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Highly Enantioselective Mutant Carbonyl Reductases Created via Structure-Based Site-Saturation Mutagenesis

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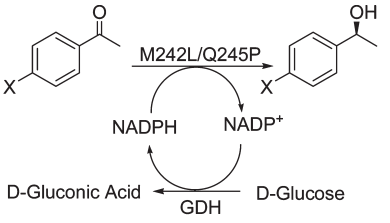

X=Cl, Br, Me, OMe, Me_3C, CF_3, ee ≥ 99%; X= F, ee = 92%

A carbonyl reductase from *Sporobolomyces salmonicolor* reduced para-substituted acetophenones with low enantioselectivity. Enzyme−substrate docking studies revealed that residues M242 and Q245 were in close proximity to the para-substituent of acetophenones in the substrate binding site. Site-saturation mutagenesis of M242 or Q245, and double mutation of M242 and Q245 were performed in order to enhance the enzyme's enantioselectivity toward the reduction of para-substituted acetophenones. Three Q245 mutants were obtained, which inverted the enantiopreference of product alcohols from (*R*)- to (*S*)-configuration with high ee values (*Org. Lett.* **2008**, *10*, 525−528). Four M242 mutant enzymes also showed greater preference for the formation of (*S*)-enantiomeric alcohols than the wild-type enzyme, but to a much less extent than Q245 mutants. M242/Q245 double variations not only greatly affect the enantiomeric purity of the product alcohols, but also invert the enantiopreference, demonstrating that these residues play a critical role in determining the enantioselectivity of these ketone reductions. The kinetic parameters of these mutant enzymes indicated that residues 242 and 245 also exert an effect on the catalytic activity of this carbonyl reductase. Highly enantioselective mutant carbonyl reductases were created by site-saturation mutagenesis, among which the one bearing double mutations, M242L/Q245P, showed the highest enantioselectivity that catalyzed the reduction of the tested para-substituted acetophenones to give (*S*)-enantiomeric products in ≥99% ee with only one exception of *p*-fluoroacetophenone (92% ee).

Introduction

Enantiomerically pure chiral alcohols are important intermediates in the fine chemical and pharmaceutical industries. In recent years, biocatalytic reduction of ketones has increasingly grown as a general method of choice for the synthesis of chiral alcohols and efficiently complements other approaches.[1−5] The information about the substrate specificity and enantioselectivity of enzymes is very valuable

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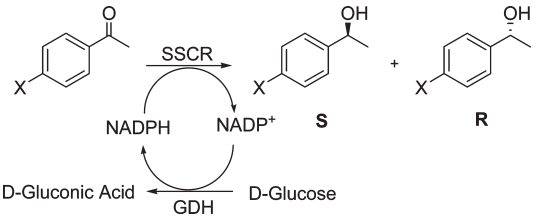
and instructive in guiding the selection of appropriate biocatalysts for a specific transformation.[6−9] Correlating the substrate profile of an enzyme with its sequential and structural information not only provides valuable insight into the understanding of how enzymes control activity and enantioselectivity but also facilitates our efforts to (semi) rationally design novel enzymes with desired substrate specificity and enantioselectivity.[10−13] Holding these in mind, we have studied substrate specificity and enantioselectivity of several carbonyl reductases from different origins that catalyze reduction of ketones of diverse structures. To our delight, these carbonyl reductases are capable of converting aryl/alkyl ketones and α-/β-ketoesters to the corresponding chiral alcohols with excellent enantiomeric purity.[14−20] In the course of our studies on a carbonyl reductase from *Sporobolomyces salmonicolor* (**SSCR**), it has been found that **SSCR** showed an unusually broad substrate range including aliphatic, aromatic ketones, α- and β-ketoesters, and sterically bulky aryl alkyl ketones and diaryl ketones.[15,16,20] This enzyme showed low enantioselectivity for the reduction of para-substituted acetophenones (14−59% ee), although it catalyzed the reduction of other ketones to the corresponding chiral alcohols with excellent enantiomeric purity.[15,16] Recently, the X-ray structures of this carbonyl reductase and its complex with a coenzyme, NADPH, have been determined.[21] A substrate−enzyme docking study of *p*-methoxyacetophenone into the crystal structure of **SSCR** was performed with ICM-Pro 3.4.9d[22−24] in order to better understand the enantioselective versatility in this ketone reduction. During these simulations, two opposite orientations of *p*-methoxyacetophenone, which yield (*S*)-alcohol isomer or the (*R*)-counterpart, respectively, have been found to be energetically close to each other in the high-scoring docking conformations. In both conformations, residues

