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Improving and Inverting C_β-stereoselectivity of Threonine Aldolase via Substrate-Binding-Guided Mutagenesis and a Stepwise Visual Screening

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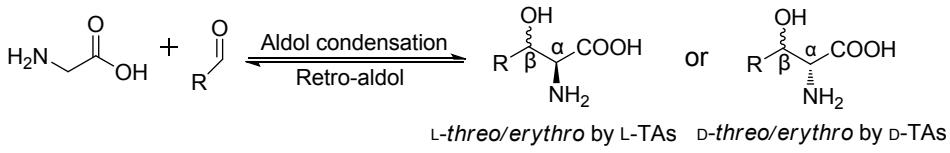
ABSTRACT: Threonine aldolase-catalyzed aldol condensation provides one of the powerful tools for C-C bond formation under mild conditions, but the low C_β-stereoselectivity has hampered its wide application. A stepwise visible screening method was developed to measure the activity and stereoselectivity of threonine aldolase-catalyzed aldol condensation by employing a stereoselective phenylserine dehydratase, enabling direct selection of mutants with higher C_β-stereoselectivity. Mutants of L-PsTA from *Pseudomonas* sp. with improved or inverted stereoselectivity towards aromatic aldehydes were obtained by simultaneously mutating the amino acid residues which interact with the amino and hydroxyl groups of the substrate and

screening the resulting mutant libraries with this method. The mutation and enzyme-substrate docking studies provided some insights into the regulation of the C_β-stereoselectivity by the enzyme-substrate interactions. This study offers a tool and useful guidance for further engineering of TAs to address the C_β-stereoselectivity problem.

KEYWORDS: Threonine aldolase, High-throughput screening, Stereoselectivity, Enzyme Engineering, C-C bond formation

INTRODUCTION

Threonine aldolases (TAs), a class of pyridoxal 5'-phosphate (PLP)-dependent enzymes, catalyze the aldol condensation of aldehydes with glycine, providing one of the useful tools to construct two stereogenic centers in a single reaction under mild conditions.[1-2] The resulting products β-hydroxy-α-amino acids are valuable precursors for the active ingredients of pharmaceuticals and agrochemicals such as thiamphenicol, L-3,4-dihydroxyphenylserine.[3-4] Several TAs from different origins have been isolated and characterized.[4-11] L-TAs catalyze the aldol addition of glycine to aldehydes to give both L-*threo* and L-*erythro* products, while a mixture of D-*threo*/D-*erythro* products are obtained for D-TAs (Scheme 1).[12,13] They are generally stereoselective for the α-carbon (>99% ee), but suffer from moderate to poor stereoselectivity at the β-carbon.[12] The insufficient C_β-stereoselectivity has limited the application of TAs in organic synthesis.[14]



Scheme 1. Aldol and retro-aldol reaction catalyzed by L- or D-TAs

Although enzyme engineering is a powerful strategy for generating mutants with excellent properties,[15,16] it has been rarely applied to improve the C_β-stereoselectivity of TAs with limited success.[6,14] This is largely due to the lack of suitable screening method for determining the enzyme activity and the C_β-stereoselectivity at same time, and the variation of C_β-stereoselectivity during the reaction process of kinetic or thermodynamic control.[13] Given the importance of TAs in potential industrial applications, several efforts have been made to increase the enzyme stability by protein engineering or enzyme immobilization. In these studies, the screening methods were based on detecting the released aldehyde in the retro-aldol reaction of β-hydroxy-α-amino acids [6,17-19] or the consumption of toxic aldehyde in the aldol reaction, and the mutants with higher activity or stability could be selected.[20] Following the First Law of Directed Evolution-'you get what you screen for',[21] the direct measurement of the stereoselectivity in the TA-catalyzed formation of β-hydroxy-α-amino acids would be very important. There is no high-throughput screening (HTS) method for the selection of TA mutants based on the stereoselectivity, presenting the experimental bottleneck in the engineering of this enzyme. At the initial stage of TA-catalyzed aldol condensation, the reaction is under kinetic control and a higher C_β-stereoselectivity can be obtained. However, as the reaction proceeds, it often leads to a thermodynamically controlled low ratio of product diastereomers. This variation presents another great challenge to identify desired C_β-stereoselective TAs.

In this context, we have developed a stepwise visual screening (SVS) method that incorporates a stereoselective phenylserine dehydratase (PSDH) for determining both the conversion and the de value of the product. By employing this SVS method, mutant enzymes with improved or inverted C_β-stereoselectivity and enhanced activity towards aromatic aldehyde were obtained from an L-TA of *Pseudomonas* sp.

