論文の内容の要旨

論文題目

Structural Basis of the Different Substrate Preferences of Two Old Yellow Enzymes from Yeasts in Asymmetric Reduction of Enone Compounds (キラル化合物合成に応用可能な 2 種の旧黄色酵素の結晶構造解析とその異なる基質特異性の構造基盤) に関する研究

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Old yellow enzymes (OYEs) are a group of enzymes that catalyze the reduction of C=C bonds in α,β-unsaturated carbonyl compounds and/or C=C bonds in α,β-unsaturated nitro compounds by using flavin mononucleotide (FMN) as a cofactor. OYE was first isolated from the brewer bottom yeast, *Saccharomyces pastorianus*, in the 1930s, and since that time OYEs have been found in bacteria, yeasts and plants. A strictly conserved histidine/asparagine or histidine/histidine pair and a tyrosine residue are involved in the catalysis of the asymmetric reduction; a hydride derived from $FMNH_2$ is stereoselectively transferred to C^{β} of the bound α,β-unsaturated carbonyl compound, and a tyrosine residue adds a proton to C^{α} of the α,β-unsaturated carbonyl compound from the opposite side.

Some OYEs display excellent enantio-selectivity in the asymmetric reduction of the C=C bonds of α,β-unsaturated carbonyl compounds. For example, two yeast OYEs, CYE and TYE, isolated from the yeasts *Candida macedoniensis* AKU4588 and *Pichia* sp. AKU4542 (formerly, *Torulopsis* sp.), respectively, can be used to obtain an industrially important chiral compound, (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone [(4*R*,6*R*)-actinol], which is applicable for the synthesis of xanthoxin, zeaxanthin and related compounds. These OYEs can reduce 3,5,5-trimethyl-2-cyclohexene-1,4-dione [ketoisophorone] to yield (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione [(6*R*)-levodione] in an enantio-selective manner (Figure 1). In addition, TYE can reduce 4-hydroxy-2,6,6-trimethyl-2-cyclohexanone [(4*S*)-phorenol] to yield (4*R*,6*R*)-actinol, although CYE hardly reduces this substrate.

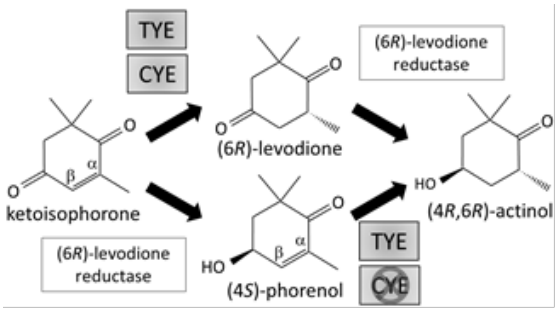

Figure 1. Synthesis of (4*R*,6*R*)-actinol from ketoisophorone

To reveal the structural basis for the different substrate preferences between CYE and TYE, we have solved the crystal structures of these OYEs in the presence or absence of a substrate analog, *p*-hydroxybenzaldehyde (*p*-HBA). We have revealed that Loop 5 (located between β_5 and α_5) and Loop 6 (located between β_6 and α_6) of CYE and TYE play pivotal roles in determining their substrate preferences and demonstrated that some artificial OYEs with mutation(s) in these loops can be applicable for the asymmetric reduction of various enone compounds.

Binding Model of (4*S*)-phorenol to CYE based on the crystal structures of CYE–*p*-HBA

To gain insights into the different substrate preferences between CYE, the binding model of (4*S*)-phorenol to CYE was built based on the crystal structures of the CYE–*p*-HBA complex (Figure 2). The binding model of (4*S*)-phorenol to CYE implies that the bulky dimethyl group at C6 of (4*S*)-phorenol would not be favored by CYE because the dimethyl group in (4*S*)-phorenol, and Phe^{250} (Loop 5) and Pro^{295} (Loop 6) in CYE would collide.

