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Ser67Asp and His68Asp Substitutions in *Candida parapsilosis* Carbonyl Reductase Alter the Coenzyme Specificity and Enantioselectivity of Ketone Reduction▽†

Rongzhen Zhang,[1] Yan Xu,[1,4]* Ying Sun,[1] Wenchi Zhang,[2] and Rong Xiao[3]

Key Laboratory of Industrial Biotechnology of Ministry of Education and School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China[1]; National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, People's Republic of China[2]; Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, New Jersey 08854[3]; and State Key Laboratory for Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China[4]

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A short-chain carbonyl reductase (SCR) from *Candida parapsilosis* catalyzes an anti-Prelog reduction of 2-hydroxyacetophenone to (*S*)-1-phenyl-1,2-ethanediol (PED) and exhibits coenzyme specificity for NADPH over NADH. By using site-directed mutagenesis, the mutants were designed with different combinations of Ser67Asp, His68Asp, and Pro69Asp substitutions inside or adjacent to the coenzyme binding pocket. All mutations caused a significant shift of enantioselectivity toward the (*R*)-configuration during 2-hydroxyacetophenone reduction. The S67D/H68D mutant produced (*R*)-PED with high optical purity and yield in the NADH-linked reaction. By kinetic analysis, the S67D/H68D mutant resulted in a nearly 10-fold increase and a 20-fold decrease in the k_{cat}/K_m value when NADH and NADPH were used as the cofactors, respectively, but maintaining a k_{cat} value essentially the same with respect to wild-type SCR. The ratio of K_d (dissociation constant) values between NADH and NADPH for the S67D/H68D mutant and SCR were 0.28 and 1.9 respectively, which indicates that the S67D/H68D mutant has a stronger preference for NADH and weaker binding for NADPH. Moreover, the S67D/H68D enzyme exhibited a secondary structure and melting temperature similar to the wild-type form. It was also found that NADH provided maximal protection against thermal and urea denaturation for S67D/H68D, in contrast to the effective protection by NADP(H) for the wild-type enzyme. Thus, the double point mutation S67D/H68D successfully converted the coenzyme specificity of SCR from NADP(H) to NAD(H) as well as the product enantioselectivity without disturbing enzyme stability. This work provides a protein engineering approach to modify the coenzyme specificity and enantioselectivity of ketone reduction for short-chain reductases.

Recently some members of the short-chain dehydrogenase/reductase (SDR) family have been described (3, 9, 22, 24). These enzymes include a large number of prokaryotic and eukaryotic enzymes with different specificities for coenzymes and substrates (8), and they are involved in the metabolism of various aliphatic and aromatic carbonyl compounds and secondary alcohols (15, 17). The enzyme in this family contains two important typical structural motifs. The first conserved domain of SDR is present on the interior surface of an α-helix and contains the universally conserved sequence Ser-Tyr-Lys, which is demonstrated to be the active site. The second is located in the vicinity of the N terminus with the Gly-X-X-X-Gly-X-Gly sequence (also named glycine-rich domain), which is found in not only SDR but also dehydrogenase in general (2). The region around the glycine-rich domain forms a βαβ-fold that is characteristic of the coenzyme-binding fold in the SDR family, as shown by site-directed mutagenesis and X-ray crystallography studies (26). But the NAD(H)- and NADP(H)-dependent dehydrogenases in the family have different fingerprint sequences for coenzyme binding. The cofactor specificity is attributed in part to an aspartic acid residue, which correlates with its presence at the C terminus of the second β-strand of the coenzyme-binding fold with NAD(H) and its absence with NADP(H) (2). The X-ray crystallography studies of NADH-dependent SDRs has suggested that the Asp residue forms a bifurcated hydrogen bond to the adenine ribose for the NAD(H)-dependent enzymes (5, 25), and Thr38 of the carbonyl reductase at a position corresponding to the Asp residue forms a hydrogen bond to the 2′ phosphate of NADPH through a water molecule (24). In other families of dehydrogenase for the NADP(H)-dependent enzymes, the third Gly of the NAD(H)-binding fingerprint is replaced by Ala, and a positively charged residue is usually included in the neighborhood of the C terminus of the βαβ-fold (2, 13, 26), whereas the residue(s) responsible for NADP(H) specificity remains unknown. For industrial applications, converting the cofactor specificity of an enzyme from NADPH to NADH would be of great significance since NADH is much cheaper than NADPH. It was reported that amino acid substitution of arginine 80 in 17β-hydroxysteroid dehydrogenase type 3 had an effect on NADPH cofactor binding and oxidation/reduction kinetics (12). Glutamate 115 determines the specificity of human 11β-hydroxysteroid dehydrogenase type 2 for the cofactor NAD^+ (1). Nakan-

* Corresponding author. Mailing address: School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China. Phone: 86 510 85918201. Fax: 86 510 85864112. E-mail: biosean@yahoo.com.cn.

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ishi reported that the coenzyme specificity of mouse lung carbonyl reductase was switched from NADPH to NADH by replacing threonine 38 with aspartic acid (15).

