Mechanistic investigation of domain specific unfolding of human serum albumin and the effect of sucrose

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Received 12 June 2013; Revised 27 July 2013; Accepted 16 August 2013

DOI: 10.1002/pro.2357

Published online 26 August 2013 proteinscience.org

Abstract: This study is devoted to understand the unfolding mechanism of a multidomain protein, human serum albumin (HSA), in absence and presence of the sucrose by steady-state and time-resolved fluorescence spectroscopy with domain specific marker molecules and is further being substantiated by molecular dynamics (MD) simulation. In water, the domain III of HSA found to unfold first followed by domains I and II as the concentration of GnHCl is increased in the medium. The sequential unfolding behavior of different domains of HSA remains same in presence of sucrose; however, a higher GnHCl concentration is required for unfolding, suggesting stabilizing effect of sucrose on HSA. Domain I is found to be most stabilized by sucrose. The stabilization of domain II is somewhat similar to domain I, but the effect of sucrose on domain III is found to be very small. MD simulation also predicted a similar behavior of sucrose on HSA. The stabilizing effect of sucrose is explained in terms of the entrapment of water molecules in between HSA surface and sucrose layer as well as direct interaction between HSA and sucrose.

Keywords: unfolding mechanism; multidomain protein; denaturation; stabilization; sugar; human serum albumin

Introduction

The characterization of folding pathway of a multidomain protein is a complex process by virtue of the presence of different domains, that folds/unfolds independently, and their mutual interactions to form the globally stabilized tertiary structure.^{1–8} The complete understandings of the unfolding process require a detailed domain specific study and thus establish the complete denaturation landscape. Human serum albumin (HSA, Scheme 1) is such a multidomain protein with three different distinguishable domains and has been studied widely in the last few decades.^{4–14} It consists of 585 amino

acids residue and is the major protein found in our circulatory system.¹⁵ The principal function of HSA is to transport a variety of metabolites and drugs in human body.^{16–22} The three dimensional structure of HSA has been determined crystallographically by He *et al.* followed by Curry *et al.* and characterized by three structurally similar α -helical sub-units named as domains I, II, and III, which assemble to form a heart-shaped structure. Each domain is divided into two sub-domains (denoted as A & B) possessing some common structural motifs.^{22–25} Among these domain IIA and IIIA are identified as the major drug binding subdomains.^{23,24} The single tryptophan (Trp, Scheme 1) unit of HSA is located in domain II and often the Trp fluorescence was used to study this domain selectively.^{15,23,24} However, the selective study of domain III is rare in the literature as no intrinsic fluorophore is present in this domain.^{26–28} Bagatolli *et al.* studied the binding of 8-anilino-1-naphthalenesulphonate (ANS, Scheme 1) in HSA and found that it selectively bind to the domain III with very high binding constant of

Additional Supporting Information may be found in the online version of this article

Fellowship to RY, University Grant Commission, Government of India. Grant sponsor: Board of Research in Nuclear Sciences (BRNS), Department of Atomic Energy (DAE), Government of India.

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Scheme 1. (a) Schematic representation of human serum albumin (HSA), showing domains I, II, and III and the location of hemin, Trp, ANS therein.^{15,16} (b) Structure of hemin, (c) Structure of 8-anilinonaphthalene-1-sulphonic acid ammonium salt (ANS), and (d) Structure of tryptophan (Trp). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$0.87 \times 10^6 \text{ M}^{-1}$ and proposed ANS as marker for domain III.²⁹ However, hemin (Scheme 1) is known to bind to domain I and can be used for the selective characterization of domain I.^{30,31}

