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A Study of Caffeine Binding to Human Serum Albumin

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Binding of caffeine to human serum albumin (HSA) was investigated with the aim of describing the binding parameters of the interaction. It was found that the results obtained by fluorescence spectroscopy are influenced by the non-negligible artifact, known as the inner filter effect due to the absorption of caffeine at the excitation wavelength (290 nm). Therefore, a suitable correction of the obtained data was performed and the binding constant for caffeine binding to HSA was estimated, revealing low affinity of caffeine for HSA $K_s = (12 \pm 1) \times 10^3$ mol^{-1} dm^3. Further, electron paramagnetic resonance (EPR) spectroscopy, using three different positional isomers of spin labeled stearic acid, doxyl stearates, was applied to study the caffeine-HSA interaction in further detail. It was found that upon caffeine binding, the hyperfine splitting decreases for HSA labeled with 5-doxylstearate. This phenomenon may indicate either an increase in mobility or a local change in polarity sensed by reporter groups upon caffeine binding. These observations may be important for the functional characteristics of HSA.

Keywords
HSA
EPR
fluorescence spectroscopy
caffeine
binding

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in the circulatory system (0.6 mmol dm^{-3}) and one of the most extensively studied proteins. HSA tertiary structure at 2.8 Å resolution reveals a relatively large, heart-shaped molecule.[1] This contains 585 amino acids that are assembled in 3 domains, each consisting of two subdomains. It is a typical polyfunctional protein, with the maintenance of the colloidal osmotic pressure of the plasma[2] and binding of different compounds of internal or external origin being its two most important functions. Subdomains IIA and IIIA form the principal binding regions for most of the HSA ligands. The primary role of HSA is the transport of lipophilic plasma components, especially fatty acids, which bind to different binding sites.[3] No final consensus about the number of fatty acid binding sites has been reached so far. Under normal physiological conditions, 3–4 molecules of fatty acids combine with each HSA molecule (*i.e.*, high affinity, low capacity binding sites). However, as many as 30 fatty acid molecules can be transported by HSA (high capacity and low affinity sites) when the need for fatty acids is extreme.[2] HSA has also important binding capacities for different drugs that are transported through the circulatory system,[4,5] and therefore it has an important impact on drug pharmacokinetics.

Caffeine (Figure 1a) belongs to a class of compounds called methylxanthines. It is an abundant alkaloid of exo-

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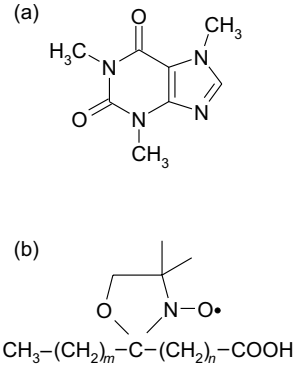

m	*n*	*N*-DSA = (*n*+2)-DSA
12	3	5-DSA
10	5	7-DSA
4	11	13-DSA

Figure 1. Structure of the caffeine molecule (a) and the different spin label positional isomers of the doxylstearic acid (b).

genous origin in human plasma.[6] Stimulation of the central nervous system, cardiac muscle, respiratory system and diuresis are only some of the numerous physiological effects that it exerts.[7,8] Extensive research on caffeine connected to various diseases has not identified any health hazard of normal caffeine consumption.[9,10]

Very few studies of caffeine binding to biological macromolecules have been reported in the literature. Therefore, the aim of our present study was to characterize caffeine binding to HSA using intrinsic fluorescence spectroscopy. There has been an attempt to address the same issue, but the reported data suffer from the non-negligible artifact, described as the inner filter effect.[11] In this study, we performed a suitable correction of the HSA fluorescence intensities and were able to provide an estimation of the binding constant, K_s, describing the caffeine binding to HSA. Further, we present the results of an independent method, electron paramagnetic resonance (EPR) spectroscopy, applied to study the caffeine-HSA interaction combined with three spin labeled stearic acid analogs, *N*-DSA, (Figure 1b) where the distance of doxyl group from carboxylate is varied.