In our previous work, a novel short-chain carbonyl reductase (SCR) was isolated from *Candida parapsilosis*, and its coding gene *scr* was cloned and overexpressed in *Escherichia coli* (16, 27); the characteristics of the special protein became available recently (16, 27). This enzyme catalyzes the reduction of 2-hydroxyacetophenone to produce valuable optically active 1-phenyl-1,2-ethanediol (PED) with high productivity and chiral purity and exhibits a high degree of coenzyme preference for NADPH over NADH (27). It is a member of the SDR family and shares more than 30% similarity with mannitol-2-dehydrogenase from *Agaricus bisporus* (7) and the *R*-specific alcohol dehydrogenase from *Lactobacillus brevis* (20). The crystal structure of SCR was determined recently by a molecular replacement method in our labs (28). In this work, in order to explore the possibility of converting SCR from a NADPH-dependent enzyme into an NADH-dependent one, we designed mutations with different combinations of Ser67Asp, His68Asp, and Pro69Asp substitutions inside or adjacent to the so-called phosphate-binding loop between βB and αC. We compared their kinetic and thermodynamic properties of coenzyme binding to the wild-type (WT) SCR and its variants. The results show that the double point mutation Ser67Asp and His68Asp (S67D/H68D) successfully alters the coenzyme specificity of SCR from NADPH to NADH as well as the enantioselectivity of ketone reduction without disturbing enzyme stability. This work will provide a new example of altering the coenzyme specificity and product stereoselectivity in SDRs and will likely have valuable industrial applications.

MATERIALS AND METHODS

Organisms and materials. *C. parapsilosis* strain CCTCC M203011 was obtained from the American Type Culture Collection. The organisms were cultivated as described previously (16, 27). The cofactors were purchased from the Sigma-Aldrich Chemical Co., Inc. The restriction enzymes were purchased from Takara Shuzou Co. (Kyoto, Japan). The substrate β-hydroxyacetophenone was prepared using the method described by Liese et al. (10). All other chemicals were of the highest grade that could be obtained commercially.

Site-directed mutagenesis and expression. The gene *scr* (GenBank accession no. DQ675534) was expressed in *E. coli* as described by Nie et al. (16). The mutants were generated using a modified overlap extension technique (6) with plasmid pETSCR used as the template. The mutations were verified by DNA sequencing. The primer pairs used are listed in Table S1 in the supplemental material (where altered bases are underlined).

Purification of recombinant enzymes. All recombinant proteins were expressed in *E. coli* strain BL21(DE3) as His_6-tagged proteins. First, the protein was purified by affinity chromatography on an Ni^{2+}-Sepharose column (His-Trap Kit; Pharmacia). Second, the pooled fractions were further loaded on a Resource Q column (1 by 1 cm) equilibrated with buffer (20 mM Tris-HCl, pH 8.5) with an ÄKTA Protein Purifier system (Pharmacia, Uppsala, Sweden). Finally, the fractions were applied to a Superdex 200 (HiLoad 26/60; preparation grade) column for chromatography in a buffer containing 20 mM Tris-HCl (pH 8.5) and 150 mM NaCl. When necessary, 0.5 mM NADH or NADPH was added into the buffer. The homogeneity of purified enzymes was judged by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

Enzyme assay and kinetic determination. Enzymatic activities of SCR and its variant enzymes for reduction of 2-hydroxyacetophenone were measured at 35°C by recording the rate of change in NAD(P)H absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation and oxidation of 1μmol of NAD(P)H per minute under measurement conditions. Kinetic parameters were measured and calculated using a Beckman DU-7500 spectrophotometer equipped with a Multicomponent/SCA/Kinetics Plus software package and a thermostated circulating water bath. Various concentrations of substrate 2-hydroxyacetophenone (0.5 to 20 mM), enzyme (10 to 200 μM), and cofactors NAD(P)H (0.5 to 5.0 mM) in 100 mM phosphate buffer (pH 7.5) were used. The reported values represent the average of at least three independent measurements. All standard errors of fits were less than 15%.

Biotransformation and analytical methods. The biotransformation reaction was carried out as described previously (16) with minor modification. For asymmetric reaction with the recombinant *E. coli* cells, the reaction mixture in a 2-ml volume consisted of 0.1 M potassium phosphate buffer (pH 6.5), 5 g/liter 2-hydroxyacetophenone, and 0.3 g of washed wet cells. When the purified protein was used as a biocatalyst, the reaction mixture in a 2-ml volume consisted of 0.1 M potassium phosphate buffer (pH 6.5), 2 mmol/liter NADH or NADPH, 5 g/liter 2-hydroxyacetophenone, and an appropriate amount of purified protein. The reactions were carried out at 30°C for 48 h and 8 h with shaking at 150 rpm, respectively, when the wet recombinant cells and purified enzyme were used as biocatalysts. At the end of the reaction, the product (*R*)-PED was extracted with ethyl acetate, and the organic layer was used for analysis. The optical purity and yield of product were determined by high-performance liquid chromatography on a Chiralcel OB-H column (Daicel Chemical Ind. Ltd., Japan).

AUC. SCR variants at a concentration of 0.5 to 1.0 mg/ml were used for analytical ultracentrifugation (AUC) analysis with a buffer of 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. Sedimentation experiments were performed using an XL-A analytical ultracentrifuge (Beckman Coulter) equipped with a four-cell An-60 Ti rotor at 20°C. Sedimentation velocity analysis was performed at 58,000 rpm. Data were analyzed with the SEDFIT program (18).

