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EPR and Circular Dichroism Solution Studies on the Interactions of Bovine Serum Albumin with Ionic Surfactants and β-Cyclodextrin

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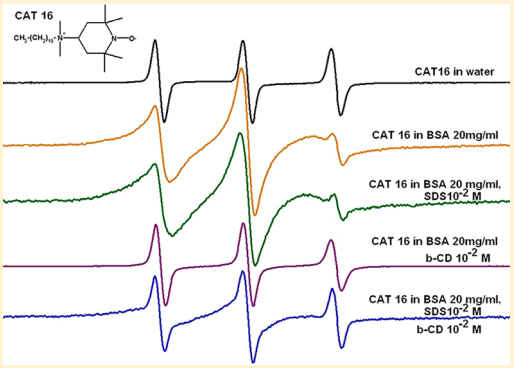
Supporting Information

ABSTRACT: The interactions of bovine serum albumin (BSA) with ionic surfactants (sodium dodecyl sulfate, SDS, and cetyltrimethylammonium bromide, CTAB) and β-cyclodextrin (β-CD) have been investigated by electron paramagnetic resonance (EPR) and circular dichroism measurements. The spin probe selected to report on the interaction of albumin with surfactants and/or β-CD was 4-*N*,*N*-dimethyl hexadecyl ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT16), on account of (a) its balance between electrostatic and hydrophobic character and (b) the ability of BSA to form complexes with various organic molecules. The distribution of the spin probe among different environments in solutions containing only BSA was confirmed by the existence of two components in the EPR spectra: one revealing a restricted mobility of the spin probe, attributed to the protein−spin probe complex, and another one showing free movement, attributed to the spin probe in solution. The presence of surfactants and/or β-CD alters the distribution of CAT16 between various compartments in each system. Formation of protein aggregates as a result of thermal denaturation was evidenced by the appearance of an immobilized component in the EPR spectrum. This component is not present in the EPR spectra of CAT16 in protein/surfactant or protein/cyclodextrin solutions. Circular dichroism spectra of BSA provided information about changes in the secondary structure of the protein induced by the presence of surfactants and/or cyclodextrin in solution. The results demonstrate that β-CD hinders the interaction between the employed surfactants and the protein. The cationic surfactant (CTAB) induces changes in protein conformation at a lower concentration compared to the anionic surfactant (SDS).



INTRODUCTION

A large number of studies have been dedicated to understanding the interactions of globular proteins with various ligands and how these interactions influence their biological function.[1−4] In this respect, serum albumins have been intensively studied with respect to their role in the transport of physiological ligands and drugs. Specifically, physicochemical studies targeting the interaction between albumins and fatty acids or corresponding surfactants are of interest because of their potential applications in cosmetics, foods, pharmaceuticals, or physiological systems.[5−7] Each protein structure has a spectrum of binding sites with different affinities for a specific ligand. Thus, in the case of human serum albumin (HSA), crystallographic studies on its complexes with fatty acids have shown that the protein exhibits up to seven distinct hydrophobic pockets that can accommodate molecules such as long-chain fatty acids.[8−11] The high affinity of albumins for compounds such as fatty acids or surfactants bearing hydrophobic tails is reflected through the values of the binding constants reported in the literature,[12−14] which fall in the range of 10^6-10^7 M^{-1}.

Addition of a surfactant to an albumin solution induces changes in its native globular conformation, leading to an unfolded state while concurrently preventing protein aggregation.[15−17] Recovery of the initial conformation of the protein is possible if the surfactant molecules can be stripped from their complex with the protein. A representative class of molecules capable of modulating protein−surfactant interactions is the cyclodextrins (CDs),[18] which exhibit a relatively nonpolar cavity in their structure in juxtaposition to water, thereby allowing formation of inclusion complexes with organic molecules, including surfactants.[19,20]

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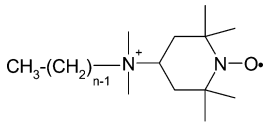
A broad range of physicochemical methods is usually employed in the study of protein−surfactant systems, including calorimetric techniques, spectroscopic and electronic microscopy techniques, and tensiometric and viscosity measurements.[21,22] Of those, circular dichroism reveals the effects of the interaction of ionic surfactants with bovine serum albumin (BSA) reflecting (a) the extent of α-helical secondary structure of the protein and (b) how this interaction is influenced by the presence of cyclodextrin. This method has been used to investigate changes induced in protein structure in the presence of surfactants.[1,5] Electron paramagnetic resonance (EPR) spectroscopy, on the other hand, is sensitive to the local structure in the vicinity of the paramagnetic center, and molecular dynamics on the nanosecond time scale can be used as a tool for studying protein−surfactant interactions. In fact, EPR spectroscopy has also been used, in some studies, to analyze protein−surfactant or protein−fatty acid interactions.[11,23−26] In view of the fact that BSA is an EPR-silent species, it was necessary to introduce into the system spin probes, such as *n*-doxyl stearic acids, that can report indirectly on the changes taking place at the molecular level. These spin probes have a structure similar to that of fatty acids and were used to obtain information about the affinity of such molecules for the protein and their mobility in the arising protein complexes through EPR spectral analysis. Furthermore, the EPR spin probe method has been used in the past to investigate the albumin−fatty acid binding capacity in patients with benign and malignant colorectal diseases, with the results showing that changes occurring in the albumin conformation induced by these diseases are reflected in the shape of the EPR spectra of the spin probe employed.[27] In a previously reported study, it was also shown by EPR spectroscopy that the ability of albumin to bind fatty acids is modified in patients with tumors because the tumor cells generate proteins that can bind albumin, thereby leading to structural changes in the albumin.[28]

Poised to explore the interactions of BSA with ionic surfactants in-depth, we report herein on an investigation of the interactions of bovine serum albumin (BSA) with ionic surfactants [sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB)], the effect of β-cyclodextrin (β-CD) on the protein−surfactant complex, and the effect of thermal denaturation of BSA at 70 °C by EPR spectroscopy and circular dichroism measurements. As spin probes, 4-*N*,*N*-dimethylalkylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT*n*) (Scheme 1) probes were selected, as their general structure consists of (a) a hydrophobic tail, allowing interaction with the hydrophobic sites of the protein structure, and (b) ionic groups, which can contribute to a weak interaction with albumin while concurrently favoring a stronger interaction with surfactant micelles. Very likely, such spin probes operate through binding to hydrophobic sites on the surface of albumin.

