J Fluoresc (2009) 19:783–791
DOI 10.1007/s10895-009-0475-x

ORIGINAL PAPER

A Study of the Interaction Between Malachite Green and Lysozyme by Steady-State Fluorescence

Fei Ding · Wei Liu · Feng Liu · Zhi-Yuan Li · Ying Sun

Received: 21 November 2008 / Accepted: 23 February 2009 / Published online: 31 March 2009

Abstract The interaction of a *N*-methylated diaminotriphenylmethane dye, malachite green, with lysozyme was investigated by fluorescence spectroscopic techniques under physiological conditions. The binding parameters have been evaluated by fluorescence quenching methods. The results revealed that malachite green caused the fluorescence quenching of lysozyme through a static quenching procedure. The thermodynamic parameters like ΔH and ΔS were calculated to be -15.33 kJ mol^{-1} and 19.47 $Jmol^{-1}K^{-1}$ according to van't Hoff equation, respectively, which proves main interaction between malachite green and lysozyme is hydrophobic forces and hydrogen bond contact. The distance *r* between donor (lysozyme) and acceptor (malachite green) was obtained to be 3.82 nm according to F rster's theory. The results of synchronous fluorescence, UV/vis and three-dimensional fluorescence spectra showed that binding of malachite green with lysozyme can induce conformational changes in lysozyme. In addition, the effects of common ions on the constants of lysozyme-malachite green complex were also discussed.

Keywords Malachite green · Lysozyme · Fluorescence quenching · Thermodynamic parameter

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Introduction

Malachite green (CAS 633-03-4, structure shown in Fig. 1), an important water-soluble *N*-methylated diaminotriphenylmethane dye, which is most widely used in the aquaculture industry worldwide as a fungicide, parasiticide and disinfectant, as well as in silk, wool, jute, cotton, leather, paper and acrylic industries as a dye [1, 2]. Furthermore, it is also employed as a food coloring agent, food additive and anthelminthic [3]. The powerful antimicrobial activity of malachite green has been attributed to inhibition of intracellular enzymes, intercalation into DNA, and/or interaction with cellular membranes [4]. However, malachite green has now become a highly controversial compound due to its reported toxic properties which are known to cause carcinogenesis, mutagenesis, teratogenecity and respiratory toxicity in humans [5, 6]. Its oral consumption is also hazardous and carcinogenic. Though the use of this dye has been banned in several countries and not approved by U.S. Food and Drug Administration, it is still being used in many parts of the world due to its low cost, ready availability and high efficacy [7, 8]. Owing to malachite green is used for different applications and has been widely used in the world indicating potential toxic risk to humans, it is necessary to investigation the interaction of malachite green with protein (including enzymes), as it can provide important insight into the transport and metabolism process of the toxic dye.

Lysozyme, also called muramidase, a small monomeric globular protein first discovered by Alexander Fleming in 1922 [9], is a basic protein belongs to the class of enzymes that lyse the cell walls of bacteria by hydrolyzing the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan. Lysozyme is antimicrobial protein widely distributed in various biological fluids and tissues

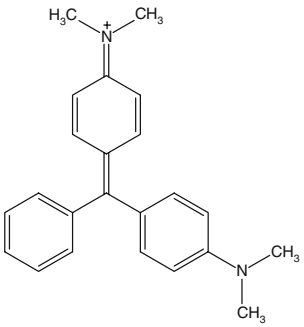

Fig. 1 Chemical structure of malachite green

including avian egg and animal secretions, human milk, tears, saliva, airway secretions, and secreted by polymorphonuclear leukocytes [10]. Besides its antimicrobial activity, it has many other biological functions including anti-inflammatory, anti-viral, immune modulatory, anti-histaminic and anti-tumor activities [11–16]. Intriguingly, another important function of lysozyme is their ability to carry ligands. Therefore, lysozyme is extensively used in the pharmaceutical and food fields. In previous work, Zhang and colleagues have probed the interaction of malachite green with bovine serum albumin [17], yet the investigation on binding interaction of malachite green to lysozyme have not been reported. In this paper, we designed to examine the effect of malachite green on the solution structure of lysozyme using fluorescence, UV/vis and three-dimensional fluorescence spectroscopy at different temperatures under physiological conditions for the first time. Attempts were made to investigate the binding mechanism of malachite green to lysozyme with respect to the binding constants, the binding sites, the thermodynamic functions and the conformational changes of lysozyme molecules.

