Structural insights into stereospecific reduction of α, β-unsaturated carbonyl substrates by old yellow enzyme from *Gluconobacter oxydans*

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We report the crystal structure of old yellow enzyme (OYE) family protein Gox0502 (a.a 1–315) in free form at 3.3 Å. Detailed structural analysis revealed the key residues involved in stereospecific determination of Gox0502, such as Trp66 and Trp100. Structure-based computational analysis suggested the bulky side chains of these tryptophan residues may play important roles in product stereoselectivity. The introduction of Ile or Phe or Tyr mutation significantly reduced the product diastereoselectivity. We hypothesized that less bulky side chains at these critical residues could create additional free space to accommodate intermediates with different conformations. Notably, the introduction of Phe mutation at residue Trp100 increased catalytic activity compared to wild-type Gox0502 toward a set of substrates tested, which suggests that a less bulky Phe side chain at residue W100F may facilitate product release. Therefore, Gox0502 structure could provide useful information to generate desirable OYEs suitable for biotechnological applications in industry.

Key words: crystal structure; structural models; old yellow enzyme; *G. oxydans* Gox0502; stereoselective production

Old yellow enzyme (OYE) is a large family of flavin mononucleotide (FMN)-containing, NAD(P)H-dependent oxidoreductases, which catalyze C=C bond reduction of α, β-unsaturated ketones and aldehydes in a high substrate and stereospecific manner.1–3 As opposed to metal catalysts, OYEs catalyze the reduction of C=C bond with enantiomeric excess values up to 99%.2,4–8 Hence, OYEs represent a group of enzymes with industrial interests. Although many OYE family members have been identified from yeast, bacteria, and plants recently9–15 and their potential metabolic functions have been suggested, the exact physiological functions of OYEs are largely unknown.11,19–21

*Gluconobacter oxydans* is regarded as one of the most important industrial bacteria due to its unique ability to incompletely oxidize a wide range of carbohydrates and alcohols with excellent efficiency and regio-, stereo-, and enanto-selectivity under natural conditions.22–28 Genome sequence analysis of *G. oxydans* has revealed 12 putative cytosolic flavin-associated proteins.29 Notably, we and others have identified and characterized two OYE homologs from *G. oxydans*, Gox0502 and Gox2684,30–32 which are able to reduce (S)-carvone to (1R, 4S)-dihydrocarvone and (R)-carvone to (1R, 4R)-dihydrocarvone in a regio-specific manner and stereospecific manner.31

Interestingly, both Gox0502 and Gox2684 are able to reduce 2-cyclohexen-1-one at significant rate rather than 2-cyclopenten-1-one.31 However, Gox0502 displays ~2-fold higher catalytic activity compared to Gox2684, although Gox0502 and Gox2684 share 67.4% identities and 83% similarities in sequence. The impact of small differences in the sequence and substrate size on enzymatic activities prompted us to speculate that the marginal differences of the substrate binding pockets in Gox0502 and Gox2684 may play a significant role in substrate selection and reduction.

To investigate the structural insights into stereospecific substrate binding and reduction by OYEs from *G. oxydans*, we report the crystal structure of Gox0502 (a.a. 1–315) in free form at 3.3 Å resolution. Similar to other OYE proteins,33–35 Gox0502 folds into an eight-stranded parallel αβ barrel with approximate dimensions 45 Å × 45 Å × 40 Å. Structural superimposition of Gox0502 in free form with OYE proteins in complex with FMN and substrate as well as the structural model of Gox2684 revealed residues involved in catalysis (His172, Asn175, and Tyr177), substrate selection (Trp66 and Trp100), and product stereoselectivity determination (Trp66 and Trp100), respectively. These results are further validated by mutagenesis analysis. Strikingly, structure-guided engineering attempts targeting the conserved residues at the substrate binding pocket of Gox0502 have produced a gain-in-function
W100F mutant displaying high catalytic activities on previously non-preferred substrates.

Taken together, our Gox0502 structure and rational design efforts may provide a well-characterized structural template to validate the structural principles toward constructing "tailor-made" OYE to catalyze any industrially important α, β-unsaturated ketones/alddehydes with "super" stereoselectivity and enhanced catalytic activity.

Materials and methods

General. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Centrifugations were carried out using centrifuges 5415D (Eppendorf), Avanti J-E, and Proteome Lab™ XL-A (Avestin). Restriction enzymes, Taq DNA polymerase and T4 DNA ligase, were purchased from New England Biolabs.

