Archives of Biochemistry and Biophysics 606 (2016) 151–156

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

I86A/C295A mutant secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* has broadened substrate specificity for aryl ketones

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ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form
29 July 2016
Accepted 1 August 2016
Available online 3 August 2016

Keywords:
Mutagenesis
Substrate specificity
Stereospecificity
Alcohol dehydrogenase
Thermophilic

ABSTRACT

Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase (SADH) reduces aliphatic ketones according to Prelog's Rule, with binding pockets for small and large substituents. It was shown previously that the I86A mutant SADH reduces acetophenone, which is not a substrate of wild-type SADH, to give the anti-Prelog *R*-product (Musa, M. M.; Lott, N.; Laivenieks, M.; Watanabe, L.; Vieille, C.; Phillips, R. S. *ChemCatChem* **2009**, 1, 89–93.). However, I86A SADH did not reduce aryl ketones with substituents larger than fluorine. We have now expanded the small pocket of the active site of I86A SADH by mutation of Cys-295 to alanine to allow reaction of substituted acetophenones. As predicted, the double mutant I86A/C295A SADH has broadened substrate specificity for *meta*-substituted, but not *para*-substituted, acetophenones. However, the increase of the substrate specificity of I86A/C295A SADH is accompanied by a decrease in the k_{cat}/K_m values of acetophenones, possibly due to the substrates fitting loosely inside the more open active site. Nevertheless, I86A/C295A SADH gives high conversions and very high enantiomeric excess of the anti-Prelog *R*-alcohols from the tested substrates.

1. Introduction

Two alcohol dehydrogenases, one with a specificity for primary alcohols, and a secondary alcohol dehydrogenase, were purified from *Thermoanaerobacter ethanolicus*, which was isolated from hot springs in Yellowstone National Park [1,2]. The wild-type secondary alcohol dehydrogenase (SADH) follows Prelog's rule in the reduction of ketones, adding the *R*-hydride of the NADPH to the *re*-face of the ketone [3]. The substrate specificity of wild-type SADH is restricted to primarily linear aliphatic and alicyclic ketones [4–9]. Fig. 1 shows the active site of SADH from *Thermoanaerobacter brockii* (formerly *Thermoanaerobium brockii* [10]), which is identical with the enzyme from *T. ethanolicus* [11]. Thus, a number of active site residues have been mutated in order to broaden the substrate specificity of SADH and make it useful in biocatalytic applications. These mutant SADHs, including S39T, C295A, W110A, and I86A, were found to have altered substrate specificity and stereoselectivity [9,12–14].

The I86A mutation of SADH not only expands substrate specificity to include acetophenone, which is a very poor substrate for wild-type SADH, but also reverses the usual preferred stereochemistry to produce the anti-Prelog *R*-product [14]. Unfortunately, I86A SADH exhibits limited reactivity with substituted acetophenones, fluorine being the only tolerable substituent found in the initial study. Homochiral 1-arylalkanols are useful intermediates in the preparation of pharmaceuticals. Thus, it was of interest to expand the active site further to allow the reduction of substituted acetophenones. The C295A mutant SADH was studied previously by Heiss and coworkers, and was found to increase the size of the alkyl group which can bind in the “small pocket” by one carbon atom [12]. Due to the proximity of Cys-295 to Ile-86 (Fig. 1), we predicted that having both mutations in the active site would expand the size of the small pocket to allow binding and reaction of

Abbreviations: ADH, alcohol dehydrogenase; $NADP^+$, nicotinamide adenine dinucleotide phosphate; SADH, *Thermoanaerobacter ethanolicus* secondary ADH.

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http://dx.doi.org/10.1016/j.abb.2016.08.002
0003-9861/

