Improved Enantioselectivity of a Lipase by Rational Protein Engineering

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A model based on two different binding modes for alcohol enantiomers in the active site of a lipase allowed rational redesign of its enantioselectivity. 1-Halo-2-octanols were poorly resolved by Candida antarctica *lipase B. Interactions between the substrates and the lipase were investigated with molecular modeling. Unfavorable interactions were found between the halogen moiety of the fast-reacting* S *enantiomer and a region situated at the bottom of the active site (stereoselectivity pocket). The lipase was virtually mutated in this region and energy contour maps of some variants displayed better interactions for the target substrates. Four selected variants of the lipase were produced and kinetic resolution experiments were undertaken with these mutants. Single point mutations gave rise to one variant with doubled enantioselectivtiy as well as one variant with annihilated enantioselectivity towards the target halohydrins. An increased volume of the stereoselectivity pocket caused a decrease in enantioselectivity, while changes in electrostatic potential increased enantioselectivity. The enantioselectivity of these new lipase variants towards other types of alcohols was also investigated. The changes in enantioselectivity caused by the mutations were well in agreement with the proposed model concerning the chiral recognition of alcohol enantiomers by this lipase.*

KEYWORDS:

enzyme catalysis · kinetic resolution · lipases · protein engineering · rational design

Introduction

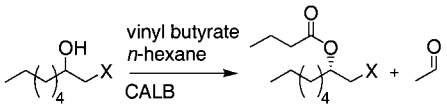
The need for new enantioselective catalysts has increased over the last decade. At the same time, biocatalysis has emerged as one of the options when facing the challenge of producing single enantiomers.[1–4] However, biocatalysts are complex catalysts, whose mechanisms of action and enantiorecognition have often remained unclear. Furthermore, due to their natural function, many enzymes have a narrow substrate specificity. Understanding and redesigning their selectivity for unnatural substrates still remains a challenge.

Lipase-catalyzed asymmetric transformation is one of the best methods of preparing optically active compounds.[5, 6] Resolution of racemates with hydrolases is the most common biocatalytic strategy for industrial production of enantiomerically pure fine chemicals.[7]

Protein engineering is a promising approach for the creation of tailor-made biocatalysts. To date, only a few lipases/esterases have been improved as regards to their enantioselectivity through the use of protein engineering. These altered biocatalysts have been obtained by directed evolution,[8–11] gene shuffling,[12] site-directed mutagenesis,[13, 14] and chemical modification.[15] In most cases, the modified enzymes have reached only moderate enantioselectivity. The strategies used can be divided into two groups—random and rational approaches.

The ability of an enzyme to distinguish between two enantiomers is defined by the enantiomeric ratio *E*.[16] This is the ratio of the specificity constants (k_{cat}/K_m) of the enzyme for the competing enantiomers.

Candida antarctica lipase B (CALB), which has been used in the academic world as well as in industry,[1, 6] is an efficient catalyst for asymmetric transformation of *sec*-alcohols and related compounds.[17] Moreover, its three-dimensional structure has been determined.[18] However, halohydrins such as 1-bromo-2-octanol (**1**) and 1-chloro-2-octanol (**2**) are poorly resolved by CALB compared to 3-nonanol (**3**; Scheme 1).[19] This report



1 X = Br $E = 6.5$
2 X = Cl $E = 14$
3 X = CH_3 $E = 200$

Scheme 1. *Enzymatic kinetic resolution of* sec*-alcohols with* Candida antarctica *lipase B (CALB).*

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presents the design and production of CALB mutants displaying better enantioselectivity towards these substrates.

Our group has previously proposed a model for explaining the mechanism of enantiorecognition by CALB as regards *sec*-alcohol enantiomers.[20, 21] This model was based on molecular modeling of the transition state of the fast- and slow-reacting enantiomers, as well as the experimental kinetic resolution of *sec*-alcohols. The model is briefly described here. Figure 1 depicts

