ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2017, *62*(8-9), 637-643

Dedicated to the memory of
Professor Victor-Emanuel Sahini (1927–2017)

THE EXTENT OF ALBUMIN DENATURATION INDUCED BY ALIPHATIC ALCOHOLS: AN EPR AND CIRCULAR DICHROISM STUDY

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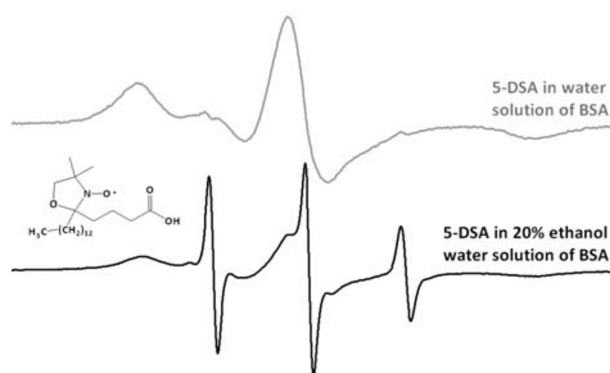
Received December 14, 2016

The aim of this study was to investigate the changes induced by aliphatic alcohols on the conformation and binding properties of bovine serum albumin (BSA), as revealed by the spin probe method of electron paramagnetic resonance (EPR) spectroscopy and by circular dichroism. It is well known that fatty acids and compounds of similar structure bind strongly to albumins. The two spin probes used for EPR measurements, 5-doxyl stearic acid (5-DSA) and 4-(N,N'-dimethyl-N-hexadecyl)ammonium-2,2',6,6'-tetramethylpiperidine-1-oxyl iodide (CAT16), have fatty acid-like structure, and thus show high affinity for BSA. The analysis of their EPR spectra revealed that, in the presence of some alcohols, the spin probes have lower affinity for the protein. This can be the result of either alcohol-induced conformational changes in BSA or competitive binding of alcohols and spin probes. Circular dichroism measurements were used to distinguish between these two mechanisms.



INTRODUCTION

Albumin is a plasma protein playing a major role in the transport of exogenous compounds like drugs and anaesthetics, and endogenous metabolites and hormones.[1] Albumin binds fatty acids strongly,[2,3] and carries them through blood between fat tissues and other tissues or organs.[4] The carrier function of albumin is highly dependent upon the protein conformation. Furthermore, the hydration shell of the protein plays important roles in the protein–ligand interaction and protein dynamics.[5]

Conformational changes of proteins, induced by components of water solutions, can perturb the protein function. The influence of alcohols on the protein conformation has been extensively investigated over the last few decades.[6–14] Some studies indicate that low concentrations of short-chain aliphatic alcohols stabilize the native state of proteins, while high concentrations promote protein unfolding through an increase in the α-helix content.[7,15–17] Such alcohol-induced conformational changes can, in time, lead to metabolic diseases associated to extensive exposure to alcohols.[18] The effect of ethanol is most often taken into account, due to its daily use and presence as metabolite. Nonetheless, several higher alcohols can also be found in

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pharmaceuticals,[19] and thus it is worth to investigate the influence of structural changes induced by alcohol molecules as a function of the length of their alkyl chain.

At molecular level, alcohol molecules can induce changes in the polar solvent structure, due to the ability of their hydroxyl groups to form hydrogen bonds with water molecules from the protein hydration shell. Two mechanisms for protein–alcohol binding have been considered, both of them claiming structural changes in the water network upon binding.[5] The first mechanism considers that alcohol molecules replace the water molecules surrounding the binding sites of the protein, while the second states that water molecules remain at the binding sites and form hydrogen bridges linking the protein and alcohol.[5,20,21] The interference of alcohol molecules in the hydration shell increases the hydrophobicity of the peptide/ solvent interface, which determines the increase of the helical content through an enhancement of intramolecular hydrogen bonding.[16,17]

