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Evidence of changes in hydrophilic/hydrophobic balance and in chemical activity of HSA induced by thermal treatments

Research Article

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Abstract: Samples of human serum albumin (HSA) obtained as a result of heat denaturation followed by refolding controlled by a cooling of the protein solution were studied by several methods: chromatographic measurements, kinetic of the reaction with a water soluble free radical and by electron paramagnetic resonance (EPR) spectroscopy. In this context the interaction of this protein with β-cyclodextrin (β-CD) and sodium dodecyl sulfate (SDS) was also investigated. Reversed phase thin layer chromatography (RP-TLC) showed changes in lipophylicity of HSA, which are related with the existence of different ensembles of conformers. The UV-Vis absorption spectra had shown the broadening of absorption band of the protein and a hyperchrom effect in the presence of SDS; β-CD reduces the effect of SDS on protein UV-Vis spectra.

Kinetic measurements related to the reaction of HSA with a water soluble DPPH type free radical provided evidence that reactivity of the HSA denaturated conformers is higher compared with the natural conformer. The affinity of SDS to the albumins surface and the effect of β-CD on the SDS/protein aggregates were also evident by changes in the EPR spectra of the spin probe CAT16.

Keywords: *Human serum albumin • Thermal denaturation • Cyclodextrin • Sodium dodecyl sulphate • DPPH radical*

1. Introduction

Scientific interest in the structural changes in proteins arises from various aspects connected with these changes: understanding of the protein folding, relationship between various conformations and biologic activity, and functionality of these materials (as therapeutic agents, catalysts or materials) [1,2]. The protein solutions represent ensembles of conformers, and the equilibrium between them can be perturbed by changing various physical or chemical factors (temperature, pH, inorganic salts, organic solvents, detergents, pressure) [3-5]. An important number of studies regarding the dependence of protein conformation on temperature are present in the literature, and this is rationalized by the fact that different kind of interactions between amino acids residues are temperature dependent [2,6]. Albumins were used extensively as a protein model for folding and binding studies considering their biological role. For example, Human Serum Albumin (HSA), the most abundant plasma protein, plays various functions (bind, store, and transport a variety of drugs, ligands with different hydrophobic/hydrophilic balance, maintain osmotic pressure), and is involved in coagulation and thrombosis processes [7,8]. In some studies, the association between conformational changes of HSA due to the temperature effect with some neurodegenerative, hepatic and nephritic diseases are mentioned [9,10].

The purpose of this work is to analyze the effect of thermal denaturation (at 70ºC and in some cases at 40ºC) induced on hydrophobic/hydrophilic balance at a protein surface and on protein reactivity with a persistent radical (Fig. 1), for some HSA solutions. Although among the physico-chemical methods, fluorescence and circular dichroism techniques are often used for studying conformational changes of proteins in

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solutions, our experiments refer to the reversed phase – thin layer chromatography (RP-TLC), UV-Vis and EPR measurements. While the RP-TLC method allows for the possibility to evaluate the overall hydrophobic property of protein molecule, EPR measurements lead to information about local changes in the microenvironment polarity around paramagnetic center. EPR and UV-VIS spectra were recorded for solutions of HSA containing β-cyclodextrin (β-CD), sodium dodecyl sulfate (SDS) and mixture β-CD/SDS, respectively. A study of the effect of β-CD and/or SDS, superposed to the thermal effect is justified by the application of these compounds in protein technologies. The system SDS/β-CD was used in the sequential refolding method for proteins [5,11], while SDS is a detergent used on a large scale for purification and characterization of proteins [12,13]. Due to their hydrophobic cavity, CDs form inclusion complexes in aqueous solutions with small organic molecules or part of macromolecules and can be used as protective or carrier agents for various compounds [14-16]. Particularly, studies on CDs /protein systems were earlier reported as CDs may serve as carriers for some peptides, proteins and oligonucleotides [17], increasing their stability [18-20]. Also, CDs can act as protective agent by enhancing protein refolding from denaturated or even aggregated states [21-25].