CD. Circular dichroism (CD) measurements were carried out using a Jasco J720 spectropolarimeter (Jasco, Inc., Easton, MD). Wavelength scan data were collected from 190 to 260 nm in a phosphate buffer (pH 7.5) with the following instrument settings (for an average of 30 scans): response, 1 s; sensitivity, 100 millidegrees; speed, 50 nm/min. Scans were repeated every 2°C or 5°C between 20°C and 70°C. The protein concentration was about 3 μM in 50 mM Na/K phosphate buffer (pH 6.8). The decrease in CD signal with the increase of temperature was recorded at the local minimum of 209 nm.

Thermal and urea stability. For thermal inactivation, the enzymes (0.2 mg/ml) were incubated at 40°C in a buffer containing 0.1 M potassium phosphate buffer, pH 7.5, and 150 mM KCl in the presence or absence of NAD(P)(H) or substrate. Aliquots of 50 ml from each sample were taken and assayed for dehydrogenase activity at different times. For denaturation by urea, the enzyme (20 mg/ml) was incubated at 35°C for 2 h in 0.1 M Tris-HCl buffer, pH 8.0, containing 0 to 6 M urea in the presence or absence of NAD(P)H or substrate. The enzyme activity was expressed as a percentage of that in the absence of urea.

Fluorescence experiment. The nucleotide binding to SCR was measured by monitoring the quenching of intrinsic enzyme fluorescence upon incremental addition of nucleotides. Emission spectra (300 to 500 nm) were recorded on an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at a 280-nm excitation. The samples for measurements were prepared at the final concentration of 3 μM enzyme and 30 to 400 μM NAD(P)H in 50 mM Na/K phosphate buffer (pH 7.5). The K_d value was determined by the nonlinear least-squares method (19, 23). The model is expressed by a simple Hill equation with consideration of the Hill coefficient h and dissociation constant K_d: $Y = [L]^h/(K_d + [L]^h)$, where Y is the fractional saturation of the enzyme (i.e., number of occupied sites/total number sites), and $[L]$ is ligand concentration. The nonlinear fitting using this model was performed with the software Origin, version 7.5.

RESULTS AND DISCUSSION

Overexpression and purification of WT and mutants. Short-chain dehydrogenases constitute a large protein family. Although many SDR enzymes exhibit residue identities at only the 15 to 30% level, they display similar Rossmann folds. Enzymes in the SDR superfamily have specificity for substrates, such as primary and secondary alcohols, steroids, and prostaglandins. However, they all use either NAD(H) or NADP(H) as the coenzyme. In NADH-dependent SDRs, there is a reserved aspartate residue (Fig. 1) (Asp37 in *Drosophila lebanonensis* alcohol dehydrogenase) that regulates the specificity for coenzyme (NAD or NADH) binding (21). By tertiary structure-based sequence alignment (Fig. 1), the Ser67, His68, and Pro69 residues of SCR are possibly involved in coenzyme binding. Na-

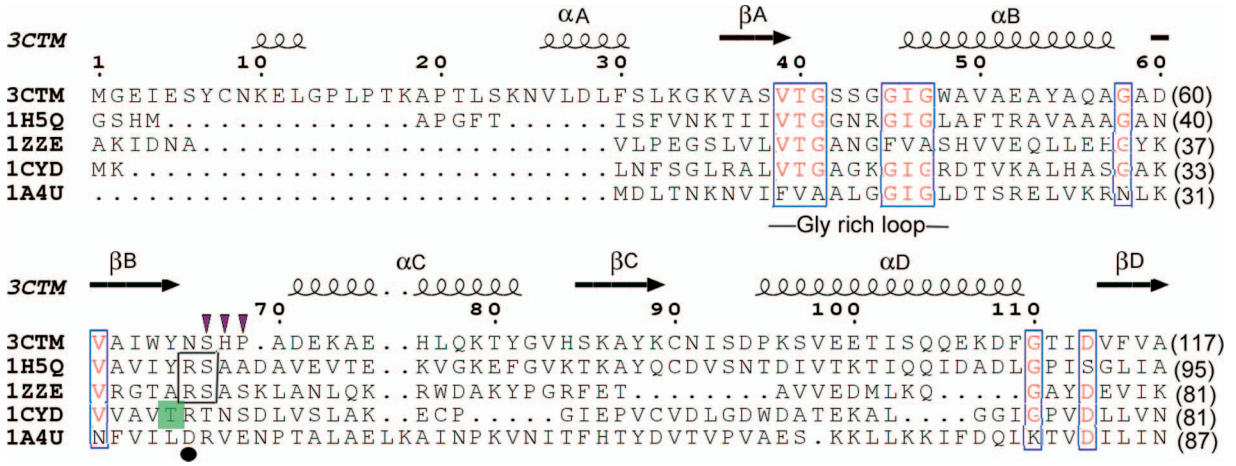

FIG. 1. Partial structural alignment of the SCR with selected members of the SDR family. Left columns contain the Protein Data Bank accession codes of the structures. 3CTM, (*S*)-1-phenyl-1,2-ethanediol dehydrogenase from *Candida parapsilosis*; 1H5Q, mannitol-2-dehydrogenase from *Agaricus bisporus*; 1ZZE, carbonyl reductase from *Sporobolomyces salmonicolor*; 1CYD, mouse lung carbonyl reductase; and 1A4U, *Drosophila lebanonensis* alcohol dehydrogenase. Conserved residues are boxed with blue lines. Selected residue numbers of the SCR are labeled above the sequence. Secondary structure elements of the SCR are marked at the top of the alignment, and the glycine-rich consensus sequence is indicated. The residues (Arg43 and Ser44 in 1H5Q and Arg44 and Ser45 in 1ZZE) in contact with the pyrophosphate bridge of NADPH-dependent SDRs is boxed in black. The Thr38 residue in 1CYD corresponding to the hydrogen bonds of the 2′ phosphate of NADPH is highlighted in green, and the Thr38Asp substitution alters the 1CYD preference from NAD(P)H to NAD(H). The Asp37 in 1A4U responsible for NAD(H) specificity is marked with a filled circle. The three residues for mutations to convert coenzyme specificity in 3CTM are marked with solid triangles. This figure was prepared with the program Espript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi/).

