (353 K), whereas in the case of domain-III, this unfolding occurs more at higher temperature than at lower temperature. This also leads to a surprising scenario where the

Table 4. Solvent Response Time Components, Average Solvation Times, Dynamic Stokes Shifts, and Degree of Folding of NPCE-Tagged HSA in the Presence of Different Concentrations of GnHCl at Different Temperatures

| [GnHCl] (M) | temperature (K) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | observed Stokes shift (cm$^{-1}$) | missing component (%) | degree of folding calculated from emission spectra |
|-------------|----------------|---------------|---------------|---------------|-------------------------------|----------------------|---------------------------------------------|
| 0           | 298           | 0.05 (0.23)   | 0.97 (0.77)   | 0.76          | 225                           | 85                   | 1.00                                         |
| 0           | 328           | 0.08 (0.62)   | 0.36 (0.38)   | 0.19          | 290                           | 81                   | 0.95                                         |
| 0           | 348           | 0.08 (0.61)   | 0.74 (0.39)   | 0.34          | 350                           | 81                   | 0.80                                         |
| 2           | 298           | 0.08 (0.52)   | 1.15 (0.48)   | 0.59          | 370                           | 77                   | 0.64                                         |
| 2           | 328           | 0.07 (0.55)   | 0.55 (0.45)   | 0.29          | 340                           | 78                   | 0.54                                         |
| 2           | 348           | 0.08 (0.43)   | 0.93 (0.57)   | 0.56          | 240                           | 87                   | 0.29                                         |
| 5           | 298           | 0.15 (0.51)   | 1.69 (0.49)   | 0.91          | 160                           | 90                   | 0.03                                         |
| 5           | 328           | 0.11 (0.59)   | 2.31 (0.41)   | 1.01          | 220                           | 87                   | −0.01                                       |
| 5           | 348           | 0.99 (0.34)   | 1.55 (0.66)   | 1.36          | 130                           | 93                   | −0.05                                       |

Figures 12. Decay of the solvent response function of NPCE tagged to domain-III of HSA in the presence of 0, 2, and 5 M GnHCl (a) at 298, (b) at 328; and (c) at 348 K, respectively.

3. DISCUSSION

It is well-known that definite interactions between amino acid residues guide a protein to fold into its characteristic native 3D structure that determines its functional specificity.49–51 The secondary structures of proteins are stabilized by hydrogen bonding, whereas other noncovalent interactions such as electrostatic interactions and hydrophobicity are responsible for the formation of the tertiary structure of the proteins.49–51 Under the action of denaturants, these interactions are broken. Temperature-, pH-, and chemical-induced denaturation pathways of various proteins have been extensively studied by several groups.12–19,52,53 In the case of GnHCl, this unfolding is driven by the attractive interaction of the protein surface with the GnHCl molecule. When this interaction overcomes the hydrogen bonding network of the protein, which is responsible for the stabilization of the native state, the protein gets denatured.54 To understand the thermal unfolding of the protein in aqueous solution, a proper understanding of the role of water molecules is necessary. Inside an aqueous solution, the hydrophobic protein molecules tend to disrupt the intermolecular hydrogen bond network between water molecules. In order to retain the network, the water molecules tend to form cagelike ordered structures around the hydrophobic core at the cost of high entropic loss. In order to minimize the effect of unfavorable entropy change, the protein molecules tend to fold so as to reduce the surface area of contact with the water molecules. An interesting fact is that this entropy change associated with the hydrophobic effect depends on the temperature. With an increase in temperature, the ordered cagelike structure tends to break down, thus leading to a decrease in a negative entropy effect. This leads to the gradual unfolding of the protein with increasing temperature.55 The course of denaturing action of GnHCl is related with their capacity to break down the salt bridges that are responsible to stabilize the folded structure of the protein.