SCHEME 1. Enzymatic Reduction of Para-Substituted Acetophenones Catalyzed by SSCR Mutant Enzymes with a Cofactor Regeneration System of D-Glucose Dehydrogenase and D-Glucose



M242 and Q245 are in close proximity to the para-substituent of the acetophenones with hydrogen bonding or hydrophobic interactions.[25,26] These simulation results are qualitatively consistent with the observed low enantio selectivity in the **SSCR**-catalyzed reduction of para-substituted acetophenones, and such close interaction was conjectured to have played significant roles in determining the enzyme's enantioselectivity. Therefore, the residues M242 and Q245 in the catalytic site were identified as the mutation targets to improve the enzyme enantioselectivity. Single and double saturation mutagenesis of residues M242 and Q245 were performed, and the resulting mutant libraries were screened for enhanced enantioselectivity toward the reduction of para-substituted acetophenones. The preliminary results of site-saturation mutagenesis of residue Q245 were previously communicated,[26] herein a detailed account of these studies are presented.

Results and Discussion

Focused libraries of mutants were created by means of site-saturation mutagenesis at residues M242 and Q245 in the catalytic cavity of the carbonyl reductase from *S. salmonicolor*. The resulting mutant libraries were screened with *p*-methoxyacetophenone as substrate to select colonies which showed close or higher activity than the wild-type **SSCR** enzyme. The enantioselectivity of these selected colonies was then measured by chiral gas chromatography. The preliminary results obtained from the screening of the site-saturation mutagenesis library at residue Q245 were previously reported in a communication[26] and are not repeated here. In screening the saturation mutagenesis library of residue M242, four mutants were selected with higher activity than others in the NADPH-adjuvant reduction of *p*-methoxyacetophenone. Among these mutant enzymes, M242Y, M242C, and M242G maintained the same stereo preference (*R*) with the wild-type **SSCR** but they catalyzed the reduction with lower enantioselectivity than the latter. However, M242D produced (*S*)-1-(*p*-methoxyphenyl)ethanol with ee values of 39%.

These mutant **SSCR** enzymes were further studied to determine their enantioselectivity toward the reduction of other para-substituted acetophenones (Scheme 1). The cofactor NADPH was regenerated with D-glucose dehydrogenase and D-glucose. The results are summarized in Table 1. It can be seen that when compared to the wild-type **SSCR**,

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TABLE 1. Reduction of Para-Substituted Acetophenones with M242 Mutant Enzymes of SSCR[a]

substrate	SSCR-WT ee (%)	M242Y ee (%)	M242D ee (%)	M242C ee (%)	M242G ee (%)
p-H	42 (*R*)	12 (*S*)	82 (*S*)	13 (*S*)	54 (*S*)
p-F	46 (*R*)	41 (*S*)	90 (*S*)	54 (*S*)	70 (*S*)
p-Cl	14 (*R*)	36 (*S*)	77 (*S*)	27 (*S*)	62 (*S*)
p-Br	42 (*R*)	22 (*S*)	61 (*S*)	4 (*R*)	52 (*S*)
p-CH_3	59 (*R*)	21(*R*)	43 (*S*)	38 (*R*)	4 (*S*)
p-OCH_3	57 (*R*)	7 (*R*)	39 (*S*)	18 (*R*)	6 (*R*)
p-$C(CH_3)_3$	31 (*R*)	93 (*S*)	> 99 (*S*)	96 (S)	90 (*S*)
p-CF_3	17 (*S*)	28 (*S*)	37 (*S*)	5 (*S*)	17 (*S*)

