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ORIGINAL PAPER

Study on the Interaction Mechanism of Lysozyme and Bromophenol Blue by Fluorescence Spectroscopy

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Abstract The interaction of lysozyme with bromophenol blue (BPB) in acetate buffer (pH 6.0) was studied by fluorescence quenching method for the first time. It was found that BPB could conspicuously quench the fluorescence of lysozyme by the static quenching process, possibly due to the binding on the active site near Trp62. The binding parameters including the binding constant and the number of binding site were calculated. The thermodynamic parameters $\Delta H°$, $\Delta S°$ and $\Delta G°$ at different temperatures were obtained. The formation of lysozyme–BPB complex depended on the cooperation of the hydrophobic and electrostatic forces. And the binding average distance between lysozyme and BPB was determined. The effect of common metal ions on the binding constant of lysozyme–BPB was also examined.

Keywords Lysozyme · Bromophenol blue · Fluorescence quenching

Introduction

Lysozyme is a compact globular protein that takes part in the first barrier of defense. As an enzyme it is possible to study the functionality of lysozyme through its lytic activity. Lysozyme, with low molecular weight (14.4 kDa) and high stability, owning six tryptophan and three tyrosine residues in its structure, is a photobiological active protein [1, 2]. The combination of these factors makes lysozyme an ideal macromolecule for studying the influence of preferential interactions on its activity [3]. The photobiological investigations on the interaction of lysozyme with different small molecules were reported, including organometallic complexes [4, 5], Ni^{2+} [6], Red-120 and Blue-4 reactive dyes [7], 4-(2-pyridylazo)-resorcinol [8], singlet oxygen [9] and tetracycline analogue drugs [10]. The binding of metacycline [5] and tetracycline [10] to lysozyme was studied by fluorescence quenching procedure. It was showed that the interaction between lysozyme and small molecules attributed to the formation of nonradiative compounds, in which the binding parameters and binding average distance were given.

The interaction of lysozyme with bromophenol blue (BPB) was also reported, in which the binding site of BPB on lysozyme was studied by different spectral methods such as ^1H NMR [11, 12], X-ray [13], ultraviolet and circular dichroism [14]. In this work, the interaction between lysozyme and BPB was studied using fluorescence quenching method, and it was found that lysozyme could be bound by BPB forming nonradiative complex. The interaction parameters including mode of interaction, association constant and number of binding site were evaluated. The thermodynamic parameters $\Delta H°$, $\Delta S°$ and $\Delta G°$ at different temperatures were calculated. The binding constant of lysozyme–BPB was decreased in the presence of metal ions. The distance from the tryptophan residue (donor) to the bound BPB (acceptor) in lysozyme was obtained according to Förster nonradiative energy transfer theory for the first time.

Experimental section

Materials

Lysozyme was obtained from Sigma Chemical Company (St. Louis, USA). BPB was purchased from Xi'an Chemical Reagent Plant (Xi'an, China) in the study. The stock so-

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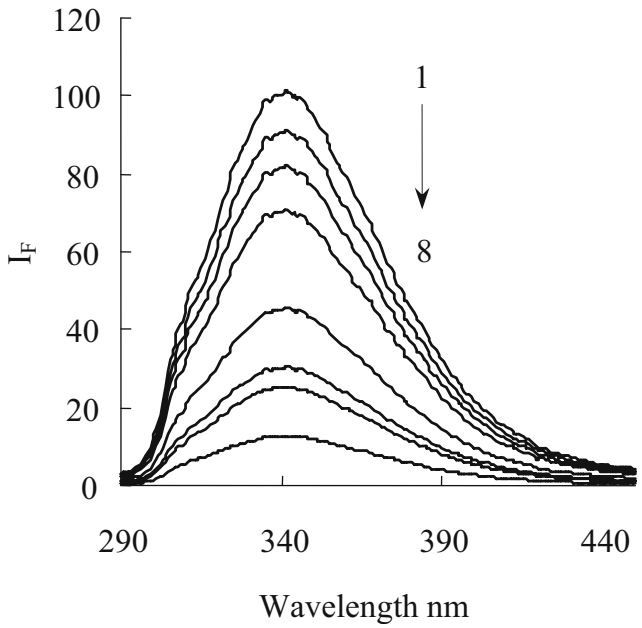

