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ORIGINAL ARTICLE

Binding of brilliant red compound to lysozyme: insights into the enzyme toxicity of water-soluble aromatic chemicals

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Abstract The non-covalent interaction of brilliant red (BR) with lysozyme was investigated by the UV spectrometry, circular dichroism (CD) and isothermal titration calorimetry (ITC). The thermodynamic characterization of the interaction was performed and the assembly complexes were formed: lysozyme$(BR)_{17}$ at pH 2.03, lysozyme$(BR)_{15}$ at pH 3.25 and lysozyme$(BR)_{12}$ at pH 4.35, which corresponded to the physiological acidities. The ionic interaction induces a combination of multiple non-covalent bonds including hydrogen bond, hydrophobic interaction and van der Waals force. The two-step binding model of BR was found, in which one or two BR molecules entered the hydrophobic intracavity of lysozyme and the others bound to the hydrophilic outer surface of lysozyme. Moreover, BR binding resulted in change of the lysozyme conformation and inhibition of the lysozyme activity. The possible binding site and type of BR and the conformational transition of lysozyme were speculated and illustrated. This work provided a useful approach for study on enzyme toxicity of aromatic azo chemicals.

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Introduction

Knowledge of biomolecular interactions such as protein–protein and protein–ligand binding (Fang et al. 2008; Bergamini 2007), enzyme catalysis and inhibition is important for understanding cellular processes including signal transduction, gene regulation and enzyme reactions (Kraut et al. 2003). Biophysical techniques are able to detect such category of bindings sensitively and characterize them accurately. Therefore, they are powerful tools in enzymology, rational drug design and toxicology in which conventional molecular spectrometric methods such as utilization of fluorescent probes, UV and circular dichroism (CD). Recently, binding mechanisms have been studied intensively using X-ray crystallography, nuclear magnetic resonance, isothermal titration calorimetry (ITC), surface plasmon resonance biosensors and so on (Eichmuller et al. 2001; Jones et al. 2006). Lysozyme (EC 3.2.1.17) also called muramidase or N-acetyl muramide glycanohydralase is ubiquitously found in human bodies and animals, including tears, saliva, latex humors, skin, liver, and gastric and lymphatic tissues. It is also found at an extraordinarily high concentration in chicken egg-white. Its structure which contains a single chain of 129 amino acid residues was characterized by X-ray analysis (Diamond 1974). Under normal physiological conditions, lysozyme is folded into a compact, globular structure with a long cleft in the protein surface. It has many pharmacologic functions such as antiseptic, antiphlogistic, repercussive, antiviral and antineoplastic actions as it is effective against gram-positive bacterial cells by

hydrolyzing their polysaccharide components. It also improves the human blood circulation and enhances the human immunity. However, binding with any chemical substance is likely to affect the hydrolytic activity of lysozyme, either enhancing it (Marolia and D'Souza 1999) with potential medical significance, or inhibiting it (Miller et al. 2005) if an organic contaminant or toxin is involved.

One representative of aromatic azo compounds (structured in Fig. 1), brilliant red (BR) is extensively used as the textile dye for cloth coloring. Due to its bright red color, and fast fixation, sometimes it is even added illegally into food and skin cosmetics. Thus, it may enter human bodies by food intake and skin absorption. Azo compounds with aryl group can be reduced by azo reductases produced by intestinal bacteria and, to a lesser extent, by enzymes of the cytosolic and microsomal fractions of the liver to produce aromatic amines, some of which are known carcinogens (Skipper and Tannenbaum 1994). Although such azo compounds may not be directly carcinogenic, they may bind and aggregate directly with proteins, such as enzymes by molecular interactions (Pible et al. 2006; Nelson et al. 2006), altering protein function or enzyme activity and thus causing toxicity. Thus, BR will cause potential toxicity to human health if it exposes in food and the skin cosmetics. The objective of the present work is to understand the general principle resulting in enzyme toxicity and human health risk by investigating the effect of non-specific binding of an anionic aromatic compound BR on the structure and activity of lysozyme in the physiological pH media by using UV-VIS, CD, and ITC.

Materials and methods

Instruments and materials

The absorption spectra of BR and protein solutions were recorded with a Model Lambda-25 spectrometer (Perkin-Elmer, USA) equipped with a thermostatic cell holder attachment to link with a Model TS-030 water-circulated thermostatic oven (Yiheng Sci. Technol. Shanghai, China).