RESULTS AND DISCUSSION

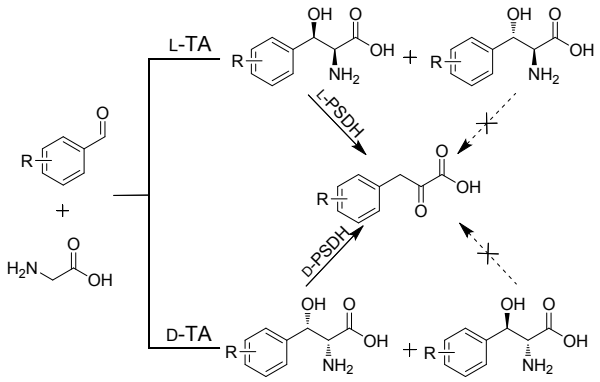
In our search for potential catalysts for asymmetric synthesis of β-hydroxy-α-amino acids, twenty L-TAs from different origins were cloned, out of which eighteen L-TAs were expressed as soluble proteins (Table S1 and Figure S1). Whole cell biotransformations were performed using benzaldehyde and glycine, but all the L-TAs showed poor stereoselectivity at β-carbon (14-46% de).

A suitable HTS method greatly contributes to acquisition of the mutants with high activity and stereoselectivity against particular substrates.[22,23] We attempted to develop a HTS method, that could measure the activity and stereoselectivity of TAs, by employing a stereoselective enzyme involved in the transformations of β-hydroxy-α-amino acid. According to the analysis of functional groups, β-hydroxy-α-amino acid can undergo dehydrogenation, dehydration and decarboxylation (Scheme S1). Phenylserine dehydrogenase[24] and decarboxylase[25,26] are deemed not suitable for this purpose because they are not able to differentiate the chirality at the β-carbon of β-hydroxy-α-amino acids. The PLP-dependent phenylserine dehydratase catalyzes the dehydration reaction of phenylserine to form phenylpyruvate. This enzyme is stereoselective at β-carbon, and only D- or L-*threo* isomer can be dehydrated.[27,28] Therefore, phenylserine dehydratase might be coupled with aldol condensation reaction to develop a HTS method for screening TA mutants based on the C_β-stereoselectivity.

The bacterial strains available in our laboratory were screened for the dehydratase activity and the strain *Paraburkholderia xenovorans* (DSM 17367) was found to have dehydration activity towards DL-*threo*-phenylserine. Based on the *P. xenovorans* genome information, the dehydratase gene (GenBank: AIP32383) was amplified and expressed (Figure S2). The stereoselectivity measurement of the recombinant dehydratase PxPSDH showed that only L-

threo-phenylserine was completely dehydrated (Figure S3), indicating that it is a promising enzyme for our purpose. Substituted phenylserine derivatives were produced in high conversion (50%) and ee (>99%) for substrates with *ortho*- and *para*-substituents (*o*-F, Cl, Br, *p*-F, Cl, Br), although *meta*-substituted phenylserines (*m*-F, Cl, Br) were obtained with modest ee (Table S2). As such, L-PxPSDH possessed a relatively wide substrate scope and was selected as a tool enzyme for developing the screening method.

L-TA from *Pseudomonas* sp. (L-PsTA) was chosen to demonstrate the reliability of the HTS method because it had a wide range of substrate specificity towards aromatic aldehydes, and would be a good template for the further engineering.[7] The aldol condensation reaction of benzaldehyde (10 mM) and glycine (100 mM) was carried out with purified L-PsTA. After the reaction was terminated and the reaction mixture was extracted with dichloromethane, the dehydration reaction was performed by adding L-PxPSDH into the aqueous solution. The unreacted benzaldehyde and the generated phenylpyruvate were detected by the 2,4-dinitrophenylhydrazine (DNPH) method after 40 times dilution to quantitatively measure the carbonyl group of the molecules (Figure S4).[29] HPLC analysis was also carried out to measure the amounts of the L-β-phenylserine. The results measured by colorimetric assay and HPLC analysis indicated that they followed a similar trend. Accordingly, a stepwise screening method was established for determining conversion and product de value of TA-catalyzed aldol reaction by coupling with the dehydration (Scheme 2), where the consumption of aldehyde and the production of phenylpyruvate could be measured in terms of the yield of L-*threo/erythro*-phenylserine and L-*threo*-phenylserine, respectively. The screening assay of D-TAs can be established in the same way by using the reported D-PSDH which specifically catalyzes dehydration of D-*threo*-phenylserine.[27]



Scheme 2. Principles of screening assay coupled with stereoselective PSDH

Restricted by the interference between aldehyde carbonyl and ketone carbonyl and the detection range of DNPH method, purpald and Fe^{3+} were investigated as alternative reagents for the determination of benzaldehyde and phenylpyruvate, respectively.[30,31] As phenylpyruvate can form a coplanar enol structure, it can react with Fe^{3+} to form a complex with cyan color (Figure 1A). Multi-wavelengths scanning showed that 540 and 640 nm were suitable detection wavelength for the purpald and Fe^{3+} colorimetric assays, respectively (Figure 1B). Furthermore, the target detection compounds could be measured without the dilution and extraction operations as the detection ranges were up to 10 mM (Figure 1C-D). The aldol addition reactions were carried out by whole cell biotransformation of eighteen newly cloned L-TAs and the results of conversion and *de* measured by this method followed a similar trend as those obtained by HPLC analysis (Figure 1E). Because the purpald and Fe^{3+} complexes display distinct colors (Figure 1D), this stepwise visible screening (SVS) method offers a rapid HTS, in which the entire processing-time of one 96 deep-well plate is no more than 5.5 h compared to 30 h by HPLC (Figure S5). It should be noted that by choosing suitable stereoselective dehydratases of diverse substrate profiles, this screening method could be useful in the screening of TAs toward a wide range of target substrates.

