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Investigation on the interaction of cefonicid sodium with lysozyme by fluorescence quenching spectroscopy

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ABSTRACT

Under simulative physiological conditions, the interaction between lysozyme (LYSO) and cefonicid sodium (CID) was investigated using fluorescence quenching and synchronous fluorescence spectroscopy at different temperatures (298, 310 and 318 K). The results indicated that CID could quench the intrinsic fluorescence of LYSO strongly,and the quenching mechanism was a static quenching process. The order of magnitude of binding constants (K_a) was 10^4, the number of binding site (n) in the binary system was approximately equa to 1. The negative enthalpy changes and positive entropy changes implied that electrostatic interaction might play a main role in the interaction between CID and LYSO. The values of Hill's coefficients were more than 1ÿit indicated that drugs or proteins had a very weakly positive cooperativity in the system. Studies on the interaction between LYSO and drug will facilitate interpretation of the drug's metabolism and transporting process in the blood.

KEYWORDS

Lysozyme;
Cefonicid sodium;
Fluorescence quenching spectroscopy;
Synchronous fluorescence spectroscopy;
Interaction;
Quench.

INTRODUCTION

Lysozyme (LYSO) is a small monomeric low molecular weight of about 14.6 kDa and consisting of 129 amino acid residues with four disulûde bonds[1]. It has six tryptophan (Trp) and three tyrosine (Tyr) residues. The six Trp residues are located at the substrate binding sites, out of which two are in the hydrophobic matrix box, while the lone Trp residue is separated from the others. Among the six Trp residues, Trp62 and Trp108 are considered to be the most dominant ûuorophores in LYSO[2,3]. LYSO is used to carry drugs, such as, antibiotics to treat inflammation, abscess, stomatitis and rheum[4].

Cefonicid sodium[5] (CID) (the structure shown in Figure 1) is a second-generation cephalosporins. cefonicid sodium is effective against a wide range of gram-positive and gram-negative bacteria, and it is useful for anaerobic infections, gonorrhea and diabetic foot infections. At present, the molecular interactions between protein and many ligands have been investigated successfully in biochemistry domain. However, the interaction between CID and LYSO has not been investigated. In this report, we took LYSO as the representative protein and provided investigations on the interaction of CID with LYSO by using

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fluorescence quenching and synchronous fluorescence spectroscopy under simulative physiological conditions. This study can provide a useful clinical reference for future combination therapy.

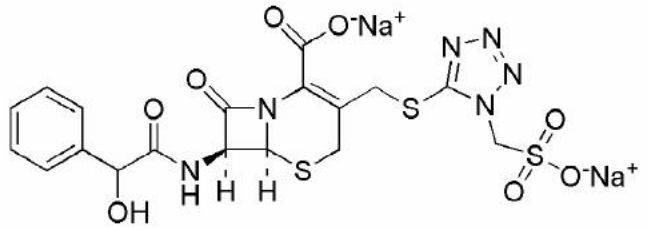

Figure 1 : Chemical structure of cefonicid sodium

EXPERIMENTAL

Apparatus

All fuorescence spectra were recorded with a Shimadzu RF-5301PC spectroûuorophotometer. All pH measurements were carried out with a pHS-3C precision acidity meter (Leici, Shanghai, China). All temperatures were controlled by a CS501 superheated water bath (Nantong Science Instrument Factory).

Materials

Cefonicid sodium (CAS#,61270-78-8) was obtained from Monitor of Chinese Veterinary Medicine (the purity grade inferior 99%). Lysozyme (LYSO) was purchased from Sigma Co (the purity grade inferior 99%). Stock solutions of LYSO (2.0×10^{-5} mol·L^{-1}) and CID (1.0×10^{-3} mol·L^{-1}) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris–HCl buffer solution containing NaCl (0.15 mol·L^{-1}) was used to keep the pH of the solution at 7.4. NaCl solution was used to maintain the ionic strength of the solution. All other reagents were of analytical grade, and all aqueous solutions were prepared with newly double-distilled water and stored at 277K.

In order to eliminate the inner Flter effects of protein and ligand, absorbance measurements were performed at excitation and emission wavelengths of the fluorescence measurements. The fluorescence intensity was corrected using the following equation[6]:

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \quad (1)$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively. A_{ex} and A_{em} are the absorbance values of CID at excitation and emission wavelengths, respectively. The fluorescence intensity used in this article was corrected.