EXPERIMENTAL

Materials and Solutions

HSA was obtained from Sigma, Germany, and caffeine from Kemika, Zagreb. 5-doxylstearic acid (5-DSA), 7-doxylstearic acid (7-DSA) and 13-doxylstearic acid (13-DSA) were prepared according to published procedures.[12] Concentrated solutions (10 mmol dm^{-3}) of three different positional isomers of spin labeled stearic acid, 5-DSA, 7-DSA and 13-DSA, were prepared in ethanol.

For fluorescence spectroscopy measurements, HSA was dissolved in 0.05 mol dm^{-3}, pH = 7.4, phosphate buffer (PB1) with the final concentration of 2.5 μmol dm^{-3}. Caffeine was prepared as 1 mmol dm^{-3} stock solution in the same buffer.

For the EPR measurements, the stock solutions of HSA and caffeine were 1 mmol dm^{-3} and 10 mmol dm^{-3}, respectively, in 0.01 mol dm^{-3}, pH = 7.4, phosphate buffer (PB2).

Fluorescence Spectroscopy Measurements

In order to reinvestigate the data reported in the literature addressing caffeine binding to HSA, fluorescence measurements were designed to follow the experimental conditions used by Gonzalez-Jimenez *et al*. Titration of HSA with caffeine was performed at 298 K and 310 K. Steady state intrinsic fluorescence spectra were recorded using a Varian Cary Eclipse spectrofluorimeter with excitation at 290 nm and the data sampling interval of 0.5 nm using quartz cuvettes (1 cm).

Correction of Fluorescence Spectroscopy Data. – For each HSA sample in the titration experiment with caffeine, absorbances of caffeine at 290 nm (wavelength of fluorescence excitation) and 340 nm (wavelength of the maximum of fluorescence emission) were recorded using a Varian Cary 50 UV-Vis spectrophotometer. Measurements were performed using quartz cuvettes (1 cm).

Fluorescence intensities of HSA samples were corrected for respective absorbances of caffeine at the wavelength of fluorescence excitation (290 nm) according to the following equation:[13]

$$F_c = F * \text{antilog } \{OD_{290} / 2\}, \quad (1)$$

where F and F_c are experimentally measured and corrected fluorescence intensities, respectively, and OD_{290} is the optical density of caffeine at 290 nm. The absorbances of the corresponding HSA samples at the wavelength of maximal intrinsic fluorescence emission (340 nm) are low (< 0.007) and could be neglected (data not shown).

Estimation of the Binding Constant. – Corrected HSA fluorescence intensities, F_c, in titration experiments were plotted *versus* caffeine concentration, and the binding curves for caffeine binding to HSA were obtained. These data were analyzed according to a simple model in which the protein is treated as a monomer, M, and the interactions between the binding sites for the ligand, L, are ignored. The binding constant, K_s, was estimated for the equilibrium M + L $\rightleftharpoons$ ML, by fitting the binding curve to the equation:

$$F_c = F_0 * (K_d / (K_d + [L])) + F_b * ([L] / (K_d + [L])),$$

$$K_s = 1 / K_d \quad (2)$$

where F_0 is the fluorescence intensity in the absence of the ligand, F_b is the fluorescence intensity with the maximal concentration of the ligand, [L] is ligand concentration, and K_d is the dissociation constant of the complex, ML.

Quenching of HSA Fluorescence by Caffeine. – The fluorescence intensity data of HSA samples in the absence/presence of caffeine were processed according to the Stern-Volmer formalism described by the equation:[13]

$$F_0 / F_c = 1 + K_{SV}[L], \quad (3)$$

where K_{SV} is the Stern-Volmer constant.[13]

EPR Spectroscopy Measurements

Prior to spin labeling of HSA, the ethanol solution of DSA was diluted with PB2 so that the final concentration of ethanol in the spin labeled HSA solution did not exceed 5 % (vol. fraction). The amount ratio of spin label : HSA = 1.2 : 1 was prepared with immediate binding of the spin label to the protein.