Scheme 1



CAT n

EXPERIMENTAL SECTION

Materials. BSA was purchased from Fluka, whereas sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and β-cyclodextrin (β-CD) were purchased from Alfa Aesar and used without further purification. The spin probe CAT16 [4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl] was obtained from Molecular Probes (Scheme 1). Phosphate buffer solution (pH 7.4) was purchased from Merck.

Instruments. EPR spectra were recorded on a JEOL FA 100 spectrometer at room temperature, with a frequency modulation of 100 kHz, a microwave power of 0.998 mW, a sweep time of 480 s, a modulation amplitude of 1 G, a time constant of 0.3 s, and a magnetic field scan range of 100 G.

Circular dichroism spectra were recorded on a JASCO J-815 CD spectropolarimeter at room temperature. The time constant, scan speed, bandwidth/resolution, and sensitivity of the device were set at 4 s, 100 nm/min, 1 nm, and 1000 mdeg, respectively. Each spectrum was signal-averaged three times.

Sample Preparation. Albumin Solutions. All samples investigated were prepared in buffer solution (mixture of $NaH_2PO_4{\cdot}H_2O$ and Na_2HPO_4) at pH 7.4. BSA solutions were prepared at room temperature by dissolving 2, 10, 20, or 40 mg of protein per milliliter. The concentrations of SDS and CTAB were 10^{-3}, 10^{-2}, or 10^{-1} M, whereas β-CD was present at a concentration of 10^{-2} M. These solutions were used for EPR measurements. For experiments targeting the investigation of the heat effect on the protein structure, in each case, 1 mL of solution was heated at 70 °C for 15 min in a water bath.

For EPR measurements, a stock solution of 10^{-2} M CAT16 was prepared in ethanol. To prepare samples for EPR measurements, in each case, an appropriate volume of ethanol solution was evaporated from a vial under a stream of nitrogen gas. Subsequently, an aqueous solution of albumin (or β-CD, SDS, etc.) was added to reach a spin probe concentration of approximately 10^{-4} M. To record EPR spectra, solutions containing CAT16 were transferred to glass capillaries and sealed. Deconvolution of EPR spectra showing two components was carried out using the Specview program. The rotational correlation times of the spin probe showing an isotropic dynamic regime were determined using the equation

$$\tau_c = 6.51 \times 10^{-10} \Delta H_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} + \left(\frac{h_0}{h_{+1}} \right)^{1/2} - 2 \right] \quad (1)$$

where ΔH_0 is the peak-to-peak width (in Gauss) of the central line and h_{-1}, h_0, and h_{+1} are the heights of the low-field, central, and high-field lines, respectively.[29]

The CAT16/complex is characterized by slow dynamics, and the corresponding EPR spectra were simulated using the software developed by Budil et al.[30]

For circular dichroism measurements, a stock solution of 10^{-6} M BSA was prepared in buffer solution at pH 7.4. This was used to prepare protein solutions containing 10^{-2} M β-CD, 10^{-2} M SDS, and 10^{-3} M SDS or a mixture of SDS/β-CD at the aforementioned concentrations. Similar solutions containing 10^{-4} M CTAB were also prepared.

RESULTS AND DISCUSSION

Binding of ionic surfactants to a protein surface is accompanied by changes in the secondary structure of the protein and occurs through specific and nonspecific phases involving electrostatic

and/or hydrophobic interactions.[13] Ionic surfactants induce unfolding of proteins at millimolar concentrations or below and are considered more efficient denaturants than urea or guanidinium salts, which induce similar changes at higher concentrations.[23,31,32] The recovery of the initial conformation of the protein can be achieved by adding cyclodextrin molecules, which strip the surfactant away from the protein surface and form inclusion complexes with the surfactant. Circular dichroism is a method, which can provide information on changes in the secondary structure of a protein induced by the interaction with other molecules.

In our experiments, the concentration of BSA used for circular dichroism measurements was lower than those used in EPR experiments.

Circular Dichroism Measurements. The circular dichroism spectra of various solutions of 10^{-6} M BSA in the absence and presence of ionic surfactants and/or β-CD were recorded in the wavelength range of 200−260 nm. The surfactants (SDS and CTAB) and β-CD do not exhibit signals in this wavelength range. Therefore, the emerging spectrum is due to the BSA contribution only. The two negative peaks at 209 and 222 nm originate in $n \rightarrow \pi^*$ transitions of the amide groups in the peptide bond and reflect the characteristic signal of the α-helical conformation of the protein. The intensity of the circular dichroic signal was expressed as mean residue ellipticity (MRE), defined as[33]

$$\text{MRE (deg·cm}^2\text{·dmol}^{-1}) = \frac{\theta}{10rl[\text{BSA}]} \quad (2)$$

in which θ is the observed (experimental) dichroism (in millidegrees), r represents the number of amino acid residues in BSA (582), l is the optical length of the cell (in cm), and [BSA] is the molar concentration of albumin.