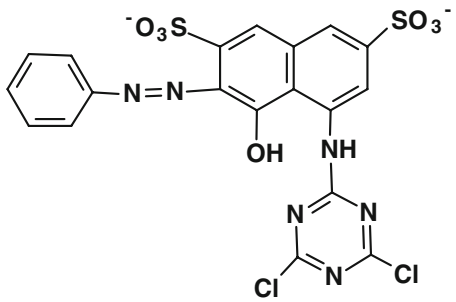

Fig. 1 Chemical structure of BR

The spectrometer was computer-controlled using UV WinLab software (Version 2.85.04). The ITC experiments were carried out on a MSC VP-ITC microcalorimeter (MicroCal LLC, Northampton, USA) with VPViewer 2000 which provides the user interface to control the VP-ITC instrument and conduct the experiment. A Model J-715 CD Spectropolarimeter (JASCO Instrum., Japan) was used to measure protein conformation. Egg-white lysozyme (0.200 mg/ml) (Shanghai Chemical Reagents, China Med. Group) was dissolved in deionized water and it was stored at less than 4°C. BR raw material (approximately 70% of content, Shanghai Dyestuff Factory, China) was recrystallized for three times in ethanol to yield the pure BR crystals (over 98% of content), which was examined with HPLC. Thus, a standard BR solution (0.350 mM) was prepared in deionized water. A series of Britton-Robinson (B-R) buffers, pH 2.03, 2.21, 2.78, 3.25, 3.88, 4.35, 5.04 and 5.68, were prepared to adjust the acidity of solution. An electrolyte solution (5.0 M $NaNO_3$) was prepared to adjust the ionic strength of solution. A set of commercial reagent packets (Jiancheng Bioengineering Institute, Nanjing, China) with four units of *Micrococus lysodeikticu* bacterial powder (5 mg per unit) as substrate and one bottle of the bacterial powder's solvent (100 ml) was used for determination of the lysozyme activity. The bacterial powder's solvent is the pH 6.2 phosphate buffer consisting of 1.17% NaH_2PO_4, 0.786% Na_2HPO_4 and 0.0392% EDTA.

Photometric determination of the BR-lysozyme interaction

All studies were carried out in a 10.0 ml calibrated flask containing a known volume of lysozyme solution, 1.0 ml of B-R buffer (pH 2.03, 3.25, and 4.35) and a known volume of 0.350 mM BR. The solution was diluted to 10.0 ml with deionized water and mixed thoroughly. After reacting for 5 min, the absorbances $A_{\lambda 2}$ and $A_{\lambda 1}$ of the lysozyme-BR solutions and $A^0_{\lambda 2}$ and $A^0_{\lambda 1}$ of the reagent blank (without lysozyme) were measured at 539 nm (λ_2) and 571 nm (λ_1) against water by UV-VIS spectrophotometry.

Isothermal titration calorimetry

The VP-Viewer 2000 software controlled the operation of the VP-ITC calorimeter. It is within VP-Viewer 2000 that all ITC experimental parameters are entered, runs are controlled and data was saved to the hard disk of the Computer Controller. ITC experiments were carried out as follows. The lysozyme solution (0.010 mM in B-R buffer) was placed in the sample-cell (1.4685 ml) of ITC device and the solution was kept at 25°C. The BR solution (1.75 mM in B-R buffer) was taken in the injector (300 μl) and it was injected into the isothermal sample-cell for

67 times in 4-μl increments at 3-min intervals. The stirring rate 310 rpm was set up for this ITC experiment. The program automatically added. ITC for an extension name, so that the raw data file was recognized by ORIGIN (version 7.0) as a VP-ITC data file. Besides, the B-R buffer without lysozyme was placed in the isothermal cell instead of the above lysozyme solution and the same operation was carried out. It was used to correct the heat of dilution of the BR solution. Finally, the Origin 7.0 was used for plotting the data received from the VP-ITC instrument. Corrected heats were divided by the number of moles injected and analyzed or fitted.

CD measurement

CD spectra were recorded over the range 190–250 nm on a spectropolarimeter. Buffer (1 ml, pH 2.03, 3.25 or 4.35) was mixed with 0.010 mM lysozyme; 0, 0.040, 0.080, and 0.120 mM BR were added to four flasks. Samples were allowed to equilibrate for 15 min, and CD spectra were measured in a 0.1-cm light path cell. The mean residue ellipticity (MRE) was calculated. Simultaneously, the reagent blank without BR was measured for correcting MRE of lysozyme. Three replicate measurements of each were performed. The relative contents of secondary structure forms of lysozyme, α-helix, β-pleated sheet, β-turn and random coil—were calculated in all the solutions.

Assay of lysozyme activity

The conventional turbidimetric method (Lee and Yang 2002) is used to determine the lysozyme activity in presence of BR at pH 2.03, 3.25 and 4.35. According to the user manual provided by the manufactory, both the *Micrococus lysodeikticu* bacterial powder (5 mg) and the bacterial powder's solvent (20.0 ml) were mixed to prepare the bacterial suspension (0.25 mg/ml). A standard lysozyme solution (0.010 mg/l) and a sample lysozyme solution with BR were prepared and placed in a constant temperature water bath at 37°C for more than 5 min. 0.2 ml of each was placed into a colorimetric cell and then 2.0 ml of the bacterial suspension was added immediately. Five and 125 sec later, the transmittancies (T_{S0} and T_{S2} for the standard lysozyme and T_0 and T_2 for the sample lysozyme) of a colorimetric liquid were measured against water. The measurement wavelength was fixed at 700 nm but not 530 nm to avoid the interference of light-absorption of BR. The sample's lysozyme activity (A) was calculated by the relation:

$$A = \frac{T_2 - T_0}{T_{S2} - T_{S0}} \times 10 \times 20.$$

Results

pH dependence of the BR-lysozyme interaction

BR is an aromatic azo compound that can form a bright red solution when dissolved in water and it is able to react with lysozyme to form a violet product. The absorption spectrum of the product shows an obvious red-shift from the original BR spectrum. The lysozyme-BR solutions were measured in various pH media and their absorption spectra are shown in Fig. 2a. Only little peak-valley difference was observed in neutral pH. The interval between the positive peak and the negative trough increases with increasing acidity of solution. This may be explained reasonably from the occupancy of the acidic AARs (Glu and Asp). There are nine acidic AARs (Glu and Asp) and 18 basic AARs (Lys, His and Arg) (Diamond 1974) in 129 AARs of egg-white lysozyme. The dissociation constants (K_a) of the side groups (R) of these AARs are 10.53 for Lys, 6.00 for His, 12.48 for Arg, 3.65 for Asp and 4.25 for Glu. The distribution of $-R^-$ (negatively charged side groups of Glu and Asp) and $-RH$ groups (side groups of Glu and Asp without charge) is that $-R_{Glu}H/-R_{Glu}^- > 4$, $-R_{ASP}H > -R_{ASP}^-$ when pH is less than 3.65. In such an acidic media, almost all of the side groups of the basic AARs are positively charged. It is favorable for BR anions to bind to lysozyme. On the contrary, there $-R_{Glu}^- > -R_{Glu}H$ and $-R_{ASP}^-/-R_{ASP}H > 4$ if the pH is over 4.25. Entry of BR will

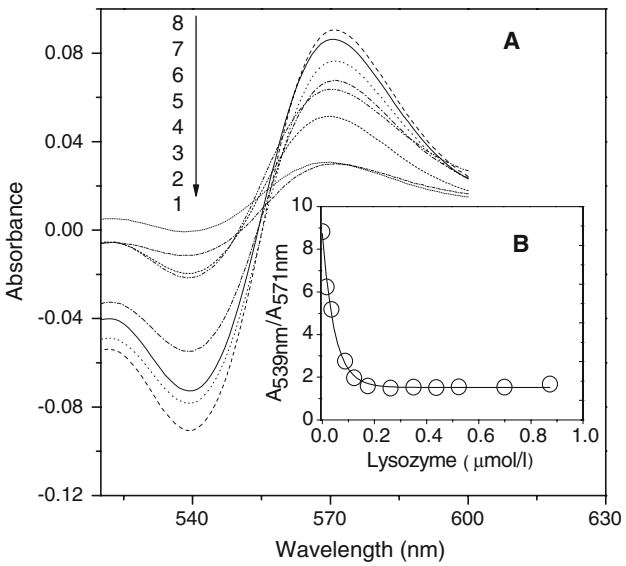

Fig. 2 **a** Absorption spectra of the lysozyme-BR solutions containing 0.035 mM BR and 18.0 mg/l lysozyme (**a**) at pH, from 1 to 8: 2.03, 2.21, 2.78, 3.25, 3.88, 4.35, 5.04 and 5.68, all measured against the reagent blank, **b** variation of the absorbance ratio ($A_{539\ nm}/A_{571\ nm}$) of the lysozyme-BR solution (**b**) at pH 2.03, where BR was 0.018 mM

therefore be impeded by strong charge repulsion. The change in BR structure from L^{2-} to HL^{-} then to neutral H_2L in an acidic solution, e.g., less than one of pH is unfavorable for the interaction of BR with lysozyme. Thus, there is an optimal pH at which the maximum number of BR molecules binds to lysozyme. From the curves in Fig. 2a, the binding between BR and lysozyme is the strongest at pH 2.03.

From Fig. 2a, the binding of BR to lysozyme occurred in a wide pH scope from 2.03 to 5.68. It is possible that the presence of BR would not cause an obvious change of the lysozyme structure and activity in neutral pH. The lysozyme activity is stable in acidic media (Johnson et al. 1968). In fact, pH is remarkable in various tissues of human body, for example, for example pH 1–3 in gastro, pH 4–5 on skin and pH 7.4 in blood. Thus, the effect of BR on the lysozyme activity in gastric tissues would be most hazardous if BR were used as a food additive. From curve six in Fig. 2a where pH 4.35 corresponds to the physiological acidity of normal skin, the difference between peak and valley indicates that BR may largely affect the lysozyme structure and activity of skin tissues as well. Certainly, BR could result in health risks when it was used as the dye for clothing or as an additive of a beauty skin agent. In this work, pH 2.03, 3.25 and 4.35 were used as the experimental media to investigate the lysozyme-BR interaction because these pHs are representative of the physiological conditions in human bodies.