Experimental

Materials

Lysozyme and malachite green were used as received from Sigma Chemical Company, St. Louis, USA. A molecular weight of 14,700 was taken for lysozyme. Lysozyme was dissolved in the pH7.40 buffer solution (4.0×10^{-5}mol L^{-1}) and lysozyme stock solution was kept in the dark at 277 K. The concentration of lysozyme was determined spectrophotometrically using ε_{280}(lysozyme)=37,646 L$mol^{-1}cm^{-1}$ [18]. All other reagents were of analytical reagent grade. The Milli-Q ultrapure water was used throughout the experiments. NaCl (1.0 mol L^{-1}) solution was used to maintain the ionic strength at 0.1. Tris (0.2 mol L^{-1})-HCl (0.1 mol L^{-1}) buffer solution containing NaCl (0.1 mol L^{-1}) was used to keep the pH of the solution at 7.40.

Apparatus and methods

All fluorescence spectra and intensity measurements were performed on F-4500 spectrofluorimeter (Hitachi, Japan) equipped with a 1.0 cm quartz cell, both excitation and emission bandwidths set on 5 nm. The UV/vis absorption spectra were carried out on a Lambda-25 double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer, USA). All pH measurements were made with a Orion-868 digital pH-meter (Orion, USA) with a combined glass electrode.

Fluorometric titration experiments: A 2.5 mL solution, containing appropriate concentration of lysozyme, was titrated by successive additions of a 2.5×10^{-4}mol L^{-1} stock solution of malachite green (to give a final concentration of 4.0×10^{-5}mol L^{-1}). Titrations were done manually by using trace syringes. The fluorescence spectra were then measured (excitation at 295 nm and emission wavelengths of 250–500 nm) at three temperatures (298, 304 and 310 K). The temperature of sample was kept by recycled water throughout the experiment.

The three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength was recorded between 200 and 500 nm, the initial excitation wavelength was set to 200 nm with increment of 10 nm, the number of scanning curves was 16, and other scanning parameters were just the same as those of the fluorescence quenching spectra.

The UV/vis absorbance spectra were recorded at room temperature in the range of 200–400 nm using a quartz cuvette with 1 cm path length.

For the research on the effect of common ions, $AlCl_3$, $CaCl_2$, $FeCl_3$, KCl, $MgCl_2$, $NiCl_2$ and $ZnCl_2$ were used as foreign substances. The anions were all chloride ion, which did not affect the lysozyme fluorescence. The concentration of metal ions were fixed at 5.0×10^{-5}mol L^{-1}. The fluorescence spectra of lysozyme-malachite green system were recorded in the presence of various ions in the range of 250–500 nm upon excitation at 295 nm.

Principles of fluorescence quenching

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule, such as excited-state reactions, energy transfer, ground-state complex formation and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching [19]. The dynamic quenching, resulting from collisional encounters between the fluorophore and quencher, static quenching is due to the formation of a ground-state complex between the fluorophore and quencher. In both cases, molecular contact is required between the fluorophore and quencher for the

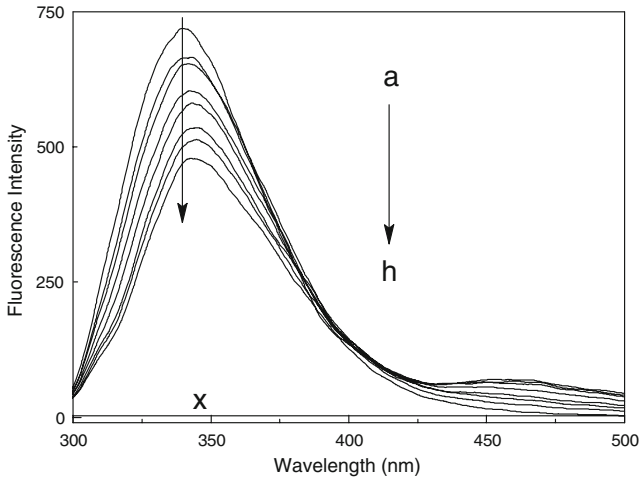

Fig. 2 The fluorescence emission spectra of the lysozyme-malachite green system. (a) 1.6×10^{-6}mol L^{-1} lysozyme; (b→h) 1.6×10^{-6}mol L^{-1} lysozyme in the presence of 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, 4.0×10^{-5}mol L^{-1} malachite green; (x) 0.5×10^{-5}mol L^{-1} malachite green. pH=7.40, T= 298 K

fluorescence quenching occur. For dynamic quenching, the decrease in intensity is usually described by the well-known Stern-Volmer equation (1) [20]:

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

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_{SV} is the Stern-Volmer quenching constant and $[Q]$ is the concentration of quencher. k_q is the quenching rate constant of the biomolecule and $k_q=K_{SV}/\tau_0$. τ_0 is the average lifetime of the biomolecule without quencher and the fluorescence lifetime of the biopolymer is 10^{-8}s [21]. For dynamic quenching, the maximum scatter collision quenching constant of various quencher is 2.0×10^{10}L $mol^{-1}s^{-1}$ [22].

