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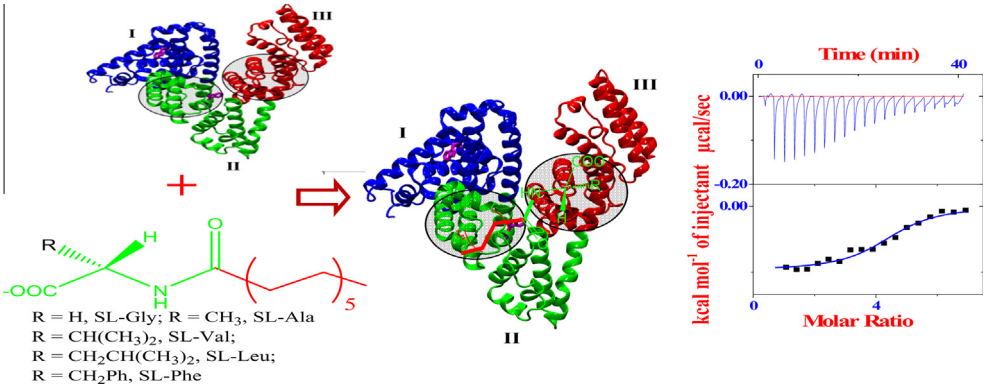
Interaction of bovine serum albumin with N-acyl amino acid based anionic surfactants: Effect of head-group hydrophobicity

Subhajit Ghosh, Joykrishna Dey *

Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur 721 302, India

GRAPHICAL ABSTRACT



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ABSTRACT

The function of a protein depends upon its structure and surfactant molecules are known to alter protein structure. For this reason protein–surfactant interaction is important in biological, pharmaceutical, and cosmetic industries. In the present work, interactions of a series of anionic surfactants having the same hydrocarbon chain length, but different amino acid head group, such as L-alanine, L-valine, L-leucine, and L-phenylalanine with the transport protein, bovine serum albumin (BSA), were studied at low surfactant concentrations using fluorescence and circular dichroism (CD) spectroscopy, and isothermal titration calorimetry (ITC). The results of fluorescence measurements suggest that the surfactant molecules bind simultaneously to the drug binding site I and II of the protein subdomain IIA and IIIA, respectively. The fluorescence as well as CD spectra suggest that the conformation of BSA goes to a more structured state upon surfactant binding at low concentrations. The binding constants of the surfactants were determined by the use of fluorescence as well as ITC measurements and were compared with that of the corresponding glycine-derived surfactant. The binding constant values clearly indicate a significant head-group effect on the BSA–surfactant interaction and the interaction is mainly hydrophobic in nature.

1. Introduction

The study of protein–surfactant interactions has drawn attention in the past few decades because of their relevance to the fields of detergents, cosmetics, foods and pharmaceuticals [1–7]. The surfactant molecules upon binding to proteins are known to alter protein structure [8–10] that determines its function. It is reported that surfactant binding to proteins can either stabilize the structure of the latter or denature it. Indeed, ionic surfactants, particularly anionic ones, are known to interact strongly with proteins and denature them at very low concentrations. Thus, they are more

* Corresponding author.
E-mail address: joydey@chem.iitkgp.ernet.in (J. Dey).

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efficient than common chemical denaturants, such as urea, and guanidine hydrochloride [11]. Surfactants are also known to influence protein aggregation. For example, amyloidogenic proteins are known to aggregate and have been associated with diseases like Alzheimer's disease [12,13]. Therefore, a detailed understanding of the nature of protein–surfactant interactions as a function of the chemical structure of the surfactant is essential for the advancement of the fields of pharmaceutical and cosmetic research [14,15]. In fact, the ability to control interactions between proteins and surfactants is necessary to develop new drug delivery systems for improved medical treatments [16–18].

However, until now, most studies have focused on interactions of bovine serum albumin (BSA) with common surfactants, such as sodium dodecyl sulfate (SDS), cetyltrymethylammonium chloride (CTAC), and cetyltrymethylammonium bromide (CTAB) [19–24] because serum albumin (SA) is an abundant protein in the blood plasma. The binding of cationic surfactant (e.g., CTAC) to HSA was found to be much weaker than that of anionic surfactant (e.g., SDS) [19,25]. On the other hand, neutral surfactants, such as Triton X-100 hardly interact with proteins. In fact, most nonionic surfactants, except a few, usually do not denature proteins [26]. The SAs are transport proteins, which carry steroids and fatty acids (FAs), serve as an important source of metabolic energy [27]. They are also known to bind many low-molecular-mass metabolites, drugs and various organic/inorganic ligands and transport them in the blood stream of mammals including humans [28]. Crystallographic studies of the HSA complexed with long-chain (C16 and C18) FAs revealed the existence of seven binding sites [29–32]. Further studies using medium-chain (C10–C14) FAs indicated the presence of four more binding sites [31]. This means there are a total of eleven different binding sites in HSA protein. The crystal structure of HSA with the stearic acid molecules bound to it is displayed in Fig. 1. It can be seen that the hydrocarbon tail of the FA is accommodated into the hydrophobic pocket, while the carboxyl moiety is bound to two or three polar amino acid residues through electrostatic/polar interactions in the sites 1–5. [31,33] Interestingly, for sites 6 and 7, no clear evidence of polar interactions of the carboxylate head group was obtained. This means that these two binding sites are the low affinity FA binding sites.

