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Competitive Process of Binding Between the Anionic Surfactants Sodium Dodecyl Sulfate and Sodium Cholate in Bovine Serum Albumin

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Summary: In this study sodium cholate (NaC) was used as a representative bile salt for the competitive binding between NaC and sodium dodecyl sulfate (SDS) in bovine serum albumin (BSA), in 0.02 M tris-HCl buffer solution at pH 7.50 and 25 °C. The NaC and SDS associations with BSA were monitored at low surfactant concentrations where only this specific binding process can develop. The applied method to monitor the binding was based on the analysis of the effect of SDS and NaC concentrations and their mixtures upon the fluorescence intensity of the BSA tryptophan residues. This consists of the measurement of the surfactant monomer partitioning between the dispersion medium and the *microaggregates* on the protein molecule where the binding is indicated by the quenching of the fluorescence chromophores. Experimentally, varying the protein concentration, the surfactant concentration needed to reach a given I_o/I ratio (I_o and I are the intensities with and without protein, respectively) was measured. The analyses, based on the average number of surfactant molecules bound on the protein, indicated that the SDS is a more efficient quencher than the bile salt. The need for 4–6 NaC bound molecules to give the same protein quenching efficiency by a single molecule of SDS was estimated. We concluded that the differences in the competitive binding on the protein are exclusively related to the quenching efficiency in the formation of the nonfluorescent fluorophore-quencher complex via a physical contact and static quenching process.

Keywords: anionic surfactant mixtures; bovine serum albumin; fluorescence quenching; protein-surfactant interaction; sodium cholate

Introduction

Serum albumins are proteins with the important function of incorporating and transporting lipids such as fats, cholesterol and derivatives, into the lymph or bloodstream. Bovine serum albumin (BSA) is the protein commonly used for research purposes and as a reference in clinical analyses and biochemistry research because of its stability, water solubility and the wide capacity for binding bioorganic components. In the cycle of cholesterol metabolism, it is transported by lipoproteins to the liver where the bile salts are synthesized. Bile salts are biosurfactants synthesized in the liver, stored in gall bladder, and then secreted into the small intestine where they assist the digestion of fats. They have the remarkable function of facilitating the cleavage of nutrient fats by lipases and phospholipases, through bringing them together and solubilazing them in emulsion aggregates.[1]

The effects of surfactant molecules on proteins depend on the nature of both the surfactant and protein component. The features resulting from the surfactants bound to the proteins provide information

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with respect to the binding and denaturation capacity of a given surfactant, on the one hand, and the protein stability, on the other. Sodium dodecyl sulfate (SDS) binds strongly to proteins and is well known for its role in their denaturation processes.[2] This capacity can be attributed to their ability to associate micelle-like aggregates on the surface of the protein promoting unfolding and consequent protein structure modifications.[3] However, some proteins have been reported to bind significant amounts of SDS only. Examples are pepsin, papain and glucose oxidase which are not denatured by SDS.[4]

However, proteins exhibit low selectivity in relation to the surfactant type. In fact, BSA has the ability to bind cationic,[5–8] anionic and zwitterionic[6,9] and also nonionic surfactants.[10] Depending on the experimental conditions such as protein concentration and surfactant type, the process as a whole can exhibit different binding characteristic, such as specific, noncooperative and cooperative binding.[2,11] Specific binding is the most important pathway observed at low surfactant concentrations at levels of micromolar unity while the cooperative process becomes the most important at higher surfactant concentrations close to the surfactant cmc. At these levels of specific binding competitivity occurs, i.e., the presence of an additional molecule competes in order to occupy the protein binding sites. The binding of small molecules can be induced by hydrophobic or electrostatic means but it depends on the characteristics of the molecular structure and experimental conditions. For instance, the electrostatic process being pH dependent is important for anionic and cationic surfactant binding.[12]

This study aims to gain new insights into the features of the specific interaction of sodium cholate (NaC) to the native BSA competing with SDS. Note that NaC is here a representative bile salt. Procedures to estimate the extension of additive association on proteins have been widely used[11,13] However, spectroscopic techniques which use the intrinsic fluorescence of the protein are, undoubtedly, the most widely applied principally in the monitoring the tryptophan residue fluorescence changes induced by protein-bound additives.[6,12,14] In this study the evaluation of the SDS and NaC binding and their competition was monitored by following the changes in the intensity of the intrinsic BSA fluorescence, after SDS, NaC and their mixture additions.