Calculation of binding parameters

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the numbers of binding sites (n) can be found from the following equation (2) [23]:

$$\log\frac{F_0 - F}{F} = n\log K_b + n\log\left(\frac{1}{[Q_t] - \frac{F_0-F}{F_0}[P_t]}\right) \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, $[Q_t]$ and $[P_t]$ are the total quencher concentration and the total protein concentration, respectively. By the plot of $\log(F_0-F)/F$ versus $\log(1/([Q_t]-(F_0-F)[P_t]/F_0))$, the binding constant ($K_b$) and the number of binding sites (n) can be obtained.

Results and discussion

Fluorescence quenching

According to the X-ray crystallographic measurements [24], lysozyme possesses a relatively rigid structure, including α-helix, β-sheet, turns and loops, and formed by 129 amino-acid residues, containing 6 tryptophanes (Trp), 4 disulfide bridges and 3 tyrosine residues (Tyr), in which Trp-62 and Trp-108 are the most dominant fluorophores, both being located at the substrate binding sites [25]. The effect of malachite green on Trp residues fluorescence intensity is show in Fig. 2. As the data show, the fluorescence intensity of lysozyme decreased regularly with the increasing concentration of malachite green, which indicates that malachite green can bind to lysozyme. Otherwise, a faint red shifts (from 340 to 343 nm) could be deduced that conformational changes induced by the interaction lead to the polarity around the Trp residues was increased and the hydrophobicity was decreased. Furthermore, an isoactinic point at 400 nm was observed in Fig. 2, which indicated that the existence of both bound and free malachite green at equilibrium [26].

In order to confirm the quenching mechanism, the fluorescence quenching was analyzed according to the well-known Stern-Volmer equation (1). Figure 3 displayed the Stern-Volmer plots of the quenching of lysozyme tryptophan residues fluorescence by malachite green at different temperatures. The plot shows that within the investigated concentrations, the results agreed with the Stern-Volmer equation (1). Table 1 summarizes the calculated K_{SV} and k_q at each temperature studied. The results

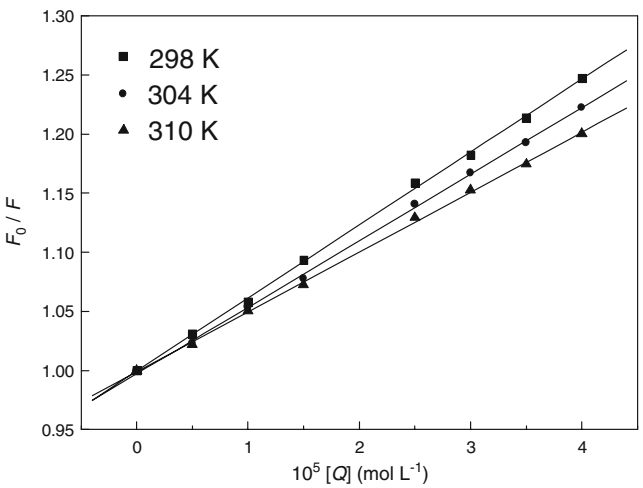

Fig. 3 Stern-Volmer plots for the lysozyme-malachite green system at three different temperatures. c(lysozyme)=1.6×10^{-6}mol L^{-1}; pH= 7.40

Table 1 Stern-Volmer quenching constants for the interaction of lysozyme with malachite green at three different temperatures

T (K)	K_{SV} ($\times 10^{-3}$ L mol^{-1})	k_q ($\times 10^{-11}$ L mol^{-1} s^{-1})	R^a	S.D.[b]
298	6.171	6.171	0.9996	+0.003
304	5.627	5.627	0.9996	+0.003
310	5.065	5.065	0.9995	+0.002

[a] R is the correlation coefficient

[b] S.D. is the standard deviation

showed that the Stern-Volmer dynamic quenching constant K_{SV} was inversely correlated with temperatures and the values of k_q are much larger than the maximum scattering collision quenching constant (2.0×10^{10} L mol^{-1} s^{-1}), which indicated that the probable quenching mechanism of fluorescence of lysozyme by malachite green was not initiated by dynamic collision but compound formation [27]. In other word, the fluorescence quenching of lysozyme results from complex formation was predominant, while from dynamic collision could be negligible.