Fig. 1 The fluorescence spectra of lysozyme with increasing concentration of BPB: *1* 0 μmol l^{-1}, *8* 20 μmol l^{-1}; lysozyme 0.1 μmol l^{-1}

lutions of lysozyme (50 μmol l^{-1}) and BPB (1 mmol l^{-1}) were prepared in dark glass bottles, respectively. Acetate buffer (pH 6.0) and NaCl solutions were used to adjust the acidity and ion strength of the system. Solutions of common metal ions (1 mmol l^{-1}) were prepared by $CuSO_4$, $Fe(NO_3)_3$, $MgCl_2$, $CrCl_3$, $CoCl_2$, $NiCl_2$, $ZnSO_4$ and $AlCl_3$, respectively, and the final concentration in fluorescence titration was 10 μmol l^{-1}. All materials were of analytical reagent grade unless specialized and doubly deionized water made by Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

Apparatus

A Hitachi F-4500 fluorophotometer (Tokyo, Japan) was applied for the fluorescence measurements, with 5.0/5.0 nm slit width and 700 V PMT. A Lambda-40 UV–Vis spectrophotometer (Perkin Elmer, USA) was employed for acquiring the absorption spectra. A pHs-3C digital pH meter (Shanghai Leici Device Works, China) was used for pH measurements. The temperature of the solutions was controlled (T±0.1°C) in a water bath.

Procedures

At room temperature, 1.0 ml acetate buffer solution, appropriate volume of lysozyme and a series of BPB standard solutions were added to the 10.0 ml comparison color tube in order, diluted by doubly deionized water and homogenized for determination. The fluorescence emission spectra were obtained in the range of 290–450 nm (λ_{max} 340 nm) with excitation wavelength at 280 nm under different temperatures 290, 298, 308, and 318 K.

Results and discussion

Fluorescence spectra of lysozyme–BPB

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions including excited-state reaction, molecular rearrangement, energy transfer, forming ground state complex and collisional quenching, which is called fluorescence quenching. The fluorescence spectra of lysozyme–BPB were shown in Fig. 1, with λ_{max} 340 nm. And it was obvious that the fluorescence intensity (IF) of lysozyme was decreased regularly with increasing concentration of BPB (0.05–20 μmol l^{-1}) but no wavelength shift on the peak occurred. It was reported that 80% of lysozyme fluorescence was due to Trp62 and Trp108, and the oxidation of either Trp62 or Trp108 was accompanied by a drastic decrease in fluorescence intensity. In addition, Trp62 emission was in a higher proportion than that of Trp108 when lysozyme molecule was excited at 280 nm [15]. Therefore it was reasonably proposed that BPB quenched the fluorescence of both Trp62 and Trp108 residues of lysozyme.

Binding constant and number of binding site for lysozyme–BPB

According to the Stern–Volmer law [16], the quenching equation is:

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

where F_0 and F is the fluorescence intensity of fluorophore in the absence and in the presence of quencher, respective-

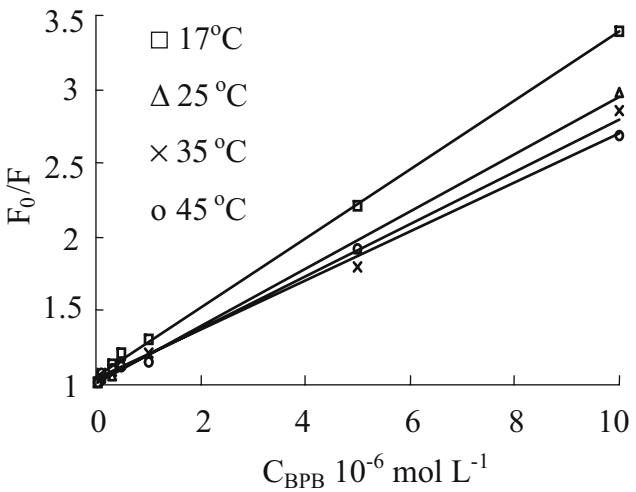

Fig. 2 Plots of F_0/F vs BPB concentration under different temperatures. lysozyme 0.1 μmol l^{-1}

