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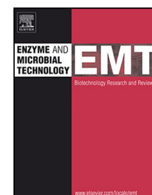

Second generation engineering of transketolase for polar aromatic aldehyde substrates

Panwajee Payongsri [a], David Steadman [b], Helen C. Hailes [b], Paul A. Dalby [a,*]

[a] Department of Biochemical Engineering, University College London, Gordon Street, London WC1H 0AH, UK
[b] Department of Chemistry, University College London, 20 Gordon Street, London WC1H 0AJ, UK

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Transketolase has significant industrial potential for the asymmetric synthesis of carbon—carbon bonds with new chiral centres. Variants evolved on propanal were found previously with nascent activity on polar aromatic aldehydes 3-formylbenzoic acid (3-FBA), 4-formylbenzoic acid (4-FBA), and 3-hydroxybenzaldehyde (3-HBA), suggesting a potential novel route to analogues of chloramphenicol. Here we evolved improved transketolase activities towards aromatic aldehydes, by saturation mutagenesis of two active-site residues (R358 and S385), predicted to interact with the aromatic substituents. S385 variants selectively controlled the aromatic substrate preference, with up to 13-fold enhanced activities, and K_M values comparable to those of natural substrates with wild-type transketolase. S385E even completely removed the substrate inhibition for 3-FBA, observed in all previous variants. The mechanisms of catalytic improvement were both mutation type and substrate dependent. S385E improved 3-FBA activity via k_{cat}, but reduced 4-FBA activity via K_M. Conversely, S385Y/T improved 3-FBA activity via K_M and 4-FBA activity via k_{cat}. This suggested that both substrate proximity and active-site orientation are very sensitive to mutation. Comparison of all variant activities on each substrate indicated different binding modes for the three aromatic substrates, supported by computational docking. This highlights a potential divergence in the evolution of different substrate specificities, with implications for enzyme engineering.

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1. Introduction

Asymmetric carbon—carbon bond formation is a powerful method in organic synthesis [1–8] and new routes could provide access to a wide range of novel and natural compounds that serve as building blocks for further synthesis [2]. Enzymes such as transketolase (TK) (EC 2.2.1.1) have been shown to catalyse asymmetric carbon-carbon bond formation with considerable synthetic potential due to their high selectivity and specificity [1,9–13]. TK belongs to the thiamine diphosphate (ThDP) dependent enzyme family, and plays two crucial roles in the non-oxidative pentose phosphate pathway and Calvin cycle [14]. It catalyses the reversible transfer of a C_2-hydroxyketone group from a ketol donor such as ketose sugars, to an aldehyde [15–17]. TK-catalysed reactions produce an α,α′-dihydroxy ketone group which is found in a wide range of natural compounds such as carbohydrates and corticosteroids [18]. Hydroxypyruvate has been extensively used as a ketol donor instead of the natural ketose sugar substrates, due to the production of carbon dioxide, which can drive the reaction to completion. The use of TK could provide advantages over chemical synthesis routes which tend to suffer from low yields due to multistep procedures [18] and racemic products or low stereoselectivities when a single step method is applied [19,20]. So far, transketolases from several organisms have been exploited in new synthetic routes to carbohydrates and sugar analogues [21–24], (+)-*exo*-brevicomin [25], N-hydroxypyrrolidine [26], furaneol [27], and the glycosidase inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol [28]. By coupling a transaminase (TAm) step as a TK-TAm pathway, chiral amino-alcohols can also be synthesised enantioselectively [29]. Extending the acceptance of this pathway to include aromatic aldehydes would open up routes to chiral aromatic amino-alcohols such as chloramphenicol antibiotics, norephedrine, and also their analogues with alternative aromatic substituents.

Although wild-type transketolases can accept a wide range of aliphatic, aromatic, and cyclic aldehydes [8,17,21,23,30], short

Abbreviations: 3-FBA, 3-formylbenzoic acid; 3-HBA, 3-hydroxybenzaldehyde; 4-FBA, 4-formylbenzoic acid; HPA, hydroxypyruvic acid; TFA, trifluoroacetic acid; ThDP, thiamine diphosphate; TK, transketolase.

* Corresponding author at: Department of Biochemical Engineering, University College London, Gordon Street, London WC1H 0AH, UK. Tel.: +44 02076799566.
E-mail address: p.dalby@ucl.ac.uk (P.A. Dalby).

