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Inverting the Enantioselectivity of a Carbonyl Reductase via Substrate–Enzyme Docking-Guided Point Mutation

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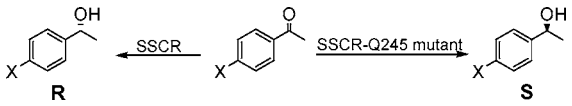
ABSTRACT



Substrate-enzyme docking-guided point mutation of a carbonyl reductase from *Sporobolomyces salmonicolor* led to mutant enzymes, which reversed the enantiopreference and enhanced the enantioselectivity toward the reduction of *para*-substituted acetophenones. Such a dramatic change in the enantioselectivity indicates that the 245 residue in the catalytic site plays a critical role in determining the enantioselectivity of these ketone reductions, providing valuable insight into our understanding of how residues involved in substrate binding affect the orientation of bound substrate and thus control the reduction stereoselectivity.

Because of the increasing importance of enantiomerically pure compounds to the fine chemical and pharmaceutical industries, there is a growing demand for reliable, efficient stereoselective synthetic methods. Biocatalysis is an attractive tool in asymmetric synthesis and efficiently complements traditional chemical methods. Naturally occurring enzymes usually catalyze a given reaction with high specificity and enantioselectivity under their physiological conditions. Enantioselectivity of biocatalysts is substrate-dependent and not always high enough for unnatural substrates. Improvement in enzyme activity and enantioselectivity is often necessary to meet the ever-increasing needs of synthetic chemists. Although several groups have used both directed evolution and rational design approaches to increase, or even to reverse the enantioselectivity of lipases,[1] esterases,[2] and other enzymes,[3] improving the stereoselectivity of an enzyme reaction for synthetic advantage still represents a difficult problem for the enzyme designers whether using rational design or directed evolution methods. This is especially the case for the carbonyl reductases, where it is difficult to implement a high-throughput method of determining enantioselectivity in the ketone reduction to allow the rapid screening of a large number of mutants.[4]

Sometimes, mutation of active site residues can yield greater effects.[5] For example, Leadlay and co-workers have recently reported that mutation of residues in motifs I and II in ketoreductase domains eryKR$_1$ and eryKR$_2$ from the erythromycin polyketide synthase altered the stereochemical outcome in reduction of (2*R*,*S*)-2-methyl-3-oxopentanoic acid

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(1) For some recent examples, see: (a) Droege, M. J.; Boersma, Y. L.; van Pouderoyen, G.; Vrenken, T. E.; Rueggeberg, C. J.; Reetz, M. T.; Dijkstra, B. W.; Quax, W. J. *Chem. Bio. Chem.* **2006**, *7*, 149. (b) Reetz, M. T.; Carballeira, J. D.; Peyralans, J.; Hobenreich, H.; Maichele, A.; Vogel, A. *Chem.—Eur. J.* **2006**, *12*, 6031. (c) Koga, Y.; Kato, K.; Nakano, H.; Yamane, T. *J. Mol. Biol.* **2003**, *331*, 585.

(2) For some recent examples, see: (a) Horsman, G. P.; Liu, A. M. F.; Henke, E.; Bornscheuer, U. T.; Kazlauskas, R. J. *Chem.—Eur. J.* **2003**, *9*, 1933. (b) Schmidt, M.; Hasenpusch, D.; Kahler, M.; Kirchner, U.; Wiggenhorn, K.; Lange, W.; Bornscheuer, U. T. *ChemBioChem* **2006**, *7*, 805. (c) Park, S.; Morley, K. L.; Horsman, G. P.; Holmquist, M.; Hult, K.; Kazlauskas, R. J. *Chem. Biol.* **2005**, *12*, 45.

