J Fluoresc (2013) 23:889–895
DOI 10.1007/s10895-013-1199-5

ORIGINAL PAPER

Interaction of the Iron(II) Cage Complexes With Proteins: Protein Fluorescence Quenching Study

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Received: 9 November 2012 / Accepted: 24 February 2013 / Published online: 4 April 2013

Abstract Interaction of the iron(II) mono- and bis-clathrochelates with bovine serum albumin (BSA), β-lactoglobulin, lysozyme and insulin was studied by the steady-state and time-resolved fluorescent spectroscopies. These cage complexes do not make significant impact on fluorescent properties of β-lactoglobulin, lysozyme and insulin. At the same time, the monoclathrochelates strongly quench a fluorescence intensity of BSA and substantially decrease its excited state lifetime due to their binding to this protein. This occurs due to the excitation energy transfer from a tryptophan residue to a cage molecule or/and to the change of the tryptophan nearest environment caused by either clathrochelate binding or an alteration of the BSA conformation. The effect of the iron(II) bis-clathrochelate on BSA fluorescence is much weaker as compared to its monomacrobicyclic analogs as a result of an increase in its size.

Keywords Clathrochelates · Protein fluorescence · Fluorescence lifetime · Protein-ligand complex · Albumin

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Introduction

The cage complexes with a metal ion encapsulated in the cavity of a macrobicyclic ligand (clathrochelates) possess unique physicochemical properties [1–16]. In such complexes, a metal ion is almost completely isolated from external factors; so most of the clathrochelates demonstrate unprecedented chemical and photochemical stability [1] as well as low toxicity [17]. These chemical and physicochemical properties open the ways for practical biochemical and medical applications of these compounds [18]. In particular, these macrobicyclic complexes and their hybrids with *closo*-borate anions are prospective molecular targets for the boron-neutron capture therapy [4, 19–21], the carriers of the radioactive ions for both the diagnostics and radiotherapy [18, 22], and the paramagnetic probes for MRI [23]. Moreover, the iron(II) clathrochelates have been recently recognized as very efficient transcription inhibitors [17]. Hence, the study of an interaction of the cage metal complexes with the biological molecules, mainly proteins, seems to be very important for the design of biologically active clathrochelates with given structure and properties.

Protein intrinsic fluorescence is widely used to study their folding processes as well as the protein—ligand interactions [24]. These interactions may affect the fluorescence of the tryptophan residues either by its quenching or by binding of the macrobicyclic molecules to a fluorophore that changes the polarity of its environment and/or its accessibility for the solvent molecules. Besides, this ligand may bind with an allosteric site that is remote from the tryptophan residue but can induce the conformational changes of the protein and affect tryptophan microenvironment [24]. The observed changes in the protein fluorescence

intensity and/or a shift of its emission spectrum allow observing a formation of the protein—ligand supramolecular complex.

Here, we report the interaction of the iron(II) mono- and bis-clathrochelates (Chart 1) with a series of the model proteins (bovine serum albumin (BSA), β-lactoglobulin (BLG), lysozyme and insulin) studied by the steady-state and time-resolved fluorescent spectroscopies.

Materials and Methods

Materials

The cage complexes **1**–**3** were prepared as described in [16, 17]. BSA, lysozyme from chicken egg white, BLG from bovine milk and human insulin were obtained commercially (Sigma-Aldrich Co. and Private Joint Stock Company «On the production of insulin «Indar»). 0.05 M Tris–HCl buffer aqueous solution with pH 7.9 was used as a solvent.

Preparation of the Solutions

The protein solutions with concentration 0.2 gL^{-1} were prepared by dissolving the weighted amount of the corresponding protein in 0.05 M Tris—HCl buffer aqueous solution. An aliquot of 1×10^{-3} or 2×10^{-3} M DMSO solution of the cage complex under study was added to an aqueous solution of the corresponding protein. As the amount of the DMSO solution added was small, the protein concentration remains practically unchanged, while the concentration of these complexes varied from 0.5, 1.5, 2.5, 5, 7.5, 10, 15 to 25 μM; the concentration of the bis-clathrochelate **3** was provided as those of its monoclathrochelate units.