Caffeine and spin labeled HSA were mixed in 1 : 1 volume ratios. The final concentrations of HSA and the spin labels were 0.24 mmol dm^{-3} and 0.29 mmol dm^{-3}, respectively, and that of caffeine 0.3 and 5 mmol dm^{-3}.

Measurements were performed at 298 K and 310 K in glass capillaries (1 mm inner diameter) on a X-Band Varian E-109 EPR spectrometer at microwave power 10 mW and modulation 0.1 mT. Data acquisition was based on the supplied software.[14]

RESULTS

Fluorescence and Absorption Measurements

The steady state intrinsic fluorescence spectra of free HSA and HSA exposed to 0.1 mmol dm^{-3} caffeine are presented in Figure 2. The observed spectra display a single broad band centered at 340 nm. It can be clearly seen that the presence of caffeine induces a decrease in fluorescence intensity without a shift in the wavelength of the emission maximum. Upon correction due to the absorption of caffeine at 290 nm, the displayed maximal fluorescence intensity of HSA exposed to caffeine is *ca.* 18 % quenched with respect to the HSA fluorescence in the absence of caffeine. However, if not corrected, the spectrum of HSA in the presence of caffeine gives rise to a decrease in the maximal fluorescence intensity of *ca.* 33 %.

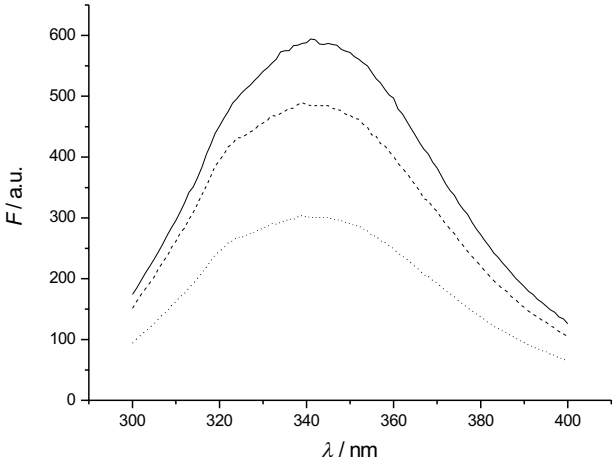

Figure 2. Steady state intrinsic fluorescence spectra of HSA in the presence of caffeine measured at 298 K. Full line denotes the spectrum of HSA (2.5 μmol dm^{-3}) in the absence of caffeine, the dotted line the non-corrected spectrum of HSA (2.5 μmol dm^{-3}) in the presence of 0.1 mmol dm^{-3} caffeine and dashed line the corrected spectrum of HSA (2.5 μmol dm^{-3}) in the presence of 0.1 mmol dm^{-3} caffeine. Fluorescence intensity (F) is expressed in arbitrary units (a.u.).

