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PAPER

Novel anti-Prelog stereospecific carbonyl reductases from *Candida parapsilosis* for asymmetric reduction of prochiral ketones†

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The application of biocatalysis to the synthesis of chiral molecules is one of the greenest technologies for the replacement of chemical routes due to its environmentally benign reaction conditions and unparalleled chemo-, regio- and stereoselectivities. We have been interested in searching for carbonyl reductase enzymes and assessing their substrate specificity and stereoselectivity. We now report a gene cluster identified in *Candida parapsilosis* that consists of four open reading frames including three putative stereospecific carbonyl reductases (*scr1*, *scr2*, and *scr3*) and an alcohol dehydrogenase (*cpadh*). These newly identified three stereospecific carbonyl reductases (SCRs) showed high catalytic activities for producing (*S*)-1-phenyl-1,2-ethanediol from 2-hydroxyacetophenone with NADPH as the coenzyme. Together with CPADH, all four enzymes from this cluster are carbonyl reductases with novel anti-Prelog stereoselectivity. SCR1 and SCR3 exhibited distinct specificities to acetophenone derivatives and chloro-substituted 2-hydroxyacetophenones, and especially very high activities towards ethyl 4-chloro-3-oxobutyrate, a β-ketoester with important pharmaceutical potential. Our study also showed that genomic mining is a powerful tool for the discovery of new enzymes.

Introduction

The NAD(P)H-dependent carbonyl reductases catalyze reduction of a variety of endogenous and xenobiotic carbonyl compounds, including biologically and pharmacologically active substrates.[1] There is considerable interest in the use of carbonyl reductases in the pharmaceutical and fine chemicals industries for the production of chiral alcohols, important building blocks for the synthesis of chirally pure pharmaceutical agents.[2] For the production of chiral auxiliaries from their corresponding prochiral ketones, carbonyl reductases have inherent advantages over chemo-catalysts in terms of their highly chemo-, enantio-, and regioselectivities. These features make stereospecific carbonyl reductases very interesting from both scientific and industrial perspectives.[3]

Stereospecific carbonyl reductases are ubiquitous in nature and have been characterized from diverse sources including bacteria,[4] yeasts,[5] plants,[6] and tissues from several mammalian species.[7] However, despite the diversity of stereospecific oxidoreductases,[8] their range of applications remains modest. This situation may be attributed to several perceived limitations including the stereospecificity and availability of the enzymes. In addition, research into the molecular mechanisms of oxidoreductases is still in its infancy. So far, most enzymes catalyzing asymmetric reductions generally follow the Prelog's rule in terms of stereochemical outcomes,[9] while enzymes with anti-Prelog stereospecificity, such as *Pseudomonas* sp. ADH and *Lactobacillus kefir* ADH, are still limited among the carbonyl reductases classified to four stereochemical patterns concerning hydride transfer from reduced cofactor (NAD(P)H) to ketone.[10] In particular, the precise mechanism of enzymatic anti-Prelog stereo-preference in asymmetric reduction is not yet fully understood.

Although most oxidoreductases possessing anti-Prelog selectivity have been identified in bacteria, such as *Geotrichum* sp.,[11] *Lactobacillus brevis*,[12] *Lactobacillus kefir*,[13] and *Pseudomonas* sp.,[14] *Candida* yeast species are attractive as sources of highly-stereospecific oxidoreductases.[15] In a previous study, an NADPH-dependent alcohol dehydrogenase of anti-Prelog type has been discovered from *C. parapsilosis* that catalyzes asymmetric reduction of 2-hydroxyacetophenone into (*S*)-1-phenyl-1,2-ethanediol (PED),[16,17] a versatile chiral building block for the synthesis of pharmaceuticals, agrochemicals, and liquid crystals. PED is also a precursor for the production of chiral biphosphines and a chiral initiator for stereoselective polymerization.[18]

As a result of recent advances in genomics, proteomics, and bioinformatics, the area of biocatalysis has developed

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significantly, and the availability of genome sequences from large numbers of microorganisms allows scientists to discover novel enzymes with potential applications.[19] The genome sequence of *C. parapsilosis* has been completed recently by the Wellcome Trust Sanger Institute Pathogen Genomics group (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/), which provided us with an opportunity to look into the genome of *C. parapsilosis* in extensive detail. We have identified three open reading frames (ORFs) in the 960-kb contig005802 of *C. parapsilosis* coding for putative stereospecific carbonyl reductase genes *scr1*, *scr2*, and *scr3*, respectively. These ORFs have been cloned and expressed, and the encoded proteins were purified to homogeneity and their functions were confirmed as stereospecific carbonyl reductases (SCR1, SCR2, and SCR3), with anti-Prelog selectivity that converts 2-hydroxyacetophenone to (*S*)-PED. These oxidoreductases have unique specificities that are useful for fine biochemical synthesis.