kanishi et al. reported that the coenzyme specificity was successfully switched by substitution of threonine 38 with aspartic acid (15). So the mutations were designed such that the Ser67, His68, and Pro69 residues of the WT SCR were all replaced by Asp residues. Six different mutants with different combinations of the above mutations were constructed.

Construction of expression plasmids of the WT SCR and mutants was performed using standard techniques as described in Materials and Methods. The expression plasmids obtained were named pETSCR, pETS67D, pETH68D, pETP69D, pETS67D/H68D, pETS67D/P69D, and pETH68D/P69D. Nucleotide sequencing has confirmed that mutations leading to variants are present in the entire gene. When *E. coli* BL21(DE3) cells carrying expression plasmids were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C, SDS-PAGE analysis showed that a predominant band corresponding to the expected size (30 kDa) of the target recombinant enzyme was observed in all recombinant cell extracts (see Fig. S1 in the supplemental material). All mutant proteins were overproduced at levels equal to that of the WT (see Fig. S1 in the supplemental material) with about 30% of the total soluble proteins in the intracellular fraction. None of the mutations had an effect on cell growth properties or protein expression. The overexpressed proteins in soluble form allowed further enzyme purification and examination of the effects of different mutations on functions.

The recombinant enzymes with C-terminal His_6 tags were purified from the cell extract of *E. coli* transformants using a HisTrap HP affinity column, followed by Resource Q and Superdex 200 chromatography. The proteins were purified to apparent homogeneity by SDS-PAGE. Calculations of the corresponding A_{280} value and SDS-PAGE analysis revealed that all mutant proteins were produced at more than 40 mg per liter of culture.

Enantioselectivity alteration of ketone reduction by mutagenesis. It has been reported that the WT SCR efficiently reduces 2-hydroxyacetophenone into (*S*)-PED (16, 27). The enzyme-mediated reduction was often enantioselective. When the whole cells of variants were used as catalysts, the optical purity of PED catalyzed from 2-hydroxyacetophenone was detected by high-performance liquid chromatography on a chiral column. The results (Table 1) showed that the product (*R*)-PED was formed by all variants at different transformation efficiencies. The variants S67D, H68D, P69D, S67D/P69D, and H68D/P69D produced (*R*)-enantiomer with low optical purity (39.8% to 45.9%) and yield (26.5% to 31.7%). The product catalyzed by S67D/H68D transformants was confirmed to comprise only the (*R*)-enantiomer with a high optical purity of 95.4% and a yield of 83.1% (Table 1).

For further investigation the biotransformation was performed using purified mutant enzymes for reducing 2-hydroxyacetophenone in the presence of 2 mmol/liter NADH or NADPH. The results showed that different cofactors had an effect on the stereoselectivity of product by mutant proteins. In the presence of NADPH the S67D, H68D, P69D, S67D/P69D, and H68D/P69D mutant proteins produced (*R*)-PED with higher optical purity and yield than their corresponding transformants, while in the presence of NADH, PED was not detected by their purified enzymes. The influence of two cofactors on biotransformation was also detected by the S67D/H68D protein. In the NADH-linked reaction the S67D/H68D protein produced (*R*)-PED with an optical purity of 97.1% and a yield of 85.2% (Fig. 2 and Table 1), while PED was not catalyzed in NADPH-linked reaction. Based on these results, it

TABLE 1. Bioreduction of 2-hydroxyacetophenone by the SCR and its variants

SCR type	Bioreduction results with the indicated catalyst								
	Transformants[a]			Enzyme and NADH[b]			Enzyme and NADPH[b]		
	Absolute configuration	Optical purity (%)	Yield (%)	Absolute configuration	Optical purity (%)	Yield (%)	Absolute configuration	Optical purity (%)	Yield (%)
WT	*S*	96.2 ± 1.5	83.8 ± 1.2	NT	NT	NT	*S*	97.6 ± 1.9	85.4 ± 1.7
S67D	*R*	45.9 ± 0.7	31.7 ± 0.5	NT	NT	NT	*R*	53.5 ± 0.9	42.2 ± 0.8
H68D	*R*	45.2 ± 0.4	30.3 ± 0.3	NT	NT	NT	*R*	52.4 ± 1.0	41.8 ± 0.6
P69D	*R*	39.8 ± 0.7	26.7 ± 0.8	NT	NT	NT	*R*	50.5 ± 0.9	37.8 ± 0.5
S67D/H68D	*R*	95.4 ± 1.2	83.1 ± 0.5	*R*	97.1 ± 0.7	85.2 ± 0.9	NT	NT	NT
S67D/P69D	*R*	39.9 ± 0.6	26.5 ± 0.5	NT	NT	NT	*R*	48.7 ± 1.2	35.8 ± 0.5
H68D/P69D	*R*	41.8 ± 0.4	28.1 ± 0.6	NT	NT	NT	*R*	51.8 ± 1.6	37.9 ± 0.7

[a] Recombinant *E. coli* BL21(DE3) was cultured until the turbidity of the culture at 600 nm reached 0.6 to 0.8, and cells were cultured for another 8 h after the addition of 1 mM I IPTG at 30°C.