Our approach to evidence the effect of alcohols on the binding properties of bovine serum albumin (BSA) was to combine EPR spectroscopy with circular dichroism. We have previously reported a series of EPR studies on the interaction of BSA with ionic surfactants (sodium dodecyl sulphate and cetyltrimethylammonium bromide) and β-cyclodextrin or Pluronic block copolymers.[22–24] These results prompted us to extend the use of EPR spectroscopy, which offers local information by analysing the behaviour of spin probes, combined with circular dichroism spectroscopy, known for providing global information on the secondary structure of proteins, in order to get insight into the protein–alcohol interaction. Since albumins are silent EPR species, we introduced two spin probes, 5-doxyl stearic acid (5-DSA) and 4-(N,N'-dimethyl-N-hexadecyl)ammonium-2,2',6,6'-tetramethyl-piperidine-1-oxyl iodide (CAT16) having similar structure to fatty acids (Fig. 1). These probes bind to BSA, which leads to a restricted, anisotropic motion that is reflected in the features of their EPR spectra. The alcohols taken into consideration in this study were members of the linear aliphatic series from methanol to 1-dodecanol; 2-propanol, ethylene glycol and glycerol have also been considered, in order to investigate the effects of chain branching and multiple hydroxyl groups.

RESULTS AND DISCUSSION

EPR spectroscopy

The EPR spectra of CAT16 and 5-DSA in water show a fast motion for both spin probes (Fig. 2, spectra *a1* and *b1* for CAT16 and 5-DSA, respectively). Differently, in BSA aqueous solution, the EPR spectra reveal the existence of two components: a fairly immobilized component in high proportion (95.9% for CAT16 and 99.5% for 5-DSA, as revealed by the simulation of the EPR spectra), which corresponds to the BSA-bound spin probe, and a fast component in low proportion, corresponding to the free spin probe. This is most evident in the case of 5-DSA (Fig. 2, spectrum *b2*). The different binding behaviour of the two spin probes can be explained by their structural particularities. Both probes contain a long alkyl tail, with the paramagnetic moiety placed either as headgroup (CAT16) or embedded near the middle of the chain (5-DSA). In a previous work correlating EPR and molecular docking data,[23] we have shown that CAT16 binds preferentially to solvent-exposed sites from the protein surface, whereas 5-DSA binds within hydrophobic pockets of BSA.

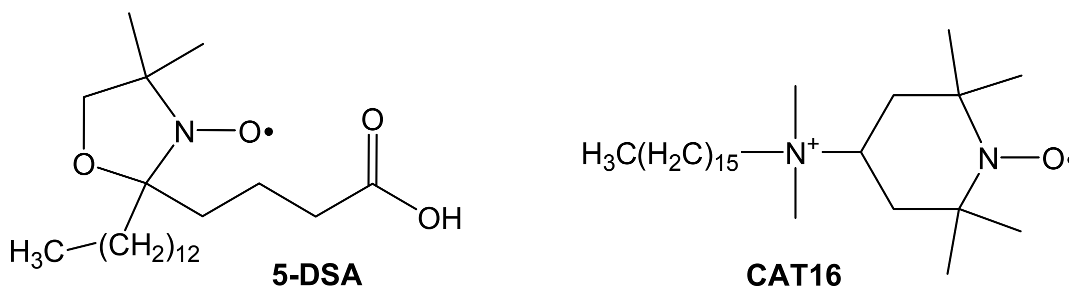

Fig. 1 – Molecular structures of the fatty acid-like spin probes.