Results and discussion

Identification of putative stereospecific carbonyl reductases-encoding genes

Bioinformatic analysis based on sequence-similarity with CPADH, a known stereospecific alcohol dehydrogenase,[17] in the *C. parapsilosis* genome revealed additional homologous ORFs, named here as *scr1*, *scr2*, and *scr3* coding for putative stereospecific carbonyl reductases (SCRs). As shown in Fig. 1, these three ORFs, as well as the *cpadh* gene, are located in the 960-kb contig005802 of

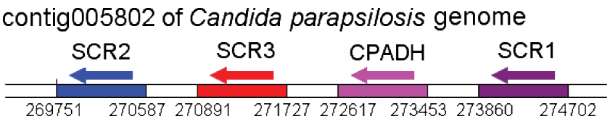


Fig. 1 Map of contig005802 of *Candida parapsilosis* genome including the four open reading frames, *scr1*, *scr2*, *scr3*, and *cpadh*.

the *C. parapsilosis* genome. The *scr1*, *scr2*, *and scr3* genes comprise 846, 840, and 840 bp, encoding polypeptides of 281, 279, and 279 amino acid residues with the calculated molecular masses of 30 061, 29 993, and 30 097 Da, respectively, and thus there are no introns found in these ORFs. Multiple sequence alignment of these four ORFs (Fig. 2) revealed high sequence identity between CPADH and SCR1 (68%), SCR2 (88%), and SCR3 (84%). From the amino acid sequence and secondary structure prediction, the three putative enzymes exhibit a classic α/β Rossmann-fold structure, the cofactor-binding motif, Gly^{43}-X-X-X-Gly^{47}-X-Gly^{49} in SCR1 and Gly^{41}-X-X-X-Gly^{45}-X-Gly^{47} in SCR2 and SCR3, and the catalytic triad, Ser^{174}-Tyr^{189}-Lys^{193} in SCR1 and Ser^{172}-Tyr^{187}-Lys^{191} in SCR2 and SCR3.[20]

To gain insights into the proposed mechanism of cofactor binding, structure models of SCRs were obtained by homology modeling based on the X-ray structure of CPADH (PDB code: 3ctm). Because of the high degree of sequence identity, it can be expected that these four proteins including CPADH and SCRs are very similar in overall conformation, except for some loop regions due to amino-acid sequence difference. Thus, the cofactor-binding domain is expected to fold into the classic Rossmann-fold structure, a seven-strand parallel β-sheet flanked on both sides

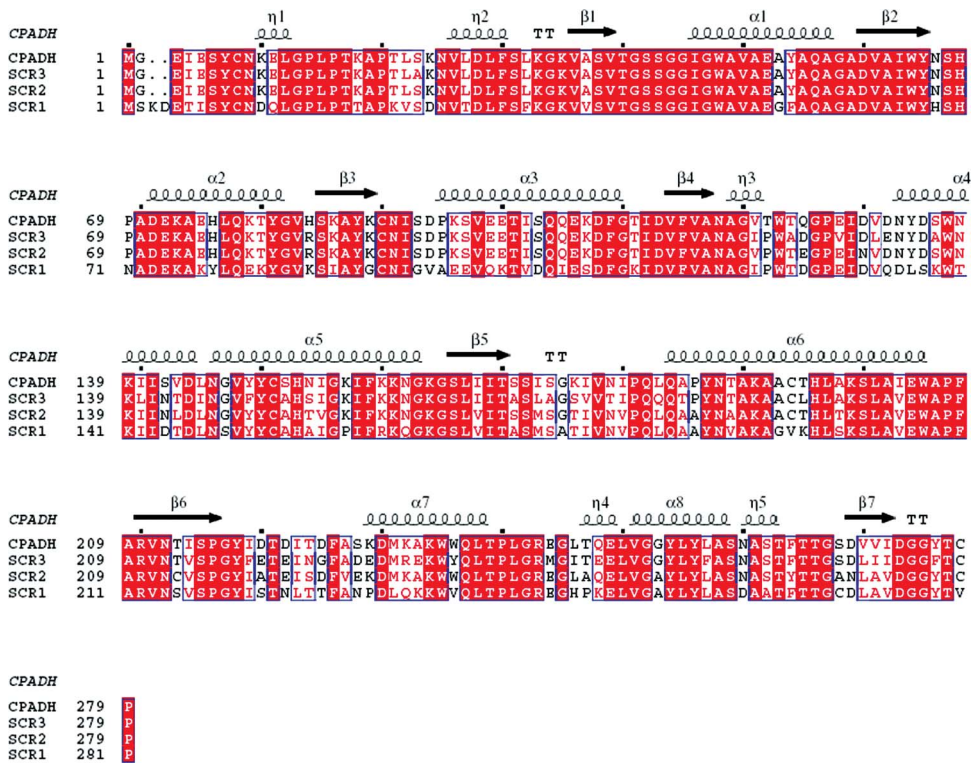


Fig. 2 Amino acid sequence alignment of CPADH (GenBank accession number DQ675534), SCR1 (GenBank accession number FJ939565), SCR2 (GenBank accession number FJ939563), and SCR3 (GenBank accession number FJ939564) from *C. parapsilosis*. Gaps in the aligned sequences are indicated by dashes. Identical amino acid residues are enclosed in boxes.

by α-helices.[21] Based on the highly conserved cofactor-binding domain, putative enzyme-cofactor docking was performed with NADPH and NADH, respectively (Fig. 3). In both lowest-energy conformational ensembles, structure comparison with different cofactors suggested that the steric conformation in the cofactor-binding domain is more favorable to NADPH.