The remarkable ability of HSA to bind FAs has motivated us to undertake this work to investigate interaction of bovine serum albumin (BSA) with fatty acid amide amphiphiles with different amino acid head groups. Many researchers have used BSA as a model protein because of its structural homology with HSA. BSA

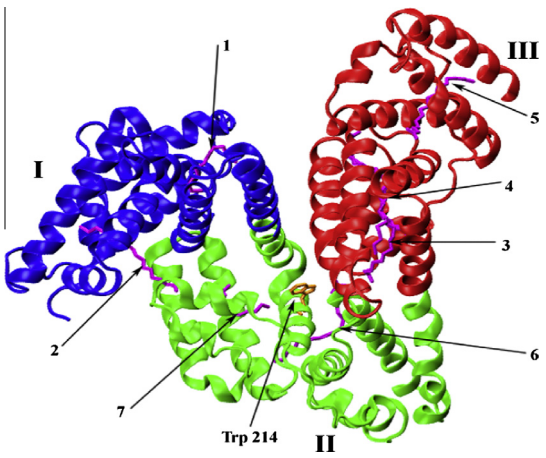

Fig. 1. The structure of HSA showing the location of the seven binding sites for stearic acid (1–7). The figure was obtained from the PDB file mentioned in Ref. [31] and modified.

is a α-helix-rich [34,35] protein with molecular weight of 66 kDa. The polypeptide chain containing 583 amino acid residues is made up of three homologous domains and 17 disulfide bridges. Each domain is further divided into two sub-domains (A and B). BSA has two tryptophan residues Trp-134 and Trp-213 which are responsible for intrinsic fluorescence of the protein. Trp-134 located on the surface of the protein and Trp-213 is located in the hydrophobic pocket of the protein 3D structure [35]. Despite marked similarity between BSA and HSA the latter has only one Trp residue (Trp-214). Although X-ray crystal structure of BSA has recently been solved, the structures of BSA/surfactant complexes at low and high surfactant concentrations remain unresolved till to date because the solution structure of BSA is not available.

Interaction of BSA with cationic, anionic and nonionic, and gemini surfactants has been widely studied in the past [24,26,36–44]. However, except a few, most of these studies concerned protein denaturation at surfactant concentrations much above their critical micelle concentration (CMC). The association process highly depends upon surfactant concentration. In low concentrations, surfactants show protecting effect toward heat- and urea-induced denaturation of proteins [45]. The reason for this is yet to be known. Only a few studies on the protein–surfactant interaction at concentrations less than the CMC value have been reported [46]. At low surfactant concentration (<CMC) the protein–surfactant interaction is specific in nature and only high affinity sites of the protein molecule are occupied by surfactant molecules. However, at high concentration (>CMC) the protein–surfactant interaction is non-specific in nature and large number of surfactant molecules may attach to a single molecule of protein. Despite numerous reports on binding of FAs and surfactants to BSA [36–44], a complete understanding of these interactions is not yet well established. Indeed the structural information on the binding of ligand to BSA is sparse, but indirect evidences of binding of surfactant to HSA suggest that strongly bound surfactant molecules occupy the hydrophobic patches on the surface of the protein molecule [24,29]. ^{13}C NMR studies [47,48] reported earlier have suggested that there is hydrogen-bonding (H-bonding) interaction between the polar residues of BSA and carboxylate anion of FAs, which might be due to the presence of Lys-His-Lys residue in the subdomain IIA.

However, there are no investigations reported on the interaction of BSA with N-acyl amino acid surfactants (NAASs) that have carboxylate head group. Recently, we have reported [49] the interactions of BSA with two NAASs, sodium N-laurylsarcosinate (SL-Sar) and sodium N-laurylglycinate (SL-Gly) having sarcosine and glycine as head groups, respectively. The fluorescence, CD and ITC data clearly indicated a significant effect of the surfactant head-group structure on binding to BSA. The methylation of the amide nitrogen of SL-Gly was observed to have a profound effect on the surfactant binding to BSA. To further shed light on these, we have carried out a systematic investigation of the BSA–surfactant interactions for a series of NAASs, sodium N-lauryl-L-alaninate (SL-Ala), sodium N-lauryl-L-valinate (SL-Val), sodium N-lauryl-L-leucinate (SL-Leu) and sodium N-lauryl-L-phenyl-alaninate (SL-Phe) (see Chart 1 for structures) with L-alanine (Ala), L-valine (Val), L-leucine (Leu) and L-phenylalanine (Phe), respectively, as head groups. Though the surfactants employed in this work are anionic with the same carboxylate ($-COO^-$) head-group, the hydrophobicity of the head groups decreases in the order SL-Leu > SL-Phe > SL-Val > SL-Ala > SL-Gly [50]. The objective of this work is to monitor conformational changes around its binding site in response to addition of each of the anionic carboxylate surfactant. The rational selection of the head group should enable selectivity with respect to physicochemical, toxicological, and morphological properties of the protein/NAAS complex. The hydrophobicity of the amino acid head group may play

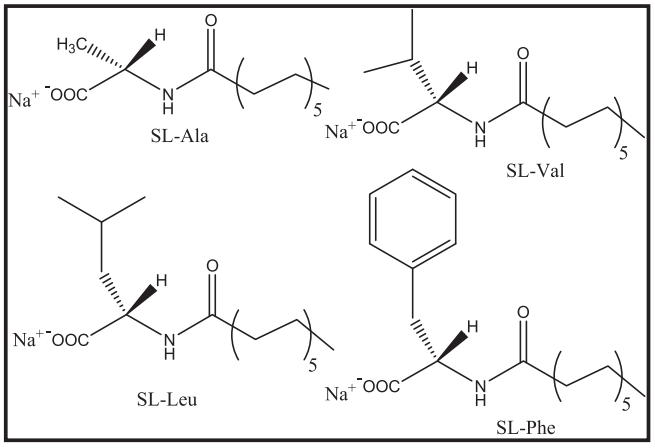

Chart 1. Chemical structure of SL-Ala, SL-Val, SL-Leu and SL-Phe surfactants.

an important role in the surfactant binding to BSA. Therefore, the binding and thermodynamic data of these surfactants at low surfactant concentrations were compared with those of SL-Gly that has a least hydrophobic head group. We have used fluorescence and circular dichroism (CD) spectroscopy and isothermal titration calorimetry (ITC) to study the BSA–surfactant interactions.

