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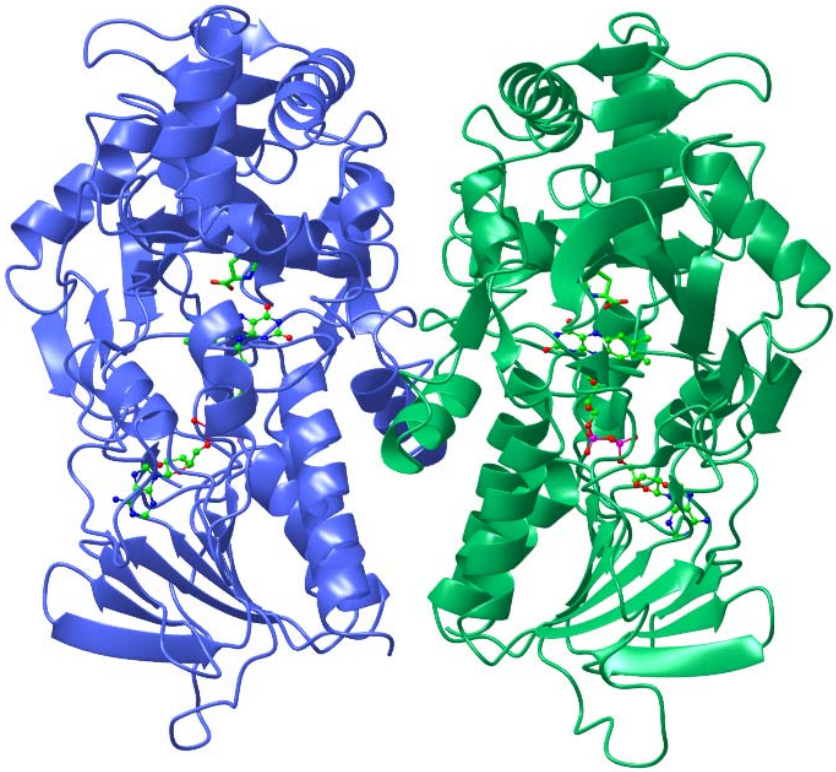

Rational creation of mutant enzyme showing remarkable enhancement of catalytic activity and enantioselectivity toward poor substrates†‡

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Received 26th January 2010, Accepted 23rd March 2010
First published as an Advance Article on the web 9th April 2010
DOI: 10.1039/c001561j

Catalytic activity and enantioselectivity of lipase toward poor substrates bearing bulky substituents on both sides have been dramatically improved by rational design; the *E* value for a poor substrate was increased from 5 (wild-type enzyme) to >200 (I287F/I290A double mutant) with an acceleration of the reaction rate.

Improvement of the catalytic function of enzymes is important from scientific and industrial viewpoints. Although lipases are widely recognized as synthetically useful biocatalysts,[1] the kinetic resolution of secondary alcohols bearing bulky substituents on both sides remains difficult. This drawback may be overcome by altering the enzyme structure.[2] Whereas directed evolution based on random mutagenesis and high-throughput screening has become the most popular method for the alteration of biocatalysts,[3–6] rational approaches based on site-directed mutagenesis are also growing steadily.[7–13]

We have previously succeeded in controlling (both increasing and decreasing) the enantioselectivity of a *Burkholderia cepacia* lipase toward 1-phenylethanol by a single mutation on the basis of the mechanism.[13] In that study, enantioselectivity was controlled by modulation of the steric repulsion between the enzyme and the slower-reacting (*S*)-enantiomer. To achieve a more remarkable improvement, a mutation that enhances the reactivity of the (*R*)-enantiomer and that suppresses the reactivity of the (*S*)-enantiomer should be effective. Here we report the rational creation of mutant enzymes showing the remarkable enhancement of both catalytic activity and enantioselectivity toward poor substrates bearing bulky substituents on both sides.

We employed 1-phenyl-1-hexanol (**1a**) as a poor substrate and performed manual docking with reference to the transition-state model (Fig. 1a).[14] We set up a working hypothesis that the I287F mutation (Fig. 1b and c) would enable attractive/repulsive dual-mode interactions with (*R*)/(*S*)-**1a**. As shown in Fig. 1c, Phe287 appears to make favorable contact with the alkyl chain of (*R*)-**1a** in the transition state, which will accelerate the acylation of (*R*)-**1a**. On the other hand, Phe287 is expected to serve as a steric barrier to the phenyl group of (*S*)-**1a**, which will hinder the acylation of (*S*)-**1a**, as is the case for (*S*)-1-phenylethanol.[13]