Expression, purification, and crystallization of Gox0502. The full length gox0502 gene was PCR amplified from the genomic DNA of G. oxydans 621H purchased from ATCC by the following primers (5′-primer: 5′-GGTTAGCCATGCGCCCAAACCTGGTTCAAG-3′; 3′-primer: 5′-GGTCCTCTGAGGTGAGGCGGAGG-3′). The plasmids encoding recombinant proteins were generated by the insertion of the PCR products into pET28b vector (Novagen) with a C-terminal His-tag. Mutant constructs were prepared using KOD-Plus-Mutagenesis Kit (TOYOBO) and verified by sequencing.

Recombinant Gox0502 and its mutants were expressed in Escherichia coli (BL21/D3E) overnight at 20 °C induced by 0.4 mM isopropyl β-d-thiogalactoside (IPTG). The bacterial culture was harvested by centrifugation and re-suspended in ice-cold lysis buffer (25 mM Tris pH 7.4, 25 mM KH2PO4, 500 mM NaCl, 10% glycerol, and 1 mM DTT). Cell suspensions were crushed by cell disruptor (Homogenizers, High-Pressure EmulsifiC3, Avestin) four times and centrifuged at 38,000 rpm for 1 h at 4 °C. Proteins were purified through Ni2+ affinity column, followed by HiLoad Superdex S-75 26/60 column (GE-healthcare). The purified proteins were dialyzed against stabilization buffer (20 mM Tris–HCl pH 7.0 and 100 mM NaCl) and concentrated to 20 mg/mL before crystallization.

Crystals of Gox0502 were grown at 20 °C by mixture of 1.0 μL of protein with 1.0 μL of reservoir containing 20% PEG 400, 50 mM potassium chloride, and 100 mM sodium cacodylate pH 6.5. These crystals grew to a maximum size of 0.2 mm × 0.1 mm × 0.1 mm over the course of 3 days.

Data collection and structural determination. Crystals were transferred into the above reservoir solution supplemented with 0.1 mM mercury chloride and incubated for 12 h prior to being flash frozen (100 K) in the cryo-protection buffer (35% PEG 400, 50 mM potassium chloride, and 100 mM sodium cacodylate pH 6.5). The diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline U17. A total of 360 frames of a data-set with 1° oscillation at 1.009 Å wavelength were collected. The data were processed by HKL2000 (www.hkl-xray.com). The structure was determined by SAD using SHARP/auto SHARP.36 The model was built using the program O (http://www.biochem.ucl.ac.uk/strucsoft) and refined using REFMAC/CCP4 (www.ccp4.ac.uk) to 3.3 Å. There is one Gox0502 molecule per asymmetric unit with solvent content of ~75%. The model of Gox0502 comprises residues 1–315. The crystallographic statistic details of these structures are listed in Table 1.

Structure models of Gox0502 and its mutants in complex with FMN and substrates were made using Discovery Studio 3.5 (Accelrys Inc.). All the models were subjected to minimization of 5000 steepest-descent steps using Discovery Studio 3.5 smart minimization protocol via an all-atom general purpose CHARMM force-field.37

Analytical ultracentrifugation (AUC). The AUC velocity experiment was performed to determine the molecular mass of Gox0502 in solution. Sedimentation velocity (SV) experiment was conducted at 20 °C on a Beckman XL-A analytical ultracentrifuge, equipped with absorbance optics and an An60-Ti rotor. Gox0502 was diluted to 1 mg/mL in PBS buffer at pH 7.4. The rotor speed was set at 60,000 rpm for the highest resolution. The sedimentation coefficient and f/f0 were obtained with c(s) method using the SediFit software.

Biocatalysis assay and data analysis. Biotransformations were typically performed in a standard reaction system containing 30 μg/mL enzyme, 2 mM substrate, 2.1 mM NADPH, 100 mM PBS buffer pH 7.0, and 10% (v/v) ethanol to a final volume of 0.5 mL. The mixtures were incubated in an orbital shaker at 150 rpm at 30 °C for 1 h. Reactions were terminated by extraction through chloroform (0.5 mL) containing 0.15% (v/v) 2-octanone (as internal standard). After centrifugation, the lower organic phase samples were taken for GC (Gas chromatography analysis).

