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PARAMAGNETIC REPORTERS FOR ALBUMIN/Zn(II) INTERACTION

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In this work we investigated the interaction between albumin and zinc-Zn(II) using indirect measurements on a DPPH water soluble derivative decay in the presence of albumin and from analysing of EPR spectra of CAT16 and 5DSA in albumin solution. We found that in the presence of Zn(II), the DPPH is reduced faster. CAT16 and 5-DSA are spin probes with different hydrophobic/hydrophilic balance which bind different sites of the protein. The spin probe CAT16 has an ionic character and is more sensitive to the presence of other ionic species in a system. The EPR spectra of CAT16 in albumin solution show two components corresponding to the free spin probe and to the complex with albumin. The EPR measurements revealed that the ratio between these two components is changed in the presence of Zn(II).

Key words: BSA, Zn(II), DPPH, EPR spectroscopy, UV-Vis.

INTRODUCTION

In the last decades some studies showed that transitional metal ions like Zn, Fe, Cu are involved in development of neurovegetative diseases (Navarra et al., 2009, 1729; Bush et al., 1994). Binding of these metal ions to protein induces conformational changes of the bio molecules and can be followed by aggregation, especially when the metal ions act as bridges between protein chains. It was also noticed that at certain ratio metal ion/protein, the aggregation process leads to formation of protein gels (Navarra et al., 2009; Navarra et al., 2009, 437). These interactions can be easily monitored by various physicochemical methods like DLS measurements, IR, fluorescence, UV-Vis spectroscopy. A number of studies have been reported about the interaction between Zn (II) and the widely used transport model protein – bovine serum albumin (BSA) (Navarra et al., 2009; Giroux et al., 1981; Ohyoshi et al., 1999). BSA exhibits more than one metal binding sites and the affinity of Zn(II) for the primary site the binding constant, defined by log K, is approximately 7.5 (Navarra et al., 2009; Ohyoshi et al., 1999).

In this work we investigated how the presence of Zn(II) influences the BSA properties such reactivity against a DPPH derivative or ability to bind stable

radicals like 5-DSA and CAT16, using UV-Vis and EPR (electron paramagnetic resonance) measurements.

MATERIALS AND METHODS

Bovine serum albumin – fraction V (BSA) – was purchased from Fluka and used as supplied without further purification. $Zn(NO_3)_2 \cdot 4H_2O$ was obtained from (Merck). Spin probes 5-DSA and CAT 16 were obtained from Molecular Probes (Fig. 1a and b). The free radical 2-(p-phenylsulphonic acid)-2-phenyl-1-picrylhydrazyl ($NaSO_3$DPPH•, Fig. 1c) was prepared by method described in literature (Ionita et al., 2000).

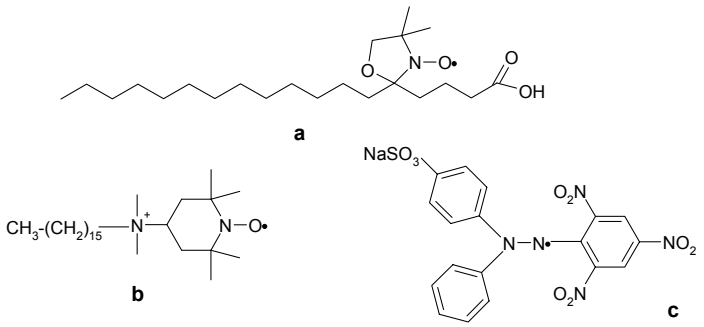


Fig. 1. The paramagnetic species used in the study: a) 5-doxylstearic acid (5-DSA); b) 4-(N, N-dimethyl-N-hexadecyl) ammonium-2, 2, 6, 6-tetramethyl-piperidine-1-oxyliodine (CAT16); c) 2-(p-phenylsulphonic acid)-2-phenyl-1-picrylhydrazyl ($NaSO_3$DPPH•).

UV-Vis spectra were recorded on Lamda 35 spectrometer (Perkin-Elmer) at room temperature in the range 240- 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.

