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Elucidation of the Enantioselective Enzymatic Hydrolysis of Chiral Herbicide Dichlorprop Methyl by Chemical Modification

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ABSTRACT: Up to 25% of the current pesticides are chiral, the molecules have chiral centers, but most of them are used as racemates. In most cases, enantiomers of chiral pesticides have different fates in the environment. Knowledge of the function of amino acids of enzymes involved in enantioselective behaviors contributes to the understanding of the enantioselectivity of chiral pesticides. In this work, *Aspergillus niger* lipase (ANL, EC3.1.1.3) was chemically modified using bromoacetic acid (BrAc), 2,3-butanedione (BD), *N*-bromosuccinimide (NBS), and methanal. The enantioselectivity of the enzymatic hydrolysis of 2,4-dichlorprop-methyl (DCPPM) was investigated by chiral GC. The results have suggested that histidine, arginine, and tryptophan are essential for lipase activity and might be involved in the catalytic site of ANL. In addition, histidine and lysine play an important role in determining the observed enantioselective hydrolysis of chiral herbicide dichlorprop methyl. The molecular modeling study revealed that the essential hydrogen bonds formed between DCPPM and catalytic residues of ANL might be responsible for the enantioselectivity of DCPPM. The loss of enantioselectivity can also arise from the fact that the modification of the amino acids may cause changes in both the nature of the ANL enzyme conformation and the binding pattern of DCPPM. Our study provides basic information for the exploration of the enantioselective interaction mechanism of enzymes with chiral pesticides.

KEYWORDS: Chemical modification, lipase, enantioselectivity, hydrolysis, pesticide

INTRODUCTION

Up to 25% of the current pesticides are chiral, the molecules have a chiral center and nonsuperimposable mirror images called enantiomers, but most of them are used as racemates.[1,2] Although the enantiomers have identical physical−chemical properties, in most cases, enantiomers behave different from biochemical processes, the length of lag phases as well as the degradation rates. It is also found that environmental factors can affect the chiral preference of pesticides.[3,4] Therefore, the effects and the environmental fate of the enantiomers of chiral pesticides need to be investigated separately.[5]

Nowadays, most environmental research on chiral pesticides has thus far been limited to investigating the enantioselectivity of degradation and toxicity.[6−10] Little is known about the mechanism of enantioselectivity of chiral pesticides in the environment. As we know, enantioselective degradation implies that one or more enzyme reactions involved in different degradation steps must be able to differentiate between the enantiomers, and only subtle differences in the enzymatic active sites determine their enantioselectivities. Thus, for a better understanding of enantioselective degradation and toxicity, more detailed studies of enzymes involved in enantioselective behaviors need to be conducted.[2,11−13]

Hydrolysis is an important degradation approach of pesticides in environments, and the presence of hydrolytic enzymes largely promotes the degradation of pesticides.[14] Among the hydrolytic enzymes, the lipases (EC 3.1.1.3) are a group of important hydrolytic enzymes in water and soils and are widely distributed throughout the organisms of different nature (some microorganisms are known to have the ability to produce extracellular lipases). Because of their central role in the soil environment, soil lipase activities are attractive as indicators for monitoring various impacts on soils.[15] It should be noticed that lipases are a group of hydrolytic enzymes that couple a wide specificity to a high regio- and enantioselectivity and specificity.[16,17] Thus, when pesticides are released into environments, they can interact with lipases. However, although lipase is an intriguing enzyme with diverse roles, our knowledge on its progress of stereoselective transformation is still rather limited.

As a means of identifying the functional roles of critical residues of enzymes, chemical modification of amino acids has been exploited for many years, and the chemistry is well established.[18,19] However, there are few reports to provide unequivocal information on the mechanism of the chiral interactions between lipase and pesticide by using chemical modification. Therefore, in the present work, the amino acid residues chosen for study were histidine, arginine, tryptophan, and lysine, which are known to be often catalytically essential. We performed chemical modification on *Aspergillus niger* lipase (ANL, EC3.1.1.3) and investigated the effects of chemical modifiers on ANL-catalyzed enantioselective hydrolysis of 2,4-dichlorprop-methyl (DCPPM). DCPPM is a highly effective, broad-spectrum chiral herbicide with low toxicity. Its racemate of DCPPM contains a pair of (*R*)- and (*S*)-DCPPM isomers (Figure 1), with only the (*R*)-form being herbicide active. The enantioselective