For the evaluation of the surfactant performances on the binding processes, we use the methodology developed by Lissi et al.[5,9] The method consists of the measurement of the surfactant monomer partitioning between the dispersion medium and the *microaggregates* on the protein molecule where the binding is indicated by the quenching of the BSA tryptophan residues. Experimentally, the surfactant concentration needed to reach a given I_o/I ratio for different protein concentrations was measured. Here, I_o and I represent the fluorescence intensities in the absence and presence of the surfactants, respectively. This plot gives a linear correlation where the molar surfactant concentration outside the protein *microaggregates* is estimated from the intercept and the slope gives the average number of surfactant monomers bound per mole of protein. The measurements were carried out at 25.0 °C in 0.02 M (tris/HCl) buffer solutions, pH 7.50.

Experimental Part

Materials

BSA (fraction V, 99% purity) and sodium dodecyl sulfate (SDS) (99% purity) were supplied by Sigma and used without further purification. The purity of SDS had already been tested by surface tension and no minimum in the surface tension-[surfactant] profile had been observed. Sodium cholate (NaC) and sodium deoxycholate were purchased from Sigma. The (D)-tryptophan 98% was supplied by Sigma. Stock solutions of 0.02 M tris(hydroxymethyl)aminomethane (tris-HCl) buffer pH 7.50, were prepared from reagent grade Aldrich 99% and were titrated with standard HCl solution at 25.0 °C, monitored on

a Beckman pHmeter model Φ71, equipped with a combined glass electrode. The stock BSA aqueous solutions (Milli-Q water) were routinely prepared in 0.02 M tris/HCl buffer, pH 7.50, with magnetic stirring for at least 12 h. The stock solutions of the bile salt were prepared by dissolving the appropriate amounts in the same buffer solution. Water employed was conductivity grade, previously distilled and demineralized by a Millipore (mili-Q) water purification system.

Electrical Conductivity

Electrical conductivity data were acquired by means of a water-jacketed flow dilution cell, with a model 170 ATIORION conductometer, at 25.0 °C. In order to emphasize the details of conductivity changes at low surfactant concentration, the specific conductivity versus [Surfactant] plots for SDS and NaC were constructed in a low surfactant concentration range of 0–4 mM with ≈60 experimental measurements. The procedure for the measurements consisted of the addition of a surfactant stock solution into the water-jacketed flow dilution cell with an appropriate volume of distilled and demineralized water with continuous stiring.

Steady-State Fluorescence

Fluorescence spectra and intensities were recorded on a Hitachi F4500 Spectrofluorimeter equipped with a thermostated cell holder set at 25.0 °C and the sample was continuously stirred in a quartz cell of 10 mm path length. Measurements of steady-state fluorescence of BSA were performed in 20 mM tris/HCl buffer at pH 7.50. Both slits, of excitation and emission monochromators were adjusted to 5 nm. The tryptophan chromophore was excited with an excitation wavelength at 280 nm and fluorescence spectra were recorded from 300 to 400 nm.

Results

Fluorescence Emission of BSA

The fluorescence quenching experiments were performed in the tris/HCl solution at pH 7.50. It should be noted that, since the BSA isoelectrical point occurs at ≈4.9, under this condition the protein is in a partially negative form.