2. Experimental Procedure

2.1. Materials

HSA was purchased from Fluka and used as supplied, without further purification. TLC plates (0.2 mm layers of silica gel 60) were purchased from Merck. Solvents (chloroform, ethanol, and acetone, all from Chimopar, Romania) and paraffin oil (Aldrich) were used as received. β-CD, SDS were purchased from Aldrich. The free radical 2-(p-phenylsulphonic acid)-2-phenyl-1-picrylhydrazyl ($NaSO_3DPPH^{\cdot}$, Fig. 1a) was prepared by method described in literature [26,27]. Spin probe CAT16 was obtained from Molecular Probes (Fig. 1). Buffer solutions were purchased from Merck.

2.2. Instruments

UV-Vis spectra where recorded on Lamda 35 spectrometer (Perkin-Elmer) at room temperature with a 0.2×1×4 cm quartz cell in a range from 250 to 325 nm.

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

2.3. Sample preparation

2.3.1. Albumin solutions

HSA solutions were prepared at room temperature by dissolving the protein in distilled water at a concentration of 10 mg mL^{-1}. This solution (labelled HSA1) was used as a reference for the denaturated samples. Two HSA samples were prepared by heating an aqueous solution to 70ºC (HSA2 and HSA3) and two additional samples were heated to 40ºC (HSA4.1 and HSA4.2), for 15 min to induce denaturation, followed by different cooling procedures, in order to eventually obtain different conformers ensembles of the protein. The procedures followed were reported in literature [28]. HSA2 and HSA4.1 were obtained after a slow cooling with an approximate rate of 1ºC min^{-1}, while in case of HSA3 or HSA4.2 the cooling was fast. Rapid cooling was achieved by the quick transfer of heated protein sample to a large bath water solvent at 5ºC.

2.3.2. EPR measurements

For EPR measurements, a stock solution of CAT16 10^{-2} M was prepared in ethanol. To prepare samples for EPR measurements, in each case, an appropriate volume of ethanol solution was evaporated from a vial. After that, 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. Simulations of EPR spectra were carried out using the Winsim program.

2.3.3. Preparation of TLC plates

RP-TLC plates were impregnated by overnight predevelopment with 95:5 (V/V) chloroform-paraffin oil. After an application of the albumin samples (natural and denaturated at 70ºC) to TLC plates, then these were developed with buffer solutions at pH 4, 7, 12, or with various mixture of acetone/*i*-propanole/water; in the case of samples denaturated at 40ºC, the eluent with a buffer solution, pH 7. In each case two independent parallel determinations were performed.

2.4. Kinetic measurements

Kinetic measurements were made in an aqueous solution at 293 K under conditions of excess of each albumin sample over the free radical. The concentration of β-CD was 10^{-2} M. The kinetics was followed by monitoring the decreasing of solution absorption at 520 nm which is due to the presence of $NaSO_3DPPH\bullet$; at this wavelength the HSA, β-CD and reaction products do not absorb. UV-Vis measurements were carried out for two half lives, and