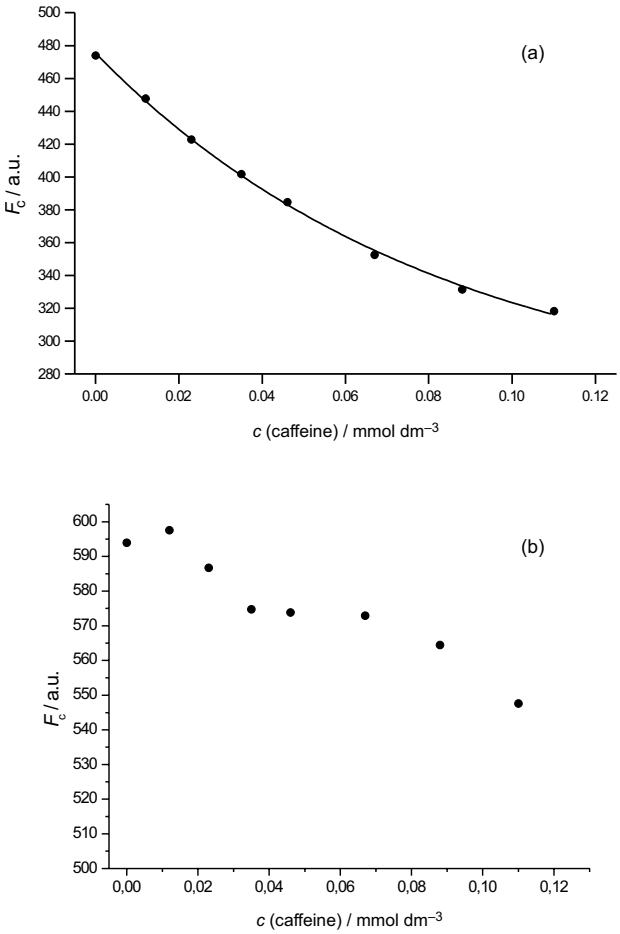

Figure 3. Decrease in the HSA intrinsic fluorescence intensity in the presence of increasing concentrations of caffeine. HSA sample (2.5 μmol dm^{-3}) is titrated with 1 mmol dm^{-3} caffeine at T = 310 K (a) and T = 298 K (b). Solid line denotes the best fit of the experimental data of caffeine binding to HSA. Fluorescence intensity is corrected for the inner filter effect (F_c).

Following the increase of the caffeine concentration in the sample, the sequential increase in the absorbances can be detected at the wavelength used for the excitation of HSA intrinsic fluorescence (290 nm). Corrected fluorescence intensities, F_c, are calculated according to Eq. (1) using these absorbances. By plotting the corrected fluorescence intensities of HSA samples *versus* increasing concentrations of caffeine, the sequential decrease of HSA fluorescence intensity was measured at 310 K (Figure 3a). The presented curve reaches saturation at caffeine con-

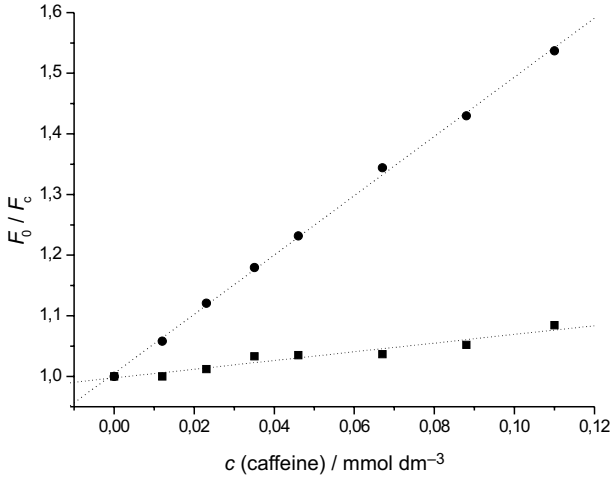

Figure 4. Quenching of HSA intrinsic fluorescence at 340 nm (excitation at 290 nm) with increasing concentrations of caffeine, corrected for the caffeine absorption at 290 nm. Measurements were performed at 298 K (■) and 310 K (●). Fluorescence intensities (F_c) are normalized with respect to fluorescence intensities without the quencher (F_0).

centrations higher than 0.11 mmol dm^{-3}. The binding constant derived from the experimental data using Eq. (2) provided an estimation of $K_s = (12 \pm 1) \times 10^3$ mol^{-1} dm^3. As can be seen in Figure 3b, the decrease in fluorescence intensity upon caffeine addition at 298 K is rather small (9 %) in the same concentration range of the ligand. As a consequence, following the same methodology in modeling, the binding data was hampered. It should be noted that fluorescence intensities are decreased at 310 K with respect to the ones at 298 K.