Table 1 The dynamic quenching constant of lysozyme–BPB

Temperature (°C)	$K_q/10^{13}$ (l mol^{-1}s^{-1})	Linear coefficient (R)
17	2.35±0.01	0.9990
25	1.95±0.01	0.9986
35	1.78±0.02	0.9975
45	1.67±0.02	0.9966

ly, [Q] the concentration of quencher, K_{SV} the Stern–Volmer constant corresponding to the slope of the plot for F_0/F vs [Q], K_q the quenching rate constant for biomolecule, and τ_0 the average fluorescence lifetime of biomolecule without quencher. The quenching efficiency of BPB on lysozyme fluorescence was tested using 0.05–10.0 μmol l^{-1} BPB at different temperatures, as Fig. 2 showed. It was observed that the Stern–Volmer plot was in a good linearity and the slope decreased with increasing temperature. The interaction between lysozyme and BPB ascribed to the static quenching model. In order to confirm the view, supposing the quenching process to be the dynamic model, K_q was calculated. It was easily obtained

$$K_{SV} = K_q \tau_0 \quad (2)$$

Because the fluorescence lifetime of biomacromolecule was 10^{-8} s [17], the quenching constant K_q was calculated from the slope and listed in Table 1. The maximum of K_q for various quenchers with biomacromolecule is 2×10^{10} l mol^{-1}s^{-1} [18]. Obviously, the values of K_q for lysozyme–BPB quenching procedure were greater than that of the scatter procedure. It was indicated that the quenching procedure of lysozyme–BPB was not initiated by the dynamic collision but forming nonradiative complex. From the equation [19],

$$\log(F_0/F - 1) = \log K + n \log [Q] \quad (3)$$

the binding constant K and the number of binding site n could be calculated, as summarized in Table 2. K value was at 105 level and n value about 0.84, indicating that the binding force of lysozyme–BPB was strong with the binding site of one. According to the analysis of the fluorescence spectra of lysozyme–BPB, it was possible for BPB to quench the fluorescence of Trp62 and Trp108. And combined with n value it was educed that BPB possibly bound the active site near Trp62 in lysozyme.

Table 2 Binding constant and number of binding site for lysozyme–BPB at different temperatures

Temperature (°C)	Binding constant ($K/10^4$)	Number of binding site (n)	Linear coefficient (R)
17	4.02±0.01	0.8456	0.9989
25	3.62±0.01	0.8648	0.9994
35	2.74±0.01	0.8411	0.9985
45	2.17±0.02	0.8277	0.9966

Table 3 Effect of common metal ions on the binding constant of lysozyme–BPB at 298 K

Ion	Binding constant ($K'/10^4$)	Linear coefficient (R)	K'/K
Without	3.62	0.9994	–
Cu^{2+}	0.87	0.9993	0.241
Fe^{3+}	1.01	0.9971	0.279
Mg^{2+}	0.79	0.9982	0.218
Cr^{3+}	1.46	0.9976	0.403
Co^{2+}	0.94	0.9982	0.260
Ni^{2+}	0.54	0.9977	0.149
Zn^{2+}	1.27	0.9984	0.351
Al^{3+}	1.17	0.9982	0.324

Effect of common metal ions on the binding constant of lysozyme–BPB

The influence of the common metal ions on the binding reaction of lysozyme with BPB was examined at 298 K, with the results listed in Table 3. The fluorescence intensity of lysozyme–BPB at 340 nm was decreased in the presence of metal ions Cu^{2+}, Fe^{3+}, Mg^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Al^{3+}. The competition between common metal ions and lysozyme on BPB led to the lysozyme–BPB binding constant dropped by 59.7–85.1%. The results showed that the addition of metal ions reduced the binding of