[a]The ee values were determined by chiral GC analysis.[27]

TABLE 2. Reduction of Para-Substituted Acetophenones with M242/Q245 Double Mutant Enzymes of SSCR[a]

substrate	SSCR-WT ee (%)	M242L/Q245P ee (%)	M242F/Q245T ee (%)	M242C/Q245L ee (%)	M242L/Q245T ee (%)
p-H	42 (*R*)	74 (*S*)	42 (*S*)	17 (*S*)	58 (*S*)
p-F	46 (*R*)	92 (*S*)	90 (*S*)	16 (*S*)	94 (*S*)
p-Cl	14 (*R*)	99 (*S*)	94 (*S*)	50(*S*)	98 (*S*)
p-Br	42 (*R*)	> 99 (*S*)	89 (*S*)	24 (*S*)	99 (*S*)
p-CH_3	59 (*R*)	99 (*S*)	72 (*S*)	20 (*S*)	95 (*S*)
p-OCH_3	57 (*R*)	99 (*S*)	92 (*S*)	36 (*S*)	97 (*S*)
p-$C(CH_3)_3$	31 (*R*)	> 99 (*S*)	95 (*S*)	99 (*S*)	99 (*S*)
p-CF_3	17 (*S*)	> 99 (*S*)	98 (*S*)	94 (*S*)	99 (*S*)

[a]The ee values were determined by chiral GC analysis.[27]

which catalyzed the reduction to give (*R*)-enantiomer, these mutant enzymes (M242Y, M242D, M242C, and M242G) disfavored the formation of (*R*)-configurated chiral alcohols for reductions of all para-substituted acetophenones to such an extent that the (*S*)-enantiomer of product alcohols was obtained in most cases. Especially for *p*-*tert*-butylacetophenone, these mutant **SSCR** enzymes exhibited inverted enantiopreference and enhanced enantioselectivity. It is clear that the residue 242 in the catalytic cavity plays an important role in determining the enantioselectivity for the reduction of the para-substituted acetophenones, but to a less extent than residue Q245. Among the four mutants, M242D exerts the greatest effect on the enzyme enantioselectivity. If the substituent amino acids are lined up according to their hydrophobicity as M > C, Y, G ≫ D, it is intriguing to observe that the replacement of hydrophobic methionine with polar amino acids cysteine, tyrosine, and glycine has altered the enzyme spatial preference toward the *S* configuration. This tendency is first reflected on the decrease in the number of substrates with *R* preference (**SSCR**-WT = 7, M242C = 3, M242Y = 2, M242G = 1 and M242D = 0). Second, this hydrophobicity effect was also reflected in enantiomeric values, e.g., for *p*-methoxyacetophenone, the ee value toward the *R* enantiomer dropped from 57% (the wild-type) to 6% (M242G). The replacement with negative charged aspartic acid then led *S* isomer alcohols as predominant products for all eight substrates. For *p*-trifluoromethylacetophenone, the major product was (*S*)-enantiomer with wild-type and mutant enzymes as biocatalyst; mutation of residue M242 did not greatly affect the enzyme enantioselectivity.

It has been demonstrated that mutation of either residue Q245 or M242 greatly tuned the enantioselectivity of carbonyl reductase **SSCR** toward para-substituted acetophenones. A question has arisen as to whether a highly enantioselective mutant carbonyl reductase could be created by double mutation of residues M242 and Q245? With interest in searching for novel highly enantioselective carbonyl reductases, we constructed a focused double mutant library by site-saturation mutagenesis at both residues 242 and 245. The library was screened with *p*-methoxyacetophenone as the substrate for higher activity than the wild-type enzyme, (*S*)-1-(*p*-Methoxyphenyl)ethanol was the major enantiomer of the product alcohol for all four mutant enzymes in which three of them exhibited high enantioselectivity with greater than 92% ee.