Since the function of proteins depends on their three-dimensional structure, several researchers put on their efforts to found suitable agent which combat the denaturation process under adverse condition.^{32–37} Several sugars are found to act as a stabilizing agent for proteins and have been a subject of study for last few decades.^{35–49} It has also been noticed that different sugars, for example, trehalose, sucrose, maltose, fructose, glucose, etc., have different stabilizing power for different proteins. There are some hypothesis regarding the stabilization of protein in presence of sugar given by different authors as: (i) The water replacement hypothesis; according to which the stabilization occurs via the formation of hydrogen bonds between the sugar and the biomolecules,^{38,39} (ii) The water-entrapment hypothesis; in this case, trapping of water molecules in between protein surface and sugar layer is the main stabilizing mechanism, which slows down the biological water,⁴⁰ (iii) Mechanical-entrapment hypothesis; here the entrapment of a particular biomolecular conformation takes place in a high viscous sugar matrix⁴⁸ and, (iv) Broken glass hypothesis; in this case, the direct interaction between protein and sugar takes place as well as water entrapment occurs in the system.⁴⁹ Alfonso *et al.* demonstrated the stabilizing effect of trehalose on beta-lactoglobulin (BLG) by using fluorescence lifetime and fluorescence anisotropy measurements. They revealed that addition of trehalose shifts the midpoint of BLG denaturation profiles towards higher denaturant concentration with a 60% increase in the free energy of denaturation.⁴² Their conclusion indicates the mechanical-entrapment hypothesis is in operation in this case. MD simulations of aqueous lysozyme in presence and

absence of trehalose have been performed by Lins *et al.* and support the second hypothesis of water entrapment for the stabilization.⁴³ Whereas in 2010 Fedorov *et al.* showed that trehalose forms patches on the surface of lysozyme and support fourth hypothesis i.e. broken glass hypothesis.⁴⁹ It has been observed that the stabilization power of different sugars varies for different proteins. Trehalose was found to be a better stabilizing agent than sucrose for BLG, carboxy myoglobin, etc.,^{50,51} whereas, for azurin, the stabilizing power of sucrose is found to be higher than trehalose.³⁵ Sola-Penna and Roberto suggested that trehalose is more effective because it occupies at least 2.5 times larger volume than other sugars⁴⁶ and this stabilization is fully explained by the mechanical entrapment hypothesis.⁴³ In general, the stabilization effect of sucrose is almost same of the trehalose, if concentration of sucrose is almost three times higher than that of trehalose.⁴⁶

Nevertheless, for a multidomain protein the unfolding pathway of different domains in absence and presence of sucrose is expected to be different. However, such a study has not been performed in the past to the best of our knowledge and we intend to pursue this using HSA as the model multidomain protein. In this study, we used guanidine hydrochloride as the denaturant to investigate the effect of sucrose on the denaturation profile of three different domains of HSA. We also have performed atomistic molecular dynamics (MD) simulations and calculate the fluctuation of different domains of HSA to have a molecular level picture.

Results

Study of domain III

Domain III of HSA has been studied by using the extrinsic fluorophore ANS.^{29,52–62} Bagatolli *et al.*