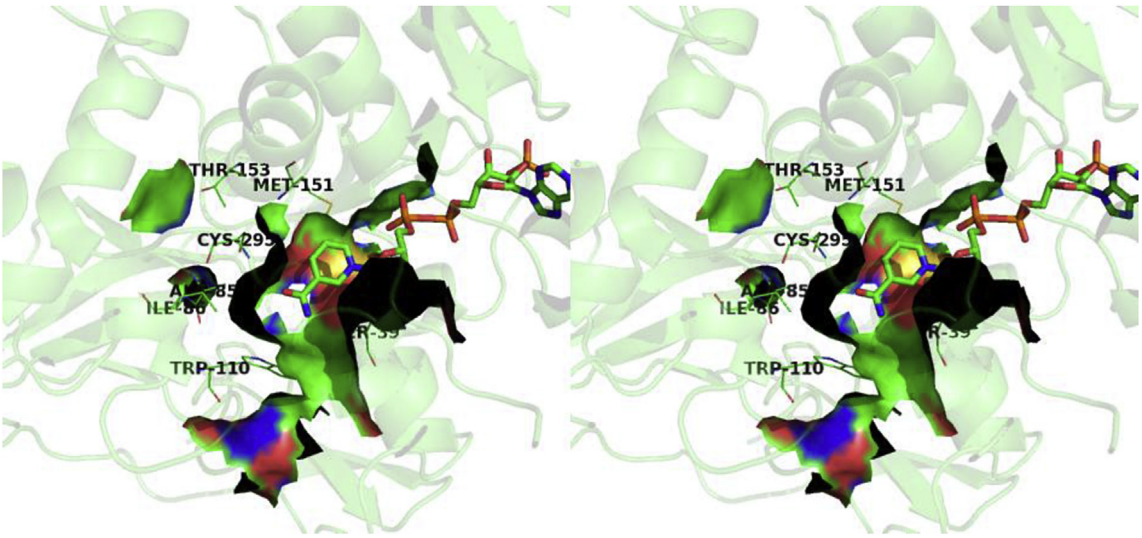

Fig. 1. Stereoview of TbSADH, with residues of interest labeled. $NADP^+$ shown in stick-form and zinc as a cyan sphere. This image was prepared with Pymol (The PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC) using the PDB file (1YKF). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ring-substituted acetophenones with subsitutents larger than fluorine. To test this hypothesis, we have now prepared the I86A/C295A double mutant of SADH, and we have found, as expected, that it has broadened specificity for substituted acetophenones.

2. Materials and methods

2.1. General methods

Gas chromatography was performed with a Varian 3300 GC (Agilent Tech; Santa Clara, CA) using a Supelco (Sigma Aldrich; St. Louis, MO) β-Dex 120 cyclodextrin chiral column (30 m, 0.25 mm [i.d.], 0.25 μm film thickness) with He as the carrier gas and equipped with a flame ionization detector. Kinetic experiments and assays were performed on a Varian Cary 100 UV–visible spectrophotometer (Agilent Tech; Santa Clara, CA) equipped with a Peltier thermoelectric temperature-controlled 12-cell holder. ^1H and ^{13}C NMR analyses were collected on a Varian 400 MHz spectrometer with $CDCl_3$ as the solvent at room temperature with tetramethylsilane or the solvent peak as the reference.

2.2. Materials

Substrates were used as purchased from commercial suppliers with the exception of 3'-bromoacetophenone, which was prepared by a published procedure [15]. Acetophenone was purchased from Fisher Scientific (Waltham, MA). 2′,4′-Difluoroacetophenone was bought from Acros (Geel, Belgium). The 2-acetylpyridine was purchased from Pfaltz and Bauer (waterbury, CT). The rest of the substrates tested were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile was a product of Burdick and Jackson (Morristown, NJ). NADPH was purchased from Acros (Geel, Belgium). The antibiotics, kanamycin and ampicillin, were bought from Roche (Indianapolis, IN) and Fisher Biotech (fair lawn, NJ), respectively. Commercial grade solvents were used without further purification. The RedA-agarose was obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Mutagenesis

The I86A plasmid, made as reported previously [14], was isolated from the host *E. coli* DH5α cells. Using the Quikchange method, the pADHB25-I86A plasmid was further mutated with the following forward and reverse primers, with the mutation indicated in bold. The resulting plasmid was sequenced to confirm the presence of the C295A mutation.

C295A-F (ATAAAAGGCGGGCTA**GCC**CCCGGTGGACG).
C295A-R (TTTCCGCCCGAT**CGG**GGGCCACCTGCAGA).

2.4. Purification of secondary alcohol dehydrogenase

The protein was expressed and purified based on our previously published method [9]. Modifications made to this method include the use of 50 mM Tris HCl, pH 8.0, buffer containing 5 mM DTT and 10 μM $ZnCl_2$, a 5 mL RedA-agarose column, and optimization of the low salt (0.02 M $NaClO_4$) and high salt (0.2 M $NaClO_4$) wash and elution solutions. No further purification was needed after the RedA-agarose column as polyacrylamide gel electrophoresis showed a single band. The pooled fractions were stored at −80 °C.

2.5. Enzyme assays

The enzyme was assayed as previously described [14]. The enzyme assays were performed at 50 °C in triplicate. The enzyme activity was measured in 50 mM potassium phosphate buffer (pH 6.5 at 50 °C) with 0.4 mM NADPH to follow ketone reduction. The initial velocity was recorded on a Varian Cary 100 UV/Vis spectrophotometer at 340 nm for 10 min by monitoring NADPH consumption (ketone reduction). The enzyme was preincubated in 50 mM buffer solution with 5 mM DTT and 10 μM $ZnCl_2$ at 50 °C in the UV/Vis sample compartment for 10 min before addition to the assay mixtures. The substrate stock solutions were prepared in acetonitrile. Each assay contained not more than 5% acetonitrile, since higher concentrations were found to inhibit the enzyme activity. The kinetic data were fit to the Michaelis-Menten equation (Equation (1)) with the HYPERO program of Cleland [16]. The enzyme concentrations were determined from the A_{280} value of 0.82 for a 0.1% solution, and using the subunit molecular weight of 37.7 kDa.

$$v = V^*_{max}[S]/(K_m + [S]) \quad (1)$$