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N-acetylcysteamine thioester.[6] In the study to confirm the hypothetical tropinone binding mode, Nakajima et al. substituted five substrate-binding residues of one tropinone reductase (TR-1) with those found in the corresponding positions of the other tropinone reductase (TR-2) in the biosynthetic pathway of tropane alkaloids, resulting in the switch of stereospecificity of TR-1 into that of TR-2 and vice versa.[7] Herein, we report the first example of enzyme−substrate docking-guided point mutation of the substrate-binding residues in a carbonyl reductase, generating mutant enzymes with reversed enantiopreference and enhanced enantioselectivity toward the reduction of *para*-substituted acetophenones. These results provide some insight into the fundamental question of how substrate-binding residues affect substrate binding orientation and thus control the reaction stereoselectivity.

Although the carbonyl reductase from *Sporobolomyces salmonicolor* (**SSCR**) catalyzes the reduction of various ketones to the corresponding chiral alcohols in excellent enantiomeric purity, it shows low enantioselectivity for the reduction of *para*-substituted acetophenones (14−59% ee).[8] To better understand the enantioselective versatility in this ketone reduction, an initial substrate−enzyme docking study of 4′-methoxyacetophenone into the crystal structure of SSCR[9] was performed using ICM-Pro 3.4.9d.[10] During these simulations, two opposite conformations which are energetically close to each other have been found in the high scoring docking conformations. Figure 1 shows the opposite conformations of 4′-methoxyacetophenone, which yield the (*S*)-enantiomer of product alcohol (Figure 1A) or (*R*)-counterpart (Figure 1B), respectively. From these docking studies, which are qualitatively consistent with the low observed enantioselectivity, it was also seen that residues Q245 and M242 are in close proximity to the *para* substituent of the acetophenones in both conformations. We reasoned that such close interaction might be responsible for the observed low enantioselectivity in the **SSCR**-catalyzed reduction of *para*-substituted acetophenones, and that mutation of the residues Q245 and M242 in the catalytic site might improve the enzyme enantioselectivity, with hydrogen bonding or hydrophobic interactions playing significant roles. In the present study, the residue Q245 was mutated to all other 19 amino acids, and the mutants were screened for enhanced enantioselectivity toward the reduction of *para*-substituted acetophenones.

A focused library of mutants was created by saturation mutagenesis of the residue Q245 in the catalytic cavity of the carbonyl reductase from *S. salmonicolor*. The resulting mutant library was screened using 4′-methoxyacetophenone as substrate. The colonies which showed higher activity than wild-type **SSCR** enzyme were selected to further determine their enantioselectivity. Surprisingly, five colonies were found to catalyze the reduction of 4′-methoxyacetophenone to (*S*)-1-(4′-methoxyphenyl)ethanol with ee values of 79−98%, while (*R*)-1-(4′-methoxyphenyl)ethanol was obtained in 57% ee with the wild-type **SSCR** enzyme. No colony showing higher activity than the wild-type **SSCR** enzyme was found to catalyze the reduction of 4′-methoxyacetophenone to (*R*)-1-(4′-methoxyphenyl)ethanol. Sequencing of the five colonies revealed three colonies showing the same mutation, Q245L, while the other two colonies showed mutations Q245H and Q245P, respectively.

These mutant **SSCR** enzymes were further screened to determine whether they also inverted enantioselectivity toward the reduction of other *para*-substituted acetophenones. The results as summarized in Table 1 show that when the residue Q245 was replaced with H, P, or L (Q245H, Q245P and Q245L), reductions of all other *para*-substituted acetophenones gave (*S*)-configurated chiral alcohols in greater than 90% ee, while the unsubstituted acetophenone was reduced in a relatively lower enantioselectivity. When compared to the wild-type **SSCR**, which catalyzed the reduction to give (*R*)-enantiomer in 14−59% ee, these mutant **SSCR** enzymes exhibited inverted enantiopreference and enhanced enantioselectivity. Therefore, the residue 245 in the catalytic cavity plays an important role in determining the enantioselectivity for the reduction of the *para*-substituted acetophenones. Furthermore, this residue affected the enzyme activity for the reduction of acetophenones. For example, compared to the wild-type **SSCR** and mutant Q245L, mutants Q245H and Q245P greatly improved the enzyme activity toward the reduction of acetophenones when the *para* substituent was Cl or Br.