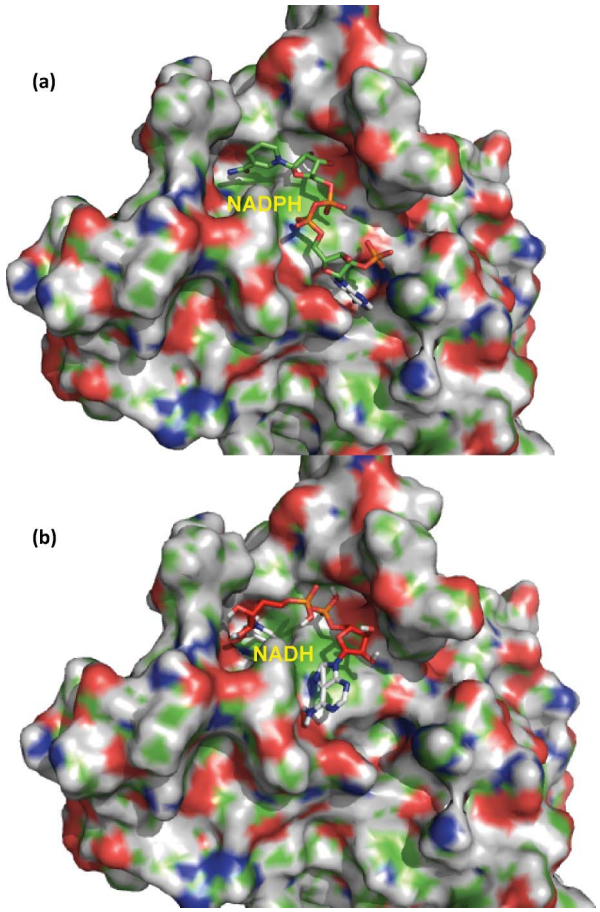


Fig. 3 Stereoview of the overall model structure of enzyme–cofactor docking with NADPH (a) and NADH (b), respectively, illustrated by the PyMOL program.

Additionally the positively charged residue His (His[70] in SCR1, His[68] in SCR2 and SCR3) is expected to play a role in the affinity of the enzyme for NADPH, rather than NADH, by forming a salt-bridge with the phosphate moiety at the 2′-position of AMP.[22]

According to the catalytic properties and primary structure information, stereospecific oxidoreductases including alcohol dehydrogenases and carbonyl reductases are mainly classified into three different groups: the zinc-dependent alcohol dehydrogenase, the short-chain dehydrogenase/reductase (SDR), and the aldo-keto reductase (AKR).[8] All four proteins share sequence motifs characteristic of the SDR superfamily, including the cofactor-binding motif Gly-x-x-x-Gly-x-Gly (x denotes any amino acid) and the catalytic triad of Ser-Tyr-Lys, and further the extended tetrad of Asn-Ser-Tyr-Lys observed in the majority of SDRs.[23] In addition, the SCRs also have the conserved sequence motifs of secondary structure elements and key positions for assignment of coenzyme specificity of the cP2 subfamily in classic SDRs, except that the conserved basic residue Lys/Arg responsible for binding phosphate group in NADPH is replaced by weak basic residue His,[24] indicating that the cofactor dependence of the putative carbonyl reductases might be not rigidly restricted to NADPH and these enzymes still show somewhat low activity with NADH. These highly-conserved characteristic sequence motifs suggested that the SCRs belong to the cP2 subfamily of the classical SDR superfamily, one of the three NADPH-dependent subfamilies.[24]

Cloning, expression, and purification of SCRs

The sequence and structure information involving the overall protein structure analysis and the conserved SDR structural characteristics revealed that the identified genes and *cpadh* show the absence of introns and the encoded proteins are all not glycoproteins. We then attempted to express these genes in the *Escherichia coli* system. From the nucleotide sequence of ORF, the *scr1*, *scr2*, and *scr3* were amplified by PCR from genomic DNA of *C. parapsilosis* CCTCC M203011, and the PCR products were inserted into pET21c vector by ligation-independent cloning to construct the recombinant plasmids. These three plasmids, pET21-SCR1, pET21-SCR2, and pET21-SCR3, were then transformed into expression host *E. coli* BL21(DE3) pMgK cells, and recombinant SCR1, SCR2, and SCR3 were produced in *E. coli* as fusion proteins containing a C-terminal His_6 tag. All three recombinant enzymes were expressed at very high levels. Of them, SCR1 and SCR3 were expressed as soluble forms at yields of 50 mg L^{-1} broth and 46 mg L^{-1} broth, respectively, while SCR2 has relatively low solubility with a yield of 5 mg L^{-1} broth (Fig. 4).

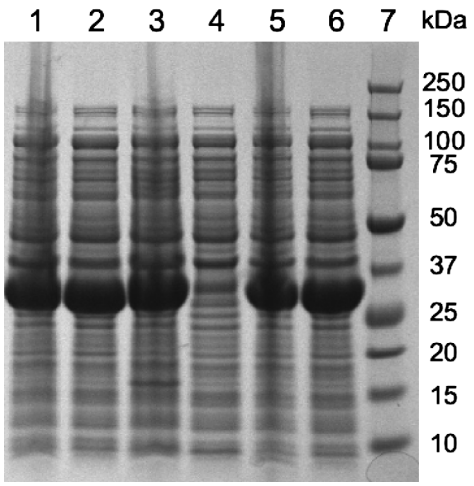


Fig. 4 Analysis of the overexpression of SCR1, SCR2, and SCR3. The proteins were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. Lane 1, total protein for SCR1; Lane 2, soluble fraction for SCR1; Lane 3, total protein for SCR2; Lane 4, soluble fraction for SCR2; Lane 5, total protein for SCR3; Lane 6, soluble fraction for SCR3; Lane 7, molecular mass standard.

The three recombinant enzymes were purified to homogeneity as judged by Coomassie Brilliant Blue staining of SDS-PAGE (Fig. 5) by Ni affinity purification followed by gel filtration chromatography. The relative molecular masses of the SCR1 and SCR3 were estimated to be 124.6 kDa and 123.4 kDa by analytical gel filtration and static light scattering using the same low salt buffer,[25,26] but SCR2 was detected as aggregated form. Since the

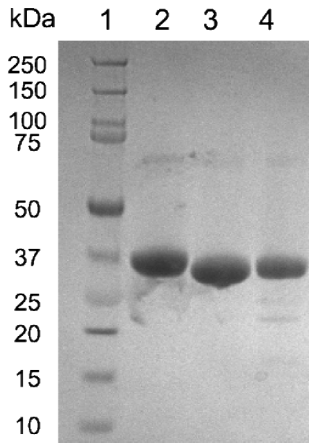


Fig. 5 SDS-PAGE analysis of purified enzymes. The purified proteins were resolved by SDS-PAGE on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. Lane 1, molecular mass standard; Lane 2, purified SCR1; Lane 3, purified SCR2; Lane 4, purified SCR3.

relative molecular mass of the monomer of the recombinant enzymes should be around 30 kDa based on their amino acid composition, these results suggested that both SCR1 and SCR3 have tetrameric structures.