Concentrations and diastereomeric excesses (de) were determined by a GC-6890 N equipped with a 30 m × 0.25 mm DB-5 column (Agilent Technologies). The injection volume was 1 μL with a split ratio of 10: 1. The following program was applied for GC analysis: 40 °C for 3 min; 10 °C/min increase to 220 °C and maintained at 220 °C for 3 min. For (S)-carvone’s catalysis experiment, the corresponding retention times were as follows: (S)-carvone 14.28 min, (1R, 4S)-dihydrocarvone 13.60 min, and (1S, 4S)-dihydrocarvone 13.72 min. For (R)-carvone’s catalysis experiment, the corresponding retention times were as follows: (R)-carvone 14.28 min, (1R, 4R)-dihydrocarvone 13.72 min, and (1S, 4R)-dihydrocarvone 13.60 min. The concentrations of the reaction products were calculated based on absorption. 2-Octanone was used as an internal standard, which had been subjected to chloroform during the extraction step.

Results and discussion

Overall structure of Gox0502

Gox0502 was expressed in E. coli with a C-terminal His-tag and purified to high homogeneity. Similar to
other OYE proteins, Gox0502 was expressed together with the bound FMN cofactor, suggested by its yellow color, and confirmed by UV spectrometry (data not shown). However, we were only able to build the Gox0502 model from residues 1–315. There was no extra density observed after a.a. 315 even using the low contour value for density map display. Apparently, the C-terminal fragment of Gox0502 (a.a. 316–361) could be trimmed during crystal incubation. Consistent with the observation, the SDS-PAGE gel of Gox0502 protein dissolved from the crystals showed a band with relatively smaller molecular weight compared to the freshly-purified Gox0502 protein. Therefore, our Gox0502 structure was crystallized in free form, and the FMN cofactor was released from the protein core together with the cleaved C-terminal fragment.

Different OYE s were reported to adopt different oligomerization forms, ranging from monomer, dimer, to tetramers. The different oligomerization forms are determined by the sequence variable regions, which are involved in the formation of the potential oligomerization interface.2,38) Gox0502 forms monomers in solution as determined by analytic ultracentrifugation (AUC) assay (Fig. S1). Consistent with AUC data, Gox0502 crystallizes as a monomer with unusually high solvent content (~75%), which could be one of the reasons for the low-resolution diffraction power of Gox0502 crystals.

Gox0502 structure was determined by single wavelength anomalous diffraction method (SAD) on a native crystal soaked with 1 mM mercury chloride. The structure of Gox0502 was refined to 3.3 Å with very good density qualities (Fig. S2). Similar to other OYE structures, the overall structure of Gox0502 resembles the traditional hydrolase structure, which consists of a single domain fold comprising an eight-stranded parallel α/β barrel with approximate dimensions of 45 Å × 45 Å × 40 Å (Fig. 1). Gox0502 structure starts with a short β hairpin (β1 and β2), followed by eight alternating α-helices and β-strands along the backbone, in which the eight-stranded parallel β-sheets are surrounded by eight α-helices. The N-terminal short β hairpin is located at the bottom of the β barrel and serves as a lid to cover the β barrel (Fig. 1(B)). About 30% of the total Gox0502 structure adopts well-refined random coil structures connecting the alternating central β-strands with adjacent α-helices. Notably, these loops are proposed to play critical roles for substrate selection and stereoselectivity determination due to their significant diversities in sequence, length, and conformation (Fig. 1).

Notably, the conserved two short α-helices (a.a. 315–320 and a.a. 323–330) serving as the structural plug to seal the FMN-binding pocket are missing due to the truncation of Gox0502 at its C-terminus. Nevertheless, the preformed FMN-binding pocket is formed by strands β8 and β9, and the conserved residues located around this pocket, such as Arg224, Arg261, Asn292, and Asp294, are proposed to be involved in the recognition of phosphate and isoalloxazine moieties of FMN. In addition, the invariable residues Trp100, His172, Asn175, and Tyr177 from the carboxyl termini of strands β5 and β8, respectively, function as the active residues for substrate binding and activation for hydride transfer (Figs. 2, S2).

### Structures of similar proteins

To investigate the structural uniqueness of Gox0502 and initiate the efforts to search for the authentic/optimal substrates of Gox0502, we performed the 3D search using the DALI-server (http://ekhidna.biocenter.helsinki).