Changes in the tryptophan microenviroment can be identified by monitoring the fluorescence emission band in the range of 320–360 nm.[6,10] Usually, the addition of a surfactant to BSA solutions produces a decrease in the intensity of the tryptophan fluorescence, and in most cases, it is followed by a blue shift of the emission band. In this context, SDS exhibits both effects: at low concentrations it induces only a decrease in the protein emission band but, at higher concentrations, this effect is concomitant with a blue shift.[6,10,15]

Differently to the well-known SDS effects on the fluorescent emission spectra of BSA, for the BSA concentrations studied here, we found that the effect of sodium cholate produces only the decrease in intensity with a maximum emission at 240 ± 1 nm without displacement of the band up to ≈2.0 mM NaC. Moreover, above this [NaC], the spectra are not sensitive to an additional amount of NaC (data not shown). For NaC concentrations where the quantum yield decreases with a constant emission maximum, the existence of a typical static florescence quenching on the protein via physical contact between the quencher and tryptophan fluorophores can be assumed, thus, forming a nonfluorescent fluorophore-quencher complex. This approach indicates that this phenomenon is a measurement of the surfactant capacity to bind to the specific high-energy sites on the protein. On the other hand, for SDS the binding developed at low surfactant concentrations, had already suggested that this is a specific binding process.[9]

Stern-Volmer Plots

In this study, we will focus the analysis on the region where the surfactant specific binding occurs, i.e., below 0.2 mM of surfactants. In this region, the observed static quenching fluorescence promoted by surfactants can be analyzed using the

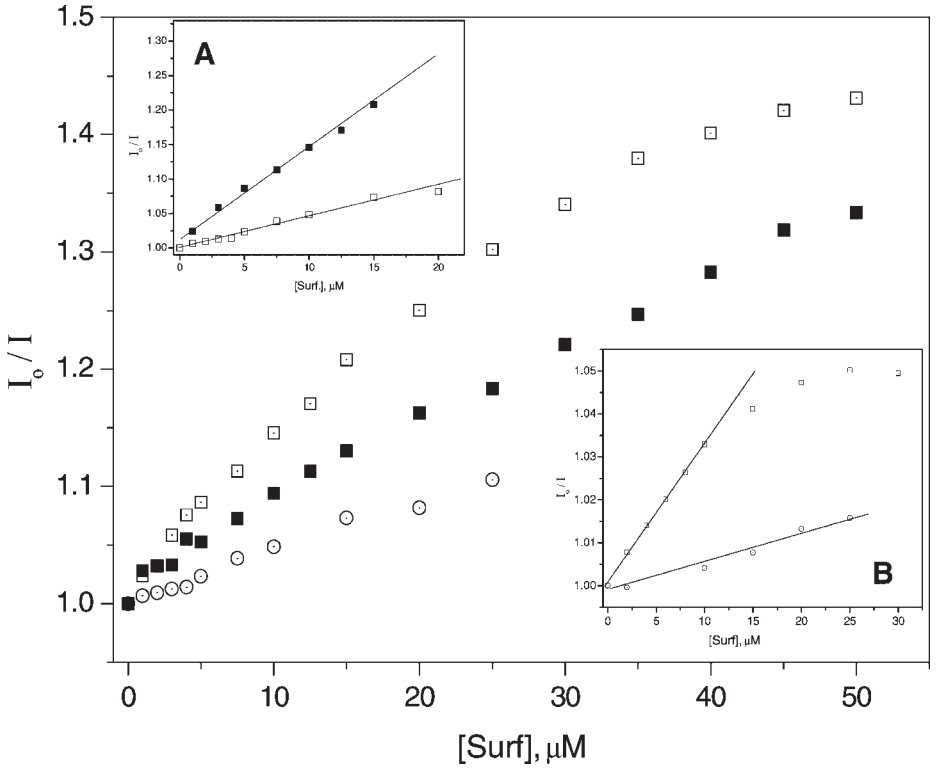

Figure 1.
Variation of the I_o/I ratio as a function of the following surfactant concentrations at 0.02 M tris-HCl buffer solution, at pH 7.50 and 25.0 °C: (□) SDS, (○) NaC and (■) NaC-SDS (0.75 NaC molar fraction). Inset (A) magnifies the linear I_o/I vs [Surf] plots for (■) SDS and (□) NaC. Inset (B) shows the effect of SDS and NaC concentrations on the fluorescence emission of the (D)-tryptophan used here as the "molecule model".

classical Stern-Volmer equation,[6,16]

$$I_o/I = 1 + K_{sv}[Q] \quad (1)$$

where I_o and I are the fluorescence intensities in the absence and presence of the quencher, K_{sv} is the Stern-Volmer quenching constant and [Q] is the molar SDS and NaC concentrations.