Having determined the HSA fluorescence quenching by caffeine, the experimental data were analyzed according to the Stern-Volmer formalism described by Eq. (3). Figure 4 presents the Stern-Volmer plot of HSA fluorescence quenching by caffeine derived from the corrected fluorescence intensities. The quenching occurs in a concentration dependent manner at both 298 K and 310 K. Stern-Volmer analysis of the fluorescence quenching data reveals the Stern-Volmer constants, K_{SV} of $(0.72 \pm 0.08) \times 10^{-3}$ mol^{-1} dm^3 at 298 K and $(4.88 \pm 0.07) \times 10^{-3}$ mol^{-1} dm^3 at 310 K.

EPR Measurements

Caffeine binding to HSA was studied by three different doxylstearic acids bearing the paramagnetic doxyl group at C-5 (5-DSA), C-7 (7-DSA) and C-13 (13-DSA) atoms of the fatty acid alkyl chain. At 298 K, no spectral changes were observed and all the presented results refer to the temperature of 310 K.

The EPR spectra of 5-DSA bound to HSA are presented in Figure 5. The observed anisotropic type of spectra can be assigned to the interaction of doxyl fatty acid with HSA, thus experiencing a hindered rotational mobility. In the absence of caffeine (spectrum a), the maximal hyperfine splitting of $2A_{max} = 6.34 \pm 0.03$ mT can be estimated, indicating a strong binding of 5-DSA to HSA. Only one population of 5-DSA is detected. The EPR spectrum of 5-DSA bound to HSA is changed in the presence of 5 mmol dm^3 caffeine (spectrum b). We found that caffeine induces a decrease in hyperfine splitting ($2A_{max} = 6.18 \pm 0.01$ mT, $\Delta = -0.16$ mT). The change of the spectral peak proportions that accompanies the change of the hyperfine splitting amounts to 6 %.

The EPR spectra of HSA labeled with 7-DSA are presented in Figure 6. Two spectral components can easily be noted. In the absence of caffeine (spectrum a), the component with larger hyperfine splitting ($2A_{max} = 6.22 \pm 0.02$ mT) can be assigned to the spin label incorporated

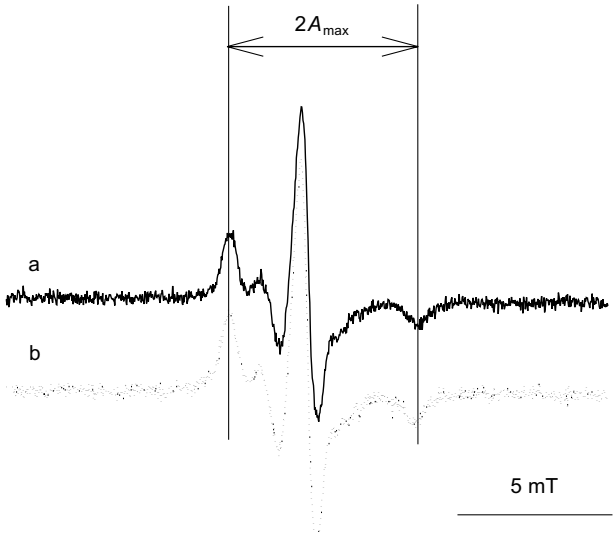

Figure 5. The EPR spectra of HSA (0.24 mmol dm^{-3}) labeled with 5-doxylstearate in the absence (a) and presence (b) of 5 mmol dm^{-3} caffeine measured at 310 K. Maximal hyperfine splittings, $2A_{max}$, are indicated. The ratio of spectral amplitudes between the low field (I_1) and central peak (I_2) is $I_1/I_2 = 0.33$, (a) and $I_1/I_2 = 0.35$, (b).