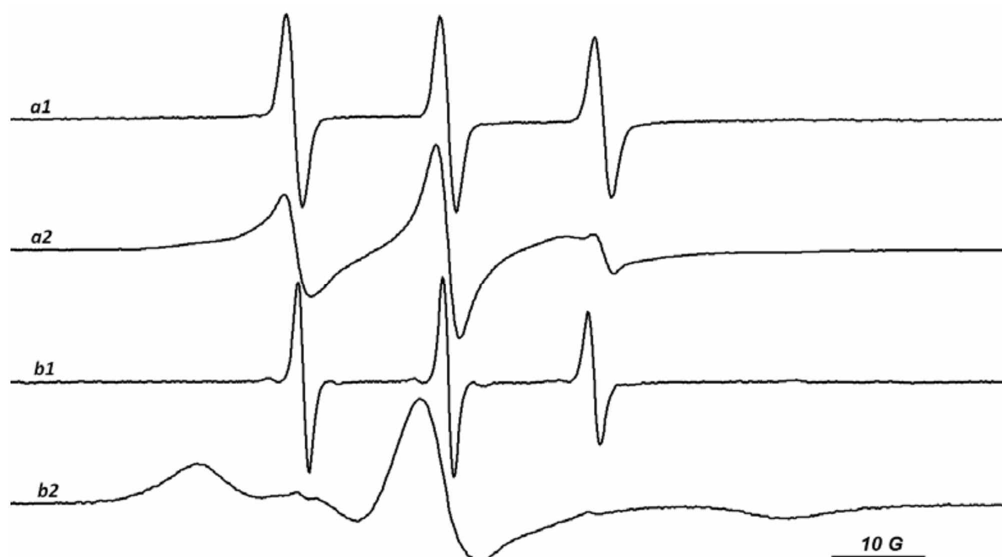

Fig. 2 – The EPR spectra of CAT16 (*a*) and 5-DSA (*b*) in water (*1*) and in aqueous solution of BSA (*2*).

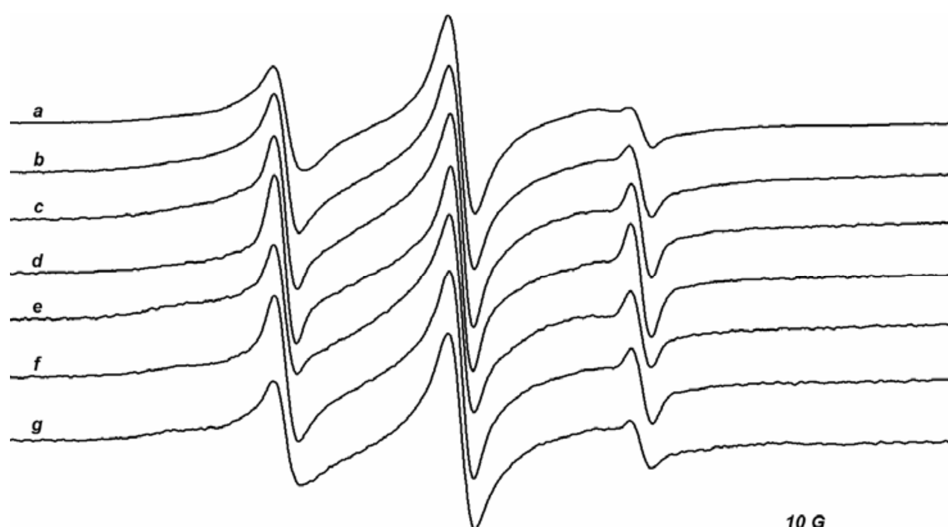

Fig. 3 – The EPR spectra of CAT16 in aqueous solution of BSA, in the absence (*a*) and in the presence of alcohol: 1-butanol 5% (*b*) and 1-pentanol (*c*), 1-hexanol (*d*), 1-octanol (*e*), 1-decanol (*f*), 1-dodecanol (*g*) at saturation.

Based on these distinct spectral features of the spin probes, we reason that EPR measurements can be used to evidence the effect of alcohols on spin probe binding to albumin, by analysing the changes in the spectral parameters and proportion of free and bound probes, as follows.

The EPR spectra of CAT16 in BSA solution are not sensitive to the presence of 5% methanol, ethanol, 1-propanol or 2-propanol. From 1-butanol to 1-decanol, a two- to three-fold increase in the proportion of the fast component is noticed (Fig. 3). This represents proof that, by increasing alcohol hydrophobicity, it is possible to displace CAT16 from the albumin binding sites. In the case of 1-dodecanol, a broadening of the EPR signal is observed, attributed to the protein–spin probe complex. Due to the extremely low solubility of 1-dodecanol in water (see Experimental section), its effect on the protein is small, and thus the ratio between the fast and slow components is similar to that corresponding to BSA in water (Fig. 3, spectra *a* and *g*).