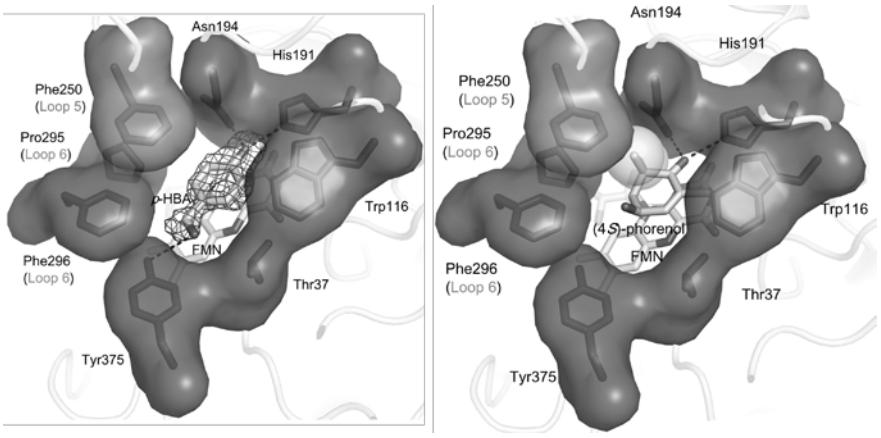

Figure 2. Left: crystal structure of CYE–*p*-HBA, Right: binding model of CYE–(4*S*)-phorenol.

The Pro^{295} in Loop 6 Plays Pivotal Roles in Substrate Preferences

. The putative binding model of (4*S*)-phorenol to CYE suggests that the dimethyl group at C6 may collide with Pro^{295} in Loop 6 and Phe^{250} on Loop 5 (Figure 2). To examine the effect of Pro^{295} on the substrate preference of CYE, CYE (P295G) was constructed to avoid the possible collision between the

dimethyl group in (4*S*)-phorenol and Pro^{295} in CYE. Furthermore, to investigate the effect of Phe^{296} on Loop 6 on the substrate preference of CYE, mutants of CYE (F296X) and CYE (P295G/F296X), where X represents one of 20 amino acid residues, were also constructed, and a series of mutational analyses were performed. However, no remarkable tendency was toward ketoisophorone and (4*S*)-phorenol. Each point mutant of CYE (P295G/F296X) exhibits an enhanced catalytic activity toward both ketoisophorone and (4*S*)-phorenol compared to the corresponding point mutant of CYE (F296X), demonstrating that Pro^{295} on Loop 6 of CYE does affect the catalytic activity of CYE toward ketoisophorone and (4*S*)-phorenol, probably via a collision with the dimethyl group of ketoisophorone and (4*S*)-phorenol. Thus, Pro^{295} on Loop 6 of CYE acts as a substrate filter. Phe^{296} does not have a significant effect on the catalytic activity when Pro^{295} is replaced with Gly^{295}.

To investigate further, a series of chimeric mutants of CYE were constructed and mutational analyses were performed. The mutants used were named as follows: CYE (CYE Loop 6→Gly$_n$) means the CYE mutant where the Loop 6 of CYE (^{289}-EPRVTDPFLPEFEKWFKEGT-^{308}) is replaced with a poly-glycine linker Gly$_n$, where n = 2–5. When Loop 6 of CYE is replaced with a poly-glycine linker Gly$_n$, where n = 2–5, CYE (CYE Loop 6→Gly-Gly), CYE (CYE Loop 6→Gly-Gly-Gly), CYE (CYE Loop 6→Gly-Gly-Gly-Gly) and CYE (CYE Loop 6→Gly-Gly-Gly-Gly-Gly) exhibit higher catalytic activity toward (4*S*)-phorenol (Figure 3). These results support the hypothesis that the Loop 6 plays a significant role in substrate preferences.

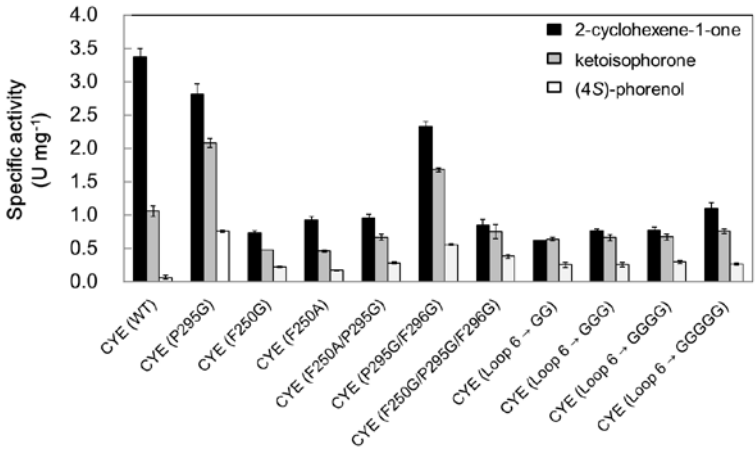

Figure 3. Specific activity of CYE mutants toward 2-cyclo-hexene-1-one, ketoisophorone and (4*S*)-phorenol.

