Rational control of enantioselectivity of lipase by site-directed mutagenesis based on the mechanism†

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The enantioselectivity of a *Burkholderia cepacia* lipase toward secondary alcohols could be both increased and decreased rationally by introducing only a single mutation on the basis of the mechanism proposed previously.

Despite the remarkable advances in enzyme science and technology,[1,2] the "rational control" of enantioselectivity of an enzyme still remains difficult because of the complexity of the enzymatic reaction. Although directed evolution, using random mutagenesis combined with a high-throughput screening system, can improve the enantioselectivity of an enzyme without knowledge of the structure and mechanism, a huge number of mutants need to be screened.[3–5] On the other hand, once the mechanistic aspect has been clarified, site-directed mutagenesis based on rational design can also be effective for changing the enantioselectivity.[6–9] Although it is desirable that the random and rational approaches should be complementary to each other to create a biocatalyst showing higher performance, in reality, the latter seems to be more difficult and inefficient than the former because of unknown factors in biocatalysis. In this context, a semi-rational and semi-random approach has also been examined.[10,11] Obviously, the mechanistic basis of biocatalysis needs to be strengthened further.[12] Here we report for the first time that the enantioselectivity of a *Burkholderia cepacia* lipase toward secondary alcohols could be both increased and decreased rationally and easily by mutating only one amino acid residue in the proximity of the active site on the basis of a well-defined mechanism.

Previously, we have proposed a stereo-sensing mechanism of lipases toward secondary alcohols as shown in the transition-state model (Fig. 1a, an expanded version is shown).[13] Enantioselectivity results principally from the conformational requirements and repulsive interactions in the transition state, and no attractive interactions between the enzyme's pockets and the substrate's polar/nonpolar substituents are involved. This mechanism has been proved by kinetic and thermodynamic analyses,[13,14] by using a gigantic secondary alcohol, 5-[4-(1-hydroxyethyl)-phenyl]-10,15,20-triphenylporphyrin,[15] and by highly enantioselective reactions at high temperatures up to 120 °C.[16] Enantioselectivity results from the suppression mechanism working on the slower-reacting enantiomer in the transition state. Therefore, if the steric repulsion between the enzyme and the larger substituent (R^2) of the slower-reacting enantiomer is increased by means of site-directed mutagenesis (Fig. 1a), the enantioselectivity will increase (and *vice versa*). The I287F mutation, which increases the steric bulkiness (Fig. 1c) as compared with the wild-type enzyme (Fig. 1b), will suppress the reaction of the slower-reacting enantiomer and improve the enantioselectivity. On the other hand, the I287A mutation, which decreases the steric hindrance (Fig. 1d), will facilitate the reaction of the slower-reacting enantiomer and diminish the enantioselectivity.

The gene encoding the mature lipase was PCR-cloned from the genomic DNA of *Burkholderia cepacia* NBRC 14595 and over-expressed in *E. coli*.[17] The denatured lipase was subjected to the *in vitro* refolding in the presence of an activator and 8 M urea.[18] After the refolded lipase was purified to homogeneity by hydrophobic chromatography and ion-exchange chromatography,