[b] The purified enzyme was used as a catalyst by the addition of a 2 mM concentration of either NADH or NADPH. NT, no detectable transformation.

was strongly suggested that the S67D/H68D variant can catalyze the anti-Prelog stereospecific reduction of prochiral carbonyl compounds at the WT levels (27) while producing different enantiomers with high optical purity and yield. The biotransformation by the purified S67D/H68D protein also confirmed similar results. In addition, cofactor requirements for biotransformation by the S67D/H68D protein and the WT SCR were different. The product (*S*)-PED was catalyzed by the WT SCR in the presence of NADH (27), while the (*R*)-enantiomer was produced by the S67D/H68D protein in the presence of NADH, with high optical purity and yield. This means that this enzyme requires different cofactors for ketone reduction to produce different enantiomers by site-directed mutagenesis.

Kinetics demonstrates that the S67D/H68D mutation alters the cofactor preference from NADPH to NADH. All mutant proteins present different biotransformation results in the presence of NADH or NADPH compared to the WT SCR. So the kinetic effect of the mutations was assessed in the direction of 2-hydroxyacetophenone reduction using different cofactors between WT SCR and its variants. The kinetic parameters were determined with 2-hydroxyacetophenone at different concentrations (0.5 to 20 mM) and cofactors in varied concentrations (0.5 to 5 mM). The kinetic and coenzyme binding data obtained are summarized in Table 2. Compared with WT SCR, all mutant enzymes showed changes in K_m values but maintained k_{cat} values essentially at the same levels. The most striking alteration resulting from the mutagenesis is that the ratio of the K_m of the NADH-linked to the NADPH-linked reaction is 13.37 and 0.07 for WT SCR and the S67D/H68D variant, respectively. The S67D/H68D mutation resulted in a nearly 10-fold increase in the k_{cat}/K_m value for NADH but a 20-fold decrease in the k_{cat}/K_m ratio for NADPH. The k_{cat}/K_m ratio between NADH and NADPH changes from 0.068 for the WT to 13.8 for the mutant. The K_m values of other variants were increased several times over the premutation value in the NADPH-linked reaction but changed slightly in the NADH-linked reaction. Their k_{cat}/K_m values decreased significantly in the NADPH-linked reaction compared to WT SCR. Meanwhile, as a complementary approach, the fluorescence titration method was used to measure the formation of the enzyme-cofactor binary complexes for the WT and all variants, and the fluorescence was quenched by the addition of different coenzymes. The data were analyzed by a modified model from a simple Hill equation (19). The results are summarized in Table 2. The S67D, H68D, P69D, S67D/P69D, and H68D/P69D variants showed higher K_d values for NADPH and lower values for NADH than the WT SCR. Values greater than 1 for the ratio of the K_d with NADPH to that with NADH suggested that these mutants favor NADPH over NADH, which indicated that the mutations caused no changes in cofactor selectivity. The reaction catalyzed by the SCR is a sequential type, with the coenzymes binding to the free form of the enzyme and subsequently to the substrates according to the kinetics of the interaction (14). The change in the binding to the substrate may be due to the altered binding of NADPH and NADH. The increased K_d and decreased k_{cat}/K_m values (Table 2) caused a low biotransformation efficiency for all of the variants except the S67D/H68D mutant. The ratio of the K_d value with NADH to that with NADPH for the S67D/H68D mutant was 0.28, indicating a stronger preference for NADH; in contrast, the ratio became 1.9 for the WT, indicating a weaker binding for NADH (Table 2). These spectroscopic results indicate that the substituted Asp67 and Asp68 may be beneficial to the formation of hydrogen bonds with the hydroxyl groups of adenine ribose of the coenzyme, as with other NADH-dependent SDRs (4, 24), and improve the process of removing the product (*R*)-PED. The change in the binding of NADP(H) was also observed in a computer modeling study of the mouse lung carbonyl reductase (CR) structure (15). Tanaka et al. thought that the altered cofactor specificity of the mouse lung CR-NADPH complex may be due to the loss of the hydrogen bridge between Thr38 (i.e., WT) (24) and the 2′-phosphate of NADP(H) by the mutation (24). Calculation of free-energy changes also confirmed a significant role of the substituted Asp for NAD(H) specificity in the mouse lung enzyme (15). Masuda et al. reported a change in enantioselectivity in bufuralol 1′-hydroxylation by a Phe120Ala mutation in cytochrome P450 2D6 (11). The double point mutation S67D/H68D inside or adjacent to the coenzyme binding pocket converted the coenzyme specificity of the SCR from NADP(H) to NAD(H) and altered the enantioselectivity of product without affecting transformation function.

