Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology 134 (2008) 240–245
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Directed evolution of transketolase substrate specificity towards an aliphatic aldehyde

Edward G. Hibbert [a], Tarik Senussi [a], Mark E.B. Smith [b], Sean J. Costelloe [a], John M. Ward [c], Helen C. Hailes [b], Paul A. Dalby [a,*]

[a] *Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK*
[b] *Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK*
[c] *Department of Chemistry, University College London, 20 Gordon Street, London WC1H 0AJ, UK*

Received 11 July 2007; received in revised form 9 January 2008; accepted 21 January 2008

Abstract

Mutants of transketolase (TK) with improved substrate specificity towards the non-natural aliphatic aldehyde substrate propionaldehyde have been obtained by directed evolution. We used the same active-site targeted saturation mutagenesis libraries from which we previously identified mutants with improved activity towards glycolaldehyde, which is C2-hydroxylated like all natural TK substrates. Comparison of the new mutants to those obtained previously reveals distinctly different subsets of enzyme active-site mutations with either improved overall enzyme activity, or improved specificity towards either the C2-hydroxylated or non-natural aliphatic aldehyde substrate. While mutation of phylogenetically variant residues was found previously to yield improved enzyme activity on glycolaldehyde, we show here that these mutants in fact gave improved activity on both substrate types. In comparison, the new mutants were obtained at conserved residues which interact with the C2-hydroxyl group of natural substrates, and gave up to 5-fold improvement in specific activity and 64-fold improvement in specificity towards propionaldehyde relative to glycolaldehyde. This suggests that saturation mutagenesis can be more selectively guided for evolution towards either natural or non-natural substrates, using both structural and sequence information.

Keywords: Directed evolution; Biocatalysis; Phylogenetics; Protein engineering; Transketolase; Saturation mutagenesis

1. Introduction

Transketolase catalyses the transfer of a two-carbon ketol unit from D-xylulose-5-phosphate, to either D-ribose-5-phosphate or D-erythrose-4-phosphate (Sprenger et al., 1995). The enantioselective carbon–carbon bond forming ability of transketolase, and the ability to yield an irreversible reaction when using β-hydroxypyruvate (HPA) as the ketol donor, makes it very attractive as a biocatalyst in industrial synthesis (Demuynck et al., 1991; Hobbs et al., 1993; Morris et al., 1996; Schenk et al., 1998; Turner, 2000; Shaeri et al., 2006; Ingram et al., 2007). Although *Escherichia Coli* transketolase can tolerate a range of non-hydroxylated aliphatic aldehyde substrates, the activity is typically only 5–35% of those for α-hydroxylated substrates such as glycolaldehyde (Dalmas and Demuynck, 1993). To achieve industrial viability in large-scale processes, the specific activity of transketolase must be improved for the biocatalytic conversion of aliphatic substrates such as propionaldehyde.

We have recently used saturation mutagenesis targeted to the enzyme active site to significantly enhance transketolase activity towards the C2-hydroxylated substrate glycolaldehyde (Fig. 1) (Hibbert et al., 2007). As the natural substrates for transketolase are also C2-hydroxylated (Fig. 1) screening for variants with improved activity did not necessarily result in altered substrate specificity. Here we have screened the same active-site libraries but now towards the non-hydroxylated aldehyde acceptor substrate, propionaldehyde (PA) (Fig. 1), to identify novel *E. coli* transketolase mutants which have improved activity for the production of 1,3-dihydroxypentan-2-one (DHP). This screen

Abbreviations: DSW, deep square well; Ery, erythrulose; E4P, erythrose-4-phosphate; GA, glycolaldehyde; HPA, hydroxypyruvate; DHP, 1,3-dihydroxypentan-2-one; TK, transketolase; TPP, thiamine pyrophosphate; TPTC, 2,3,5-triphenyltetrazolium chloride.

* Corresponding author.
E-mail address: p.dalby@ucl.ac.uk (P.A. Dalby).

0168-1656/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2008.01.018

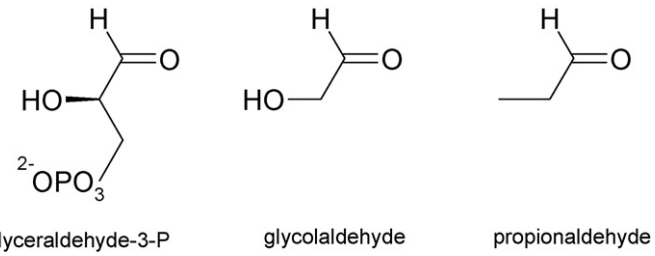

Fig. 1. Progression from natural phosphorylated aldehyde acceptor substrate, to the non-phosphorylated glycolaldehyde studied previously (Hibbert et al., 2007) and the non-hydroxylated propionaldehyde studied here.

employed a TLC-based and also a recently developed colorimetric high-throughput assay for the non-hydroxylated substrate reaction with TK (Smith et al., 2006). Comparison of the specific activities of the new mutants and also those obtained previously, towards both the C2-hydroxylated glycolaldehyde and the non-hydroxylated propionaldehyde substrates, reveals distinctly different subsets of active-site mutations. Previously it was observed that improvements in activity towards the C2-hydroxylated (and non-phosphorylated) substrate were found only for mutations at phylogenetically variant sites, and at conserved sites that interact with the phosphate group in natural substrates. However, in the present study, a new and distinct cluster of residues was found to produce the greatest improvements in activity towards propionaldehyde. More notably, these mutants also resulted in a significant shift in substrate specificity in contrast to the previously identified residues which mostly improved the overall enzyme activity towards both substrates.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma and used as supplied, except β-hydroxypyruvate (HPA) which was prepared as the lithium salt by modification of a previously described procedure (Morris et al., 1996).