Fluorescence Quenching Studies

The fluorescence spectra were obtained at room temperature on a Cary Eclipse fluorescence spectrophotometer (Varian) in quartz cell (1×1 cm). To study the quenching of the protein fluorescence by the iron(II) mono- and bis-clathrochelates, the fluorescence spectra of the initial protein and those upon a successive addition of clathrochelate DMSO solutions were registered. Fluorescence was excited at 280 nm, and its intensity value was registered at λ_{max} of the corresponding free protein.

Fluorescence Lifetime Measurements

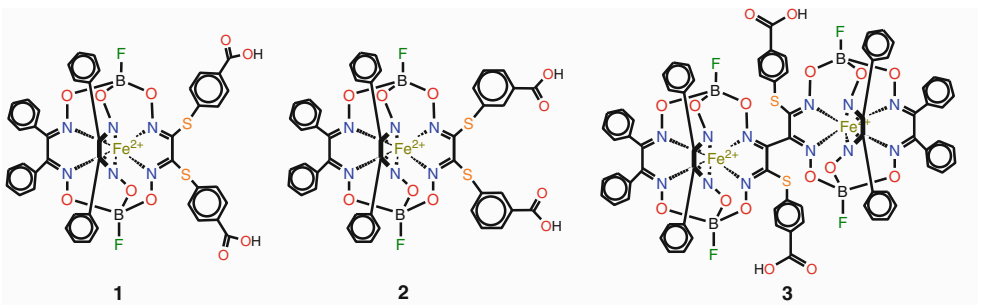
The fluorescence intensity decay curves of the proteins studied were obtained using a Fluorolog 3 modular spectrofluorimeter (Horiba Jobin Yvon). The fluorescence emission was excited using NanoLED with pulse duration approximately 40 ps at 282 nm and registered at 350 nm (for BSA, BLG and lysozyme) and at 320 nm (for insulin). The decay curves obtained were analyzed using a DAS6 v.6.4 program. The approximations by two exponents gave the χ^2 values from 1.18 to 1.58, and their deviations from the experimental curves are clearly observed in the range of the short lifetimes. At the same time, the approximations by three exponents described the experimental data more precisely with the χ^2 parameters close to unity (Table 2) and the calculated curves were close to those registered experimentally. In order to generally characterize the decay rate for each of these curves, the average decay times τ were calculated using a following equation: $\tau=(A_1\times\tau_1+ A_2\times\tau_2+ A_3\times\tau_3)/100$, where τ_1, τ_2 and τ_3 are the lifetimes of the components of the approximated decay curves; A_1, A_2 and A_3 are the corresponding relative amplitudes.

Results and Discussion

Quenching of the Protein Fluorescence by Iron(II) Mono- and Bis-Clathrochelates

To study an interaction of the cage complexes **1**–**3** with proteins, the Stern—Volmer plots of the intrinsic fluorescence intensity of BSA, BLG, lysozyme and insulin versus their concentrations were used (Fig. 1). The