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**Table 1. X-ray data and refinement statistics.**

| Data collection |  |
|-----------------|---|
| Space group     | P6(1)22 |
| PDBID           | 3WJS |
| Cell dimensions a, b, c (Å) | 138.89, 138.89, 145.09 |
| Resolution (Å)* | 50–3.3 (3.36–3.3) |
| Rsym (%)        | 7.7 (49.7) |
| l(σ(l))         | 36.6 (12.5) |
| Completeness (%)* | 100.0 (100.0) |
| Redundancy      | 23.1 (23.2) |
| Initial figure of merit | 0.54 |
| Refinement      |  |
| Resolution range (Å) | 50–3.3 (3.38–3.3) |
| No. reflections | 12,332 |
| Rwork (Rfree) (%) | 17.4/24.1 (25.1/36.7) |
| No. atoms       |  |
| Protein         | 2,404 |
| Hg              | 1 |
| PEG             | 17 |
| B-factors (Å²)  |  |
| Protein         | 35.3 |
| Hg              | 78.5 |
| PEG             | 44.0 |
| R.m.s. deviations |  |
| Bond lengths (Å) | 0.018 |
| Bond angles (°)  | 1.98 |
| % favored (disallowed) in Ramachandran plot | 87.1 (0) |

*Values for the highest-resolution shell are in parentheses.
As expected, Gox0502 shares high structural similarities to many FMN-binding reductases with sequence identities of ~40% (Fig. 1(A)). The best matches are OYE1 (Z-score 37.8, r.m.s. 2.4 Å, 302 Ca, PDBID 4GWE), OPR3 (Z-score 41.2, r.m.s. 1.7 Å, 304Ca, PDBID 3HGS), and OPR1 (Z-score 39.4, r.m.s. 1.7 Å, 294 Ca, PDBID 3HGR) (Fig. 2).

Strikingly, Gox0502 shares the highest structural and sequence similarity (~70% identity) to the recently determined “ene” nicotinamide-dependent cyclohexenone reductase structure from Zymomonas mobilis (NCR, Z-score 44.2, r.m.s. 1.4 Å, 300 Ca, PDBID 4A3U) (Fig. 2). Furthermore, both Gox0502 and NCR display monomeric form in solution, which are confirmed by analytic ultracentrifugation (AUC) (Fig. S1) and dynamic light scattering (DLS) experiments, respectively. The key residues involved in catalytic activity, such as His172, Asn175, Tyr177, and key residues involved in substrate determination, such as Trp66 and Trp100, are highly conserved in both Gox0502 and NCR structures (Fig. 1(A)). In addition, the critical residues Pro245, Asp337, and Phe314, whose mutations could enhance the catalytic activities in NCR, are all conserved in the Gox0502 sequence. Such observations suggest that Gox0502 might be an authentic NCR targeting to cyclohexenone with specific “R” selectivity.

However, significant sequence variations are observed between Gox0502 and NCR at their C-terminal (a.a. 330–340), in which the Gox0502 structure was trimmed during crystallization incubation.

Fig. 1. Crystal structure of Gox0502.

Notes: (A) Sequence alignment of Gox0502 with OYEIs. Secondary structure elements are drawn on the basis of structures of Gox0502 and shown at the top of the aligned sequences. β-Sheets are shown as arrows in blue, whereas α-helices are shown as bars in red. Residues involved in enzyme catalysis are highlighted in green stars at the bottom of the aligned sequences, whereas the proposed key residues involved in substrate preference are highlighted in red triangle at the bottom of the aligned sequences. Gox0502, NCR, OYE1, OPR3, OPR1, and Gox2684 are the representations of Gluconobacter oxydans oxidoreductase 0502 (GI: 58038972), Z. mobilis ene reductase NCR (GI: 409107016), Saccharomyces cerevisiae OYE 1 (GI: 417431), Solanum lycopersicum 12-oxophytodienoate reductase 3 (GI: 350539279), Solanum lycopersicum 12-oxophytodienoate reductase 1 (GI: 350536879), and Gluconobacter oxydans oxidoreductase 2684 (GI: 58038436). (B) Stereoview of Gox0502.
FMN-binding site and active-site residues

Gox0502 crystal structure was crystallized in free form, and the attempts for cocrySTALLization of Gox0502 with FMN and substrate failed. However, the high structural and sequence similarity between Gox0502 and NCR prompted us to make the structural model of Gox0502 in complex with FMN for structural and functional analysis (Fig. 3(A) and (B)).

