Journal of Molecular Catalysis B: Enzymatic 115 (2015) 155–159

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase mutants with improved racemization activity

Musa M. Musa[a,*], Jay M. Patel[b], Christopher M. Nealon[b], Chang Sup Kim[c], Robert S. Phillips[b,d], Ibrahim Karume[a]

[a] Department of Chemistry, King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia
[b] Department of Chemistry, University of Georgia, Athens, GA 30602, USA
[c] Department of Chemical and Biological Engineering, Hanbat National University, Daejeon 305-719, South Korea
[d] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA

ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form 10 February 2015
Accepted 21 February 2015
Available online 2 March 2015

Keywords:
Alcohol dehydrogenase
Racemization
Redox reactions
Secondary alcohols
Site-directed mutagenesis

ABSTRACT

Controlled racemization of enantiopure alcohols is a key step in dynamic kinetic resolution. We recently reported the racemization of enantiopure phenyl-ring-containing alcohols using W110A *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (W110A TeSADH), which relies on selectivity mistakes. Trp-110 lines the large pocket of the active site of TeSADH, which allows W110A TeSADH mutant to accommodate phenyl-ring-containing substrates in Prelog mode, albeit with selectivity mistakes. Here, we report the racemization of enantiopure phenyl-ring-containing alcohols using several Trp-110 mutants of TeSADH in the presence of the oxidized and reduced forms of nicotinamide–adenine dinucleotide. We observed a noticeable enhancement in racemization efficiency when W110G TeSADH was used compared with W110Q, W110M, W110L, W110I, and W110V. This observation was anticipated because the W110G mutation is expected to open the large pocket of the active site to a greater extent compared to other mutants of TeSADH at W110. Both enantiomers of 1-phenyl-2-propanol and 4-phenyl-2-butanol were fully racemized by W110G TeSADH. We also constructed a triple mutant of TeSADH, W110A/I86A/C295A, by site-directed mutagenesis with the aim of opening the two pockets of the active site of TeSADH. The W110A/I86A/C295A mutant was employed to racemize enantiopure phenyl-ring-containing alcohols. The current study demonstrates that W110G and W110A/I86A/C295A TeSADH are more efficient catalysts for the racemization of enantiopure secondary alcohols than the previously reported mutant W110A TeSADH [6].

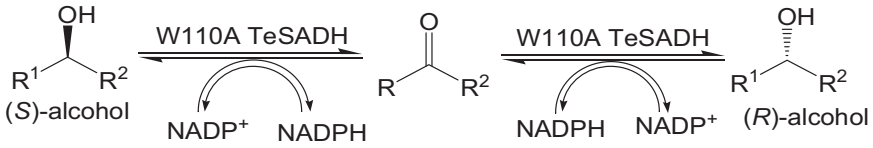
1. Introduction

Deracemization of enantiomers is an ideal alternative to the well-known industrial kinetic resolution (KR) approach, which is limited to 50% chemical yield with high enantiomeric purity and therefore requires separation of starting materials from products. By contrast, dynamic kinetic resolution (DKR) is a deracemization method that involves KR and in situ racemization of the slowly reacting enantiomer, thus enabling complete conversion of a racemic starting material to a single enantiopure product. Developing mild controlled racemization approaches is crucial to enabling the development of new DKR methods. Metal catalysts have been used for controlled racemization in DKR [1,2]. Most established racemization methods involve harsh conditions that use acids or bases and are therefore unsuitable for use in DKR. Modern advances in biotechnology allow enzymes to be altered to improve their effectiveness in organic synthesis [3]. Because nature has a limited requirement for racemization, there is no known enzyme with physiological racemase activity for secondary alcohols that is not accompanied by another functionality [4]. Alcohol dehydrogenases (ADHs) have been employed to racemize enantiopure alcohols [5–8]. Enzyme-catalyzed racemization represents a greener alternative to metal-catalyzed racemization. For effective racemization, an ADH with low stereoselectivity is required. However, only a few non-selective ADHs are known because the primary goal was to search for highly selective enzymes [5].

Secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH; EC 1.1.1.2), a nicotinamide–adenine dinucleotide phosphate ($NADP^+$)-dependent ADH, has high thermal

* Corresponding author. Tel.: +966 13 860 7343; fax: +966 13 860 4277.
E-mail address: musam@kfupm.edu.sa (M.M. Musa).

http://dx.doi.org/10.1016/j.molcatb.2015.02.012
1381-1177/



Scheme 1. W110A TeSADH-catalyzed racemization of enantiopure phenyl-ring-containing secondary alcohols.

stability and tolerance to elevated concentrations of organic solvents, making it a favorable biocatalyst for organic synthesis [9–12]. Several mutants of TeSADH have been designed with expanded substrate specificity compared to the wild type enzyme [13,14]. Temperature [15,16], pressure [17], mutation [18,19] and reaction medium [20] influence the stereospecificity of TeSADH. We recently reported a racemization approach for enantiopure phenyl-ring-containing alcohols using W110A TeSADH in the presence of reduced and oxidized forms of cofactor (i.e., NADPH and $NADP^+$), shown in Scheme 1 [6]. TeSADH follows Prelog's rule; the stereoselectivity toward *S*-configured alcohols is higher if the bulkier alkyl group of the alcohol has higher Cahn–Ingold–Prelog priority. Although ADHs are known for their high enantioselectivities, selectivity mistakes can occur, leading to lower enantioselectivities. The accumulation of selectivity mistakes leads to the formation of racemic mixtures under appropriate conditions and time. Racemization is a thermodynamically downhill process ($\Delta G \approx -RT \ln 2$) because of the entropy increase associated with the formation of two enantiomers from one [21].