The constant K_{sv} values are given from the plot of I_o/I vs [Q]. The inset of Figure 1 shows a typical Stern-Volmer plot obtained up to 20 μM of SDS and NaC, at 16 μM BSA. Since the magnitude of the Stern-Volmer constant represents also the efficiency of the binding process, we can anticipate that, from the plots of inset (A) of Figure 1, K_{sv} values are 15 000 ± 500 M^{-1} and 4 500 ± 200 M^{-1} for SDS and NaC, respectively. For this protein concentration, it can be seen that SDS in the quenching process is ca. three to four times more effective.

Quenching of (D)-Tryptophan

In the absence of the protein, we used the dextrorotatory configuration of the tryptophan as the model compound in order to compare the quenching efficiency of the bile salt and SDS surfactants, under conditions of minimal fluorophore/surfactant interactions, in buffer solution pH 7.50. Inset (B) of Figure 1 shows the changes in the I_o/I vs. [NaC] and [SDS] in tris-HCl buffer solution containing 0.0083 M (D)-tryptophan. Note that the plots are quite linear in the interval of 0 to 20 μM surfactant. Since the extent of quenching is small, the estimated uncertainties are closer to 20%. For instance, at 25 μM surfactant the

extent of quenching does not exceed ≈10% and ≈1.5% for SDS and NaC, respectively. The Stern-Volmer constants obtained were $3\,200 \pm 200$ and 700 ± 50 M^{-1}, respectively. These values show that the SDS is circa 4.5 times more effective in the quenching process than the NaC. Moreover, we controlled the effect of the (D)-tryptophan concentration on the I_o/I ratio and found that it is insensitive to changes in the interval of 1×10^{-3} to 1×10^{-6}M.

Mixtures of SDS, NaC and BSA

In order to explain the effect of the surfactants on the protein we chose 0.1% as a representative BSA concentration. The competitive binding process on the protein between SDS and NaC can be observed in Figure 1 where the I_o/I ratio is plotted against the surfactant concentrations. This Figure indicates that SDS is a more effective quencher than NaC. Figure 1 exhibits also the profile for a mixture of SDS and NaC of a 0.75 NaC molar fraction. A preliminary analysis shows that the effect on the I_o/I ratio suggests a mechanism of binding where both surfactants competitively bind to specific sites on the protein.

This competition is much more clearly observed in Figure 2 where the I_o/I ratio was plotted against the SDS molar fraction at fixed surfactant concentrations of 5 and 15 μM in the presence of 16 μM BSA. Three characteristics from the isotherms can be observed: (i) considering both plots, at molar fractions up to ≈0.4, the I_o/I ratios do not exceed 10% of the final I_o/I ratio; (ii) above ≈0.4, however, a massive increase in the ratio occurs indicating a SDS monomer enhancement at the protein binding sites and (iii) above ≈0.9 molar fraction, a plateau of saturation occurs.

