Interactions of Lysozyme with 6-Amino-4-aryl-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitriles: a Fluorescence Quenching Study

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The interactions between 6-amino-4-aryl-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile and lysozyme (LYSO) were investigated by using tryptophane fluorescence quenching and 6-amino-4-(2-hydroxyphenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile (**1**) was studied in detail because of its high water solubility. At different temperatures, the quenching constants K_{sv} , the binding constants K and the binding sites n of LYSO with **1** were determined and the thermodynamic parameters were calculated. The distance r between tryptophane residues and **1** was obtained according to the Forster mechanism of non-radiation energy transfer. Furthermore, synchronous fluorescence spectroscopy data indicated that the association between **1** and LYSO changed LYSO's conformation and that the hydrophobic interaction played a major role in **1**-LYSO association. It was proved that the fluorescence quenching of LYSO by **1** was related to the formation of a **1**-LYSO complex and to a non-radiation energy transfer.

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Introduction

Drugs bring the action of medicine *via* the storage and transport of blood plasma and proteins can combine with many antibiotics. Therefore, it is important to study the interactions between drugs and proteins. The interactions between drugs and human serum albumin (HSA) have already been widely reported,^{1,2} but similar studies about lysozyme (LYSO) were very infrequent. LYSO is formed by 129 tactic amino residue and contains 6 tryptophanes and 3 tyrosines³ and is used to treat inflammation, abscess, stomatitis and rheum, *etc.* *via* the binding with antibiotics in clinic. So, its favorable cooperative and synergistic actions with antibiotics show a very important practical value in the medical area. However, studies on LYSO have mainly focused on its separation and purification. Therefore, studies on the interactions between small molecules and LYSO in different aspects will help to explain the transport and metabolism processes of the small molecules, the relation of structure and function of LYSO, and the chemical essences of the interactions between biomacromolecules and small molecules.

The structures containing the pyrazolo[3,4-b]pyridine system have shown many interesting biological and pharmacological properties such as antitubercular activity,⁴ active antagonistic of adrenocorticotrophic hormone (ACTH) and corticotropin releasing factor (CRF).⁵ It was proved that they could also cure several diseases such as hypochondrium, gastroenteritis, anorexia nervosa, and irritable bleeding.^{6,7}

In this work, 6-amino-4-aryl-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitriles (Fig. 1) was synthesized, and their interactions with LYSO were subsequently studied.

The results indicated that all of these compounds could quench the fluorescence emission from LYSO and 6-amino-4-(2-hydroxyphenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile (**1**) was studied in detail because of its high water solubility. At different temperatures, the binding distance between acceptor and donator and the mechanism were determined, the binding constants and sites were also calculated. Those results provide useful clinical information and aid the usage of drugs *via* the reaction mechanisms from the molecular level.

Experimental

Reagent and apparatus

LYSO ($\geq 99\%$) were purchased from Sigma. 6-Amino-4-aryl-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitriles were synthesized from aromatic aldehyde, malononitrile and 3-methyl-1-phenyl-1H-pyrazol-5-amine according to the

Fig. 1 Molecular structure of 6-amino-4-aryl-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitriles. R = H, 2-OH, 4-NO₂, 4-CN, 4-Br, 4-Cl, 4-CH₃, 2-Cl, 4-OCH₃, 3-NO₂, 4-N(CH₃)₂.

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Fig. 2 Fluorescence quenching spectra of LYSO ($T = 290$ K). Concentrations: LYSO, 1.0×10^{-5} mol/L; **1**, 1.0×10^{-5} mol/L. 1–13: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4.

Fig. 3 Fluorescence quenching F_0/F -[Q] figure of LYSO.

literature⁸ (recrystallized 2 times with 95% ethanol, normalized purity (HPLC) 99.50–99.86%). The Tris(hydroxymethyl-aminomethane) buffer (> 99.5%) and other agents were of analytical purity. Redistilled water was used throughout.

An F-4500 spectrophotometer (Hitachi) was used; the slit width was 5 nm. Cell dimensions are $1 \times 1 \times 4$ cm³. The absorption spectrum was measured with a Tu-1201 ultraviolet spectrophotometer (Shimadzu). The pH values were measured with an Aiwang (Shanghai, China) pH meter.

