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MINI-REVIEW

The molecular basis for lipase stereoselectivity

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Abstract

Lipases are among the most applied biocatalysts in organic synthesis to catalyze the kinetic resolution of a wide range of racemic substrates to yield optically pure compounds. Due to the rapidly increased demands for optically pure compounds, deep understanding of the molecular basis for lipase stereoselectivity and how to obtain lipases with excellent asymmetric selectivity have become one of primary research goals in this field. This review is focused on the molecular factors that have impacts on the stereoselectivity of lipases including the steric complementarity between the lipase topological structure and its substrate, the regional structural flexibility, the hydrogen bonds between the residues around the catalytic site and the tetrahedral intermediates, and the electrostatic interactions between surface residues. Moreover, the synergistic effects of these structural factors on the catalytic properties including stereoselectivity, activity, and stability are also discussed.

Keywords Lipase · Stereoselectivity · Steric exclusion · Structural flexibility · Hydrogen bond · Electrostatic interaction

Introduction

Enantiomerically pure compounds are of rapidly increasing importance for chemical industries as they are widely used in production of pharmaceuticals, agrochemicals, flavors, and fragrances (Liebeton et al. 2000; Meyer et al. 2013; Sharma and Kanwar 2014). Enzymatic resolution of racemic mixtures represents an important method of choice (Höhne and Bornscheuer 2009). In particular, lipases are among the most applied biocatalysts in organic synthesis to catalyze the kinetic resolution of a wide range of substrates for generation of optically pure compounds, because they are cheap, stable, and cofactor-independent enzymes with broad substrate spectra, high activities, as well as great stereoselectivity (Guieysse et al. 2008; Tomić et al. 2004). Consequently, the molecular basis for the stereoselectivity of lipases and how to achieve the perfect asymmetric selectivity of a lipase have been the primary research focus in this field (Berglund 2001; Bordes et al. 2009; Guieysse et al. 2008).

For the chiral recognition of secondary alcohol enantiomers by a lipase, there has been an empirical "Kazlauskas" rule (Fig. 1), by which the enantiomers recognition relies on the match between the size of substituents at the stereocenter and the size of the corresponding binding pockets (Kazlauskas et al. 1991; Schulz et al. 2001). This empirical rule has played an important role in predicting favorable enantiomers in many lipase-catalyzed reactions (Ahmed et al. 1994; Magnusson et al. 2005; Rotticci et al. 1998). However, it does not provide comprehensive structural mechanisms for the dynamic process of enantiomers recognition. With the development of computational simulation technologies and protein crystallography, these important subjects have been extensively re-visited and more molecular factors that are related to the discrimination of

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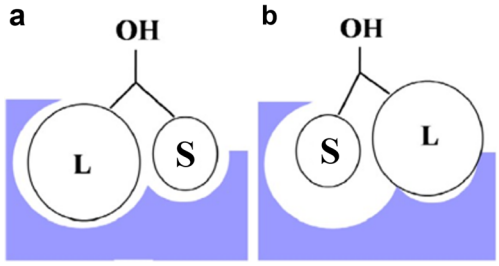

Fig. 1 The structural basis of "Kazlauska rule" for discrimination of different enantiomers of secondary alcohols. **a** The fast reacting enantiomer. **b** The slow reacting enantiomer. L and S represent the substituents with large and small size, respectively

enantiomers or diastereomers have been identified. These factors include the complementarity between the topological structure of a lipase active site and its substrate mainly based on steric hindrance which is the structural basis of Kazlauskas rule (Bordes et al. 2009; Kobayashi et al. 2010), the regional structural flexibility (Rariy and Klibanov 2000), the hydrogen bonds between the residues surrounding the catalytic site and the tetrahedral intermediates (Chen et al. 2014b; Meng et al. 2014), and the electrostatic interactions between surface residues that are distant from the catalytic site (Xu et al. 2015). Furthermore, intensive investigations on the chiral recognition mechanism of lipases have significantly advanced the understanding on their structure-activity relationship, aided the rational design of these industrially important enzymes to achieve improved asymmetric selectivity, and broadened the application of these biocatalysts in chiral chemicals manufacturing (Piamtongkam et al. 2011; Wang et al. 2010; Yao et al. 2013).