Some other para-substituted acetophenones were also studied with these double mutant enzymes to examine their enantioselectivity (Table 2). As shown in Table 2, all four double mutant enzymes greatly increased the (*S*)-enantiomeric preference to an extent that the absolute configuration of major products was inverted from (*R*) to (*S*). Among them, mutant M242L/Q245P possessed the highest enantioselectivity in the reduction of these ketones, which was higher than those of the mutant Q245P, suggesting that replacements of M242 with L and Q245 with P had a synergic effect on preferable formation of (*S*)-enantiomeric products. Compared to Q245L, mutant M242C/Q245L showed much lower enantioselectivity in most cases. The preference for the (*S*)-enantiomer formation of these related mutant enzymes followed the order wild-type < M242C < M242C/Q245L < Q245L, indicating that M242C had a negative effect on the (*S*)-enantiomeric preference of Q245L. In contrast to this, M242C had a positive effect on the (*S*)-enantiomeric preference of wild-type enzyme as described above. Mutant M242L/Q245T exhibited higher enantioselectivity than mutant M242F/Q245T. The synthetic application of these mutant enzymes was demonstrated by the reduction of *p*-chloroacetophenone with mutant M242L/Q245P at preparative scale, giving (*S*)-1-(*p*-chlorophenyl)ethanol in 92% yield.

The kinetic parameters of these mutant carbonyl reductases were measured and their overall catalytic efficiencies (k_{cat}/K_m) toward these para-substituted acetophenones are presented in Figures 1−3. From Figure 1, it can be seen that the overall catalytic efficiencies of mutant carbonyl reductases M242Y, M242D, and M242G were lower than wild-type enzymes, while for mutant M242C, the k_{cat}/K_m values were comparable with those of the wild-type enzyme for most substrates with the exceptions of *p*-chloro (twice higher) and the unsubstituted acetophenone (half high).

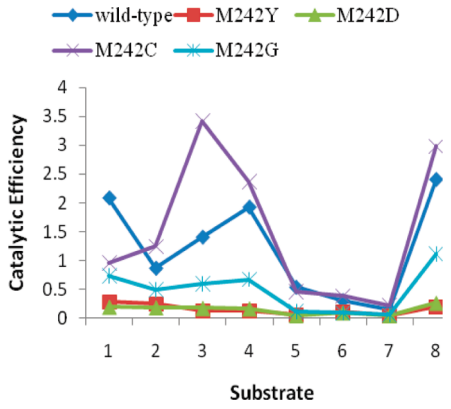

FIGURE 1. The catalytic efficiency (k_{cat}/K_m, $min^{-1}\cdot mM^{-1}$) of **SSCR** wild-type and M242 mutant enzymes toward reduction of acetophenones: (1) unsubstituted, (2) *p*-fluoro, (3) *p*-chloro, (4) *p*-bromo, (5) *p*-methyl, (6) *p*-methoxy, (7) *p*-*tert*-butyl, and (8) *p*-trifluoromethyl.

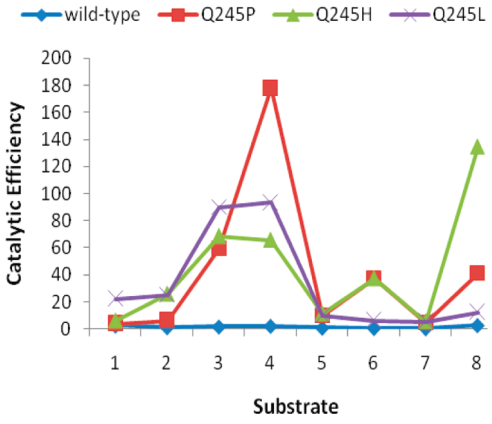

FIGURE 2. The catalytic efficiency (k_{cat}/K_m, $min^{-1}\cdot mM^{-1}$) of **SSCR** wild-type and Q245 mutant enzymes toward reduction of acetophenones: (1) unsubstituted, (2) *p*-fluoro, (3) *p*-chloro, (4) *p*-bromo, (5) *p*-methyl, (6) *p*-methoxy, (7) *p*-*tert*-butyl, and (8) *p*-trifluoromethyl.