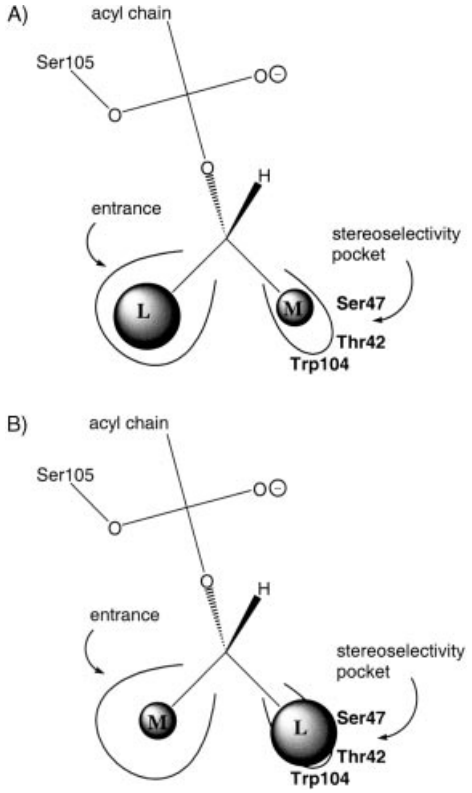

Figure 1. *Representation of the productive docking modes for* sec*-alcohol enantiomers in their transition states in the active site of* Candida antarctica *lipase B. A) Docking mode 1, which is optimal for the fast-reacting enantiomer. B) Docking mode 2, which is optimal for the slow-reacting enantiomer.* ***L*** *and* ***M*** *represent the large- and the medium-sized substituents at the stereocenter of the alcohol. The pockets indicate the binding site for the alcohol moiety.*

the proposed transition states of *sec*-alcohol enantiomers in the active site of CALB. The large pocket represents the entrance of the active site and the stereoselectivity pocket represents a region of limited volume situated at the bottom of the active site. For the reaction to be catalyzed, the fast- and slow-reacting enantiomers bind in different productive docking modes (docking modes 1 and 2, Figure 1). When the large substituent at the stereocenter is bigger than an ethyl group, the selectivity is dramatically increased due to the lack of space to accommodate the large substituent of the slow-reacting enantiomer in the stereoselectivity pocket.

Steric interactions are not the only important factors for the differentiation between enantiomers. CALB also shows quite different enantioselectivity towards aliphatic alcohols and their almost isosteric halohydrin analogues, as shown in Scheme 1. This difference in enantioselectivity shown towards halohydrins and their nonhalogenated analogues is suggested to be due to unfavorable interaction between the halogen atom and the stereoselectivity pocket of the enzyme.[22] The difference was experimentally determined to exceed 1.5 kcal mol^{-1}.

This article presents an example of site-directed mutagenesis of a lipase, based on rational design, that led to the creation of mutants which were more enantioselective than the wild-type lipase.

Results and Discussion

Molecular modeling was undertaken in order to clarify the possible presence of unfavorable interactions between the enzyme and the halogenated substrates. The affinity of the alcohol-binding site for 1-bromo-2-octanol (**1**) was evaluated by means of the program GRID.[23] The methyl and organic bromine probes were used to simulate the interactions of the large and the medium moiety at the stereocenter, respectively. The stereoselectivity pocket did not display any favorable interactions for the halogenated moiety (CH_2Br) according to the energy contour map when compared to the large pocket at the entrance of the active site (Figure 2 A). This marked preference of the halogenated moiety for the entrance of the binding site will disfavor the productive docking mode 1 for the fast-reacting enantiomer of **1**. This would explain the poor enantioselectivity for halohydrins bearing the halogen on their medium-sized substituent and also why the reversed effect is observed when the halogen is placed on the large substituent as in 1-bromo-2-propanol.[19] Improving the affinity of the stereoselectivity pocket for the halogenated substituent (CH_2X) at the stereocenter should result in a higher enantioselectivity towards compounds **1** and **2**.

Thr40, Thr42, Ser47, and Trp104 define the stereoselectivity pocket of CALB. Only three of these four residues were good candidates for site-directed mutagenesis. Thr40 was not mutated due to its importance for the stabilization of the oxyanion formed in the transition state. In order to maintain the stereoselectivity of CALB, the size of the stereoselectivity pocket should not increase so as to facilitate the slow-reacting enantiomer to bind in its productive docking mode 2 (Figure 1). Further, the affinity for docking of the CH_2Br and the CH_2Cl moieties should be increased to favor docking mode 1 of the fast-reacting enantiomer, that is, the electrostatic potential of the pocket should be altered. The residues Thr42, Ser47, and Trp104 were virtually mutated with molecular modeling, followed by a short molecular dynamics simulation and energy minimization of the mutated residue in order to find more stable conformations. Only small amino acids were considered as candidates due to the limited space in the pocket (about the size of an ethyl group). The removal of electronegative groups or the introduction of basic amino acids in this region might increase the affinity for the halogen atom.