Therefore, the fluorescence data were further examined using modified Stern-Volmer equation (3) [28]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a f_a [Q]} \tag{3}$$

where K_a is the effective quenching constant for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. Figure 4 displays the modified Stern-Volmer plots and the corresponding results of K_a values at different temperatures were shown in Table 2. The decreasing trend of K_a with increasing temperatures was in accordance with K_{SV}'s dependence on temperature as mentioned above. The K_a values show that the binding between lysozyme and malachite green was moderate, which indicated that a reversible lysozyme-malachite green complex formation and malachite green can be carried by lysozyme in the body.

Analysis of binding equilibria

Figure 5 is the plots of $\log(F_0-F)/F$ versus $\log(1/([Q_t]-(F_0-F)[P_t]/F_0))$ for the lysozyme-malachite green system at different temperatures obtained from the fluorescence titration. The calculated results at different temperatures were presented in Table 3. It is observed that K_b decreased with the increasing of temperature, which indicated the forming of an unstable compound. The unstable compound would be partly decomposed with the rising temperature. The values of n approximately equal to 1 indicated the existence of just a single binding site in lysozyme for malachite green. In lysozyme, the Trp-62 being the more exposed to the polarity environment [29], so from the value of n, it may be inferred that malachite green most likely binds to the Trp-62 and quench its intrinsic fluorescence.

Binding mode

Generally speaking, the force between small organic molecule and biological macromolecule includes hydrogen bond, van der Waals force, electrostatic and hydrophobic interaction [30]. The signs and magnitudes of thermodynamic parameters for protein reactions can account for the main forces contributing to protein stability. If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then its value can be determined from the van't Hoff equation (4):

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

K is the effective quenching constant at temperature T and R is gas constant. The value of ΔH and ΔS were obtained from linear van't Hoff plot. The value of ΔG was calculated from the equation (5):

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

From the linear relationship between ln$K$ and the reciprocal absolute temperature (Fig. 6), the value of ΔH and ΔS can be obtained and presented in Table 2. As shown in Table 2, ΔH and ΔS for the binding reaction between lysozyme and malachite green are found to be −15.33 kJ mol^{-1} and 19.47 Jmol^{-1}K^{-1}, which indicated that the binding processes were an exothermic reaction. The negative sign for ΔG means that the spontaneity of the binding of malachite green with lysozyme. Ross and Subramanian [31] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of

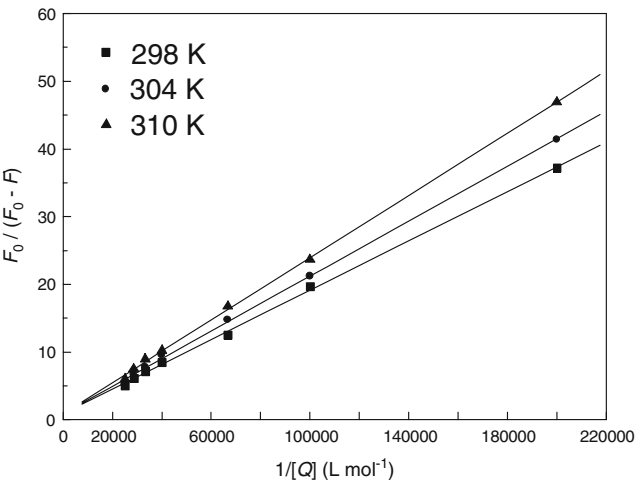

Fig. 4 Modified Stern-Volmer plots for the binding of lysozyme with malachite green at three different temperatures. c(lysozyme)=1.6×10^{-6}mol L^{-1}; pH=7.40

Table 2 Modified Stern-Volmer association constants K_a and relative thermodynamic parameters of the lysozyme-malachite green system

T (K)	K_a ($\times 10^{-3}$L mol^{-1})	R[a]	S.D.[b]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1}K^{-1})
298	5.027	0.9994	+0.22	−15.33	−21.12	19.47
304	4.546	0.9994	+0.22		−21.29	
310	3.955	0.9996	+0.17		−21.35	

[a] R is the correlation coefficient

[b] S.D. is the standard deviation

interaction that may take place in protein association processes. From the point of view of water structure, a positive ΔS value is frequently taken as typical evidence for hydrophobic interaction, because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic reaction. Further, the negative ΔH value (−15.33 kJ mol^{-1}) observed can't be mainly attributed to electrostatic interactions since for electrostatic interactions ΔH is very small, almost zero. Negative ΔH value is observed whenever there is hydrogen bond in the binding. From the negative values of ΔH and ΔS observed in the present study, it can be concluded that the acting force are mainly hydrophobic forces and hydrogen bonding.