2. Experimental details

2.1. Materials

Lauroyl chloride, warfarin, ibuprofen and BSA were purchased from Sigma–Aldrich ((St. Louis, MO, USA) and were used directly from the bottle. The amino acids, L-alanine (Ala), L-valine (Val), L-leucine (Leu) and L-phenylalanine (Phe) were purchased from SRL (Mumbai). The fluorescent probe N-phenyl-1-naphthyl amine (NPN) was obtained from Sigma–Aldrich (St. Louis, MO, USA) and was purified by repeated recrystallization from acetone/water (80:20 v/v) mixture. The purity of NPN was tested by measuring fluorescence excitation spectra at different emission wavelengths. The surfactants employed in the work were synthesized and purified according to methods reported in the literature [49]. The synthetic procedure is described under "Supporting Information" (SI). The surfactant molecules were chemically identified by ^{1}H and ^{13}C NMR spectra (Fig. S1 and Fig. S2). All organic solvents, including tetrahydrofuran (THF), alcohol, acetone and trimethylamine (TEA) were purchased locally and were used after purification and drying before use. A 20 mM phosphate buffer of pH 7.0 was prepared in Milli Q water (~18 MΩ) and all solutions were prepared using this buffer.

2.2. Methods and instrumentation

2.2.1. Fluorescence measurements

Steady-state fluorescence measurements were carried out with a Perkin–Elmer LS-55 luminescence spectrometer equipped with a filter polarizer and a thermostating cell holder. The temperature was controlled using a circulating bath (Thermo Neslab, RTE 7).

The CMC values of the surfactants were determined by fluorescence titration method using NPN as a probe molecule. An aliquot of the stock solution of NPN (10^{-3} M) prepared in dry acetone was taken in a series of volumetric flask (5 mL) and evaporated slowly to dryness by using a stream of N_2. Surfactant stock solution (10^{-2} M) was prepared in phosphate buffer (20 mM, pH 7). A known volume of this surfactant solution was added to each of the 5 mL volumetric flasks to obtain surfactant solutions in the desired concentration range. The final concentration of NPN (~10^{-5} M) was maintained constant in all the surfactant solutions. Surfactant solutions containing NPN probe were excited at 340 nm and fluorescence spectra were recorded in the range of 370–600 nm using a fixed excitation and emission slit widths of 2.5 nm and 4 nm, respectively. The fluorescence spectra of all samples were blank subtracted. All measurements started 2–3 h after the sample preparation. The fluorescence measurements were performed at 298 K using 1 cm^2 quartz cuvette.

Aqueous solutions of BSA (15 μM) was prepared in phosphate buffer (20 mM, pH 7) containing surfactant in the concentration range between 0 and 0.2 mM. BSA solutions were excited at 295 nm and the spectra were recorded between 310 and 440 nm using a fixed excitation and emission slit widths of 2.5 nm and 3.2 nm, respectively. The intensity was measured at the emission maximum (350 nm). The samples containing warfarin were excited at 325 nm and the spectra were recorded from 350 to 500 nm. For the experiment using ibuprofen the BSA solutions were excited at 295 nm. The fluorescence measurements of BSA solutions were performed at 298 K using a quartz cuvette of path length 5 mm.

2.2.2. Circular dichroism spectra

A Jasco J-810 spectropolarimeter was used to measure the circular dichroism (CD) spectra using quartz cells of 1 mm path length. For each spectrum, an average of three scans was taken under the conditions of 1 nm bandwidth, 2-s response time, and 50 nm/min scan speed. Each spectrum was baseline corrected using the appropriate reference solution. All measurements were carried out at 298 K. The spectrum was recorded in the range of 190 nm to 260 nm. An accumulation of three scans with a speed 50 nm/min was performed and data were collected. The CD results expressed in terms of the mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ can be defined as [51]:

$$\mathrm{MRE} = \theta_{\mathrm{obs}}/(10 \times l \times C \times N) \quad (1)$$

where θ_{obs} is the circular dichroism in milli degree, l is the path length (1 mm), C is the molar concentration, and N is the number of amino acid residues (583). Now from the MRE value at 208 nm the α-helix content can be calculated using the following equation [52,53]:

$$\alpha\text{-helix}\ (\%) = [(-\mathrm{MRE}_{208} - 4000)/(33,000 - 4000)] \times 100 \quad (2)$$

where MRE_{208} is the observed MRE at 208 nm, 4000 is the MRE value of β – form and random coil at 208 nm and 33,000 is the MRE value of pure α-helix conformation at 208 nm.

2.2.3. Isothermal titration calorimetry (ITC)

The ITC experiments were carried out in a Microcal iTC200, (made in U.S.A) at 298 K. Titration of surfactant against a protein (BSA) was carried out by injecting 0.5 mM SL-Ala and 1 mM SL-Phe, SL-Val or SL-Leu and same surfactant against phosphate buffer. BSA concentration was 15 μM in each case. The total number of injection was 20 and the cell temperature was 298 K. Reference power and initial delay were set to 5, and 60 s, respectively. A string speed of 600 rpm and spacing of 120 s was used for the ITC measurements.

3. Result and discussion

3.1. CMC of surfactants

The CMC values of SL-Ala, SL-Val, SL-Leu and SL-Phe surfactants were determined in phosphate buffer (20 mM, pH 7) by steady-state fluorescence technique using NPN probe. NPN is a well-known hydrophobic probe molecule, which is poorly soluble

in water, but gets solubilized within the hydrophobic interior of the surfactant aggregates. This is indicated by the large blue shift of the emission maximum (λ_{max}) along with a huge rise of fluorescence intensity in the presence of surfactants at concentrations above CMC. The blue shift ($\Delta\lambda = \lambda_{water} - \lambda_{surfactant}$) is indicative of solubilization of NPN in a less polar environment of the aggregates. The plots of variation of $\Delta\lambda$ with the surfactant concentration are shown in Fig. S3. The concentration corresponding to the onset of rise of $\Delta\lambda$ was taken as the CMC value of the surfactant. The CMC values thus determined were 1.91, 2.40, 1.15 and 0.84 mM for SL-Ala, SL-Val, SL-Leu and SL-Phe, respectively.