We have reported earlier[23] that, differently from the case of CAT16, 5-DSA binds deeply within the hydrophobic pockets of BSA, and this accounts for why the effect of alcohols in low concentration on the protein cannot be sensed by this spin probe. The spectra of 5-DSA presented in Fig. 4 evidence that the presence of up to 5% alcohol in BSA solution has no effect on the spin probe dynamics. Thus, this is proof that, at 5% concentration or lower, alcohols do not affect the binding of 5-DSA to BSA.

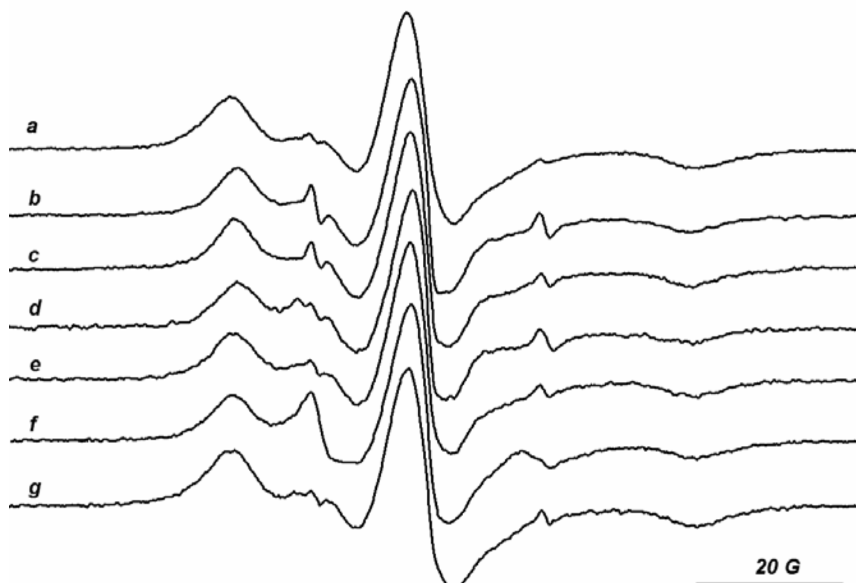

Fig. 4 – The EPR spectra of 5-DSA in aqueous solution of BSA, in the absence (*a*) and in the presence of alcohol: 1-butanol 5% (*b*) and 1-pentanol (*c*), 1-hexanol (*d*), 1-octanol (*e*), 1-decanol (*f*), 1-dodecanol (*g*) at saturation.