To gain insight into how the single mutation at residue 245 results in such a drastic change of enantioselectivity for the reduction of *para*-substituted acetophenones, in silico mutagenesis of Q245 to H and docking of a variety of *para*-

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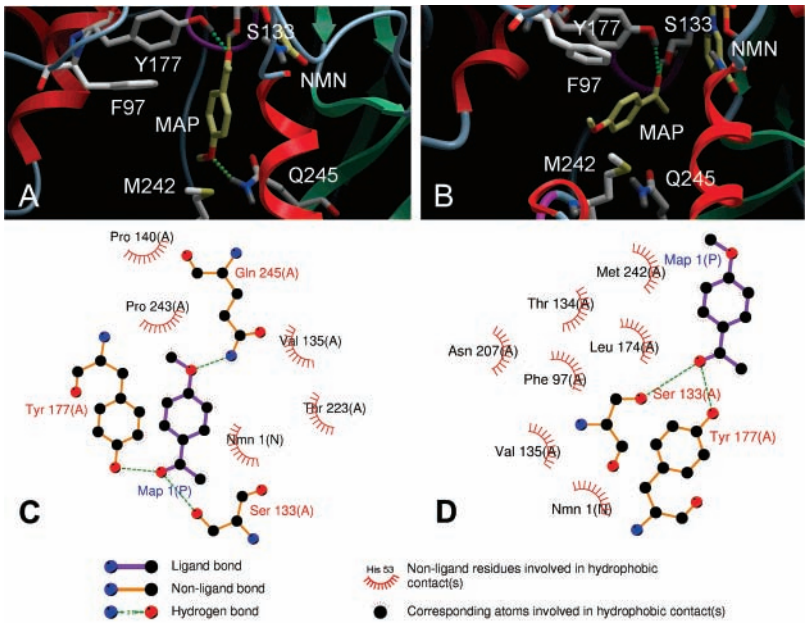
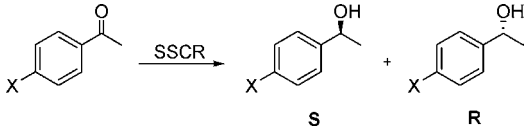


Figure 1. Substrate 4′-methoxyacetophenone docked into the active site of the carbonyl reductase from *Sporobolomyces salmonicolor* (**SSCR**). (A) The substrate (MAP) is shown at the center of the binding site, oriented with the *Re* face toward the cofactor, NMN, at the upper right of the pocket. Catalytic residues S133 and Y177 are shown at the upper left of the pocket, Q245 is at the lower right, with additional residues F97 and M242 shown. Hydrogen-bonding interactions are shown as dotted lines. For clarity, only polar hydrogens are shown. Rendered with ICM-Pro 3.4.9d. (B) The substrate (MAP) is shown at the center of the binding site with the *Si* face toward the cofactor, NMN. Residues and cofactor labeled as in (A). (C) Ligplot[11] showing significant binding interactions between the flexibly docked substrate in (A) and **SSCR**. (D) Ligplot[11] showing significant binding interactions between the flexibly docked substrate in (B) and **SSCR.**

substituted acetophenone substrates to the mutant Q245H were performed. The substrate typically adopted an energetically preferred conformation where the NMN ring of the cofactor was optimally positioned at the *Re* face of the substrate, and the carbonyl oxygen atom of the substrate formed a hydrogen bond with Y177 or S133. This orientation was facilitated by the hydrophobic contacts between the 4′-methoxy group and the mutated residue H245 (Figure 2). In this configuration, protonation and subsequent hydride attack would lead to selective reduction to the corresponding (*S*)-1-(4′-methoxyphenyl)ethanol. The docking of *para*-substituted acetophenone substrates into the mutants Q245P and Q245L showed that the substrates also adopted the energetically prefered *Re* face conformation. Thus, structural details provided by the docking study validate the data shown in Table 1, and our hypothesis that Q245 affects enantioselec-

Table 1. Asymmetric Reductions of *Para*-Substituted Acetophenones Catalyzed by **SSCR** and Its Mutants