Chart 1 The mono- and bis-clathrochelates studied



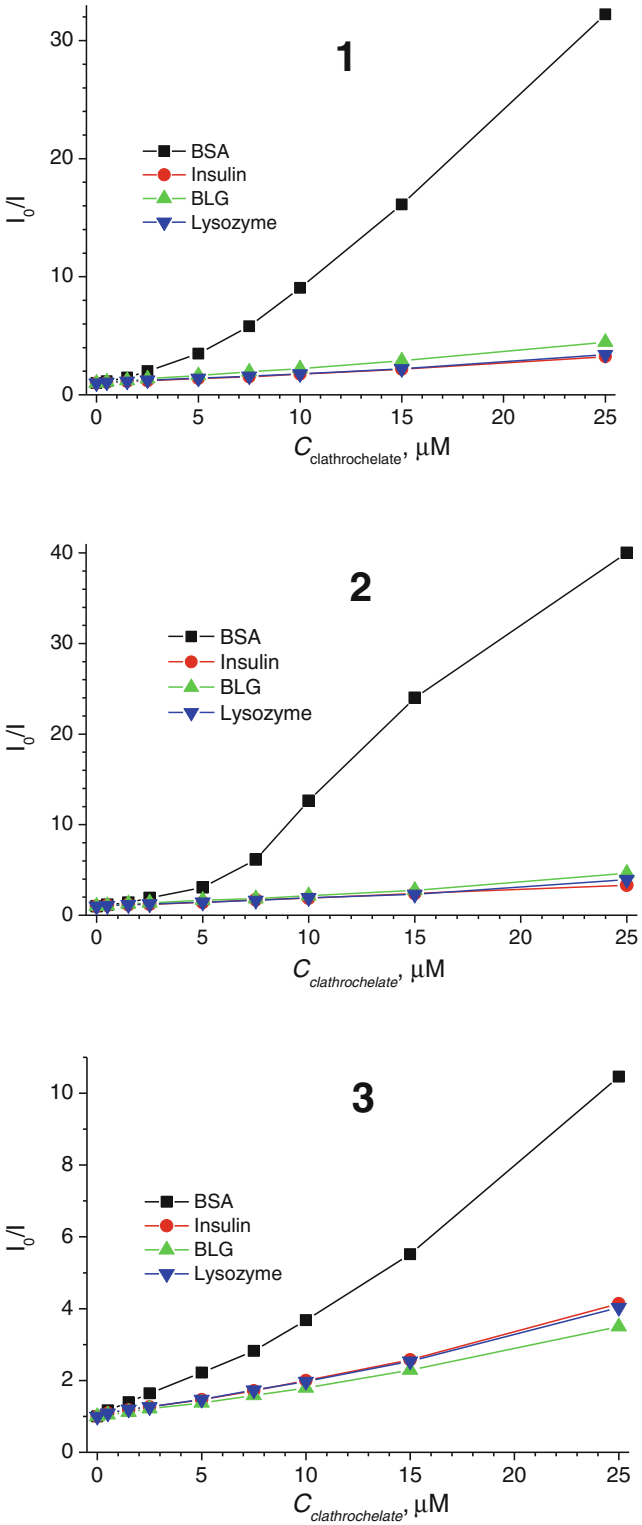


Fig. 1 Plots of the fluorescence intensity of the proteins studied at their concentration equal to 0.2 gL^{-1} in the Stern—Volmer coordinates versus the concentration of the cage compounds **1**–**3**. I_0 and I are the fluorescence intensities in the absence and in the presence of these complexes, respectively

fluorescence intensities of the buffer solutions of BLG, lysozyme and insulin were slightly affected by the presence of the clathrochelates: a 3.2–4.6 times decrease in a fluorescence intensity was observed at their 25 μM concentrations. This result may be explained by the inner filter effect (i.e., an absorption of the excitation light by these compounds; this effect caused approximately 50–100 % of a quenching according to our estimation) together with weak non-specific interactions of the cage complexes with proteins or by an influence of DMSO on the protein conformation.

In the case of BSA, the results were quite different: the addition of the monoclathrochelates **1** and **2** leads to the strong quenching of BSA fluorescence intensity (by 32 and 40 times, respectively, at 25 μM concentrations of these complexes), whereas the bis-clathrochelate **3** quenches the protein fluorescence only by 10.5 times at the same concentration of the monoclathrochelate units. This could be explained by strong interactions of **1** and **2** with BSA. As for the complex **3**, the not so strong quenching could result partially from binding of only one of its two macrobicyclic entities to BSA. Meanwhile, as the quenching of BSA emission in the presence of 25 μM of **3** is three times weaker than that for **1**, we can conclude that a formation of the bis-clathrochelate framework makes a negative impact on an ability of the cage complexes to bind to BSA, probably, due to an increase in a size of their molecules.