The recombinant lipases were prepared as reported previously,[13] and the lipase-catalyzed kinetic resolutions of **1** were conducted with vinyl acetate in dry *i*-Pr_2O at 30 °C (Scheme 1). Enantioselectivity was evaluated by the *E* value (the ratio of the rate constants for both enantiomers).[15] The results are summarized in Table 1. Because of the poor reactivity of **1a** as compared with 1-phenylethanol,[13] higher catalyst loadings and longer reaction times were needed. To our delight, the conversion and the *E* value for the I287F mutant toward **1a** were approximately 2-fold and 6-fold higher, respectively, than those for the wild-type enzyme (entries 1 and 2). A similar trend was observed for homolog **1b** (entries 7 and 8). It should be noted that the I287F mutant has previously shown a decrease in conversion (nearly half) and only 2-fold enhancement in the *E* value for 1-phenylethanol as compared with the wild-type enzyme.[13] In contrast to the I287F mutation, the I287A mutation, diminishing the steric hindrance at position 287, did not improve the conversion of **1a** (entry 3). These results suggest that the I287F mutant has a

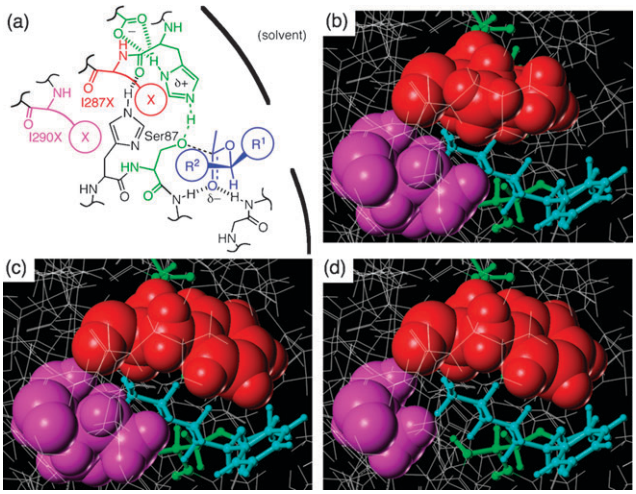

Fig. 1 (a) The transition-state model to rationalize the enantioselectivity in the lipase-catalyzed kinetic resolution of secondary alcohols (residues 287 and 290 are added to the original version). The catalytic triad residues, the ester being produced, residue 287, and residue 290 are shown in green, blue, red, and magenta, respectively. Typically, the (*R*)-enantiomer reacts faster because, in this favorable conformation shown in blue, the larger substituent (R^1) can be directed toward external solvent without severe strain and/or steric hindrance. (b)–(d) The active sites of (b) the wild-type enzyme, (c) the I287F mutant, and (d) the I287F/I290A double mutant. (*R*)-**1a** connected to Ser87 in the tetrahedral intermediate is shown in light blue.

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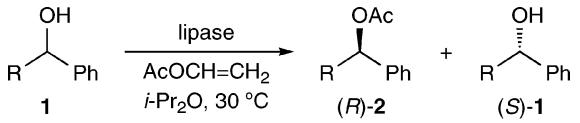
† This article is part of the 'Enzymes and Proteins' web-theme issue for ChemComm.

‡ Electronic supplementary information (ESI) available: Site-directed mutagenesis, synthesis of **1**, lipase-catalyzed kinetic resolution of **1**, determination of kinetic constants, design of mutants, and copies of NMR spectra. See DOI: 10.1039/c001561j



a: R = $(CH_2)_4CH_3$ **b**: R = $(CH_2)_5CH_3$
c: R = $(CH_2)_4CF_3$ **d**: R = $(CH_2)_2(CF_2)_2CF_3$

Scheme 1

mechanism accelerating (*R*)-**1a**, such as the attractive interaction between Phe287 and the alkyl chain of (*R*)-**1a**, which was supported by the kinetic constants and the experiments with partially fluorinated substrates as described later.

Next, we tried to create a second-generation biocatalyst. Manual docking suggested that Ile290 comes into contact with a part of the alkyl chain of (*R*)-**1a** (Fig. 1c). We expected that the I290A mutation would make space to accommodate (*R*)-**1a** nicely in the transition state (Fig. 1d), leading to higher activity. As expected, the I287F/I290A double mutant exerted much higher activity and enantioselectivity for **1a** and **1b** (Table 1, entries 4 and 9). In both cases, the conversions reached almost 50% within a few hours, and the *E* values exceeded 200. In sharp contrast with the I287F/I290A double mutant, the I287F/I290F double mutant showed very poor activity and enantioselectivity for **1a** (entry 5). The I290A mutant had catalytic activity and enantioselectivity intermediate between those of the wild-type enzyme and the I287F/I290A double mutant, as is the case for the I287F mutant (entry 6). It is therefore most likely that the second I290A mutation eliminated the steric hindrance to enhance the reactivity of (*R*)-**1a** or (*R*)-**1b**.