The Q245 mutant and M242/Q245 double mutant enzymes showed higher overall catalytic efficiencies than the wild-type enzyme, especially for the acetophenones with electron-withdrawing groups such as chloro, bromo, and trifuoromethyl (Figures 2 and 3). These results suggest that residues 242 and 245 not only play an important role in determining the enzyme's enantioselectivity, but also affect the catalytic activity toward the reduction of para-substituted acetophenones, and residue 245 exerts a much greater influence on both enantioselectivity and catalytic activity.

Naturally occurring enzymes are not always highly active and/or stereoselective for unnatural substrates. Improvement in enzyme activity and enantioselectivity is often necessary to meet the synthetic needs. Up to now, reports on the enantioselectivity engineering of carbonyl reductase are still very limited. Recently, Philips's group has reported that secondary ADH from *Thermoanaerobacter ethanolicus* (**TeSADH**) bearing a single mutation I86A catalyzed the reduction of benzylic and heteroaryl ketones to give the

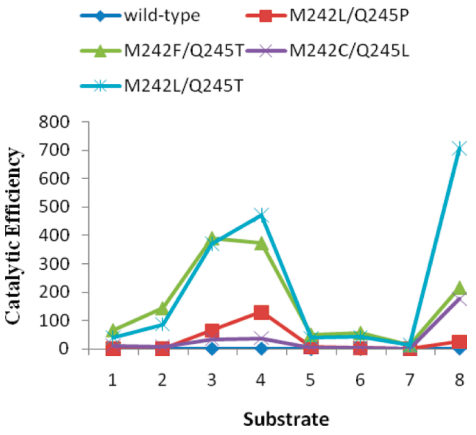

FIGURE 3. The catalytic efficiency (k_{cat}/K_m, $min^{-1}\cdot mM^{-1}$) of **SSCR** wild-type and M242/Q245 double mutant enzymes for the reduction of acetophenones: (1) unsubstituted, (2) *p*-fluoro, (3) *p*-chloro, (4) *p*-bromo, (5) *p*-methyl, (6) *p*-methoxy, (7) *p*-*tert*-butyl, and (8) *p*-trifluoromethyl.

corresponding "anti-Prelog" (*R*)-alcohols. As a comparison, the wild-type enzyme was not able to reduce these ketones and provided (*S*)-alcohols as the major products for the reduction of smaller ketones.[28] Other examples are the substrate-binding residue exchange of stereocomplementary ketoreductase domains leading to the reversion of stereochemistry. For example, mutation of residues in motifs I and II in ketoreductase domains eryKR1 and eryKR2 from the erythromycin polyketide synthase altered the stereochemical outcome in reduction of (2*R*,*S*)-2-methyl-3-oxopentanoic acid *N*-acetyl-cysteamine thioester.[29,30] In the study to confirm the hypothetical tropinone binding mode, replacement of 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 resulted in the switch of stereospecificity of TR-1 into that of TR-2 and vice versa.[31] These structure-based mutageneses revealed some insights into how these enzymes control their stereoselectivity. The scarcity of such study might be due to the difficulty of implementing a high-throughput method to determine enantioselectivity in the ketone reduction that allows the rapid screening of a large number of mutants. The present study demonstrates that enzyme−substrate docking-guided site-mutagenesis is a practical approach to access highly enantioselective carbonyl reductase enzymes.

Conclusions

Although a carbonyl reductase from *Sporobolomyces salmonicolor* catalyzed the reduction of various ketones with diverse structures to highly enantiomerically pure products, it reduced para-substituted acetophenones with low enatioselectivity. Enzyme−substrate docking studies