General process

The following reagents were added into a 10 mL tube in the order indicated: 1.0 mL of protein (1.0×10^{-4} mol/L), adequate **1** and 2.0 mL of Tris-HCl (0.05 mol/L, pH 7.4) buffer solution. After adding 0.9% NaCl aqueous solution (to control ionic intensity) to 10 mL tube and mixing, we measured the absorption spectra and fluorescence spectra at 290, 300, 310, 318 and 324 K. Fluorescence measurements were taken at excitation and emission wavelengths of 281 and 300–500 nm, respectively.

Principles of fluorescence quenching

Fluorescence quenching is described by the well-known Stern-Volmer equation:⁹

$$F_0/F = 1 + K_{SV}[Q]$$

Table 1 Quenching constants K_{SV} , binding constants K and binding sites n for the interaction of LYSO with **1**

pH	T/K	$K_{SV}/\times 10^{-4} \text{ L mol}^{-1}$	$K/\times 10^{-5} \text{ L mol}^{-1}$	n
7.4	290	4.409	5.65	1.24
	300	4.277	7.47	1.27
	310	4.005	3.16	1.20
	318	3.809	2.79	1.19
	324	3.735	2.61	1.19

Table 2 Relative thermodynamic parameters of the system of **1**-LYSO

T/K	$\Delta G/\text{kJ mol}^{-1}$	$\Delta H/\text{kJ mol}^{-1}$	$\Delta S/\text{J mol}^{-1}$
290	-31.93	0	110.1
300	-33.73		112.4
310	-32.64		105.3
318	-33.15		104.2
324	-33.60		103.7

where F_0 and F denote the steady-state fluorescence intensities in the absence and in the presence of quencher **1**, respectively, K_{SV} is the Stern-Volmer quenching constant, and $[Q]$ is the concentration of the quencher. Hence, the equation was applied to determine K_{SV} by linear regression of a plot of F_0/F against $[Q]$.

Results and Discussion

Fluorescence quenching spectra

The fluorescence quenching spectra of LYSO with varying concentrations of **1** is shown in Fig. 2.

We found that the endogenic fluorescence of LYSO was reduced by the gradual concentration augment of **1**; the emission peak also red-shifted continuously. Such results indicated that there were interaction and energy transfer between them. To discuss the results within the linear concentration range, we carried out experiments about the linear part of the Stern-Volmer dependence (F_0/F against $[Q]$). In such experiments, concentrations of LYSO solution were stabilized at 1.0×10^{-5} mol/L, and the concentration of **1** varied from 0 to 2.4×10^{-5} mol/L. The fluorescence quenching Stern-Volmer figures of LYSO with **1** are shown in Fig. 3 at different temperatures: 290, 300, 310, 318 and 324 K. The curves had excellent linear relations ($r = 0.9983, 0.9985, 0.9986, 0.9967, 0.9972$), and the quenching constant K_{SV} decreased with increasing temperature (Table 1). And if the quenching phenomenon of the style is dynamic quenching, k_q (rate constant in quenching proceeding) should far more than $2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$.⁹ The data showed an opposite result.

So we suppose the quenching reason was because a new stable complex which has no fluorescence was formed with a static quenching process:

$$\log(F_0 - F)/F = \log K + n \log [Q]$$

The binding constants K and binding sites n could be calculated from Fig. 4 ($r = 0.9977, 0.9996, 0.9977, 0.9991, 0.9986$), and the results calculated from Table 1 indicated that it has approximately one binding site after a strong binding between **1** and LYSO.

Fig. 4 Plots of $\log[(F_0 - F)/F] - \log[Q]$.