The Gly^{250} in Loop 5 Plays Pivotal Roles in Substrate Preferences

The binding model of (4*S*)-phorenol to CYE implies that the bulky dimethyl group at C6 of (4*S*)-phorenol would not be favored by CYE because the dimethyl group in (4*S*)-phorenol, and Phe^{250} in CYE would collide in addition to Pro^{295} in Loop 6. To examine the effect of Phe^{250} on the substrate preferences of CYE, CYE (F250G), CYE (F250A) mutants were constructed to avoid the possible collision between dimethyl group in (4S)-phorenol and Phe^{250} in CYE. CYE (F250G) and CYE (F250A), which would have larger substrate binding pockets than CYE (WT), show increased catalytic activity toward (4*S*)-phorenol (Figure 3), indicating that the substrate binding pocket of CYE (WT) would hardly accommodate

(4*S*)-phorenol. Mutations to Loop 5 and Loop 6, CYE (F250A/P295G) and CYE (F250G/P295G/F296G), have also been constructed, and catalytic activities were assayed toward 2-cyclohexene-1-one, ketoisophorone and (4*S*)-phorenol, and the catalytic data were shown in Figure 3. The data indicates that the CYE (P295G) mutant shows the most increased catalytic activities among them.

The Grafting Mutant of Loop 6 Showed Highest Catalytic Activity.

No electron density of Loop 6 in TYE was observed (Figure 4). To investigate the role of Loop 6 of TYE, grafting mutant, CYE (CYE Loop 6→TYE Loop 6) mutant where the Loop 6 of CYE (^{289}-EPRVTDPFLPEFEKWFKEGT-^{308}) is replaced with the Loop 6 of TYE (^{286}-EPRVNGIADAPENSED-^{301}), was constructed. CYE (CYE Loop 6→TYE Loop 6) mutant showed the highest catalytic activity compared to other CYE mutants (Figure 5), and this indicates that Loop 6 plays pivotal roles in substrate preferences and catalytic efficiencies.

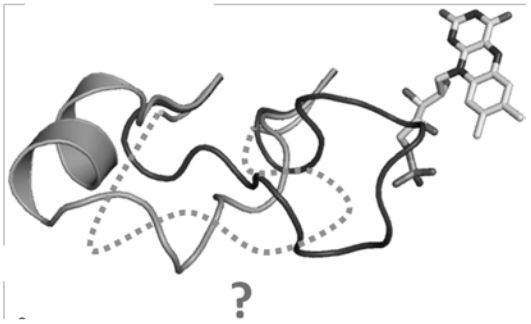

Figure 4. The electron density of Loop 6 was not observed.

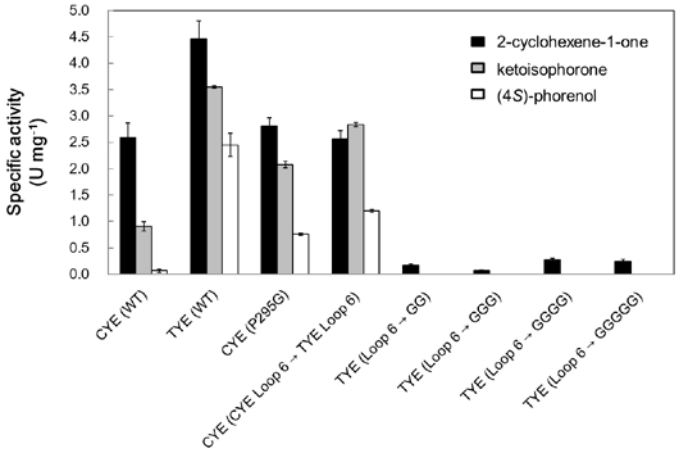

Figure 5. Specific activity of CYE and TYE mutants toward 2-cyclohexene-1-one, ketoisophorone and (4*S*)-phorenol.

Conclusions

In this study, we have designed and constructed mutants in Loops 5 and 6 of CYE and TYE based on their crystal structures, and succeeded in obtaining a CYE mutant, CYE (CYE Loop6 → TYE Loop 6), which shows remarkably higher catalytic activities than the wild-type CYE (3- and 300-fold toward ketoisophorone and (4*S*)-phorenol, respectively). Although the relative activities of this CYE mutant are still lower than those of the wild-type TYE (0.6- and 0.5-fold toward ketoisophorone and (4*S*)-phorenol, respectively), the present mutational data demonstrate the importance of Loops 5 and 6 in determining the substrate preferences of OYEs for the first time and suggest that mutations in these loops could improve the catalytic efficiencies of OYEs and/or confer a new substrate preference to OYEs.