Lipases are sophisticated molecular machines with complicated regulatory mechanisms for their structure-activity relationship. The molecular factors which determine the lipase chiral selectivity can also influence other catalytic properties such as activity and stability (Santos et al. 2001; Wu et al. 2013). However, it remains a great challenge to design and engineer a new type of lipase with excellent chiral selectivity, activity, and stability.

This review article presents an overview of different molecular factors which can impact the stereoselectivity of a lipase. In some cases, their influences on enzyme activity and stability are discussed as well. By summarizing a select number of representative results, we expect that this review can provide a useful reference for the future research efforts on designing and bioengineering of robust lipases with greatly enhanced chiral selectivity, thus enabling more industrial application.

The effect of steric exclusion on lipase chiral selectivity

The steric exclusion effect of the catalytic pocket often plays an important role in lipase chiral selectivity. During the induced-fit process, the topological structure of the catalytic pocket would restrict the orientation of the substituents at the substrate stereocenter through steric exclusion effects. Only the substrate with the configuration that complements the contour of catalytic pocket can be transformed into the productive tetrahedral intermediate and hence the final product (Park et al. 2016; Reetz 2012). In this regard, the catalytic pocket of a lipase also has its own chirality, which is the intrinsic cause of Kazlauskas rule.

For a lipase with low chiral selectivity, spacious regions are often identified around the substrate stereocenter in its catalytic pocket. This indicates that the substrate binding pocket is unable to provide sufficient steric exclusion effects to limit the orientation of chiral substitutes (Chen et al. 2014a) (Fig. 2a). Therefore, many efforts have been made to improve lipases' chiral selectivity through modification of the topological structure of their catalytic pockets. For example, based on the analysis of the enzyme-substrate complex structure obtained from substrate docking and molecular dynamic simulation, Chen et al. revealed that the size of the amino acids at position 180 and 272 could be crucial for the diastereoselectivity of the lipase from *Pseudomonas alcaligenes* toward the racemic diastereomeric mixture of menthyl propionate, which has three stereocenters and eight isomers as substrates. For wild-type *P. alcaligenes* lipase, the catalytic pocket does not provide sufficient steric exclusion effects to restrict the orientation of the methyl and isopropyl groups at C5 and C2 stereocenters, respectively, thus causing low diastereopreference. A double mutant V180L/A272F, in which the mutated residues leucine and phenylalanine confer stronger steric exclusion effects due to their larger size (Fig. 2b), displayed significantly improved diastereoselectivity. At approximately 50% substrate conversion rate, the ratio between (2*R*, 5*S*) L-neomenthol and (2*S*, 5*R*) L-menthol (dr1) and the ratio between (2*R*, 5*R*) D-isoneomenthol and (2*S*, 5*R*) L-menthol (dr2) of this double mutant were 4.7- and 2.1-fold, respectively, higher than those of wild-type enzyme. The mutant lipase specifically recognized a substrate with the 2*R*- and 5*S*-configuration (Chen et al. 2014a). Moreover, when a larger chemical modifier, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), was conjugate at the position 272 to further restrict the orientation of the *S*-configured methyl group at C5 stereocenter, the diastereoselectivity was further improved. Specifically, when the conversion ratio of (*2S*, *5R*) L-menthyl propionate achieved 100%, the de_p (diastereomeric excess of product) of the target product (*2S*, *5R*) L-menthol was still higher than 90%. (Chen et al. 2013).

In some cases, a lipase could even be forced to recognize the substrate with reversed configuration by dramatically modifying the topology of its catalytic pocket (Cambon et al. 2010; Ivancic et al. 2007; Wu et al. 2013), for instance, the Lip2p lipase from *Yarrowia lipolytica*, which