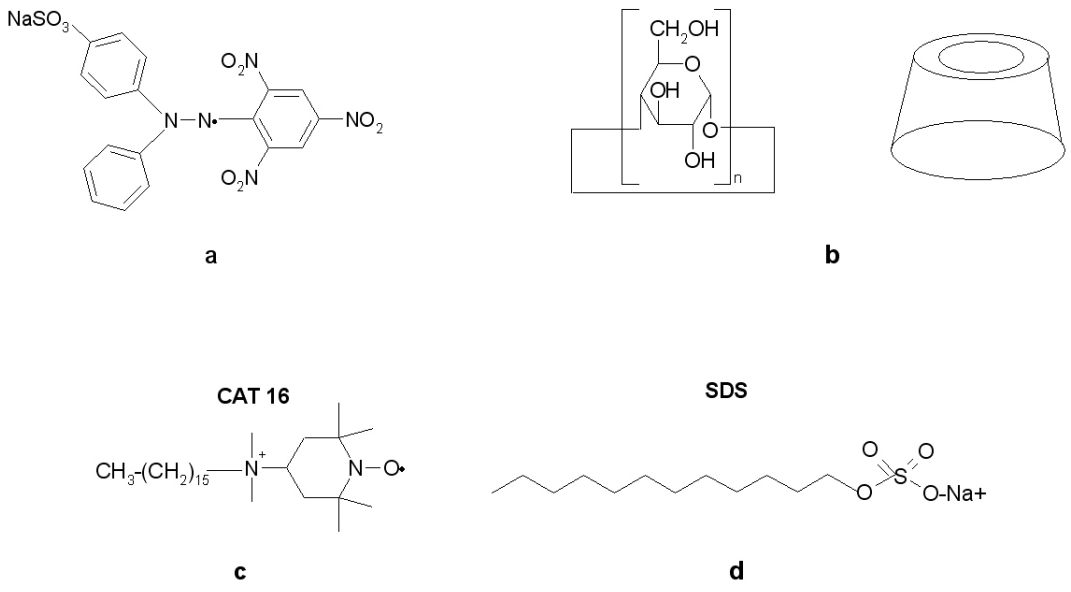

Figure 1. (a) Structure of the free radical, (b) structure of β-CD and schematic representation of a CD, (c) structure of spin probe CAT16, and (d) structure of SDS

the rate constants were evaluated from linear plots of logarithm of absorbance against time.

3. Results and Discussion

Protein refolding depends upon the folding pathway and intermediates and for this reason in the performed experiments, HSA solutions were denaturated by heating at two different temperatures, which affect in a different ways the interactions between amino acid residues and as a result the protein conformations.

According to the literature data, heating of a protein solution at 70ºC leads to a more advanced denaturation. The refolding process might be slower and the resulted ensembles conformers are significantly different from their natural state.

In the second series of experiments albumin solutions were heated at 40ºC and maintained for 15 minutes at this temperature. In some pathologic states this temperature value can be reached by human bodies. If some changes in physico-chemical properties in albumin solutions can be evidenced at this temperature we can intuitively assume that febrile episodes can affect in time also the biological protein functions.

3.1. Reverse phase thin layer chromatography

The parameter used to characterise the behaviour of a compound in TLC experiment is the retention factor (R_f) which represents the distance from the origin to the centre of the separated zone divided by the distance from the origin to the solvent front [29,30]. Another parameter, derived from R_f and used to characterise the hydrophilic/hydrophobic balance of a compound is the molecular lipophylicity (R_M), defined by the Eq. 1:

$$R_M = \log\left(\frac{1}{R_f} - 1\right) \quad (1)$$

This equation is often used in RP-TLC in order to evaluate the lipophilic character of organic compounds with biological activities. The significance of this value for albumin samples will give information about changes of the hydrophilic character of the protein surface, due to the reorientation of amino acids residues induced by thermal treatment.

In Fig. 2, the molecular lipophylicities of HSA1, HSA2 and HSA3 samples are presented as a function of eluent (water) pH. The results are presented in Fig. 2 and correspond to the chromatographic system silica gel impregnated with paraffin oil/water (buffer).

It can be noticed that for samples HSA1 and HSA2, the R_M variations with pH are similar, while in case of HSA3 the R_M values are constant. This result suggests that the hydrophilic/hydrophobic balance of protein conformers differ considerably from sample HSA2 to HSA3. Sample HSA3 was obtained using a “shock” thermal procedure leaving probably the hydrophobic residues of amino acids from protein chain oriented toward the solvent; the hydrophobic effect thus become more important, as R_M does not change with the pH. In contrast, a slow cooling rate, like in case of HSA2 sample, favours the protein chain returning to the

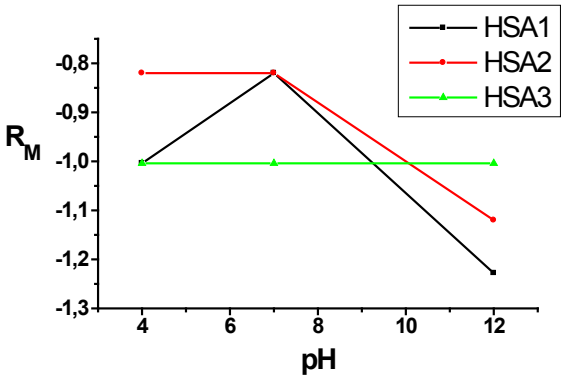

Figure 2. R_M dependence of HSA 1, HSA2, HSA 3 with pH.

