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Time-Resolved Fluorescence Studies on Bovine Serum Albumin Denaturation Process

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The denaturation of Bovine Serum Albumin (BSA) by a chaotropic agent, guanidinium hydrochloride (GuH^+Cl^-) was studied by fluorescence lifetime analysis. The BSA was labelled with 1-anilino-8-naphthalene sulfonate (ANS) at two different molar ratios (1:1) and (1:10). The non-exponential fluorescence kinetics of the BSA-ANS complex at different stages of denaturation is analysed using three different models: a discrete tri-exponential sum, stretched exponential, and Gaussian lifetime distribution. In all cases, the fluorescence decay times decreased with protein denaturation. The results from the models show that there are at least two different binding sites located in the BSA protein with different water accessibility.

KEY WORDS: Bovine serum albumin; 1-anilino-8-naphthalene sulfonate; denaturation; fluorescence lifetime distribution.

INTRODUCTION

A major endeavour in the field of biophysical chemistry is focused on studying the mechanisms of protein folding/unfolding/refolding, i.e., the transformation of a protein from a polypeptide chain to a 3-D native structure state [1]. Defects in protein folding to the native state may be the molecular basis for a wide range of human genetic disorders e.g. rare neurodegenerative illnesses such as Alzheimer's, and Parkinson's diseases [2,3]. Misfolded proteins and partially unfolded states take place in competition with the normal folding process [4]. Substantial attention has, thus, been devoted to characterizing partially unfolded states in order to gain insight on the possible steps in the mechanism of protein folding.

The intermediate states for the unfolding/refolding mechanism can be studied in the denaturation of protein [5]. The denaturation process has been followed by different techniques using mainly kinetic and thermodynamic approaches [5,6]. Fluorescence spectroscopy became a valuable tool in the study of proteins due to its great sensitivity. In general, the denaturation process can be followed via intrinsic (e.g. fluorescence due to tryptophan residue) or extrinsic (fluorescent probes) attached to the protein [7].

Human serum albumin (HSA) is the most abundant of all proteins in blood plasma, and accounts for about 60% of the total serum protein content [8]. Bovine serum albumin (BSA) and HSA are characterized by a high homology in the sequence and similar conformation. The primary structure of serum albumin is unusual among extracellular proteins in possessing a single cysteine group (Cys-34), and low tryptophan content. The secondary structure of the polypeptide chain consists of about 580 amino acids with approximately 67% present as an alpha-helix without beta-sheets. The protein is composed of three homologous domains (I, II, and III) which are divided into two sub-domains (A and B). In addition, there are nine loops and 17 disulphide bridges which make a heart-shaped 3D structure of the protein molecule [8]. The principal function of serum albumin is to transport a wide variety of fatty acids and metabolites via the main binding regions located in sub-domains IIA and IIIA [9].

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1-anilino-8-naphthalene sulfonate (ANS) is a known "hydrophobic probe" for proteins [10, 11]. In the present work, ANS has been used as an extrinsic fluorophore to study the BSA denaturation process. ANS exhibits a large fluorescence enhancement on binding to proteins in the native form [10]. Moreover, the fluorescence quantum yield of ANS in water is very low (0.3%) [12], so fluorescence is profoundly affected if a change in protein hydration or ANS accessibility to water occurs during binding or denaturation. The Guanidine hydrochloride salt (GuH^+Cl^-) is a chaotropic denaturant agent, which is used to induce protein unfolding [5].

In this work, we gain more insight into the denaturation process of the BSA protein induced by Guanidine hydrochloride. The structural changes are monitored spectrophotometrically via protein fluorescence time-resolved emission from ANS bound to BSA in two different molar ratios.

EXPERIMENTAL PART

Materials

Both bovine serum albumin (BSA) of purity 99+% (catalogue no. A7638) and 1-Anilino-8-naphtalenesulfonic acid hemi-magnesium salt hydrate (ANS) of purity 90+% were purchased from Sigma and Fluka, respectively. All reagents were used without further purification. Phosphate buffered saline tablets for 0.01 M phosphate buffer (PBS), 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4 were purchased from Aldrich. All solutions were made up in purified water from a Milli-Q Millipore system (18 MΩ cm).