	SSCR-WT		SSCR-Q245H		SSCR-Q245P		SSCR-Q245L	
X	specific activity[a]	ee (%) abs config	specific activity[a]	ee (%) abs config	specific activity[a]	ee (%) abs config	specific activity[a]	ee (%) abs config
4′-OCH_3	20	57 (*R*)	16	79 (*S*)	62	98 (*S*)	20	96 (*S*)
4′-H	28	42 (*R*)	85	78 (*S*)	39	64 (*S*)	86	82 (*S*)
4′-F	14	46 (*R*)	72	92 (*S*)	25	90 (*S*)	36	93 (*S*)
4′-Cl	20	14 (*R*)	238	90 (*S*)	309	96 (*S*)	67	96 (*S*)
4′-Br	13	42 (*R*)	203	92 (*S*)	403	98 (*S*)	47	97 (*S*)
4′-CH_3	11	59 (*R*)	25	95 (*S*)	45	96 (*S*)	20	95 (*S*)
4′-$C(CH_3)_3$	11	31 (*R*)	32	96 (*S*)	84	99 (*S*)	9	99 (*S*)

[a] The specific activity is defined as nmol·min^{-1}·mg^{-1}.

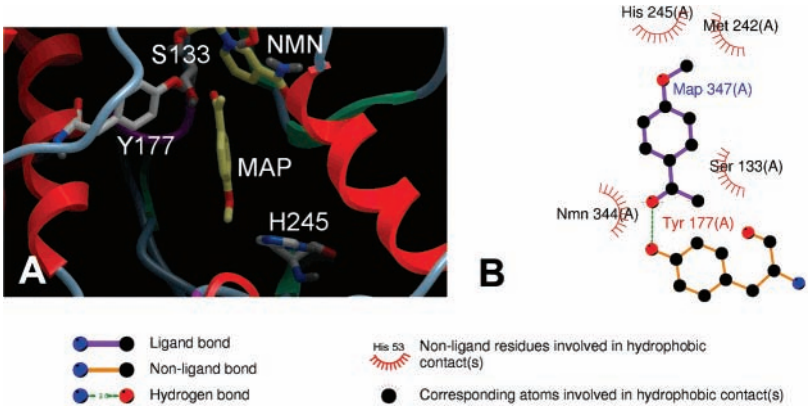


Figure 2. Substrate 4′-methoxyacetophenone docked to the Q245H mutant of SSCR. (A) The substrate is shown at the center of the binding site, with catalytic residues S133 and Y177 shown at the left of the pocket. H245 is at the lower right. The NMN is shown at the upper right. For clarity, only polar hydrogens are shown. Rendered with ICM-Pro 3.4.9d. (B) Ligplot[11] showing significant binding interactions between the flexibly docked substrate and SSCR.

tivity toward reduction of *para*-substituted acetophenones is substantiated.

In summary, based on the knowledge of the crystal structure and enzyme−substrate docking studies, residues Q245 and M242 in the catalytic cavity of the carbonyl reductase from *S. salmonicolor* were identified to closely interact with the *para* substituent of acetophenone substrates. Saturation mutagenesis of residue Q245 coupled with screening generated three mutant **SSCR** enzymes, which showed reversed enantio-preference and enhanced enantioselectivity toward the reduction of *para*-substituted acetophenones. It is remarkable that a single mutation results in such dramatic change in the enantioselectivity. It is clear that residue 245 in the catalytic site plays a critical role in determining the enantioselectivity of these ketone reductions. Single-crystal X-ray diffraction analysis of the Q245H mutant in complex with substrates are in progress, and studies on how the residue 242 affects the enantioselectivity are currently underway in our laboratories. Furthermore, the in silico docking-guided semirational approach has shown to be a very valuable methodology for discovery of new enzymes with synthetic advantage, particularly carbonyl reductases, where high-throughput methods for rapid screening of large numbers of mutants to determine enantioselectivity are difficult to implement.

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Supporting Information Available: Flexible docking and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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