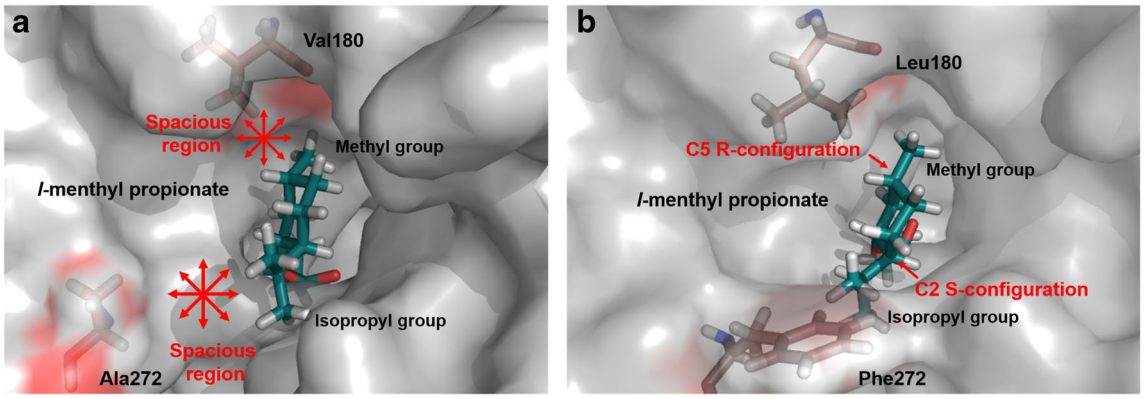

Fig. 2 Models of L-menthyl propionate covalently bound to *P. alcaligenes* lipase and the match between the tetrahedral intermediate of L-menthyl propionate and the topological structure of the lipase catalytic pocket. **a** Wild-type *P. alcaligenes* lipase and L-menthyl propionate. **b** The mutant V180L/A272F and L-menthyl propionate

demonstrates a low *S*-enantioselectivity (*E* value = 5) during the hydrolytic kinetic resolution of 2-bromo-phenyl acetic acid octyl esters. Using a double mutant D97A/V232F, the enantiomeric preference was reversed from *S*- to *R*-enantioselectivity. The *E* value of *R*-enantioselectivity was shown to be higher than 200. Mechanistically, the V232F mutation likely exerts stronger steric exclusion to limit the orientation and enables favorable stacking for the phenyl group of *R*-enantiomer (Cambon et al. 2010).

Notably, a number of studies of *Burkholderia cepacia* lipase and *Candida antarctica* lipase B showed that the steric exclusion could take effect not only in catalytic pocket, but also in substrate access channel (Guieysse et al. 2008; Lafaquière et al. 2009; Marton et al. 2010). The study of pseudo-molecular dynamics under constraints (Guieysse et al. 2003) indicated that the structure-based discrimination of enantiomers, which is possibly mediated by the hydrophobic residues with pivoting side chains, might happen at the stage of substrate access prior to the catalytic stage. This study also suggested that the fast reacting enantiomer could encounter less steric hindrance than the slow reacting one when accessing the catalytic site. Later, Guieysse et al. (2008) and Lafaquière et al. (Lafaquière et al. 2009) developed the path-planning algorithm (Cortés et al. 2005) to tackle the influence of substrate access on the enantioselectivity of *B. cepacia* lipase toward a group of enantiomer pairs derived from (*R*, *S*)-bromophenylacetic acid ethyl ester. Through the substrate access pathway analysis and the algorithm for collision detection, the residues L17 and V266 were identified, which comprise the bottleneck of the substrate access channel and exert higher steric hindrance toward the slow reacting enantiomer during substrate entrance. Subsequently, these two residues were set as mutation hot spots to construct mutation library. The effective regulation of *B. cepacia* lipase enantioselectivity can be achieved by obtaining the mutants with different size at these two important positions.

The effect of regional structural flexibility on lipase chiral selectivity

Since lipases are structurally flexible biomacromolecules, changing the regional flexibility of these enzymes would have profound influences on their biological function (Celej et al. 2003; Peters and Bywater 1999). So far, the published literature has presented contradictory examples on whether the enhanced flexibility of a lipase might increase or decrease the enantioselectivity (Castillo et al. 2010; Foresti et al. 2009).

In many cases, enzymes with higher structural flexibility usually display lower enantioselectivity, as they are more capable of accepting both substrate enantiomers and tolerating the less reactive one which experiences greater steric hindrance in the enzyme-bound transition state (Ke and Klibanov 1999; Rariy and Klibanov 2000). In a previous study (Yang et al. 2017), the results of molecular dynamic simulation indicated that the low enantioselectivity of *Candida Antarctica* lipase B (CALB) toward racemic 3-*t*-butyl-dimethyl-silyloxy glutaric acid methyl monoesters at 30 °C might be due to the increased structural flexibility of the regions around the CALB catalytic pocket. The increased flexibility likely leads CALB to better accommodate the *S*-enantiomer, thereby enhancing the catalytic rate of the undesired reaction. Thus, a D223V/A281S double mutant was designed and constructed to decrease the regional flexibility. This mutant exhibited much higher *R*-enantioselectivity at 30 °C with the ee_p (enantiomeric excess of product) value of product increased from 8% to > 99%.