Sample Preparation

In all experiments, a fresh protein stock solution ($\sim 5 \times 10^{-5}$ M) in PBS at pH = 7.4, which was kept in a refrigerator (at 4 °C) in the dark was used. A stock aqueous solution of ANS was prepared. The BSA and ANS concentration was calculated using molar extinction coefficients $\varepsilon_{280} = 43600\ M^{-1}\ cm^{-1}$ [10] and $\varepsilon_{350} = 5000\ M^{-1}\ cm^{-1}$ [10], respectively. The final protein concentrations used in the fluorescence measurements were $\sim 2 \times 10^{-6}$ M.

Apparatus

Absorption spectra were recorded with a Shimadzu UV-1601 UV-visible spectrophotometer in 1 cm quartz cells, with the sample held at room temperature. Magic-angle fluorescence decays were recorded using a Time Correlated Single Photon Counting (TCSPC) system (Fluotime 200 from Picoquant GmbH). The excitation source was a 405 nm pulsed diode LDH (LDH-400 Picoquant GmbH). The repetition rate of the excitation laser was set to 5 MHz and decays obtained using 70 ps time resolution. Typical full widths at half-maximum obtained for instrument response function are in the order of a hundred picoseconds, and were obtained using a diluted Ludox aqueous solution.

Data Analysis

The decays were analysed using different models to interpret the fluorescence intensity decays. The first model is a discrete multiexponential function (MEF) which is described by:

$$I(t) = \sum_i a_i \exp(-t/\tau_i) \tag{1}$$

where a_i values are the amplitudes to the corresponding lifetime τ_i.

The second model is a stretched exponential (SE). In this model the decays are analysed using a sum of exponential terms with a_i amplitudes and stretched lifetime τ_i by an empirical parameter β_i (Eq. (2)):

$$I(t) = \sum_i a_i \exp(-t/\tau_i)^{\beta_i} \tag{2}$$

The third model is a Gaussian lifetime distribution (GLD). In this model, the decays are fitted using a continuous multimodal Gaussian distributed exponential (Eq. (3)):

$$I(t) = \int_0^\infty \rho(\tau) e^{-t/\tau}\, d\tau \tag{3}$$

where

$$\rho(\tau) = \sum_i a_i \frac{1}{\sigma_i \sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{\tau - \tau_i}{\sigma_i}\right)^2\right] \tag{4}$$

The parameter a_i is the amplitude of ith distributed component, τ_i is the medium lifetime of the ith distribution and σ_i is proportional to full width at half maximum of distribution.

The MEF model was applied using the FluoFit software program (PicoQuant GmbH, ver 3.2). The other models were implemented in the analysis program through a nonlinear least-squares procedure based on the Marquardt-Levenberg algorithm and combined with an

iterative reconvolution method based on the fast Fourier transform (FFT) to account for distortions of the decay signal from the instrument response function. The quality of the fittings was judged using the usual statistical criteria: symmetrical distribution of weighed residuals for the autocorrelation function and the chi squared (χ^2) parameter.

RESULTS AND DISCUSSION

ANS fluorescence increases substantially when there are more hydrophobic binding sites on the protein. Because of such high efficiency of fluorescence emission when ANS is bound to proteins and low fluorescence quantum yield in water (0.003) [12], ANS is one of most widely used fluorescence probes in the study of proteomic processes [7,10,11].

The ANS fluorescence decay is mono-exponential in homogeneous solutions with a lifetime ranging from 250 ps in water to 14 ns in ethanol [13]. Although, the photophysical mechanism is still controversial [14], there is a consensus that the quantum yield and lifetime data in water-dioxane mixtures show the presence of at least two emissions that depend on water content in the mixture.[3]

ANS fluorescence is significantly enhanced when it is adsorbed into hydrophobic sites in proteins. The fluorescence decays of ANS associated to other proteins such as human oxyhemoglobin [15] and β-lactoglobulin [16] show tri-exponential decay kinetics with lifetimes of 12.4–15.1 ns, 2.7–5.5 ns, and 0.25–0.5 ns. Bi-exponential decays with lifetimes of 10.1–17.3 ns and short lifetimes of 2.3–6.1 ns were reported for other proteins [17]. Using the multiexponential function model (Eq. (1)) the fluorescence decay kinetic parameters of BSA-ANS in different concentrations of guanidinium hydrochloride were recovered. Three lifetimes are extracted from the multi-exponential function analysis, and our results agree (as displayed in Tables I and II and Fig. 1) with the results reported for these other proteins.