used diazepam and aspirin as a marker and revealed that ANS binds to domain IIIA with high affinity ($K = 0.87 \times 10^6 \text{ M}^{-1}$) and to domain IIA with low affinity ($K = 0.079 \times 10^6 \text{ M}^{-1}$).⁴³ Thus it is important to carefully choose the relative concentration of ANS to HSA, because at higher concentration it can also bind to the lower affinity site. At a relative concentration of ANS to HSA $< 10^3$, it preferentially binds to domain III⁵⁹ and in this study ANS/HSA ratio is maintained at 0.5. The concentration of HSA is maintained at $10 \mu\text{M}$ for all the studies. This is to mention that at this ANS to HSA concentration ratio, the secondary structure of HSA remains unchanged (Supporting Information Figure S1). ANS in water is characterized with absorption maximum at 374 nm and emission maximum at ~ 520 nm with a very low fluorescence quantum yield of 0.003.²⁹ On binding to HSA, the fluorescence property of ANS changes dramatically with a large blue shift of its emission maximum to 465 nm and increase in fluorescence quantum yield to 0.67, without much affecting its absorption characteristics. It is to be noted that the ANS have two emissive states; nonplanar (NP) state where ANS remains NP and charge transfer (CT) state where molecule become planar and charge transfer takes place. In polar media, the locally excited (LE) state readily formed the non-emissive CT state and thus the fluorescence quantum yield of ANS in water is very low. In low polarity media, the emission predominantly occurs from the LE state which results in the blue shift of the emission maximum and increase in the fluorescence quantum yield.⁵² On denaturation the absorption spectrum of ANS does not change much, however, the fluorescence response of ANS is found to be depends strongly on denaturation. Until 1.5M GnHCl, the fluorescence quantum yield is found to decrease slowly with a mere change of its maximum. On further increase in GnHCl concentration, the fluorescence intensity decreases rapidly with a notable red shift of the emission maximum (Fig. 1). To have a first look on the effect of sucrose on denaturation, we repeat the same experiment in presence of 1M sucrose. The emission maximum of ANS in HSA with 1M sucrose was observed at 464.5 nm with fluorescence quantum yield of 0.63 in native state, which is almost similar in absence of sucrose. However, the effect of GnHCl is found to be completely different (Fig. 1). In presence of sucrose, the emission maximum of ANS remains unchanged till 2.5M GnHCl. To get a better picture on the effect of sucrose on denaturation pathway of domain III, we measured the fluorescence transients of ANS bound to HSA at different GnHCl concentration in absence and presence of sucrose, which are shown in Supporting Information Figure S2a and S2b, respectively. The average lifetime of ANS bound to HSA with increase in the GnHCl concentration in absence

Figure 1. Plot of ANS emission maxima in the absence (●) and in presence (▲) of 1M sucrose as a function of GnHCl concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and presence of sucrose are shown in Figure 2 and are tabulated in Table I. ANS displays a triple exponential fluorescence decay behavior with three distinct lifetime components of 0.75 ns (0.01), 7.47 ns (0.15) and 18.13 ns (0.84) in the native state of HSA when no sucrose is present in the system with an average lifetime of 16.36 ns. On denaturation, the fluorescence lifetime of ANS in HSA keeps on decrease monotonically and at 3.2M GnHCl, the three fluorescence lifetime components are 0.26 ns (0.27), 2.51 ns (0.44), and 7.05 ns (0.29) with an average lifetime of 3.22 ns. In presence of 1M sucrose, the average lifetime of ANS in native HSA is found to be 16.18 ns, which is almost same as in absence of sucrose. However, at 3.2M GnHCl the fluorescence lifetime of ANS is measured as 4.03 ns, which is 1.25 times higher compared with when sucrose is not present in the medium. On further increasing the GnHCl concentration to 4M , the average lifetime of ANS bound to HSA becomes 3.36 ns, which is very similar to the average lifetime of ANS

Figure 2. Plot of Average lifetime of ANS in HSA (●) in the absence and (▲) in the presence of 1M sucrose as a function of GnHCl concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Fluorescence Lifetimes of ANS in HSA for Different Concentration of GnHCl and Sucrose

Sucrose (M)	GnHCl (M)	τ_1^a ns (a_1)	τ_2^a ns (a_2)	τ_3^a ns (a_3)	$\langle\tau\rangle^a$ (ns)
0	0	0.75 (0.01)	7.47 (0.15)	18.13 (0.84)	16.36
0	1	0.63 (0.02)	6.21 (0.17)	16.72 (0.81)	14.61
0	2	0.35 (0.21)	3.36 (0.32)	10.12 (0.47)	05.90
0	3.2	0.26 (0.27)	2.51 (0.44)	07.05 (0.29)	03.22
0	4.5	0.23 (0.28)	2.03 (0.43)	07.29 (0.29)	03.06
1	0	0.52 (0.01)	5.73 (0.15)	18.23 (0.84)	16.18
1	1	0.53 (0.03)	5.05 (0.18)	16.03 (0.79)	13.58
1	2	0.39 (0.22)	3.45 (0.29)	11.32 (0.49)	06.63
1	3.2	0.31 (0.24)	2.19 (0.37)	08.07 (0.39)	04.03
1	4.5	0.32 (0.25)	2.04 (0.39)	07.22 (0.36)	03.47

$$\langle\tau\rangle = a_1 \tau_1 + a_2 \tau_2 + a_3 \tau_3.$$

^a ± 0.2 .

in 3.2M GnHCl when sucrose is absent. It shows that the denaturation profile of domain III of HSA in absence and presence of 1M sucrose are different and a relatively high concentration of denaturant is required to unfold the domain III of HSA in presence of 1M sucrose.