Procedures

Fluorescence measurements

In a typical fluorescence measurement, 1.0mL of pH = 7.40 Tris-HCl, 0.2 mL LYSO solution (2.0×10^{-5} mol·L^{-1}) and different concentrations of CID were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static at different temperatures (298, 310and 318 K). The excitation wavelength for LYSO was 280nm (or295nm) with the excitation and emission slit widths set at 5nm. The solution was subsequently scanned on the fluorophotometer and determined the fluorescent intensity at 341nm.

Synchronous fluorescence measurements

Solution preparation was as detailed above; we recorded the fluorescence spectra of the LYSO-CID system when the $\Delta\lambda$ value between the excitation and emission wavelengths was stabilized at 15 and 60 nm, respectively.

RESULT AND DISCUSSION

Fluorescence quenching spectra of LYSO–CID system

Proteins are considered to have intrinsic fluorescence due to the presence of amino acids, mainly Trp and Tyr. When the excitation wavelengths were at 280 nm and 295 nm, the emission peaks for LYSO were both located at 341 nm. Figure 2 shows the fluorescence emission spectra of LYSO in the presence of different concentrations of CID. It can be seen from Figure 2 that the fluorescence intensity of LYSO decreased regularly with the addition of CID (similar to 295 nm), this result implied that CID could quench the intrinsic fluorescence of LYSO strongly,and the quenching of LYSO fluorescence by CID was due to the formation of the LYSO–CID ground-state complex[7].

In order to confirm the quenching mechanism, the fluorescence quenching data were analyzed by the

Stern–Volmer equation[8]

$$R \ln K = \Delta S - \Delta H / T \quad (2)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively.τ_0 is the average lifetime of fluorescence without quencher and is 10^{-8} s. K_{sv} is the Stern–Volmer quenching constant. K_q is the quenching rate constant of biomolecule, and [L] is the concentration of the quencher. Based on the linear fit plot of F_0/F versus [L], the K_q values can be obtained. The calculated results were shown in TABLE 1. The quenching mechanism[9] is generally classified as either dynamic or static. Dynamic and static quenching can be distinguished by their different dependence on temperature. Quenching rate constants decrease with increasing temperature for the static quenching, but the reverse effect is observed for dynamic quenching. As shown in TABLE 1, The results revealed that the values of K_{sv} and K_q decreased with increasing temperature, and the K_q were much greater than the maximum scatter collision quenching constant of various quenchers (2×10^{10} L·mol^{-1}·s^{-1}). Those all suggested that the quenching mechanism was due to static quenching in each case. In other words, the quenching of the LYSO fluorescence by CID was due to specific complex formation[10].

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher could be usually described by equation (3) to obtain the binding constant (K_a) and the number of binging sites (n) in most of paper[11]:

$$\log\left(\frac{F_0 - F}{F}\right) = n \log K_a + n \log\left\{[D_t] - n\frac{F_0 - F}{F_0}[B_t]\right\} \quad (3)$$

where [Dt] and [Bt] are the total concentrations of MET and LYSO, respectively. On the assumption that n in the bracket is equal to 1, the curve of log $(F_0$ -F)/F versus log{$[D_t]$-$[B_t](F_0$ -F)/F_0}is drawn and fitted linearly, then the value of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of log $(F_0$ -F)/F versus log{$[D_t]$-$[B_t](F_0$ -F)/F_0} is drawn again. The above process is repeated again and again till n obtained is only a single value or a circulating value. Based on the n obtained, the binding constant K_a can be also obtained. In the work, a calculation program was developed. The calculation process can be finished with calculator based on the simple program, and the calculating results can be obtained by inputting F, $[D_t]$ and $[B_t]$. The calculated results were shown in TABLE 1. As seen in TABLE 1, the values of n were approximately equal to 1 at different temperatures, indicating there is one binding site in LYSO for CID.