Catalytic properties of recombinant SCRs

Because SCR1, SCR2, and SCR3 showed high homology to CPADH, which exhibits catalytic activity towards 2-hydroxyacetophenone,[17] the enzymatic activities of these three SCRs were investigated for reduction of 2-hydroxyacetophenone. Under the assay conditions, SCR1 gave the highest specific activity of 5.16 U mg^{-1}, and SCR3 had catalytic activity of 4.23 U mg^{-1}, while SCR2 had a lower specific activity of 1.55 U mg^{-1}. In addition, corresponding to the results from the cofactor-binding model and secondary structure prediction and analysis, the SCRs all displayed catalytic activity with NADPH as the coenzyme, but very low activities with NADH, indicating that these three enzymes, like CPADH,[17] are all NADPH-dependent oxidoreductases.

Since environmental pH value can have an influence on the stereochemistry of enzymatic reactions,[27] the effect of the reaction pH on the activities of SCRs catalyzing 2-hydroxyacetophenone reduction was also investigated. All three enzymes exhibited the highest activity at pHs ranging from 5.0 to 6.0 (Fig. 6). Subsequently, the enzymes were evaluated under their individual optimal pH, and apparent kinetic parameters were further measured by double reciprocal Lineweaver–Burk plots at various 2-hydroxyacetophenone concentrations with fixed NADPH concentrations. As shown in Table 1, for reduction of 2-hydroxyacetophenone, these three enzymes exhibited different kinetic parameters.

Stereoselectivity to prochiral carbonyl group

Using 2-hydroxyacetophenone as the substrate, optically pure 1-phenyl-1,2-ethanediol (PED) as (*S*)-enantiomer (>99% e.e.) was produced by each of SCR1, SCR2, and SCR3, respectively (Fig. 7). These three enzymes are capable of catalyzing asymmetric reduction of prochiral carbonyl compounds and are all (*S*)-specific

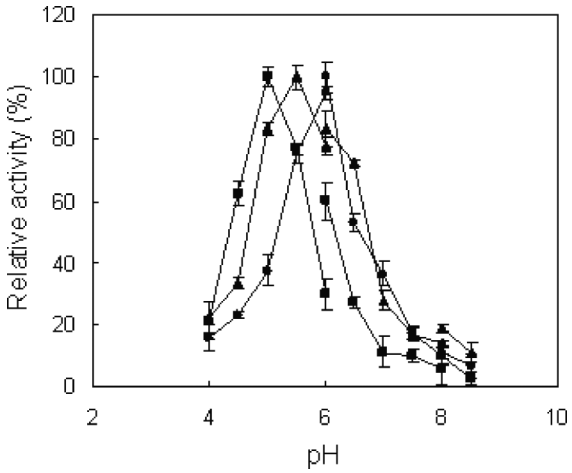


Fig. 6 pH dependence of SCR1, SCR2, and SCR3 catalyzing 2-hydroxyacetophenone reduction. The enzyme activities of SCR1 (■), SCR2 (▲), and SCR3 (●) were measured in 0.1 M acetate buffer (pH 4.0 to 6.0) or 0.1 M sodium phosphate buffer (pH 6.0 to 8.0) or 0.1 M Tris-HCl buffer (pH 8.0 to 8.5) with 2-hydroxyacetophenone as the substrate and NADPH as the cofactor. Maximal enzyme activity observed was set as 100% relative activity for each enzyme.

Table 1 Activities and kinetic parameters for reduction of 2-hydroxyacetophenone (2-HAP) by SCR1, SCR2, SCR3, and CPADH, using NADPH as cofactor

Enzyme	pH[a]	Specific activity / U mg^{-1}	K_m /mM	V_{max}/μmol min^{-1} mg^{-1}	K_{cat}/s^{-1}
SCR1	5.0	5.16	9.83	42.0	21.04
SCR2	5.5	1.55	4.81	8.83	4.41
SCR3	6.0	4.23	4.68	32.3	16.20
CPADH	4.5	2.70	5.83	18.3	9.18

[a] Activity assay was carried out at the optimum pH for each enzyme

carbonyl reductases towards 2-hydroxyacetophenone. Of them, however, SCR2 is not as efficient as the other two enzymes, corresponding to its lower activity. Like the homologous anti-Prelog specific alcohol dehydrogenase, CPADH, these data demonstrate that the analogous SCR1, SCR2, and SCR3 enzymes are anti-Prelog-type stereospecific carbonyl reductases.[17,28]

Oxidoreductases perform a wide variety of asymmetric reductions, differing in stereospecificity and substrate specificity, and have been used for producing optically active alcohols from various prochiral ketones, ketoacids, and ketoesters. The SCRs catalyze (*S*)-specific reduction of 2-hydroxyacetophenone, an anti-Prelog type reaction.[28] Therefore, these new enzymes complement the stereospecific oxidoreductases described to date for catalysis of the reduction of prochiral carbonyl compounds to the corresponding optically pure alcohols with anti-Prelog stereopreference.