The difference in activation free energy between both enantiomers (chiral recognition energy) can be calculated from $\Delta_{R-S}\Delta G^{\ddagger} = -RT\ln E$, and the difference in the chiral recognition energy between enzymes A and B can be calculated from $\Delta_{A-B}\Delta_{R-S}\Delta G^{\ddagger} = -RT\ln E_A/E_B$.[5,13] For example, the chiral recognition energies of the I287F mutant and the I287F/I290A double mutant toward **1a** are greater by 1.1 and at least 2.2 kcal mol^{-1}, respectively, than that of the wild-type enzyme. The *E* value for **1a** was changed from 1.4 to >200 by introducing single or double mutations, which amounts to an energetic difference of at least 3.0 kcal mol^{-1}.

The degree of catalytic activity can be estimated roughly from the total turnover number (TTN) and the turnover frequency (TOF). TTN is defined as the number of substrate molecules converted by one enzyme molecule, and TOF is the turnover number per unit time. One can calculate these values from the data in Table 1. For example, the TTN value for the wild-type enzyme toward **1a** (entry 1) is calculated to be 1080 from TTN = 0.5 mmol × 0.23/(700 mg × 0.005/33 000), and the TOF value is calculated to be 26 h^{-1} where 33 000 is the molecular weight of the enzyme. These values for the poor substrate **1a** are much smaller than those reported for the same enzyme toward a good substrate, 1-phenylethanol (TTN = 7800 in 4.5 h; TOF = 1700 h^{-1}).[13] On the other hand, Table 1 indicates that the I287F/I290A double mutant has much higher values for **1a** (entry 4) than the wild-type enzyme; TTN = 2300 in 2.5 h and TOF = 940 h^{-1}.

To gain a deeper insight, we next determined the kinetic constants for the wild-type and mutant enzymes toward each enantiomer of **1a**. The kinetic constants determined are summarized in Table 2, which are helpful for understanding the effect of each mutation on the enzymatic activity. First, the enantioselectivity resulted mainly from the difference in the V_{max} value (in other words, the transition state) as demonstrated previously.[14] Importantly, the mutations altered the V_{max} values (the transition state) rather than the K_m values (the substrate-binding step). Moreover, the I287F mutant had a V_{max} value for (*R*)-**1a** that was ~3-fold higher than that of the wild-type enzyme, while the I287F mutant had a V_{max} value for (*S*)-**1a** that was ~2-fold lower than that of the wild-type enzyme. These results clearly support our proposal on the synergic effect that Phe287 contributes to both acceleration of (*R*)-**1a** and deceleration of (*S*)-**1a**. Furthermore, the I287F/I290A double mutant had a V_{max} value for (*R*)-**1a** that was ~5-fold greater than that of the I287F mutant, which supports our expectation that the second I290A mutation would eliminate the steric hindrance to enhance the reactivity of (*R*)-**1a**. The kinetic constants in Table 2 thus indicate that the transition state was manipulated successfully as we expected.

Using these recombinant enzymes, we investigated the above-mentioned attractive interaction between Phe287 and the alkyl chain of (*R*)-**1a** in more detail. We synthesized partially fluorinated analogs **1c** and **1d** and used them as

Table 1 Kinetic resolution of **1** with wild-type and mutant enzymes[a]

Entry	1	Lipase	Time/h	Conversion (%)[b]	E[c]
1	**1a**	Wild-type	41	23	5
2	**1a**	I287F	41	46	32
3	**1a**	I287A	41	20	1.4
4	**1a**	I287F/I290A	2.5	50	>200
5	**1a**	I287F/I290F	41	10	4
6	**1a**	I290A	41	41	79
7	**1b**	Wild-type	41	39	9
8	**1b**	I287F	22	47	71
9	**1b**	I287F/I290A	4	47	>200
10	**1c**	Wild-type	41	34	14
11	**1c**	I287F	22	47	55
12	**1c**	I287F/I290A	4	50	>200
13	**1d**	I287F/I290A	75	19	10

[a] Conditions: immobilized lipase (700 mg, 0.5% (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 mL), 30 °C. [b] Conversion calculated from c = ee(**1**)/(ee(**1**) + ee(**2**)). [c] Calculated from $E = \ln[1 - c(1 + \text{ee}(\mathbf{2}))]/\ln[1 - c(1 - \text{ee}(\mathbf{2}))]$.