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with *p*-methoxyacetophenone revealed that the observed low enantioselectivity was consistent with two energetically close conformations of substrate in the binding site. Residues M242 and Q245 in the substrate binding site were identified as the key residues for enzyme engineering to create highly enantioselective carbonyl reductases for the reduction of para-substituted acetophenones. Site-saturation mutagenesis of single point mutation at M242 or Q245 and of double point mutation of these two residues was performed. Four M242 variants, three Q245 variants, and four M242/Q245 double mutant enzymes were obtained by screening of these focused mutant libraries. All these mutant enzymes showed stronger preference for the formation of (*S*)-configurated product alcohols in the reduction of para-substituted acetophenones than the wild-type **SSCR**, leading to the configuration invertion of the major product alcohols from (*R*) to (*S*) in most cases. Especially for the Q245 mutant and the double mutant enzymes, (*S*)-alcohols were obtained in a very high enantiomeric purity. Similarly, residues M242 and Q245 also alter the enzyme activity, but M242 has less effect than Q245, as shown in Figures 1−3. These studies indicated that residues M242 and Q245 not only play a crucial role in determining the enantioselectivity of these ketone reductions, but also exert some effect on the enzyme's catalytic activity. The structure-based site-saturation mutagenesis created highly enantioselective mutant carbonyl reductases. Among them, double mutant enzyme M242L/Q245P showed the highest enantioselectivity, which catalyzed the reduction of the tested para-substituted acetophenones to give (*S*)-enantiomeric products in ≥99% ee, and the only exception was *p*-fluoroacetophenone with 92% ee. As such, the *in silico* docking guided semirational approach should be a very valuable methodology for discovery of new enzymes with synthetic advantages.

Experimental Section

M242 Mutant Library. The carbonyl reductase gene from *Sporobolomyces salmonicolor* (**SSCR**) was cloned as described before.[15] The plasmid was used as the template for the Quick Change site-directed mutagenesis polymerase chain reaction (PCR). The forward primer was 5′-TTCCCCGGCTCTGGCTCTGNNKCCACCGCAGT ACTACGTT-3′ and the reverse primer was 5′-AACGTAGTACTGCGGTGGMNNCAGAGCCAGAGCCGGGGAA-3′. PCR was carried out with *pfx* polymerase under the following conditions: the reaction was started at 94 °C (2 min), followed by 30 cycles: 94 °C (30 s), 55 °C (45 s), 68 °C (6 min 48 s), with a final extension at 68 °C (10 min). The reaction was carried out in 100 μL reaction volume containing 0.5 pM of both primers, 210 ng genomic DNA, 1.0 mM $MgSO_4$, 0.2 mM dNTP, and 3.7 U of *pfx* polymerase. The PCR product was digested with DpnI restriction enzyme and electronically transformed to *E. coli* competent cell BL21(DE3).

M242 and Q245 Double Mutant Library. The double mutant library construction followed the similar procedure described above for M242. The forward primer was 5′-TTCCCCGGCTCTGGCTCTGNNKCCACCGNNKT-3′, and the reverse primer was 5′-AACAGCGGAAACGTAGTAMNNCGGTGGMNNCA-3′.

Screening of M242 Mutant Library. Each of the 92 mutant colonies along with 4 colonies expressing wild-type **SSCR** gene were grown in 150 μL of Luria−Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C for 12 h in a 96-well microplate. For each colony, 4 μL of the overnight culture was diluted into 2 mL of LB medium containing 100 μg/mL ampicillin and the culture was incubated at 37 °C with shaking at 150 rpm until the optical density reached 1.2 (2 h). The cell cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM), and incubated at 30 °C for 6 h. The cells were harvested by centrifugation at 4100 rpm for 30 min and lysed with 400 U of Ready-Lyse lysozyme (Epicenter, Inc.) in 50 μL of TES buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The lysis reactions were incubated at room temperature for 30 min, and 300 μL of potassium phosphate buffer (100 mM, pH 6.5) was added to the mixture. The diluted cell-free extract thus obtained after centrifugation was used in the activity assays, which were carried out by spectrophotometrically monitoring the disappearance of NADPH at 340 nm with 4′-methoxyacetophenone as the substrate. The colonies, which showed activity close to or higher than the wild-type **SSCR** enzyme, were cultivated in 15 mL of LB media containing 100 μg/mL ampicillin at 37 °C for 12 h. The cell-free lysates were prepared as described above and used in the 1 mL scale reactions to determine the enantioselectivity. The reaction mixtures containing 300 μL of lysate, 0.5 mg of NADPH, 12.5 mM 4′-methoxyacetophenone, 0.5 mg of GDH, and 4.0 mg of glucose in 1 mL of potassium phosphate buffer (100 mM, pH 6.5) were shaken at room temperature for 12 h. The ee value of the product alcohol was measured by chiral GC analysis. The plasmid DNAs from the clones, which showed some enantioselectivity, were isolated and sequenced. Confirmed plasmid DNAs were transformed into *E. coli* competent cell Rosetta 2(DE3)pLysS.