In addition, the finding of stereospecific carbonyl reductases capable of catalyzing (*S*)-specific reduction of 2-hydroxyacetophenone from the same host would give us a profound knowledge of the reaction mechanism of *C. parapsilosis* whole-cell mediated stereoinversion, which involves two steps, the oxidation step of (*R*)-PED to the intermediate (2-hydroxyacetophenone) and the reduction step of the intermediate to (*S*)-PED.[16] The deracemization of racemic PED to the (*S*)-enantiomer by

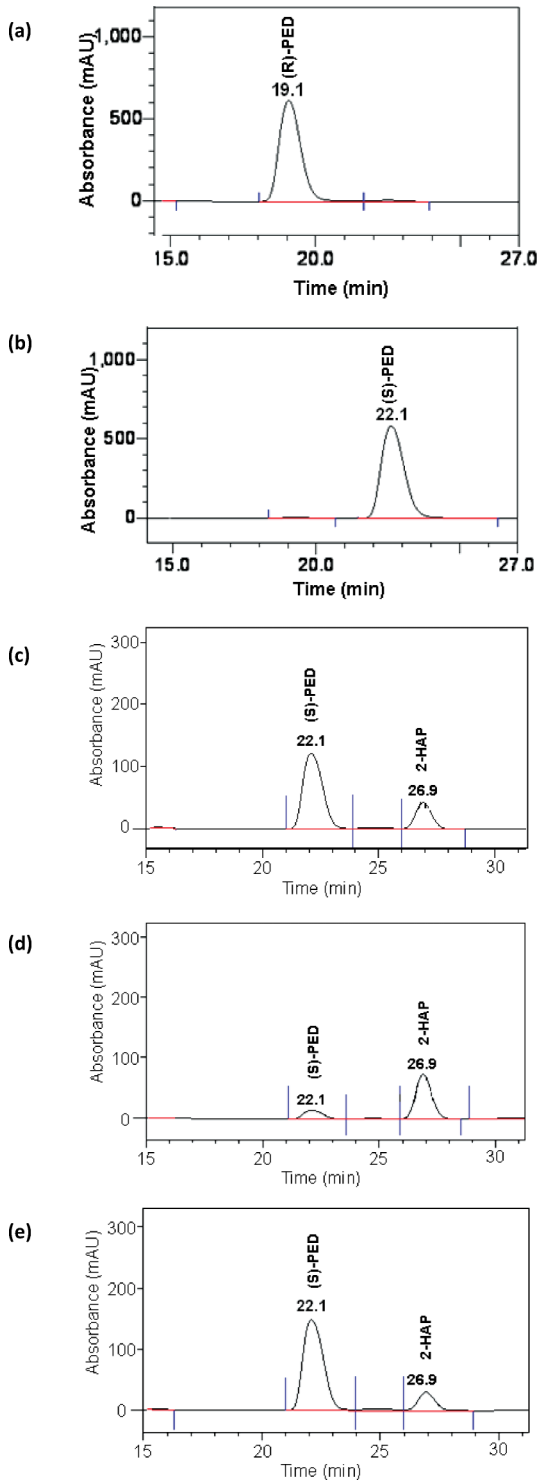
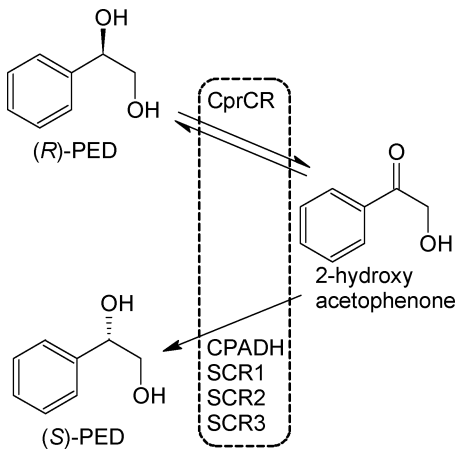


Fig. 7 Asymmetric reduction of 2-hydroxyacetophenone (2-HAP) to 1-phenyl-1,2-ethanediol (PED) enantiomer by SCR1, SCR2, and SCR3, respectively. (**a**) Standard sample of (*R*)-PED. (**b**) Standard sample of (*S*)-PED. (**c**) SCR1 catalyzed asymmetric reduction of 2-HAP. (**d**) SCR2 catalyzed asymmetric reduction of 2-HAP. (**e**) SCR3 catalyzed asymmetric reduction of 2-HAP.

C. parapsilosis whole cells should be considered as a one-pot concurrent tandem process involving at least four stereoselective alcohol dehydrogenases and carbonyl reductases, not just a single alcohol oxidase and a single carbonyl reductase (Scheme 1).[17,29] In addition, the contribution of newly discovered SCRs to the whole-cell mediated PED stereoinversion can be further proved by comparing the kinetic parameters such as the maximum initial reaction rates and Michaelis constants between the two steps involved in the one-pot redox reaction, in which the maximum initial reaction rate of reduction is almost 10 times higher than the oxidation reaction.[30] Thus, the reduction step catalyzed by CPADH and SCRs would drive the whole reaction process to the product formation and the oxidation step is the restrictive step in the sequential two-step reaction.

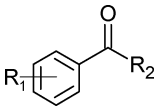
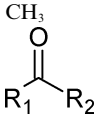
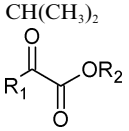
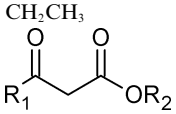


Scheme 1 One-pot concurrent tandem stereoinversion of 1-phenyl-1,2-ethanediol by *Candida parapsilosis* involving CPADH and SCRs.