The BSA-ANS in PBS at pH 7.4 show $\tau_1 \sim 21$ ns, $\tau_2 \sim 12$ ns, and $\tau_3 \sim 2.4$ ns as mentioned above these may be related to ANS binding sites with different degrees of exposure to aqueous environment. The addition of a denaturant agent induces a reduction in all these values, which is independent of the molar ratio of the BSA-ANS complex. Although, there are reports of complexed ANS in other proteins, even assigning the short decay time to free ANS in solution, we still do not detect the corresponding lifetime of ANS (∼250 ps) even at highest concentration of denaturant, as can be observed in Fig. 1b where the free ANS decay (in dotted line) is shorter than the shortest fluorescence decay in the (1:10) BSA-ANS complex. This may suggest that ANS molecules are still bound to BSA while being quite exposed to the water medium due to denaturation. In addition, the pre-exponential factors of the short component increases and the pre-exponential factor of long components decrease with addition of guanidinium hydrochloride in both systems (Tables I and II), that is, the contribution of the short lifetime component increases while the contribution of the long lifetime decreases with denaturation. Furthermore, both lifetime values decrease with the increasing denaturant concentration. The denaturation of protein, leads to an increased level at water exposure of original hydrophobic pockets where ANS molecules are bound, and therefore it is expected that the contribution of such bound ANS in the total fluorescence decay will lead to a faster fluorescence decay.

In the multi-exponential model, however, the number of exponentials may not necessarily represent the number of binding sites. The Stretched exponential (Eq. (2)) and Gaussian lifetime distribution (Eq. (3)) were applied in the BSA-ANS denaturation studies in order to take into account the heterogeneous features that can be involved in the binding sites where the fluorescence probe is located. The results from stretched exponential and Gaussian lifetime distribution models' fitting to the fluorescence decays are summarised in Tables III–VI. Although, Fig. 1 shows the fluorescence decays analysed by MEF, the decay fits are also an example of fluorescence decays and the typical fitting obtained for the other two models which do not differ much from MEF model. This may also be observed by reviewing the chi-squared values (Tables III–VI).

In the stretched exponential model, two time decay constants were needed to fit the data for both systems (Tables III and IV). In addition, β values for the long lifetime component in both systems are equal to 1, that is, the fluorescence decays for both systems can be represented by a sum of one single exponential ($\beta = 1$) and a stretched exponential ($\beta < 1$). β is an empirical parameter that shows the degree of heterogeneity present. In other words, the stretched exponential can be used to analyse complex fluorescence decays with different emission sources in different microenvironments, which can lead to a distribution of decay times. In fact, the stretched exponential function is correlated to a continuous distribution of lifetimes [18].

[3] Lifetime of 263 ps from the fluorescence decay of ANS in PBS buffer solution at pH = 7.4 with 405 nm excitation, and emission collected at 530 nm was obtained here.

Table I. Decay Parameters of Multi-Exponential Fit of (1:1) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+]$ (M)	τ_1(ns)	τ_2(ns)	τ_3(ns)	a_1	a_2	a_3	χ^2 §
0	21.80	13.53	2.43	0.25	0.53	0.23	1.091
0.5	22.48	13.77	2.50	0.21	0.50	0.29	1.104
1.0	21.59	12.62	2.36	0.24	0.41	0.35	1.120
1.5	21.34	11.93	2.15	0.21	0.35	0.44	1.324
2.0	21.10	9.51	1.94	0.08	0.25	0.67	1.291
2.5	10.75	5.04	1.71	0.11	0.10	0.79	1.164
3.0	16.72	6.87	1.72	0.02	0.14	0.84	1.175
3.5	12.54	6.17	1.59	0.02	0.11	0.87	1.213
4.0	17.55	7.77	1.69	0.00	0.10	0.90	1.265
4.5	13.62	6.84	1.66	0.02	0.12	0.87	1.173
5.0	17.11	7.42	1.67	0.01	0.09	0.90	1.245

§The average value is 1.199.