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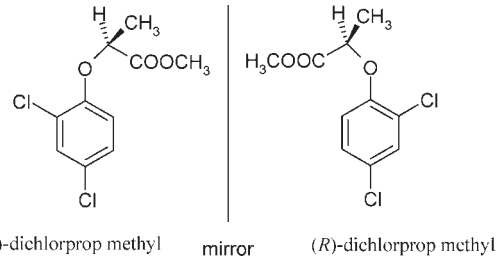

Figure 1. Enantiomers of dichlorprop methyl ester.

Table 1. Modification of Lipase by Chemical Modifiers

modifier	modified amino acid	modified group	ref
bromoacetic acid	histidine	imidazole	22
2,3-butanedione	arginine	guanidyl	23
N-bromosuccinimide	tryptophan	indolyl	24
methanal	lysine	amido	25

degradation and toxicity of DCPPM are observed.[8,20] On the basis of the chemical modification, the molecular modeling method was applied to explore the binding mode of (*R*)- and (*S*)-DCPPM isomers to the lipase. Combining the chemical modification and molecular modeling, we have identified the important role of four amino acids in the enantioselectivity of ANL. This study provides basic information for the exploration of the enantioselective hydrolysis mechanism of ANL with the chiral herbicide.

MATERIALS AND METHODS

Materials and Reagents. ANL was supplied by Shenzhen Leveiking biological engineer Co. Ltd., with a reported activity of 10 $KLUg^{-1}$. Bromoacetic acid (BrAc), 2,3-butanedione (BD),and *N*-bromosuccinimide (NBS) were supplied by Sigma Co. Ltd. as modifiers of lipase. (*R*)-Dichlorprop methyl ester, (*S*)-dichlorprop methyl ester, and (*Rac*)-dichlorprop methyl ester with 99% purity were synthesized according to Camps's method.[21] *n*-Hexane, NaH_2PO_4, Na_2HPO_4, and Na_2SO_4 were analytically pure and used without further purification.

Chemical Modification of Lipase. Histidine (His), arginine (Arg), tryptophan (Try), and lysine (Lys) residues in lipase were modified separately by different modifiers according to the slightly modified literature procedures listed in Table 1. All modification of amino acid residues were carried out in the dark in 5 mL total reaction volume (25 °C) and in 50 mM sodium phosphate buffer, pH 7.00. First, the 4 mg lipase dry powder was added into the buffer solution. Then, different concentrations of chemical modifiers were added, and the solutions were incubated. After 20 min, aliquots (1 mL) were removed and assayed for residual enzyme activity.

Enzymatical Hydrolysis. Two methods were used to carry out the lipase-catalyzed hydrolysis reactions. In the first method, fluorescein diacetate (FDA) was used as the lipase substrate to assay the hydrolytic activity of lipase. First, phosphate buffer solution (pH 7.0) was added to the tubes to make a constant volume of 5 mL and 0.5 mM FDA, and then 0.8 mg lipase was added to start the reaction. For all determinations of FDA hydrolytic activity, fluorescence emission spectra of fluorescein released during the assay was used. The scanning parameters were as follows: scanning time of 3 min, excitation wavelength of 492 nm, emission wavelength of 514 nm, and an experimental temperature of 25 °C. The fluorescence intensity at 200 s was recorded. Each treatment was sampled in triplicate, and the enzyme-free samples were included as controls for the analytical background of enzyme activity.