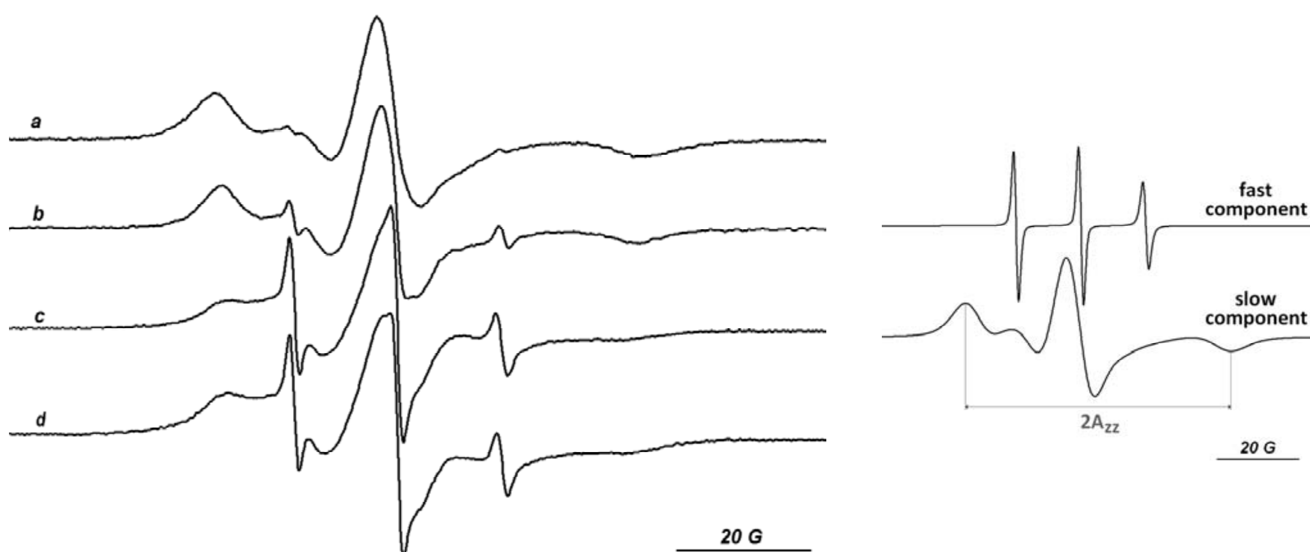

Fig. 5 – The EPR spectra of 5-DSA in aqueous solution of BSA, in the absence (a) and in the presence of 1-butanol 5% (b), 10% (c) and saturated solution (d). Inset: Simulated EPR spectra of the fast and slow components in experimental spectrum (a).

Table 1

The $2A_{zz}$ values for 5-DSA in aqueous solution of BSA, in the absence and in the presence of alcohol 20% (methanol, ethanol, 1-propanol, 2-propanol) or at saturation (1-butanol, 1-pentanol, 1-hexanol, 1-octanol, 1-decanol)

Alcohol	$2A_{zz}$ (G)
Blank	63.97
Methanol	63.36
Ethanol	63.44
1-Propanol	62.68
2-Propanol	62.62
1-Butanol	59.48
1-Pentanol	62.16
1-Hexanol	61.83
1-Octanol	62.40
1-Decanol	63.37

Table 2

Rotational diffusion rates (R_{prp}, R_{pll}, R_{bar}) of CAT16 and 5-DSA in aqueous solution of BSA, in the absence and in the presence of 20% ethylene glycol or 20% glycerol

Alcohol	5-DSA			CAT16		
	Slow component		Fast component	Slow component		Fast component
	R_{prp}	R_{pll}	R_{bar}	R_{prp}	R_{pll}	R_{bar}
Blank	7.4	6.1	9.0	7.9	8.0	9.4
Ethylene glycol	6.5	6.3	9.0	7.7	8.0	9.4
Glycerol	6.5	6.6	9.0	7.7	8.0	9.4

By increasing the alcohol concentration up to 20% for miscible aliphatic alcohols, it was noticed that the ratio between the fast component, attributed to the free spin probe, and the slow component, attributed to the bound probe, significantly increased for both CAT16 and 5-DSA. The EPR spectra of 5-DSA in BSA aqueous solution in the absence and in the presence of 1-butanol are depicted in Fig. 5A in order to evidence the effect of the alcohol concentration on the spin probe–albumin interaction. The proportion of fast component increases from 0.5% in aqueous solution of BSA to 4.5% in aqueous solution of BSA saturated in 1-butanol. In this case, it can be concluded that higher concentrations of alcohol affect not only the hydration shell of the protein and the conformation of the binding site, but also displace the spin probes from their complexes in a significant proportion. High concentrations of alcohol determine an increase in the hydrophobicity of the microenvironment surrounding the spin probe, as revealed by the decrease in the value of the A_{zz} hyperfine tensor component, as shown in Table 1 ($2A_{zz}$ is the distance between the outer peaks in the EPR spectrum, and is depicted in the inset of Fig. 5).