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chain aliphatic aldehydes tend to give faster reactions than more sterically-challenging substrates [21,23,30]. In addition, most of the synthetic products previously reported used aliphatic hydroxylated aldehydes as starting materials. Expansion of the substrate range accepted by transketolases will increase their potential use for the synthesis of novel dihydroxy ketone compounds. Indeed, protein engineering of WT *E. coli* TK has been successfully used to improve its activity towards aliphatic aldehydes [31], with both enhanced and reversed stereospecificity [32–35], and also improved activity towards D-ribose and D-glucose [36]. By contrast, aromatic aldehydes still suffer from low reaction yields [33], except 3-formylbenzoic acid (3-FBA) and 4-formylbenzoic acid (4-FBA) which introduce a beneficial binding interaction between their carboxylic acid group and the transketolase phosphate-binding residues [37]. The *E. coli* TK variant D469T was found previously to have the highest activity towards the carboxylated aromatic substrates 3-FBA and 4-FBA, from all TK variants tested [37]. However, the best activity was still low compared to any natural substrates, and for 3-FBA, substrate inhibition was also always present.

Saturation mutagenesis at residues that interact directly with substrates have the greatest potential to improve enzyme activity and stereospecificity in a single round of evolution, compared to mutation of residues at more distant locations [38]. We have therefore applied a targeted saturation mutagenesis strategy to further improve the activity and yield towards the substituted benzaldehydes 3-FBA, 4-FBA and additionally, 3-hydroxybenzaldehyde (3-HBA) (Scheme 1).

Previously, the variant D469T/R520Q was found to retain most of the activity of D469T, towards 3-FBA and 4-FBA. The saturation mutagenesis was targeted into D469T/R520Q, as it is also more readily able to accommodate further mutations due to stabilisation of the enzyme in terms of protection from the formation of insoluble aggregates [39]. Residue S385 is within one of two cofactor-binding loops (residues 383–393) present in each active-site of *E. coli* TK, and not structured in the apo-enzyme [40]. The side chain hydroxyl group of S385 can form a hydrogen bond with the phosphate moiety of natural substrates, as determined from several *E. coli* TK crystal structures with substrates (2R5N, 2R8O, 2R8P) [41]. Residue R358 is also involved in binding the phosphate moiety of natural substrates [41], as confirmed by selected mutagenesis of the yeast TK at the equivalent site, such that R359A resulted in a 2-fold and 38-fold increase in K_M for xylulose 5-phosphate and ribose 5-phosphate, respectively [42]. Previous mutations, R358P and R358L also indicated an analogous interaction with the carboxylate moieties of 3-FBA and 4-FBA [37]. In our previous work, saturation mutagenesis of S385 and R358, along with 18 other sites within the wild-type enzyme, aimed to improve activity towards non-aromatic aldehydes, glycolaldehyde and propanal [31,43]. While several other sites gave significantly improved activities, S385 gave no variants of interest, and R358 yielded only R358I with a 2-fold improvement of activity towards each substrate. Here, S385 and R358 have again been mutated to all possible amino acids, but within D469T/R520Q instead of wild-type TK, resulting in improved activities towards substituted aromatic aldehydes.

2. Materials and methods

2.1. Chemicals and reagents

All chemical reagents were purchased from Sigma–Aldrich (Aldrich® Chemistry, UK), otherwise stated. Lithium hydroxypyruvate was prepared according to the previous protocol [10].

2.2. Transketolase library

Mutations were constructed on D469T/R520Q due to its stability [39], and initial activity on the aromatic aldehydes [37]. The transketolase D469T/R520Q variant gene and all further variants were expressed under the control of *tktA* gene promoter in the plasmid pQR791 in XL-10 Gold (Stratagene) [40]. Target residues for saturation mutagenesis were from those determined to be in the first shell [43]. Mutagenesis was targeted only to those involved in binding phosphate groups within natural substrates, as determined by previous mutagenesis and crystal structure analysis [41]. These included S385, R358, H461 and R520. R520 was already mutated to R520Q in the parent variant D469T/R520Q, and so not mutated further. H461 was also excluded from further mutation as it was previously found to lead to stability issues [37,39]. Mutagenesis was carried out using Quikchange site directed mutagenesis (Stratagene). The *dpnI*-digested PCR product was transformed into XL-10 gold competent cells (Stratagene). The quality and diversity of the PCR was checked by DNA sequencing prior to screening. The primers used for additional variant construction were listed below (codon underlined, mutations in bold, and N is an equal mix of all four bases, H is 33% A, 33% C and 33% T, and S is 50% G and 50% C).

R358X GAAAATCGCCAGC**NHN**AAAGCGTCTCAGAATG

S385X GCTGACCTGGCGCCG**NHS**AACCTGACCCTGTGG

The NHN and NHS codons both exclude Cys, Trp, Arg and Gly amino-acids.

