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# Interaction of Lysozyme with 6-Amino-4-aryl-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole: A Fluorescence Auenching Study

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The interactions between 6-amino-4-aryl-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole and lysozyme (LYSO) were studied by using tryptophan fluorescence quenching and 6-amino-4-(2-hydroxyphenyl)-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c] pyrazole (**1**) was studied in detail because of its best quenching efficiency and biggest water solubility. At different temperatures, quenching constants $K_{SV}$, binding constants K and binding sites n of LYSO with **1** were determined and 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 suggested that the association between **1** and LYSO changed the molecular conformation of LYSO and the hydrophobic interaction played a major role. It was proved that the fluorescence quenching of LYSO by **1** was related to the formation of a 1-LYSO complex and the nonradiation energy transfer.

**Key Words: Lysozyme, 6-Amino-4-aryl-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole, Fluorescence quenching, Non-radiation energy transfer.**

## INTRODUCTION

Drugs bring the action of medicine *via* the store and transport of blood plasma and protein can combine with many drugs. Therefore, it is important to study the interactions between drugs and protein. Earlier studies, showing the interactions between small molecules and human serum albumin (HSA)[1], revealing the interactions between small molecules and lysozyme (LYSO)[2], have established the generation of an immunomodulatory complex as a result of the interaction between LYSO and 4*H*-pyran derivatives[3]. But, the detailed biophysical aspect of interaction between LYSO and 4*H*-pyran derivatives has not yet been reported. In this study, an attempt is made to explore the mechanism of interaction between LYSO and 4*H*-pyran derivatives using mainly fluorescence quenching.

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4*H*-Pyran derivatives have many biological activities and pharmacology activities. They are the structural units of natural products which have been reported on anti-cancer and antianaphylactin[4]. In this work, 6-amino-4-aryl-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole (Fig. 1) was synthesized and studied the interactions of them to LYSO. Due to the best quenching efficiency and biggest water solubility, 6-amino-4-(2-hydroxyphenyl)-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole (**1**, Fig. 1) was studied in detail. The results indicated that it can quench the fluorescence of LYSO by forming a new complex and non-radiation energy transfer. In the experiment the binding mechanism was discussed, binding distance of acceptor and donor was determined and meanwhile the quenching constants, binding constants, binding sites and thermodynamic parameters at different temperatures were calculated. Those results provide useful clinical information to the compatibleness and usage of small molecules *via* the reaction mechanism from molecule level.

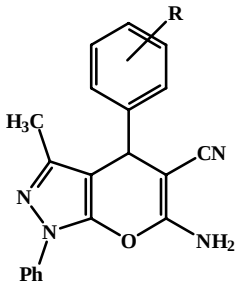


Fig. 1. Molecular structure of pyrazole
[R = H, 2-OH(1), 4-$NO_2$, 4-CN, 4-Br, 4-Cl, 4-$CH_3$, 2-Cl]

## EXPERIMENTAL

Lysozyme (≤ 99 %) was purchased from Sigma Company. 6-Amino-4-aryl-5-cyano-3-methyl-1-phenyl-1,4-dihydropyrano[2,3-c]pyrazole was synthesized from aromatic aldehyde, malononitrile and 5-amino-3-methyl-1-phenylpyrazole according to the literature[5] (recrystallized 2 times with 95 % ethanol, normalized purity (HPLC) 99.50 %) and dissolved by DMF:$H_2O$ = 1:19. The *tris* ((hydroxymethyl)amino-methane) buffer had a purity of *ca.* 99.5 % and NaCl, HCl, *etc.* were all of analytical purity. The samples were dissolved in *tris*-HCl buffer solution (0.05 mol/L *tris*, 0.9 % NaCl, pH 7.4). Redistilled water was used. An F-4500 spectrophotometer (Hitachi) was used and the cell dimension is 1 × 1 × 4 $cm^3$, the slit width was 5 nm. The absorption spectrum was measured by Tu-1201 ultraviolet spectrophotometer (Shimadzu). The pH value was measured by a Aiwang (Shanghai) pH meter.