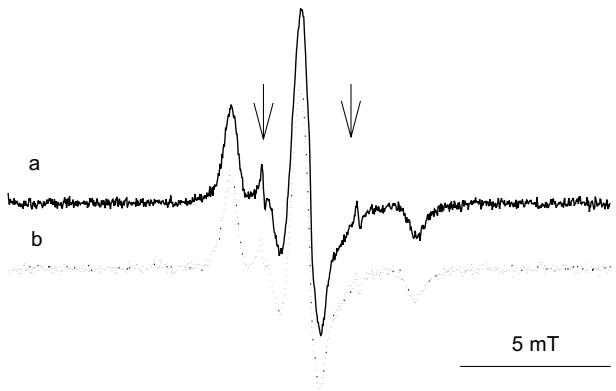

Figure 6. The EPR spectra of HSA labeled with 7-doxylstearate in the absence (a) and presence (b) of 5 mmol dm^{-3} caffeine measured at 310 K. Arrows indicate the spectral component indicative of the free spin label in solution.

Table I. Maximal hyperfine splittings, $2A_{max}$, of different *N*-DSA bound to HSA (0.24 mmol dm^{-3}) in the absence/presence of caffeine measured at 310 K. The significant change of $2A_{max}$ in the presence of caffeine has been deduced for $\Delta(2A_{max}) > 0.1$ mT

DSA	$2A_{max}$(HSA)	$2A_{max}$(HSA+caffeine)
	mT	mT
5-DSA	6.34 ± 0.03	6.18 ± 0.01
7-DSA	6.22 ± 0.02	6.18 ± 0.03
13-DSA	5.99 ± 0.03	5.90 ± 0.02

into the protein binding sites. Smaller hyperfine splitting ($2A_{max}$ = 3.28 ± 0.01 mT), a characteristic of the other spectral component, is indicative of the free spin label in the solution. In the presence of caffeine (spectrum b), we did not observe any significant change in the maximal hyperfine splitting or line shape of 7-DSA bound to HSA. In addition, no change in partitioning between free and bound spin label molecules could be deduced.

When HSA was labeled with 13-DSA, the spectrum of HSA exerted no changes when exposed to caffeine (Table I).

DISCUSSION

The aim of this study was to investigate the binding of caffeine to HSA. Such experiments, based on the application of fluorescence spectroscopy, were previously reported by Gonzalez-Jimenez *et al.*, but suffered from incorrect evaluation of experimental data.

Namely, an increase in the absorbance of the sample at the wavelength of the fluorescence excitation (290 nm) due to caffeine absorption is indicative of the artifact known as the inner filter effect, which should be taken into account and corrected. This effect may decrease the intensity of the exciting light, and being concentration dependent, it can easily masque the real fluorescence quenching of the protein due to the ligand binding. Emphasis should be placed on the fact that the conclusion about caffeine quenching of HSA fluorescence can be made only after the correction of fluorescence intensities is performed in the described manner. Therefore, the HSA fluorescence intensities are corrected for the contribution of caffeine absorption by applying Eq. (1). The corrected data obtained at 310 K verify that HSA fluorescence intensities indeed decrease upon exposure to caffeine. The binding curve reached saturation at caffeine concentrations higher then 0.11 mmol dm^{-3}, which is in the physiological concentration range of caffeine consumers (up to 0.5 mmol dm^{-3}). The best fit of experimental data provided the binding constant, assuming the model of ligand binding to macromolecule with all binding sites equivalent. Considering the caffeine binding to HSA in terms of its binding constant, it could hardly indicate a specific type of binding. Rather, low affinity binding of the caffeine molecule to certain local protein regions might be proposed. Among other ligands of HSA, with their chemical structure similar to caffeine, a wide range of values of binding constants can be found in the literature. For instance, bilirubin, whose transport through the circulation is facilitated by HSA, displays a binding constant of the order of magnitude 10^7 mol^{-1} dm^3.[15] On the other hand, herbicides like atrazine or 2,4-dichlorophenoxyacetic acid bind to HSA with their binding constants in the range from 10^3 to 10^4 mol^{-1} dm^3.[16]

A decrease in the HSA fluorescence intensity upon caffeine addition has also been detected at 298 K, but small spectroscopic changes hampered any further analysis of this set of data. This finding, together with the observation that the maximal fluorescence intensity of HSA in the absence of caffeine at 298 K is higher than the one at 310 K, may indicate that the protein displays different surface properties at these two temperatures. The structure of HSA at 310 K may present certain binding sites that are otherwise hidden at 298 K.

