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Comparative Studies on the Interaction Between the Acriflavine and Bovine Serum Albumin by Fluorescence Spectroscopy, Synchronous Fluorescence

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Under simulated physiological conditions, we studied the reaction mechanism of acriflavine (ACF) with bovine serum albumin (BSA) at different temperature by utilizing fluorescence quenching method and synchronous fluorescence method, respectively. The results indicate that acriflavine could quench the intrinsic fluorescence of bovine serum albumin strongly and the quenching mechanism was a static quenching process; the electrostatic force played an important role on the conjugation reaction between acriflavine and bovine serum albumin. The results obtained by the two methods were consistent, which indicated synchronous fluorescence spectroscopy can replace traditional fluorescence quenching method to study reaction mechanism of dyes with proteins.

Keywords: Fluorescence spectroscopy, Synchronous fluorescence, Acriflavine, Bovine serum albumin, Interaction.

INTRODUCTION

Serum albumin, the most abundant protein constituent in blood plasma, can be combined with a lot of endogenous and exogenous compounds and plays a fundamental role in the disposition and transportation of various molecules. Therefore, investigating the binding mechanism of endogenous or exogenous compounds and serum albumins has very significant implications for the life sciences, chemistry, pharmacy and clinical medicine. Synchronous fluorescence spectrometry technology was first proposed by Lloyd[1] and the biggest difference between it and fluorescence measurement method is scanning the excitation and emission monochromators simultaneously. Comparing with conventional fluorescence spectroscopy, synchronous fluorescence method has some advantages such as good selectivity, high sensitivity, less interference *etc*[2] and it can be used for the simultaneous determination of multicomponent mixture[3]. However, research of the binding constant and binding sites between the protein and the dye with this method has reported less.

Acriflavine (ACF) also called acriflavinium chloride, is a kind of topical antiseptic agent derived from acridine, first synthesized in 1912 by Paul Ehrlich, a German medical researcher and was used in the early 20th century, during the First World War, as topical antibacterial and against sleeping sickness[4]. In this study, we study the binding mechanism of dyes and proteins by utilizing traditional fluorescence quenching method and synchronous fluorescence method, whose research object is acriflavine, respectively. The results of two methods are consistent. The new method is a useful supplement to the fluorescence spectroscopy's application about the research of combination mechanism of proteins and dyes.

EXPERIMENTAL

All fluorescence spectra were recorded on a Shimadzu RF-5301PC spectro-fluorophotometer. All spectrophotometric measurements were made with a Shimadzu UV-265 spectrophotometer. All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by CS501 super-heated water bath (Nantong Science Instrument Factory).

Bovine serum albumin was purchased from Sigma (the purity grade inferior 99 %) and stock solutions (10 μM) were prepared by doubly distilled water. Acriflavine (2 mM) were prepared by doubly distilled water, respectively. All the stock solutions were further diluted as working solutions prior to use. The *tris*-HCl buffer (0.05 M, pH = 7.40) containing 0.15 M NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. All other reagents were of analytical reagent grade and double-distilled water was used during the experiment. And all the stock solutions were stored at 277 K.

Fluorescence spectra and synchronous fluorescence spectra: In the experiment we use the 1 mL of pH 7.40 *tris*-HCl, a certain amount of 10 μM bovine serum albumin solution

and different concentrations of acriflavine (0.1 mM) was added into 10 mL colourimetric tube sequentially. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking and kept static for 0.5 h. The fluorescence emission spectra were measured at 293, 303 and 310 K with the width of the excitation and emission slit adjusted at 5 and 5 nm, respectively. The synchronous fluorescence spectra were recorded at $\Delta\lambda$ = 15 and 60 nm in the absence and presence of various amounts of acriflavine over a wavelength range of 280-400 nm.

Determination of the binding sites: At 293 K, different concentrations (10 μM) of site marker I (warfarin, WF), II (ibuprofen, IB), or III (digoxin, DG) were added to the mixture of BSA-ACF systems.

RESULTS AND DISCUSSION

Fluorescence quenching spectra of BSA-ACF system: The quenching mechanism of fluorescence can be classified into static quenching and dynamic quenching[5]. Dynamic quenching is mainly caused by collisional encounters between the fluorophore and the quencher, static quenching is mainly resulted from the formation of stable compound between fluorophore and quencher[6]. Fig. 1 shows the fluorescence emission spectra of bovine serum albumin in the presence of various concentrations of acriflavine at 293 K. The fluorescence emission intensity of bovine serum albumin decreased regularly with the gradual addition of acriflavine. This result indicates that acriflavine can interact with bovine serum albumin and quench its intrinsic fluorescence, changing the microenvironment of the fluorophores.