3.2. Fluorescence studies

In order to examine whether the surfactants bind to BSA, fluorescence measurements were carried out. The emission spectra of BSA in the absence and in the presence of the different surfactants are depicted in Fig. S4 (a–d) under SI. BSA shows a strong emission band at λ_{max} = 350 nm when the excitation wavelength is fixed at 295 nm. The intrinsic fluorescence of BSA (due to Trp residues) decreased regularly with the addition of surfactant accompanied by a large blue shift of the λ_{max} from 350 nm to 336 nm. The representative fluorescence spectra of BSA in the presence of the NAASs and BSA alone are shown in Fig. 2. The spectral shift is also accompanied by the decrease of fluorescence lifetime of the Trp residues. The fluorescence lifetime data are collected in Table S1 under SI. In our previous publication [49], we have shown that decrease of fluorescence intensity and lifetime upon addition of SL-Gly or SL-Sar is not due to any excited state process, but is a consequence of the change of protein conformation. In the case of present surfactant systems also, an in-depth analysis of the fluorescence emission spectra along with the fluorescence lifetime values has ruled out the possibility of any dynamic quenching (see discussion under SI). Thus the fluorescence spectral change of BSA, upon addition of the NAASs must be due to binding of the surfactant molecules to the protein leading to conformational change. The conformational change of the protein is further confirmed by the CD spectra of the BSA/surfactant complexes as discussed below. Upon surfactant binding the microenvironment of the Trp residues of BSA becomes less polar causing a blue shift and intensity reduction of the fluorescence spectrum.

3.3. CD spectral study

To investigate if there is any conformational change of BSA upon surfactant binding, we have measured the CD spectra of BSA in the presence of NAASs. Because the peptide bonds in proteins are asymmetric and molecules without a plane of symmetry show CD spectrum, the secondary structure of a protein can be derived from its CD spectrum. Consequently, CD spectra are widely used to monitor the structure, conformation and stability of protein in solution. In this study, the CD spectrum of BSA was used to obtain the fraction of α-helix, β-sheet, and random coil structures. The CD spectra (Fig. 3) of all the BSA/surfactant complexes, including BSA alone were measured to examine the conformational change of BSA upon surfactant binding. The CD spectrum of pure BSA exhibits two minima at 222 and 208 nm, the intensity of which increases upon addition of surfactant. However, the intensity increases in the order SL-Phe < SL-Ala < SL-Val = SL-Leu. That is, the surfactant with most hydrophobic head group is more efficient in changing the conformation. However, despite having head group with relatively higher hydrophobicity [50], SL-Phe has the least effect. This might be due to the anion-π interaction of the phenyl ring of the amino acid side chain that inhibits binding to the protein. Thus, it can be concluded that the binding of SL-Leu to BSA is stronger than those with less hydrophobic head group. We have also calculated the fraction of α-helix, β-sheet, and random coil structures using the intensity of the 208 nm band. The data collected in Table S2 show that the α-helix content of the protein increases in the order SL-Phe < SL-Ala < SL-Val = SL-Leu upon interaction with the surfactant molecules, which means the protein goes to the more ordered state. This suggests that the stability of BSA increases in the order SL-Phe < SL-Ala < SL-Val = SL-Leu upon binding with the surfactant molecules. In the case of SL-Leu, the ordered structure of BSA is more in comparison to SL-Gly, which implies that the hydrophobic side chain (which is absent in the case of SL-Gly) of the former helps binding to BSA.

3.4. Determination of binding site(s) of the NAASs

There are two Trp residues in BSA, one (Trp-134) in subdomain IB and another (Trp-213) in subdomain IIA. Surfactant molecules can interact with any of these Trp residues or both. To identify the binding site of the surfactants we have performed site marker experiments using fluorescence spectroscopy. Since warfarin is considered to be a subdomain IIA binder, it was chosen as a site marker. In this experiment, both BSA and warfarin concentrations were kept at 2×10^{-6} M and warfarin was selectively excited at 325 nm. The fluorescence spectra of BSA-bound warfarin in the absence and presence of each of the surfactants are depicted in Fig. 4. It is observed that the intensity of warfarin fluorescence is decreased in the presence of 0.2 mM SL-Phe, SL-Ala, SL-Val or

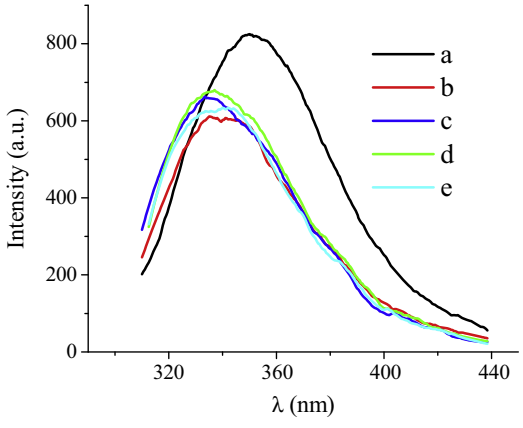

Fig. 2. Fluorescence spectra of BSA (15 μM) in phosphate buffer (20 mM, pH 7) in the (a) absence and presence of 0.2 mM (b) SL-Ala, (c) SL-Val, (d) SL-Leu, and (e) SL-Phe surfactants at 298 K.

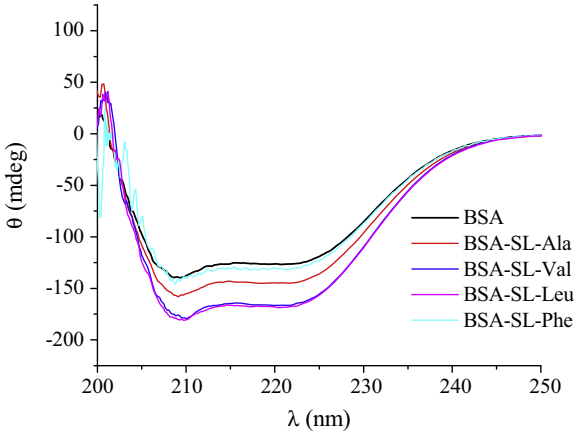

Fig. 3. CD spectra of BSA and BSA/surfactant complexes in phosphate buffer (20 mM, pH 7) at 298 K; [surfactant] = 0.2 mM.