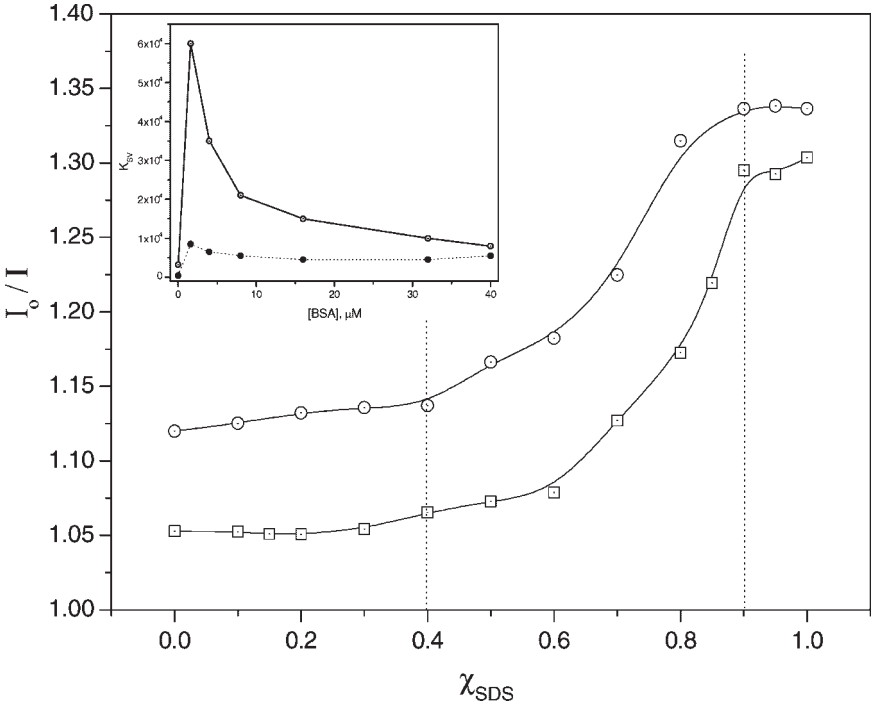

Figure 2.
Variation of I_o/I ratio as a function of SDS molar fraction measured at (○) 5 μM and (□) 15 μM of total surfactant concentration (SDS + NaC) obtained at 0.02 M tris-HCl buffer solution, at pH 7.50 and 25.0 °C. The lines indicate the threshold molar fraction necessary to obtain the massive increase in the I_o/I ratio and the saturation, respectively. The inset magnifies the variation in the Stern-Volmer constant (K_{sv}, M^{-1}) in the presence of SDS (○) and NaC (●) as a function of the BSA concentration. The data are given in Table 1.

Interestingly, the slight increase in the I_o/I ratio at a lower SDS molar fraction, up to ≈0.4, seems to characterize a threshold molar fraction necessary to obtain the massive increase in (ii). As expected, these results are in agreement with the SDS efficiency in the protein fluorescence quenching comparatively with the activity of NaC. Comments on the increased mentioned in (ii) will be presented below with the estimates, from the different I_o/I ratios, for the parameters such as the free surfactant monomers $[S]_f$ and average number of surfactant monomers bound per protein molecule (n).

Evaluation of the Surfactant Binding on BSA

Several procedures have previously been used to estimate the binding of a surfactant on proteins. We use here a simple method developed by Lissi et al.[9] which is based on the measurement of the intensity of protein intrinsic fluorescence as a function of surfactant concentration. In this study, this methodology was applied at low surfactant concentrations where, it is assumed that, only the specific process of binding to specific sites of the protein is occurring.

According to the method, the dependence on the BSA concentration is used to evaluate the extent of surfactant binding from the plots of I_o/I versus surfactant concentration at different [BSA] (plots not shown). Table 1 lists the Stern-Volmer constant values obtained from these plots.

Table 1.
Stern-Volmer constants (K_{sv}, M^{-1}) for SDS and NaC at different protein concentrations.

BSA	BSA	SDS	NaC
μM	% wt		
None[a)]	None	3 200 ± 200	700 ± 50
1.6	0.01	60 000 ± 500	8 500 ± 300
4.0	0.025	35 000 ± 400	6 500 ± 100
8.0	0.05	21 000 ± 500	5 500 ± 300
16	0.10	15 000 ± 500	4 500 ± 200
32	0.20	10 000 ± 500	4 500 ± 200
40	0.25	8 000 ± 500	5 500 ± 200

[a)] Values given from the quenching of the molecule model [(D)-tryptophan] in water.

The estimation of K_{sv} was performed at low surfactant concentrations up to 20 μM surfactant where the Stern-Volmer plots exhibit linear correlations. Note that, the K_{sv} values clearly indicate that the SDS is a more efficient quencher than the bile salt whose order of magnitude depends on the protein concentration. It can be noted also that the K_{sv} values decrease with the increase in BSA concentration, indicating a spontaneous binding process and quencher distributions with the protein molecules in solution. From this dependence, it can also be concluded that the quencher efficiency is a function of the performance of the local surfactant concentration bound on the protein molecule.