The fluorescence spectra of BSA in the absence and in the presence of the clathrochelate **1** are shown in Fig. 2. An increase in the concentration of **1** causes not only quenching of the BSA fluorescence but also the short-wavelength shift of its maximum from 347 nm (for the initial BSA aqueous solution) to 317 nm at high concentration of this complex. This shift could be explained by much stronger quenching of the tryptophan

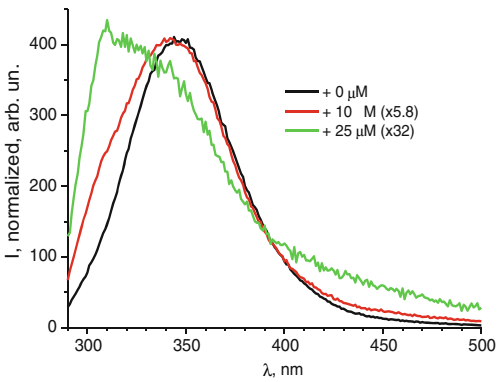


Fig. 2 Fluorescence spectra of BSA ($c_{protein}$=0.2 gL^{-1}) in the absence and in the presence of the clathrochelate **1** ($c_{clathrochelate}$=10 and 25 μM, the fluorescence intensity was multiplied by 5.8 and 32, respectively)

emission than that of tyrosine and is observed also in the case of **2**. At the same time, the presence of the cage complexes **1**–**3** does not affect the shapes of the fluorescence spectra of BLG, lysozyme and insulin.

Time-Resolved Fluorescence Studies

A decay of the fluorescence intensities of BSA, BLG, lysozyme and insulin in the absence and in the presence of the complexes **1**–**3** was studied to characterize the interaction of the latter with these proteins. The tryptophan fluorescence emission was excited at 282 nm and registered at 350 nm for BSA, BLG and lysozyme; that of tyrosine at 320 nm was measured for insulin because this protein contains no tryptophan residues. The average decay times τ, which are characteristic of the decay curves, are given in Table 1.

In the case of BLG, lysozyme and insulin, the τ values in the presence of the cage complexes studied remain practically unchanged. Therefore, their macrobicyclic molecules either do not bind to these proteins or this binding does not affect the tryptophan residues (or tyrosine ones for insulin) as well as their nearest environment. Besides, no excitation energy transfer from tryptophan to a cage molecule takes place. As some quenching of the lysozyme, BLG and insulin fluorescence in the presence of these macrobicycles is observed (*vide supra*), a formation of the non-fluorescent supramolecular complexes of the mono- and bis-clathrochelates **1**–**3** with tryptophan or tyrosine residues could not be excluded as well.

In the presence of 10 μM of the monoclathrochelates **1** and **2**, the average decay time of the BSA fluorescence intensity exhibits 2.4 and 2.9 times decrease, respectively, whereas in the case of the bis-cage **3** only 1.2 times decrease is observed (Table 1). This suggests the strong interactions between BSA and these monoclathrochelates: the substantial decrease in the τ values could be explained by the excitation energy transfer from the tryptophan residues to the polyene macrobicyclic molecule or/and by the changes in the tryptophan closest environment. The interaction of the bis-clathrochelate **3** with BSA is less efficient as compared with those for **1** and **2**. This correlates with the data on quenching of the fluorescence intensity (*vide supra*).

The Influence of the Mono- and Bis-Clathrochelates on the Components of the BSA Emission Intensity Decay

To study a quenching of the BSA emission in the presence of the cage complexes in details, the fluorescence intensity decay curves were analyzed in the three-exponents approximation. For initial BSA aqueous solution, this approximation yielded the lifetimes of τ_1=3.3, τ_2=6.8 and τ_3=0.54 ns (Table 2). The addition of the monoclathrochelates **1** and **2** led to the substantial decrease (up to 3 times) in the τ_1 value, whereas τ_2 changes only slightly. At the same time, the relative amplitude A_2 decreases up to three times in the presence of these compounds (Table 2 and Fig. 3). In the case of the bis-clathrochelate **3**, the components of BSA fluorescence intensity decay demonstrate the same changes, but they are less pronounced as compared to its monoclathrochelate analogs (Table 2).