Based on the τ_i and β_i values, from the stretched exponential model we can interpret the existence of at least a common binding site in the (1:1) and (1:10) BSA-ANS complexes where the complex fluorescence decays follow a mono-exponential function with decay times described by τ_2 values. The denaturation of BSA-ANS shows a continuous decrease of either τ_2 (from ∼16 ns to ∼8 ns) or the pre-exponential factor, a_2. Assuming that the mono-exponential components are attributed to ANS bound to a more hydrophobic site, this may indicate that this site is gradually being exposed to a more hydrophilic environment, which leads to a reduction of fluorescence lifetime and fluorescence intensity, during the unfolding process. In the case of the other binding site represented by the stretched exponential parameters, τ_1 and β_1 values, it should be noted that the amplitude values (a_1) increase with the progress of denaturation in both molar ratios. The decay times τ_1 are reduced with increase of guanidinium salt, however variations in τ_1 values and in β_1 values are dependent on the complex composition. At zero concentration of denaturant, the β_1 values in (1:1) and (1:10) complexes are close to ∼0.5. But the decay time τ_1 of the (1:1) complex is approximately half the value of τ_1 found in the (1:10) complex. This can be interpreted in terms of the degree of water accessibility of the binding sites occupied by ANS molecules. β_1 values are roughly around 0.5–0.6 for guanidinium salt concentrations up to approximately 1.0–1.5 Molar concentration. Also, no large changes are observed in the corresponding decay time values. However, with a denaturant concentration above 1.5 Molar, both β_1 and τ_1 values change abruptly. In general, the τ_1 values decrease in both complexes, but, β_1 value increases in (1:1) complex while it decreases in (1:10) complex. The reduction of decay time values is due to the exposure of the original binding sites to a more hydrophilic environment. The observed β_1 values increase in the (1:1) complex which can mean that the bound ANS are more exposed to water, and making the sites become

Table II. Decay Parameters of Multi-Exponential Fit of (1:10) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+]$(M)	τ_1(ns)	τ_2(ns)	τ_3(ns)	a_1	a_2	a_3	χ^2§
0	19.79	10.95	2.52	0.42	0.44	0.13	1.132
0.5	19.74	10.89	2.28	0.43	0.44	0.13	1.074
1.0	19.98	10.65	1.69	0.43	0.43	0.14	1.088
1.5	19.59	10.00	1.59	0.44	0.38	0.18	1.101
2.0	15.96	6.76	1.05	0.16	0.30	0.54	1.124
2.5	12.03	4.23	0.91	0.13	0.23	0.65	1.050
3.0	10.66	3.16	0.77	0.08	0.21	0.71	1.084
3.5	10.53	3.27	0.82	0.05	0.17	0.78	1.090
4.0	10.16	2.93	0.80	0.04	0.16	0.80	1.076
4.5	8.54	2.16	0.64	0.04	0.20	0.76	1.192
5.0	9.52	2.92	0.88	0.03	0.14	0.82	1.150

§The average value is 1.106.