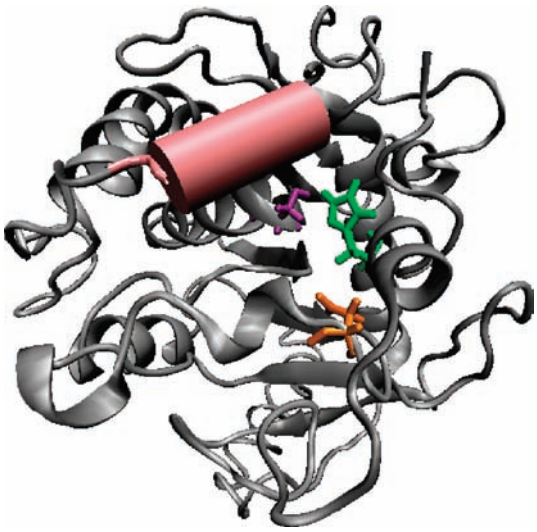

Figure 2. Tertiary structure of ANL generated by homology modeling. The catalytic triad was composed of Ser173 (purple color), Asp228 (orange color), and His285 (green color). This canonical Ser/Glu/His catalytic triad was located in a cavity with a lid composed of seven residues spanning from Ile113 to Asp119.

In the second method, DCPPM was used as the hydrolytic substrate to assay the enantioselectivity of lipase to DCPPM by slightly modifying the literature procedure.[26] The lipase-catalyzed hydrolysis of DCPPM was carried out in the dark. Ten milliliters of sodium phosphate buffer (pH 7.0) was added to 0.2 mL of substrate (1.0 mg/mL) and 0.5 mg of lipase. The mixture was incubated at 25 ± 1 °C in a rotary shaker at 120 rpm for 5 h. After the completion of the reaction, aliquots (0.2 mL) of the reaction mixture were withdrawn and extracted with 2 mL of *n*-hexane, followed by drying over anhydrous Na_2SO_4. The samples were analyzed using chiral gas chromatography. Each treatment was sampled in triplicate, and the enzyme-free samples were included as controls for the analytical background of enzyme activity.

Enantioselectivity Gas Chromatography Separation. DCPPM was analyzed by gas chromatography (GC-2010 Shimadzu), equipped with a chiral glass capillary column (β-cyclodextrin BGB-176, 30 m length, outer diameter 0.35 mm, inner diameter 0.25 mm, and film thickness 0.25 μm; BGB Analytik AG). The oven temperature of GC was 140 °C for 50 min, whereas that of the injection port and the detector were 200 and 230 °C, respectively. The analytes were monitored by electric capture detection. Peaks were identified by comparison of the retention times of (*R*)-DCPPM and (*S*)-DCPPM, respectively. The enantiomeric ratio (ER) was expressed as the peak area of the (*R*)-isomer divided by that of the (*S*)-isomer.

Spectrometry Experiments. The spectra of lipase were measured as reports.[2] Fluorescence emission spectra were recorded using a Hitachi F2500 spectrofluorimeter. UV spectra were measured using a Shimadzu UV-2401PC spectrophotometer.

Homology Modeling. The amino acid sequence of ANL (GenBank accession number ABG37906.1) was used for the structural construction. The homology modeling for the tertiary structure of ANL was performed with the I-TASSER server.[27,28] The initial template was identified using the LOMETS threading and the following structure assembly, model selection, and refinement, and structure-based functional annotation were performed with the reported protocols.[29] The quality of the obtained model was further evaluated by the Prosa web server.[30] The knowledge-based potentials of mean force were used to evaluate the model accuracy following the reported protocol.[31]

Molecular Docking. To build the structures for the complex of ANL with with (*R*)- and (*S*)-DCPPM isomers, automated molecular