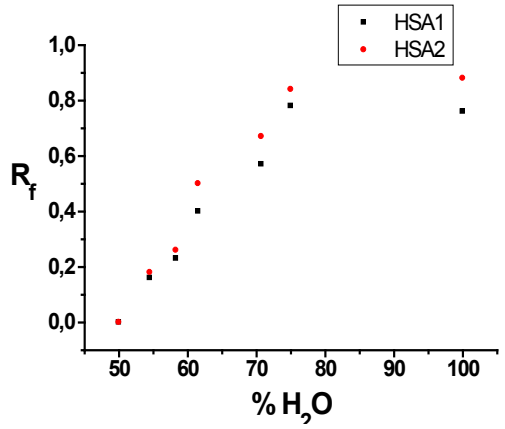

Figure 3. R_f values for albumin samples HSA1 and HSA3 corresponding to chromatographic systems silica gel impregnated with paraffin oil; eluent: water/acetone/2-propanol.

conformation close to the initial one. Samples HSA1 and HSA2 represent conformational ensembles in which functional groups sensitive to pH are more exposed to the solvent.

Sensitivity to changes of the eluent polarity for albumin samples HSA1-3 was evidenced in the TLC experiments using as an eluent a water/acetone/*i*-propanol mixture. The ratio between organic solvents was kept constant, while the ratio between water and organic solvent was varied. The R_f values of natural albumin (HSA1) and the denaturated one (HSA3) as a function of water content in eluent are shown in Fig. 3. Differences in the chromatographic behaviour of HSA1 and HSA3 were noticed for the chromatographic systems with water content in eluent mixture higher than 60%.

For samples of albumin denaturated at 40ºC, small differences were also noticed in R_f values of the system silica gel impregnated with paraffin oil/water, suggesting that conformers are slightly different related to hydrophobicity. For instance, in case of HSA1, the R_f was 0.78, while for HAS4.2 it was 0.72. Smaller values of R_f, in case of HSA42, indicates a more hydrophobic surface.

The differences between the chromatographic parameters corresponding to these HSA samples are small, but all chromatographic experiments revealed that proteins exhibits a more hydrophobic surface after thermal denaturation, sustaining the model of defolded conformers in denaturated states, with some hydrophobic amino acids residues exposed to the solvent.

3.2. Kinetic of HSA oxidation with a water soluble DPPH radical

In our previous papers we reported results regarding oxidation of amino acids and bovine serum albumin (BSA) with the free radical $NaSO_3DPPH^{\cdot}$, in the absence and in the presence of CDs [18,31,33]. In addition, the thermal denaturation effect on BSA reactivity with this radical had been reported [32]. Those studies [18,31,32] revealed that β-CD protects amino acids or BSA against oxidation by $NaSO_3DPPH\bullet$ (Fig. 1a) and showed that the structure of amino acid also influences the rate of oxidation. Oxidation of aromatic amino acids by $NaSO_3DPPH\bullet$ was sensibly faster compared to one of the aliphatic amino acids [18,31].

In this study we investigated in a similar way, the reaction of the radical $NaSO_3DPPH\bullet$ with various HSA samples. In the structure of HSA there are present a total number of 66 aromatic amino-acids [7], and we can assume that their residues might have an important contribution to the overall reaction with $NaSO_3DPPH\bullet$. The constant rates corresponding to the oxidation of HSA by $NaSO_3DPPH\bullet$ in the absence and in the presence of β-CD (at a total concentration of 10^{-2} M) are shown in Table 1. In the absence of HSA, the radical itself decomposed slowly following a first order kinetic pathway. Therefore, the disappearance of the radical $NaSO_3DPPH\bullet$ in the presence of HSA is, in fact, a result of two parallel processes: 1) reaction with water (in the absence or in the presence of β-CD) – characterized by a constant rate k^w, respectively $k^{w'}$ and 2) reaction with protein - characterized by a constant rate k. The experimental constant rate (k^{exp}), determined for each sample of HSA, is a sum of these constants (Eq. 2):

$$k^{exp} = k^w + k \qquad (2)$$

The rate constant observed for the radical decomposition in water (k^w) is 1×10^3 min^{-1}, while in the presence of β-CD 0.24×10^3 min^{-1} ($k^{w'}$). These results can

Table 1. Rate constants corresponding to reaction of radical with HSA samples in the absence (k) and in the presence of β-CD (k_β)

Sample ($\times 10^3$ min^{-1})	HSA1	HSA2	HSA3	HSA41	HSA42
k	3.21	2.62	4.37	2.19	2.51
k_β	2.65	3.04	2.90	1.68	1.78

be related to a protection of the radical by complexation with β-CD. The values of rate constant, *k*, were obtained taking into account Eq. 2.