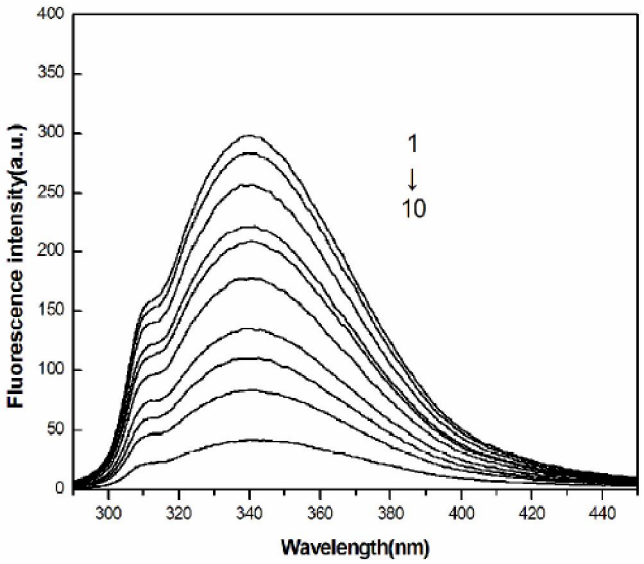

Figure 2 : Fluorescence emission spectra of LYSO-CID(T=298K,λ_{ex}=280); C_{LYSO}=4.0×10^{-7} mol·L^{-1}, 1~10: C_{CID}= (0, 0.4, 1.0, 2.0, 3.0 4.0, 6.0, 8.0, 10, 16) ×10^{-5} mol·L^{-1}

TABLE 1 : Quenching reactive parameters of CID and LYSO at different temperatures

λ_{ex}(nm)	T/(K)	K_q/(L·mol^{-1}·s^{-1})	K_{sv}/(L·mol^{-1})	r_1	K_a/((L·mol^{-1})	n	r_2
	298	2.83×10^{12}	2.83×10^{4}	0.9929	5.01×10^{4}	1.21	0.9938
280	310	2.34×10^{12}	2.34×10^{4}	0.9941	4.12×10^{4}	1.13	0.9957
	318	2.02×10^{12}	2.02×10^{4}	0.9965	3.58×10^{4}	1.05	0.9932
	298	2.79×10^{12}	2.79×10^{4}	0.9939	4.98×10^{4}	1.18	0.9989
295	310	2.32×10^{12}	2.32×10^{4}	0.9921	4.09×10^{4}	1.11	0.9923
	318	1.99×10^{12}	1.99×10^{4}	0.9978	3.57×10^{4}	1.05	0.9934

***K_q* is the quenching rate constant; *K_a* is the binding constant; *n* is the number of binding site;*r_1* is the linear relative coefficient of *F_0/F~[L];r_2* is the linear relative coefficient of log$(F_0$-$F)/F$~log{$[D_t]$-*n*$[B_t](F_0$-$F)/F_0$}**

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Meanwhile, binding constants Ka were reduced with the rising temperature,further suggested that the quenching was a static process[12].

The participation of amino acid residue studies in LYSO-CID system

At 280 nm wavelength, the Trp and Tyr residues in LYSO are excited, whereas the 295 nm wavelength excites only Trp residues[13]. Based on the Stern–Volmer equation, comparing the fluorescence quenching of LYSO excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system[14]. As seen in Figure 3, in the presence of CID, the quenching curves of LYSO excited at 280 nm and 295 nm overlap. This showed that only Trp residue played an important role in the interaction of CID with LYSO. From TABLE 1, at the same temperature, K_a values at excitation wavelengths of 280 nm and 295 nm are of the same order of magnitude and similar, which also suggests that only Trp residues played an important part in the LYSO-CID system.

Synchronous fluorescence spectra studies in LYSO-CID systems

Synchronous fluorescence spectra are used to investigate the protein conformational change,as it has been shown to give narrow and simple spectra[15]. When Δλ between the excitation and emission wavelengths is stabilized at either 15 or 60 nm, the synchronous Fuorescence spectrum can give the characteristics of Tyr residues or Trp residues[16]. It can be seen from Figure 4 that when Δλ was 60 nm, the synchronous fluorescence intensities of LYSO–CID decreased regularly with obviously red shifted, indicating that Trp residues played an important role in the interaction of LYSO with CID, but, when Δλ was 15 nm, the ûuorescence intensities in LYSO-CID showed no obvious decrease and the emission maximum didn't significant shift. It indicated that the conformation of LYSO was changed,the polarity around the Trp residues was increased and the hydrophobicity was decreased. This may be due to the changes of residue microenvironment with the interacion of CID.