The following mutants were modeled: Ser47Ala, Ser47Asn, Ser47His, Thr42Asn, Thr42Asp, Thr42His, Thr42Val, Trp104His, and Thr42Val – Ser47Ala. Upon mutation for a histidine, both the protonated and unprotonated forms were considered. The

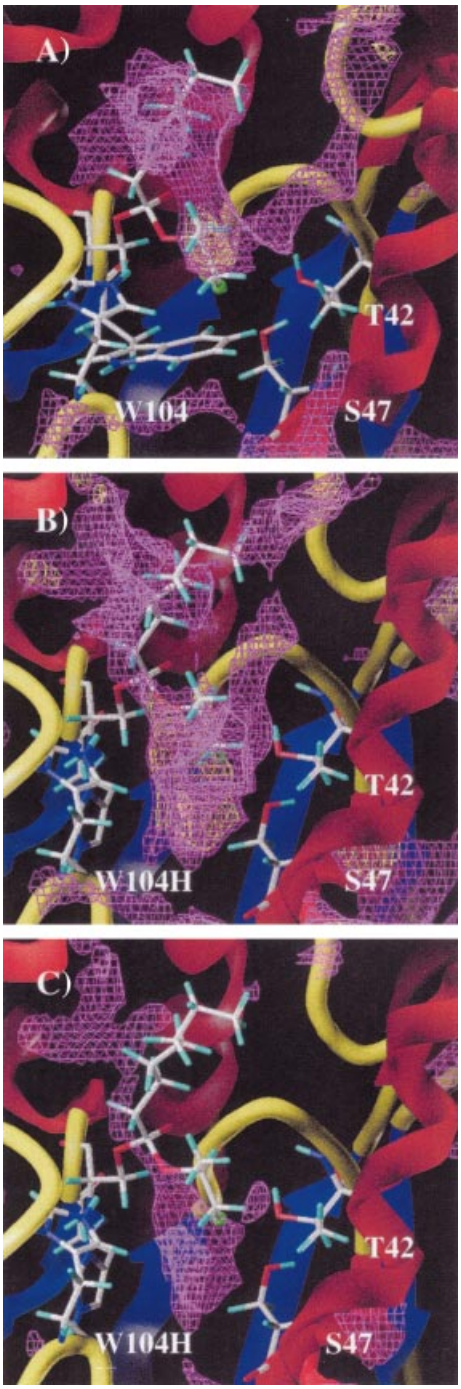

Figure 2. *The energetically favorable binding sites for the organic bromine atom and methyl group. Close-up views of models of the active sites of the wild-type lipase (A) and the mutant Trp104His (B, C) are displayed. The favorable interactions for an organic bromine atom (A, B) and a methyl group (C) were calculated with the program GRID. The contour maps (energy levels of* -3 *kcal mol*$^{-1}$ *in magenta and* -6 *kcal mol*$^{-1}$ *in yellow) are shown. The substrate, (S)-1-bromo-2-propyl octanoate, is shown as a stick representation and was positioned according to previous modeling on 3-methyl-2-butyl octanoate in order to help the reader to visualize the hypothetical position of the substrate.*[26] *The green atom represents the bromine atom in the stereoselectivity pocket. W = Trp, H = His, S = Ser, T = Thr.*

resulting structures were inspected visually for catalytic relevance. Irrelevant structures often displayed a major rearrangement of the loop containing Thr40, that is, the loop had flipped away from where the oxyanion must be placed. The energy contour maps obtained for the mutations at positions 42 and 47 were very similar to the one from the wild-type lipase and only the energy contour map of the mutants Ser47Ala, Thr42Val–Ser47Ala, and Thr42Asn suggested a subtle increase in affinity for the CH_2Br group in the vicinity of the stereoselectivity pocket. Calculation for the Trp104His mutation exhibited a favorable binding site for the organic bromine in this region (Figure 2B), but a large cavity was also created (Figure 2C). This cavity should favor the binding of the large substituent of the slow-reacting enantiomer in the stereoselectivity pocket. As a result, this mutation would facilitate the binding of both the fast- and slow-reacting enantiomers in their productive docking modes.