2.3. Library screening

After the transformation, 24 colonies for each library were inoculated into 12 ml LB media with 150 μg/ml ampicillin, incubated for 18 h at 37 °C, shaking at 250 rpm and then 2 ml of the culture was centrifuged at 13,000 rpm for 10 min. The probability of coverage and possible number of amino acid replacements was calculated using the online tool: GLUE-IT [44] (currently maintained at http://guinevere.otago.ac.nz/cgi-bin/aef/glue-IT.pl). From each codon mix used, the two libraries could produce 16 possible variants and so 11.6 distinct amino acids were expected within 24 randomly picked colonies. The diversity of mutations was confirmed by DNA sequencing of at least 6 random colonies from each library.

Cell pellets were resuspended in 200 μl of 2× cofactor solution (4.8 mM ThDP and 18 mM $MgCl_2$ in 50 mM Tris.HCl, pH 7.0), incubated for 20 min, and 150 μl then transferred to a borosilicate microplate (Radleys, Essex, UK). Screens were performed on whole cells to avoid the influence of uneven cell disruption on TK release and the bioconversion rate. Final yields of products using whole cells and clarified lysates, were found to correlate well (supplementary information Figs. S1 & S2). Reactions were started by the addition of 150 μl of 2× substrate solution (100 mM 3-FBA and 100 mM HPA, 80 mM 4-FBA and 80 mM HPA, or 30 mM 3-HBA and 60 mM HPA, each in 50 mM Tris buffer, pH 7.0). All reactions were performed at 22 °C with shaking at 200 rpm, 3 mm amplitude, to avoid cell sedimentation, and sealed to prevent evaporation (Thermo Scientific Nunc). Conversion to product was determined in 3-FBA samples taken at 1 h and 24 h after the reaction was started. Conversion at 1 h provided a comparison of activities, whereas conversion at 24 h provided final product yields. For the slower reactions with 4-FBA and 3-HBA, the libraries were screened for conversion after 18 h to identify variants with improved activities.

At each time-point, 20 μl of the reaction sample was added to 380 μl 0.1% TFA, centrifuged at 13,000 rpm for 3 min, and supernatants analysed by HPLC with an ACE5 C18 reverse phase column (150 × 4.6 mm). The 3-FBA and 4-FBA reaction samples were analysed as previously described [37] using two mobile phases 0.1% TFA and 100% acetonitrile, and a flow rate of 1 ml/min (see SI for further details). The 3-HBA reaction samples were analysed by gradient elution as follows. Using a mobile phase of 0.2 M acetic acid and 80% methanol at the flow rate of 1 ml/min, the flow profile was separated into 3 phases: 1st phase 5 min using 90% 0.2 M acetic acid/10% methanol (80% v/v); 2nd phase ramped linearly to 40% 0.2 M acetic acid/60% methanol (80% v/v) over 14 min, and the last phase maintaining 40% 0.2 M acetic acid/60% methanol (80% v/v) for 6 min. The column was then re-equilibrated for 3 min using 90% 0.2 M acetic acid/10% methanol (80% v/v). The retention time of the product from the 3-HBA reaction was 4.7 min and that for 3-HBA was 14.5 min.

2.4. Cell culture and protein quantification for detailed enzyme kinetics

Glycerol stocks of selected variants were re-streaked on 150 μg/ml ampicillin-LB plates and incubated at 37 °C for 18 h. Single colonies were inoculated into 20 ml LB with 150 μg/ml ampicillin in 250 ml shake flasks, incubated at 37 °C with shaking at 250 rpm for 18 h, then harvested by centrifugation. Supernatant-free cell pellets were resuspended in Tris buffer, pH 7.0 and sonicated on ice (MSE Soniprep 150 probe, Sanyo) with 10 s on, 15 s off for 10 cycles. Cell debris was removed by centrifugation at 17,700 × g for 10 min at 4 °C. The clarified lysate was aliquotted and stored at −80 °C. Total protein concentration in the clarified lysate was determined using the Bradford assay with BSA as a standard protein. The lysate was further analysed by SDS-PAGE and densitometry as previously described [43] to determine the TK concentration which was always over-expressed to above 20% of the total protein. The plasmids of these variants were extracted and sequenced to identify the mutation. The whole procedure was performed in triplicate for each variant.

2.5. Detailed enzyme kinetics

Detailed enzyme kinetic analyses were carried out on clarified lysates, as this is the form most relevant to industrial biocatalytic processes, either as an isolated enzyme or within a de novo pathway [45]. TK enzyme purification has also

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