The α-helical content was calculated using the MRE values at 222 nm, where the spectral changes are most pronounced, according to the equation[33]

$$\alpha\text{-helix (\%)} = \frac{-\text{MRE}_{222\text{nm}} - 2340}{30300} \times 100 \quad (3)$$

Figure 1 illustrates the changes in the circular dichroism spectrum of BSA induced by the presence of SDS at a concentration of 10^{-3} M [a concentration below the critical micelle concentration (cmc) of this surfactant] and CTAB and the capacity of β-CD to remove the surfactants from their complexes with the protein. When β-CD was added to the SDS (10^{-3} M)/BSA (10^{-6} M) or CTAB (10^{-4} M)/BSA (10^{-6} M) solution at a 10^{-2} M concentration, the recovery of the initial α-helical contribution to the secondary structure of the protein was almost complete. These changes are in line with data reported in the literature on protein/surfactant/cyclodextrin interactions.[5]

The effect of SDS on the ellipticity of BSA depends on the concentration of the surfactant, but not in a linear fashion. Thus, when the concentration of SDS in solution was 10^{-2} M, the α-helical content at 222 nm was 41.1%, quite similar to that at 10^{-3} M SDS (42.5%). However, the ability of cyclodextrin to facilitate the recovery of the initial ellipticity of the protein depends on the molar ratio between the surfactant and β-CD. To this end, when the SDS:β-CD ratio was 1:1, recovery of the initial ellipticity was not achieved.

The circular dichroism spectra presented in Figure 1 show that CTAB is more efficient in unfolding BSA than SDS, as it induces a stronger change in the α-helical content of the protein structure at a lower concentration.

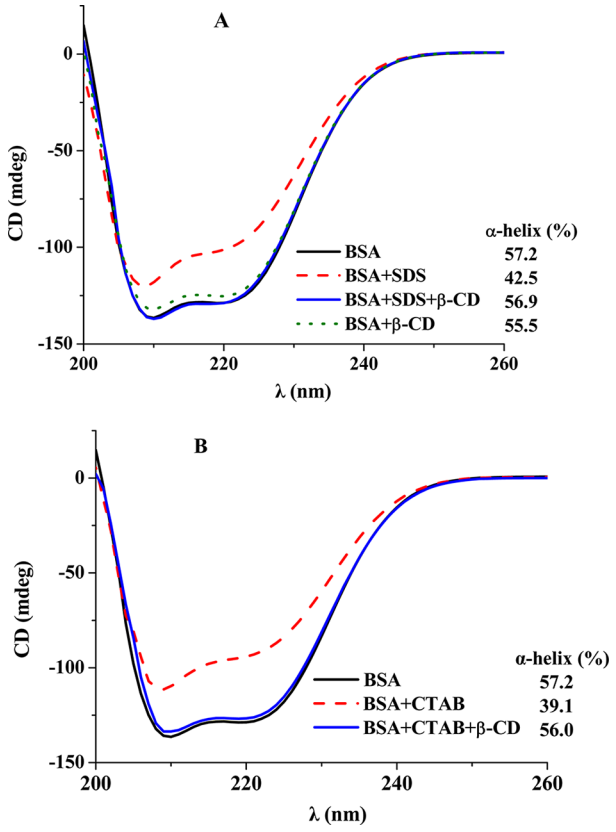


Figure 1. Circular dichroism spectra of BSA (10^{-6} M) in the absence and presence of (A) SDS (10^{-3} M) and/or β-CD (10^{-2} M) and (B) CTAB (10^{-4} M) and β-CD (10^{-2} M).

These findings can be rationalized as follows: At concentrations lower than the cmc, the surfactant binds to high-affinity binding sites on BSA, with electrostatic and hydrophobic interactions operating cooperatively to favor the process.[34] The more pronounced effect of CTAB on the BSA secondary structure upon binding can be ascribed to stronger electrostatic interactions arising between the cationic surfactant and BSA, which is negatively charged at pH 7.4 .[35]

The presence of β-CD hinders both BSA−CTAB and BSA−SDS interactions. This effect can be attributed to the stronger and more specific CD−surfactant hydrophobic interactions coming into play.[5] β-CD does not bind BSA,[36] but its nonpolar cavity has a very high affinity for binding the hydrophobic chains of the surfactant,[37] thus preventing surfactant binding to BSA. Moreover, β-CD is able to extract the surfactant molecules bound to the protein, as that is amply shown in the circular dichroism spectra by the recovery of the native state of BSA upon β-CD addition to the BSA−surfactant systems.

EPR Measurements. *CAT16 in BSA Solutions.* The hydrophilicity/hydrophobicity balance in CAT*n* spin probes is determined by the length of the alkyl chain and influences the strength of their interactions with proteins, such as albumins. Albumins can interact with molecules of the CAT*n* family through (a) hydrophobic forces involving the alkyl chain and (b) electrostatic forces involving the cationic group of the spin probe. Furthermore, the presence of the cationic group in the spin probe structure favors a stronger interaction with surfactant micelles. In this regard, changes in the EPR spectra

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of albumins facilitate determination of the mobility of the ionic spin probe bearing a hydrophobic tail on the protein surface and derivation of indirect information on the competition between β-CD and protein for molecules such as ionic surfactants.

Preliminary experiments showed that the CAT1 spin probe had no affinity for BSA, as EPR parameters were not affected by the presence of the protein in solution, irrespective of its concentration. In the case of spin probe CAT4, small changes in the EPR spectra were observed, but the balance between the hydrophobic and ionic characters was not the right one to provide relevant information on the interaction of BSA with surfactants and β-CD (Figure 1 of the Supporting Information). Spin probe CAT16 represents a suitable reporter of changes occurring in solutions of BSA, because it offers a good balance between hydrophobic and electrostatic forces in its structure. Equally important for this study, BSA not only exhibits hydrophobic pockets, but also has an inhomogeneous distribution of charges on its three domains (which have −11, −7, and +1 individual charges).[38] CAT16 has in its structure a hydrophobic alkyl chain tail, which is responsible for the interaction with the hydrophobic domains in the albumin structure. The positive charge of CAT16 plays a role in the interaction with the protein, likely orienting itself toward binding sites located on the surface of the protein. Figure 2