In our experiments where we have monitored the denaturation of HSA at a temperature range 283–353 K and in the presence of 0–6 M GnHCl, we could find that the temperature has a less effect to cause the denaturation of the two domains of the protein as compared to GnHCl. In the absence of GnHCl, the degree of folding of both domain-I and domain-III was found to decrease from 1.0 at 298 K to ∼0.7 at 353 K (Figure 5). However, the overall unfolding of the protein was found to be more sensitive to temperature as the degree of folding was found to vary from 1.0 at 298 K to ∼0.4 at 353 K (Figure 2). Also, at any given concentration of GnHCl, the degree of folding due to the change in temperature was not found to vary to a great extent. On the contrary, the degree of folding of both the domains as well as the overall protein decreased from 1 to 0 with an increase in the concentration of GnHCl from 0 to 6 M (by definition, the degree of folding is 1 at 0 M GnHCl, 298 K, and is 0 at 6 M GnHCl, 298 K).

The two domains were also found to show some differences in their nature of unfolding because of GnHCl. In the case of domain-I, the extent of unfolding due to GnHCl was more pronounced at lower temperature (283 K) than at higher temperature (353 K), whereas in the case of domain-III, this unfolding occurs more at higher temperature than at lower temperature. This also leads to a surprising scenario where the
most unfolded state of domain-I of HSA in our temperature–GnHCl combinations occurs near 283 K, 6 M GnHCl instead of 353 K, 6 M GnHCl. However, in the case of domain-III, the most unfolded states occurred near 353 K, 6 M GnHCl as expected. The overall denaturation of the protein above 298 K follows an intermediate path, followed by domain-I and domain-III. The stabilizing effect of domain-I at higher temperature suggests that the DACIA molecule, which is bound to the Cys-34 position of the domain-I of HSA, is slightly pushed into a more hydrophobic environment because of the movement of protein side chains near that site of the protein at higher temperatures.

The associated free energy change of different parts of HSA was evaluated by considering either a two-step or a three-step process as given below.

$$Y = \frac{Y_N + Y_U \times e^{-x}}{1 + e^{-x}}$$  \(6\)

$$Y = \frac{Y_N + Y_I \times e^{-x} + Y_U \times e^{-y}}{1 + e^{-x} + e^{-y}}$$  \(7\)

$$x = \frac{(\Delta G^0 - m[\text{denaturant}])}{RT}$$  \(8\)

$$y = \frac{\Delta G^0_{1} + \Delta G^0_{2} - (m_1 + m_2)[\text{denaturant}]}{RT}$$  \(9\)

In the above equation, \(Y\) is the degree of folding and \(Y_N, Y_I,\) and \(Y_U\) are the degree of folding for the native, intermediate, and unfolded states, respectively. \(\Delta G^0\) is the free energy change associated with the concerned transition and \(m\) denotes the slope of the free energy change plotted against the denaturant. \(R\) and \(T\) are the universal gas constant and temperature in K, respectively.

Using the above model, we have calculated the free energy change associated with the domain-specific unfolding of HSA by either temperature or GnHCl. A three-state model was adopted for the temperature-induced unfolding of domain-I of HSA, and for all the other cases, a two-state model was sufficient to fit the data (see Figure 13). The corresponding \(\Delta G^0\) are tabulated in Table 5. The change in free energy associated with each of the unfolding processes is positive, indicating that none of these processes happens spontaneously. It could also be noted that the free energy change associated with the GnHCl-induced unfolding is much lesser than that of the temperature-induced unfolding, showing that GnHCl is much more effective for HSA denaturation and domain-III is more vulnerable than the domain-I of HSA.

Solvation times of DACIA tagged to domain-I of HSA at different temperatures and different concentrations of GnHCl are illustrated in Figure 14. The solvation time is found to generally decrease with an increase in temperature or in GnHCl concentration. This change is expected as the denaturation of HSA results in DACIA being located in a less hydrophobic environment away from the protein core, where the dynamics of water molecules are faster. As a result, the average solvation time of DACIA tagged to domain-I of HSA decreased by 0.1 ns from 0.39 to 0.29 ns when the

**Table 5. Change in Free Energy Associated with the Domain-Specific and Overall Unfolding of HSA**

|          | domain-I of HSA | domain-III of HSA | overall HSA |
|----------|-----------------|-------------------|-------------|
| \(\Delta G^0\) associated with GnHCl-induced unfolding (kcal mol\(^{-1}\)) | 2.8             | 1.7               | 1.8         |
| \(\Delta G^0\) associated with temperature-induced unfolding (kcal mol\(^{-1}\)) | 25.4, 4.7       | 18.6              | 22.2        |