Based on the molecular modeling the following mutations were performed. The two amino acids Thr42 and Ser47, containing electronegative hydroxy groups, were replaced in the stereoselectivity pocket by valine and alanine, respectively. The mutation Trp104His was also carried out.

The kinetic resolution experiments were performed with immobilized enzyme preparation of the wild-type lipase and the four mutants in hexane at low water activity ($a_w = 0.1$). *E* values were experimentally determined from the enantiomeric excesses of the product and the substrate at several different conversions.[24] As can be seen in Table 1, the enantioselectivity towards the targets 1-bromo-2-octanol (**1**) and 1-chloro-2-octanol (**2**) was doubled when the mutant Ser47Ala was used instead of wild-type lipase. The mutation Thr42Val did not affect the enantioselectivity and the double mutation led to a catalyst that was more enantioselective than the wild-type lipase, but not as good as Ser47Ala. This suggested that only the mutation Ser47Ala was important for the enantioselectivity.

Table 1. *Enantioselectivity of* Candida antarctica *lipase B variants towards target substrates* ***1*** *and* ***2****.*

CALB	*E*[a]	
	1	**2**
wild-type	6.5 ± 0.4	14 ± 2
Thr42Val	6.7 ± 0.3	15 ± 2
Ser47Ala	12.4 ± 0.4	28 ± 3
Thr42Val – Ser47Ala	9.4 ± 0.8	20 ± 2
Trp104His	1.7 ± 0.1	2.0 ± 0.1

[a] Enantiomeric ratio values with standard deviation.

The mutation Trp104His increased the size of the stereoselectivity pocket, which could now accommodate both the large- and the medium-sized substituents of **1** and **2**. Consequently, not only the fast-reacting enantiomer but also the slow one could bind in its productive docking mode (Figure 1). The loss of enantioselectivity for this mutant supported our proposed mechanism for the enantiorecognition of *sec*-alcohol enantiomers by CALB and was in agreement with the low enantioselectivity reported by Patkar et al. for this mutant towards aliphatic *sec*-alcohols.[25]

The specific activities of the lipase variants are presented in Table 2. All the variants lost some specific activity towards tributyrin, compared to the wild-type lipase. The most interest-

Table 2. *Specific activity of the* Candida antarctica *lipase B variants toward tributyrin.*

CALB	Activity[a] [s^{-1}]
wild-type	253 ± 12
Thr42Val	45 ± 2
Ser47Ala	144 ± 14
Thr42Val – Ser47Ala	84 ± 8
Trp104His	102 ± 13

[a] Enzymatic activities were measured with the free lipases in aqueous media. Active site concentrations were determined with methyl *p*-nitrophenyl hexylphosphonate as an irreversible inhibitor.[30]

ing variant (Ser47Ala) with doubled enantioselectivity towards **1** and **2** retained 60% of the specific activity of the wild-type lipase. It is worth noting that the Trp104His variant showed higher activity than the wild-type enzyme at low and high pH values (data not shown).

Kinetic resolution of other types of alcohols was carried out in order to evaluate the scope of CALB mutants (Table 3). The Trp104His mutant displayed the lowest enantioselectivity toward all the substrates tested, probably due to the increased

Table 3. *Enantioselectivity of* Candida antarctica *lipase B variants towards other alcohols.*

CALB	E[a]		
	1-bromo-2-butanol	2-butanol	*meso*-2,3-butanediol
wild-type	88 ± 2	6.5 ± 0.4	11 ± 2
Thr42Val	58 ± 1	6.1 ± 0.3	14 ± 1
Ser47Ala	60 ± 1	6.5 ± 0.3	13 ± 2
Thr42Val – Ser47Ala	61 ± 1	5.7 ± 0.1	13 ± 1
Trp104His	1.7 ± 0.1	1.4 ± 0.1[b]	2.0 ± 0.1[b]

[a] Enantiomeric ratio values with standard deviation. [b] Opposite enantiopreference to the wild-type lipase.

volume of the stereoselectivity pocket (Figure 2C). Interestingly this mutant showed a switch in enantiopreference for 2-butanol and *meso*-2,3-butanediol. However, the enantioselectivity for the Trp104His was poor towards these substrates, but this mutation might be used as a starting point for the future creation of *S*-enantioselective CALB mutants.