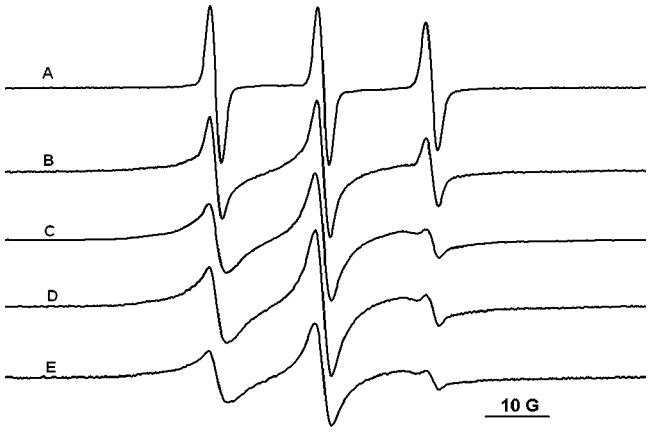

Figure 2. EPR spectra of CAT16 in (A) buffer solution at pH 7 and solutions containing BSA at different concentrations: (B) 2, (C) 10, (D) 20, and (E) 40 mg/mL.

shows the EPR spectra of CAT16 in buffer solution at pH 7.4 and in BSA solutions with concentrations ranging between 2 and 40 mg protein/ml. The EPR spectra of CAT16 in BSA solutions reflect a superposition of (a) a component with narrow lines corresponding to the free CAT16 in solution, and (b) a component with broader lines due to restricted mobility, corresponding to the spin probe "bound" to albumin. The interaction of CAT16 with BSA is comparable to that of CAT16 with HSA, as the spectrum of CAT16 in a solution of 10 mg/mL HSA exhibits similar features.[39] The experimental spectra of CAT16 in BSA solutions can be deconvoluted by subtracting the spectrum of CAT16 in buffer solution from the spectrum recorded in BSA (Figure 3). The difference spectrum corresponds to the complex CAT16/BSA and reveals an anisotropic motion. The fraction of the mobile component can then be evaluated from the ratio of the double integrals of the deconvoluted spectra. In this approach, the assumption made

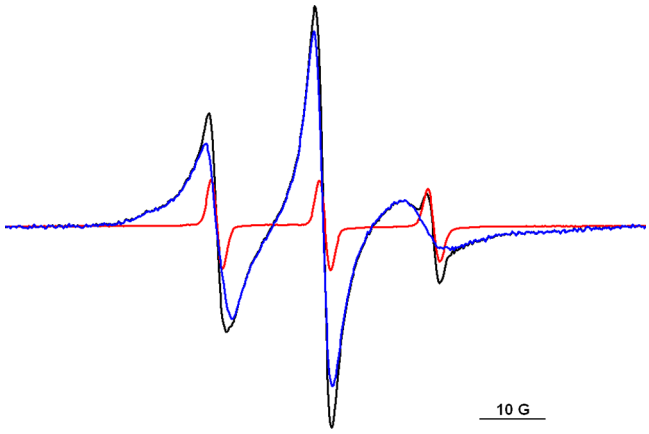

Figure 3. Deconvolution of the CAT16 spectrum in a solution containing 20 mg/mL BSA. The experimental spectrum (black), the mobile component (red), and the restricted-motion component (blue) are shown.

relies on the approximation that the parameters of the mobile component are always the same, that is, the CAT16 spectrum.

The contribution of the mobile component to the EPR spectra, attributed to free CAT16 in albumin solutions, decreases with increasing concentration of BSA (Table 1).

Table 1. Proportion of the Mobile Component in EPR Spectra of CAT16 as a Function of BSA Concentration

[BSA] (mg/mL)	content of mobile component (%)
2	32.83
10	24.39
20	13.34
40	5.03

The τ value corresponding to CAT16 in water is 1.33×10^{-10} s, whereas the τ value corresponding to bound CAT16 to the protein is 22×10^{-10} s. From Table 1, it can be seen that the ratio between the mobile and the less-mobile components decreases with increasing BSA concentration. Compared to data previously reported[11,26] on the interaction between the 5-doxyl stearic spin probe and albumins, which showed that this spin probe has a restricted motion in the protein complex, our data suggest that CAT16 in the BSA complex is characterized by higher mobility. Considering that (a) albumin has several binding sites for hydrophobic molecules and (b) CAT16 and 5-doxyl stearic acid have different hydrophobicities, it could be assumed that their binding is different. When both 5-doxyl stearic acid and CAT16 spin probes are present in albumin solutions, regardless of the order in which the spin probes are introduced in the system, the resulting EPR spectrum represents the sum of the individual spectra of CAT16 and 5-doxyl stearic acid. This suggests that these two spin probes do not compete for the same hydrophobic site of the protein (Figure 2 of the Supporting Information).

CAT16 in BSA/Ionic Surfactant Solutions. The interactions between albumins and ionic surfactants were studied over time, using different techniques. In particular, SDS/albumin systems were investigated in detail. The binding isotherm of SDS to BSA depends on the surfactant concentration, exhibiting four regions: At low concentration, the surfactant binds only to the specific (hydrophobic) sites. In the second part, a slow rise of SDS concentration appears until a plateau is reached. Then, a

third region exhibits an abrupt increase of SDS concentration bound to BSA. Finally, a fourth region corresponds to the saturation process where no further binding occurs (and in solution, free SDS micelles are present).[23] Various models for the surfactant−protein complex have been proposed corresponding to the high concentration of surfactant (above the cmc): a necklace and bead structure at low concentration of SDS, a rodlike prolate ellipsoidal aggregate model, and a flexible capped helical cylindrical model.[23,38]

Because we wanted to observe minute changes in binding between protein and surfactant as a function of concentration, we employed EPR spectroscopy as a technique known for its sensitivity. In each case investigated, the effect of SDS or CTAB at 10^{-3} M, 10^{-2} M, and 10^{-1} M concentration on the EPR spectra of CAT16 in albumin solutions was examined. In the case of SDS, a 10^{-3} M concentration is below the cmc,[40−42] and the EPR parameters of the CAT16 spectrum were not significantly affected. There was no change in the nitrogen hyperfine splitting (a_N value), and the rotational correlation time increased slightly, possibly as a result of the interaction of the cationic spin probe with the negative charge of the dodecyl sulfate anion. As the concentration of SDS increased, the EPR spectra showed line broadening due to the restricted motion of the spin probe and a decrease in a_N (0.62 G) in SDS micelles at 10^{-2} M and 10^{-1} M concentrations.