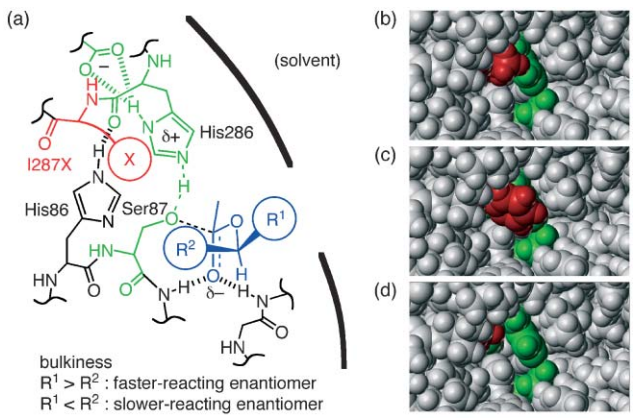

Fig. 1 (a) Transition-state model to rationalize the enantioselectivity in the lipase-catalyzed kinetic resolution of secondary alcohol: an expanded version of the model (residue 287 is added to the original version). (i) The C–O bond of the substrate takes the *gauche* conformation with respect to the breaking C–O bond, which is due to the stereoelectronic effect. (ii) The hydrogen atom attached to the stereocenter in the substrate is *syn*-oriented toward the carbonyl oxygen atom. Enantioselectivity is explained by the conformational requirements and repulsive interactions and/or strains caused in the transition state. The catalytic triad residues, the ester being produced, and the mutation site are shown in green, blue, and red, respectively. The amino acid numbers for *Burkholderia cepacia* lipase are shown. (b)–(d) Space-filling representations of (b) the wild-type enzyme, (c) the I287F mutant, and (d) the I287A mutant. The catalytic triad residues and residue 287 are shown in green and red, respectively. These structures are viewed from the right side of Fig. 1a. The crystal structure of a *Burkholderia cepacia* lipase (PDB code 1OIL) having 96% sequence identity with that used in this study was used after the thirteen different residues had been replaced. The structures were drawn with SYBYL 6.4 (Tripos Inc.).

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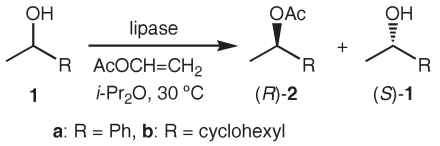
† Electronic supplementary information (ESI) available: Construction of plasmids, site-directed mutagenesis, expression and purification of lipase and kinetic resolution. See http://dx.doi.org/10.1039/b508244g



Scheme 1

Table 1 Enantioselectivity of the wild-type lipase and mutants[a]

Entry	Lipase	Alcohol	Time/h	c[b]	TTN[c]	Ee (%) (*R*)-**2**	Ee (%) (*S*)-**1**	*E* value[d]
1	Wild-type	**1a**	4.5	0.473	7800	94.1	84.4	88
2	I287F	**1a**	7	0.457	7500	96.8	81.5	156
3	I287L	**1a**	9	0.438	7200	95.8	74.6	105
4	I287M	**1a**	3.5	0.484	8000	88.4	82.8	42
5	I287A	**1a**	12.5	0.491	8100	52.6	50.8	5
6	Wild-type	**1b**	9	0.460	7600	90.0	76.8	44
7	I287F	**1b**	28	0.443	3700	96.3	76.5	123
8	I287L	**1b**	16	0.477	7900	90.9	82.8	54
9	I287M	**1b**	14	0.411	6800	95.2	66.3	81
10	I287A	**1b**	42	0.349	5800	87.5	46.9	24

[a] Conditions: lipase (100 mg except for entry 7 (200 mg), 1% (w/w) enzyme/Toyonite-200M), **1** (0.50 mmol), vinyl acetate (1.0 mmol), molecular sieves 3 Å (three pieces), dry *i*-Pr_2O (5.0 mL), 30 °C. [b] Conversion calculated from c = ee(**1**)/(ee(**1**) + ee(**2**)). [c] TTN is the total number of moles of the product formed per mole of the enzyme. Calculated from TTN = 0.5 × c × 33000 (molecular weight of the lipase). [d] Calculated from $E = \ln[1 - c(1 + \mathrm{ee}(\mathbf{2}))]/\ln[1 - c(1 - \mathrm{ee}(\mathbf{2}))]$.

it was immobilized on the porous ceramic called Toyonite-200M.[16] Site-directed mutagenesis was introduced by the overlap-extension PCR method.[19] The lipase-catalyzed kinetic resolutions of **1** were conducted with vinyl acetate in dry *i*-Pr_2O at 30 °C (Scheme 1). The results are listed in Table 1. The enantioselectivities were compared by using the *E* value.[20]

As shown in Table 1, the mutants catalyzed the transesterification of **1** more slowly than the wild-type enzyme did in most cases. The mutation at position 287 seems to perturb the catalytic action to some degree. Nevertheless, the total turnover numbers (TTN) of the mutants were high enough, ranging from 3700 to 8100 (Table 1), which are comparable to those for the wild-type enzyme (TTN 7600 or 7800) and that reported for a commercially available lipase (TTN 5000).[21] Importantly, the *E* value increases as the amino acid residue at position 287 is more bulky: Phe > Leu ≈ Met ≈ Ile > Ala. This trend is consistent with the prediction described above. The *E* values for the I287F mutant toward **1a** and **1b** were 1.8- and 2.8-fold higher, respectively, than the corresponding values for the wild-type enzyme. On the other hand, the *E* values for the I287A mutant toward **1a** and **1b** were 17.6- and 1.8-fold lower, respectively, than the corresponding values for the wild-type enzyme. The *E* values for the I287F mutant toward **1a** and **1b** were 31- and 5-fold higher, respectively, than those for the I287A mutant. The 31-fold difference in *E* value amounts to the energetic difference of −2.0 kcal mol^{-1}, according to the equation $\Delta_{F-A}\Delta_{R-S}\Delta G^{\ddagger} = -RT\ln E_F/E_A$, which clearly represents the effectiveness of the single amino acid substitution at position 287.