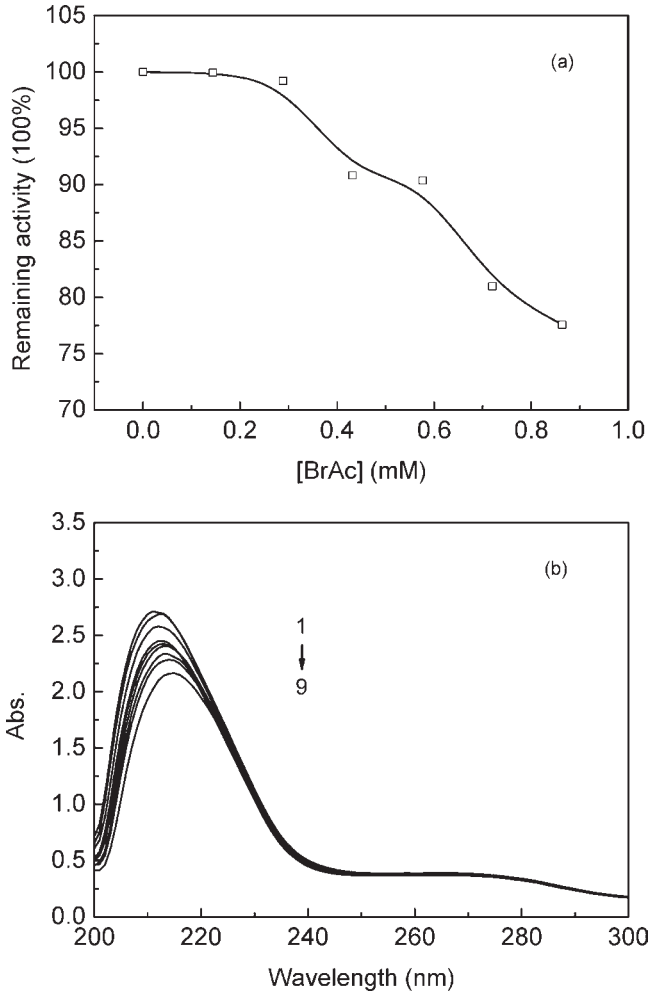

Figure 3. (a) Remaining activity of lipase incubated with different concentrations of BrAc. (b) The UV absorption spectra of lipase in the presence of BrAc. c(BrAc)/mM: 1, 0; 2, 0.144; 3, 0.288; 4, 0.432; 5, 0.576; 6, 0.72; 7, 0.864; 8, 1.008; 9, 1.152.

docking soft Gold 5.0 was used.[32] The binding pocket was defined as a sphere with a radius of 5 Å to cover the ligands. Twenty times of genetic algorithm runs were carried out during the molecular docking process. The energetic evaluations were performed using the GoldScore fitness function. Ten poses of each isomer were finally generated, and the best pose with the highest docking score was finally chosen.

RESULTS AND DISCUSSION

To better characterize the biological functions of ANL, its tertiary structural details are essential. Considering the lack of an experimentally determined structure of ANL, the tertiary structure of ANL was modeled with the established I-TASSER server, and the obtained model was verified by the Prosa web server.

The structure of ARL shares a canonical topology with the lipase family (Figure 2). Its catalytic triad was composed of Ser173 (purple color), Asp228 (orange color), and His285 (green color). This canonical Ser/Glu/His catalytic triad was located in a bowl-shaped cavity with a lid (pink color) composed by seven residues spanning from Ile113 to Asp119.

Modification of Histidines. Histidine, an essential amino acid, has as a positively charged imidazole functional group. The imidazole makes it a common participant in enzyme catalyzed

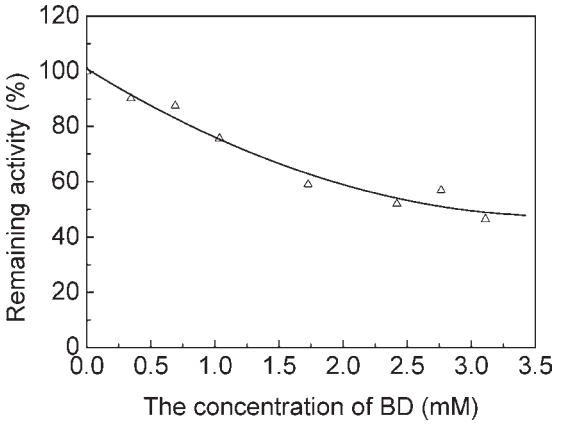

Figure 4. Remaining activity of lipase incubated with different concentrations of BD.

reactions. The modeled tertiary structure of ANL showed that the catalytic His285 locates at the active site, which is in line with the experimental study of the modification of lipase by dye-sensitized photooxidation.[33] Considering BrAc is a specific modifier for protein histidine residues and is being used widely in enzyme modification,[22,34] by modification of histidine residue with BrAc, we can understand whether the amino acids at (or near) the active center of the enzyme take part in the progress of stereoselective transformation of the chiral herbicide DCPPM.