Literature data[43,44] indicate that BSA has up to 70 sites capable of binding SDS, of which some 10−11 bind more strongly than the others. In the presence of SDS (10^{-3} M) in BSA solutions of 2 and 10 mg/mL, the EPR spectra of CAT16 show only one component (Figure 4). Possibly, these sites can

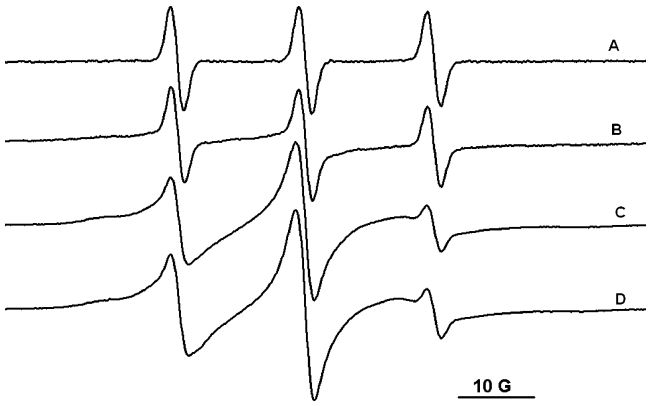

Figure 4. EPR spectra of CAT16 in solutions containing 10^{-3} M SDS and BSA at (A) 2, (B) 10, (C) 20, and (D) 40 mg/mL.

interact with other molecules through hydrophobic forces. As SDS and CAT16 bear a hydrophobic tail and an ionic group in their structures, they compete for similar protein binding sites. At 10^{-3} M concentration, however, SDS is in large excess compared to BSA or CAT16, and in view of the fact that SDS has a high affinity for protein binding sites, formation of a protein−CAT16 complex is restricted. As the concentration of BSA increased to 20 or 40 mg/mL, the EPR spectra of CAT16 showed a multicomponent feature. Beyond the two components observed in Figure 2 in solutions of BSA (20 or 40 mg/mL), in this case, the appearance of another component with a high degree of immobilization can be observed. This is somewhat surprising, but can be explained by the formation of some protein aggregates following conformational changes induced by the surfactant.

When the concentration of surfactant was 10^{-2} M, SDS micelles were present in the solution interacting with CAT16. As a result, the EPR spectrum of CAT16 in 10^{-2} M SDS reveals a restricted motion compared to the almost isotropic motion in water or in 10^{-3} M SDS solution. The EPR spectrum shape did not change significantly when the SDS concentration increased to 10^{-1} M. The rotational correlation time τ was found to be 14.48×10^{-10} s for a solution of 10^{-2} M and 16.95×10^{-10} s for a solution of 10^{-1} M. The distribution of CAT16 among different microenvironments in SDS/BSA solutions depends on the molar ratio between protein and surfactant. Hence, potential processes arising when SDS micelles labeled with CAT16 interact with BSA include the following: (1) Some SDS can bind to binding sites with strong affinity for SDS molecules, leading to dissolution of the micelles and release of the spin probe in solution. (2) SDS micelles can form complexes with BSA, with CAT16 located in the micelle. The molar ratio between BSA and SDS influences the (a) distribution of the spin probe between the protein and SDS micelles and (b) consequences on the EPR spectra of CAT16 in the corresponding solutions. Figure 5 shows the EPR spectra of

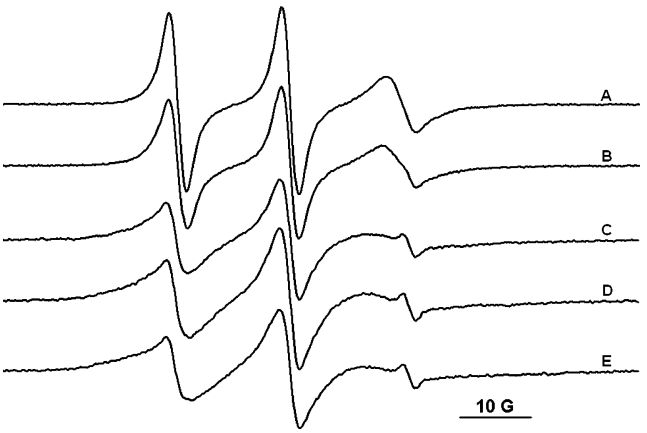

Figure 5. EPR spectra of CAT16 in solutions containing (A) 10^{-2} M SDS and BSA at (B) 2, (C) 10, (D) 20, and (E) 40 mg/mL.

CAT16 in solutions of BSA and SDS at 10^{-2} M. At the lowest concentration of BSA (2 mg/mL), it can be observed that the shape of the EPR spectrum was the sum of two components, showing that CAT16 was distributed between the SDS micelles and the solvent. The EPR spectrum was broader because some of the SDS micelles containing CAT16 were in a complex with the protein. The presence of free CAT16 in solution is probably due to the release from micelles after some of them were adsorbed on the hydrophobic region of the protein. Upon increasing the BSA concentration, the EPR spectra of CAT16 became similar to those observed in the absence of SDS. However, the observed lines were much broader as CAT16 was "attached" to the SDS micelles that formed the complex with the protein.

At the highest concentration of SDS (10^{-1} M) used in this study, the EPR spectra of CAT16 suggest that CAT16 was located in the SDS micelles, even in the presence of BSA. As the protein concentration in solution increased, only a change in the rotational correlation time of CAT16 was noticed, without being accompanied by a change in the dynamic range of the spin probe (Figure 3 of the Supporting Information). This behavior is due to a higher concentration of micelles than BSA and probably to the saturation of the protein surface with

SDS micelles. The increasing values of τ with the concentration of albumin (Table 2) are the result of the increased viscosity of

Table 2. τ Values for CAT16 in a Solution Containing 10^{-2} M β-CD and BSA

[BSA] mg/mL	τ
0	2.75
2	2.81
10	3.29
20	3.52
40	4.01

the solution. The EPR spectrum of CAT16 in solutions of BSA (40 mg/mL) and SDS (10^{-1} M) is the sum of two components, suggesting that, at this concentration, the SDS/BSA ratio is under the saturation limit.