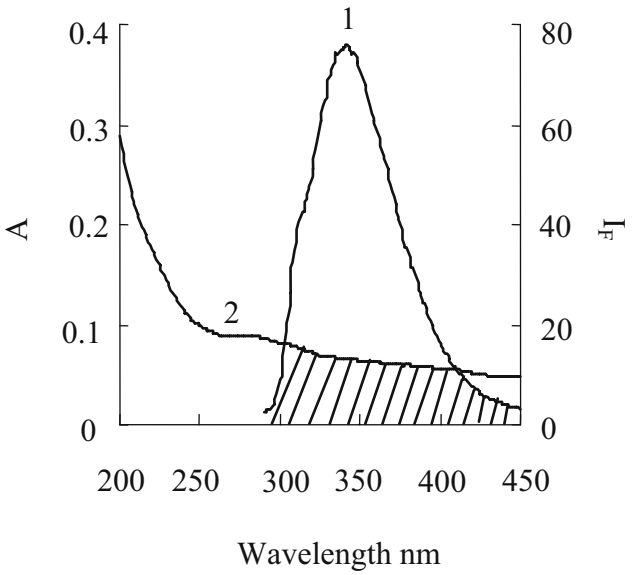

Fig. 3 The overlap of lysozyme fluorescence spectrum *1* and BPB absorption spectrum *2* lysozyme 0.1 μmol l^{-1}; BPB 0.1 μmol l^{-1}

lysozyme–BPB, which would lead to the need for more doses of BPB to achieve the desired effect.

Binding mode of lysozyme–BPB

The acting forces between biomolecule and a dye may include hydrophobic effect, hydrogen bond, van der Waals force and electrostatic attraction and so on. According to the van't Hoff equation,

$$RT\ln K = -\Delta H^\circ + T\Delta S^\circ$$

$$\Delta G^\circ = -RT\ln K = \Delta H^\circ - T\Delta S^\circ \quad (4)$$

ΔH° and ΔS° were obtained as −16.58 kJ mol^{-1} and 31.16 J $K^{-1}mol^{-1}$. It was obvious that $\Delta H^\circ<0$ and $\Delta S^\circ>0$. According to the relationship between the signs of the thermodynamic parameters and the interaction between protein and ligands [20], it was educed that the acting force of lysozyme–BPB was mainly the cooperation of hydrophobic association and electrostatic attraction.

Energy transfer between BPB and lysozyme

From Förster's theory [21], a lot of useful information concerning the molecular details of donor–acceptor can be obtained; for instance, the efficiency of the energy transfer can be used to evaluate the distance between the ligand and tryptophan residue in protein molecule. It was certain that there should be the proper overlap for the emission spectrum of protein (donor) and the absorption spectrum of ligand (acceptor). The energy transfer rate depends on the extent of the overlap, the relative orientation of the donor and acceptor transition dipoles, and the distance between the donor and the acceptor. The energy transfer effect (E) is related not only to the distance (r) between the donor and acceptor but to the critical energy transfer distance (R_0), expressed as following [22, 23]

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (5)$$

where F and F_0 is the relative fluorescence intensity of the donor in the presence and absence of acceptor, respectively; R_0 is a characteristic distance, called the Förster distance or critical distance when the efficiency of energy transfer is 50%; and r is the distance between the donor and acceptor. R_0 can be denoted as below

$$R_0^6 = 8.8\times10^{-25}K^2N^{-4}\Phi J \quad (6)$$

where K^2 is the spatial orientation factor of the dipole, N the refractive index of the medium; Φ is the fluorescence quantum yield of the donor in the absence of acceptor and the overlap integral J expresses the degree of spectral overlap between the donor emission and the acceptor absorption. J can be given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (7)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at the wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient at wavelength λ. The spectral overlap of the fluorescence emission spectrum of lysozyme and the absorption spectrum of BPB was shown in Fig. 3. So J can be evaluated by integrating the spectra in Fig. 3 for λ=290–450 nm of 1.67×10^{-14} cm^{-3} l mol^{-1}. In this case, R_0 was calculated at 2.71 nm from Eq. 5 using K^2=2/3, N=1.336 and Φ=0.14 [24], the energy transfer effect E=0.152, and the distance between Trp62 residue and the bound BPB in lysozyme was obtained r=3.62 nm. The donor-to-acceptor distance r was less than 7 nm, and nonradiative energy transfer was detected [25]. In the present study, $0.5R_0<r<1.5R_0$ was observed, which revealed the presence of the static quenching mechanism to a large extent [26, 27].

Conclusions

It was provided an approach for studying the binding of BPB to lysozyme. The results showed that lysozyme fluorescence was quenched by BPB through static quenching process. The spectral data revealed the conformational changes of lysozyme upon interaction with BPB. The decrease in the binding constant of lysozyme–BPB was observed when metal ion was added. The distance between tryptophan residue and bound BPB in lysozyme was given for the first time.

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