Lipases are a class of enzymes that are able to catalyze transesterification reactions in organic solvents. Since the hydrophobicity and dielectric constant of the working organic solvent can significantly affect the structural flexibility of the lipase, the selection of a specific organic solvent is a simple but effective way to regulate the chiral selectivity. In a solvent with high hydrophobicity and low dielectric constant, lipases usually exhibit higher structural rigidity. Thus, improved

stereoselectivity in such organic solvents were reported (Carrea and Riva 2000; Klibanov 2001; Nishigaki et al. 2008; Ueji et al. 2003a). Unusually, an interesting example was that Watanabe et al. used *Candida rugosa* lipase MY (*Candida rugosa*: Meito Sangyo Co. Ltd.) to catalyze the esterification of 2-(4-substituted phenoxy) propionate acids with 1-butanol in hexane (Watanabe et al. 2004). Addition of variant amounts of water was adopted to adjust the structural flexibility of the lipase MY. The 0.4% (*v*/*v*) water treatment was revealed to be the optimum percentage, at which the flexibility of *C. rugosa* lipase MY was increased to approximately five times higher than that without water addition, and the *E* value was improved from 1 to 78 when the substituent of substrate was a methyl group. The increase of flexibility upon water addition was proposed to enable the rapid sampling of a large repertoire of enzyme conformations, thus enhancing the probability of reaching a conformational state that is able to bind and to convert an *R*-enantiomer of the substrate used (Broos et al. 1995).

Furthermore, the replacement of a lipase residue with a larger sized one could also decrease the regional structural flexibility surrounding the mutation site due to the reduced rotation freedom and better hydrophobic packing of the bulkier side chain. The effect of regional structural flexibility and the effect of steric exclusion could be synergistic, thus together preventing hydrolysis of the slower reacting enantiomers (Chen et al. 2014a; Chen et al. 2013).

The effect of hydrogen bonds on lipase chiral selectivity

In a lipase catalyzed reaction, the tetrahedral intermediate of the substrate must form four catalytically critical hydrogen bonds with corresponding enzyme residues: the two from the oxyanion oxygen of the tetrahedral intermediate to the two N-H groups in the oxyanion hole, and the other two from the N -H of the catalytic histidine ($H_{N\varepsilon}$) to the alcohol oxygen (O_{alc}) of the tetrahedral intermediate, and to O_γ of the active site serine (Fig. 3). The conformations containing all four hydrogen bonds are considered catalytically productive, while those missing one or more hydrogen bonds were treated to be non-productive (Colton et al. 2011). Previous studies indicated that the geometric parameter $d(H_{N\varepsilon}\text{-}O_{alc})$ and the formation of hydrogen bond between $H_{N\varepsilon}$ and O_{alc} were essential factors for enantiomer discrimination (Cygler et al. 1994; Schulz et al. 2000). In one study (Chen et al. 2014b), the *P. alcaligenes* lipase was employed to resolve racemic D,L-menthyl propionate, which exhibits excellent enantioselectivity (*E* value > 200). The structural analysis (Fig. 4) indicated that the isopropyl substituent of D-menthyl propionate points toward the catalytic His271 when the lipase bound to the slow reacting enantiomer (D-menthyl propionate). The steric requirements of

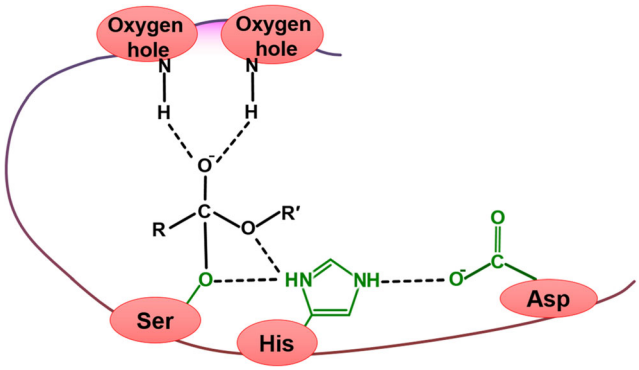

Fig. 3 The essential hydrogen bonds for the productive binding between the tetrahedral intermediate of the substrate and the lipase catalytic residues including the two N-H bonds of the oxyanion hole to the oxyanion oxygen of the tetrahedral intermediate, and the two from N -H of the catalytic histidine ($H_{N\varepsilon}$) to the alcohol oxygen (O_{alc}) of the tetrahedral intermediate, and to O_γ of the active site serine