Study of domain II

The fluorescence properties of single Trp residue in domain II of HSA is used to probe the unfolding behavior of this domain selectively. The absorption spectrum of HSA have the contribution from this single Trp solely around 290 nm region with its characteristic emission centered at 347 nm with a 295 nm excitation. On denaturation, the emission maximum of Trp changes with decrease in the fluorescence intensity. Until 1.75M GnHCl, the emission maximum shows a 5.5 nm blue shift and then it starts shifting to the longer wavelength as is shown in Figure 3. For 4.5M GnHCl the maximum is observed at 357 nm. To investigate the effect of sucrose on the denaturation profile of domain II of HSA, same experiment has been performed in the presence of 1M sucrose. In presence of 1M sucrose, the Trp emission of native HSA is characterized

Figure 3. Trp emission maxima in the absence (●) and in the presence (▲) of 1M sucrose as a function of GnHCl concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with a maximum at 348 nm, which is almost similar as in the absence of sucrose, although there is a small decrease in the fluorescence intensity. Like in absence of sucrose, an initial blue shift of the emission maximum of Trp is also observed with increase in GnHCl concentration until 2.25M. On further increasing the GnHCl concentration, the emission maximum start shifting to the red and at 4.5M GnHCl concentration the emission maximum is found to be at 359 nm (see Fig. 3). At a first glance, this observation indicates sucrose also stabilizes the domain II of HSA. To better understand this behavior we measure the lifetime of Trp for different GnHCl concentration by recording the fluorescence transients. In bulk water, the fluorescence decay of Trp in HSA is biexponential in nature with two time constants of 3.12 ns (0.37) and 7.10 ns (0.63), having an average lifetime of 5.62 ns. The lifetime of Trp decreases monotonically with the increase in the GnHCl concentration. At 4.5M GnHCl, the fluorescence decay can be best fitted with two time constants of 1.39 ns (0.46) and 3.51 ns (0.54) with an average lifetime of 2.54 ns. In presence of 1M Sucrose, the average lifetime of Trp in HSA is found to be 6.63 and 3.08 ns in the native and unfolded (with 4.5M GnHCl) state, respectively. The observed fluorescence transients are shown in Supporting Information Figure S3a and S3b and the data are tabulated in Table II. Figure 4 shows the variation of the average lifetime of Trp in HSA as a function of GnHCl concentration. It can be readily seen that in absence of sucrose, domain II become fully denatured at 4.5M GnHCl, which remains unchanged till 6M of GnHCl. In presence of 1M sucrose, the average lifetime of Trp in HSA with 4.5M GnHCl is clearly high compared with in the absence of sucrose. This indicates the protective nature of sucrose for domain II of HSA.

Study of domain I

To study of domain I, we have used hemin as quencher which selectively binds to the domain I of HSA.^{27,30,31} Dockal *et al.* showed that hemin

Table II. Fluorescence Lifetimes of Trp in HSA for Different Concentration of Sucrose and GnHCl

Sucrose (M)	GnHCl (M)	τ_1^a ns (a_1)	τ_2^a ns (a_2)	$\langle\tau\rangle^a$ (ns)
0	0	3.12 (0.37)	7.10 (0.63)	5.62
0	1	2.88 (0.34)	6.61 (0.66)	5.33
0	2	2.25 (0.43)	6.01 (0.57)	4.39
0	3	1.80 (0.52)	4.97 (0.48)	3.32
0	4.5	1.39 (0.46)	3.51 (0.54)	2.54
0	6	1.40 (0.47)	3.34 (0.53)	2.42
1	0	2.46 (0.24)	6.63 (0.76)	5.63
1	1	2.28 (0.27)	6.39 (0.73)	5.28
1	2	1.99 (0.33)	5.80 (0.67)	4.54
1	3	1.83 (0.41)	5.51 (0.59)	4.00
1	4.5	1.21 (0.38)	4.22 (0.62)	3.08

$$\langle\tau\rangle = a_1 \tau_1 + a_2 \tau_2.$$

^a ± 0.2 .