2.2. Saturation mutagenesis libraries

The fully randomised saturation mutagenesis libraries have been described previously (Hibbert et al., 2007), and are targeted to a total of seventeen active-site residues with low phylogenetic sequence entropy (0–0.96): H26, H100, I189, H261, R358, S385, H461, D469, R520, and high phylogenetic sequence entropy (0.76–1.98): A29, N64, M159, S188, D259, A383, P384, and V409. Each library contains 90 randomly picked variants and three wild-type controls where transketolase is expressed from the self-promoting tktA gene in the plasmid pQR711 in TOP10 or XL10 (Stratagene) *E. coli* cells. Replicated reaction plates used here contain 100 μl of each overnight culture in 96-well polypropylene plates. High-throughput screening of a total of 1800 random variants was chosen rather than first using DNA sequencing to identify all the possible variants. The latter approach statistically requires more than 3-fold oversampling to obtain all 400 possible variants, and thus at least 4800 DNA sequences to cover the 2 kb TK gene where each sequence gives 500 additional base-pairs.

2.3. Screening of libraries for activity

Reaction plates were thawed twice from −80 °C resulting in freeze–thaw lysis of the 100 μl of cell culture. A 12× cofactor stock solution (25 μl of 28.8 mM TPP, 108 mM $MgCl_2$ in 50 mM Tris–HCl, pH 7.0) and β-HPA stock (70 μl of 200 mM LiHPA in 50 mM Tris–HCl, Ph 7.0) was added then incubated for 20 min, prior to initiation of the reaction with propionaldehyde (PA) (100 μl of 150 mM PA in 50 mM Tris–HCl, pH 7.0). Reactions were sealed using a Uniseal polypropylene mat (Whatman, Brentford UK). After one hour at 25 °C, reactions were analysed by both TLC and a colorimetric assay described previously (Smith et al., 2006). TLC on silica plates was carried out with a 1:1 ethyl acetate:hexanes mobile phase, and 1,3-dihydroxypentan-2-one product (Rf = 0.3) visualised with phosphomolybdic acid and then heating. For the colorimetric assay, 50 μl of reaction product was transferred to a fresh 96-well plate containing 50 μl of 50 mM Tris–HCl, pH 7.0, then 20 mg of MP-carbonate scavenger resin (Biotage) was added using a resin loader (Radleys). The plate was allowed to stand for 3 h before a further 100 μl 50 mM Tris–HCl, pH 7.0, was added to each well and 50 μl of the mix was then transferred from each well into a fresh plate. A plate reader (Fluostar, BMG-labtech) fitted with an autoinjector was then used to add 20 μl of 2,3,5-triphenyltetrazolium chloride (TPTC) solution (0.2% solution in methanol), followed by 10 μl of 3 M NaOH. The plate was shaken for 10 s, allowed to sit for 60 s and then an absorbance reading of each well was taken at 485 nm. A total of 1530 clones were screened.

2.4. High-accuracy characterisation of selected mutants

Glycerol stocks of potentially interesting mutants were streaked from the library master plate onto LB agar containing 150 μg ml^{-1} ampicillin. Individual colonies were selected and grown in 10 ml LB with 150 μg ml^{-1} ampicillin for 16 h at 200 rpm, 37 °C. 8 ml of culture was centrifuged at 10,000 *g* for 10 min and resuspended in 2 ml of 5 mM sodium phosphate buffer, pH 7.0, before sonication for 10 cycles of 10 s. The lysate was clarified by centrifuging at 10,500 rpm for 10 min. 33% (v/v) of lysate was used in triplicate reactions with a final volume of 300 μl. The reactions were performed at 25 °C in sealed glass vials. 100 μl of lysate was added to 25 μl of cofactor solution (28 mM TPP, 108 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.0) and 75 μl of ketone solution (200 mM LiHPA, 50 mM Tris–HCl, pH 7.0), then allowed to stand for 20 min. 100 μl of aldehyde solution was then added (150 mM propionaldehyde or glycolaldehyde, 50 mM Tris–HCl, pH 7.0) to initiate the reaction. Samples from the reactions were quenched by 1:9 dilution in 0.1% TFA, and product was determined by HPLC using a 15 cm C18 column and a 15 min isocratic protocol of 0.1% TFA in 5% acetonitrile with a flow rate of 0.6 ml min^{-1}, and detection of product by UV at 200 nm. Standard curves