Substrate specificity

Since the three new enzymes showed distinct (*S*)-specific carbonyl-reducing activity, we then further examined the substrate specificity of these enzymes towards various carbonyl compounds including aryl ketones, aliphatic ketones, α- and β-ketoesters. As shown in Table 2, on the one hand, the enzymes all exhibited higher catalytic activity towards ketoesters than towards alkyl and aromatic ketones, while compared with the other two enzymes, SCR2 generally showed lower activities towards keto substrates, corresponding to the observation in activity and kinetics evaluation of these enzymes catalyzing 2-hydroxyacetophenone reduction; on the other hand, the enzymes exhibited diversity in specificity towards aryl ketones and ketoesters, respectively. For both substituted acetophenone and 2-hydroxyacetophenone derivatives, bearing chloro or methyl at various positions of the phenyl ring, the *ortho* substituted derivatives were poor substrates for these enzymes, indicating that the substitution at *ortho* position might have a steric effect on the hydrogen attack from electron donator NADPH to the carbonyl group and therefore significantly influence the reactivity of the enzymes. However, SCR3 was more specific to *p*-Cl-2-hydroxyacetophenone, while SCR1 had higher activity towards *m*-Cl-2-hydroxyacetophenone. Concerning the aryl ketones, despite its low activities towards the substrates, SCR2 was more active towards acetophenone and its derivatives than towards 2-hydroxyacetophenone, which would be a trend observed different from SCR1 and SCR3. For ketoesters, the enzymes exhibited high activity towards those with small groups, but compared with SCR3, SCR1 was more active towards bulky ketoesters with

Table 2 Substrate specificity and stereoselectivity of SCR1, SCR2, and SCR3 towards various carbonyl compounds

	Substrate		Relative activity[a]/%			
	R_1	R_2	SCR1	SCR2	SCR3	Product configuration
	Aryl ketones					
1	H	CH_3	1.27	3.36	2.57	*R*
2	H	CH_2OH	2.84	0.78	3.08	*S*
3	2′-Cl	CH_2OH	0.60	1.74	0.19	*S*
4	3′-Cl	CH_2OH	14.61	2.21	5.91	*S*
5	4′-Cl	CH_2OH	2.96	2.97	18.49	*S*
6	4′-OCH_3	CH_2OH	—	—	—	—
7	H	CH_2CH_3	6.31	0.32	2.08	*R*
8	H	$(CH_2)_2CH_3$	10.90	0.16	0.81	*R*
9	H	$(CH_2)_3CH_3$	1.96	—	0.93	*R*
10	H	$(CH_2)_4CH_3$	—	—	—	—
11	2′-CH_3	CH_3	1.65	0.42	0.12	*R*
12	3′-CH_3	CH_3	0.05	0.57	2.50	*R*
13	4′-CH_3	CH_3	1.63	0.51	7.29	*R*
14	2′-Cl	CH_3	1.88	1.21	0.36	*R*
15	3′-Cl	CH_3	0.88	1.68	2.33	*R*
16	4′-Br	CH_3	2.02	3.87	6.54	*R*
17	4′-OCH_3	CH_3	—	—	—	—
	Aliphatic ketones					
18	CH_3	CH_2CH_3	0.93	—	0.91	—
19	CH_3	$(CH_2)_2CH_3$	0.65	—	0.10	—
20	CH_3	$(CH_2)_3CH_3$	0.86	0.83	0.40	*R*
21	CH_3	$(CH_2)_4CH_3$	0.15	0.52	0.28	*R*
22	CH_3	$(CH_2)_5CH_3$	1.39	—	0.52	*R*
23	CH_2CH_3	$CH(CH_3)_2$	0.37	—	0.82	—
	α-Ketoesters					
24	CH_3	CH_3	67.12	18.92	74.12	*R*
25	Phenyl	CH_3	32.93	5.76	3.82	*S*
26	CH_3	CH_2CH_3	65.32	19.89	80.72	*R*
27	Phenyl	CH_2CH_3	19.15	2.78	0.19	*S*
	β-Ketoesters					
28	CF_3	CH_2CH_3	29.51	15.24	11.86	*S*
29	CH_3	CH_3	29.31	9.88	4.96	*R*
30	CH_2CH_3	CH_3	9.44	7.82	5.05	*R*
31	4-Fluorophenyl	CH_3	14.12	5.62	3.89	*S*
32	CH_3	CH_2CH_3	45.43	8.17	21.31	*R*
33	CH_2CH_3	CH_2CH_3	8.79	7.16	20.85	*R*
34	Chloromethyl	CH_2CH_3	100.00	17.58	53.20	*S*
35	Phenyl	CH_2CH_3	7.01	—	0.22	*S*
36	3,4-Dimethoxyphenyl	CH_2CH_3	4.44	—	0.47	*S*

[a] The enzyme activities of SCRs towards various substrates and the configuration of produced alcohols were measured as described in the text. Maximal enzyme activity observed was set as 100% relative activity for the enzymes towards various substrates.

phenyl ring and generally showed higher activities towards β-ketoesters. For stereoselective reduction of carbonyl compounds with different chemical structures, these three enzymes followed the anti-Prelog rule for their behaviors in catalyzing asymmetric reduction of 2-hydroxyacetophenone, although chiral alcohol enantiomers were produced in different absolute configurations, affording aryl diols, phenylhydroxyl esters, ethyl 4-trifluoro-3-hydroxybutyrate, and ethyl 4-chloro-3-hydroxybutyrate in (*S*)-configuration, whereas (*R*)-antipode for others due to the switch in CIP priority.