also binds to other secondary binding sites located in domain II and III of HSA at the molar ratio of hemin/HSA as 2.³⁰ Hence, in this study hemin/HSA ratio is maintained at 0.5. CD spectra confirm that at this low hemin concentration the secondary structure of HSA remains unchanged (Figure S1 Supporting Information). Hemin quenches the intrinsic Trp fluorescence of HSA, which depends on the relative distance between them.^{30,31} In native state, the relative fluorescence quenching of Trp with hemin is found to be 34.8%. On denaturation, the relative fluorescence quenching of Trp by hemin decreases monotonically and at 4.5M GnHCl it is found to be 6.8%, which remain unchanged on further increase of GnHCl concentration. To understand the effect of sucrose on denaturation profile of domain I, we repeat the same experiment in presence of 1M sucrose. The hemin mediated relative fluorescence quenching of HSA as a function of GnHCl shows a

Figure 4. Plot of Average lifetime of Trp in HSA (●) in the absence and (▲) in the presence of 1M sucrose with increase in the GnHCl concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. Relative Trp fluorescence quenching by hemin (●) in the absence and (▲) in the presence of 1M sucrose as a function of GnHCl concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

different behavior in presence of 1M sucrose compared with otherwise as shown in Figure 5 and Table III. In presence of 1M sucrose, the relative fluorescence quenching of native HSA is measured to be 35.8% and become 13.3% at 4.5M GnHCl. This suggests that at 4.5M GnHCl the domain I of HSA is less denatured in presence of sucrose compared with when sucrose is not present in the medium.

MD simulations

To have a molecular level understanding of the effect of sucrose on different domains of HSA, MD simulation has been performed. It is to mention that during the course of the simulation, HSA remained in the folded state. The variation of potential energy and kinetic energy with time satisfied the energy conservation criteria in MD simulation and there was no change detected after an initial drift in total energy of the system for both

Table III. Relative Fluorescence Quenching (%) at 340 nm of HSA for Different Concentration of GnHCl and Sucrose

Sucrose (M)	GnHCl (M)	Relative fluorescence quenching (%) ^a
0	0	34.8
0	1.25	30.1
0	2	25.0
0	3	12.7
0	4.5	6.8
0	6	6.1
1	0	35.8
1	1	32.6
1	2	28.0
1	3	20.2
1	4.5	13.3

^a $\pm 2\%$.

Figure 6. (a) Root mean square atomic positional deviation (RMSD) of α -C atoms of HSA as a function of time and (b) root mean square atomic positional fluctuations (RMSF) of α -C atoms of different amino acid residues of HSA averaged over 1.5–2.5 ns of MD simulation, in the absence (—, red line) and in the presence (—, blue line) of 1M sucrose. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the cases under investigation. The protein radius of gyration as well as solvent accessible surface area has also been calculated to see the compactness of protein structure in absence and presence of sucrose. Supporting Information Figure S4 shows the similar values of radius of gyration for HSA in absence and presence of sucrose as a function of time. Solvent accessible surface area for HSA has been calculated as 32,238 Å² and 31,456 Å² in absence and presence of sucrose, respectively, by using probe radius as 1.4 Å. The root mean-square atomic positional deviation (RMSD) as well as root mean-square atomic positional fluctuations (RMSF) have been calculated for α -carbon atoms of the protein after a proper alignment. The calculated RMSD and RMSF are shown in Figure 6(a,b), respectively. It has been observed that RMSD is small when sucrose is present in the system and it can be regarded as the stabilization of HSA by sucrose. The observed fluctuations in all residues are not uniform in either case. Radial distribution functions corresponding to the water in absence of sucrose as well as water and sucrose in presence of sucrose around