As shown in Figure 3a, the enzyme activity remained constant at low concentration (0−0.288 mM). This result is consistent with Liu's result,[35] since it was impossible to label histidine residues in the low range of modifier concentration. At high concentration of BrAc (0.288−0.864 mM), the loss of enzyme activity increased quickly with an increase of modifier concentration and the enzyme activity remained 77.59% in the 0.864 mM BrAc. These data indicated the possible involvement of histidine residues in the catalytic mechanism of action of the enzyme.

Protein UV absorption is mainly due to the electronic excitation of aromatic amino acids such as tryptophan and tyrosine, followed by phenylalanine and histidine. The absorption spectra of these chromophores changes with varying solution conditions. In the UV differential spectrometry experiments, the absorption peak of lipase was approximately 210 nm with an absorption intensity of 0.27. Differential spectra of lipase with different concentrations of BrAc were shown in Figure 3b. As illustrated, it was found that the absorption spectrum of lipase underwent changes when BrAc was added. It can be seen that the maximum absorption of lipase decreases regularly with a red shift with increasing concentrations of BrAc (from 0 to 1.152 mM). This indicates that lipase forms complexes with BrAc and that this complex produces observable changes in the absorption spectra. The decrease in the UV−vis differential spectral intensity and red shift in the absorption maximum may be attributed to the perturbation of the microenvironment of histidine residues at higher concentrations of BrAc. These results further indicated the involvement of histidine residues in the catalytic mechanism of action of the enzyme.

To further reveal the role of histidine in enantioselectivity of the enzyme, the enzymatical hydrolysis of racemate DCPPM was investigated. It was found that ER value decreased from 2.93 to 1.77 after histidine residues were modified by BrAc and was only 60.4% that of native lipase. This result showed that the histidine residue of lipase was essential for its enantioselective catalytic

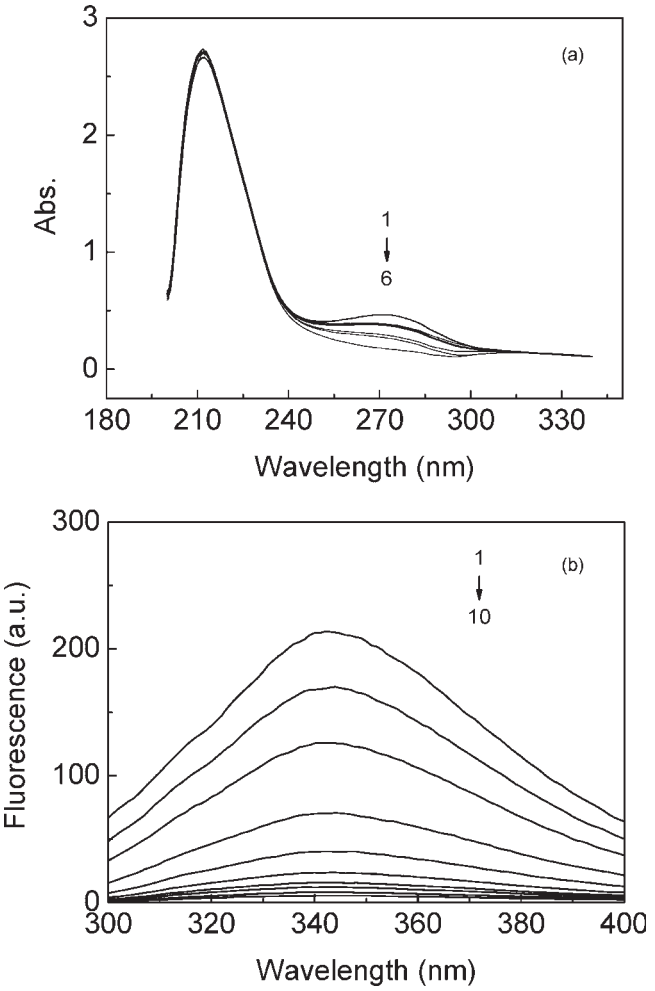

Figure 5. (a) Changes in the UV spectra of lipase induced by BD. (b) Changes in the fluorescence emission spectra of lipase induced by BD. (a) c(BD)/mM: 1, 0; 2, 0.14; 3, 0.28; 4, 0.41; 5, 0.55; 6, 0.70. (b) c(BD)/mM: 1, 0; 2, 0.35; 3, 0.69; 4, 1.04; 5, 1.38; 6, 1.73; 7, 2.07; 8, 2.42; 9, 2.76; 10, 3.11.