Table 2 Kinetic constants for the lipase-catalyzed acylations of **1a**[a]

Lipase	1a	V_{max}/M min^{-1} mg(lipase)$^{-1}$	K_m/M
Wild-type	(*R*)	$(5.0 \pm 0.4) \times 10^{-7}$	$(1.9 \pm 0.4) \times 10^{-1}$
Wild-type	(*S*)	$(5.9 \pm 0.8) \times 10^{-8}$	$(4.9 \pm 1.0) \times 10^{-1}$
I287F	(*R*)	$(1.6 \pm 0.1) \times 10^{-6}$	$(3.5 \pm 0.3) \times 10^{-1}$
I287F	(*S*)	$(2.7 \pm 0.4) \times 10^{-8}$	$(3.7 \pm 0.7) \times 10^{-1}$
I287F/I290A	(*R*)	$(7.7 \pm 0.5) \times 10^{-6}$	$(1.1 \pm 0.2) \times 10^{-1}$
I287F/I290A	(*S*)	$(1.0 \pm 0.1) \times 10^{-8}$	$(3.1 \pm 0.5) \times 10^{-1}$

[a] For the detailed procedure, see ESI. Because of the heterogeneous reaction, the nonlinear least-squares method was applied to the Michaelis–Menten type of equation: $v_0 = V_{max}(E)_{mg}[S]_0/(K_m + [S]_0)$. The V_{max} value, which is normalized by the weight of the immobilized enzyme powder $(E)_{mg}$, corresponds to the k_{cat} value in homogeneous enzymatic reactions.

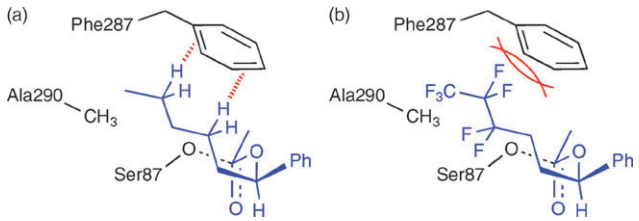


Fig. 2 (a) The CH/π interactions in the transition-state of the I287F/I290A double mutant-catalyzed acylation of (*R*)-**1a**. (b) The steric repulsion in the transition-state of the I287F/I290A double mutant-catalyzed acylation of (*R*)-**1d**.

probes. For example, the CH/π interaction[16] cannot take place at the fluorinated moiety of the substrate, which may enable us to specify the kind and position of the attractive interaction. As shown in Table 1, the I287F mutant showed higher activity and enantioselectivity for **1c** than the wild-type enzyme (entries 10 and 11), and the I287F/I290A double mutant achieved much higher values (entry 12). This trend is quite similar to that observed for the non-fluorinated counterpart **1a**, which strongly suggests that the terminal methyl group of **1a** experienced no specific, attractive interactions. In contrast, the catalytic activity and enantioselectivity of the I287F/I290A double mutant toward **1d** were found to be very low (entry 13), and a clear difference was observed between **1d** (entry 13) and **1a** (entry 4) or **1c** (entry 12), which strongly suggests that the fluorinated methylene moiety of (*R*)-**1d** underwent a severe steric repulsion (Fig. 2b). Based on these results and computer representations, we suppose that the hydrogen atoms at the ω − 1 position (next to the terminal methyl group) and more weakly at the ω − 3 position of (*R*)-**1a** participate in the CH/π interaction with the phenyl group of Phe287 as shown in Fig. 2a.

In summary, only two mutations (I287F/I290A) dramatically enhanced both catalytic activity and enantioselectivity toward poor substrates **1a–1c** by the synergic effect: Phe287 contributed to both acceleration of the (*R*)-enantiomer and deceleration of the (*S*)-enantiomer, while Ala290 made space to facilitate the acylation of the (*R*)-enantiomer. To the best of our knowledge, this is the first example of introducing the CH/π interaction in the transition state to promote the enzymatic reaction. Almost all the mutant enzymes that we have so far prepared are presented here, and the enzyme structure has not yet been optimized, for example, by a random approach such as saturation mutagenesis.[3–6] Nevertheless, the impact of the mutations was found to be great enough. Therefore, the present results clearly demonstrate the efficiency and power of the rational design approach to the creation of an excellent biocatalyst.

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and by The Sumitomo Foundation. We are grateful to the SC-NMR Laboratory of Okayama University for the measurement of NMR spectra.