Among the tested β-ketoesters, additionally, SCR1 produced the highest activity towards ethyl 4-chloro-3-oxobutyrate, an

important chiral building block. The enzyme–cofactor–substrate docking model, as shown in Fig. 8, revealed that, in the lowest-energy conformational ensemble, the substrate-binding domain is favorable to this kind of substrate, and the carbonyl carbon is in a position proximal to the C4 atom of the nicotinamide ring of NADPH. These results suggested that, for enzymatic reactions, the enzyme structure generally has a significant influence on the enzyme activity and affinity towards substrates. The steric conformation of the active site in the catalytic domain determines the substrate recognition, binding, and orientation in the enzyme.[31]

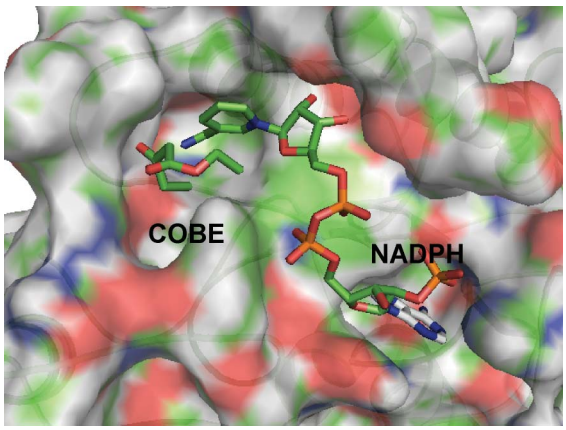


Fig. 8 Stereoview of the model structure of SCR1–NADPH–ethyl 4-chloro-3-oxobutyrate (COBE) docking, illustrated by the PyMOL program.

It is worth noting that, as shown above, SCR1 catalyzes the reduction of a broad spectrum of ketones including aryl ketones, aliphatic ketones, and α- and β-ketoesters, and shows a particular substrate specificity towards ethyl 4-chloro-3-oxobutyrate, a precursor for the synthesis of ethyl 4-chloro-3-hydroxybutyrate which is an important pharmaceutical intermediate.[32] Therefore, the newly discovered stereospecific carbonyl reductases should be useful enzymes with potential applications.

Experimental

Materials

C. parapsilosis strain CCTCC M203011 was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). *E. coli* XL-10 gold cells were used for gene cloning and plasmid preparation, and *E. coli* BL21 (DE3) pMgK competent cells, a rare codon-enhanced strain, were used for gene expression. High-fidelity PCR kit including DNA polymerase was purchased from FINNZYMES (Finland). The plasmid pET21c was obtained from Novagen (USA). (*R*)- and (*S*)-1-phenyl-1,2-ethanediol, all the aliphatic ketones and ketone esters, aryl ketones including acetophenone and its derivatives, 2-hydroxyacetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, and coenzymes including NAD(P)H and $NAD(P)^+$ were purchased from Sigma-Aldrich (USA). All other 2-hydroxyacetophenone derivatives including *o*-Cl-2-hydroxyacetophenone, *m*-Cl-2-hydroxyacetophenone, *p*-Cl-2-hydroxyacetophenone, and *p*-CH_3O-2-hydroxyacetophenone were prepared using the method described by Itsuno.[33] All other chemicals used in this work were of analytical grade and commercially available.

Cloning and expression of genes encoding stereospecific reductases

The genes encoding SCR1, SCR2, and SCR3 were amplified by polymerase chain reaction from *C. parapsilosis* genomic DNA. PCR-amplified DNA products were purified by QIAquick PCR Purification Kit (QIAGEN, USA) and inserted into pET21c expression vector (Novagen, USA) by ligation-independent cloning (LIC) using In-Fusion PCR Cloning Kit (Clontech, USA) for construction of recombinant plasmids. The infusion reaction mixtures were used to transform *E. coli* XL-10 gold cells. The plasmids isolated from these transformants were verified by DNA sequence analysis using BigDye Terminator cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The plasmids with the correct inserts, pET21-SCR1, pET21-SCR2, and pET21-SCR3, were transformed into *E. coli* BL21(DE3) pMgK competent cells for the production of SCRs. These plasmids provide SCRs with a six-His tag fused at the C-terminus.

E. coli BL21 (DE3) pMgK transformants were cultivated at 37 °C in Luria broth (LB) medium in the presence of ampicillin (100 μg mL^{-1}) and kanamycin (50 μg mL^{-1}). When the optical density of the culture at 600 nm reached 0.6, the temperature was changed to 17 °C and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to give a final concentration of 1 mM for induction of gene expression. After an additional incubation of 20 h at 17 °C, cells harvested by centrifugation were disrupted by sonication, and expressions of the recombinant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For expression of SCR2, expression conditions involving a lower concentration of IPTG (0.1 mM) and a shorter induction time (12 h) were adopted, besides the above standard expression conditions.

Purification of recombinant enzymes

The cells were suspended in binding buffer (20 mM Tris-HCl, pH7.5, 0.3 M NaCl, 40 mM imidazole, 1 × protease inhibitors, 1 mM Tris (2-carboxyethyl) phosphine (TCEP)) and disrupted on ice by sonication. The supernatant of the cell lysate was collected by centrifugation at 26 000 × *g* for 40 min at 4 °C and purified by an AKTAxpress system using HisTrap HP affinity column followed by Superdex 75 gel filtration column (GE Healthcare, USA), and the purified fractions were exchanged into low salt buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.02% NaN_3, 5 mM D,L-dithiothreitol).[25,26] The final recombinant enzymes were purified with an apparent homogeneity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gels. Their molecular masses were measured by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystems, USA), and their oligomerization states were determined by analytical gel filtration using Agilent 1200 series HPLC system followed by static light scattering (Wyatt Technology, USA). These final preparations of purified SCRs were used in all of the experiments in this study.