Using the cationic surfactant CTAB, a behavior similar to that of CAT16 in a solution containing protein and SDS was observed. The affinity of CAT16 for CTAB micelles is weaker than that for the SDS micelles. As circular dichroism measurements suggest, CTAB induces changes in protein structure at a lower concentration than SDS. The EPR spectra of CAT16 in solutions containing CTAB and BSA provide information on the spin probe distribution between different environments based on its affinity toward them. Compared to the case of CAT16−SDS systems, CAT16 interacts more weakly with CTAB micelles, and the EPR spectra of CAT16 in CTAB solutions show evidence for a two-component feature. When BSA is present in solution, CAT16 interacts with protein sites rather than with CTAB micelles. As in the case of SDS, at the lowest concentration of CTAB in albumin solution, a third component appears in the EPR spectrum with an immobilization of the spin probe. By increasing the concentration of CTAB, it appears that the aggregation process is prevented, in a fashion similar to that of SDS. In contrast to SDS, however, saturation of binding sites is not reached even in solutions containing 20 mg/mL. The complex between the protein and CTAB micelles is observed in EPR spectra at a high concentration of albumin. As the free component of CAT16 is present in all EPR spectra of the BSA−CTAB system, it is suggested that the sites on the protein surface responsible for binding positively charged ligands are occupied (Figure 4 of the Supporting Information).

Effect of β-CD on CAT16/BSA and CAT16/Surfactant Systems. The ability of cyclodextrins to prevent or slow a protein aggregation process or otherwise inhibit the interaction between a protein and hydrophobic molecules has already been reported.[13,45] Cyclodextrins are capable of complexing hydrophobic moieties, which might be present in some cases at the surface of a protein. In our previous studies on the radical scavenging activity of BSA and HSA, a strong interaction between β-CD and albumins was not revealed.[39,40]

In the present study, the systems investigated are complex and offer multiple possibilities for host−guest complexation. The hydrophobic chain of the CAT16 molecule is a good candidate for the cyclodextrin cavity, and EPR measurements can easily demonstrate such a process. The τ value corresponding to CAT16 increases in buffer solutions of 10^{-2} M β-CD (pH 7) from 1.23×10^{-10} to 2.75×10^{-10} s. The increase of the τ value is the result of spin probe complexation by the β-CD cavity. The binding constant between β-CD and CAT16 is 5×10^2 M^{-1} and was calculated from the variation of τ values with the concentration of cyclodextrin (Figure 5 of the Supporting Information). In BSA solutions, in the presence of β-CD (10^{-2} M), the EPR spectra of CAT16 reveal only an isotropic motion, independent of the protein concentration. No significant changes were observed in the EPR parameters, as the increase of the τ value (Table 2) with the concentration of BSA was attributed to the altered solution viscosity. This result shows that the association between β-CD and CAT16 inhibits the interaction between the latter molecule and BSA, when cyclodextrin is in large excess compared to albumin. The distribution of CAT16 between the complexes with albumin and β-CD is determined by the albumin/β-CD concentration ratio and the binding constants of the complexes. Figure 6

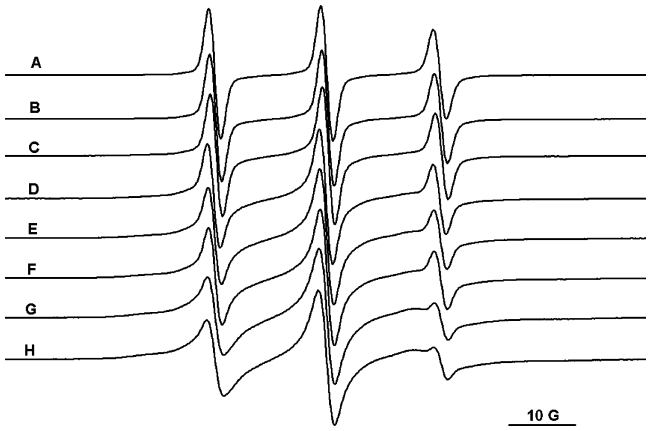

Figure 6. EPR spectra of CAT16 in BSA (20 mg/mL) and β-CD at (A) 10^{-2}, (B) 6×10^{-3}, (C) 3×10^{-3}, (D) 10^{-3}, (E) 6×10^{-4}, (F) 5×10^{-5}, (G) 10^{-5}, and (H) 0.

shows the EPR spectra of CAT16 in BSA (20 mg/mL) at various molar concentrations of β-CD. As can be seen, only at β-CD concentrations higher than 3×10^{-3} M is CAT16 completely removed from the albumin complex (Figure 6A−C). The value of the binding constant for the BSA/CAT16 complex is 1.31×10^3 M^{-1}. This was calculated by considering the binding constant for the β-CD/CAT16 complex and the proportion between the components of the EPR spectrum of CAT16 in a solution of BSA (20 mg/mL) and β-CD (10^{-3} M). SDS or CTAB molecules compete with CAT16 for the β-CD cavity. The EPR spectra of CAT16 in surfactant/β-CD solutions depend on the concentration of the surfactant (Figure 6 of the Supporting Information). At 10^{-3} M surfactant concentration, the EPR spectrum of CAT16 is the same as that observed in a solution of β-CD. When both cyclodextrin and surfactant concentrations are the same, the spectra exhibit a two-component feature due to the distribution of CAT16 between the cyclodextrin cavities and the surfactant micelles. The EPR spectrum corresponding to a surfactant (10^{-1} M)/β-CD (10^{-2} M) mixture indicates the presence of the spin probe in a micelle environment in each case.