The method uses a plotting approach related to the surfactant concentration necessary to reach an I_o/I ratio for different protein concentrations. Figure 3 and 4 show the typical linear plots. From the intercepts one estimates the surfactant free monomer concentrations ($[S]_f$) remaining in the aqueous pseudophase and the slopes give the average surfactant molecule bound to each protein molecule (n). From the above parameters, one can obtain a pseudo-association constant (K), according to Equation (2).[9] The K values obtained from this method for different I_o/I ratios are given in Table 2.

$$K = \frac{n}{[S]_f} \tag{2}$$

Discussion

Effect of BSA Concentration on Protein Fluorescence

The quenching of the fluorophore excitation state can occur through several mechanisms and, in the data treatment, it may be important to take into account the mechanism involved. Basically, the dynamic and static quenching processes are applied to interpret the fluorescence changes of a protein by quenchers. The important point, however, is the fact that in both pathways the molecular contact between the fluorophore and quencher is

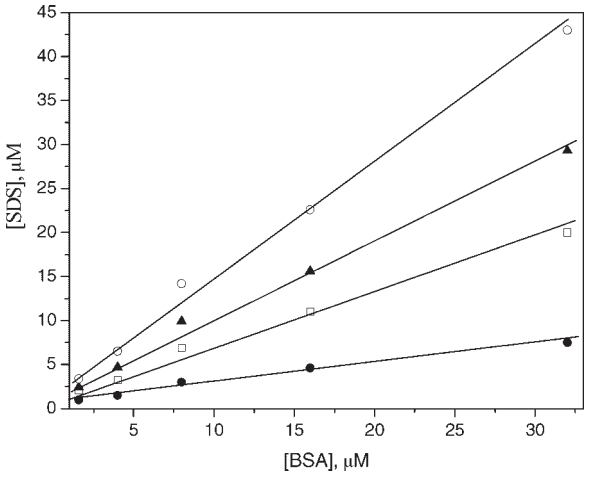

Figure 3.
Plots of SDS concentration as a function of BSA concentration needed to give the following I_o/I ratio: (○) 1.28; (▲) 1.20; (△) 1.15 and (●) 1.08.

a requirement. In this study, we used the simplified Stern-Volmer equation [Equation (1)] without distinguishing between the two processes. The formal argument supporting the methodology used here is based on the fact that the K_{sv} values obtained for SDS listed in Table 1 are, in order of magnitude, similar to those previously reported for the BSA-SDS system.[9]

The quencher efficiency of SDS compared to the weaker performance of NaC was mentioned above. The inset of Figure 2 highlights the dependence of K_{sv} on the protein concentration for both surfactants. In fact, the Stern-Volmer constants are higher at the lower [BSA], although for NaC, this tendency, qualitatively, is much less pronounced. For SDS with 16 μM BSA, the difference in the quenching efficiency is approx. ten-fold (Table 1). We can speculatively attribute this observed difference to the fact that the SDS has a higher capacity to quench the ''molecule model'' (D)-tryptophan. Based on the K_{SV} values

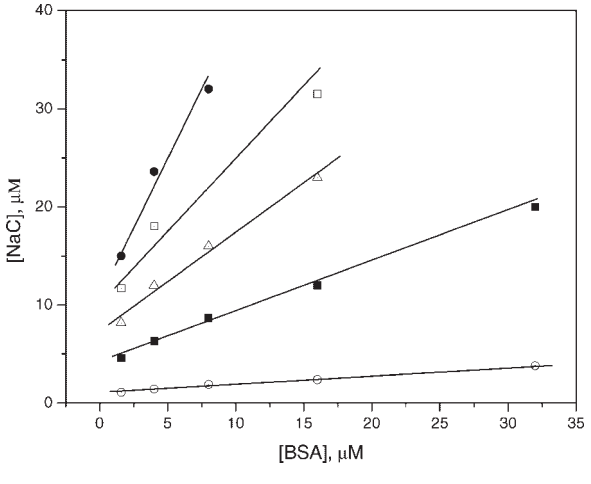

Figure 4.
Plots of NaC concentration as a function of BSA concentration needed to give the following I_o/I ratio: (●) 1.15; (□) 1.12; (△) 1.08; (■) 1.05 and (○) 1.01.