Energy transfer from lysozyme to malachite green

Fluorescence resonance energy transfer (FRET) is a distance dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecule (donor) to another molecule (acceptor) without emission of a photon from the former molecular system. According to the F rster non-radiative resonance energy transfer theory [32], the rate of energy transfer depends on the following conditions: (i) the extent of overlap between the donor emission and the acceptor absorption, (ii) the orientation of the transition dipole of donor and acceptor, and (iii) the distance between the donor and the acceptor. Here the donor and acceptor are lysozyme and malachite green, respectively. There was a spectral overlap between the fluorescence emission spectrum of free lysozyme and UV/vis absorption spectrum of malachite green (Fig. 7). The efficiency of energy transfer between the donor and acceptor, E, could be calculated by the following equation (6):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{6}$$

where r is the binding distance between donor and acceptor, and R_0 is the critical distance at 50% transfer efficiency,

$$R_0^6 = 8.8 \times 10^{-25} k^2 \cdot n^{-4} \cdot \varphi \cdot J \tag{7}$$

where k^2 is the orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor. The dipole orientation factor, k^2, is the least certain parameter in calculation of the critical transfer distance, R_0. Although theoretically k^2 can range from 0 to 4, the extreme values require very rigid orientations. If both the donor and acceptor are tumbling rapidly and are free to assume any orientation, then k^2 equals 2/3. n is the average refractive index of medium in the wavelength range where spectral overlap is significant, φ is the fluorescence quantum yield of the donor, J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{8}$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$, $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. In the present

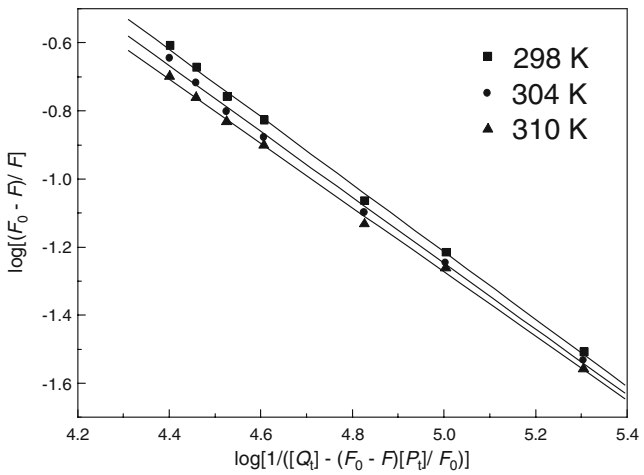

Fig. 5 The plots of $\log(F_0-F)/F$ versus $\log(1/([Q_t]-(F_0-F)[P_t]/F_0))$. c(lysozyme)=1.6×10^{-6}mol L^{-1}; pH=7.40

Table 3 Binding parameters and binding sites n at different temperatures

T (K)	K_b ($\times 10^{-3}$L mol^{-1})	n	R[a]	S.D.[b]
298	5.433	0.99	0.9993	+0.014
304	3.831	0.97	0.9989	+0.017
310	2.733	0.94	0.9992	+0.014

[a] R is the correlation coefficient

[b] S.D. is the standard deviation for the K_b values

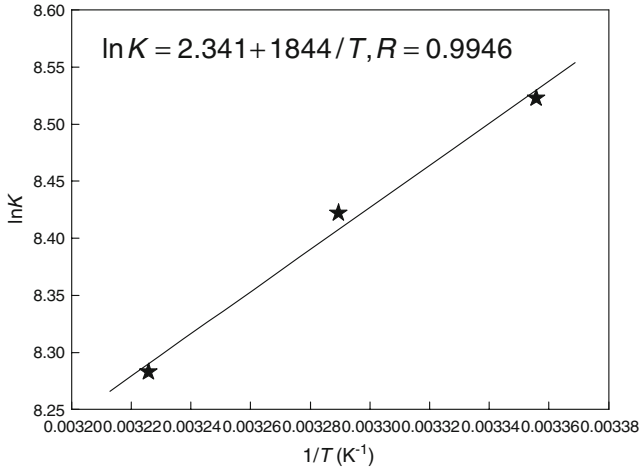

Fig. 6 Van't Hoff plot for the interaction of lysozyme and malachite green in Tris-HCl buffer, pH=7.40