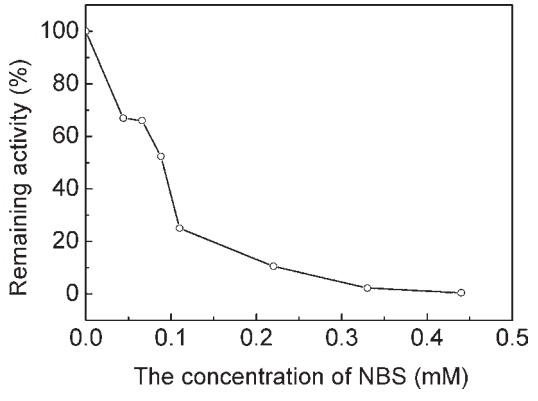

Figure 6. Remaining activity of lipase incubated with different concentrations of NBS.

activity, and the modification of histidine residue by BrAc led to the loss of enantioselectivity of lipase.

Modification of Arginines. BD has been exploited widely for the modification of arginine residues.[23,35] Figure 4 shows the remaining activity of lipase incubated with different concentrations

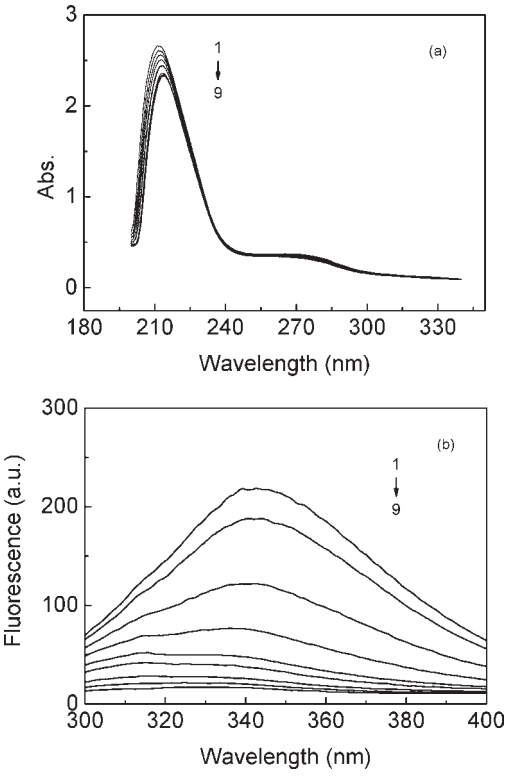

Figure 7. (a) Changes in the UV spectra of lipase induced by NBS. (b) Changes in the fluorescence emission spectra of lipase induced by NBS. (a) c(NBS/mM: 1, 0; 2, 0.045; 3, 0.90; 4, 0.13; 5, 0.18; 6, 0.22; 7, 0.27; 8, 0.32; 9, 0.36. (b) c(NBS)/mM: 1, 0; 2, 0.11; 3, 0.22; 4, 0.33; 5, 0.44; 6, 0.55; 7, 0.66; 8, 0.77; 9, 0.88.

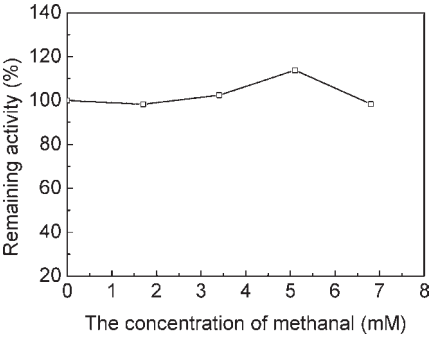

Figure 8. Remaining activity of lipase in the presence of methanal.

of BD. Similar to the result of the modification of the histidine residue, the enzyme activity decreased with the increase of modifier concentration (0−3.11 mM). The chemical modification of lipase was executed for 20 min in the presence of BD. Incubation of lipase with 3.11 mM BD to labeling arginines decreased the enzyme activity 50%. This result also indicated the possible involvement of arginine residues in the catalytic mechanism of action of the enzyme.