Figure 7. Radial distribution function for water around the center of geometry of HSA in absence of sucrose (—, red line), for water around the center of geometry of HSA in presence of sucrose (—, black line), and for sucrose around the center of geometry of HSA (—, blue line). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the centre of geometry of HSA have also been calculated and shown in Figure 7. It shows that some water molecules in presence of sucrose come closer to the protein surface than when sucrose is absent in the system. The average number of hydrogen bonds between the amino acid residues and water in different domains has also been calculated for both the cases. It shows that there was 27, 40, and 38% increase in number of hydrogen bonds for domain I, II, and III, respectively in presence of sucrose.

Discussion

The main aim of the present work is to investigate the unfolding mechanism of different domains of HSA and to study the effect of sucrose therein. As mentioned above, we have used different marker molecules for different domains, namely hemin for domain I, intrinsic Trp-214 for domain II and ANS for domain III, respectively. The change in the fluorescent response of a fluorophore in a protein matrix during its unfolding process is a well known phenomenon in the literature and explained in terms of the increase in the nonradiative pathway of the fluorophore in more polar solvents (generally water) when exposed due to the unfolding of the protein which is the case for Trp and ANS in domain II and III, respectively.^{28,52–57} For domain I the relative fluorescence quenching of Trp by hemin, which depends on their relative distance, has been used to monitor the unfolding process.

Using Eqs. (1) and (2) first we have calculated the change in the free energy of unfolding for different GnHCl concentrations and consequently calculated $[D]_{1/2}$ (concentration of denaturant for 50% unfolding) using Eqs. (3) and (4). The variation of ΔG as a function of GnHCl concentration for domain

Figure 8. Free energy change during denaturation for domain I (a, d), domain II (b, e), and domain III (c, f) as a function of GnHCl concentration (●) in the absence and (▲) in the presence of 1M sucrose. Solid lines represent the best fit with Eq. (3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

I, II, and III is shown in Figure 8(a–c), respectively. The linear nature of ΔG depends on GnHCl concentration also confirms the two step unfolding process of HSA and is the case for individual domains. The estimated values of $[D]_{1/2}$ for domain I, II, and III are 2.3, 2.4, and 1.6M GnHCl, respectively. This clearly indicates that the whole protein does not unfold uniformly and our results also suggest that domain III of HSA is most unstable and denatured first followed by almost simultaneous denaturation of domains I and II. This is to mention here, we have used the relative fluorescence quenching of Trp by hemin (reside in domain I of HSA) to study the unfolding behavior of domain I. Obviously, the change in the tertiary structure of domain II has also contribute to the domain I probing. In presence of sucrose, the $[D]_{1/2}$ values are also calculated by same fashion and found to be 3.1, 3.0, and 1.7M GnHCl for domains I, II, and III, respectively [see Fig. 8(d–f)]. Here, the unfolding of domain III happens first followed by domains II and I. However, it is interesting to note that, the value of $[D]_{1/2}$ in presence of sucrose is found to be more compared with in the absence of sucrose, which is an indication of the sucrose mediated stabilization of HSA towards

its unfolding by GnHCl. The difference in the $[D]_{1/2}$ values ($\Delta[D]_{1/2} = [D]_{1/2}^{\text{sucrose}} - [D]_{1/2}^{\text{water}}$) for domain I, II, and III are calculated to be 0.8, 0.6, and 0.1M GnHCl, respectively. Understandably, the stabilization provided by sucrose for different domains of HSA are not same. Domain I get more stability by sucrose followed by domain II and III. The low value of $\Delta[D]_{1/2}$ for domain III (0.1M GnHCl) suggests a mere gain in stabilization for this domain, which is not the case for other domains.