The three mutations at the positions 42 and 47 affected only the stereoselectivity towards the halohydrins. 1-bromo-2-butanol has two substituents of approximately the same size, so that the E value reflects the electrostatic contribution to the enantioselectivity for *vic*-brominated substrates. The enantioselectivity of CALB mutants was reduced with this substrate due to a decrease of electrostatic repulsions induced by the mutations. These results showed that nonsteric interactions can be important in enzymatic kinetic resolutions and that one can take advantage of these interactions to modify the enzyme enatioselectivity. All three mutations at positions 42 and 47 had a negative effect on the enantioselectivity for 1-bromo-2-butanol while only two of these mutations had a positive effect on the enantioselectivity of CALB towards **1** and **2**.

These results, achieved by site-directed mutagenesis, support the model proposed for molecular recognition of *sec*-alcohol enantiomers by CALB and show that rational design of enantioselectivity is possible if based on thorough models. The prospects of rational design may increase in the future due to an increasing knowledge in enzyme catalysis and structural biology. Molecular modeling of both enantiomers in their transition states will also be essential for such design. Rational design may also help to find regions important for enzyme substrate selectivity, thus narrowing the huge DNA sequence space involved in directed evolution.

Experimental Section

General: All reagents and solvents were acquired from the Aldrich chemical company (unless otherwise stated). Medium-pressure liquid chromatography was performed on silica gel (Merck KGaA, Germany) using a SEPARO AB setup (Bæckström, Sweden). ^{1}H NMR spectra were recorded at 400 MHz on a Bruker Advance 400 with $Si(CH_3)_4$ as the standard. Gas chromatography (GC) analyses were carried out on Hewlett Packard MS Chemstation GC/FID 6890 and MS 5973 (70 eV). Enzymatic activities were performed with a Radiometer pH-stat equipped with an ABU91 auto burette.

Modeling: All the simulations were performed on a Silicon Graphic Workstation Octane. Energy minimizations and molecular dynamics simulations were carried out with the software package SYBYL 6.5 (Tripos Inc., St Louis, MO, USA) using the all atoms Kollman force field. The binding site of the wild-type and mutant lipases was investigated with the methyl and organic bromine probes of the GRID program, Version 17, and the resulting contour maps were displayed with SYBYL.

Molecular modeling was performed with the Protein Databank (PDB) file entry 1TCA after removal of the sugar units. This structure was prepared for the subsequent calculations as previously described by Raza et al.[26] The residues of interest were then virtually mutated and allowed to relax in the enzyme through energy minimization and molecular dynamics (MD). The virtually mutated residues were heated by 50 K per 1 ps to 300 K, followed by a 10-ps simulation at 300 K and subsequent energy minimization. The whole enzyme was then subjected to the same procedure (minimization+MD+minimization). Upon mutation into a histidine, both the protonated and the unprotonated forms were considered. The resulting structures were inspected visually for catalytic relevance. After removal of all of the water molecules, the energy contour maps of the active site were calculated by means of the program GRID and the resulting contour maps were displayed.

Site-directed mutagenesis: Thr42Val, Ser47Ala, and Thr42Val – Ser47Ala mutants where obtained by using the overlap polymerase chain reaction (PCR) technique.[27] The following primers where used to insert the desired mutations: fCALBT42V 5′-GGAACCGGCGTCACAGGTCC, rCALBT42V 5′-GGACCTGTGACGCCGGTTCC, fCALBS47A 5′-TCCACAGGCTTTCGACTCGAA, and rCALBS47A 5′-GTTCGAGTCGAAAGCCTGTGG. The CALB gene was inserted into the plasmid YpDC541 using *Xho* I and *Not* I restriction sites; the lipase gene was placed directly after the α-factor secretion signal sequence, as previously described by Rotticci-Mulder et al.[28] For the Trp104His mutant, the Transformer site-directed mutagenesis kit was used as described by the manufacturer (Clontech Laboratories Inc., USA). The plasmid YpCALB was mutated with the mutagenic primer 5′-GCTTCCCGTGCTTACCCACTCCCAGGGTGG and the selection primer