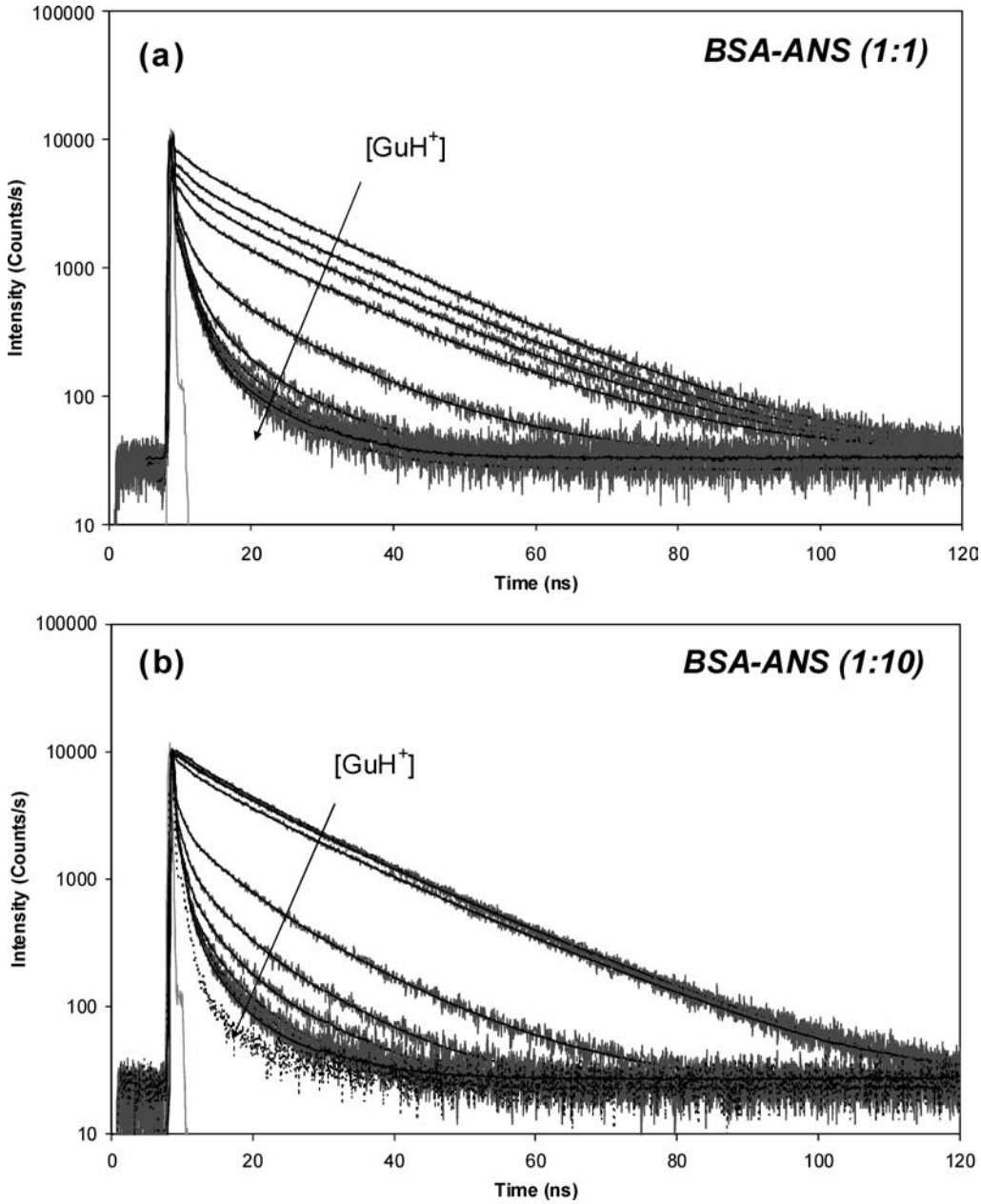

Fig. 1 Fluorescence decays of BSA-ANS (1:1) (a), and BSA-ANS (1:10) (b), excited at 405 nm and probed at 480 nm with different concentration of Guanidinium hydrochloride in PBS buffer of pH = 7.4. Dotted line decay in (b) is free ANS in 5.0 M of GuHCl.

more similar to each other. In fact, when the protein is fully denatured the β_1 values are close to unity. In the case of the (1:10) complex where the β_1 values decrease, we can suppose that different hydrophilic binding sites are being probed by ANS, since there are 10 times more ANS molecules than in the (1:1) complex. It is important to note that, in the (1:10) complex, the β_1 values start to increase gradually with [GuH^+] above 2.0 M, that is, the binding sites probed by ANS are becoming similar to each other, however, without achieving the same degree of similarity as observed in the (1:1) complex for β_1 close to unity.