Biocatalysts are behind artificial catalysts, such as chiral ligand–metal complexes and chiral organocatalysts, in rational design approaches, in the latter of which enantioselectivity can be tuned rationally by altering the structures of catalysts.[22,23] Here we have succeeded in controlling (both increasing and decreasing) the enantioselectivity of the lipase rationally by mutating only one amino acid residue on the basis of the mechanism, which is rational enough as compared with the alteration of artificial catalysts. Further work is currently under way to evolve the enzyme rationally.

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Notes and references

1 *Enzyme Catalysis in Organic Synthesis*, ed. K. Drauz and H. Waldmann, Wiley-VCH, Weinheim, 2nd edn, 2002.
2 U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis*, Wiley-VCH, Weinheim, 1999.
3 M. T. Reetz, *Tetrahedron*, 2002, **58**, 6595–6602.
4 G. P. Horsman, A. M. F. Liu, E. Henke, U. T. Bornscheuer and R. J. Kazlauskas, *Chem. Eur. J.*, 2003, **9**, 1933–1939.
5 R. Carr, M. Alexeeva, M. J. Dawson, V. Gotor-Fernández, C. E. Humphrey and N. J. Turner, *ChemBioChem*, 2005, **6**, 637–639.
6 F. Manetti, D. Mileto, F. Corelli, S. Soro, C. Palocci, E. Cernia, I. D'Acquarica, M. Lotti, L. Alberghina and M. Botta, *Biochim. Biophys. Acta*, 2000, **1543**, 146–158.
7 D. Rotticci, J. C. Rotticci-Mulder, S. Denman, T. Norin and K. Hult, *ChemBioChem*, 2001, **2**, 766–770.
8 C. Gentner, R. D. Schmid and J. Pleiss, *Coll. Surf. B: Biointerfaces*, 2002, **26**, 57–66.
9 Y. Ijima, K. Matoishi, Y. Terao, N. Doi, H. Yanagawa and H. Ohta, *Chem. Commun.*, 2005, 877–879.
10 Y. Koga, K. Kato, H. Nakano and T. Yamane, *J. Mol. Biol.*, 2003, **331**, 585–592.
11 S. Park, K. L. Morley, G. P. Horsman, M. Holmquist, K. Hult and R. J. Kazlauskas, *Chem. Biol.*, 2005, **12**, 45–54.
12 T. Ema, *Curr. Org. Chem.*, 2004, **8**, 1009–1025.
13 T. Ema, J. Kobayashi, S. Maeno, T. Sakai and M. Utaka, *Bull. Chem. Soc. Jpn.*, 1998, **71**, 443–453.
14 T. Ema, K. Yamaguchi, Y. Wakasa, A. Yabe, R. Okada, M. Fukumoto, F. Yano, T. Korenaga, M. Utaka and T. Sakai, *J. Mol. Catal. B: Enzym.*, 2003, **22**, 181–192.
15 T. Ema, M. Jittani, K. Furuie, M. Utaka and T. Sakai, *J. Org. Chem.*, 2002, **67**, 2144–2151.
16 T. Ema, M. Kageyama, T. Korenaga and T. Sakai, *Tetrahedron: Asymmetry*, 2003, **14**, 3943–3947.
17 S. Aoyama and N. Yoshida, *Eur. Pat. 0657535A2*, 1995.
18 D. T. Quyen, C. Schmidt-Dannert and R. D. Schmid, *Appl. Environ. Microbiol.*, 1999, **65**, 787–794.
19 S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen and L. R. Pease, *Gene*, 1989, **77**, 51–59.
20 C.-S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294–7299.
21 T. Ema, S. Maeno, Y. Takaya, T. Sakai and M. Utaka, *J. Org. Chem.*, 1996, **61**, 8610–8616.
22 R. E. Gawley and J. Aubé, *Principles of Asymmetric Synthesis*, Elsevier, Oxford, 1996.
23 A. Berkessel and H. Gröger, *Asymmetric Organocatalysis*, Wiley-VCH, Weinheim, 2005.