5′-GCTGTTCCAGGGATCGCAGTGGTGAG. The selection primer allowed a silent mutation in the plasmid that removed the unique restriction site *Sma* I. All different CALB mutants were sequenced on both strands by use of Sanger sequencing reactions[29] with dye terminators and were analysed on an ABI Prism 377 DNA sequencer (Perkin-Elmer, Wellsley, USA).

Lipase expression, purification, and immobilization: The methylotrophic yeast *Pichia pastoris* was previously found to be a suitable host for the overexpression of active CALB.[28] The plasmids containing the desired mutated lipase gene were transformed into *P. pastoris* (SMD1168) by electroporation. The resulting yeast colonies were screened for lipase production by colony blotting. After production in shaking flasks, the culture medium containing the lipases was purified by hydrophobic interaction chromatography and gel-filtration chromatography as described for the wild-type enzyme.[28] The resulting lipase solutions displayed a single band of 35 kDa by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE); this band was identified by Western blot analysis with rabbit anti-CALB antibodies to be CALB. The lipase variants were immobilized onto Accurel EP100 (5 lipase units per mg of carrier) as previously described by Holmquist et al.[13]

Active site titration and lipase activity towards tributyrin: Methyl *p*-nitrophenyl *n*-hexylphosphonate was used for the determination of the active site concentration of the lipase variants in water.[30] The procedure used was previously described by our group. The enzymatic activity towards tributyrin was measured using a pH-stat at 25 °C and pH 7.5. The substrate solution (0.2 M tributyrin, 2% gum arabicum, 0.2 M $CaCl_2$) was emulsified and the reaction was started by adding the enzyme to the emulsion.

Chemical syntheses: 1-Bromo-2-octanol (**1**) and 1-chloro-2-octanol (**2**) were prepared essentially as previously described.[31]

1-Bromo-2-butanol: A dark liquid containing 1-bromo-2-butanol and 2-bromo-1-butanol was purchased from Tokyo Kasei Kogyo (TCI) Co. Ltd. This liquid was subjected to distillation and medium-pressure chromatography on silica gel (C_6H_{14}/EtOAc) to afford the colorless bromohydrin. ^{1}H-NMR (400 MHz): $\delta = 3.75$ (m, 1H); 3.55 (dd, $J = 10.3$, 3.6 Hz, 1H), 3.39 (dd, $J = 10.3$, 7.0 Hz, 1H), 2.12 (br. s, 1H), 1.59 (q, $J = 7.3$ Hz, 2H), 0.98 (t, $J = 7.3$ Hz, 3H).

Kinetic resolution experiments: The enzyme preparations were equilibrated against a saturated aqueous solution of LiCl for 24 hours ($a_w = 0.1$; a_w = water activity). In a sealed vial, the racemic alcohol (0.5 mmol) was added to the immobilized lipase (7 – 30 mg of carrier) suspended in hexane (1 mL, previously dried over molecular sieves) at 23 °C. Vinyl butyrate (0.5 mmol, 57 mg) was added to the solution and samples were taken regularly. The enantiomeric excess values for the substrate and the product, ee_s and ee_p, were monitored by chiral GC (J&W Scientific CycloSil-B column, 30 m × 0.32 mm). The enantiomeric ratio E was based on four to six measurements at different conversions, all lower than 50%.

Desymmetrization of *meso*-2,3-butanediol: In a stoppered vial, *meso*-2,3-butanediol (1 – 2 mmol) was added to the CALB preparation (7 mg of carrier, $a_w = 0.1$) suspended in methyl *tert*-butyl ether (previously dried over molecular sieves). The mixture was stirred at 500 rpm in a thermostated multistirrer at 23 °C for 30 minutes. Vinyl butyrate (2 equiv, 2 – 4 mmol) was added to the solution and samples were taken regularly. The ee_p value was monitored by chiral GC (J&W Scientific CycloSil-B column, 30 m × 0.32 mm). The E values were based on four to six measurements.

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