EPR Spectra of CAT16 in BSA, Surfactant, and β-CD Solutions. The results in the previous section show that cyclodextrin can strip the CAT16 spin probe from the complex with the protein and the surfactant. In this section, the behavior of CAT16 in the ternary BSA/surfactant/β-CD system is described. At low concentration of SDS or CTAB, the EPR spectra of CAT16 in ternary systems show an isotropic motion corresponding to its inclusion complex with β-CD, whereas in the case of a high concentration of surfactant (10^{-1} M), the

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CAT16 spin probe gives rise to spectra similar to those in corresponding surfactant solutions or mixtures of surfactant (10^{-1} M) with BSA. In the case of systems containing surfactant and β-CD at the same concentration, 10^{-2} M, the distribution of the spin probe among different environments is reflected in the shape of the EPR spectrum. To this end, the spectra of CAT16 in solutions containing BSA at a concentration of 20 mg/mL, SDS, and β-CD are shown in Figure 7. In the presence of 10^{-3} M SDS, β-CD (10^{-2} M) is

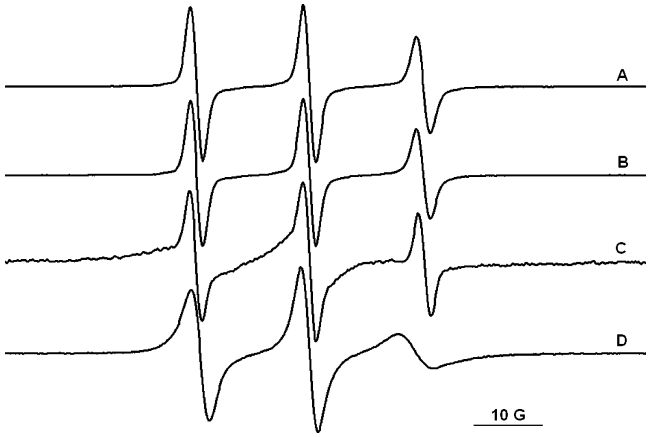

Figure 7. EPR spectra of CAT16 in (A) BSA (20 mg/mL)/β-CD (10^{-2} M) and ternary BSA (20 mg/mL)/SDS/β-CD (10^{-2} M) systems at (B) 10^{-3}, (C) 10^{-2}, and (D) 10^{-1} M SDS.

able to extract the spin probe from the protein complex completely. As a result, the EPR spectrum corresponds to a single species, the complex CAT16/β-CD. As the concentration of SDS rises to 10^{-2} M, what is observed is that CAT16 is not completely removed from the complex with the protein. In this case, CAT16 is distributed between the protein complex and β-CD (or solvent). However, in the presence of β-CD, the proportion of CAT16 bound to the protein decreases compared to the case of a solution containing surfactant/BSA. At 10^{-1} M concentration of SDS, β-CD does not affect the surfactant micelles and its complex with protein. In conclusion, the experiments suggest that β-CD can efficiently strip out the hydrophobic molecules bound to the protein surface only if it is present in a large excess.

Because the CTAB molecules and CAT16 have similar structures, with a hydrophobic tail attached to a positive charge, they compete for the same sites on the protein complex. When the concentrations of β-CD and CTAB were equal, the proportion of the spin probe/β-CD complex in the ternary system increased compared to similar systems containing 10^{-2} M SDS (Figure 7 of the Supporting Information). Probably, the specific binding sites for SDS and CTAB on the BSA structure, involving mainly electrostatic interactions, are not the same. EPR data obtained for solutions containing β-CD (10^{-2} M) and CTAB (10^{-2} M) at various concentrations of BSA suggest that the spin probe can be easily removed from the protein complex compared to data emerging from experiments involving SDS/β-CD mixtures. For example, in the case of a series of solutions with BSA (20 mg/mL)/CTAB/β-CD, CAT16 was complexed with β-CD, and only in the case of a 10^{-1} M CTAB was it located specifically in surfactant micelles (see the Supporting Information).

Even when β-CD was added to the already-formed protein/CAT16/surfactant system or CAT16 was added after the ternary system β-CD/BSA/surfactant, the EPR spectra were the same.

Effect of Thermal Denaturation on Solutions Containing BSA. Role of Surfactants and Cyclodextrins. Changes in albumin conformation as a function of temperature were studied using physicochemical methods, including FTIR spectroscopy,[46,47] circular dichroism,[48] and differential scanning calorimetry (DSC).[49] Thermal denaturation of BSA induces reversible changes in the secondary structure below 50 °C and only partial reversible changes of the α-helix in the range of 50−75 °C.[48] Above 60 °C, unfolding of the α-helix progresses, favoring formation of a β-sheet conformation, which induces gel formation in the protein solution. In this respect, we were interested in finding out (a) if the EPR spectra of CAT16 can provide information on structural changes in the protein structure induced by thermal treatment at 70 °C and (b) what the influence is of surfactants and/or cyclodextrins in protein solutions. As was mentioned above, EPR measurements at room temperature demonstrated that, at low concentrations of surfactant (independent of its nature), CAT16 spectra exhibit a component with a high degree of immobilization. Heat-induced aggregation of BSA at 70 °C might affect the affinity of the protein for the spin probe or change the dynamic regime due to the complex with protein aggregates. Indeed, as a general behavior, following thermal treatment of BSA solutions, the EPR spectra of CAT16 indicate the emergence of a third

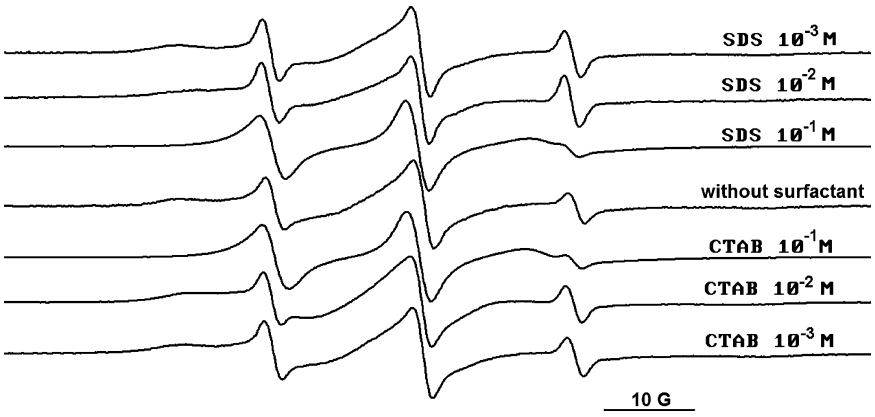

Figure 8. EPR spectra of CAT16 in BSA (40 mg/mL) solutions in the absence and presence of SDS or CTAB following denaturation at 70 °C.

component with a more restricted motion (immobilized) that can be explained by the appearance of protein aggregates.