The S67D/H68D mutation induces no changes in protein folding or conformational stability. Although the S67D/H68D mutant enzyme and the WT SCR showed identical subunit molecular masses, the pI value is 5.2 for the S67D/H68D vari-

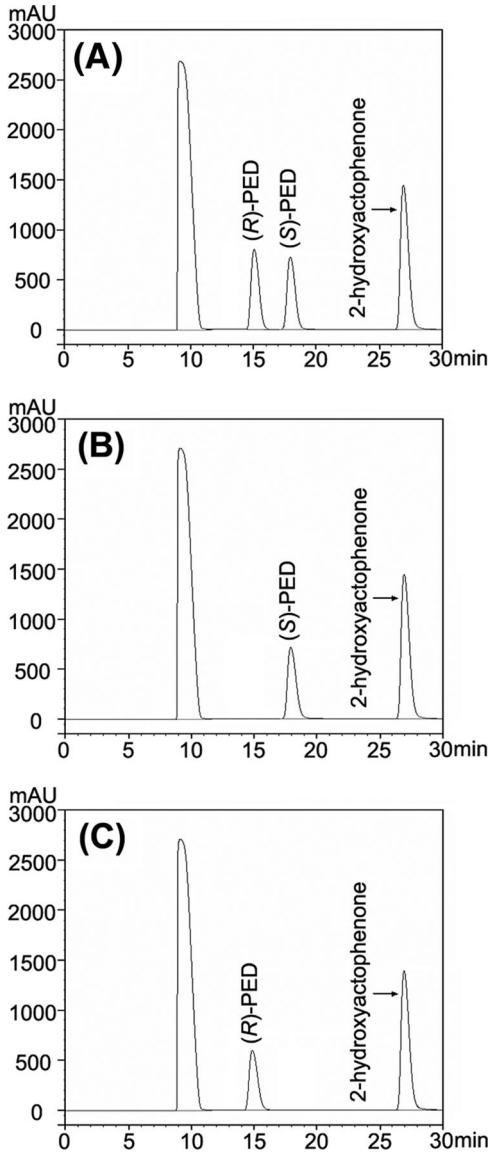

FIG. 2. Asymmetric reduction of 2-hydroxyacetophenone using purified SCR and S67D/H68D proteins. (A) Retention times of standard samples are as follows: (*R*)-PED, 15.0 min; (*S*)-PED, 18.3 min; 2-hydroxyacetophenone, 27.1 min. (B) Reaction products catalyzed by the SCR in the presence of NADPH. (C) Reaction products catalyzed by the S67D/H68D protein in the presence of NADH. AU, arbitrary units.

ant enzyme, which is lower than that of 5.6 for the WT due to the introduction of two Asp residues with acidic charges. So it was necessary for the S67D/H68D variant protein to be analyzed with regard to folding, oligomerization, and stability for industrial application. In a CD spectroscopy assay, the WT SCR and the S67D/H68D mutant form displayed essentially identical spectral characteristics, indicating their similar secondary structure properties (Fig. 3A). Thermal stability of the variant was analyzed using a CD temperature scan; the molar residue ellipticity at 209 nm was used as a measurement of structural transition during the thermal denaturation. In the CD thermal denaturation assay, the recombinant protein of the S67D/H68D mutant showed thermal stability similar to that of the WT SCR (Fig. 3B). The S67D/H68D variant resulted in a 50°C melting temperature, only 2°C lower than that of the WT SCR (Fig. 3B). Judging from these experiments, no significant differences between the WT and S67D/H68D mutant protein could be detected, indicating their similarity in folding and conformational stability.

The purified S67D/H68D protein had a molecular mass distribution in AUC analysis showing a single tetramer peak (~110 kDa) similar to the WT SCR (see Fig. S2 in the supplemental material). The result indicates that the S67D/H68D mutant enzyme formed a tetramer in solution similar to the WT protein (eluted at the 112-kDa position). This observed tetramer formation was consistent with results from our molecular size exclusion chromatography studies, which showed that the SCR (see Fig. S3A in the supplemental material) and S67D/H68D variant both exhibited homotetramers in solution (see Fig. S3B in the supplemental material). Because the determined structure of the WT SCR is an inactive form (28), we tested the probable effects of cofactor on the tetramerization and compared size exclusion chromatography profiles between the SCR and the S67D/H68D samples in the presence or absence of NAD(P)H. The results showed that in both cases the SCR and the S67D/H68D variant enzymes formed homotetramers (see Fig. S3 in the supplemental material). It further suggested that the S67D/H68D mutation would not affect the oligomerization of the variant enzyme in the presence or absence of NADPH or NADH.

NAD(P)H provides different levels of protection for the WT and the S67D/H68D variant. Stability measurements of the

TABLE 2. Kinetic parameters for 2-hydroxyacetophenone reduction by the WT and mutant enzymes[a]