The protective role of β-CD against the action of $NaSO_3DPPH\bullet$ is enhanced in case of the thermally treated protein solutions. This observation can be explained by the existence of conformers in which aromatic residues can deeply interact with the β-CD molecules, due to a more exposure of those residues to the protein surface. Values of rate constants corresponding to samples denaturated at 40ºC are close to those obtained for samples heated at 70ºC and cooled slowly; it can be assumed that reactive functional groups oriented at protein surface are nearly the same for conformations of samples HSA2 or HSA4.1 and HSA4.2. It can also be noted that a fast cooling procedure leaves the protein in conformations which differ significantly from the initial one, even if protein solutions were heated at 70ºC or 40ºC. The effect of β-CD is more evident in case of protein solutions cooled faster, HSA3 and HSA4.2.

3.3. UV measurements for HSA samples

The interactions of HSA with β-CD and / or SDS have been studied by UV-measurements. A UV spectrum of HSA in solution shows an absorption band with a maximum around 280 nm due to the presence of the aromatic residues from amino acids chain. Our measurements show that UV spectra are not sensitive to heating/ cooling procedures. In the presence of β-CD (10^{-2} M) or/ and SDS (10^{-2} M) small differences were noticed between HSA1-3 samples in UV absorption spectra. In Fig. 4 show the UV-Vis spectra of HSA2 in the absence and in the presence of β-CD and/or SDS. The presence of β-CD (10^{-2} M) does not induce changes in the spectra of HSA1, but for samples HSA2 (Fig. 5) and HSA3 (Fig. 6), a bathochromic effect on protein absorption was noticed. Most probably, the interaction between albumin and β-CD takes place by the complexation of the hydrophobic residues from the amino acids chain. In natural conformation (as we assume for sample HSA1) the hydrophobic groups (including those responsible for absorption maxima at 280 nm) are oriented toward the interior of molecule and cannot interact properly with CD. After thermal treatment of albumin samples,

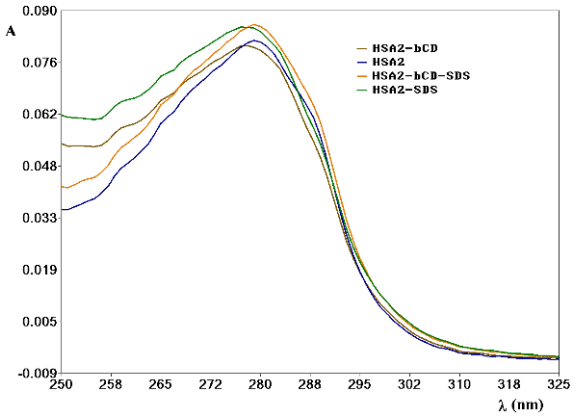

Figure 4. UV spectra of HSA2 in the presence of β-CD and/or SDS UV spectra of HSA1 (native conformer), in the presence of β-CD and/or SDS

hydrophobic residues are more exposed to the solvent and to interaction with β-CD.

In all cases, the presence of SDS leads to a broadening of absorption band of the protein and to a hyperchrom effect, as amphyphilic molecules of detergent change the polarity of environment around protein molecules and interact by hydrophobic and ionic forces with protein. In the presence of β-CD, the complex protein/SDS micelle is disturbed.

For all three HSA samples containing SDS a hypsocrom effect was noticed after adding β-CD. In all cases the maximum absorption shifts toward the initial value. From literature data it is known that CDs increases the critical micelle concentration of surfactants [34]. The changes observed in UV spectra of HSA in the presence of β-CD and/or SDS sustains a strong interaction between protein and surfactant which is perturbed by adding the CDs. These results also suggest that the presence of β-CD in 10^{-2} M concentration is not enough to strip the surfactant away from the complex with protein. Similar results were observed from EPR measurements using the spin probe method.