SL-Leu surfactant which means warfarin molecule is expelled from its binding site on BSA molecule. In other words the surfactant molecules bind to the subdomain IIA of BSA. That is the surfactant molecules bind to a region where Trp-213 is located. Since the quenching of fluorescence is accompanied by a blue shift of the λ_{max}, there is a hydrophobic interaction between the protein and the surfactant molecule, which alters the microenvironment around the Trp-213 residue. The results are similar to those obtained with SL-Gly surfactant [49]. Literature reports also suggest that the binding site of fatty acids is the same as warfarin [54].

3.5. Measurement of binding constants of NAASs

From the above studies it can be concluded that the change in the fluorescence and CD spectra of BSA is due to the binding of surfactant molecules and therefore the corresponding fluorescence data can be used to determine binding constant of the surfactant. On the assumption that there are n binding sites for a surfactant molecule S on protein, P, the quenching process can be represented as

$$nS + P \rightleftharpoons PS_n \tag{3}$$

$$K_b = \frac{[PS_n]}{[S]^n[P]} \tag{4}$$

where $[S]$ and $[P]$ are the surfactant and protein concentrations, respectively, and $[PS_n]$ is the concentration of the non-fluorescent fluorophore/quencher complex. If $[P]_o$ is the total protein concentration, then

$$[PS_n] = [P]_o - [P] \tag{5}$$

$$K_b = ([P]_o - [P])/[S]^n[P] \tag{6}$$

Since fluorescence intensity is proportional to protein concentration as described by

$$[P]/[P]_o \propto F/F_o \tag{7}$$

Results from the fluorescence measurement can be used to estimate the binding constant of protein–surfactant complex. From Eqs. (6) and (7) one obtains:

$$\log\{(F_o - F)/F\} = \log K_b + n\log[S] \tag{8}$$

If we assume that there are n same and independent binding sites in the protein, Eq. (4) takes the form [55]:

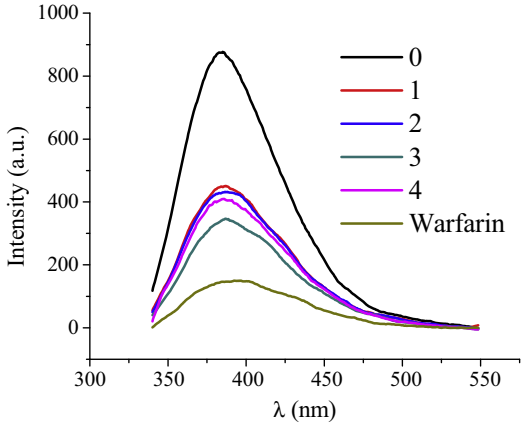

Fig. 4. Fluorescence spectra of BSA-bound warfarin in phosphate buffer (20 mM, pH 7.0) in the (0) absence and in the presence of 0.2 mM (1) SL-Ala, (2) SL-Val, (3) SL-Leu and (4) SL-Phe surfactants at 298 K; [BSA] = [warfarin] = 2×10^{-6} M, λ_{ex} = 325 nm.

$$K_b^n = \frac{[PS]_n}{[S]^n[P]} \tag{9}$$

$$\text{Since } [S]_o = [S] - [PS_n] \tag{10}$$

$$\text{And} \quad [P] = [P]_o\, F/F_o \tag{11}$$

Using Eqs. (5) and (11) we get

$$[PS_n] = \{(F_o - F)[P]_o\}/F_o \tag{12}$$

Substituting Eqs. (11) and (12) into Eq. (9), we get

$$K_b^n = \{(F_o - F)/F\}(1/\{[S]_o - (F_o - F)[P]_o/F_o\})^n \tag{13}$$

Therefore,

$$\log\{(F_o - F)/F\} = n\log K_b - n\log(1/\{[S]_o - (F_o - F)[P]_o/F_o\}) \tag{14}$$

According to Eq. (14), plot of log $[(F_o - F)/F]$ versus log $(1/\{[S]_o - (F_o - F)[P]_o/F_o\})$ produces a straight line (Fig. 5a and b) with slope n. In all the cases, the value of n is found to be close to 1. This means only one surfactant molecule binds to the binding pocket of BSA where Trp-213 is situated.

Protein–surfactant interactions are both hydrophilic and hydrophobic nature. But here we kept the hydrophobic tail as same and changed the head group of the surfactants. From the data in Table 1, a significant change in the binding constant (K_b) value is observed and the K_b value decreases in the order SL-Leu > SL-Phe > SL-Val > SL-Ala > SL-Gly. This order is same as the hydrophobicity order of the amino acids. Thus according to fluorescence data 1:1 complex is formed between the surfactant and BSA. The type of the complex formed between the protein and surfactant is almost similar with all the surfactants. The protein contains three homologous domains and the surfactant may be bound in the fatty acid binding site 1. We know that the fatty acid binding site 2 covers some area of the drug binding site I [31]. The surfactant might be bound to the BSA in such a way that the polar head group falls into the warfarin binding site and the tail remains in subdomain IA. The carboxylate group of the surfactant binds to the basic residue of the protein like Arg, Lys, etc. and the amide part may be located toward the bulk water molecules, whereas the hydrophobic side chain of the head group is located near the non-polar residue(s) of the BSA protein. As SL-Ala has a shorter side chain ($-CH_3$ group) in the amino acid head group, its binding constant is slightly greater than that of the SL-Gly which has no hydrocarbon moiety in the head group. The large binding constant value of the SL-Val and SL-Leu can be explained on the basis of hydrophobicity of the amino acid side chain. As the head-group hydrophobicity increases in going from SL-Ala to SL-Leu, the binding affinity also increases following the same order.