Fluorescence decay curves of ANS in the protein/dye complex exhibit non-single exponential characteristics as shown above. The reasons for such behaviour can be due to either a dynamic effect from rearrangements of

Table III. Decay Parameters of Stretched Exponential Fit of (1:1) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+](M)$	τ_1(ns)	τ_2(ns)	β_1	β_2	a_1	a_2	χ^2§
0	3.45	16.75	0.53	1.00	0.50	0.50	1.083
0.5	2.77	17.21	0.51	1.00	0.59	0.41	1.070
1.0	2.15	17.22	0.49	1.00	0.68	0.32	1.091
1.5	1.38	17.54	0.46	1.00	0.77	0.23	1.064
2.0	1.56	14.88	0.72	1.00	0.83	0.17	1.150
2.5	1.86	9.41	1.04	1.00	0.83	0.17	1.112
3.0	1.94	8.71	1.10	1.00	0.85	0.15	1.133
3.5	1.75	7.53	1.10	1.00	0.87	0.13	1.123
4.0	1.84	7.88	1.11	1.00	0.88	0.12	1.196
4.5	1.82	7.64	1.12	1.00	0.86	0.14	1.111
5.0	1.77	7.74	1.10	1.00	0.88	0.12	1.081

§The average value is 1.111.

the ANS probe in the binding site during the fluorescence lifetime of bound ANS or the presence of multiple interacting binding sites, in addition to different hydrophilic environments.

Lifetime distribution analysis of fluorescence decay curves can rationalize the data by taking into account such effects. The analyses reveal two types of emission sites where the parameter values found are shown in Tables V and VI. In (1:1) BSA-ANS, the short lifetimes (τ_1) can be averaged to around 1.9 ns with a very sharp distribution width (σ_1) of ~25 ps. Both parameters are not affected much in any stage of denaturation. The long lifetime component in the (1:1) complex shows different features: lifetimes (τ_2) and distribution widths (σ_2) are reduced with increases in guanidinium salt concentration. In addition, the contribution of the long lifetime component in the fluorescence decays decreases, and the short lifetime contribution increases as observed in the amplitude values displayed in Table V. Although, the short lifetime in (1:10) BSA-ANS is more scattered around the mean value (~1.3 ns), it does not show any trend with guanidinium concentration (Table VI). The same observation can be made with σ_1. These values are very dispersed showing no trend with the denaturant concentration; however, the mean σ_1 value of ~750 ps is greater than that observed for the (1:1) complex. The lifetime values for the second component (τ_2) in the (1:10) complex show the same trend observed for the (1:1) complex, that is, τ_2 decreases with GuH^+ concentration increase. By contrast to the σ_2 values for the (1:1) complex, the distribution width values in the (1:10) complex increase with denaturation. The contribution of the short fluorescence lifetime component increases and the long fluorescence lifetime component decreases with GuH^+ concentration increase as can be observed in the amplitude values a_1 and a_2 in Tables V and VI.

Table IV. Decay Parameters of Stretched Exponential Fit of (1:10) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+](M)$	τ_1(ns)	τ_2(ns)	β_1	β_2	a_1	a_2	χ^2§
0	7.05	15.84	0.64	1.00	0.53	0.47	1.164
0.5	9.00	15.61	0.71	1.00	0.56	0.44	1.055
1.0	7.95	16.02	0.66	1.00	0.52	0.48	1.069
1.5	5.67	16.29	0.60	1.00	0.54	0.46	1.120
2.0	0.12	11.77	0.31	1.00	0.92	0.08	1.088
2.5	0.11	10.39	0.34	1.00	0.97	0.03	1.092
3.0	0.12	10.49	0.38	1.00	0.98	0.02	1.149
3.5	0.19	10.04	0.44	1.00	0.98	0.02	1.230
4.0	0.24	9.77	0.49	1.00	0.98	0.02	1.041
4.5	0.16	8.98	0.46	1.00	0.99	0.01	1.233
5.0	0.52	8.27	0.64	1.00	0.97	0.03	1.144

§The average value is 1.126.