In the case of the BSA aqueous solutions, the emission at 350 nm is mainly caused by the tryptophan residues. As it has been earlier reported [25], the tryptophan fluorescence intensity decay in the aqueous solutions consists of two mono-exponential components with decay times 3.1 and 0.53 ns assigned to the emission of the different conformers of this residue: the second one belongs to the conformation with a close positioning of the π-system of tryptophan and the positively charged amino group. This results in a fast transfer of the excited π-electron to this group and, thus, the formation of the non-fluorescent charge transfer (CT) state [25, 26]. The τ component at 3.1 ns is assigned to the conformers without a CT. For the tryptophan solution in glycerol, the three components at 0.54, 2.3 and 5.4 ns have been observed [27]: first two are similar to those for the aqueous solutions, while the third component is assigned to the excimers that are formed by the excited tryptophan residues and the glycerol molecules.

Despite the fact that in many cases a correlation between the state of tryptophan residues and the lifetime components in the BSA emission intensity decay is not clear, in some systems it is nevertheless observed. For example, this decay for Ribonuclease T_1 at pH 5.5 is monoexponential with τ=3.87 ns that is characteristic of the tryptophan residues situated in the hydrophobic part

Table 1 The average decay times τ (ns) of the proteins at their concentrations equal to 0.2 gL^{-1} in the absence and in the presence of 10 μM of the iron(II) mono- and bis-clathrochelates **1**–**3**

	Free protein	Protein + **1**	Protein + **2**	Protein + **3**
BSA	5.2	2.2	1.8	4.3
BLG	2.2	2.0	2.0	1.8
Lysozyme	1.8	1.8	1.8	1.8
Human Insulin	1.3	1.3	1.2	1.2

Table 2 The average decay times τ, the three-exponential components of the decay curves τ_1, τ_2 and τ_3 (ns) and the corresponding relative amplitudes A_1, A_2 and A_3 (%) of the BSA fluorescence intensity decay as well as the corresponding χ^2 values in the absence and in the presence of the mono- and bis-clathrochelates **1**–**3** at their various concentrations

	τ_1	τ_2	τ_3	A_1	A_2	A_3	τ	χ^2
BSA	3.3	6.8	0.54	30.4	61.2	8.4	5.2	1.09
BSA+**1** (5 μM)	1.55	6.1	0.19	38.2	50.9	10.9	3.7	1.06
BSA+**1** (10 μM)	1.2	5.8	0.18	51.8	26.0	22.2	2.2	1.08
BSA+**1** (25 μM)	1.1	6.2	0.11	49.0	21.0	30.0	1.9	1.15
BSA+**2** (5 μM)	1.3	5.8	0.15	58.6	25.8	15.5	2.3	1.08
BSA+**2** (10 μM)	1.2	5.9	0.15	59.1	18.6	22.3	1.8	1.19
BSA+**3** (5 μM)	2.1	6.4	0.06	27.9	64.5	7.6	4.7	1.07
BSA+**3** (10 μM)	1.9	6.2	0.06	32.0	59.2	8.8	4.3	1.04
BSA+**3** (25 μM)	1.7	5.8	0.22	39.7	53.3	7.0	3.8	1.05

of this protein, where there are no polar groups forming the CT systems. For the human serum albumin (HSA) with only one tryptophan residue (Trp-214), the three-exponential fluorescence decay (0.49, 4.0 and 7.8 ns according to [28] and 0.79, 4.1 and 7.2 ns according to [29]) has been reported. These components have been assigned in [29] to its three possible rotamers. At the same time, in [28] the presence of two components with shortest lifetimes have been explained by tryptophan internal characteristics, which are independent on its microenvironment, whereas the third one has been assigned to an interaction of tryptophan with the nearest amino acidic residues. Our data for the double-tryptophan BSA (Trp-212 and Trp-134) are similar to those for the single-tryptophan HSA (Table 2).

