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Two "classical" Old Yellow Enzymes from *Chryseobacterium* sp. CA49: Broad substrate specificity of *Chr*-OYE1 and limited activity of *Chr*-OYE2

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ABSTRACT

Two putative Old Yellow Enzyme (OYE) homologues, *Chr*-OYE1 and *Chr*-OYE2, were identified from the genome of *Chryseobacterium* sp. CA49 as new members of the "classical" subfamily. *Chr*-OYE1 and *Chr*-OYE2 were most closely related to the SYE4 from *Shewanella oneidensis* and NerA from *Agrobacterium radiobacter* with 41% and 45% identity, respectively. Both enzymes were expressed in *Escherichia coli* in soluble form, but their catalytic abilities as ene-reductases were quite different. Among the 19 substrate tested, *Chr*-OYE1 could catalyze the reduction of 18 of them including an ynone with excellent stereoselectivity for several prochiral ones, and its specific activity was roughly 1100-fold high than *Chr*-OYE2, which only catalyzed 3 of the substrates. After restoring the conserved tyrosine, *Chr*-OYE2 remained the same substrate spectrum, but showed significantly enhanced activity and stereoselectivity.

1. Introduction

Asymmetric bioreductive routes to enantiomerically pure compounds have emerged to become a key component in the synthesis of increasingly complex pharmaceuticals that often contains multiple stereocenters [1]. The bioreductions have highly chemo-, regio-, and stereoselective, and low energy requirements because of their ambient temperature and pressure conditions [2]. In the recent decades, the great potential of the Old Yellow Enzyme (OYE) in asymmetric synthesis has been studied extensively [3–8]. They are typically used as ene-reductases to catalyze the bio-reduction of alkenes to generate up to two stereogenic centers. These NAD(P)H-dependent oxidoreductases contain a non-covalently bound cofactor flavin mononucleotide (FMN) and are capable of reducing a variety of activated alkenes, including α,β-unsaturated ketones, aldehydes, nitroalkenes, carboxylic acids and derivatives [9,10]. The products often serve as versatile synthons in pharmaceutical and fine chemical industries [7,11,12].

The OYE-catalyzed asymmetric ene-reduction is *trans*-specific addition, which is in contrary to the *cis*-hydrogenation catalyzed with transition metal-based homogeneous catalysts. The hydride transfer is from the N_5 of reduced FMN onto C_β of the substrate and a Tyr residue or solvent adding a proton to C_α from the opposite side [5,13–16]. These enzymes have in common an $(\alpha/\beta)_8$ barrel fold (TIM barrel), conserved throughout the whole protein family, and exist as a function monomer [17], dimer [15] or tetramer [18]. The catalytic site of OYEs usually consists of a His/His or His/Asn residue pair, which is responsible for hydrogen bonding with the substrate. The highly conserved tyrosine residue has been shown to be the proton donor, but in some specific cases, such as the morphinone reductase bearing a cysteine residue in the active site instead of tyrosine, the proton is inferred to come from the solvent [15,16].

OYEs have been discovered, identified and characterized from many biological sources, including plant, bacteria, and fungi. With the rapid development of genome sequencing technology, the tremendous genomic database offers an excellent opportunity for discovering novel enzymes by genome mining. Using such an approach, we identified three putative OYEs from the genome of *Chryseobacterium* sp. CA49, a strain that is involved in the *anti*-Prelog reduction of aprepitant intermediate [19]. One of the OYEs, namely *Chr*-OYE3, which belongs to the "thermophilic-like" subfamily, has been characterized [20]. In this study, two "classical" OYEs, *Chr*-OYE1 and *Chr*-OYE2, were comparatively analyzed to

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evaluate their potential as ene-reductases. *Chr*-OYE1 showed very broad substrate specificity, while *Chr*-OYE2 had limited activity. Mutants of *Chr*-OYE2 were constructed at the conserved catalytic motif, which displayed enhanced activity and selectivity.

2. Materials and methods

2.1. Materials

The strain *Chryseobacterium* sp. CA49 was deposited at the China Center for Type Culture Collection (Wuhan, China) under the acquisition number CCTCCM 2012484. Chemicals and solvents were of analytical grade or better. Substrate **15a** and corresponding racemic reduction product, and the racemic reduction products of **12a**, **13a**, and **14a** were synthesized as reported previously [21–26]. Other substrates and racemic reduction products were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Alfa Aesar (Tianjin, China). Oligonucleotdies were purchased from Invitrogen (Shanghai, China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Kanamycin and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were obtained from Amresco (Solon, OH, USA).

2.2. Cloning of chr-*oye1*, chr-*oye2* and site-directed mutagenesis

The genomic DNA was isolated from *Chryseobacterium* sp. CA49 using the MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0 (Takara Shuzo Co., Ltd., Dalian, China). DNA fragments encoding *Chr*-OYE1 and *Chr*-OYE2 (GenBank accession no. KP202696 & KP202697) were amplified by PCR with primers 5′-GAATTC ATG AGC ACA GAA TCA TTA TTT ACA C-3′ and 5′-CTCGAG TTA GTA TAA TAC GCC GAG GCT TTC-3′ (for *Chr*-OYE1), or 5′-GAATTC ATG GAA AAT ACA TTA TCA CCT CTT TTA G-3′ and 5′-AAGCTT CTA TTC AGT AAT TAA TGC TTT AGG ATA G-3′ (for *Chr*-OYE2). The PCR products were ligated into pMD19-T vector, and subcloned into pET28a (+) plasmid using the *Eco*R I and *Xho* I restriction sites (for *Chr*-OYE1), or *Eco*R I and *Hin*d III (for *Chr*-OYE2). The resulting expression plasmids, pET28a-*chr-oye1* or pET28a-*chr-oye2* were verified by DNA sequencing.