Table V. Decay Parameters of Lifetime Gaussian Distribution Fit of (1:1) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+](M)$	τ_1(ns)	τ_2(ns)	σ_1(ns)	σ_2(ns)	a_1	a_2	χ^2§
0	2.07	16.89	0.03	5.98	0.26	0.74	1.109
0.5	2.24	17.07	0.03	6.11	0.40	0.60	1.127
1.0	2.18	16.92	0.03	6.67	0.44	0.56	1.131
1.5	2.00	16.63	0.03	7.02	0.47	0.53	1.077
2.0	1.92	13.37	0.03	7.06	0.70	0.30	1.232
2.5	1.71	9.20	0.02	4.34	0.83	0.17	1.162
3.0	1.71	8.74	0.02	4.35	0.87	0.13	1.169
3.5	1.57	7.79	0.02	3.34	0.88	0.12	1.199
4.0	1.83	9.20	0.02	0.20	0.91	0.09	1.277
4.5	1.83	8.91	0.02	0.21	0.91	0.09	1.157
5.0	1.83	9.40	0.02	0.19	0.91	0.09	1.159

§The average value is 1.163.

Analysis of the time-resolved decay data in terms of a Gaussian lifetime distribution is a simpler model where the distributed component is attributed to two binding sites in the BSA-ANS complex. The widths of the lifetime distributions recovered from protein/dye complexes can be related to the extent of motion of the fluorophore, i.e., a sharp lifetime distribution indicates that the probe local mobility increases [19]. Assuming that each Gaussian lifetime distribution function corresponds to a binding site, two sites are characterized in BSA-ANS complex. The binding sites with the environment accessible to water molecules show that ANS molecules have less mobility than in the other more hydrophobic binding sites, that is, $\sigma_2 > \sigma_1$. Furthermore, the mobility of ANS bound to hydrophobic sites in the (1:1) complex decreases with denaturation while in the (1:10) complex mobility increases. This apparent contradiction is explained by the distribution of ANS molecules in the remaining hydrophobic sites in different stages of denaturation. In the (1:10) BSA-ANS, there are 10 times more ANS molecules that can be distributed to different binding sites where ANS has more mobility and also has different water accessibility, in different GuH^+ concentration. Therefore, taking the (1:10) complex at $[GuH^+] = 5.0$ M, for example, there are more ANS molecules which are less constrained or more mobile and in sites with more water exposure than in the (1:1) complex at the same guanidinium concentration.

CONCLUSIONS

The differences in values of fluorescence decay times and relative contributions of decay components revealed by the three different models in the fluorescence decay analysis clarifies the heterogeneous nature at ANS binding to BSA protein. The comparison between these models is not easy, since from a statistical point of view

Table VI. Decay Parameters of Lifetime Gaussian Distribution Fit of (1:10) BSA-ANS Fluorescence at Different Unfolded States from 405 nm Excitation and 480 nm Probe Emission

$[GuH^+](M)$	τ_1(ns)	τ_2(ns)	σ_1(ns)	σ_2(ns)	a_1	a_2	χ^2
0	3.19	16.54	1.82	6.48	0.02	0.98	1.164
0.5	2.04	16.45	0.20	6.61	0.01	0.99	1.100
1.0	1.50	16.74	0.22	6.84	0.01	0.99	1.124
1.5	1.41	16.63	0.05	7.10	0.02	0.98	1.123
2.0	0.86	11.06	0.03	8.53	0.06	0.94	1.153
2.5	0.81	9.05	1.55	6.49	0.30	0.70	1.108
3.0	0.71	5.94	1.22	8.42	0.32	0.68	1.135
3.5	0.87	2.00	0.73	9.91	0.26	0.74	1.181
4.0	0.86	2.02	0.88	9.76	0.37	0.63	1.030
4.5	0.62	2.24	1.04	8.68	0.47	0.53	1.202
5.0	1.02	2.01	0.53	8.64	0.38	0.62	1.218

§The average value is 1.140.

(chi-squared values and residuals) they are very similar. However, building on the common points extracted from them, we find that there are at least two different binding sites located in the BSA protein. In one of them, ANS is believed to be located not too deeply in the protein interior but it is located at a position where water is accessible to ANS. The other is likely to be more external where water molecules can diffuse to it and therefore give some features where the ANS probe lifetime is close but not equal to that observed in bulk water.

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