3.4. EPR measurements

EPR spectroscopy is a method suitable to provide information about changes in the environment around paramagnetic centers, molecular dynamic, structures of paramagnetic compounds [35]. The aim of an EPR investigation with the HSA samples was to find out whether by this method it is feasible to monitor processes taking place in the protein solutions (such denaturation or changes in a more complex systems containing β-CD/protein/surfactant). EPR spectroscopy has been used for investigations of albumins interactions with various surfactants or only with doxyl type spin probes [36-39]. There are a wide range of other possible

candidates to be used as spin probes in such systems, probably most well known being the TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl) derivatives. We found that the ionic spin probes from CAT family (4-N,N-dimethyl-alkyl-ammonium-2,2,6,6-tetramethyl-piperidine-1-oxyl iodide) are better reporters for such systems. Among them, spin probe CAT16 (Fig. 1c) was more sensitive to interactions with β-CD, SDS and/or albumin, as this spin probe can interact by hydrophobic forces (due to the alkyl chain) and by electrostatic forces (being a quaternary ammonium salt). The EPR parameters: hyperfine splitting constant, a_N (Fig. 5) reporting on the polarity around spin paramagnetic moiety and rotational correlation time, τ which reflect changes of dynamics spin probe [40] have been used in this study for a qualitative analysises of changes in solutions containing HSA, β-CD and/or SDS .

In the Fig. 5 spectra of CAT 16 in water (a) and in the following solutions: β-CD 10^{-2} M (b), SDS 10^{-2} M (c) and β-CD/SDS (d) are presented. In the presence of β-CD the mobility of spin probe decreases comparative with water, due to the formation of inclusion complexes between CAT16 and β-CD. EPR spectrum of CAT16 shows a decrease in the a_N value (0.13 G) in the presence of β-CD compared with the spectrum in water, suggesting that the nitroxide group cannot penetrate deep into CDs cavity and the complexation occurs more probably from the side of alkyl chain of the spin probe. The degree of complexation is reflected also by an increase of τ value from 1.46×10^{-10} s in water to 3×10^{-10} s in solution of β-CD 10^{-2} M. This behavior of CAT 16 in the presence of β-CD is similar to other amphyphilic spin probes [41].

In the solution of 10^{-2} M SDS (Fig. 5c), CAT16 is incorporated in the surfactant micelles and this fact resulted in a decrease of a_N value (from 16.85 G in water to 16.25 G in SDS solution) and a slow dynamic ($\tau = 10.2\times10^{-10}$ s). The spectrum of CAT16 in the SDS/β-CD mixture (Fig. 5d) exhibits two components and comparatively with the other spectra from Fig. 5, the lines are much broader. The presence of β-CD in solution of SDS leads to a change in the organization of the micelles. In this system β-CD can influence both SDS and CAT16. If β-CD can complex some SDS molecules in the micelle, which also incorporate CAT16, then the rotation of this system becomes much slower and will be evident by the changes in the EPR parameters. The two components of CAT16 spectrum in β-CD/SDS solution (Fig. 5d) are attributed to the free species in solution (more mobile) and to the species interacting with the complex SDS/β-CD. Simulated spectrum for CAT16 in β-CD/SDS is presented as a thin line under the experimental one (Fig. 5d)

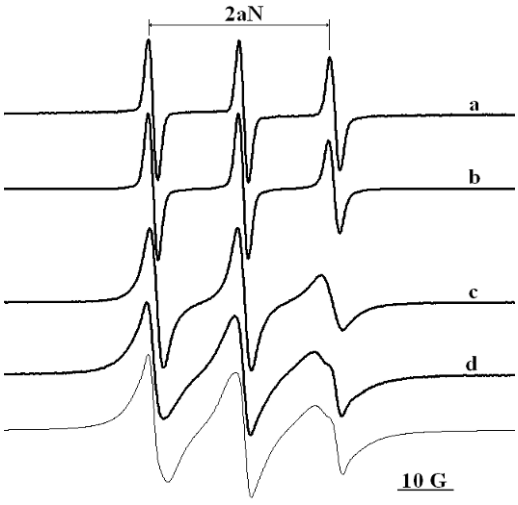

Figure 5. EPR spectra of (a) CAT16 in water, (b) β-CD (10^{-2} M), (c) SDS (10^{-2} M), (d) β-CD/SDS mixture (10^{-2} M each) and simulated one.