the isopropyl substituent of the D-menthyl propionate force an approximately 30° rotation of the imidazole ring of His271. This rotation results in an increase of $d(H_{N\varepsilon}\text{-}O_{alc})$ from 2.2 to 3.7 Å, which is too far to form a productive hydrogen bond between $H_{N\varepsilon}$ and O_{alc}, thus abolishing the efficient reaction of the slow reacting enantiomer.

In the resolution of secondary alcohols, the larger acyl moiety of the substrate is often favored to achieve higher lipase enantioselectivity (Kazlauskas et al. 1991). Amplification of the size difference between the two substituents usually improves the enantioselectivity of lipase. However, this strategy was unsuccessful for the lipase catalyzed reactions using primary alcohols as substrates (Tuomi and Kazlauskas 1999). In a previous study (Meng et al. 2014), the enantioselectivity of *P. cepacia* lipase toward 14 pairs of different chiral primary alcohol esters was evaluated. It was revealed that the enantioselectivity toward the primary alcohol esters with an oxygen atom at the non-α-position of the acyloxy group ($O^{non\text{-}\alpha}$) was much better than those without (Fig. 5a). Additionally, as the acyl moiety in the substrate increased in size, the initial reaction rates decreased correspondingly (Fig. 5b). This tendency is apparently opposite to that observed for secondary alcohols. The structural analysis of the enzyme-substrate complex showed that a new hydrogen bond between $O^{non\text{-}\alpha}$ and Tyr29-OH likely plays a critical role in the enantioselectivity. Acylation of *P. cepacia* lipase via *N*-acetylimidazole modification suggested that the weakening of the Tyr29's ability as a hydrogen bond donor decreases the enantioselectivity and vice versa. Mechanistically, a larger acyl moiety causes stronger steric hindrance of the catalytic cavity, which would result in a decreased $\{C^1_{acyl}\text{-}O_{alc}\text{-}C^\alpha_{alc}\text{-}C^\beta_{alc}\}$ dihedral angle and push the $O^{non\text{-}\alpha}$ away from the Tyr29-OH (Fig. 5a), thus decreasing the enantioselectivity. Importantly, the enantioselectivity and the $\{C^1_{acyl}\text{-}O_{alc}\text{-}C^\alpha_{alc}\text{-}C^\beta_{alc}\}$ dihedral angles exhibited a linear relationship (R^2 = 0.985) (Fig. 5b).