Type of interaction force in LYSO–CID systems

Generally, the interaction forces between the small drug molecule and biological macromolecule include

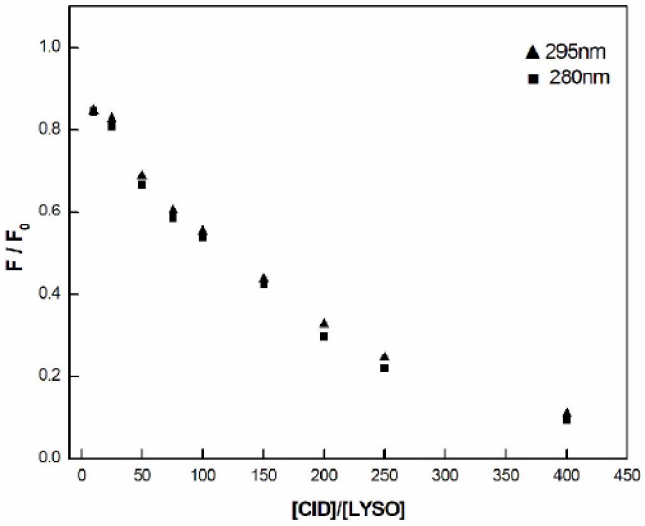

Figure 3 : Fluorescence emission spectra of LYSO-CID (T =298K);C_{LYSO} = 4.0×10^{-7} mol·L^{-1}, C_{CID}=4.0×10^{-6}~ 1.6×10^{-4} mol·L^{-1}

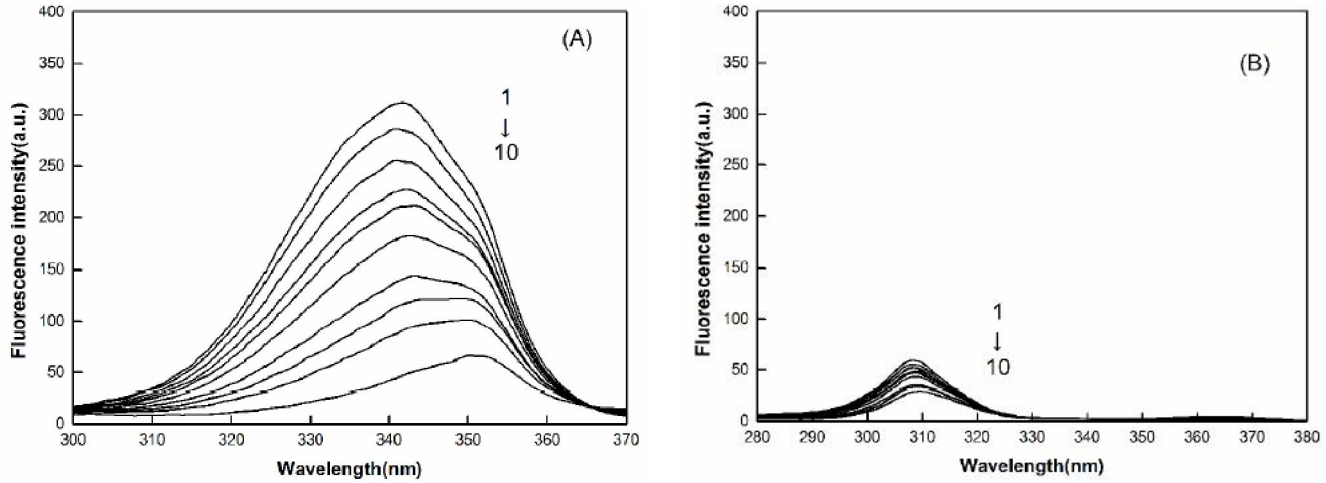

Figure 4 : Fluorescence spectrum of LYSO-CID s ystem(T = 298 K) (A)Δλ=60nmÿ(B)Δλ=15nm C_{LYSO} = 4.0×10^{-7} mol·L^{-1}; 1~10 C_{CID} = (0, 0.4, 1.0, 2.0, 3.0 4.0, 6.0, 8.0, 10, 16)×10^{-5} mol·L^{-1}