MD simulation also qualitatively represents the protective nature of sucrose on HSA unfolding, as evident from the lower and comparatively stable RMSD value of α - carbon atoms of HSA when sucrose is present in the system [see Fig. 6(a)]. From Figure 6(b), we can see that the sucrose induced stabilization of RMSF values for different residues of HSA are not uniform in nature. For the hemin binding site of domain I (residue number 102–190), the average stabilization of RMSF value is found to be 0.14 Å, and for Trp environment in domain II (residue number 197–297) the value is calculated to be 0.16 Å. This is to note that the stabilization of the above two sites are almost same. In case of ANS binding site in domain III (residue number 384–497) the stabilization of RMSF value is found to be 0.11 Å.^{22,23,31} MD simulation thus predict the lowest sucrose mediated stabilization of domain III binding site of HSA compared with the other domains and is in good agreement with our experimental observations. The average number of hydrogen bonds formed by different amino acid residues of HSA with water and sucrose is also calculated from the MD trajectory. In presence of sucrose, it has been seen that 15, 11, and 13% of total sucrose molecules involves in hydrogen bonding with domain I, II, and III, respectively and there is a 27, 40, and 38% increase in number of hydrogen bonds with water compared with when sucrose is not present in the system. Radial distribution function also clearly indicates that water as well as sucrose molecules come closer to the surface of HSA when sucrose is present in the system (see Fig. 7). This suggests that the broken glass hypothesis is in operation in this case and manifests a differential stabilization of sucrose on different domains of HSA.

In conclusion, this study reveals the domain wise unfolding mechanism of HSA by GnHCl in absence and presence of sucrose. HSA has three distinct domains I, II, and III and were studied using different marker molecules specific for each domains. It was observed that, in water, domain III of HSA first gets unfold in the denaturation process. The domain I and II unfolds thereafter almost simultaneously. The $[D]_{1/2}$ values of domain I, II, and III for GnHCl are estimated to be 2.3, 2.4, 1.6M, respectively. In presence of sucrose, the $[D]_{1/2}$ values are found to be 3.1, 3.0, and 1.7M GnHCl,

respectively. The sequence of unfolding of HSA in sucrose medium is found to be same as compared with otherwise, however the $[D]_{1/2}$ values are shifted at higher GnHCl concentration. Clearly, addition of sucrose impose restriction in the GnHCl induced unfolding process for all the three domains but with different extent. The domain I and II are found to be stabilized more by sucrose as compared to domain III. The relative change in the RMSD and RMSF values obtained from MD simulation study also supports our experimental observations. This is to conclude that the unfolding behavior of different domains of a big protein is not uniform and the stabilizing effect of a stabilizer is expected to be different for different domains. The increase in the number of hydrogen bonding between the amino acid residues and water as well as the participation of sucrose in hydrogen bonding with protein indicate that broken glass hypothesis is in operational in the stabilization process.

Materials and Methods

Experimental

HSA [Scheme 1(a)], hemin from Porcine [Scheme 1(b)] and 8-anilidonaphthalene-1-sulphonic acid ammonium salt [ANS, Scheme 1(c)] were purchased from Sigma-Aldrich and used as such. Molecular biology grade guanidine hydrochloride (GnHCl) was purchased from Spectrochem, India and sucrose from Sigma-Aldrich and used as received. The steady state absorption and emission spectra were recorded in a commercial UV-visible spectrophotometer (Schimadzu 2450) and spectrofluorimeter (Fluoromax 4, Jobin Yvon), respectively.

In all the experiments, ANS was excited at 375 nm and Trp was excited at 280 nm. Time resolved fluorescence transients were recorded at magic angle polarization using a commercial TCSPC setup (Life Spec II, Edinburgh Instruments and FluoroLog 3-21, Jobin Yvon). The full width at half maxima of the instrument response function is about 110 ps and 1.2 ns, respectively. All fluorescence transients for Trp and ANS were recorded at 350 and 470 nm, respectively. All the experiments have been done in absence and presence of 1M sucrose by using GnHCl as denaturant. The concentration of GnHCl has been varied from 0 to 6M in absence of sucrose, however in presence of 1M sucrose, it has been varied from 0 to 4.5M due to solubility problem. All the experiments have been performed at 25°C.