Enzyme assays

Carbonyl reductase activity was measured by a continuous spectrophotometric assay using 2-hydroxyacetophenone as a substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NAD(P)H per min under the assay conditions. The standard assay mixture for the enzyme activity comprised of 0.1 M potassium phosphate buffer (pH 6.5), 0.3 mM NAD(P)H, 0.7 mM 2-hydroxyacetophenone and the appropriate enzyme in a total volume of 100 μL. The decrease in the amount of the coenzyme was measured spectrophotometrically at 340 nm (extinction coefficient [ε] = 6.22 mM^{-1} cm^{-1}). Protein concentration was determined using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard. The pH dependence of the enzyme activity was determined over a pH range of 4.0 to 8.5 using the following buffers: 0.1 M acetate (pH 4.0 to 6.0), 0.1 M sodium phosphate (pH 6.0 to 8.0) and 0.1 M Tris-HCl (pH 8.0 to 8.5).

The substrate specificity of the SCRs was investigated under the same conditions as described above. Various carbonyl compounds including aryl ketones, aliphatic ketones, α- and β-ketoesters were used as the substrates with the cofactor of NADPH.

Asymmetric reduction and stereoselectivity assay

Asymmetric reduction of 2-hydroxyacetophenone by the purified enzymes was carried out at 30 °C for 6 h with shaking in a reaction mixture comprising 0.1 M potassium phosphate buffer (pH 6.5), 1 g L^{-1} 2-hydroxyacetophenone, NADPH (7 mM) and 0.5 mg of the purified enzyme in a total volume of 0.5 mL. The reaction products were extracted with ethyl acetate and the organic layer was used for analysis. The optical purity of the reaction products were analyzed by HPLC using a Chiralcel OB–H column (4.6 × 250 mm, Daicel Chemical Ind., Ltd., Japan). Enantiomers were eluted with hexane and 2-propanol (9 : 1) at a flow rate of 0.5 mL min^{-1}. The effluent was monitored at 215 nm, and the areas under each peak were integrated.[16] To further confirm the product of 1-phenyl-1,2-ethanediol but not byproducts of the reduction, the reaction product was analyzed by ^{1}H-NMR (for assay details see the ESI†).

Asymmetric reductions of various carbonyl compounds, including aryl ketones, aliphatic ketones, α- and β-ketoesters, by the enzymes were carried out at 30 °C for 6 h with shaking in a reaction mixture comprising 0.1 M potassium phosphate buffer (pH 6.5), NADPH (7 mM) and 0.5 mg of the purified enzyme in a total volume of 0.5 mL. The reaction products were extracted with ethyl acetate or hexane and the organic layer was used for analysis. The reaction products were analyzed by chiral HPLC (HP 1100, Agilent, USA) equipped with Chiralcel OB–H column (4.6 mm × 250 mm; Daicel Chemical Ind. Ltd., Japan) or chiral GC (7890A, Agilent, USA) equipped with FID detector and Chrompack Chirasil-Dex CB chiral capillary column (25 m × 0.25 mm; Varian, USA) (for assay details see the ESI†).

Homology modeling, alignment, and flexible docking

The crystal structure of CPADH (PDB code: 3ctm) was used. The homology modeling of SCRs was carried out by HOMA (Homology Modeling Automatically) (http://www-nmr.cabm.rutgers.edu/HOMA/). The structure alignment of CPADH and SCRs was performed with the Deep View Swiss-Pdb Viewer 4.0 program. All docking calculations were accomplished with AutoDock Vina 1.0.[34] A docking algorithm that took account of ligand flexibility but kept the protein rigid was employed. Docking runs were carried out using the standard parameters of the program for interactive growing and subsequent scoring (for settings of grid box dimensions and centers see the ESI†).

Nucleotide sequence accession number

The nucleotide sequence for the stereospecific carbonyl reductase genes *scr1*, *scr2*, *and scr3* have been deposited in the GenBank database under accession numbers FJ939565, FJ939563, and FJ939564, respectively.

Conclusion

In this work, we described a new gene cluster of enantioselective oxidoreductases with unusual stereospecificity in *C. parapsilosis*. We confirmed that these genes encode three unique stereospecific carbonyl reductases through cloning, expression, purification, and characterization of the corresponding gene products. We verified the enantiomer configuration of the enzymatic products from asymmetric reduction of prochiral carbonyl groups using multiple substrates. SCR1, SCR2, and SCR3, like CPADH, all exhibit a novel anti-Prelog stereospecificity in reducing prochiral carbonyl groups such as forming (*S*)-1-phenyl-1,2-ethanediol from the corresponding ketone substrate, 2-hydroxyacetophenone. The enzymes are, however, distinct in their catalytic properties, including their pH dependency and substrate specificity spectrum. The genes encoding SCR2, SCR3, CPADH, and SCR1 are sequential in the cluster (Fig. 1) with intervals of several hundred base pairs. Further studies are necessary to understand the metabolic roles of these homologues and potential synergies among them.

The discovery of novel stereospecific carbonyl reductases of anti-Prelog selectivity further demonstrates the diversity of stereospecific oxidoreductases in microorganisms. Such enzymes provide a basis for elucidating the molecular mechanism of enzyme-mediated asymmetric reactions involving stereo-recognition between protein and chiral molecule, and the mechanism of electron transfer between functional groups of chiral molecule and key amino acid residues in the enzyme. Apart from their unique value for study of the mechanism of stereospecific oxidoreduction reaction, these novel carbonyl reductases with anti-Prelog stereopreference discovered by genomic mining have much potential to generate chiral alcohols useful as intermediates in fine chemical synthesis.

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