Screening of M242 and Q245 Double Mutant Library. Three thousand colonies were picked up randomly from the double mutant library. A similar procedure described above was followed, using the wild type and mutant Q245P as references. The plasmid DNAs from the clones, which showed higher enantioselectivity, were isolated and sequenced. Confirmed plasmid DNAs were transformed into *E. coli* competent cell Rosetta 2(DE3)pLysS.

Purification of SSCR Mutant Enzymes. SSCR mutants were grown in LB medium containing 100 μg/mL ampicillin and incubated at 37 °C until the optical density reached 0.6 at 600 nm. The expression was induced by 0.1 mM IPTG and the cell culture was incubated at 30 °C for another 6 h. The cells were harvested by centrifugation at 4100 nm at 4 °C for 30 min. The cell pellet was resuspended in potassium phosphate buffer (100 mM, pH 7.4) and the cells were disrupted by a High Pressure Homogenizer. The cell-free extract was mixed with an equal volume of PEI solution (0.25% polyethyleneimine, NW 40−60 K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids. The supernatant was precipitated with 55% ammonium sulfate. The precipitate was resuspended in potassium phosphate buffer (10 mM, pH 7.4, 0.1 mM dithiothreitol), followed by diafiltration with potassium phosphate buffer (10 mM, pH 7.4, 0.1 mM dithiothreitol) to remove the excess of ammonium sulfate. The resulting lysate was lyophilized to give the **SSCR** mutant enzymes as white powders, which were used in the acetophenone reduction to measure the activity and enantioselectivity.

Kinetic Assay. The enzyme activity for ketone reduction was determined by spectrophotometrically monitoring the reduction of NADPH at 340 nm at room temperature with a molar extinction coefficient of 6220 M^{-1} cm^{-1}. The reaction was carried out in potassium phosphate buffer (100 mM, pH 7.0) with 0.25 mM NADPH. The substrate concentration range was between 0.1 and 2.4 mM. A microplate reader was applied for the measurements and SigmaPlot version11 software was used for calculation of the kinetic parameters.

Measurement of Enantioselectivity. The enantioselectivity of the enzymatic reduction of ketones was studied by using an NADPH recycle system. The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADPH (0.5 mg), the carbonyl reductase (SSCR, 0.5 mg), and ketone solution in DMSO (50 μL, 0.25 M) were mixed in a

potassium phosphate buffer (1 mL, 100 mM, pH 6.5) and the mixture was shaken overnight at room temperature. The mixture was extracted with methyl *tert*-butyl ether (1 mL). The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral GC analysis to determine the enantiomeric excess. The absolute configurations of product alcohols were identified by comparing the chiral GC data with the standard samples.[27]

Enzymatic Reduction of *p*-Chloroacetophenone (Preparative Scale). D-Glucose (20 g L^{-1}), D-glucose dehydrogenase (2 g L^{-1}), NADPH (1 g L^{-1}), and mutant M242L/Q245P enzyme (2 g L^{-1}) were dissolved in potassium phosphate buffer (100 mM, pH 7.0), and 38 mL of the resulting solution was mixed with 2 mL of *p*-chloroacetophenone solution in DMSO (0.25 M). The reaction mixture was shaken at room temperature for 24 h. The reduction was completed as shown by GC analysis. The mixture was saturated with sodium chloride and extracted with methyl *tert*-butyl ether (3 × 30 mL). The organic extract was dried over anhydrous sodium sulfate and removal of solvent gave (*S*)-1-(*p*-chlorophenyl)ethanol[14] (71.1 mg, yield 92%).

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