TABLE 2 : The thermodynamic parameters of LYSO-CID system at different temperatures in two ways

	T /(K)	K_a /(L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS/(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
λ_{ex}=280nm	298	5.01×10^4	-13.17	45.77	-26.81
	310	4.12×10^4		45.86	-27.39
	318	3.58×10^4		45.76	-27.72

hydrogen bond, Van der Waal's force, electrostatic interactions and hydrophobic force[17]. For a binding interaction, the nature of interaction forces between quencher and biomacromolecule can be learned from the thermodynamic parameters. Negative ΔH and positive ΔS indicate electrostatic interaction plays a major role in the binding reaction. Positive ΔH and ΔS are generally considered as the evidence for typical hydrophobic interactions. In addition, Van der Waal's force and hydrogen bonding formation in low dielectric media are characterized by negative values of ΔH and ΔS[18]. The thermodynamic parameters can be calculated on the basis of the following equation[19]:

$$\mathbf{R \ln K = \Delta S - \Delta H / T} \quad (4)$$

$$\mathbf{\Delta G = \Delta H - T\Delta S} \quad (5)$$

When temperature varies in a small range, the ΔH could be considered as a constant[20]. According to the binding constants K_a of CID to LYSO at different temperatures above (TABLE 1), the thermodynamic parameters were obtained conveniently. The calculated results were shown in TABLE 3. The negative values of ΔG indicated that the binding processes occurred spontaneously in all studied cases. The negative value of ΔH and positive value of ΔS showed that electrostatic attraction played a main role in the binding of CID to LYSO.

Hill's coefficient of LYSO–CID system

In biochemistry, the binding of a ligand molecule at one site of a macromolecule often influences the affinity for other ligand molecules at additional sites. This is known as cooperative binding. It is classified into positive cooperativity, negative cooperativity and non-cooperativity according to the promotion or inhibition to the affinity for other ligand molecules. Hill's coefficient provides a way to quantify this effect and is calculated graphically on the basis of the following equation[21]:

$$\mathbf{lg\frac{Y}{1-Y} = lg K + n_H \ lg[L]} \quad (6)$$

Where Y is the fractional binding saturation; K is the binding constant and n_H is the Hill's coefficient. Hill's coefficient is greater than 1, which exhibits positive cooperativity. Conversely, Hill's coefficient is less than 1, which exhibits negative cooperativity. A coefficient of 1 indicates non-cooperative reaction.

For fluorescence measurement:

$$\mathbf{\frac{Y}{1-Y} = \frac{Q}{Q_m - Q}} \quad (7)$$

Where $Q=(F_0-F)/F_0$; $1/Q_m$=intercept of the plot 1/Q versus 1/[L]. Hill's coefficients were presented in TABLE 4. As seen in TABLE 4, the values of n_H were slightly more than 1 in the systems at different temperatures, which indicated positive cooperativeness in the interaction of CID with LYSO, but they were weak. In addition, the values of n_H were inversely correlated with increasing temperature, it is illustrated that the ability of drug bounding to LYSO was decreased with the previous ligand (CID) bounding to LYSO gradually. It was also one of the reasons which led to the reduced K_a with increasing temperature[22].

TABLE 3 : Hill coefficient of LYSO-CID system at different temperatures

T/K	λ_{ex} =280 nm		λ_{ex} =295 nm	
	n_H	r_3	n_H	r_3
298	1.058	0.9956	1.048	0.9929
310	1.035	0.9963	1.032	0.9936
318	1.021	0.9927	1.015	0.9912

r_3 is the linear relative coefficient of lg[*Y*/(1-*Y*)]~lg[*L*]

CONCLUSIONS

The interaction of CID with LYSO has been investigated by using fluorescence quenching and synchronous fluorescence spectroscopy under simulated physiological conditions in this work. It was found that the quenching of LYSO fluorescence by CID takes place with the complex formation between the protein and CID with 1:1 binding ratio. Electrostatic interaction played important role in the conformational changes

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during the binding process. This study is expected to provide important insight into the interactions of the important LYSO with drugs, and it will be significant for pharmacy,pharmacology and biochemistry.

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