In order to examine whether surfactant molecules also bind to subdomain IIIA or not, a similar experiment using ibuprofen, which is a site II (i.e., the hydrophobic site of subdomain IIIA) binder of the BSA protein, was also carried out. In this experiment, both BSA and ibuprofen concentrations were kept at 1.5×10^{-5} M. The fluorescence spectra of the protein were then measured in the presence of varying concentrations of each of the surfactant molecules (Fig. S5 (a–d)). As in the case of pure protein, the fluorescence intensity of the BSA/ibuprofen complex gradually decreased with the addition of increasing concentrations of surfactant, but to a lesser extent which means the surfactant molecules compete with ibuprofen for binding to the protein. Due to this competitive binding process, the observed binding constant (K_b) should decrease. The fluorescence data were therefore analyzed using the modified Eq. (15):

$$\log\{(F_o - F)/F\} = n'\log K_b' - n'\log(1/\{[S]_o - (F_o - F)[P]_o/F_o\}) \tag{15}$$

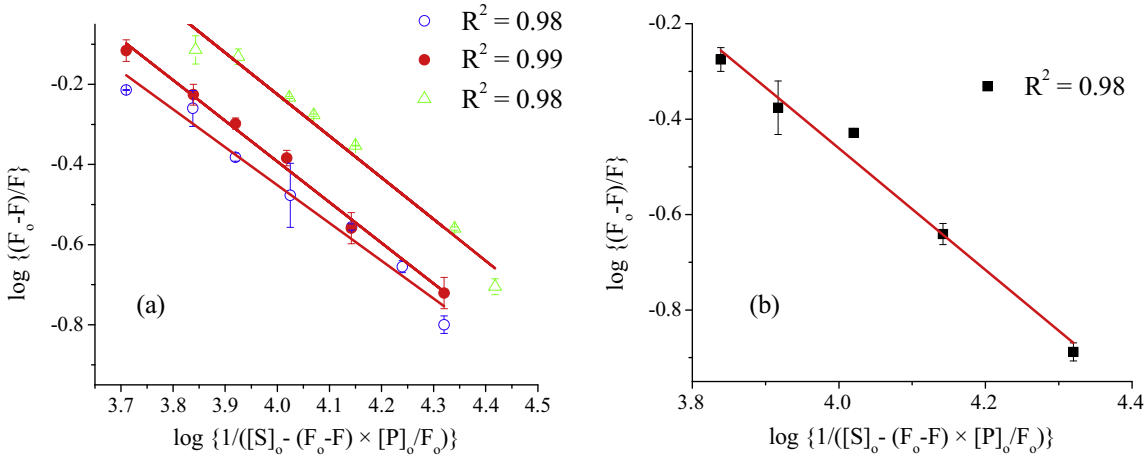

Fig. 5. (a and b). Plot of log $[(F_o - F)/F]$ versus log $(1/([S_o] - \{(F_o - F)/F_o\}\,[P_o]))$: ○ SL-Ala, ● SL-Val, △ SL-Leu, ■ SL-Phe.

Table 1
Binding constants (K_b, K_b') and binding numbers (n, n') of SL-Leu, SL-Val, SL-Ala, SL-Phe and SL-Gly surfactants in the absence and presence of ibuprofen, obtained by fluorescence titrations of BSA in phosphate buffer (20 mM, pH 7) at 298 K.

Surfactant	$K_b \times 10^{-3}\ M^{-1}$	n	$K_b' \times 10^{-3}\ M^{-1}$	n'
SL-Gly[a]	2.51 ± 0.23	1.00 ± 0.11	2.23 ± 0.32	1.26 ± 0.19
SL-Ala	3.09 ± 0.23	0.95 ± 0.06	1.66 ± 0.28	0.93 ± 0.08
SL-Val	4.26 ± 0.10	1.01 ± 0.04	1.99 ± 0.30	1.01 ± 0.07
SL-Phe	4.36 ± 0.21	1.27 ± 0.10	2.18 ± 0.64	0.88 ± 0.06
SL-Leu	6.02 ± 0.44	1.04 ± 0.07	3.80 ± 0.40	1.50 ± 0.14

[a] Data taken from Ref. [49].

where n' is the number of binding sites and K_b' is the binding constant in the presence of ibuprofen. The relevant plots are depicted in Fig. 6a and b and the corresponding binding data are included in Table 1. In all the cases, the value of n' is found to be close to 1. That the surfactant molecules compete with the ibuprofen drug is reflected by the decrease of binding constant value (K_b') (Table 1). Thus, it can be concluded that the NAASs bind simultaneously to the warfarin and ibuprofen binding sites of the BSA protein.

3.6. Thermodynamics of surfactant binding

The binding affinity can be best described by the thermodynamic equations:

$$\Delta G_b^o = -RT \ln K_b \tag{16}$$

$$\Delta G_b^o = \Delta H_b^o - T\Delta S_b^o \tag{17}$$

where K_b is the association constant, ΔH_b^o is the standard enthalpy of binding, ΔS_b^o is the standard entropy of binding, R is the gas constant and T is the absolute temperature. The binding process can be fully understood by the contribution of enthalpy and entropy from protein, ligand and the solvent water [56]. ITC measurement is one of the most sensitive techniques that permit the direct measurement of thermodynamic changes in the course of binding of surfactant to protein. Fig. 7a–d shows the binding isotherms of SL-Ala, SL-Val, SL-Leu and SL-Phe. Here 15 μM BSA was titrated by 0.5 mM SL-Ala and 1 mM SL-Phe, SL-Val and SL-Leu. Each injection shows the exothermic nature of the binding. The values of thermodynamic parameters obtained from the measurements have been included in Table 2.