*Figure 13. Change in degree of folding of (a) domain-I of HSA, (b) domain-III of HSA, and (c) overall structure of HSA due to the action of GnHCl (red circles) and temperature (blue circles). The fitting of the data using either a two-state or a three-state model is also shown.*

*Figure 14. Average solvation times of DACIA-tagged HSA at different temperatures in the presence of different concentrations of GnHCl plotted over the contour plot of extent of folding calculated from the steady-state emission spectra.*

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temperature was increased from 298 to 328 K and further decreased by 0.03 ns when the temperature was increased to 348 K, in the absence of any GnHCl. The value of dynamic Stokes shift also reduced monotonously when the temperature was changed from 298 to 348 K. In the presence of 2 M GnHCl, the solvation time decreased with an increase in temperature from 298 to 328 K, but the change was only 0.02 ns (from 0.19 to 0.17 ns) which is only 1/5th of the change we had observed for the corresponding change in temperature in the absence of GnHCl. With further increase in temperature, the average solvation time increased to 0.24 ns. This suggests that increasing the temperature to the region of 348 K would slightly result in the DACIA molecule being located in a more confined environment. Similar changes could be observed in the presence of 5 M GnHCl, where the average solvation time decreases only by 0.01 ns (from 0.16 to 0.15 ns) when the temperature is increased from 298 to 328 K and then increased to 0.26 ns at 348 K. However, at all concentrations of GnHCl, the value of dynamic Stokes shift decreases monotonously with temperature.

The solvation dynamics of NPCE tagged to domain-III of HSA was found to be more intriguing than domain-I of HSA (see Figure 15). When the temperature was increased from 298 to 328 K, the average solvation time decreased sharply by a factor of 4 and 2 in the presence of 0 and 2 M GnHCl, respectively, implying that the temperature has an immediate denaturing effect on the domain-III of HSA. However, a further increase in temperature to 348 K results in an increase in solvation time, suggesting that denaturation near the binding site does not occur anymore and the increase in temperature has a water structure making property near domain-III. In the presence of 5 M GnHCl, such a phenomenon starts even at a lower temperature, and as a result, the average solvation time increases with temperature. At 328 and 348 K, the average solvation time shows a monotonous increase with the increase in the concentration of GnHCl. At lower temperature (298 K), the solvation time decreases when the GnHCl concentration is increased from 0 M GnHCl to 2 M GnHCl and then increases in the presence of 5 M GnHCl (Scheme 1).

This study reveals that the course of denaturation for domain-I and domain-III of HSA is slightly different, which also differs from the nature of the overall denaturation of the protein. The solvation dynamics studies in the presence of denaturing agents, temperature and GnHCl, at two particular regions (domain-I and domain-III) of the protein show that the dynamics at these two regions follow quite different pathways in response to the denaturing agents. This must be due to the different confined environments in which the fluorophores are located. The much slower solvation times of the NPCE molecule tagged to domain-III of HSA suggest that relaxation of water molecules is slower inside domain-III as compared to that of domain-I. Also, in the case of DACIA-tagged HSA, denaturing agents cause a decrease in solvation time in most of the experiments we had carried out, which is expected. On the other hand, the solvation dynamics inside domain-III reveals no trend on imposing the denaturation either by the temperature of GnHCl. Increase in temperature or concentration of GnHCl sometimes resulted in more confinement in the motion of water molecules inside the protein core, which resulted in slower solvation at these sites. The confinement of water molecules could have been caused because of the orientation of side chains near the binding site of the fluorophore or the stronger bonding of water molecules with the amino acid moieties.

4. CONCLUSIONS

To conclude, we have studied the effect of GnHCl and temperature on the overall structure, domain-I, and domain-III of HSA using covalent fluorescent labels. The overall unfolding of HSA calculated using CD spectroscopy showed that the unfolding of the protein molecule is following the expected trend, where it is more unfolded at a higher temperature and in the presence of GnHCl. The domain-specific unfolding of domain-I and domain-III of HSA, calculated using steady-state emission spectroscopy, also exhibited a slightly different behavior compared to the overall unfolding. Thus, we proved that different parts of HSA do not unfold sequentially. From solvation dynamics studies, it was revealed that solvation inside the core of domain-III is much slower than that inside the core of domain-I. This difference is attributed to the different microenvironments at the core of the two domains, which restrict the motion of water molecules at the respective sites.