We recently created six mutants of TeSADH at the Trp-110 site to enhance the enantioselectivity of the reduction of phenyl-ring-containing ketones [22]. In this study, we evaluated the effect of these small alterations in the active site of TeSADH on the efficiency of the racemization reactions of enantiopure phenyl-ring-containing secondary alcohols. We also report a triple mutant of TeSADH and demonstrate its effectiveness in the racemization of enantiopure alcohols.

2. Experimental

2.1. General

Capillary gas chromatographic measurements were performed on a gas chromatograph (GC) equipped with a flame ionization detector and an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μm film thickness) using Helium as the carrier gas. Nuclear Magnetic Resonance spectra were recorded on a 500 MHz spectrometer at 500 MHz (^{1}H) and at 125 MHz (^{13}C) at room temperature using the solvent peak as an internal standard. Details of GC and GC–MS analysis have been provided in supplementary information. Commercial grade solvents were used without further purification. *Candida antarctica* lipase B (CALB, Novozyme 435), $NADP^+$, NADPH, isopropenyl acetate, (*R*)-1-phenyl-2-propanol [(*R*)-**1a**] (>99% *ee*), (*S*)-1-phenyl-2-propanol [(*S*)-**1a**] (>99% *ee*), (*rac*)-1-phenyl-2-propanol [(*rac*)-**1a**], (*rac*)-4-phenyl-2-butanol [(*rac*)-**2a**], (*R*)-4-phenyl-2-butanol [(*R*)-**2a**] (>99% *ee*), (*S*)-4-phenyl-2-butanol [(*S*)-**2a**] (>99% *ee*), 4-(4′-methoxyphenyl)-2-butanone, and 1-phenyl-2-butanone were used as purchased from commercial sources. (*R*)-**2a** (>99% *ee*), (*S*)-**2a** (72.7% *ee*), and (*R*)-1-phenyl-2-butanol [(*R*)-**4a**] (98% *ee*) were prepared by lipase-catalyzed kinetic resolution of their racemates as reported [22]. (*S*)-4-(4′-methoxyphenyl)-2-butanol [(*S*)-**3a**] (91% *ee*) was prepared by the W110A TeSADH-catalyzed reduction of the corresponding ketone as reported [23].

2.2. Methods

2.2.1. Preparation, gene expression and purification of mutants

All point mutations were introduced by PCR-amplified oligonucleotide-directed mutagenesis using a modified QuikChange site-directed mutagenesis protocol (Agilent) and confirmed by DNA sequencing. The wild-type *T. ethanolicus adhB* gene for TeSADH in the pADHB1M1-kan plasmid (a pBluescriptII KS(+)-kanamycin derivative, Burdette et al.) was used as the template [11,24]. W110G, W110V, W110I, W110Q, W110L, W110M TeSADH (W: tryptophan, G: glycine, V: valine, I: isoleucine, Q: glutamine, L: leucine, M: methionine) were prepared, expressed and purified as reported [22]. To prepare the double mutant, I86A/C295A TeSADH, a C295A mutation (C: cysteine) was introduced in the single mutant I86A [14] using the forward mutagenic primer 5′-ATAAAAGGCGGGCTA**GCC**CCCGGTGGACGTCT and its reverse complement as the reverse mutagenic primer (the mutated nucleotides are bold and underlined). To prepare the triple mutant, W110A/I86A/C295A TeSADH, an additional mutation (W110A) was introduced in the double mutant using the forward mutagenic primer 5′-TGGAATGCTGGCAGGC**GCG**AAATTTTCGAATGTA and its reverse complement as the reverse mutagenic primer. W110A/I86A/C295A TeSADH was then expressed and purified as reported [22], with the exception that the heat treatment was performed at 65 °C instead of 70 °C. Briefly, the TeSADH gene was expressed in *E. coli* DH5α under the control of the *lacZ* promoter on the pBluescriptII KS(+) plasmid. Enzyme concentrations were determined by the Bradford protein assay [25].

2.2.2. General procedure for mutant TeSADH-catalyzed racemization

Enantiopure alcohol (0.015 mmol), $NADP^+$ (0.5 mg), NADPH (1.0 mg), mutant TeSADH (0.2 mg in Tris–HCl buffer solution, 50 mM, pH 8.0), Tris–HCl buffer solution (950 μL, 50 mM, pH 8.0), and acetonitrile (50 μL) were mixed in a 1.5-mL plastic tube. The reaction mixture was shaken at 50 °C and 200 rpm for 48 h, followed by extraction with ethyl acetate (2 × 500 μL). The combined organic layer was dried with sodium sulfate and concentrated to dryness. The remaining residue was treated with pyridine (two drops) and acetic anhydride (one drop) for 1 h to convert the alcohols to their corresponding acetates. The acetate products were diluted with $CHCl_3$ prior to analysis on a GC equipped with a chiral column to determine *ee*.

2.2.3. Determination of the absolute configuration of alcohols

The racemization products were converted to the corresponding acetate derivatives, then injected in a GC equipped with a chiral stationary phase. The resulting retention time was then compared with the *R*- or *S*-acetate derivatives of standard samples of alcohols. The acetate derivatives of the racemization products of enantiopure **1a** and **2a** were co-injected with the acetate derivatives of standard samples of enantiopure alcohols. The derivatized racemization products of (*S*)-**3a** were co-injected with a sample of (*S*)-**3a** prepared by W110A TeSADH-catalyzed reduction of the corresponding ketone, which is known to produce (*S*)-**3a** [23]. The acetate derivatives of racemization products of (*R*)-**4a** were co-injected with a