Photometric characterization of the BR-lysozyme interaction

The interaction of BR (L) with lysozyme (M) can be summarized below:

$$N\mathbf{BR} + \mathbf{Lysozyme} \overset{K_b}{\rightleftharpoons} \mathbf{Lysozyme}(\mathbf{BR})_N$$

Initiation	$c_{L0}(A^0_{\lambda 2})$	c_{M0}	0
Equilibrium	$c_L = c_{L0} - Nc_{M0}(A_{\lambda 1}, A_{\lambda 2})$	$c_M \to 0$	$c_{M0}(A_c)$

Both c_{L0} and c_{M0} are the initial mole concentration of BR. The symbol c_L is the equilibrium concentration of BR, K_b the binding constant in M^{-1} unit and N the saturation binding number of BR in lysozyme. Both $A_{\lambda 2}$ and $A_{\lambda 1}$ are the absorbances of the lysozyme-BR solution, measured at wavelengths λ_2 and λ_1. The symbol $A_{\lambda 2}$ is the absorbance of BR solution at λ_2 and A_c is that of the binding product. Without doubt, A_c cannot be measured directly because of the interference of equilibrium BR in solution. In this work, the spectral correction technique was applied (Gao et al. 2004). The effective fraction (η) of BR and the molar ratio (γ) of BR bound to lysozyme can be calculated by the following relations:

$$\eta = \frac{A_c - A_{\lambda 2}}{A^0_{\lambda 2}} + 1 \quad (1)$$

and

$$\gamma = \eta \times \frac{c_{L0}}{c_{M0}} \quad (2)$$

where

$$A_c = \frac{A_{\lambda 2} - \beta A_{\lambda 1}}{1 - \alpha\beta} \quad (3)$$

where the symbols α and β are the correction constants. The absorbance ratio ($A_{539\ nm}/A_{571\ nm}$) of the lysozyme-BR solutions was measured at 539 and 571 nm, and their values are shown in Fig. 2b. The $A_{539\ nm}/A_{571\ nm}$ value decreases with increasing lysozyme concentration and approaches a constant value of 1.32 when lysozyme is over 0.3 μM. This indicates that more and more BR molecules bound to lysozyme until no excess BR existed in solution. Thus, this constant minimum could be used as the α value for characterizing the binding product. The β value of BR corresponds to the $A_{571\ nm}/A_{539\ nm}$ ratio in the absence of lysozyme, which is located at the beginning of curve B. By preparing a series of BR solutions containing known concentrations of lysozyme, we found that lysozyme (20 mg/l) precipitated when BR is more than 3 mM in the three pH media. The possible reason is that the excessive BR bridged between lysozyme via electric interaction to form a supramolecular aggregate. Therefore, BR concentration was limited in less than 0.4 mM, i.e., approximately 240 of c_{L0}/c_{M0} in this work. The A_c, η and γ were calculated according to Eqs. 1–3 above and the variation in γ of BR is shown in Fig. 3. The γ increases with increasing BR concentrations and it approaches the following maximal

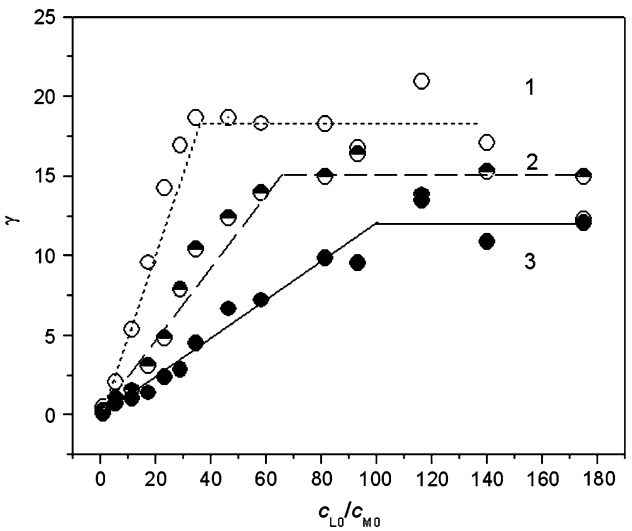

Fig. 3 Variation of γ in solutions containing 20.0 mg/l lysozyme and variable BR. *1* pH 2.03, *2* pH 3.25 and *3* pH 4.35

constants: approximately 18 (curve 1) when c_{L0}/c_{M0} is more than 30, approximately 15 (curve 2) when c_{L0}/c_{M0} is more than 50 and approximately 12 (curve 3) when c_{L0}/c_{M0} is more than 100. These maximum values directly reveal that the binding of BR with lysozyme may have reached saturation. These values are further examined and corrected in the subsequent experiments. In a more acidic solution, almost all the acidic AARs, such as Glu and Asp to form anionic side groups to favor the entry of BR to the peptide chains until saturation is reached. On the other hand, the Glu and Asp side groups would form anionic groups to obstruct the entry of BR. when the pH is higher than 4.25. Thus, *N* of BR decreases with increase in pH of solution. Interestingly, *N* of BR at pH 2.03 is just the same as the number of basic AARs in lysozyme. It indicates that the ionic interaction between BR anion and the positively charged side groups of basic AARs may induce the binding of BR to lysozyme, i.e., ion-pair attraction plays the position-fixing action of BR.