The following reagents were added into a 10 mL tube in the order indicated: 1.0 mL of protein ($1.0 \times 10^{-4}$ 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 10 mL tube and mixed, fluorescence spectra were respectively measured at 287, 296, 303, 311 and 319 K.

## RESULTS AND DISCUSSION

**Fluorescence quenching spectra:** Fluorescence quenching is described by the well-known Stern.Volmer equation[6]:

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

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].

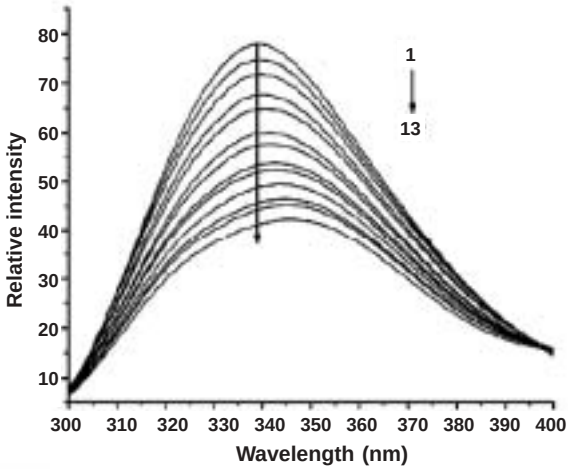


Fig. 2. The influence of different concentration of **1** on the fluorescence spectra of LYSO (T = 287 K); c(LYSO) = $1.0 \times 10^{-5}$ mol/L; c(**1**)/(10-5 mol/L):0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0,2.2, 2.4, 2.6

The fluorescence quenching spectra of LYSO were shown in Fig. 2. It is found that the endogenic fluorescence intensity reduced regularity by the gradual concentration augment of **1** and the emission peak blue-shifted ceaselessly. It indicated that there were interaction and energy transfer between them. To discuss the results within the linear concentration range, we carried out the experiment within the linear part of Stern-Volmer dependence ($F_0/F$ against [Q]). In this experiment, the concentration of LYSO solution was stabilized at $1.0 \times 10^{-5}$ mol $L^{-1}$ and the concentration of **1** varied from 0 to $2.6 \times 10^{-5}$ mol $L^{-1}$ and the Stern-Volmer figure is linear at the the concentration of **1** from 0 to $1.8 \times 10^{-5}$ mol $L^{-1}$, respectively. At different temperature 287, 296, 303, 311 and 319 K the fluorescence quenching Stern-Volmer figures of LYSO with **1** were shown in Fig. 3. It indicated that two curves had linear relation (r = 0.9951, 0.9980, 0.9984, 0.9984, 0.9980), and the quenching constants $K_{SV}$ decreased with increasing temperature (Table-1). And if the quenching phenomenon of the style was dynamic quenching, $k_q$ (rate constant in quenching proceeding) should far more than[7] $2.0 \times 10^{10}$ L $mol^{-1}$ $s^{-1}$. The result of this calculation was opposite.

TABLE-1
QUENCHING CONSTANTS $K_{SV}$, BINDING CONSTANT K AND BINDING SITES n OF LYSO WITH **1**

| pH | T (K) | $10^{-4}$ $K_{SV}$ (L $mol^{-1}$) | $10^{-5}$ K (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 |

