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Towards the computational design and engineering of enzyme enantioselectivity: A case study by a carbonyl reductase from *Gluconobacter oxydans*

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ABSTRACT

In our previous work, a NAD(H)-dependent carbonyl reductase (*Go*CR) was identified from *Gluconobacter oxydans*, which showed moderate to high enantiospecificity for the reduction of different kinds of prochiral ketones. In the present study, the crystal structure of *Go*CR was determined at 1.65 Å resolution, and a computational strategy concerning substrate-enzyme docking and all-atom molecular dynamics (MD) simulation was established to help understand the molecular basis of enantiopreference and enantiorecognition for *Go*CR, and to further guide the design and engineering of *Go*CR enantioselectivity. For the reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE), three binding pocket residues, Cys93, Tyr149, and Trp193 were predicted to play a critical role in determining the enantioselectivity. Through site-directed mutagenesis, single-point mutant W193A was constructed and proved to reduce OPBE to ethyl (*R*)-2-hydroxy-4-phenylbutyrate (*R*-HPBE) with a significantly improved *ee* of >99% compared to 43.2% for the wild type (WT). Furthermore, double mutant C93 V/Y149A was proved to even invert the enantioselectivity of *Go*CR to afford *S*-HPBE at 79.8% *ee*.

1. Introduction

Carbonyl reductases (CRs) can catalyze the asymmetric reduction of prochiral ketones to afford optically active alcohols which often serve as key building blocks for the synthesis of many pharmaceuticals (Musa and Phillips, 2011). Compared to asymmetric transfer hydrogenation (ATH) using precious metals, such as iridium, rhodium, and ruthenium as catalysts, carbonyl reductase-mediated reactions have many inherent merits such as environmental compatibility, high enantioselectivity and also mild reaction conditions, thus drawing more and more attentions from the synthetic chemists (Ni and Xu, 2012; Xu et al., 2012). To acquire carbonyl reductases of desired properties which have practical utility, a conventional strategy is to screen enzymes from natural source. This method has proven to be feasible by a large number of cases reported in the past years, and several enzymes of high substrate tolerance and at the same time excellent enantioselectivity were identified (He et al., 2015; Liu et al., 2014b; Ma et al., 2012; Matsuda et al., 2009). However, enantioselectivity of naturally occurring enzymes is often substrate-dependent and not always high enough for unnatural substrates (Ni et al., 2011; Zhu et al., 2006). Thus, improvement in enzyme enantioselectivity for a specific reaction is often necessary to meet the ever-increasing demands in asymmetric synthesis (Zhu et al., 2008).

Though directed evolution is a straightforward strategy to engineer the enantioselectivity of carbonyl reductases needing only sequence information, it is difficult to implement a high-throughput screening (HTS) method for determining the enantioselectivity of the large variant libraries which often consist of tens of thousands of mutants (Truppo et al., 2008; Zhang et al., 2015b). By contrast, structure-guided (semi-) rational design of the enantioselectivity has attracted much attention because this method is more targeted and efficient, and thus can avoid unnecessary work to some extent. Several groups have successfully increased, or even inverted the enantioselectivity of carbonyl reductases using structural information. For example, Patel et al. adopted the site-saturation mutagenesis approach to create a comprehensive mutant library at Trp110 of the alcohol dehydrogenase TeSADH from *Thermoanaerobacter ethanolicus*. Five of the designed

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Trp110 mutants were proved to reduce phenylacetone with a significantly improved *ee* (>99.9%, *S*) compared to wild type (WT, 37%, *S*) (Patel et al., 2014). In the study of carbonyl reductase SSCR from short-chain dehydrogenase/reductase (SDR) superfamily, Zhu et al. reversed its enantiopreference and enhanced the enantioselectivity toward the reduction of para-substituted acetophenones, through substrate-enzyme docking-guided point mutation (Zhu et al., 2008). For carbonyl reductase from medium-chain alcohol dehydrogenase (MDR) superfamily, Wang reported the enantioselectivity inversion of NADH-dependent carbonyl reductase RCR from Prelog to anti-Prelog-selectivity based on structure-guided design of substrate-binding pocket (Wang et al., 2014). Though these results provide valuable insights into the structure-function relationship of carbonyl reductases from different superfamilies, the molecular basis of the substrate-dependent enantiorecognition has not yet been well understood so far, and efficient design strategies of enantioselectivity are still lacking.