Thermodynamic parameters of the BR: lysozyme interaction

In order to understand the mechanism of the lysozyme, BR reaction and to assess the effect of acidity, on its specificity and stability, a group of detailed thermodynamic data is indispensable. ITC measurements provide information on thermodynamic quantities such as enthalpy during the molecular interaction based on the heat produced by reactions (Cooper et al. 1994; Goobes and Minsky 2001). Figure 4 depicts the typical isothermal titration curves obtained by injecting 1.75 mM BR into the ITC cell containing 0.010 mM lysozyme in three pH media at 25°C. From curves $X-1$ (X = A, B, C), an exothermic heat pulse is detected following each injection. Its magnitude progressively decreases until a plateau is reached corresponding to the heat of dilution of the peptide species in the B-R buffer and indicating saturation. The heat involved at each injection was corrected for the heat of dilution, which was determined separately by injecting the BR solution into the B-R buffer and then divided by the number of moles injected. The area of each peak was integrated and corrected as the enthalpy change (ΔH) of the reaction (curves *X*-2 in Fig. 4). The data appears to fit to the cooperative model in all three pH media so the binding of BR to lysozyme corresponds to a two-step sequential interaction. When c_{L0}/c_{M0} is less than six, the released heat increases with increase in c_{L0}/c_{M0}. The total ΔH values integrated in the first step were −17.0 kcal at pH 2.03, −15.8 kcal at pH 3.25 and −14.6 kcal at pH 4.35. From curves 1–3 in Fig. 3, the actual binding numbers of BR are 2 at pH 2.03, 1 at pH 3.25 and 1 at pH 4.35 when c_{L0}/c_{M0} is

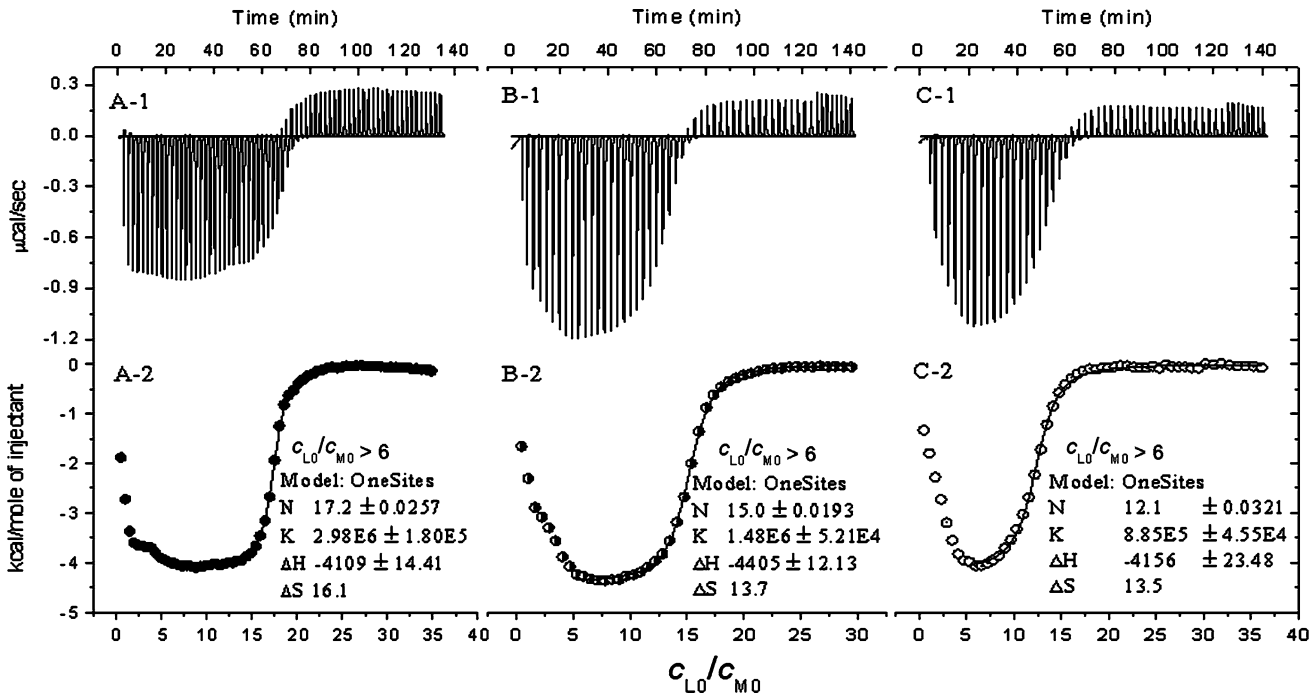

Fig. 4 *X*-1 (X = A, B, C): ITC titration profile of BR–lysozyme interaction at pH 2.03 (**a**), pH 3.25 (**b**) and 4.35 (**c**). The temperature was 25°C and all the solutions contained 20% B-R buffer. Each pulse corresponded to a 4-μl injection of 1.75 mmol/l BR into the ITC cell (1.4685 ml) containing 0.010 mmol/l lysozyme. *X*-2: The area of each peak in *X*-1 was integrated and corrected for the heat of dilution, which was estimated in a separate experiment by injecting the BR into the B-R buffer. The corrected heat was divided by the moles of injectant and values were plotted as a function of c_{L0}/c_{M0}