case, k^2=2/3, n=1.336, φ=0.14 [33]. According to equations (6, 7, 8), the values of the parameters were found to be: J=1.452×10^{-14}cm^3 L mol^{-1}, R_0=2.65 nm, E=0.0996, r=3.82 nm. The donor-to-acceptor distance (r) is smaller than 8 nm, which indicated that the energy transfer from lysozyme to malachite green occurred with high probability, while r was bigger than R_0 also revealed that malachite green could strongly quench the fluorescence of lysozyme by static quenching [34].

Conformation investigations

Synchronous fluorescence spectroscopy studies

Synchronous fluorescence spectroscopy was introduced by Lloyd in 1971 [35]. It involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval between them. In the synchronous spectra, the sensitivity associated with fluorescence is maintained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects [36]. According to the theory of Miller [37], when the D-value ($\Delta\lambda$) between excitation and emission wavelength are stabilized at 60 nm, the synchronous fluorescence gives the characteristic information of tryptophan residues. The effect of malachite green on lysozyme synchronous fluorescence spectroscopy was shown in Fig. 8. It was apparent from Fig. 8 that a faint red shift (from 280 to 282 nm) can be observed from the synchronous fluorescence spectra of interaction between malachite green and lysozyme. The faint red shift effect expresses the change in conformation of lysozyme. It is also indicated that the polarity around the tryptophan residues was increased and the hydrophobicity was decreased [38]. Moreover, the fluorescence intensity decreased regularly with the addition of malachite green, which further demonstrated the occurrence of fluorescence quenching in the binding process.

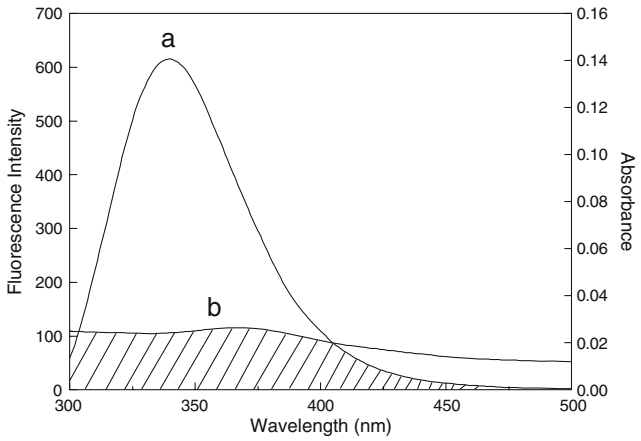

Fig. 7 Overlapping between the fluorescence emission spectrum of lysozyme (**a**) and UV/vis absorption spectrum of malachite green (**b**), c(lysozyme)=c(malachite green)=1.6×10^{-6}mol L^{-1}; pH=7.40, T=298 K

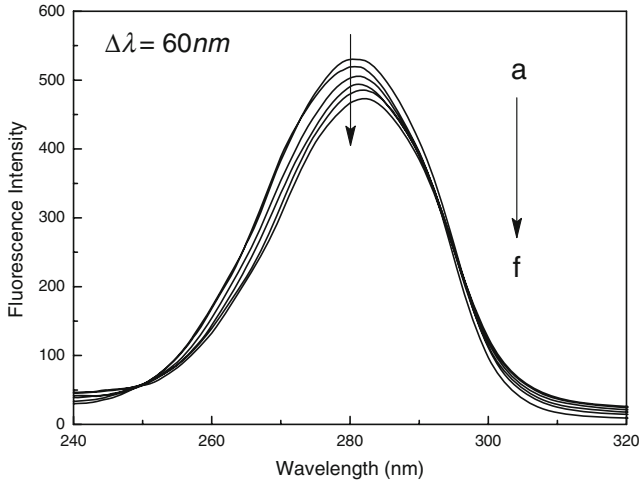

Fig. 8 Synchronous fluorescence spectrum of lysozyme in the absence and presence of malachite green (pH=7.40, T=298 K, $\Delta\lambda$=60 nm). **a** 1.6×10^{-6}mol L^{-1} lysozyme; **b**→**f** 1.6×10^{-6}mol L^{-1} lysozyme in the presence of 0.5, 1.5, 2.5, 3.0, 3.5×10^{-5}mol L^{-1} malachite green, respectively