Further, it has been experimentally verified that caffeine quenches HSA fluorescence in a concentration dependant manner at both 298 K and 310 K. Thus, the measured fluorescence intensities of HSA samples in the titration experiments with caffeine were analyzed according to the Stern-Volmer formalism. The linear Stern-Volmer plot is indicative of a single class of fluorophores, all equally accessible to the quencher. According to the estimated Stern-Volmer constants, the rate of quenching is lower at 298 K than at 310 K, implying the dynamic type of HSA fluorescence quenching by caffeine. This conclusion relies on the increased frequency of collisions between fluorophores and the quencher. The possibility of caffeine molecules colliding with tryptophan and tyrosine side-chains can be assumed. Regarding the chemical properties of caffeine, it cannot be excluded that, under the applied experimental conditions, the molecule experiences hydrogen bonding with amino acid side-chains of the protein.

In addition, we have applied EPR spectroscopy with spin labeled fatty acid reporter groups as an independent approach to probe the interaction between HSA and caffeine. Three stearic acid derivatives bearing the doxyl group at different distances from the carboxyl moiety were used to label HSA. Distances between the carbonyl carbon atom of the carboxylic group and the chain carbon atom of the doxyl ring for 5-DSA, 7-DSA and 13-DSA are 0.5 nm, 0.8 nm and 1.4 nm, respectively. This approach is suitable with respect to the physiological role of HSA, being a fatty acid transporter. Moreover, the amount ratio of the spin label to protein (*ca.* 1:1) was chosen according to normal physiological conditions. As previously reported[17] in the case of BSA, doxylstearic acid molecules occupy the binding sites normally occupied by stearic acid under physiological

conditions. Although BSA and HSA somewhat differ in structure, it is very likely that *N*-DSA molecules bind to the same binding sites also in HSA. EPR spectra of the spin labeled fatty acid analogs bound to HSA presented here are in agreement with the previously reported data.[18] The doxyl group from the applied spin labels bound to HSA detects different motional restrictions expressed as different $2A_{max}$. Binding of caffeine to HSA at 310 K can be monitored directly by 5-DSA. The maximal hyperfine splitting of the 5-DSA bound to HSA indicates that the motion of the doxyl ring is strongly restricted. Upon interaction with caffeine, the narrowing of $2A_{max}$ was measured. This observation could be explained either as a change in mobility/motional restrictions of the reporter group or as a local change in polarity induced by the presence of caffeine. Since the change of the spectral peak proportions that accompanies the change in the hyperfine splitting is small, neither possibility can be excluded. On the other hand, the results indicate that the doxyl groups of 7-DSA and 13-DSA are in the region where the binding of caffeine has no measurable effect on segmental motion or polarity of the environment of the spin labeled fatty acid. As the doxyl group moves along the alkyl chain of the fatty acid, the effect of caffeine is abolished. We have concluded that the impact of caffeine binding is mostly expressed in the region of HSA where the DSA carboxylic group is bound. This effect is spatially limited and, therefore, only 5-DSA is able to detect it. However, no spectral changes of spin labels bound to HSA were detected at 298 K. This finding is consistent with the result obtained by fluorescence spectroscopy, which detected a very small total decrease in fluorescence intensity upon exposure to caffeine at 298 K, also indicating a very low affinity, unspecific attachment of caffeine to HSA.

CONCLUSIONS

We have shown that caffeine binding to HSA can be investigated by intrinsic fluorescence spectroscopy; however, including a suitable correction due to the inner filter effect. Caffeine quenches HSA intrinsic fluorescence in a concentration dependant manner. The rate of quenching is higher at 310 K than at 298 K, meaning that a dynamic quenching of HSA fluorescence by caffeine occurs. Furthermore, the spin labeled stearic acid analogs were applied as reporter groups in the experiments using EPR spectroscopy. We have shown that an increase in the local protein dynamics and/or polarity change has been introduced upon caffeine binding. These observations may be important for the functional characteristics of HSA, which should be further investigated.