If it is assumed that the fluorescence quenching mechanism of bovine serum albumin by acriflavine is dynamic quenching process, fluorescence quenching can be described by Stern-Volmer equation[7].

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

According to the Stern-Volmer plots of F_0/F *versus* quencher concentration at different temperatures (293, 303 and 310 K). The quenching rate constant K_q was obtained and listed in Table-1. It is obvious K_q decreases with rising temperatures, revealing that the quenching is initiated by static quenching process. Moreover, the values of K_q between bovine serum albumin and acriflavine are all greater than 2×10^{10} M^{-1} s^{-1}. Therefore, acriflavine binding bovine serum albumin was a static quenching process proved to be true[8].

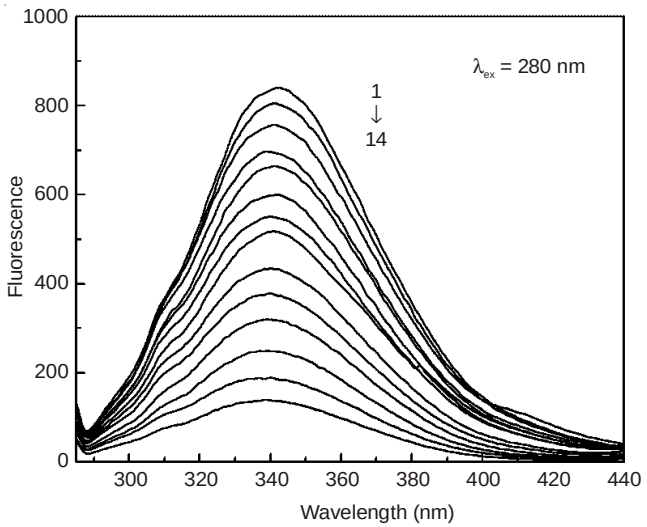

Fig. 1. Fluorescence emission spectra of BSA-ACF (T = 293 K); C_{BSA} = 0.4 μM, 1~14: C_{ACF} = (0,1.0, 2.0, 3.0, 4.0, 5.0, 8.0, 10, 15, 20, 30, 40, 50, 60) μM

For the static quenching interaction, under the assumption that there are similar and independent binding sites in the biomolecule, the binding constant and the number of binding sites can be derived from the double logarithm regression curve (eqn. 2)[9].

$$\log [(F_0\text{-}F)/F] = n\log [Q] + \log K_a \quad (2)$$

The curve of log $[(F_0\text{-}F)/F]$ *versus* log [Q] is drawn and fitted linearly, then the value of n and K_a can be obtained from the plot. Table-1 gives the corresponding calculated results. The value of *n* almost equals to 1, indicating that there is one class of binding site for acriflavine to bovine serum albumin molecule. In other words, acriflavine and bovine serum albumin form a complex with molar ratio 1:1. According to the results shown in Table-1, the binding constants of the interaction between acriflavine and bovine serum albumin decreases with the rising temperature, further suggested that the quenching was a static process[10].

Synchronous fluorescence spectra: When $\Delta\lambda$ is 15 nm, synchronous fluorescence detects characteristics of tyrosine (Tyr) residues, but when $\Delta\lambda$ is 60 nm, characteristic information from tryptophan (Trp) residues is highlighted[11]. The synchronous fluorescence spectra of BSA-ACF systems shown in Fig. 2. It can be seen from Fig. 2, the λ_{max} had red shifted when $\Delta\lambda$ = 15 and $\Delta\lambda$ = 60 nm (the λ_{max} has an obvious red shift). This indicated that the interaction of bovine serum