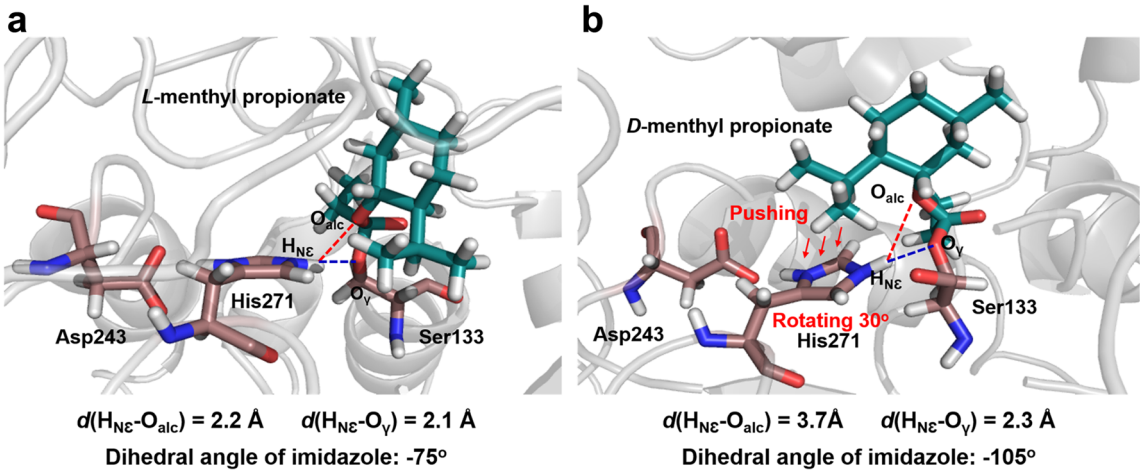

Fig. 4 The role of the hydrogen bond between $H_{N\varepsilon}$ and O_{alc} on enantiomers recognition. **a** The binding modes of L-menthyl propionate and *P. alcaligenes* lipase. **b** The binding modes of D-menthyl propionate and *P. alcaligenes* lipase

The effect of electrostatic interactions between surface residues on lipase chiral selectivity

The majority of studies on structural basis of lipase stereoselectivity have focused on the direct interactions between substrate and the catalytic center of lipase. Interestingly, a growing number of investigations have indicated that the surface residues also have impacts on the stereoselectivity of lipase. Barbosaa et al. improved the enantioselectivity of CALB toward methyl mandelate from 25.5 to 35.6 by modifying a number of surficial amino and carboxylic groups (Barbosa et al. 2012). In another study (Ueji et al. 2003b), the *C. rugosa* lipase MY modified with the benzyloxycarbonyl group gave a 15-fold increase in enantioselectivity (*E* value from 3.2 to 39) regarding the hydrolysis of racemic butyl 2-(4-ethylphenoxy) propionate in an aqueous buffer solution. The mechanism of the enantioselectivity enhancement of MY was proposed to be the surface positive charge variation. In the modified lipase, the presence of a hydrophobic cluster and the decrease of positive charges on its surface due to the replacement of lysine residues with uncharged hydrophobic groups might alter the lipase conformation and the coupled structural flexibility.

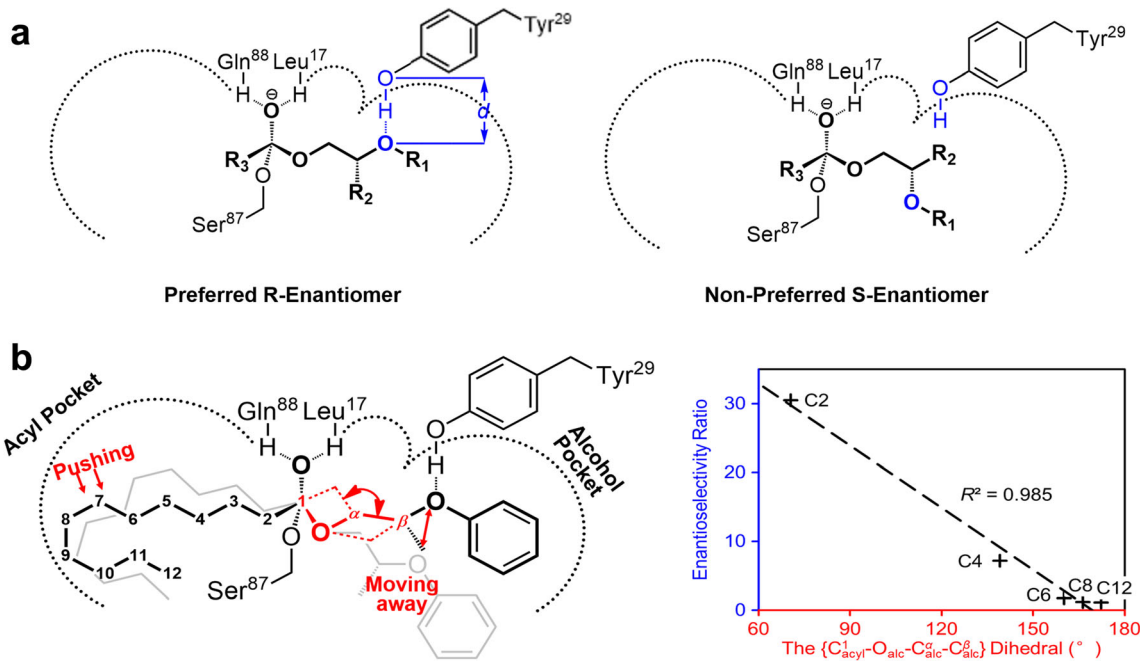

Fig. 5 **a** The role of the hydrogen bond between $O^{non\text{-}\alpha}$ and Tyr29-OH on enantiomers discrimination. **b** The relationship between enantioselectivity and the $\{C^{1}_{acyl}\text{-}O_{alc}\text{-}C^{\alpha}_{alc}\text{-}C^{\beta}_{alc}\}$ dihedral angle. The numbers on the acyl chain represent the substrate whose acyl moiety has the indicated number of carbon atoms