In our model, the FMN cofactor is embedded inside the β-barrel with the surface of the FMN molecule completely buried, whereas C4 and the pyrazine ring are accessible to solvent. The dimethyl-benzene moiety of FMN is surrounded by the hydrophobic residues Pro23 and Leu24, whereas the polar atoms of FMN are within the hydrogen bond distances to the conserved residues Thr25, Gln98, His172, and Arg224 (Fig. 3(C) and (D)). Interestingly, the FMN-binding site of Gox0502 is composed of an elongated tunnel with the dimensions of 7 Å × 10 Å × 15 Å, which is lined by aromatic and hydrophobic residues on one side, such as Trp66, Trp100, His128, and Tyr177. The flavin moiety of the bound FMN molecule is located at the bottom of the tunnel. The small cavity above the FMN could be used to accommodate the substrate with small size (indicated by red rectangle), which could form hydrogen bonds with the conserved residues Thr25, His172, and Asn175 for hydride transfer from FMN and for protonation assisted by the invariable Tyr177 (Fig. S3). Such structural arrangement strongly suggests that His172, Asn175, and Tyr177 are critical residues involved in substrate catalysis. Consistent with the structural observation, the introduction of Ala mutation at either His172 or Asn175 or Tyr177 completely disrupts the catalytic activity of Gox0502 (Table 2).

Substrate binding site

To further investigate the molecular principles of substrate selectivity and catalytic stereoselectivity determined by Gox0502, Gox0502 structure was superimposed with OYE1 mutant structures, which were cocrySTALLized with (R)-carvone and (S)-carvone, respectively, to produce structural models of Gox0502 in complex with FMN and carvone (Fig. 4). Surprisingly, the mercury atom introduced into the Gox0502 structure by soaking, superimposed perfectly with the bound carvone substrate. This mercury atom is coordinated by the side chains of His172 (4.1 Å), Asn175 (3.7 Å), Tyr177 (3.8 Å), Asp230 (4.7 Å), and Arg224 (5.1 Å) and is located near the side chains of the conserved residues Thr25 (5.8 Å) and Trp100 (5.0 Å) (Fig. 4).

In (R)-carvone model, the (R)-carvone substrate is located above the flavin moiety of FMN with the carboxyl oxygen hydrogen-bonded to His172 and Asn175. The methyl group of the (R)-carvone substrate is buried inside the hydrophobic environment formed by Ala56, Trp100, Tyr177, and the flavin moiety of FMN (Fig. 4(A) and (B)). By contrast, there is no significant interaction between the alkyl moieties of the (R)-carvone substrate with Gox0502, except the partial π-stacking contributed by the flavin moiety (Fig. 4(A)). Similarly, (S)-carvone substrate is able to easily dock into the substrate binding pocket with minimal rotation around the carbonyl group (Fig. 4(C) and (D)). These observations support the notion that both (R)-carvone and (S)-carvone are favorable substrates for Gox0502 (Table 2).

Notably, structural comparison of Gox0502 with OPR1 shows significant structural deviations at the entrance of the substrate binding pocket (Fig. 5(A)–(C)). The substrate binding pocket of Gox0502 is relatively smaller compared to that of OPR1, which suggests that Gox0502 likely prefers aromatic substrate with short alkyl chain instead of long-chain substrates (Fig. 5(B)). Consistent with the structural observation, Gox0502 displays high catalytic activity to carvone comprising of an aromatic head connected to a short alkyl tail.\(^\text{31}\)

Table 2. Reductions of carvone by Wild-Type and Mutant \(G.\ oxydans\) OYE1s.