In our previous study, a NAD(H)-dependent short-chain dehydrogenase from *Gluconobacter oxydans* (*Go*CR) was characterized (Liu et al., 2014a). *Go*CR had a broad substrate spectrum and could catalyze the reduction of aliphatic ketones, aromatic ketones, and halogenated acetophenones, to the corresponding alcohols with exclusive Prelog-selectivity (>99% *ee, S*). In particular, for the preparation of (*S*)-1-(2-chlorophenyl)-ethanol, a chiral intermediate in the synthesis of several promising PLK1 (serine-threoninekinase polo-like kinase 1) inhibitors for cancer therapy, 90% product yield and >99% *ee* were achieved at a high *o*-chloroacetophenone concentration of 500 mM (Deng et al., 2015). However, for the reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE), *Go*CR showed low enantioselectivity, producing ethyl (*R*)-2-hydroxy-4-phenylbutyrate (*R*-HPBE), an important chiral precursor to angiotensin-converting enzyme (ACE) inhibitors, at only 43.2% *ee* (Liu et al., 2014a; Shen et al., 2012). This substrate-dependent enantioselectivity of *Go*CR provides us a suitable model towards the exploration of the structural basis of enantiopreference for carbonyl reductases. Herein, we solved the crystal structure of *Go*CR. A computational strategy combing substrate-enzyme docking with all-atom molecular dynamics (MD) simulation was established to help understand the molecular basis of the enantiorecognition and enantiopreference for *Go*CR, and to further guide the design and engineering of this enzyme, with enhanced or inverted enantioselectivity toward the reduction of OPBE. The application potential of the constructed mutants was subsequently evaluated by performing the asymmetric synthesis of *R*-HPBE in a glucose dehydrogenase-coupled NADH regeneration system.

2. Materials and methods

2.1. Crystallization and refinement of the GoCR structure

Induced expression of recombinant carbonyl reductase *Go*CR was performed as described in our previous study (Liu et al., 2014a). For crystallization purpose, *Go*CR protein was purified through Ni^{2+} affinity column followed by HiLoad Superdex S-75 26/60 column. The purified protein was concentrated to 10 mg/mL in a Centriprep-30 (Amicon). Crystals were grown at 20 °C by mixing 1.0 μL of *Go*CR (~10 mg/mL) with 1.0 μL of reservoir solution containing 12% PEG 3350, 200 mM proline, and 100 mM HEPES (pH 7.6), and equilibrated over 1 mL of reservoir solution for a course of 3 days. Crystals were flash frozen (100 K) in the above reservoir solution supplemented with 30% glycerol. A single wavelength data set was collected on a native crystal at National Synchrotron Light Source (NSLS) beamline X29A and processed by HKL2000 (Otwinowski and Minor, 1997). The structure was determined via molecular replacement using CCP4/Phaser (McCoy et al., 2007) by using the crystal structure of a dehydrogenase from *Sinorhizobium meliloti* (PDB ID: 3V2G) as the search model. The model was built by using the program O (Jones et al., 1991) and refined using REFMAC/CCP4 (Murshudov et al., 1997). Statistics of the processed data are presented in Table S1.

2.2. Molecular docking

Substrate-enzyme docking was performed using the Autodock 4.2 program (Morris et al., 2009). All the ketone substrates listed in Table 1 were separately docked into the substrate-binding pocket of *Go*CR-NADH complex. 3D-coordinates (mol2 files) of substrates were downloaded from the ZINC database (Irwin et al., 2012). AutoDock tools (ADT, version 1.5.6) were used for enzyme and substrate preparation. To encompass the entire substrate-binding pocket, the docking box was set to a size of 60 × 60 × 60 grid points with a grid spacing of 0.375 Å. The box center was set exactly at the OH atom of the catalytic residue Tyr155. In each docking run, the substrate was set as flexible while receptor residues except for Tyr155 and Ser142 were set as rigid. Tyr155 and Ser142 sidechains were set as flexible to facilitate the forming of the two catalytic H-bonds between Tyr155/Ser142 and the substrate carbonyl. For each substrate, 100 different poses were generated using the Lamarckian genetic algorithm (LGA) with the maximum number of energy evaluation set to be 25,000,000. Cluster analysis of the resulted poses was then performed using an rmsd cutoff value of 2.0 Å.

2.3. Molecular dynamics simulation

MD was performed using the sander module implemented in the Amber 14 suite (Case et al., 2014), with the ff12SB force field used for the protein system, and the GAFF force field used for the ligands. ANTECHAMBER module was used to calculate ligands AM1-BCC atom charges. Hydrogen atoms and sodium ions (to neutralize the negative charges) were added to protein with the tleap utility. Each simulation system was immersed in a truncated octahedral box of TIP3P explicit water, extended 10 Å outside the protein on all sides. To start the MD simulation, initial structure of the *Go*CR-NADH-substrate ternary complex was treated as follows: (a) water

Table 1
Molecular docking results of *Go*CR using five representative substrates as ligands.

Entry	Substrates	Number of poses in targeted cluster	Number of productive poses		Binding energy[a] (kcal/mol)		Predicted config.	Experimental config.
			Pro-*S*	Pro-*R*	$\triangle G_{pro\text{-}S}$	$\triangle G_{pro\text{-}R}$		
1	Acetophenone	83	83	0	−6.38	–	*S*	*S*
2	*o*-Fluoroacetophenone	81	81	0	−5.92	–	*S*	*S*
3	*o*-Chloroacetophenone	49	9	0	−6.25	–	*S*	*S*
4	*o*-Bromoacetophenone	35	7	7	−6.79	−6.73	*R&S*	*S*
5	OPBE	19 (39)[b]	16	8	−7.83	−8.07	*R&S*	*R&S*

[a] The lowest binding energy of productive poses.
[b] For OPBE, two clusters were produced, which corresponded to the pro-*R* and pro-*S* poses, respectively.