Data analysis

The unfolding of HSA was done by GnHCl as chemical denaturant and is known to follow a two step unfolding ($N \rightleftharpoons U$), where N and U are the native and denatured states of HSA.¹⁰ The equilibrium con-

stant (K) of the native to denatured state transformation can be calculated as,^{4,6}

$$K = \frac{\tau_N - \tau}{\tau - \tau_U} \quad \text{or} \quad \frac{FQ_N - FQ}{FQ - FQ_U} \quad (1)$$

where τ_N and τ_U are the fluorescence lifetimes of protein in folded and unfolded state, respectively, whereas, FQ_N and FQ_U are the relative fluorescence quenching in the native and unfolded states, respectively. Here, τ and FQ are the fluorescence lifetimes and relative fluorescence quenching of HSA at different denaturant concentration. The observed value of K was used to calculate free energy change (ΔG) between native and unfolded states using following equation.

$$\Delta G = -RT \ln K \quad (2)$$

where R is the universal gas constant (1.987 cal K⁻¹ mol⁻¹) and T is the absolute temperature. The free energy change in the absence of denaturant (ΔG^0) can be calculated by extrapolation method by fitting the data observed in Eq. (2) as a function of denaturant using,⁴

$$\Delta G = \Delta G^0 - m[D] \quad (3)$$

where m is the slope, which measures the dependence of ΔG on denaturant. The concentration of denaturant to unfold 50% of the protein ($[D]_{1/2}$) can be written as,

$$[D]_{1/2} = \frac{\Delta G^0}{m} \quad (4)$$

MD simulations

Atomistic MD simulations at 300 K have been performed for aqueous solution of HSA in presence and absence of 1M sucrose, under the periodic boundary condition. The initial coordinate of HSA was obtained from the Protein Data Bank (PDB ID: 1HA2).⁶³ For both cases, we have used Packmol⁶⁴ for fixing the protein at the center of a box with edges $104 \times 66 \times 106 \text{ \AA}^3$ and were solvated with the TIP3P⁶⁵ water molecules. Sucrose molecules, when present, were uniformly distributed throughout the box with a distance of 5 Å from protein surface as well as from each other. The HSA-water system contains one HSA and 20,837 water molecules, whereas the HSA-water-sucrose system contains one HSA, 420 sucrose, and 6394 water molecules. For both the cases, solution was neutralized by addition of 13 sodium ions. AMBER 9⁶⁶⁻⁶⁹ and general AMBER force field⁷⁰ has been used to model the protein and sucrose molecules, respectively. First, the systems were minimized for 1000

steps by holding both the protein and sucrose for HSA-water-sucrose system and protein alone for the HSA-water system. These were followed by 2500 steps of minimization without restrain any solute. Each system was equilibrated for 20 ps with restrained the protein and sucrose (when present). In case of HSA-water-sucrose a further 30 ps equilibration has been done holding the protein alone. During this equilibration, each time steps was 1 fs and the initial temperature for both the systems was 0 K. The system reached 300 K in 6–7 ps time scale and maintained the same temperature hereafter. Finally, the MD simulation has been carried out for 2.5 ns by maintaining the temperature at 300 K with the help of Langevin dynamics.⁷¹ Isotropic position scaling⁷² was used to maintain the pressure at 1 atm with a relaxation time of 2 ps. The SHAKE⁷³ algorithm was employed to restrain the length of all covalent bonds involving hydrogen atom as well as the geometry of water molecule. A 2 fs time step was used to integrate the equation of motion and each 0.5 ps trajectory frames were recorded for analysis. Particle Mesh Ewald method was employed to obtain the long range electrostatic interactions whereas a 10 Å cutoff was used for van der Waals interactions.⁷⁴

Acknowledgment

MD simulations were performed using the computer cluster installed at the Department of Chemistry, Indian Institute of Technology Kanpur, through the DST-FIST program. The authors also thank Dr. Nisanth N. Nair, Depttment of Chemistry, IIT Kanpur for his help in MD simulation.

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