Previously, the *Rhizomucor miehei* lipase was employed as a model enzyme to resolve racemic *R*,*S*-*n*-butyl-2-phenxypropinate (Xu et al. 2015). By analyzing the structural differences between the two simulated complexes (i.e., the lipase in complex with the *R* and *S* enantiomer, respectively), it was found that the vast majority of the residues in both conformations of simulated complexes have similar B-factors (the indicator of structural flexibility), except for two regions: β1-β2 loop and α2 helix. B-factors of the residues in these domains of the (*S*)-conformation were up to six times higher than those of (*R*)-conformations (Fig. 6a), suggesting that the binding of *S*-substrate to the lipase might make the enzyme much more flexible in these regions than that of the *R*-substrate. Accordingly, a surficial-electrostatic interaction-aided stereo-recognition mechanism was proposed (Fig. 6b). Specifically, most lipases have a "lid" structure module which usually is an α-helix. When the lid is open, the lipase is activated. Otherwise, the enzyme stays inactive (Jaeger et al. 1999). For the *R. miehei* lipase, the residues Arg86 in the lid module and Asp61 in β1-β2 (Fig. 6a) loop form an electrostatic interaction which can be considered as a "lock" to keep the "lid" open. The binding of the fast reacting enantiomer (*R*-substrate) would not break this electrostatic interaction. On the contrary, the absence of such Asp61-Arg86 interaction was observed in the simulated complex of the lipase and *S*-substrate. This result indicated that the slow reacting enantiomer could break the lock, thereby destabilizing the active conformation of lipase. Consequently, hydrolysis of the *S*-enantiomer is more difficult. The authors further used 1-iodo-2,3-butanedione to connect the two aspartate residues covalently, and the enantioselectivity was increased from 6 to 45.

Variation of single structure factor may have influence on multiple catalytic properties

In industrial applications, high stereoselectivity, activity, and stability are all required for a lipase in order to improve the substrate concentration, the product optical purity, the space-time yield, and the cost-effectiveness. Currently, the optimization of a single catalytic property of a lipase by protein engineering might not be difficult. However, the structural factors related to lipase chiral selectivity have unpredictable influences on other catalytic properties, such as activity and