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REFERENCES

1. X. M. He and D. C. Carter, *Nature* **358** (1992) 209–215.
2. A. C. Guyton and J. E. Hall, *Textbook of medical physiology*, Saunders, Philadelphia, 1996.
3. D. C. Carter and J. X. Ho, *Adv. Protein Chem.* **45** (1994) 152–203.
4. G. Sudlow, D. J. Birkett, and D. N. Wade, *Mol. Pharmacol.* **11** (1975) 824–832.
5. G. Sudlow, D. J. Birkett, and D. N. Wade, *Mol. Pharmacol*. **12** (1976) 1052–1061.
6. J. J. Barone and H. Roberts, *Food Chem. Toxicol.* **34** (1996) 119–129.
7. H. R. Lieberman, R. J. Wurtman, G. G. Emde, C. Roberts, and I. L. Coviella, *Psychopharmacology* **92** (1987) 308–312.
8. M. Jarvis, *Psychopharmacology* **110** (1993) 45–52.
9. C. La Vecchia, M. Ferraroni, E. Negri, B. D'Avanzo, A. Decarli, F. Levi, and S. Franceschi, *Cancer Res.* **49** (1989) 1049–1051.
10. M. R. Joesoef, V. Beral, R. T. Rolfs, S. O. Aral, and D. W. Cramer, *The Lancet* **335** (1990) 136–137.
11. J. Gonzalez-Jimenez, G. Frutos, and I. Cayre, *Biochem. Pharmacol*. **44** (1992) 824–826.
12. W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.* **93** (1971) 314–326.
13. J. R. Lakowicz, *Principles of fluorescence spectroscopy*, Plenum Press, New York, London, 1983.
14. P. D. Morse, *Biophys. J*. **51** (1987) 440a.
15. R. A. Weisiger, J. D. Ostrow, R. K. Koehler, C. C. Webster, P. Mukerjee, L. Pascolo, and C. Tiribelli, *J. Biol. Chem.* **276** (2001) 29953–29960.
16. M. Purcell, J. F. Neault, H. Malonga, H. Arakawa, R. Carpentier, H. A. Tajmir-Riahi, *Biochim. Biophys. Acta* **1548** (2001) 129–138.
17. J. D. Morrisett, H.J. Pownall, and A. M. Gotto Jr., *J. Biol. Chem.* **250** (1975) 2487–2494.
18. M. Ge, S. B. Rananavare, and J. H. Freed, *Biochim. Biophys. Acta* **1036** (1990) 228–236.

SAŽETAK

Istraživanje vezanja kofeina na albumin iz ljudskoga seruma

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Predmet ovoga istraživanja je vezanje kofeina na albumin iz ljudskoga seruma (HSA) s ciljem određivanja točne metodologije obrade dobivenih eksperimentalnih podataka. Naime, ustanovljeno je da na rezultate iz literature dobivene uporabom fluorescencijske spektroskopije utječe učinak unutarnjega filtera, kojega nije moguće zanemariti. Učinak unutarnjega filtera prisutan je uslijed snažne apsorpcije kofeina na valnoj duljini pobude fluorescencije HSA (290 nm). Stoga je u ovome radu provedena korekcija dobivenih eksperimentalnih podataka te je procijenjena konstanta vezanja kofeina na HSA. Nadalje, za istraživanje interakcije kofeina i HSA primijenjena je spektroskopija elektronske paramagnetske rezonancije uz uporabu triju izomera spinski označene stearinske kiseline, doksil-stearata. Dobiveni rezultati ukazuju na smanjenje hiperfinoga cijepanja spektra HSA obilježenoga 5-doksil-stearatom u prisutnosti kofeina. Taj rezultat, zajedno s uočenim promjenama spektralnih amplituda, upućuje na povećanje mobilnosti spinske oznake u prisutnosti kofeina, uslijed povećanja lokalne pokretljivosti proteina.