Table 2.
The parameters, average surfactant molecule bound to the protein molecule (n) and free surfactant monomer concentrations ($[S]_f$), obtained from the plots of Figure 3 and 4.

Surfactant	I_o/I	S_{free}	n	10^3 K · M^{-1}	S_T[a)]
		μM			μM
NaC	1.01	1.0	0.07	70	2.5
	1.05	3.0	0.60	200	16.0
	1.08	6.0	1.21	201	23.0
	1.12	9.0	2.20	250	-
	1.15	10.4	2.60	250	-
SDS	1.05	0.5	0.10	192	2.3
	1.08	0.7	0.23	328	4.6
	1.12	1.0	0.45	445	8.6
	1.15	1.1	0.60	550	11.0
	1.20	1.1	0.87	791	16.0
	1.28	1.3	1.29	1 016	22.7

[a)] Analytical surfactant concentrations estimated for each I_o/I ratio.

shown in Table 1, the SDS is circa 4.5 times more efficient in the quenching process. Considering the average surfactant molecule bound to the protein molecule (n) listed in Table 2, the n_{NaC}/n_{SDS} ratios vary from four to six depending on the I_o/I ratio considered. These values represent the amount of surfactant monomer bound to the protein to give the same I_o/I ratio of that for the SDS binding. We note that the pseudo binding constants K obtained from Equation (2) and given in Table 2 are not used for discussion purposes. As has previously been observed,[9] K gives low accuracy values because of the uncertainty of the free surfactant concentration estimated from the intercepts. However, the tendency observed in Table 2, given higher K values for SDS, is real.

The above differences in quenching efficiency can be explained from a second point of view. Table 2 also lists the free surfactant monomers (S_{free}) estimated from the intercepts of the plots of Figure 3 and 4. Note that S_{free} is understood as the fraction of surfactant monomer remaining in the external medium of the protein,[9] therefore, it is that fraction which does not promote quenching. According to the values in Table 2, for a given I_o/I ratio they indicate that, the concentration of NaC free monomer is much higher than that for SDS, therefore, compared with the SDS, the NaC exhibits a higher solubility in the external medium. For instance, at $I_o/I = 1.08$ the values are 6.0 and 0.7 μM for NaC and SDS, respectively, representing an ≈nine-fold difference. Despite the uncertainty in the determination of the free monomer concentration from the plot intercept in Figure 3 and 4, the observed tendency of the values for SDS and NaC are reasonable and meaningful.

Thus, a reasonable explanation for the rapid decrease in K_{SV} (inset of Figure 2) is that there is a process of surfactant monomer redistribution among the albumin molecules to the available binding sites without contact with tryptophan residues. In this single process, however, a simple effect of surfactant dilution without changes in the Stern-Volmer constant with the BSA concentration would be expected, which is not the case here. We can conclude, therefore, that the pathway of monomer distribution among binding sites without fluorescence quenching, while the protein concentration is increased, is the most important effect which can explain the rapid decrease in K_{SV} values.

Evaluation of Quenching by SDS, NaC and their Mixtures

Despite the uncertainties above described, from Equation (2), this present methodology gives an evaluation of the average number of surfactant molecules (n) necessary to produce a given I_o/I ratio. The real meaning of this binding parameter, however, is the amount of surfactant monomer per mol of BSA which produces quenching on the protein internal probe. The results are shown in Figure 5 for SDS and NaC, and for mixtures of 0.50 and 0.75 NaC molar fractions. As was expected, the linear plots obtained explain a proportional correlation between the amount of protein in solution and the binding process represented by the I_o/I ratio. This is in agreement with the conclusion that, at the SDS and NaC concentrations studied, a net surfactant specific binding is developing and the linearity of the plots essentially indicates