| Enzyme variant | Reaction: substrate \(\rightarrow\) product | 1a \(\rightarrow\) 1b | Yield (%) | \(de\) (%) | 1a \(\rightarrow\) 1b | Yield (%) | \(de\) (%) | 1a \(\rightarrow\) 1b | Yield (%) | \(de\) (%) | 1a \(\rightarrow\) 1b |
|---------------|------------------------------------------|----------------|------------|-----------|----------------|------------|-----------|----------------|------------|-----------|----------------|
| Gox0502 WT    | 1a=(S)-carvone, 1b=(1R, 4S)-dihydrocarvone, 2a=(R)-carvone, 2b=(1R, 4R)-dihydrocarvone. 3a = citral, 3b = citronellal. | 72.9 ± 3.3 | >99 | 64.5 ± 2.0 | >99 | 64.5 ± 2.1 |
| Gox0502 W66I  | 2a=27.2 ± 4.6 | 95.2 ± 3.2 | 20.3 ± 2.2 | 91.3 ± 3.1 | 15.7 ± 4.5 |
| Gox0502 W66F  | 70.4 ± 4.7 | >99 | 65.4 ± 4.9 | >99 | 61.2 ± 1.1 |
| Gox0502 W66Y  | 69.2 ± 5.0 | 92.7 ± 1.4 | 59.7 ± 3.4 | >99 | 48.3 ± 2.2 |
| Gox0502 W100I | 50.5 ± 3.6 | 90.6 ± 1.7 | 18.3 ± 0.3 | 85.3 ± 1.9 | 9.6 ± 4.9 |
| Gox0502 W100F | 76.8 ± 0.3 | 92.6 ± 3.6 | 71.1 ± 1.0 | 93.1 ± 2.7 | 70.2 ± 6.2 |
| Gox0502 W100Y | 61.6 ± 1.3 | 96.7 ± 2.8 | 36.2 ± 1.7 | 89.0 ± 2.2 | 60.5 ± 0.5 |
| Gox0502 W66A  | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox0502 W610A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox0502 H172A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox0502 N175A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox0502 Y177A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox2684 WT    | 38.7 ± 3.5 | >99 | 30.2 ± 2.4 | >99 | 33.7 ± 4.0 |
| Gox2684 H172A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox2684 N175A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |
| Gox2684 Y177A | <1.0 | n.d. | <1.0 | n.d. | <1.0 |

Notes: All biotransformations were conducted in triplicate. \(de\) = diastereomeric excesses, n.d. = not determined.
However, dissimilar to the bulky Tyr246 in OPR1, Gox0502 employs Thr231 at the entrance of the substrate binding pocket, which yields a relatively elongated and sterically less crowded binding cavity (Fig. 5(A)). Such structural arrangement allows Gox0502’s flexibility to recognize certain non-aromatic long-chain substrates. Indeed, Gox0502 displays catalytic activity, although at a weak level, to non-aromatic long-chain substrates, such as citral.30,31 Hence, the combination of the structural and sequence arrangement of key residues along the substrate binding pocket plays a determinative role for substrate selection.

To further investigate the impacts of key residues at the substrate binding pocket in substrate selection and catalytic activity, we compared the Gox0502 structure with Gox2684 model generated by SWISS MODEL server41–43 (Fig. 5(D)–(F)). Although Gox0502 and Gox2684 share a high degree of sequence identity and both display a broad substrate spectrum, Gox0502 displayed ~2-fold activity compared to Gox2684.31 Therefore, we speculate the small differences in amino acid composition at some unidentified critical residues may have an impact on catalytic activities. As expected, the overall structure of Gox2684 is almost

![Overall structure of Gox0502 and other OYEs.](image)

**Fig. 2.** Overall structure of Gox0502 and other OYEs.

Notes: (A) Overall structure of Gox0502. The conserved catalytic triad in Gox0502, H172-N175-Y177, is shown as sticks and colored in yellow, whereas the proposed key residues involved in substrate preference, W66-W100, are shown as sticks and colored in magenta. (B) Cartoon view of crystal structure of OYE1. OYE1 is colored in green. The conserved catalytic triad and the residues involved in substrate preference are shown as sticks and indicated. (C) Cartoon view of crystal structure of OPR3. OPR3 is colored in marine. The conserved catalytic triad and the residues involved in substrate preference are shown as sticks and indicated. (D) Cartoon view of crystal structure of OPR1. OPR1 is colored in yellow. The conserved catalytic triad and the residues involved in substrate preference are shown as sticks and indicated. (E) Cartoon view of crystal structure of NCR. NCR is colored in orange. The conserved catalytic triad and the residues involved in substrate preference are shown as sticks and indicated. The critical residues enhance the catalytic activities, P245-D337-F314, are shown as sticks and labeled in blue.
identical to that of Gox0502. However, we observed that the side chain of residue 22 in Gox0502 (Ser22 in Gox0502 and Ala22 in Gox2684, Fig. 5(E) and (F)) may have the chance to form a hydrogen bond to the side chain of the conserved Arg224, while Arg224 could form a hydrogen bond to the flavin moiety of bound FMN (Fig. 5(E)). We speculate that the extra hydrogen bond formed between Gox0502, but not Gox2684, and bound FMN facilitates the electron transfer from FMN to the substrate and eventually increasing the catalytic activity.