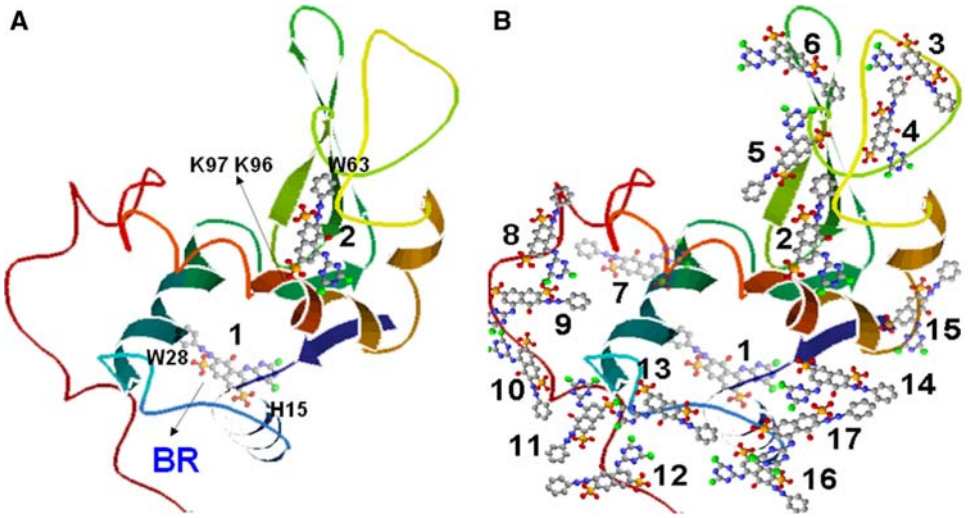

Fig. 5 Cartoon illustrating the possible binding sites of BR in lysozyme and the corresponding bonds. **a** Two binding sites of BR when c_{L0}/c_{M0} is less than 6. **b** The binding sites of BR on the outer surface of lysozyme via ion-pair attraction position-fixing

at 6. Therefore, ΔH_1 of the BR-lysozyme reaction are −8.50, −15.8 and −14.6 kcal/mol at pH 2.03, 3.25 and 4.35. Lysozyme binding BR in this step caused the decrease of entropy change (ΔS) from the falling tendency of curves *X*-2. The refolding of lysozyme occurred in the first step. As a result, BR may enter the hydrophobic intracavity of lysozyme (Collins et al. 2005) (Fig. 5a). For example, a BR molecules binds with the side group of H15 of lysozyme via ionic interaction and H-bond and bridged with that of W28 via hydrophobic π-π interaction. The other BR may interact with the side groups of K96 and K97 via ionic interaction, H-bond and bridged with that of W63 via π-π interaction (Gao et al. 2006). These interactions will contract the structure of lysozyme and cause an entropy decreasing. When c_{L0}/c_{M0} is more than six, curves *X*-2 in Fig. 4 were fitted to a sigmoid curve by a nonlinear least squares method. The K_b, N and ΔH were obtained directly; the Gibbs free energy change (ΔG) and ΔS were then calculated using the equation $\Delta G = -RT\ln(K_b) = \Delta H - T\Delta S$, where T is the temperature in Kelvin degrees and R the gas constant, 8.314 J mol^{-1} K^{-1}. The thermodynamic parameters of the BR-lysozyme interaction in the second step are summarized in Table 1 and Fig. 6. Comparison with the photometric method (Fig. 3) shows that the two measurements yield the similar N values in all cases. Because ΔH is much less than 60 kcal/mol (Yang 1998) the BR-lysozyme interaction is non-covalent, involving ionic interaction (Dominiak et al. 2007) and hydrogen bond (Panigrahi and Desiraju 2007) as

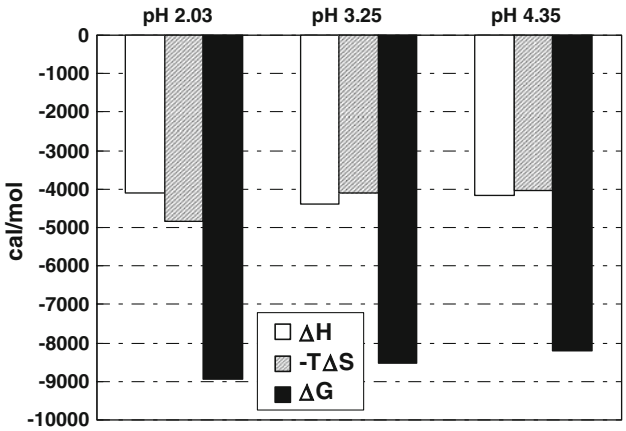

Fig. 6 Comparative distribution of ΔH (*left column*) and $-T\Delta S$ (*middle column*) in ΔG (*right column*) at pH 2.03, 3.25 and 4.35

the main contributors. The K_b values indicate that the stability of the product increases with increasing acidity of solution. The $-T\Delta S$ values always approach to ΔH ones in all three pH media (Fig. 6) so the formation of the product is driven by both enthalpy and entropy changes. In the second step, the binding numbers of BR are calculated to be 15, 14 and 11 at pH 2.03, 3.25 and 4.35. The binding of BR should be located the hydrophilic outer surface of lysozyme. The possible sites included all the side groups of the other AARs positively charged, e.g., R68, R73, R61, R45, K33, R112, R114, K116, R125, R128, R21, R5, K1, R14 and K13 (No. 3–17 in Fig. 5b), where the ionic

Table 1 Determination of the thermodynamic parameters of the lysozyme-BR reaction at pH 2.03, 3.25 and 4.35

pH	N	K_b, $\times 10^6$	ΔH, kcal/mol	ΔS, cal/(mol K)	ΔG, kcal/mol
2.03	17.2 ± 0.03	2.98 ± 0.18	−4.11 ± 0.01	16.1	−8.94
3.25	15.0 ± 0.02	1.48 ± 0.05	−4.41 ± 0.01	13.7	−8.51
4.35	12.1 ± 0.03	0.89 ± 0.05	−4.16 ± 0.02	13.5	−8.21

interaction played a position-fixing action. These hydrophilic interactions caused the entropy increase and the unfolding of lysozyme.