All samples were prepared in phosphate buffer solution at pH 7.4. For UV-Vis measurements were used samples of BSA (2 mg/ml) while concentration of Zn salt was in the range $0\text{-}10^{-3}$ M. For EPR measurements samples were freshly prepared with concentration of BSA varying in the range of 2-40 mg/ml, while concentration of Zn was kept constant at 10^{-3} M. For investigation of the heat effect on BSA samples in the presence and in the absence of Zn salt, in each case, albumin samples were heated for 15 min in a water bath at 40, 50, 60 or 70 ^{0}C. Stock solutions of 10^{-2} M 5-DSA and CAT16 were 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 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. Dynamic parameters of the spin probes CAT16 and 5-DSA were obtained either by simulations of the experimental spectra using the software NLSL developed by Budil et al. (in case of 5DSA), or using the equation (1) in case of CAT16.

$$\tau_c = 6.51\times10^{-10}\Delta H_0\left[\left(\frac{h_0}{h_{-1}}\right)^{1/2} + \left(\frac{h_0}{h_{+1}}\right)^{1/2} - 2\right] \qquad (1)$$

where ΔH_0 is the peak-to-peak width (in Gauss) of the central line, h_{-1}, h_0 and h_{+1} are the heights of the low field, central, and high field lines, respectively (Stones et al.).The kinetics was followed by monitoring the decreasing of solution absorption at 520 nm which is due to the presence of $NaSO_3DPPH$•. UV-Vis measurements were carried out for two half lives, and the rate constants were evaluated from linear plots of logarithm of absorbance against time.

RESULTS AND DISCUSSION

UV measurements for samples of BSA (2 mg/ml) evidenced that position of the absorption band with maximum around 280 nm is not shifted when Zn(II) is present. However, in time was noticed an aggregation processes, depending on the concentration of metal ion, which determine an increase of BSA absorbance around 280 nm.

At concentration of Zn(II) below or equal with that of the protein, the aggregation process is very slow. For this reason, in order to estimate the effect of Zn(II) on the albumin reactivity, the concentration of metal ion was 5×10^{-5} M in case of experiments monitoring the kinetic of $NaSO_3DPPH$• reduction.

Kinetic measurements on the reduction of $NaSO_3DPPH$• in the presence of albumin were made at 293 K, in condition of an excess of each albumin sample over free radical. In some of our previous studies we reported data on the influence of β-cyclodextrin (Ionita et al., 2001) or on the effect of thermal denaturation (Ionita et al., 2004, Sahini et al., 2011) on the reduction of this radical.

In the absence of BSA, the radical itself decomposed slowly following a first order kinetic. The decomposition rate was determined in buffer solution (pH 7.4) and in solution of Zn(II) $5x10^{-5}$ M. The decay of the radical $NaSO_3DPPH$• in the presence of BSA is, in fact, a result of two parallel processes: 1) reaction with water (in the absence or in the presence of Zn(II)) – characterized by a constant rate k^{sol}, respectively $k^{sol'}$ and 2) reaction with protein – characterized by a constant rate k (respectively k_{Zn}). The experimental constant rate (k^{exp}), determined for each sample of BSA, is a sum of these constant (relation 2):

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

It was found that the rate constant corresponding to radical decomposition in water (k^{sol}) is 0.98×10^3 min^{-1}, while in the presence of Zn(II) 1.1×10^3 min^{-1} ($k^{sol''}$). These values have been extracted from variation of ln A in time, were A represent the absorbance of $NaSO_3DPPH\bullet$ at 520 nm. In Fig. 2 is shown the variation of ln A in time for samples containing albumin together with the linear fit. The labels for each symbol correspond to those from Table 1. The experimental values of k^{exp} and the constant rates k and k_{Zn} corresponding to reaction of $NaSO_3DPPH\bullet$ with BSA are shown in Table 1. The reaction was followed for BSA solutions of freshly prepared and for solutions previously heated at 60 ^{0}C, followed by cooling at room temperature.