Scheme 1. Schematic Representation of Domain-Specific Unfolding of HSA in Double Denaturing Condition

"The nature of change of associated solvation dynamics is also represented through circular disk representation (the larger the circle the larger the solvation time)."

Figure 15. Average solvation times of NPCE-tagged HSA at different temperatures in the presence of different concentrations of GnHCl plotted over the contour plot of extent of folding calculated from the steady-state emission spectra.
This difference in local environments inside the two domains also results in different responses of their solvation dynamics to the denaturing agents, as the domain-I of HSA shows a more regular decrease in solvation time because of higher temperature or presence of GnHCl, whereas for domain-III of HSA, the change in solvation time with temperature or concentration of GnHCl is more random.

5. EXPERIMENTAL SECTION

5.1. Materials. HSA (fatty acid free) and GnHCl were purchased from Sigma-Aldrich and were used as received. DACIA was purchased from Molecular Probes Inc. and was used as received. Coumarin-343, 4-dimethylamino pyridine, N,N-dicyclohexyl carbodiimide, and 4-nitrophenol were bought from Sigma-Aldrich and were used as received for the synthesis of NPCE as reported earlier.39 Analytical grade disodium 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 cutoff) was purchased from Sigma-Aldrich and used after removing the glycerol and sulfur compounds according to the procedure given by Sigma-Aldrich. Centrifugal filter units (Amicon Ultra, 10 kDa cutoff) were purchased from Merck Millipore, Germany. HSA was tagged with DACIA and NPCE following the already reported procedure (Scheme 2).39,41 High-performance liquid chromatography grade dimethyl sulfoxide and dichloromethane were purchased from Spectrochem, India.

5.2. Instrumentation. All steady-state absorption spectra were recorded using a UV–visible spectrophotometer (Shimadzu 2450, Japan). For recording, an emission spectra spectrofluorimeter (FluoroMax-4, Jobin Yvon, USA) was used. A commercial CD spectrometer (Jasco J-815, Japan) was used for recording CD spectra. Centrifugation and lyophilization were done in Eppendorf centrifuge 5810R and Heto VR-1 setup, respectively. Unless stated otherwise, all the experiments were done at 298 K.

Time-resolved fluorescence decays were collected using a commercial time-correlated single photon counting setup (LifeSpec-II, Edinburgh Instruments, UK). Two different lasers were used for exciting the samples. A 375 nm laser, EPL-375, Edinburgh Instruments, UK, was used for exciting the samples with DACIA, and a 442 nm laser, EPL-445, Edinburgh Instruments, UK, was used for exciting the samples with NPCE. Peak counts of 8000 were collected for lifetime measurements. The emission polarizer was oriented at the magic angle (54.7°). Dilute lodox solution was used for the measurement of instrument response function (IRF), which was found to be ∼120 ps. The contribution of IRF was nullified by the iterative reconvolution method. A commercial software, Fast, Edinburgh Instruments, UK, was used for this purpose. The fluorescence transient decays were fitted using the equation

\[ I(t) = \sum_{i=1}^{n} f_i e^{-t/\tau_i} \]  

(10)

where \( I(t) \) is the fluorescence intensity at time \( t \) and \( f_i \) is the amplitude associated with the fluorescence lifetime \( \tau_i \). The sum of \( f \) values is unity. We have used Igor Pro software for further plotting and analysis.

The parameters obtained after fitting the decay transients at different wavelengths using the above equation were used for constructing the TRES. The change in peak frequency with time obtained from the TRES data was used to calculate the time-dependent Stokes shift. The solvent correlation function, \( C(t) \), constructed from the peak frequency is given by

\[ C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)} \]  

(11)

where \( \nu(0), \nu(t), \) and \( \nu(\infty) \) are the peak frequencies at time 0, \( t \), and \( \infty \), respectively.

“Fluorescent tag labeling schemes for DACIA and NPCE are also shown in the lower panel.

Scheme 2. Schematic Representation of HSA (PDB: 1HA2) Showing Three Different Domains and Tagging Sites, Cys-34 and Tyr-411“
Crystal structure of human serum albumin at 2.5 Å resolution.

Pharmacol.

Understanding protein folding via free-energy surfaces from theory and experiment.

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