**Structural flexibility of substrate binding pocket**

Our C-terminal segment trimmed Gox0502 protein was crystallized in free form without the tightly bound FMN cofactor, which provides a unique chance to look at the conformational changes corresponding to the FMN binding by structural superimposition of Gox0522 in free form with other OYEśs in complex with FMN. Structural superimposition of Gox0502 in free form with NCR, OPR1, and OPR3 in complex with FMN and substrate showed dramatic conformational changes of a loop within the FMN-binding pocket (Figs. 2 and 6). More than 10 Å movements and reshaping of two well-refined loop regions (a.a. 227–237, connecting α6 and β9, and a.a. 262–276, connecting α7 and β10) were observed in Gox0502 (Fig. 6). By contrast, the structural conformation of the small cleft for substrate binding is relatively rigid. There were no significant structural changes observed for the locations and orientations of the invariable residues involved in substrate binding pocket formation, such as Tyr177 and Asn175 (Fig. 6). Unexpectedly, in Gox0502 structure, the side chain of Asn230 is located near to the alkyl moieties of carvone group, which may participate in substrate selection in Gox0502 (Fig. 4(A) and (C)).

**Structural insights into Stereospecificity determination by Gox0502**

Surprisingly, the position of the proposed substrate filtering gate formed by Tyr and Tyr pair in OPR1 is not conserved in the Gox0502 structure. In our Gox0502 structure, the corresponding residue to OPR3 Tyr78 in sequence is Trp66, whereas the corresponding residue to OPR1 Tyr246 in sequence is Thr231.
However, structural comparison of Gox0502 in free form with OPR1 and NCR in complex with substrate showed more than 6 Å movement of Thr231 together with the loop connecting α6 and β9, which yields a wide pocket for substrate binding. Notably, the corresponding OPR1 Tyr246 residue in NCR is Ile231, which was perfectly superimposed with OPR1 Tyr246 (Fig. 5(A)). It was hypothesized that small size of the side chain of Ile or Thr at this position may allow formation of a wider substrate binding pocket, which is less favorable for enantio-restrictive selection. However, both Gox0502 and NCR showed excellent diastereoselectivity (>99% toward (R)-conformation product) for carvone reduction. Therefore, we speculate that Trp66 could be the key amino acid playing the dominant role in stereoselectivity. Consistent with this speculation, structural superimposition of crystal structures of NCR, OPR1, OPR3, and Gox0502 showed that the bound substrate is encapsulated by an aromatic-ring environment contributed by partially conserved His/Asn175, Tyr177, Trp100, and Phe/Tyr/Trp66 (Fig. 2). By contrast, the proposed Tyr246 residue in OPR1 is more than 5 Å away from the bound substrate, which may not be directly involved in product diastereoselectivity determination. Furthermore, the partially conserved Asn175 residue in Gox0502/NCR has the potential to form a hydrogen bond with the bound substrate directly, which may play a critical role for substrate anchoring (Fig. 4). Remarkably, three side chains with aromatic rings (Tyr177, Trp100, and Trp66), together with the flavin moiety of FMN, serves as a wall sealing one side of the substrate binding pocket, therefore determining the diastereoselectivity of the product (Fig. 6). Hence, we speculate that the bulky groups of the side chains of Trp66 and Trp100 play an important role for product diastereoselectivity determination. Surprisingly, the introduction of Tyr mutation at residue Trp66 significantly decreases diastereoselectivity toward (R)-conformation product from 99% to 92.7% for (S)-carvone reduction, whereas has no significant impact on diastereoselectivity for (R)-carvone reduction (Table 2). Furthermore, the introduction of Phe mutation at residue Trp66 showed no significant impact for both the (R)-carvone and (S)-carvone reductions (Table 2). We hypothesized that the introduction of Tyr instead of Phe residue at residue Trp66 may disrupt the local hydrophobic environment in this region. By contrast, the introduction of Ile at residue...
Trp66 significantly decreases the diastereoselectivity toward (R)-conformation product from 99% to 95.2% for (S)-carvone reduction and 91.3% for (R)-carvone reduction (Table 2). Remarkably, the introduction of Ala at Trp66 completely disrupted Gox0502 catalytic activity toward both (R)-carvone and (S)-carvone (Table 2), which suggested that the substrates are no longer properly positioned within the substrate binding pocket after the introduction of W66A mutation. Therefore, the bulky hydrophobic side chain at residue Trp66 indeed plays an important role for diastereoselectivity determination. Similarly, the introduction of either Ile or Phe or Tyr mutation at residue Trp100 significantly decreased the diastereoselectivity toward (R)-conformation product for (R)-carvone and (S)-carvone reductions (Table 2). The introduction of Tyr mutation at residue Trp100 decreases diastereoselectivity toward (R)-conformation product from 99% to 96.7% for (S)-carvone reduction and 89.0% for (R)-carvone reduction, whereas the introduction of Phe mutation at residue Trp100 decreases diastereoselectivity toward (R)-conformation product from 99% to 92.6% for (S)-carvone reduction and 93.1% for (R)-carvone reduction (Table 2). In addition, the introduction of Ile mutation at residue Trp100 decreases diastereoselectivity toward (R)-conformation product from 99% to 90.6% for (S)-carvone reduction and 85.3% for (R)-carvone reduction. Similarly, the introduction of Ala mutation at residue Trp100 completely disrupted Gox0502 catalytic activity (Table 2).
Conclusion