UV/vis absorbance spectroscopy analysis

UV/vis absorption measurement is a very simple method and applicable to explore the structural change [39] and know the complex formation. In the present study, the UV/vis absorption spectra of lysozyme, malachite green and lysozyme-malachite green system was recorded (Fig. 9). As can be seen from Fig. 9, the addition of increasing malachite green to the lysozyme solution led to the gradual enhancement in UV/vis intensity and exhibited a concentration-dependent relationship. The maximum peak position of lysozyme-malachite green was shifted towards

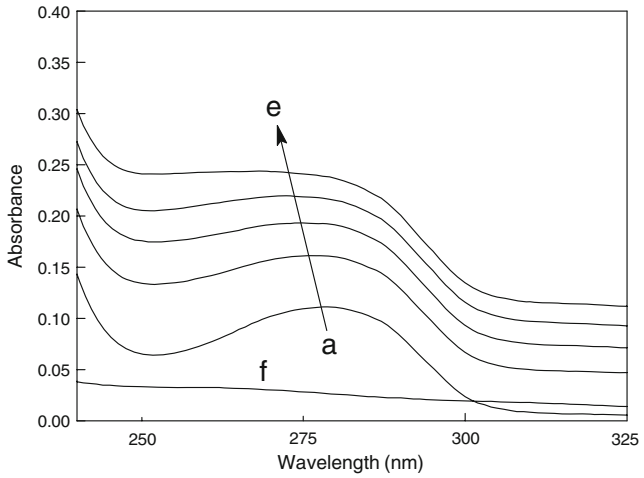

Fig. 9 UV/vis absorption spectra of lysozyme. Lysozyme concentration was at 1.6×10^{-6}mol L^{-1} (**a**) and malachite green concentration for lysozyme-malachite green system was at 0.5, 1.0, 1.5, 2.0×10^{-5}mol L^{-1} (**b**→**e**). A concentration of 0.5×10^{-5}mol L^{-1} malachite green (**f**) was used for malachite green only. pH=7.40, T=298 K

lower wavelength (from 280 to 270 nm). A reasonable explanation for the two evidences may come from the interaction between lysozyme and malachite green [40]. It also suggested that small structural change of lysozyme upon interaction with malachite green.

Three-dimensional fluorescence spectroscopy studies

The three-dimensional fluorescence spectra are a rising fluorescence analysis technique in recent years [41]. It can comprehensively exhibit the fluorescence information of the sample, which makes the investigation of the characteristic conformational change of protein be more scientific and credible. Figure 10 presented the three-dimensional fluorescence spectra of lysozyme (A) and lysozyme-malachite green (B) complex. Peak *a* is the Rayleigh scattering peak ($\lambda_{ex}=\lambda_{em}$) and with the addition of malachite green, the fluorescence intensity of peak *a* increased. The possible reason may be that a lysozyme-malachite green complex came into being after the addition of malachite green, and thus result in the scattering effect enhanced. Peak *b* is the second-ordered scattering peak ($\lambda_{em}=2\lambda_{ex}$) [42]. Peak 1 ($\lambda_{ex}$=280.0 nm, λ_{em}=335.0 nm) mainly reveals the spectral characteristic of Trp and Tyr residues, because when protein is excited at 280 nm, it mainly reveals the intrinsic fluorescence of Trp and Tyr residues, and the fluorescence of phenylalanine (Phe) residue can be negligible [43]. Peak 2 (λ_{ex}=230.0 nm, λ_{em}=335.0 nm) mainly exhibit the fluorescence characteristic of polypeptide backbone structures of lysozyme. Analyzing from the fluorescence intensity changes of peak 1 and peak 2, they decreased obviously but to different degree: in the presence and absence of malachite green, the