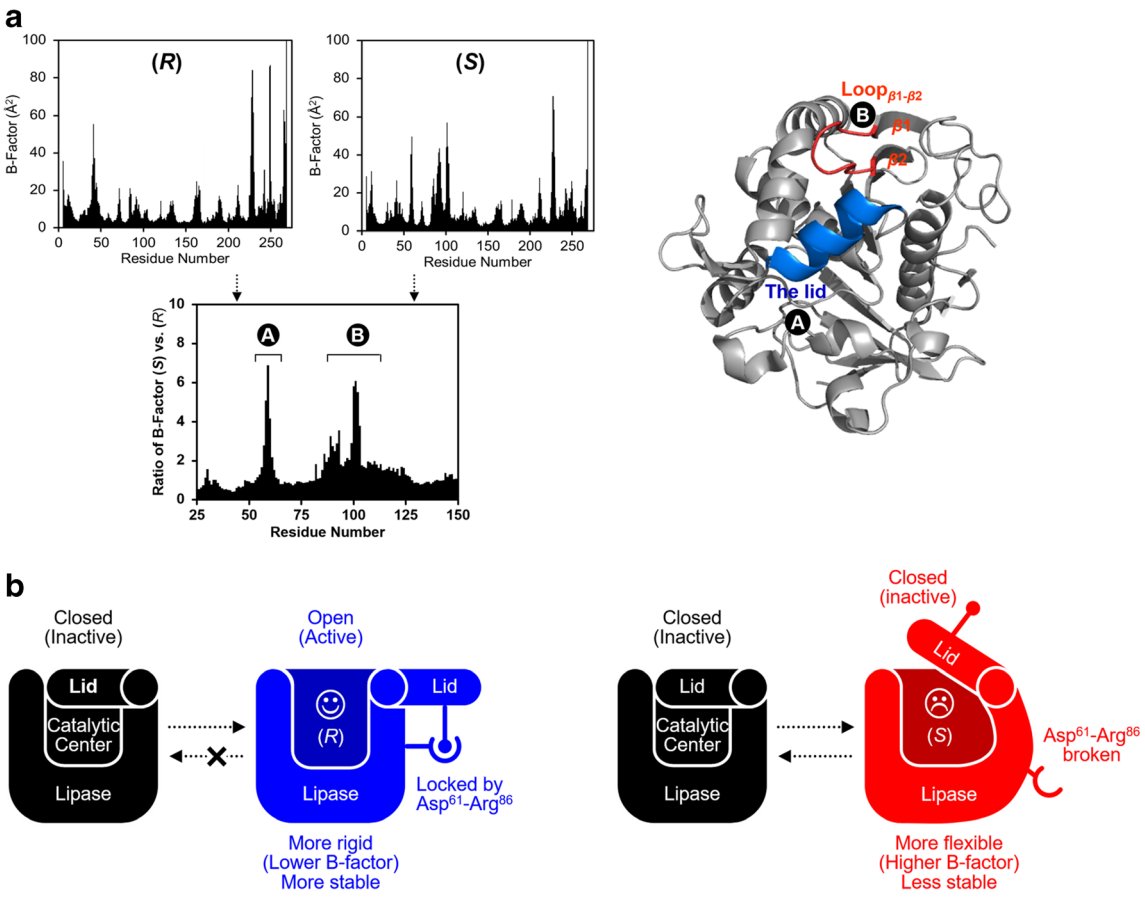

Fig. 6 **a** Ratios of the B-factors of *R miehei* lipase bound with *S*-*n*-butyl-2-phenxypropinate versus the B-factors of *R miehei* lipase bound with *R*-*n*-butyl-2-phenxypropinate (left) and the domains with the ratios significantly greater than 1 (right). **b** Cartoon of the surficial-electrostatic interaction-aided stereo-recognition mechanism

stability. For instance, the steric exclusion could affect the substrate access and the product release and hence the catalytic activity. Kobayashi et al. reported that a single mutation R48S of *Escherichia coli* acetyl esterase relieved the steric hindrance caused by the bulky alcohol moiety of tributyrin substrate, which increased the specific activity for 2.8 times by facilitating the substrate binding (Kobayashi et al. 2010). The structural flexibility has influence on both activity and thermostability. The mutant G28S of *Bacillus pumilus* lipase displayed a k_{cat} value that is four times higher than the wild-type enzyme and significantly improved thermostability (the half-life time increased from 24 to 41 min at 35 °C) due to the increased local flexibility (Bustos-Jaimes et al. 2010). The hydrogen bonds and salt bridging interactions on the surface or in interior of a lipase can also impact the stability (Park et al. 2012; Wu et al. 2015).

A noteworthy problem is that lipase engineering which improves one particular catalytic property may cause negative impacts on other catalytic properties. For example, upon obtaining higher enantioselectivity, the activity of the modified lipases decreased (Bordes et al. 2009; Chen et al. 2013). Activity improvement could also cause decreased thermostability (Shih and Pan 2011). Therefore, synergistic modulation of stereoselectivity, activity, and stability based on more comprehensive understanding of the structure-activity relationship of lipases remains a critical scientific and practical problem.

Conclusions and perspectives

Lipase-based biotransformation is an effective choice for production of chemicals with high optical purity. In the recent two decades, significant progresses have been made in understanding of the structure-activity relationship with regard to the stereoselectivity of lipases. Several structural factors which play important roles in chiral recognition have been revealed. However, the current studies are mainly dedicated to deciphering the relationship between a single molecular factor and the stereoselectivity. Essentially, the stereoselectivity of a lipase is a compositive result of multiple molecular factors. One particular factor can influence other catalytic properties. The complicated interactions between multiple molecular factors and catalytic properties are still a "gray box", if not a "black box". How to resolve the gray box by establishing efficient computational prediction and evaluation methods to synergistically improve the catalytic properties should be the focus of the future researches on these industrially important biocatalysts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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