Table 1

Rate constants corresponding to reaction of radical with BSA samples in the absence (k) and in the presence of Zn(II) (k_{Zn})

Sample	**1** BSA 2mg/ml	**2** BSA 2 mg/ml Zn (II) $5x10^{-5}$ M	**3** BSA 2mg/ml after thermal denaturation	**4** BSA 2 mg/ml Zn (II) $5x10^{-5}$ M after thermal denaturation
$k^{exp}\times10^4$ (min^{-1})	15.7 r = 0.985	-	30.3 r =0.994	
$k\times10^4$ (min^{-1})	5.9		20.5	-
$k^{exp}{}_{Zn}\times10^4$ (min^{-1})		29.4 r =0.996	-	33.7 r =0.985
$K_{Zn}\times10^4$ (min^{-1})	-	18.4		23.7

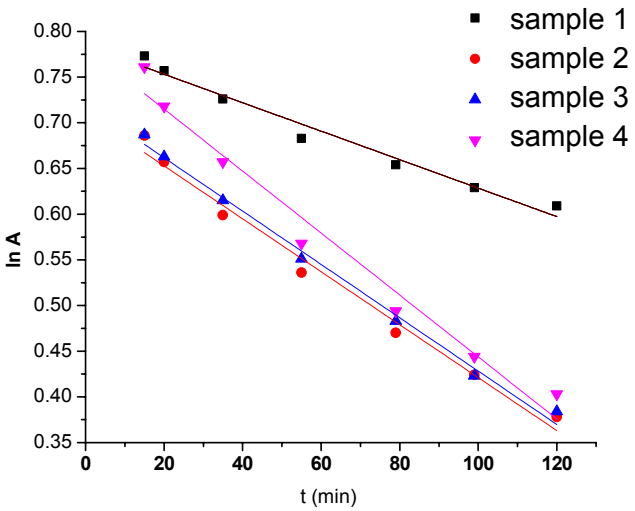
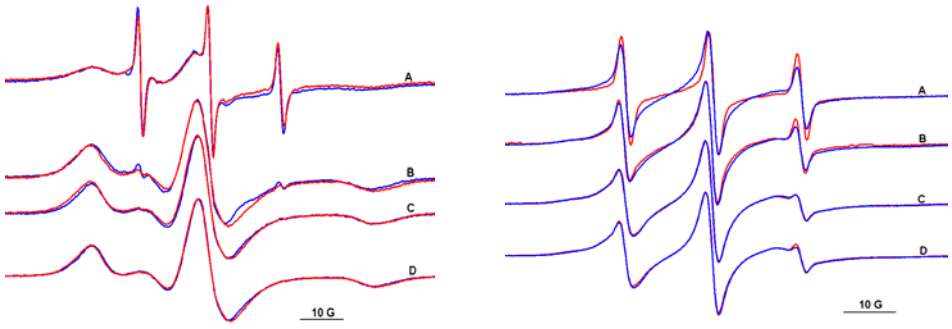


Fig. 2. Variation of $NaSO_3DPPH\bullet$ absorbance (logarithmic scale) in time in samples 1-4 (data shown only for the first 2 hours).

Data presented in Table 1 evidence that both heating the BSA sample and presence of Zn(II) induces an increase of protein reactivity relative to $NaSO_3DPPH•$. These results represent indirect evidence on conformational changes in protein conformation which lead to exposure of reactive residues of amino acids to the radical $NaSO_3DPPH•$. Exposure of BSA to Zn(II) after thermal denaturation (sample 4) does not determine an increasing reactivity of the protein, the binding constant being only slightly bigger compared with sample expose either to Zn (II) (sample 2) or to thermal treatment (sample 3).

The *EPR measurements* were performed in order to analyse if the presence of Zn(II) influences the ability of BSA to bind organic molecules with different hydrophilic/hydrophobic balance. Albumins, including BSA, exhibit a number of binding sites characterised by different affinities for the ligands like fatty acids or compounds with similar structure (like 5-DSA) (Bhattacharya et al., 2000). The EPR spectra of 5-DSA in albumin solution reveals a restricted motion. This has been reported previously (Junk et al., 2010; Muravsky et al., 2009) and was also observed in our experiments, independently on the concentration of albumin (Fig. 3a). In contrary, the EPR spectra of CAT16 in the presence of albumin indicate a motion which can be assigned to the fast regime dynamic (Fig. 3b). The spin probe CAT16 is more hydrophilic than 5DSA and for this reason is possible they do not bind the same sites of BSA.