The UV absorption spectra of lipase modified by BD are shown in Figure 5a. Increasing the BD concentration resulted in a decrease in the enzymatic absorption intensity of the 280 nm region, which suggested a change in tyrosine exposure, besides indicating contributions from the variation of the environment or the ionization of tryptophan residues, phenylalanine residues, and probably also disulfide groups.[36] It suggested that the

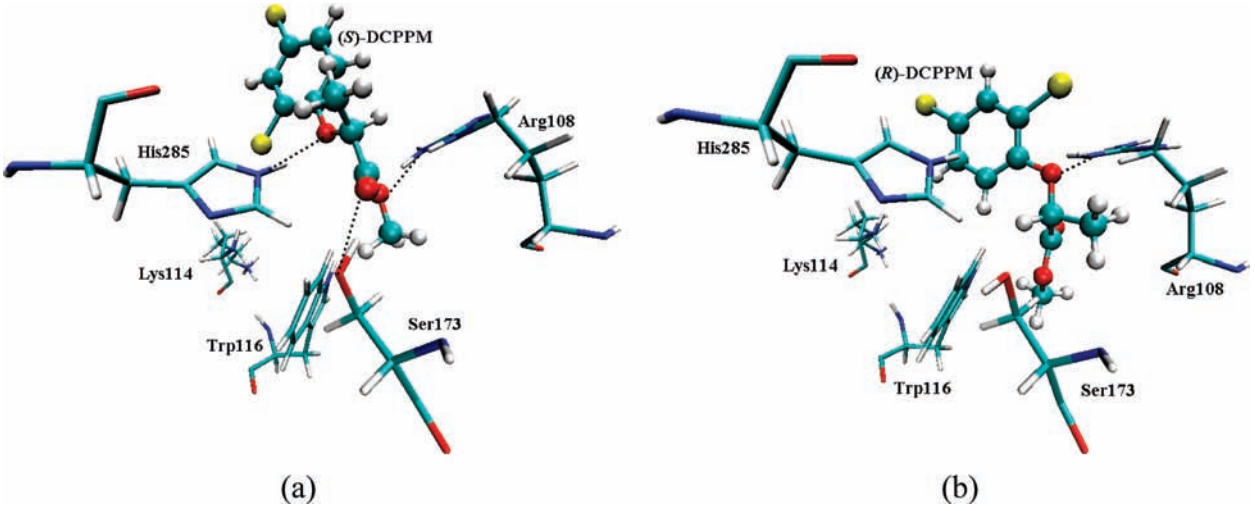

Figure 9. Binding mode of (*R*)- and (*S*)-DCPPM isomers to ANL. The dashed line indicates the hydrogen bond formed between DCPPM isomers and ANL. The DCPPM isomers are show, in stick and the residues of ANL in licorice representation.

arginine modified by BD may interact with tyrosines or tryptophans of lipase.

To further reveal the interactions between lipase and modifier BD, the fluorescence of lipase was investigated, and the fluorescence quenching spectra are shown in Figure 5b. It can be seen that increasing the concentration of BD resulted in a decrease in the fluorescence emission intensity of lipase. Since arginine was not a fluorescent amino acid, chemical modification of arginines had an effect on the microenvironment of tyrosines or tryptophans residues which were fluorescent residues of lipase.

To understant the role of arginine in the enantioselectivity of enzyme, the enzymatical hydrolysis of racemate DCPPM was also investigated. It was found that ER value remained unchanged after histidine residues were modified by BD. This result showed that the arginine residue of lipase was not essential for its enantioselective catalytic activity. But because the modification of the arginine residue by BD led to the loss of enzyme activity, these results indicated that there is no inevitable link between enzyme activity and enantioselectivity of lipase.

Modification of Tryptophans. NBS has been widely used for the specific modification of tryptophan residues, and changes in catalytic activity and loss of tryptophan residues (i.e., conversion of the indole ring into an oxindole derivative) were followed as a function of the molar ratio of NBS to enzyme.[24] In the present study, NBS was used for labeling tryptophan residues of lipase. NBS was employed initially at 0.044, 0.066, 0.088, 0.11, 0.22, 0.33, and 0.44 mM for 20 min of reaction time. From Figure 6, it can be seen that enzyme activities decrease quickly with increase of the NBS concentration. It was noticed that when the NBS concentrations increased from 0 to 0.11 mM, the enzyme activities decreased quickly. Then the enzyme activities decreased with the further increase of the NBS concentrations. At the NBS concentration of 0.33 mM, the modification by NBS caused over 99% loss of lipase activity.