Effects of electrolyte and temperature

The stability of non-covalent interaction is always affected by various environmental conditions such as pH, ionic strength and temperature (Smeller et al. 2006). The effect of pH has been discussed in detail above. The effect of electrolytes on the γ of BR in the lysozyme-BR interaction is shown in Fig. 7a. With increasing electrolyte concentrations, γ decreases in three pH media, for example in 0.2 M electrolyte it is only half of that in the absence of electrolyte. This is due to the Debye-Huckel screening, where the Debye length is inversely proportional to the square root of the ionic strength of solution (Quinn et al. 1998).

The effect of temperature was also studied. The lysozyme cleft expanded at higher temperatures, increasing the distance between the adjacent peptide chains. Thus, the effective number of BR binding sites is reduced, readily causing the desorption of BR from the peptide chains. From the curves in Fig. 7b, γ decreases in all three pH media as well. However, curve three shows that γ increases when the temperature exceeds 55°C. This is because the pH used is close to the isoelectric point of lysozyme, and only a few of the basic AARs bind to BR. At a higher temperature, the original internal ion-pair bindings in lysozyme would be destroyed by an expansion of the peptide chains and the basic AARs can be released. Thus, BR molecules may weave into the internal structure of lysozyme and bind with these released basic AARs.

Effect of BR on the secondary structure of lysozyme

The specific conformation of a protein with a particular function results from covalent and non-covalent interactions among its amino acid residues. When an organic compound such as a pollutant, drug or toxicant is added to a protein solution, the internal non-covalent interactions of the peptide chain may be altered or even destroyed. In particular, strong binding between a protein and an organic compound may cause a permanent and irreversible change in the conformation and the loss of its original function. CD spectrometry is often used to characterize the secondary structure of a protein, i.e., the fractions of β-sheet, turn, α-helix and random coil. Variation of CD spectra of the solutions is shown in Fig. 8. The β-sheet fractions of lysozyme decrease rapidly in all three pH media when BR reached 0.04 mM, i.e., c_{L0}/c_{M0} is less than 4. In contrast, the α-helix and β-turn fractions increase. For example, the β-sheet decreases by over 17% in 0.04 mM BR at pH 2.03 and the β-turn increases from 0 up to approximately 6%. The hydrophobic binding of BR to lysozyme at W63 resulted in the transition of β-pleated sheet (Fig. 5a). Similar results were obtained at pH 3.25 and 4.35. The decrease of β-pleated sheet fraction also indicated that the entropy decrease of the binding reaction (Fig. 4) and the refolding of lysozyme. When BR is more than 0.04 M, e.g., 0.08 and 0.12 mM, the α-helix fraction decreased slowly and the β-sheet increased. The hydropholic binding of BR on the outer surface of lysozyme caused the unfolding of lysozyme so that some α-helixes re-changed into the β-sheet.

Inhibition of lysozyme resulting from binding of BR

The structural transformation that occurs during protein folding and functioning is of great significance in

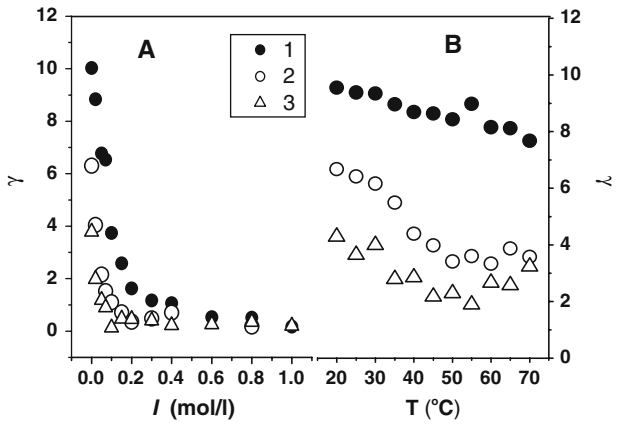

Fig. 7 Effects of electrolyte (**a**) and temperature (**b**) on γ of solutions containing 0.015 mM BR and 18.0 mg/l lysozyme at: *1* pH 2.03, *2* pH 3.25, and *3* pH 4.35