TABLE-1
QUENCHING REACTIVE PARAMETERS OF ACRIFLAVINE AND BOVINE SERUM ALBUMIN AT DIFFERENT TEMPERATURES

	Temperature (K)	K_q (M^{-1} s^{-1})	r_1	K_a (M^{-1})	n	r_2
λex = 280 nm	293	6.37×10^{12}	0.9984	5.18×10^4	0.93	0.9953
	303	5.80×10^{12}	0.9961	4.35×10^4	0.97	0.9971
	310	4.38×10^{12}	0.9988	3.47×10^4	0.92	0.9946
Δλ = 15 nm	293	3.19×10^{12}	0.9954	2.41×10^4	0.96	0.9944
	303	2.24×10^{12}	0.9969	2.12×10^4	0.98	0.9932
	310	1.98×10^{12}	0.9972	1.69×10^4	0.96	0.9970
Δλ = 60 nm	293	3.88×10^{12}	0.9953	3.34×10^4	0.89	0.9965
	303	2.86×10^{12}	0.9954	2.92×10^4	0.92	0.9937
	310	2.04×10^{12}	0.9961	2.15×10^4	0.95	0.9946

r_1 is the linear relative coefficient of F_0/F~[Q]; r_2 is the linear relative coefficient of log$[(F_0\text{-}F)/F]$~log[Q]

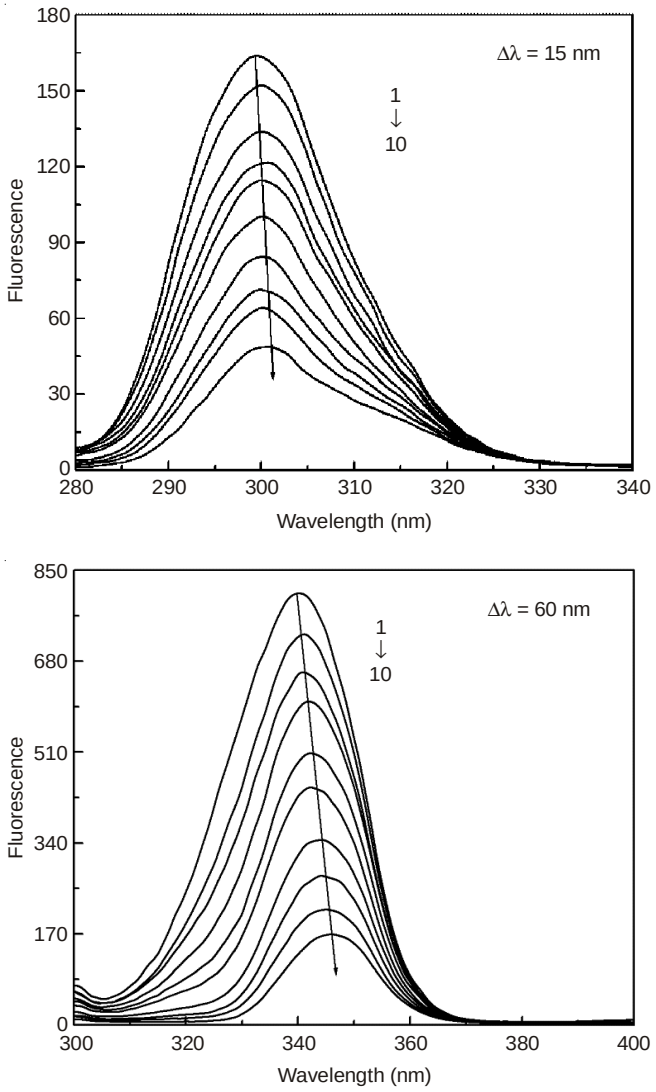

Fig. 2. Synchronous fluorescence spectra of BSA-ACF system (T = 293 K) C_{BSA} = 0.4 μM 1~10: C_{ACF} = (0, 3.0, 6.0, 10, 15, 20, 30, 40, 50, 60) μM

albumin with acriflavine changed the microenvironment of Tyr residues and Trp residues[12].

High concentrations of dyes make protein molecules extend, reducing the energy transfer between the amino acid residues and reducing their fluorescence intensity. For the quenching process, according to eqns. 1 and 2, the corresponding results are shown in Table-1. From Table-1, it can be seen the values of K_q decreased with the rising temperature in all systems, which indicated that the probable quenching mechanism of the interaction between bovine serum albumin and acriflavine was a static process. Obviously, the K_q value of protein quenching procedure initiated by acriflavine was greater than 2×10^{10} M^{-1} s^{-1}. This indicated that the quenching was not initiated from dynamic collision but from the formation of a complex. The *n* value approaches unity, suggesting that one molecule of acriflavine combines with one molecule of bovine serum albumin; the decreasing trend of K_a with the increasing temperature was in accordance with binding constants dependence on the temperature as mentioned above, which indicates that ACF-BSA would be partly decomposed when the temperature rising. It also indicates it is a static quenching. The quenching mechanism obtained by synchronous fluorescence method was coincident with the one obtained by fluorescence method. Comparing the data in Table-1, we can see that the quenching parameters obtained by two methods have the same order of magnitude.