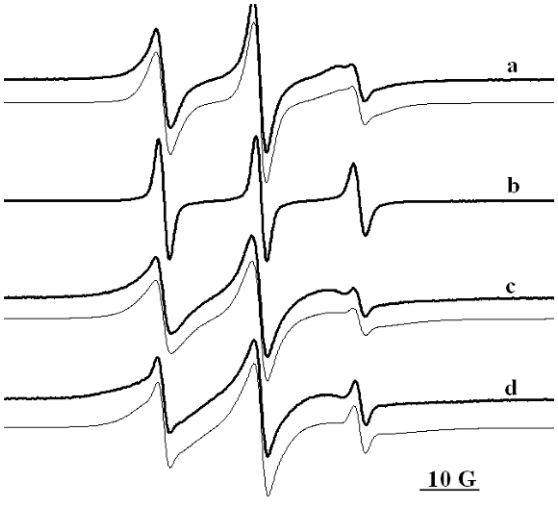

Figure 6. EPR spectra of CAT16 in solutions of (a) HSA1, (b) HSA1+β-CD, (c) HSA1+SDS, (d) HSA1 +β-CD/SDS mixture. Simulated spectra are shown as thin lines bellow the experimental ones.

Fig. 6 shows the experimental spectra of CAT16 in solution containing HSA in natural conformation (HSA1), in the absence or in the presence of SDS and β-CD and the simulated ones for those representing a sum of two components. A spectrum of CAT16 in the HSA1 sample (Fig. 6a) presents two components – one corresponding to the free species in solvent and the other one corresponding to the complex of protein with spin probe. The tumbling rate of the CAT16 in the complex with protein is significantly lower compared with free spin probe, as EPR lines are broader and their intensities vary. In the presence of SDS, protein molecules form a complex with surfactant micelles. In this case spin probe CAT16 is distributed between protein/SDS micelles

complex and the solvent. The EPR spectrum for CAT16 in protein/SDS complex reflects a much slower dynamic comparatively with protein complex, and this is evident by an increase in line broadening. In the solutions of HSA1 and β-CD (10^{-2} M) (Fig. 6b), CAT16 shows a spectrum with EPR parameters similar with those observed in the solution of β-CD, suggesting that the spin probe has a higher affinity for cyclodextrin compared to the protein. On the other hand, this is proof that β-CD can strip out the other molecules from the protein surface interacting by hydrophobic forces. In a solution of HSA1/β-CD/SDS (Fig. 6d), the spin probe is distributed between SDS/protein complex and the solvent or complex with β-CD. The spectra presented in Fig. 6 and the results of simulation suggest a stronger interaction of CAT16 with SDS/protein complex comparatively with protein only. The simulated spectra shows that the percentage of the component attributed to the complexes containing protein varies in the order: SDS/HSA1 > HSA1 > SDS/β-CD/HSA1. The concentrations of CAT16 and HSA are comparable (10^{-4} M and 1.6×10^{-4} M respectively), and it can be assumed that the broadening of the EPR spectra is not due to a spin-spin exchange interaction between CAT16 molecules interacting with the same protein chain. However, the effect of line broadening observed in EPR spectra presented in Figs. 6a, 6c and 6d might be due not only to the complexes formed in solution characterized by a slow motion, but also to the adsorbed oxygen in aqueous solutions containing SDS and/or HSA.

Similar EPR spectra of CAT 16 in solutions of HSA2 or HAS3 were also recorded. These measurements did not show a noticeable difference between the denaturated samples and HSA1. Only a slight increase of the free component spectra of CAT16 in case of denaturated samples was evident.

From the data described above it can be concluded that the EPR measurements do not reveal significant differences in the properties of HSA thermal denaturated sample compared with natural one (HSA1), but it is suitable to study interactions in the complex systems of protein, surfactant and CDs.

4. Conclusions

In this study we analyzed changes induced in some physico-chemical properties of HSA, like hydrophilic/hydrophobic balance at the molecule interface with solvent or protein reactivity, as a result of different thermal treatments on protein solution. Using RP-TLC, UV-Vis and EPR methods, small differences were evidenced between HSA samples. After thermal treatment, protein surface became more hydrophobic and some reactive groups became more exposed to the environment. The interaction of the protein with surfactants or cyclodextrin is evident and can be monitored by the spin probe method of EPR spectroscopy. The surfactant/protein interaction studied by EPR offers more possibilities that need to be explored. An extensive study on this topic can be connected with the practical application on protein purification using surfactants.

Acknowledgements

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