The EPR spectra of 5DSA or CAT16 in samples of BSA show two components with different dynamics (one for the free sample in solution and the other one corresponding to the complex with the albumin). In this case is possible to extract from spectra simulation the dynamic parameters for each component, assuming that parameters of one component are not influenced by those of the other component.



a- 5-DSA b- CAT16

Fig. 3. a) EPR spectra of 5-DSA and b) EPR spectra of CAT16 in: BSA solution A – 2mg/ml, B – 10 mg/ml, C – 20 mg/ml, D- 40 mg/ml (blue) and BSA + Zn(II) solution – 2mg/ml, B-10 mg/ml, C – 20 mg/ml, D – 40 mg/ml (red).

The presence of Zn(II) in BSA solutions does not induce changes in the spectra of 5DSA. From simulation of the 5DSA spectra were obtained the rotational diffusion rates Rrrp and Rpll (6.8 and 7.7 respectively) which led to a value of rotational correlation time 9.7×10^{-9} s. By contrast, the spin probe CAT16 is more sensitive to the presence of Zn(II), especially at lower concentration of BSA. Deconvolution of spectra presented in Fig. 3b provided the ratio between the fraction of CAT16 bound to the protein and the one of the free spin probe (Table 2). The rotational correlation times for the complex CAT16/BSA and for CAT16 unbound to the protein were 22.1×10^{-10} s and 1.3×10^{-10} s, respectively. These values were calculated applying the equation 1.

Table 2

The proportion of mobile component in EPR spectra of CAT16 in BSA samples; effect of Zn (II)

Sample	2 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml
BSA	32.83	24.39	20.35	5.03
BSA/Zn(II)	86.46	68.61	21.58	5.45

CAT16 is a cationic spin probe and the interaction with BSA has an electrostatic component. Zn(II) binds stronger to BSA compared with CAT16 as the value of logK corresponding to the complex of Zn(II) with BSA reported in literature is around 7.5 (Ohyoshi et al., 1999), while for the complex CAT16/BSA is only 3.1 (Rogozea et al. 2012). Considering the significant difference between the affinities of CAT16 and Zn(II) and data presented in Table 2, we can conclude that their primary binding sites are not the same.

Heating the BSA samples up to 50 ^{0}C, in the absence and in the presence of Zn(II) does not induce changes in the dynamic of CAT16. As temperature rises to 60 ^{0}C or 70 ^{0}C, the EPR spectra corresponding to the complex of CAT16 with BSA indicate the immobilisation of the spin probe (Fig.4).

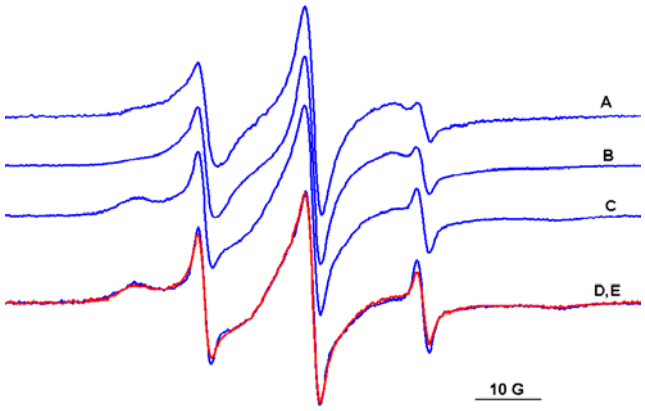


Fig. 4. EPR spectra of CAT16 in solution of BSA 40 mg/ml and Zn (II): at room temperature (A), 40 ^{0}C (B), 60 ^{0}C (C), 70 ^{0}C (D, blue spectrum) and in solution of BSA 40 mg/ml after denaturation at 70 ^{0}C (E, red spectrum).

As temperature rises, the fraction of free CAT16 increases. After heating at 60 ^{0}C or 70 ^{0}C, it was noticed the formation of protein gels in the albumin samples (at concentration higher than 10 mg/ml) and in the presence of Zn(II). In the absence of Zn (II), similar samples of BSA aggregate without to be evidenced a gel state. Nevertheless, a difference between the EPR spectra of CAT16 attributed to the complex in samples denaturated at 70 ^{0}C of BSA (40 mg/ml) in the absence and in the presence of Zn (II) was not observed (Fig. 4 D and E). As temperature of denaturation rises, the fraction of free CAT16 in solution increases slightly from 5.45 to 9.12.

In conclusion, the presence of Zn(II) induces significant changes in the reactivity of BSA, but has little influence on the ability of the protein to bind hydrophobic ligands, like 5-DSA and even CAT16.

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