Identification of the binding site: To identify the binding site on bovine serum albumin, site marker competitive experiments were carried out, using the drug which specially binds to a known site or region on bovine serum albumin. X-ray crystallography studies have shown that warfarin (WF) binds to sub-domain IIA whereas ibuprofen (IB) and digoxin (DG) are believed to bind to IIIA binder sites II and III, respectively[13]. Information about BSA-ACF binding site can therefore be obtained by monitoring changes in the fluorescence of acriflavine-bound bovine serum albumin caused by binding by site I (WF), site II (IB) and site III (DG) markers. Binding constants determined on the basis of eqn. 2 show the effect of warfarin, ibuprofen and digoxin on BSA-ACF at 293 K. Under the condition of the traditional fluorescence spectrum, it is observed that binding constants for the ternary system ($K_{BSA\text{-}WF\text{-}ACF} = 6.54 \times 10^3$, $K_{BSA\text{-}IB\text{-}ACF} = 1.19 \times 10^4$ and $K_{BSA\text{-}DG\text{-}ACF} = 1.40 \times 10^4$ M^{-1}) are lower than that for the binary system BSA-ACF ($K_{BSA\text{-}ACF} = 5.18 \times 10^4$ M^{-1}). At Δλ = 60 nm the $K_{BSA\text{-}WF\text{-}ACF} = 4.77 \times 10^3$, $K_{BSA\text{-}IB\text{-}ACF} = 1.10 \times 10^4$ and $K_{BSA\text{-}DG\text{-}ACF} = 1.16 \times 10^4$ M^{-1} are lower than the $K_{BSA\text{-}ACF} = 3.34 \times 10^4$ M^{-1} also. It can be indicating that warfarin hinders the formation of BSA-ACF and can compete for the same binding site in sub-domain IIA (site I). The conclusions of the synchronous fluorescence method were consistent with fluorescence method.

Type of interaction force of BSA-ACF systems: Basically, four main types of interactions, hydrogen bonds, electrostatic forces, van der Waals forces and hydrophobic forces play critical roles in the interactions between small molecules and macromolecules[14]. In order to characterize the force between acriflavine and bovine serum albumin, thermodynamic parameters on the temperatures were analyzed. Thermodynamic parameters can be calculated using eqns. 3 and 4[15].

$$R\ln K = \Delta S - \Delta H/T \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

If it is assumed that the enthalpy change (ΔH) nearly had no change within the investigated temperature, there should be a good linear relationship between ln K and 1/T. The values of thermodynamic parameters (ΔH, ΔS and ΔG) were obtained at 293 K. Under the condition of the traditional fluorescence spectrum, there are -17.40 KJ mol^{-1}, 31.00 J mol^{-1} K^{-1}, -26.48 KJ mol^{-1}, respectively. At Δλ = 15 nm, there are -15.26 KJ mol^{-1}, 32.01 J mol^{-1} K^{-1}, -24.64 KJ mol^{-1}, respectively. At Δλ = 60 nm, there are -18.59 KJ mol^{-1}, 23.51 J mol^{-1} K^{-1}, -25.48 KJ mol^{-1}, respectively. $\Delta H < 0$ and $\Delta S > 0$ indicate that the interaction is mainly driven by electrostatic attractions[16]. The $\Delta G < 0$ opinion that the binding interaction between bovine serum albumin and acriflavine were spontaneous. The conclusions of the synchronous fluorescence method were consistent with fluorescence method.

Conclusion

In this paper, the binding of acriflavine to bovine serum albumin under physiological conditions was studied by traditional fluorescence quenching method and synchronous

fluorescence and used the same equation for processing data, respectively. It is observed that the data obtained by both methods were in the same order of magnitude and very close to each other, quenching mechanism and type of interaction force were consistent, which indicated synchronous fluorescence spectroscopy could replace traditional fluorescence spectroscopy quenching method to study reaction mechanism of dyes with proteins.

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