From the data in Table 2 one can see that the ΔG values for SL-Ala, SL-Phe, SL-Val, and SL-Leu are negative as in the case of SL-Gly surfactant [49]. This indicates that all these five surfactant molecules form a spontaneous complex in the concentration range employed. But all the surfactant molecules, except SL-Gly, exhibit binding to a single set of sites in BSA. The binding number (n) increases from 1 to 7 in going from SL-Gly to SL-Leu, i.e., in the order of increasing hydrophobicity of the amino acid head group. In consistence with this, the binding constant values decrease in

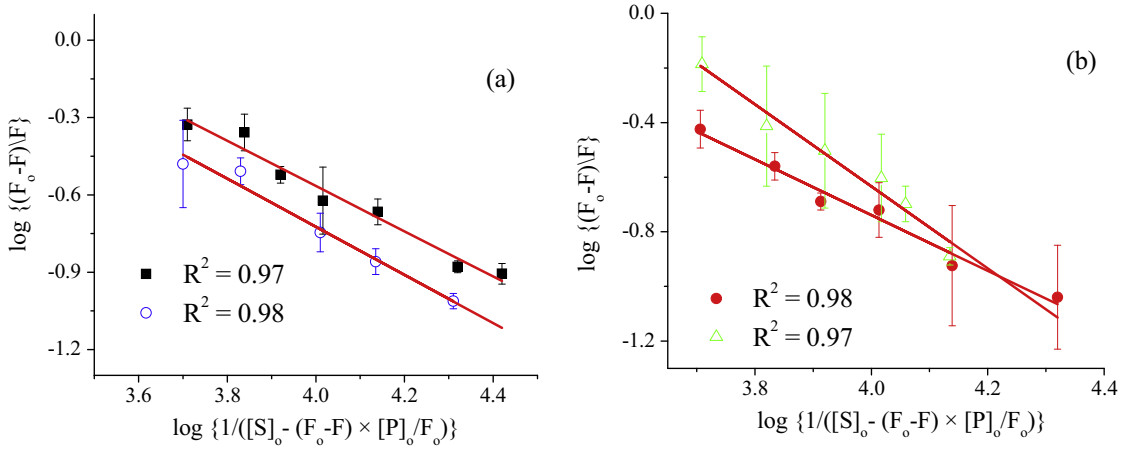

Fig. 6. (a and b). Plot of log $[(F_o - F)/F]$ versus log $(1/([S_o] - \{(F_o - F)/F_o\}\,[P_o]))$ in presence of ibuprofen: ○ SL-Ala, ● SL-Val, △ SL-Leu, ■ SL-Phe.

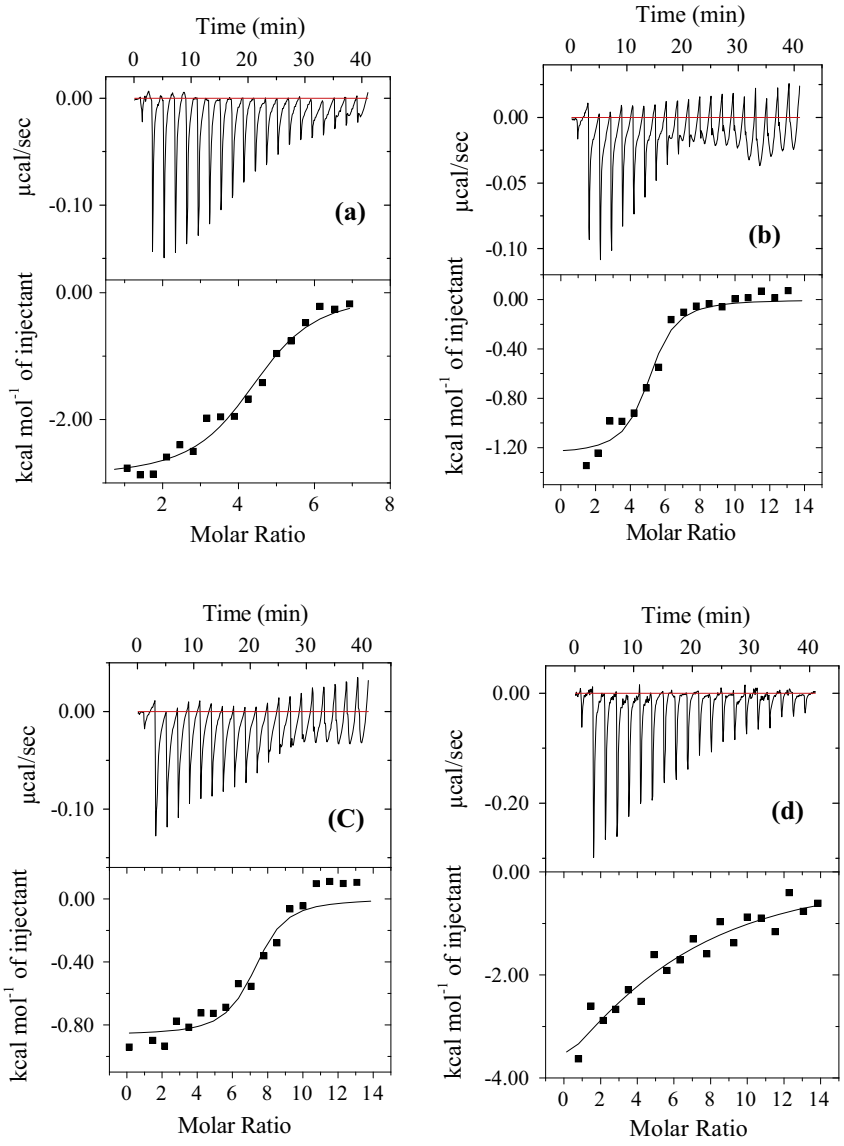

Fig. 7. ITC plots of surfactant binding with BSA at 298 K: (a) SL-Ala, (b) SL-Val, (c) SL-Leu and (d) SL-Phe.