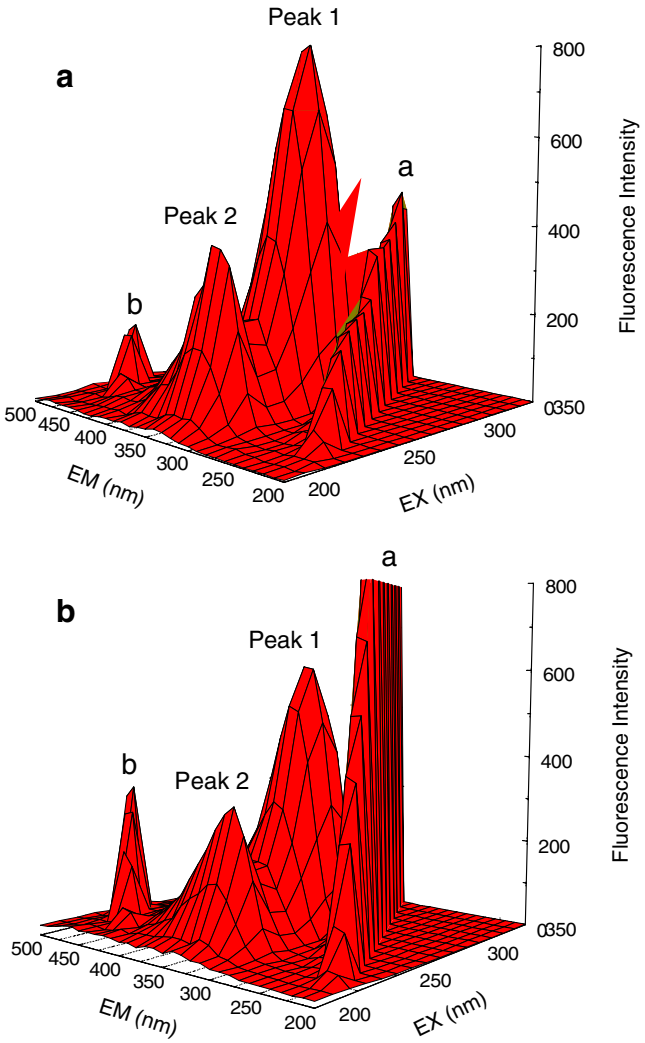

Fig. 10 The three-dimensional fluorescence spectra of lysozyme (**a**) and lysozyme-malachite green (**b**). **a**: c(lysozyme)=2.0×10^{-6}mol L^{-1}, c(malachite green)=0; **b**: c(lysozyme)=2.0×10^{-6}mol L^{-1}, c(malachite green)=2.0×10^{-6}mol L^{-1}. pH=7.40, T=298 K

Table 4 Effects of some common ions (5.0×10^{-5}mol L^{-1}) on lysozyme-malachite green system

System	Binding constant ($\times10^{-4}$L mol^{-1})
lysozyme+malachite green	0.5433
lysozyme+malachite green+Al^{3+}	4.522
lysozyme+malachite green+Ca^{2+}	2.323
lysozyme+malachite green+Fe^{3+}	2.228
lysozyme+malachite green+K^{+}	4.081
lysozyme+malachite green+Mg^{2+}	4.823
lysozyme+malachite green+Ni^{2+}	4.745
lysozyme+malachite green+Zn^{2+}	3.132

fluorescence intensity ratio of peak 1 and peak 2 was 1:1.31 and 1:1.21, respectively. The decrease of the fluorescence intensity of lysozyme in combination with the synchronous fluorescence and UV/vis spectra results, we can conclude that there was specific interaction occurring between lysozyme and malachite green, and the binding of lysozyme-malachite green induced some microenvironmental and conformational changes in lysozyme [17].

The effect of common ions on the binding constant

The common ions are widely distributed in human. Therefore, we have examined the effects of some inorganic cations on the binding constant of lysozyme-malachite green system at 298 K by recording the fluorescence intensity in the range of 250–500 nm upon excitation at 295 nm. The results were shown in Table 4. It was evident from Table 4 that the binding between lysozyme and malachite green increased in the presence of the above ions prolong the storage time of malachite green in blood plasma and decrease the maximum toxicity of malachite green [44].

Conclusions

This paper provided an approach for studying the interaction of lysozyme with malachite green using fluorescence, UV/vis and three-dimensional fluorescence spectroscopy. The results showed that the fluorescence of lysozyme has been quenched for reacting with malachite green and forming a certain kind of new compound. The quenching belonged to static fluorescence quenching type, with non-radiative energy transfer happening within single molecule. The enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be −15.33 kJ mol^{-1} and 19.47 $Jmol^{-1}K^{-1}$, which indicated that hydrophobic forces and hydrogen bond played major roles in stabilizing the complex. In the conformation investigation, the synchronous fluorescence, UV/vis and three-dimensional fluorescence spectroscopy revealed that the conformation and microenvironment of lysozyme were changed in the presence of malachite green. The binding study of malachite green to lysozyme is greatly important in toxicology. This study is expected to provide important insight into the interaction mechanism of lysozyme with malachite green, which may be a useful guideline for further toxicology investigation.

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