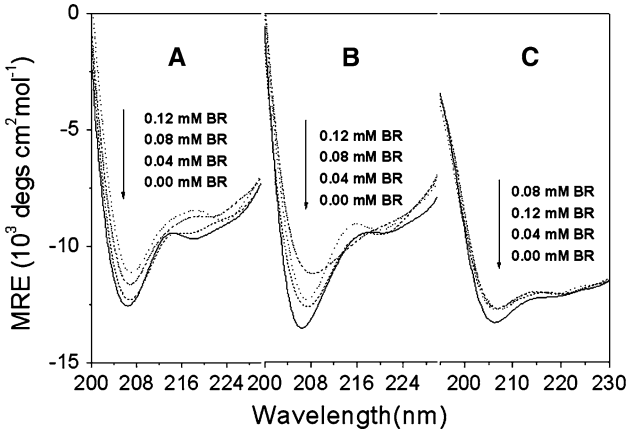

Fig. 8 CD spectra of the solutions with BR (0, 0.040, 0.080 and 0.120 mM) at pH 2.03 (**a**), pH 3.25 (**b**) and pH 4.35 (**c**). All of them contained 0.010 mM lysozyme

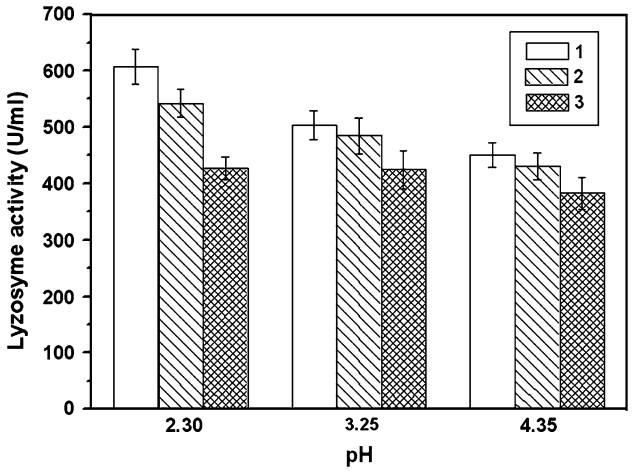

Fig. 9 Change of lysozyme activity in three pH media. *1* No BR, *2* 0.035 mM BR, and *3* 0.070 mM BR

organisms (Hu and Xu 1999). During the formation of a chemical complex, a small organic compound may insert into the inner part of the protein, regulating structure and function (Xie et al. 2005; Desai et al. 2006). Non-covalent binding is often weak and non-specific but a combination of many non-covalent bonds may alter the conformation and function of the protein (Piekarska et al. 1996). Lysozyme can attack a specific component of certain bacterial cell walls, such as the peptidoglycan composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) cross-linked by peptide bridges, by hydrolyzing the bond between NAG and NAM. This increases the permeability of the wall and causes lysis of the bacterium. Inevitably, the binding of any chemical substance with a protein or enzyme, particularly an organic compound, will affect the protein function or enzyme activity (Derham and Harding 2006; Liu et al. 2000). As an example, the polysaccharides with sulfonic group (Derham and Harding 2006), sodium dodecyl sulfate (Waehneldt 1975) and native ovalbumin (Cunningham and Lineweaver 1967) were confirmed earlier to inhibit the lysozyme activity. In this work, effects of the aromatic anions BR with two sulfonic groups on lysozyme activity were determined and the activity change is shown in Fig. 9. The activity increases with increasing solution acidity but is inhibited by the addition of BR. In addition, the inhibition by BR increases with decreasing pH. At pH 2.03, only 0.070 mM BR reduces the lysozyme activity by one third. This can be attributed to the maximal binding of BR at pH 2.03, which prevents the entry of bacterial cell wall peptidoglycan into the lysozyme cleft and thus inhibiting hydrolysis. Therefore, non-covalent binding of the organic compound severely affects enzyme activity by altering the enzyme conformation and overlapping its active sites.

Discussion

The formation of covalent complexes with specific residues in a protein is being studied increasingly in, for example, aspects of DNA repair (Liao et al. 2006), identification of enzyme active sites (Kolandaivel et al. 2006), pharmaceutical development (Yang et al. 2006) and heavy metal toxicity (Wells et al. 2006). However, non-specific non-covalent interactions are more prevalent in cells. Although a non-covalent bond is often weaker than a covalent bond, the combination of many non-covalent bonds will produce a stronger association. Despite their complicated mechanisms, increasing numbers of biochemists become interested in interactions between small molecules and biomacromolecules, because a multitude of significant biological functions depend on such type of binding. Although the crystal structures of a number of proteins have been analyzed and their possible binding regions have been identified (Kolandaivel et al. 2006), it is still difficult to distinguish all the intermediate forms or to determine single non-covalent bond energy accurately. The current work investigated the interactions of BR with lysozyme in the normal physiological acidities of skin and gastric tissues where BR often presents. A two-step binding model was found, in which only one or two of BR molecules entered the hydrophobic intracavity of lysozyme in the first step and most of BR on hydrophilic outer surface in the second step. The saturation binding number of BR is 17, 15 and 12 at pH 2.03, 3.25 and 4.35 and the possible binding sites were speculated. Such a non-specific binding resulted in a conformational change in lysozyme and inhibited its activity. On the whole, this work produced a new approach for characterizing non-specific interactions occurring in cells and organisms. It can be further extended to study the enzyme toxicity of exogenous chemical substances, e.g., aromatic pollutant and drug, and the health risk mechanism.

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