Establishing the pattern of interaction force

To elucidate the interaction between **1** and LYSO, we calculated the thermodynamic parameters:

$$\Delta H = R(T_2 - T_1) \cdot \ln(K_2/K_1)/T_1 T_2 \quad (1)$$

$$\Delta G = -RT \cdot \ln K \quad (2)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (3)$$

If ΔH is approximately a constant, we can calculate ΔH , ΔG , ΔS easily (Table 2). The negative values of free energy (ΔG) supported the assertion that the binding process was spontaneous. The enthalpy change (ΔH) and positive entropy (ΔS) values of the interaction between **1** and LYSO indicated that hydrophobic interaction played a major role in the binding reaction.¹⁰

Energy transfer from LYSO to **1**

Electron energy transfer includes radiation energy transfer and non-radiation energy transfer.¹¹ According to the Forster mechanism of non-radiation energy transfer,¹² the distance r between **1** and LYSO (tryptophan residues) could be calculated by the equations:

$$E = 1 - F/F_0 \quad (4)$$

$$E = R_0^6 / (R_0^6 + r^6) \quad (5)$$

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \quad (6)$$

$$J = \int F(\lambda) \xi(\lambda) \lambda^4 d\lambda / \int F(\lambda) d\lambda \quad (7)$$

E denotes the efficiency of transfer between the donor and the acceptor, and R_0 is the critical distance when the efficiency of transfer is 50%. K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles, and $K^2 = 2/3$ for random orientation as in fluid solution; N is the average refracted index of the medium in the wavelength range where spectral overlap is significant; Φ is the fluorescence quantum yield of the donor; and J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which can be calculated by Eq. (7). $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range from λ to $\lambda + \Delta\lambda$; $\xi(\lambda)$ is the extinction coefficient of the acceptor at λ .

Fig. 5 Spectral overlap of **1** absorption (a) with LYSO fluorescence (b). $T = 290$ K. Fig. 6 Synchronous fluorescence spectra of LYSO ($T = 290$ K, $\Delta\lambda = 60$ nm). Concentrations: LYSO, 1.0×10^{-5} mol/L; **1**, 1.0×10^{-5} mol/L. 1 - 13: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4.

In general, a transfer of energy happens under these conditions: (i) the donor can produce fluorescence light; (ii) fluorescence emission spectrum of the donor and UV-vis absorbance spectrum of the acceptor have more overlap; and (iii) the distance between donor and acceptor is getting closer and is lower than 8 nm.¹³

The overlapping spectra of absorption spectra of **1** (1:LYSO = 1:1) with fluorescence spectra of LYSO are shown in Fig. 5. So J could be calculated by Eq. (7). Under these experimental conditions, we found $J = 1.01 \times 10^{-13} \text{ cm}^3 \text{ L mol}^{-1}$, $R_0 = 3.60$ nm using $K^2 = 2/3$, $N = 1.336$, $\phi = 0.118$,¹² and the energy transfer effect $E = 0.074$, $r = 5.49$ nm. The average distance between **1** and the tryptophane residues on the 2 to 8 nm and $0.5R_0 < r < 1.5R_0$,¹⁴ indicated that the non-radiation energy transfer¹¹ from LYSO to **1** occurred with high probability.

Conformation investigation

The effect of **1** on LYSO synchronous fluorescence spectroscopy is shown in Fig. 6. It was apparent from Figs. 6 and 2 that $\lambda_{\text{max(em)}}$ and $\lambda_{\text{max(ex)}}$ red-shifted (281 to 286 nm, 339 to 346 nm) at the investigated concentration range when $\Delta\lambda = 60$ nm. The red-shift of the emission maximum suggested a more polar (or less hydrophobic) environment of the tryptophane residues. It showed that the association between **1** and LYSO changed molecular conformation of LYSO.

Conclusions

The interaction between **1** and LYSO has been verified as consistent with the static quenching procedure. We proved that the fluorescence quenching of LYSO by **1** was related to the formation of a **1**-LYSO complex and the non-radiation energy transfer. And the $\lambda_{\text{max(em)}}$ red-shifted progressively at the investigated concentration range when $\Delta\lambda = 60$ nm, which suggested a more polar (or less hydrophobic) environment of the tryptophane residues. The thermodynamic parameters $\Delta G < 0$, $\Delta H = 0$, and $\Delta S > 0$ at different temperatures indicated that the binding process was spontaneous and that the hydrophobic interaction was a major factor.

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