The concentration of the surfactant in albumin solutions modulates the effect of temperature on protein conformation and, consequently, on its complex with the spin probe. Macroscopically, it was observed that albumin solutions behave differently depending on the concentration of the surfactant. Thus, in the absence of surfactant, an aggregation process occurs irrespective of protein concentration, whereas in the case of solutions containing SDS (or CTAB) at 10^{-3} M concentration, heating induces gel formation. Upon increasing the concentration of surfactant (to 10^{-2} or 10^{-1} M), gel formation or protein aggregation was not visible. The spectral features of the spin probe in BSA solutions in the absence and presence of surfactants, following thermal treatment are shown in Figure 8 for the case of BSA (40 mg/mL). The presence of an immobilized component is noted in all spectra, with the exception of a solution of BSA and surfactant (10^{-1} M). Its pattern is the same in the absence of surfactant or presence of surfactant at 10^{-2} and 10^{-3} M concentrations. The contribution of this component to the overall spectrum decreases in the case of systems containing surfactant at 10^{-2} M concentration. The EPR spectra show that the contribution of the component to the aggregation processes is more important when CTAB is used. This can lead to the conclusion that CTAB is less efficient in preventing aggregation or associative processes involving protein chains compared to SDS. The EPR spectra of CAT16 in systems containing surfactants at 10^{-1} M concentration show no evidence of the existence of protein aggregates following thermal treatment.

To demonstrate the capacity of β-CD to prevent protein aggregation or disrupt aggregates induced by heating, thermal denaturation experiments were carried out adding β-CD before and after thermal treatment. As shown in Figure 9a, the EPR spectrum of CAT16 in a BSA solution of 20 mg/mL, following heating and cooling to room temperature, shows the immobilized component associated with the presence of protein aggregates. Adding β-CD to this system, following thermal treatment, results in the dissolution of protein aggregates. The EPR spectrum (Figure 9b) shows that CAT16 is removed from the protein complex(es) and is present as a complex with β-CD. We observed that, after β-CD had been added, the solution became clear. This demonstrates that, under the experimental conditions, β-CD was able to disrupt protein aggregates. This result appears to be different from those reported in the literature concerning refolding of MM-creatine kinase assisted by SDS and cyclodextrins.[50]

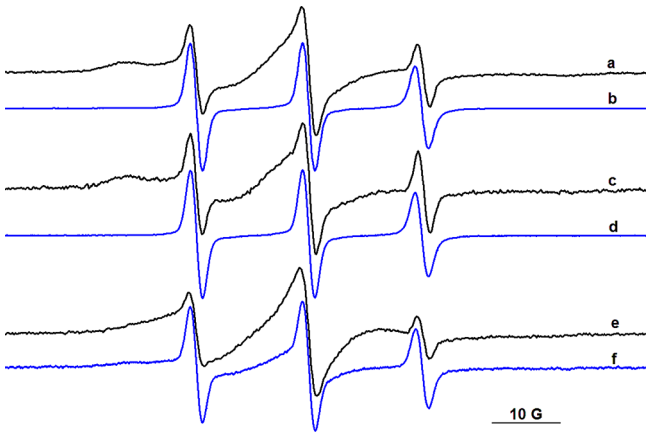

Figure 9. EPR spectra of CAT16 in denaturated BSA solutions at 70 °C: (a) 20 mg/mL BSA; (b) 20 mg/mL BSA and 10^{-2} M β-CD (added after thermal treatment); (c) 20 mg/mL BSA and 10^{-3} M SDS; (d) 20 mg/mL BSA, 10^{-3} M SDS, and 10^{-2} M β-CD (added after thermal treatment); (e) 20 mg/mL BSA and 10^{-3} M SDS; and (f) 20 mg/mL BSA, 10^{-3} M SDS, and 10^{-2} M β-CD (added after thermal treatment).

Following heating of a solution of albumin (20 mg/mL) in the presence of SDS (10^{-3} or 10^{-2} M), the EPR spectrum of CAT16 indicated the presence of an immobilized component attributed to the formation of protein aggregates (Figure 9c or e). Adding β-CD to a solution containing 10^{-3} M SDS after thermal treatment, the EPR spectra showed only that component attributed to the CAT16/β-CD complex (Figure 9d). In the case of a solution containing 10^{-2} M SDS, formation of protein aggregates was not detected by EPR spectroscopy, as the pattern was not affected by thermal treatment. Following addition of β-CD, the EPR spectrum indicated the predominant presence of a CAT16/β-CD inclusion complex and the CAT16/protein complex in solution (Figure 9f). This result demonstrates that β-CD interacts more strongly with SDS molecules than with CAT16, even after thermal treatment of the albumin solution.

CONCLUSIONS

Collectively, the present spectroscopic studies (EPR, circular dichroism) provide new evidence on (a) the interactions between proteins and surfactants and (b) the role of β-CD in the modulation of surfactant−protein interactions. The ability of β-CD to extract hydrophobic molecules from protein complexes has been confirmed by monitoring changes in the EPR spectra of the spin probe CAT16. The EPR spectra revealed that protein aggregation induced by thermal treatment is prevented when surfactants and/or β-CD are present in solutions, but their protective effect is a function of their nature and concentration. β-CD interacts more strongly with the anionic surfactant molecules than the cationic spin probe. Both EPR and circular dichroism measurements demonstrate that the ratio between β-CD and surfactant is important in the recovery of the initial conformation of the protein.

ASSOCIATED CONTENT

Supporting Information

Additional EPR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BSA, bovine serum albumin; CAT16, 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl; CD, cyclodextrin; CTAB, cetyltrimethylammonium bromide; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate

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