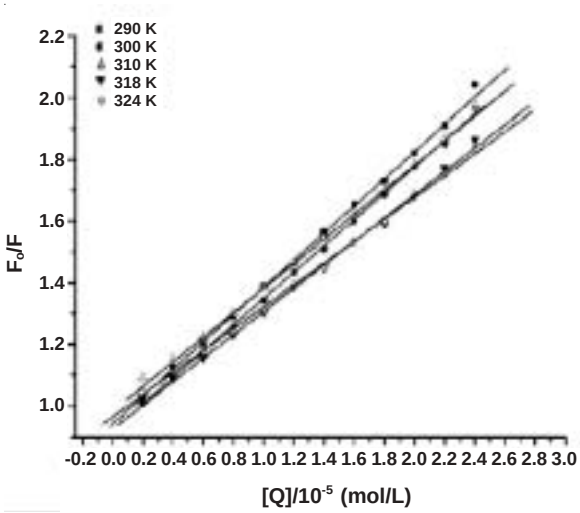


Fig. 3. Fluorescence quenching of LYSO by **1** in different temperatures (Stern-Volmer figure)

So the quenching reason was that a new steady complex which has no fluorescence was formed and it was a static quenching proceeding with the equation:

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

The binding constants K and binding sites n could be calculated from Fig. 4 (r = 0.9979, 0.9982, 0.9995, 0.990, 0.9994) and the calculated results (Table-1) indicated that it approximately had one binding site after a strong binding between **1** and LYSO.

**Establishing the pattern of interaction force:** To elucidate the interaction between **1** and LYSO, the thermodynamic parameters were calculated.

$$\Delta H = R(T_2\text{-}T_1)Ln(K_2/K_1)/T_1T_2 \quad (3)$$

$$\Delta G = -RTLnK \quad (4)$$

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

If the enthalpy change (H) is approximately a constant which is not changed along with temperature, we can calculate the results of enthalpy change (ΔH), energy

change (ΔG), positive entropy (ΔS) (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 of **1** with LYSO indicated that the hydrophobic interaction played a major role in the binding reaction[8].

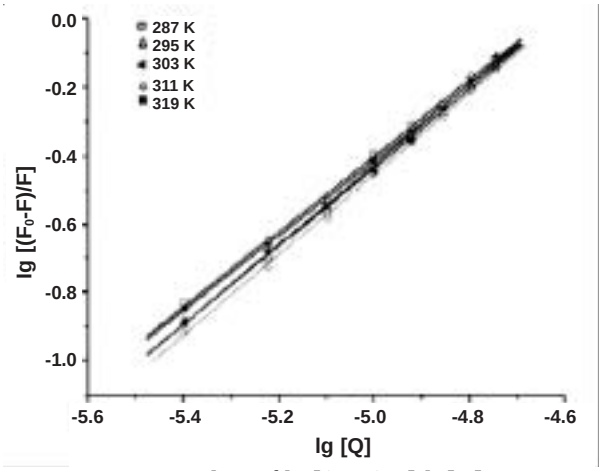


Fig. 4. Plots of lg [($F_0$-F)/F]-lg[Q]

TABLE-2
RELATIVE THERMODYNAMIC PARAMETERS OF LYSO-1 SYSTEM

| T (K) | ΔG (KJ $mol^{-1}$) | ΔH (KJ $mol^{-1}$) | ΔS (J $mol^{-1}$) |
|---|---|---|---|
| 287 | -29.64 | | 103.3 |
| 296 | -29.26 | | 98.9 |
| 303 | -29.78 | 0 | 98.3 |
| 311 | -31.72 | | 102.0 |
| 319 | -33.28 | | 104.3 |

**Energy transfer from LYSO to 1:** Electron energy transfer includes radiation energy transfer and non-radiation energy transfer[9].

According to the Forster mechanism of non-radiation energy transfer[10], the distance r between **1** and (tryptophane residues) could be calculated by the equation:

$$E = 1-F/F_0 \quad (6)$$

$$E = R_0^6/(R_0^6+r^6) \quad (7)$$

$$R_0^6 = 8.8 \times 10^{-25}\ K^2N^{-4}\ \Phi J \quad (8)$$

$$J = \Sigma F(\lambda)\xi(\lambda)\lambda^4\Delta\lambda/\Sigma F(\lambda)\Delta\lambda \quad (9)$$

where 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 the eqn. 9. F(λ) is the corrected fluorescence intensity of the donor in the wavelength range from λ to λ+λ; ξ(λ) is the extinction coefficient of the acceptor at λ.