In what follows, we were interested in determining whether the EPR spectra are sensitive to the presence of alcohols possessing multiple hydroxyl groups. For this purpose, we performed EPR measurements in the presence of a diol (ethylene glycol) and a triol (glycerol). These two polyols are often used as additives in water solutions for conducting studies at low temperatures, since they prevent ice formation during cooling. The EPR spectra recorded at room temperature for 5-DSA and CAT16 in the presence of 20% ethylene glycol or 20% glycerol were similar with those recorded in their absence. This observation is in line with other studies that have investigated the effect of glycerol, which state that this triol does not alter the protein structure.[25] The EPR parameters of the spin probes in BSA aqueous solution, obtained by simulating the EPR spectra in the absence and in the presence of polyols, are given in Table 2.

Circular dichroism

A spectroscopic method complementary to EPR is circular dichroism, which can reveal global changes occurring in the secondary structure of albumins upon alcohol addition. The shape of the BSA spectrum in the far-UV region (Fig. 6A) is typical to that of a predominantly helical protein, with two negative minima at 209 and 222 nm.[26] In the case of our BSA–alcohol systems, little to no modification of the protein conformation was recorded in the presence of alcohols. Namely, alcohols at 5% concentration had a negligible effect on the secondary structure of BSA. The α-helix content increased slightly from 60% in native BSA to 62% at 20% alcohol. A more pronounced increase in the helical content, up to 66%, was recorded in the presence of 20% glycerol (Fig. 6B). This increase in helicity occurred on the expense of both the β-sheet and random coil contributions.

Literature data support the stabilizing effect of alcohols on the helical structure of proteins.[27] At a fixed alcohol concentration, this effect was shown to increase with the alcohol chain length, degree of branching and presence of halogen atoms.[28] It was explained by the weakening of long-range hydrophobic interactions (responsible for stabilizing the native tertiary structure) and the strengthening of local polar interactions like hydrogen bonds (stabilizing the secondary structure).[29] The resulting molecular rearrangement leads to the stabilization of long, rod-like helical segments in which the hydrophobic side chains are exposed while the peptide backbone is shielded from the solvent.[30] This explains why denaturation by alcohols leads to proteins rich in α-helices, as opposed to most types of denaturation that disrupt the helical structure.[27] These structural changes may lead to the binding of alcohol molecules to hydrophobic binding sites on the protein, with or without displacement of bound endogeneous ligands,[31] or to replacing the hydration water by alcohol clusters.[29]

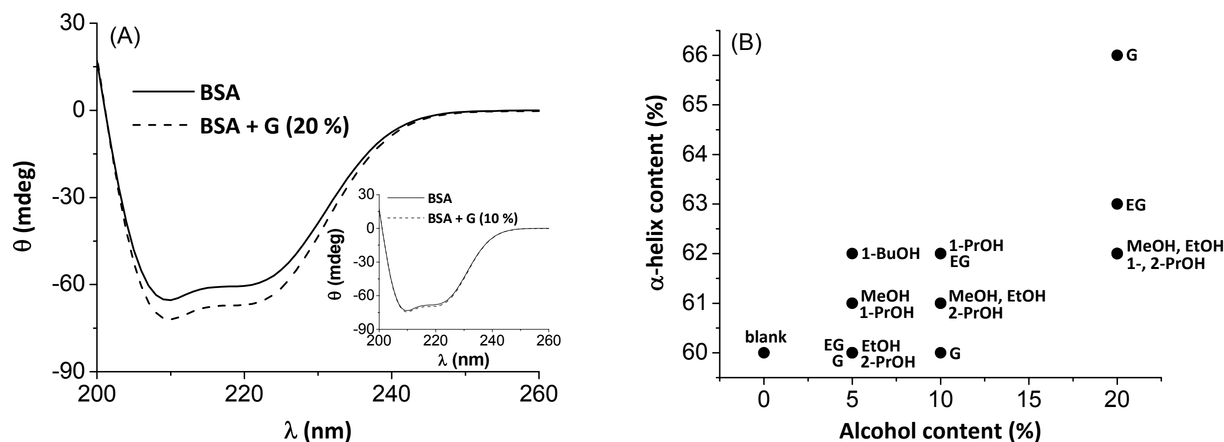

Fig. 6 – (A) Circular dichroism spectra of BSA in the presence of 20% or 10% (inset) glycerol. (B) The α-helix content of BSA determined in the absence and in the presence of different alcohol concentrations. Abbreviations: methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), 1-butanol (1-BuOH), ethylene glycol (EG), glycerol (G).