Notes and references

1 (*a*) *Enzyme Catalysis in Organic Synthesis*, ed. K. Drauz and H. Waldmann, Wiley-VCH, Weinheim, 2nd edn, 2002; (*b*) K. Faber, *Biotransformations in Organic Chemistry*, Springer-Verlag, Berlin, 5th edn, 2004; (*c*) U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis*, Wiley-VCH, Weinheim, 2nd edn, 2006; (*d*) *Industrial Biotransformations*, ed. A. Liese, K. Seelbach and C. Wandrey, Wiley-VCH, Weinheim, 2nd edn, 2006; (*e*) *Biocatalysis in the Pharmaceutical and Biotechnology Industries*, ed. R. N. Patel, CRC, Florida, 2007; (*f*) *Practical Methods for Biocatalysis and Biotransformations*, ed. J. Whittall and P. Sutton, John Wiley & Sons, West Sussex, 2010.
2 P. F. Mugford, U. G. Wagner, Y. Jiang, K. Faber and R. J. Kazlauskas, *Angew. Chem., Int. Ed.*, 2008, **47**, 8782–8793.
3 (*a*) M. T. Reetz, L.-W. Wang and M. Bocola, *Angew. Chem., Int. Ed.*, 2006, **45**, 1236–1241; (*b*) M. D. Mihovilovic, F. Rudroff, A. Winninger, T. Schneider, F. Schulz and M. T. Reetz, *Org. Lett.*, 2006, **8**, 1221–1224; (*c*) M. T. Reetz and S. Wu, *J. Am. Chem. Soc.*, 2009, **131**, 15424–15432.
4 (*a*) S. Bartsch, R. Kourist and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2008, **47**, 1508–1511; (*b*) H. Jochens, K. Stiba, C. Savile, R. Fujii, J.-G. Yu, T. Gerassenkov, R. J. Kazlauskas and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2009, **48**, 3532–3535.
5 S. Park, K. L. Morley, G. P. Horsman, M. Holmquist, K. Hult and R. J. Kazlauskas, *Chem. Biol.*, 2005, **12**, 45–54.
6 (*a*) R. Carr, M. Alexeeva, M. J. Dawson, V. Gotor-Fernández, C. E. Humphrey and N. J. Turner, *ChemBioChem*, 2005, **6**, 637–639; (*b*) N. J. Turner, *Nat. Chem. Biol.*, 2009, **5**, 567–573.
7 (*a*) D. Rotticci, J. C. Rotticci-Mulder, S. Denman, T. Norin and K. Hult, *ChemBioChem*, 2001, **2**, 766–770; (*b*) A. O. Magnusson, M. Takwa, A. Hamberg and K. Hult, *Angew. Chem., Int. Ed.*, 2005, **44**, 4582–4585.
8 C. Gentner, R. D. Schmid and J. Pleiss, *Colloids Surf., B*, 2002, **26**, 57–66.
9 R. Weis, R. Gaisberger, W. Skranc, K. Gruber and A. Glieder, *Angew. Chem., Int. Ed.*, 2005, **44**, 4700–4704.
10 (*a*) A. E. Tripp, D. S. Burdette, J. G. Zeikus and R. S. Phillips, *J. Am. Chem. Soc.*, 1998, **120**, 5137–5141; (*b*) C. Heiss, M. Laivenieks, J. G. Zeikus and R. S. Phillips, *J. Am. Chem. Soc.*, 2001, **123**, 345–346.
11 (*a*) F. P. Seebeck, A. Guainazzi, C. Amoreira, K. K. Baldridge and D. Hilvert, *Angew. Chem., Int. Ed.*, 2006, **45**, 6824–6826; (*b*) M. D. Toscano, M. M. Müller and D. Hilvert, *Angew. Chem., Int. Ed.*, 2007, **46**, 4468–4470.
12 (*a*) Y. Ijima, K. Matoishi, Y. Terao, N. Doi, H. Yanagawa and H. Ohta, *Chem. Commun.*, 2005, 877–879; (*b*) Y. Terao, K. Miyamoto and H. Ohta, *Chem. Commun.*, 2006, 3600–3602.
13 T. Ema, T. Fujii, M. Ozaki, T. Korenaga and T. Sakai, *Chem. Commun.*, 2005, 4650–4651.
14 (*a*) T. Ema, J. Kobayashi, S. Maeno, T. Sakai and M. Utaka, *Bull. Chem. Soc. Jpn.*, 1998, **71**, 443–453; (*b*) T. Ema, M. Jittani, K. Furuie, M. Utaka and T. Sakai, *J. Org. Chem.*, 2002, **67**, 2144–2151; (*c*) 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 C.-S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294–7299.
16 M. Nishio, M. Hirota and Y. Umezawa, *The CH/π Interaction: Evidence, Nature, and Consequences*, Wiley-VCH, New York, 1998.