Protein	Value for the parameter with NADH as cofactor					Value for the parameter with NADPH as cofactor				
	K_m	k_{cat}	k_{cat}/K_m	Relative k_{cat}/K_m[b]	K_d[c]	K_m	k_{cat}	k_{cat}/K_m	Relative k_{cat}/K_m[b]	K_d[c]
WT	7.89 ± 0.19	1.32 ± 0.05	0.17	100	267.72 ± 17.63	0.59 ± 0.01	1.48 ± 0.06	2.51	100	140.12 ± 15.02
S67D	1.26 ± 0.08	6.63 ± 0.13	0.19	111.8	243.43 ± 14.03	1.54 ± 0.05	2.35 ± 0.04	0.66	26.3	163.38 ± 11.05
H68D	1.35 ± 0.06	6.58 ± 0.21	0.18	105.9	230.42 ± 13.25	1.58 ± 0.03	1.95 ± 0.11	0.81	32.3	175.54 ± 13.13
P69D	1.22 ± 0.11	6.54 ± 0.03	0.19	111.8	237.24 ± 18.71	1.51 ± 0.03	2.23 ± 0.13	0.68	27.1	178.16 ± 16.75
S67D/H68D	0.76 ± 0.03	1.26 ± 0.05	1.66	976	71.31 ± 12.64	10.27 ± 0.21	1.21 ± 0.03	0.12	5	253.78 ± 13.54
S67D/P69D	1.42 ± 0.06	6.76 ± 0.14	0.21	123.5	241.95 ± 14.54	1.55 ± 0.07	2.32 ± 0.16	0.67	26.7	180.23 ± 12.83
H68D/P69D	1.46 ± 0.09	6.37 ± 0.11	0.16	94.1	232.87 ± 12.13	1.61 ± 0.06	1.72 ± 0.08	0.94	37.5	187.4 ± 11.53

[a] All reactions involved in the calculation of kinetics constants were assayed with 100 mM acetate buffer (pH 5.0) at 35°C.
[b] Relative values were compared to the WT value for the same cofactor.
[c] The K_d (dissociation constant) indicates the strength of binding between the enzyme and cofactors in terms of how easy it is to separate the enzyme-cofactor complex. Values were determined by fluorescence titrations. All experiments were repeated three to five times.

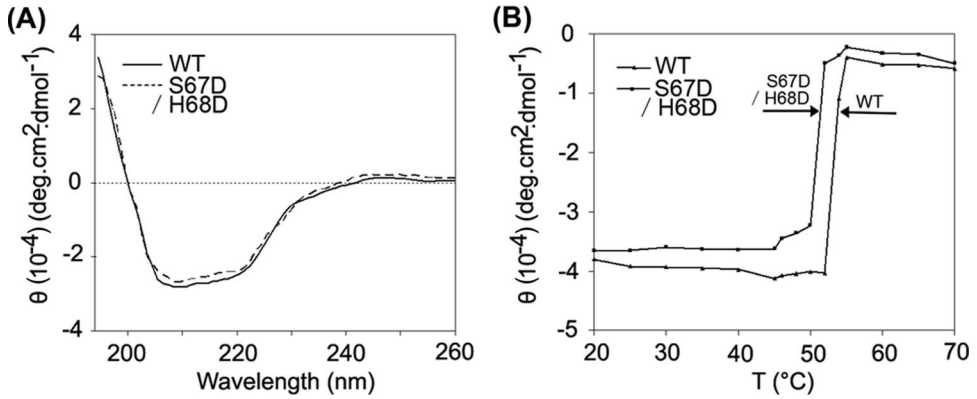

FIG. 3. CD spectra (A) and thermal denaturation (B) of the SCR and the S67D/H68D variant. The CD spectra were recorded by measuring the ellipticity as a function of wavelength at 0.1-nm increments between 195 and 260 nm at 20°C. The thermal denaturation was determined by measuring the ellipticity at 209 nm as a function of temperature (T) at increments of 2 or 5°C between 20 and 70°C.

WT enzyme and S67D/H68D variant were performed using thermal denaturation techniques and by titration with urea at different concentrations. In the thermal denaturation assay for enzyme activity, the WT SCR protein and the S67D/H68D mutant showed different thermal stability measurements with the addition of different cofactors (Fig. 4). The data shown in Fig. 4A indicated that the thermal inactivation of the WT was protected by NADPH at low concentrations. When the concentration of NADPH was 2 mM, the relative activity of the enzyme remained over 95% after the enzyme was kept at 40°C for 30 min; this result means that 2 mM NADPH almost protects the whole activity of the enzyme (Fig. 4A). Thermal inactivation was protected moderately by high concentrations of NADH for the WT enzyme, whereas NADH provided more efficient protection against the thermal inactivation of S67D/H68D than did NADPH (Fig. 4). For example, when 4 mM NADPH and 2 mM NADH were added into the S67D/H68D variant protein, enzyme activity remained at 93.7% and 78.5%, respectively, after thermal denaturation for 30 min at 40°C (Fig. 4B).

The WT SCR and the S67D/H68D form maintained only about 20% of their enzyme activity after denaturation with 4 M urea (Fig. 5) without the addition of cofactors. Similar protective effects of the coenzymes appeared with the denaturation by urea, in which S67D/H68D was slightly unstable compared to WT SCR (Fig. 5). NADPH showed greater protection against the urea denaturation of the WT than NADH (Fig. 5A). The protection of S67D/H68D from the urea denaturation afforded by the addition of NADH was noticeable (Fig. 5B). It should be noted that the substrate (5 mM 2-hydroxyacetophenone) did not show significant protection against the thermal and urea denaturation of the two enzymes, which supports the kinetics-ordered addition of the coenzymes to the free enzymes followed by the substrates. The kinetics results were also supported by differences between the S67D/H68D and WT forms in protection by the coenzymes against thermal and urea denaturation.