In general, a transfer of energy happens under 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 approached and is lower than 8 nm[11].

The overlapping spectra of the absorption spectra of **1** and LYSO (**1**: LYSO = 1:1) and the fluorescence spectra of LYSO were shown in Fig. 5. So J can be evaluated. Under these experimental conditions[10], we found $J = 5.58 \times 10^{-13}$ cm$^3$ L mol$^{-1}$, $R_0$ = 4.79 nm using $K^2 = 2/3$, N = 1.336, φ = 0.118 and the energy transfer effect E = 0.216, r = 5.94 nm. The average distance between **1** and the tryptophane residues on the 2 to 8 nm and 0.5 $R_0$ < r < 1.5 $R_0$[8] indicated that the non-radiation energy transfer[9] from LYSO to **1** occured with high probability.

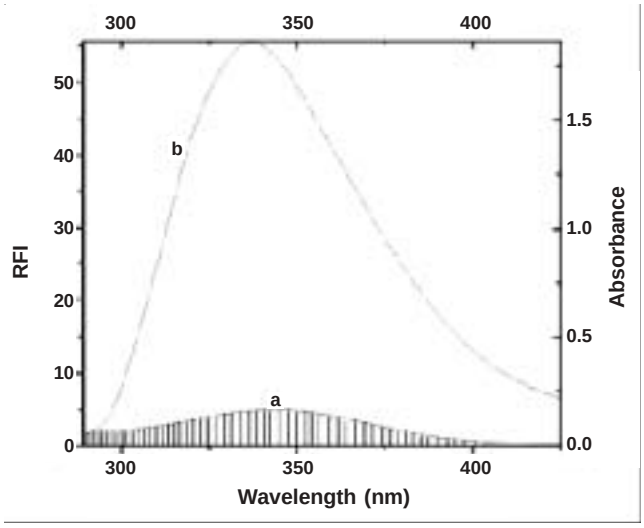


Fig. 5. Overlap of absorption of **1** (a) with LYSO's fluorescence emission (b)

**Conformation investigation:** The effect of **1** on LYSO synchronous fluorescence spectroscopy was shown in Fig. 6. It was apparent from the data of Fig. 6 and Fig. 2 that $\lambda_{max(ex)}$ and $\lambda_{max(em)}$ blue-shifted (280→282 nm, 339→337 nm) at the investigated concentration range when Δλ = 60 nm. The blue-shift of the emission maximum suggested a more polar (or less hydrophobic) environment of the tryptophane residues. It showed the association between **1** and LYSO did change molecular conformation of LYSO.

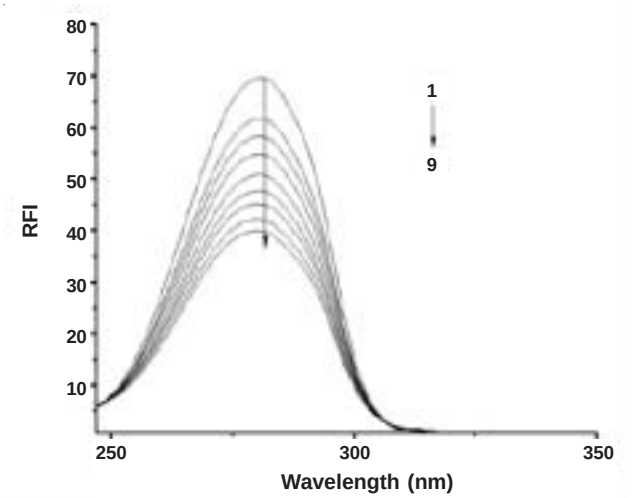


Fig. 6. Synchronous fluorescence spectrum of LYSO (T = 287 K, $\Delta\lambda$ = 60 nm)
c(LYSO) = $1.0\times10^{-5}$ mol/L; c(**1**)/($10^{-5}$ mol/L): 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8

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