We assumed that the shortest component of the BSA emission intensity decay with τ=0.54 ns appears when the positively charged group(s) is(are) positioned close to the excited tryptophan residue and, therefore, can form the non-fluorescent CT complex. The component with τ=3.3 ns is assigned to the emission of the tryptophan that forms no CT complex due to its complete isolation by the hydrophobic environment. The third component is assigned to the excimers formed by this residue with some groups of BSA or with the solvate molecules.

Thus, the effects of the mono- and bis-clathrochelates on the BSA lifetime components may be explained as follows:

- 3.3-ns component: its τ decreases either due to the excitation energy transfer from a tryptophan residue to a clathrochelate framework leading to a decrease in the excited state lifetime or due to an alteration of the tryptophan nearest environment caused by the BSA conformation changes or by exposing the tryptophan residue to a cage molecule.
- 6.8-ns component: this is assigned to the tryptophan excimers that are formed with some polar groups of BSA or with the solvate molecules. Since a decrease in both this component relative amplitude and the total BSA emission intensity was observed, we conclude that the binding of a clathrochelate molecule to a protein structure substantially decreases the possibility to form these excimers. Namely, the changes in the tryptophan environment upon clathrochelate binding hinder an access of the groups able to form such excimers. In particular, these changes could be caused by the change of the BSA conformation, or the formation of supramolecular assemblies hinder an interaction of the solvate molecules with a surface-exposed tryptophan residue.

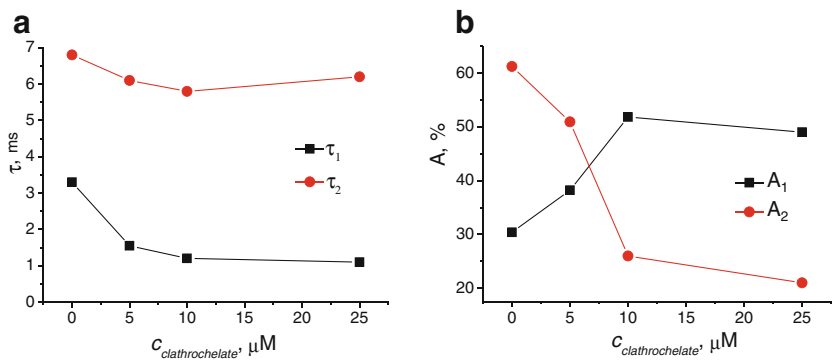


Fig. 3 Plots of the values of τ (**a**) and A (**b**) for the 3.3-ns (*squares*) and 6.8-ns (*circles*) components of the BSA decay curve versus the concentration of the clathrochelate **1**

Conclusions

We studied the effect of a series of the iron(II) mono- and bis-clathrochelates on a fluorescence of some proteins (BLG, insulin, lysozime and BSA) to characterize their supramolecular interactions. The strong changes in the fluorescent characteristics in the presence of the cage complexes were observed only in case of BSA. The binding of the monoclathrochelates to BSA leads to the substantial quenching of protein fluorescence and to decrease in its excited state lifetime. This result was explained by the formation of the supramolecular BSA—clathrochelate complexes that resulted in the excitation energy transfer from a tryptophan residue to a cage molecule, or/and in the change of the tryptophane nearest environment caused by either a close binding of the clathrochelate molecule or by an alteration of the BSA conformation. The effect of the iron(II) bis-clathrochelate on the BSA fluorescence is much weaker as compared with its monoclathrochelate analogs. This was attributed to the increase in a size of its bis-cage molecule.

Acknowledgments This study was supported by RFBR—SFFR grants 12-03-90431 / 0112U004235 and by Marie Curie IIF Scheme of the 7th EU Framework Program (grant 295160).

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