EXPERIMENTAL

Materials

Fatty acid free BSA and 5-DSA were purchased from Sigma Aldrich. CAT16 was acquired from Molecular Probes. Methanol, ethanol, 1-propanol and 2-propanol were purchased from Chimopar, while the series from 1-butanol to 1-dodecanol was from Merck. Ethylene glycol and glycerol were purchased from Alfa Aesar.

Sample preparation and instruments

EPR measurements

BSA was dissolved in double distilled water or water/alcohol solution to reach the concentration of 20 mg/mL. Alcohol concentrations were 5%, 10% and 20% (w/w) for the water miscible alcohols (methanol, ethanol, 1-propanol, 2-propanol, ethylene glycol and glycerol). Saturated aqueous solutions were employed for the other alcohols, taking into account their miscibility with water at 20°C:[32] 7.7% 1-butanol, 2.2% 1-pentanol, 0.59% 1-hexanol, 0.05% 1-octanol, 0.004% 1-decanol and 0.0004% 1-dodecanol. For each continuous wave-EPR measurement, an appropriate volume of 10^{-2} M ethanolic solution of 5-DSA or CAT16 was added into a vial followed by solvent evaporation under a stream of inert gas. Then, the solid spin probe was redissolved in appropriate volumes of BSA solution, under stirring. The final concentration of each spin probe in these solutions was 2×10^{-4} M. The EPR spectra were recorded on a JEOL FA 100 spectrometer at 20°C using the following parameters: frequency modulation of 100 kHz, microwave power of 0.998 mW, sweep time of 480 s, modulation amplitude of 1 G, time constant of 0.3 s, and a magnetic field scan range of 150 G. In simulating the slow-motion spectra showing two-component features, the program developed by Budil *et al.*,[33] based on non-linear least–squares NLLS fits, was used.

Circular dichroism measurements

Solutions of 1 mg/ml BSA in water and water/alcohol similar to those used for EPR measurements were employed. Circular dichroism spectra were recorded on a Jasco J-815 CD spectropolarimeter at 20°C, in 0.05 cm path length cuvettes, at 4 s response time, 100 nm/min scan speed, 1 nm bandwidth and 1000 milidegrees (mdeg) sensitivity. The signal characteristic for the secondary structure of BSA, expressed as ellipticity (in mdeg), was recorded in the wavelength range 200–260 nm, averaged over three scans and corrected by subtraction of the blank. The α-helix, β-sheet and random coil contributions were determined using the DichroWeb server[34–36] and K2D deconvolution algorithm.[37] All fits yielded NRMSD values lower than 0.14.

CONCLUSIONS

The results obtained through EPR and circular dichroism measurements led to the conclusion that alcohols influence the binding of spin probes to albumin. This is revealed by the EPR spectra, which show an increase in the proportion of the component corresponding to the free spin probe, characterized by fast dynamics. This effect was best observed for the spin probe–albumin systems at a concentration of miscible alcohol of 20%. Differently, the EPR spectra of 5-DSA and CAT16 in BSA solution were not influenced by the presence of glycerol or ethylene glycol. This result is of importance and finds immediate applicability for EPR investigations conducted in water solutions of proteins at low temperature. In such cases, adding glycerol (or, alternatively, ethylene glycol) prevents ice formation during the freezing procedure without altering the spin probe binding to the protein. The circular dichroism measurements have shown that the α-helix content of BSA is increased at high concentration of glycerol (20%). Contrarily, the α-helix content is not significantly influenced by the presence of miscible, monohydroxy alcohols irrespective of their concentration. This result suggests that the alcohol interaction with the protein involves

mainly the hydration shell, in which the alcohol molecules interfere.

Acknowledgement: This work was supported by a grant from the Roumanian National Authority for Scientific Research, CNCS–UEFISCDI, project number PN-II-ID-PCE-2011-3-0328.

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