The FMN containing OYEs have been studied for many years. Many phenolic compounds containing α, β-unsaturated carbonyl moiety, such as cyclohexenone and carvone, have been tested for activity. Current research focuses on OYEs that have been highlighted on industrial applications. Therefore, the structural principles of OYE-mediated reduction of α, β-unsaturated carbonyl substrates could be one of the keys to design novel OYE to catalyze a “given” α, β-unsaturated carbonyl substrate with excellent stereoselectivity.

Structural comparison of Gox0502 at free form with other OYEs in complex with substrates not only confirmed the invariable residues involved in catalysis, such as His172, Asn175, and Tyr177, but also revealed the critical residues involved in substrate accommodation, such as Trp66 and Trp100 (Fig. 2). Moreover, our structural analysis on Gox0502 crystalized in free form demonstrated that the identity of one of the proposed substrate filtering pairs located at this inner-side of the substrate binding pocket (Trp66), instead of the pairs (Trp66 and Thr231), determines the specific “R” conformation of the product (Figs. 5 and 6).

In addition to the proposed substrate filtering pairs, the invariable Trp100 residue located at the substrate binding pocket also plays an important role for substrate selection and product stereoselectivity determination. We consider that the replacement of the bulky group of Trp with less bulky group of Ile or Phe or Tyr at either Trp66 or Trp100 residue should create additional space at the active site accommodating the isopropenyl moiety of the bound carvone, which allows the flexibility for methyl group to take (S)-conformation. Consistent to this observation, introduction of Ile or Phe or Tyr residue at this position significantly reduced the product diastereoselectivity, whereas introduction of W100A or W66A mutation completely abolished catalytic activity, probably due to lost of the substrate binding affinity (Table 2). Surprisingly, the introduction of W100F mutation slightly increased catalytic activities to three substrates tested so far, which suggests that the less bulky Phe group may facilitate the product release (Table 2).

Dissimilar to other well-characterized OYEs, Gox0502 displays comparable catalytic activity on different types of α, β-unsaturated carbonyl substrates, such as non-aromatic citral substrate and aromatic carvone substrate. Remarkably, the residue identity at Trp100 seems more critical for long-chain hydrophobic substrate recognition. In citral catalysis reaction, the introductions of Tyr, Ile, and Ala at residue Trp100 reduced the catalytic activity from 8%, ~6-fold to more than 1000 fold, respectively. By contrast, the introduction of Phe at residue Trp100 increased the catalytic activity by 10% (Table 2).

Hence, structural analysis of Gox0502 provides not only the structural insights into stereospecific reduction of carvone but also the initial clues to engineer “novel” OYE specifically catalyzing non-aromatic α, β-unsaturated ketones with high stereoselectivity.

Supplemental material

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Author contribution

D. W and Y.A.Y designed the study. B.Y carried out the bulk of the experiments. J.D and L.L contributed to the Biocatalysis assay. B.Y, L.L, Y.A.Y, and D.W wrote the paper. All authors have read and approved the final manuscript.

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