Table 2
Thermodynamic parameters of BSA–surfactant interaction in phosphate buffer (20 mM, pH 7) at 25 °C.

System	n	ΔH_b^o (kJ mol^{-1})	ΔS_b^o (J K^{-1} mol^{-1})	ΔG_b^o (kJ mol^{-1})	$K_b \times 10^{-4}$ (M^{-1})
SL-Gly[a]	1.3 ± 0.1	−80.8 ± 3.6	−174.7 ± 12.1	−28.6 ± 0.2	10.0 ± 0.85
SL-Ala	4.4 ± 0.0	−12.2 ± 0.4	67.6 ± 2.41	−32.4 ± 0.6	44.0 ± 11.0
SL-Val	4.9 ± 0.2	−5.2 ± 0.2	92.8 ± 3.4	−32.9 ± 1.0	55.3 ± 22.0
SL-Phe	7.6 ± 0.8	−16.9 ± 3.2	27.0 ± 11.4	−24.9 ± 1.1	2.31 ± 1.03
SL-Leu	7.1 ± 0.3	−3.6 ± 0.2	99.1 ± 4.7	−33.2 ± 1.4	63.4 ± 35.5

[a] Data taken from Ref. [49].

the order SL-Leu > SL-Val > SL-Ala > SL-Gly which is similar to that obtained from fluorescence titrations. However, the K_b values of all the surfactants are greater than the corresponding value obtained from fluorescence studies. This is because of the inherent difference in these two methods. We know that fluorescence is an indirect technique and only those molecules that bind near the Trp residues affect its fluorescence property. Other molecules that bind to site(s) far away from the Trp residues will not affect the protein fluorescence. Consequently, both binding number and binding constant values are less than the corresponding values obtained from ITC technique. The latter method is a direct measurement of the heat change upon binding of all the surfactant molecules, irrespective of where they bind. Therefore, the results obtained by ITC method are more reliable than those obtained by fluorescence technique. The strong binding of SL-Val and SL-Leu in comparison to SL-Ala or SL-Gly reflects the effect of hydrophobicity of the surfactant head group for the BSA/surfactant complex formation. Although, the K_b value of SL-Phe as obtained from ITC measurement is less than that of the SL-Gly (least hydrophobic), both fluorescence and ITC methods give K_b values of SL-Phe that are less

than that of SL-Leu (highest hydrophobicity). This might be due to the phenyl ring in the head group SL-Phe surfactant. As mentioned before, the anion-π interaction of the phenyl ring with the anionic residues of the BSA may inhibit the binding of SL-Phe molecules to the protein as indicated by the lower values of ΔG and ΔS compared to those of the other NAASs. The anion-π interaction in complexes is well known in the literature.

For SL-Gly the ΔS_b^o is negative, which means that the protein goes to a more ordered state when it interacts with SL-Gly. It also means that SL-Gly binds to the hydrophilic region of the protein. On the other hand, the entropy change for other surfactants is positive, which means that with the increase of head-group hydrophobicity of the surfactants, the binding becomes more hydrophobic in nature. The change in enthalpy values which becomes much less negative for SL-Leu (-3.63 kJ/mol^{-1}) is consistent with the entropy change and suggests stronger hydrophobic interaction. The data in Table 2 clearly suggest that the hydrophobic interaction of the surfactant molecules with BSA gradually decreases in the order SL-Leu > SL-Val > SL-Ala > SL-Gly.

4. Conclusions

In summary, we have studied the interactions of four NAASs, SL-Ala, SL-Val, SL-Leu, and SL-Phe with BSA and compared the results with those of SL-Gly. The nature of binding is mainly hydrophobic and the head-group hydrophobicity plays an important role in stabilizing the BSA/surfactant complex. The binding of all the NAASs to BSA at low concentrations causes stabilization of the protein structure in the order SL-Phe < SL-Ala < SL-Val = SL-Leu. This is just opposite to what is reported in the literature. The consensus view is that the binding of cationic or anionic surfactants to proteins below the respective CMC causes some expansion in the protein structure, which means partial unfolding of the protein. [19,57–59] Like FAs [60] all the NAASs bind at the site I in the same orientation with the carboxylate ($-COO^-$) group H-bonded to Arg-117 residue. From the results of site marker experiments, it can be concluded that unlike fatty acids, these NAASs bind to site I of subdomain IIA and site II of the subdomain IIIA of BSA simultaneously. This conclusion is in line with the common view that binding below CMC occurs in one or two regions with specific binding of surfactants to a few high affinity sites on the protein surface via both hydrophobic and electrostatic interactions. Both fluorescence and ITC results suggest that as the hydrophobicity of the head-group increases, the binding efficiency of NAASs increases in the order SL-Gly < SL-Ala < SL-Val < SL-Leu. However, the binding constant value of SL-Phe is less than that of the SL-Gly with least hydrophobic head group, indicating some role of the phenyl ring in the head group. In fact, though the head group of SL-Phe is more hydrophobic than that of SL-Val [50] its K_b value is less than that of the latter. In other words, the stability of BSA/surfactant complex increases in the order SL-Phe < SL-Ala < SL-Val = SL-Leu upon binding to surfactant molecules. This has been attributed to the anion-π interaction of the phenyl ring with the anionic residues of the BSA which inhibits binding of the SL-Phe molecules to BSA. Interestingly, only one molecule of SL-Gly binds to the BSA, but the binding number is greater than 4 with the other NAASs and increases in the order SL-Ala < SL-Val < SL-Leu < SL-Phe.

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Appendix A. Supplementary material

Details about the synthesis of surfactants, representative ^{1}H and ^{13}C NMR spectra of SL-Ala in D_2O, plots of fluorescence titration, tables containing values of fluorescence lifetime, percentage of α-helix structure in BSA and BSA/surfactant complexes, and the fluorescence emission spectra of BSA and ibuprofen-bound BSA in the absence and presence different surfactants are available free of charge via the Internet (see DOI). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2015.07.064.

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