Site-directed mutagenesis was performed following the instructions of QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). To construct the mutants, the pET28a-*chr-oye2* was used as the template. Primers used to construct the A181H substitution were 5′-GAG GTG CAT GCT ATA GCC GGT TAC CTC ATT CCG CAA TTT CTT AG-3′ and 5′-CTA AGA AAT TGC GGA ATG AGG TAA CCG GCT ATA GCA TGC ACC TC-3′. Primers used to construct the M183Y substitution were 5′-GTT GAG GTG CAT GCT ATA CAC GGT ATG CTC ATT CCG C-3′ and 5′-GCG GAA TGA GCA TAC CGT GTA TAG CAT GCA CCT CAA C. The mutated sequences were verified by comparing the sequences of the sense and antisense strands.

2.3. Production and purification of proteins

The expression plasmid in pET28a (+) vector was transformed into *Escherichia coli* BL21 (DE3). Single colonies were grown overnight at 37 °C in Luria–Bertani (LB) media containing 50 μg kanamycin/ml. Two milliliter of overnight culture was then inoculated into 200 ml of Terrific Broth (TB) containing 50 μg kanamycin/ml in a 500 ml flask. The cultures were incubated at 37 °C for 3 h before the addition of 0.5 mM IPTG, and the incubation was continued at 30 °C for another 18 h with gyratory shaking at 220 rpm. Cell pellets were collected by centrifugation at $1 \times 10^4 \times g$ for 5 min and resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0).

The entire purification procedure was performed at 4 °C. The cells were lysed through a high-pressure homogenizer (ATS-AH100B, ATS Engineering Inc., Canada) at 60 MPa for four times, and the cell debris was removed by centrifugation at $2 \times 10^4 \times g$ for 20 min at 4 °C. The resulting supernatant was loaded onto a Ni^{2+}-nitrilotriacetic acid column (Bio-Rad). After washing with ten bed volumes of buffer B (50 mM potassium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), the target protein was eluted with buffer C (50 mM potassium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein fractions were dialyzed against 50 mM potassium phosphate buffer (pH 8.0), concentrated by ultrafiltration and stored at −80 °C before use.

Protein concentration was determined using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The molecular mass and purity of purified recombinant protein were estimated by SDS-PAGE. A Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan) was used for the spectral studies and the determination of free flavin [27]. Free flavin was released by the protein denaturation through incubating in 100 °C boiling water bath for 10 min, then the resulting supernatant was used to determine flavin content [26].

2.4. Enzyme assays

All measurements were performed in triplicate. Purified enzymes were used throughout. Substrate 2-methyl-*N*-phenylmaleimide (**12a**) was used to analyze the co-factor preference. Substrates 2-cyclohexen-1-one (**4a**) and **12a** were used to determine the pH- and temperature-dependent of activity for *Chr*-OYE1 and *Chr*-OYE2, respectively. The reactions were performed in 1-ml reaction system containing 100 mM buffer, 10 mM NADH, 10 mM **4a** or 5 mM **12a**, and 63 μg purified *Chr*-OYE1 or 500 μg purified *Chr*-OYE2. Four buffers were applied to determine the pH optima (sodium citrate (pH 4.5–5.5), potassium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.5–9.0) and sodium carbonate (pH 9.5)). The reaction was measured at 30 °C for 2 min or 2 h for *Chr*-OYE1 and *Chr*-OYE2, respectively. To determine the temperature optima, potassium phosphate (pH 7.0) was applied at varied reaction temperature ranging from 10 to 50 °C. The products were analyzed with gas chromatography (GC) or high performance liquid chromatography (HPLC) to determine the conversion and enantiomeric excess. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of products per minute under the assay conditions.

The kinetic parameters of *Chr*-OYE2-M183Y were determined in 1-ml potassium phosphate buffer (100 mM, pH 7.0) containing 1.2 mM NADH and 0.05–1.2 mM **12a**. The reaction was initiated by the addition of 500 μg purified enzyme, and a continuous spectrophotometric measurement was employed by monitoring the oxidation of NADH at 340 nm for 2 min at 25 °C on a Shimadzu UV-1800 spectrophotometer. Background consumption of NADH was determined by measuring the same reaction mixture in absence of the enzyme. Kinetic parameters were estimated according to the Michaelis–Menten equation, and data were analyzed using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA).

2.5. Bioreduction of activated alkenes and product analysis

The bioreduction of various substrates was performed in 1-ml reaction system containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM NADH, 5 mM substrate, and 50 μg purified *Chr*-OYE1 or 500 μg purified *Chr*-OYE2. After 2-min or 16-h incubation at 30 °C for *Chr*-OYE1 and *Chr*-OYE2, respectively, the reactions were terminated by extraction with ethyl acetate. The organic phase was analyzed using GC or HPLC.

Preparative-scale biotransformation was performed in 30-ml reaction system for substrates **4a**, **6a**, and **12a**–**17a** catalyzed with *Chr*-OYE1. The incubation was continued for 12 h. The extracted