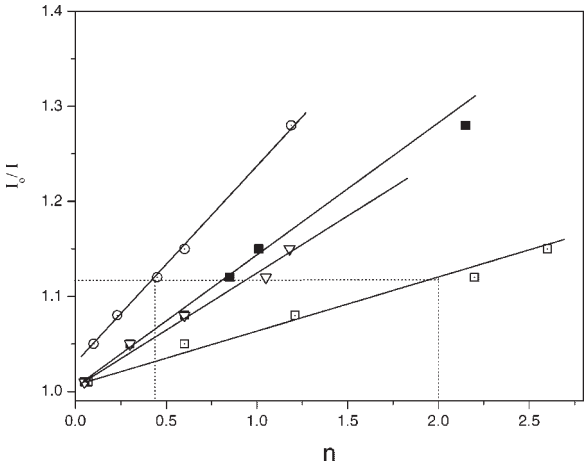

Figure 5.
Plots of I_o/I versus the average number (n) of surfactant monomers bound per molecule of BSA obtained for (○) SDS and (□) NaC, and for the molar fractions of (■) 0.50 and (△) 0.75 NaC.

static quenching by the surfactant monomers even for the mixtures.

Furthermore, additional relevant information taken from Figure 5 refers to the slope of the plots which is related to the quenching efficiency of the surfactants. The values of the slopes for SDS and NaC are 0.20 and 0.05, respectively. This difference represents the quenching efficiency of SDS being ≈four times higher than that of NaC. In other words, using the premise on which this present method of evaluation was based, we can say that the amount of NaC necessary to produce the same I_o/I ratio as the SDS is four times higher. Considering, however, the similar performance of SDS with respect to that of NaC found in the quenching process of the (D)-tryptophan, used here as molecule model (see inset B of Figure 1 and Table 1), this coincident finding leads us to conclude that the effect of surfactant monomers on the quenching of the tryptophan fluorophore residues relates to the formation of a nonfluorescent fluorophore-quencher complex via physical contact and static quenching processes.

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[1] R. H. Abeles, P. A. Frey, W. P. Jencks, "*Biochemistry*", Jones and Bartlett Publishers, Boston **1992**, p. 838.
[2] N. J. Turro, K. P. Ananthapadmanabhan, X. G. Lei, M. Aronson, *Langmuir* **1995**, *11*, 2525.
[3] S. F. Santos, D. Zanette, H. Fischer, R. Itri, *J. Colloid Interface Sci.* **2003**, *262*, 400.
[4] K. P. Ananthapadmanabhan, in: *"Interactions of Surfactants with Polymers and Proteins"*, E. D. Goddard, K. P. Ananthapadmanabhan, Eds., CRC Press, Boca Raton **1993**, p. 427.
[5] X. Díaz, E. Abuin, E. Lissi, *J. Photochem. Photobiol. A* **2002**, *6176*, 1.
[6] E. L. Gelamo, C. H. T. P. Silva, H. Imasato, M. Tabak, *Biochim. Biophys. Acta* **2002**, *1594*, 84.
[7] K. Takeda, M. Shigeta, K. Aoki, *J. Colloid Interface Sci.* **1987**, *117*, 121.
[8] Y. Nozaki, J. A. Reynolds, C. Tanford, *J. Biol. Chem.* **1974**, *249*, 4452.
[9] E. Lissi, E. Abuin, M. E. Lanio, C. Alvarez, *J. Biochem. Biophys. Methods* **2002**, *50*, 261.
[10] H. Durchschlag, K.-J. Tiefenbach, S. Gebauer, R. Jaenicke, *J. Mol. Structure* **2001**, *563–564*, 449.
[11] M. N. Jones, *Biochem. J.* **1975**, *151*, 109.
[12] E. L. Gelamo, M. Tabak, *Spectrochim. Acta A* **2000**, *56*, 2255.
[13] E. Dickinson, in: *"Interactions of Surfactants with Polymers and Proteins"*, E. D. Goddard, K. P. Ananthapadmanabhan, Eds., CRC Press, Boca Raton **1993**.
[14] C. N. Lunardi, J. B. S. Bonilha, A. C. Tedesco, *J. Luminescence* **2002**, *99*, 61.
[15] B. Schweitzer, D. Zanette, R. Itri, *J. Colloid Interface Sci.* **2004**, *277*, 285.
[16] L. Tortech, C. Jaxel, M. Vicent, J. Gallay, B. de Foresta, *Biochim. Biophys. Acta* **2001**, *1514*, 76.