For industrial applications, we constructed several variants of the SCR to explore the possibility of converting its cofactor specificity from NADPH into NADH. The double point mu-

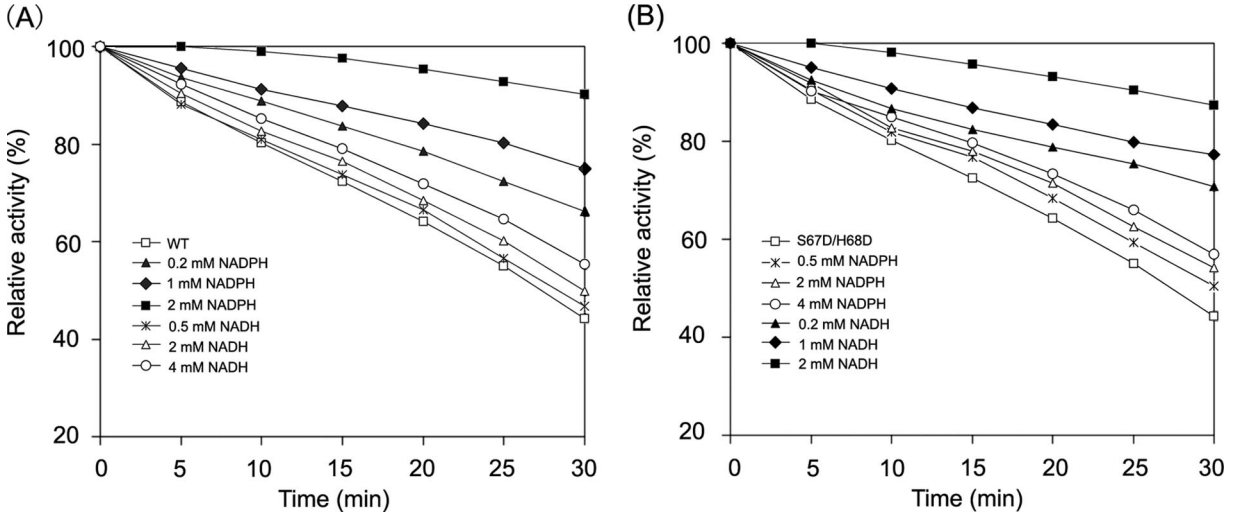

FIG. 4. Effects of coenzymes on thermal inactivation of the WT (A) and the S67D/H68D mutant (B). The WT and S67D/H68D mutant enzymes were incubated at 40°C in the presence or absence of NAD(P)H.

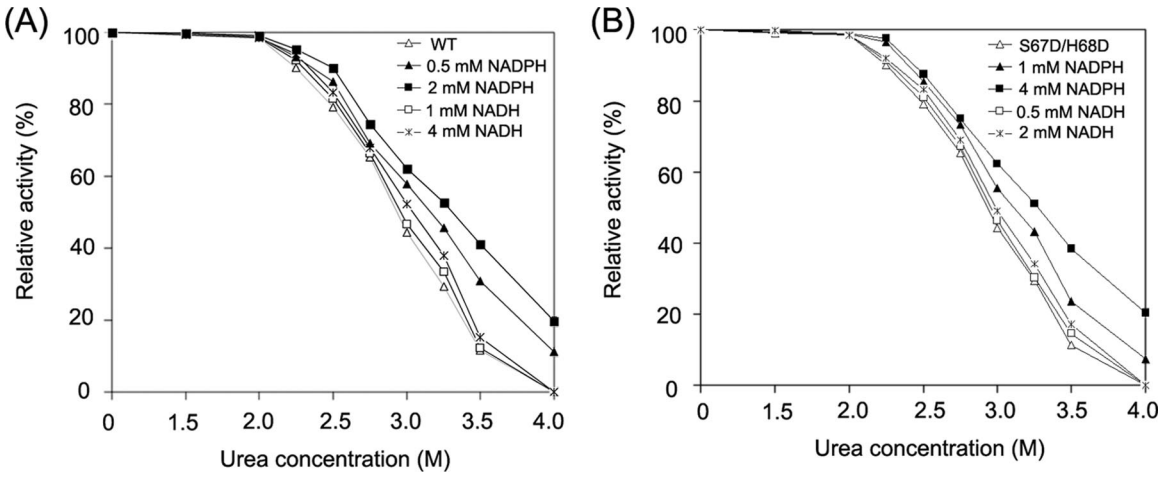

FIG. 5. Effects of coenzymes on urea denaturation of the WT (A) or the S67D/H68D mutant (B) at pH 8.0. The WT and S67D/H68D mutant enzymes were incubated at 25°C in the presence or absence of NAD(P)H.

tant S67D/H68D might make changes of the residues located in the phosphate-binding loop between βB and αC. The carefully introduced aspartic acid residues would be energetically unfavorable for binding by the negatively charged 2′ phosphate group of NADPH, thus favoring NADH over NADPH, as described by Nakanishi et al. (15). The kinetics also demonstrate that the S67D/H68D mutation alters the cofactor preference from NADPH to NADH. In the direction of 2-hydroxyacetophenone reduction, the S67D/H68D variant caused a significant shift of enantioselectivity toward the (*R*)-configuration with high optical purity and yield. The unexpectedly reversed stereoselectivity of the reaction as a result of changing the cofactor specificity may be due to structural changes in the active site. Therefore, we have successfully changed the coenzyme specificity of the SCR as well as product enantioselectivity in the direction of 2-hydroxyacetophenone reduction without disturbing the secondary structure and stability of the enzyme. The study provides a new example to modify coenzyme specificity and alter product enantioselectivity in SDRs by using the protein engineering approach, and this will have valuable industrial applications.

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