The UV absorption spectra of lipase modified by NBS are shown in Figure 7a. Alteration of the concentration of NBS produced an absorption change in the 210 nm region. Increasing the NBS concentration resulted in a decrease in the absorption intensity and a small red-shift of the 210 nm region. The fluorescent properties of modified ANL by NBS are also presented in Figure 7b. The fluorescence intensity of lipase also decreased in the presence of increasing concentrations of NBS. These results showed that the tryptophan residue was located at or near the substrate binding sites of the enzyme and that oxidation of these moieties by NBS led to conformational changes in the protein, either at the active site or at some other location which, in turn, can affect the active site.[24] The study of enzymatical hydrolysis of racemate DCPPM found that the ER value also remained unchanged after tryptophan residues were modified by NBS. This result also indicated that the tryptophan residue of lipase was not essential for its enantioselective catalytic activity. In other words, the enantioselectivity and catalytic activity of lipase are two different processes, and the amino acids between two processes are not completely the same nor identical.

Modification of Lysines. Chemical modification of lysine residues has been reported to cause the inactivation of several enzymes, and methanal is widely used as lysine modifier.[25] Figure 8 shows the remaining activity of lipase in the presence of methanal. It can be found that the enzyme activity toward FDA remained fully active for 20 min in the presence of methanal (0−6.8 mM). This finding indicated that no lysine residues appeared to be essential for lipase activity. In addition, methanal did not have effects of the UV absorption spectra and fluorescence spectra of lipase. These results indicated that the modification of lysine residues by methanal also did not lead to conformational changes in the protein, either at the active site or at some other location which, in turn, can affect the active site.

It was found that the enantioselectivity decreased by chemical modification of lysine using methanal. In the presence of 3.4 mM methanal, the ER value decreased from 1.58 to 1.18. It was conceivable that lysine residues might be involved in the enantioselective enzymatic hydrolysis of DCPPM, whereas lysine was not essential in the hydrolysis of FDA as described above.

Interactions of ANL with DCPPM. Studying the interactions between DCPPM and ANL at the atomic level is essential to the elucidation of the mechanism of enantioselective hydrolysis of DCPPM isomers catalyzed by ANL. Here, we applied the molecular docking technique to explore the interactions of ANL with DCPPM isomers. The (*R*)- and (*S*)-DCPPM isomers were separately docked into the active site of ANL using the Gold 5 program, and their binding mode to ANL was compared (Figure 9).

DCPPM isomers were located at the active site of ANL and were surrounded by several residues: His285, Ser173, Arg108, Trp116, and Lys114. (*S*)-DCPPM (Figure 9a) was located at the active site of ANL and formed three hydrogen bonds with ANL, including the catalytic hydrogen bonds formed with His285 and with Ser173. However, (*R*)-DCPPM (Figure 9b) only has one hydrogen bond with Arg108, and it did not form hydrogen bonds with catalytic His285 or Ser173. The hydrolysis experiment showed that ANL is enantioselective for (*S*)-DCPPM more than (*R*)-DCPPM. This difference in hydrolysis of (*R*)- and (*S*)-DCPPM isomers by ANL is possibly due to the lack of hydrogen bond interactions between (*R*)-DCPPM and catalytic His285 and Ser173. Therefore, the specific hydrogen bond interactions between DCPPM and catalystic residues of ANL are essential for the enantioselective hydrolysis of DCPPM. The modification of these essential residues may change the binding mode of ANL to DCPPM and cause conformational changes of the ANL enzyme, thus, leading to the loss of enantioselectivity.

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ABBEVIATIONS USED

DCPPM, 2, 4-dichlorprop-methyl; ANL, *Aspergillus niger* lipase; BrAc, bromoacetic acid; BD, 2,3-butanedione; NBS, *N*-bromosuccinimide; His, histidine; Arg, arginine; Try, tryptophan; Lys, lysine; FDA, fluoresceindiacetate.

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