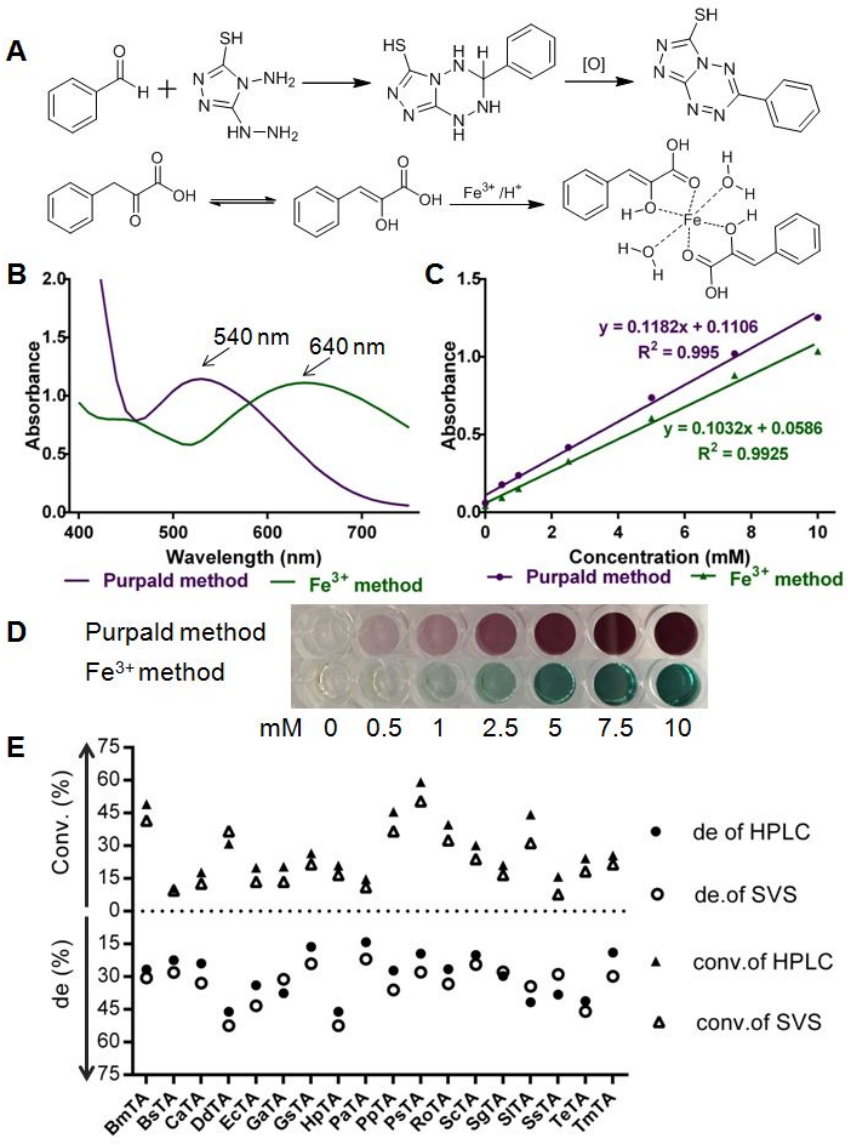


Figure 1. (A) Reactions of colorimetric assays for benzaldehyde and phenylpyruvic acid. (B) Multi-wavelength scanning. (C) Calibration curves. (D) Photos of colorimetric assays at different concentrations. (E) Conversion and de values of whole cell biotransformation with newly cloned TAs measured by HPLC and SVS assay.

This screening method was then applied in the mutation of L-PsTA in an effort to obtain the mutant enzymes with improved C_β-stereoselectivity. A structural model of L-PsTA was constructed based on the known crystal structure (PDB: 1V72) and L-β-phenylserine was docked

into the homology model using Discovery Studio 4.1 (Accelrys). Initially, the site-saturation mutagenesis of the amino acid residues lining the substrate-binding pocket (Figure 2A) was performed and the resulting mutant libraries were screened by this SVS method. The aldol condensation reactions were carried out for 12 h to ensure that the reactions were under thermodynamic control.[32,33] Unfortunately, no mutant with improved activity and stereoselectivity was obtained. Our results are consistent with the previous kinetic and crystallographic studies of *E. coli* L-TA, which suggest that the substrate preference between the *threo*- and *erythro*- isomers of L-TAs may be determined by the overall microenvironment that surrounds the substrate bound at the enzyme active site rather than by one specific residue.[34] We thus assumed that single amino residue might not exert meaningful effect on the activity and stereoselectivity. As such, to examine the cooperative effects of the residues, the adjacent residues (S10/N12, A34/Y35, H89/D93, I132/H133, T146/E147, A176/R177, T206/K207, R321/M323) were grouped together[35-37] and the combinatorial active-site saturation libraries were constructed.[38] The following screening also resulted in no mutant with drastically improved catalytic activity and stereoselectivity.

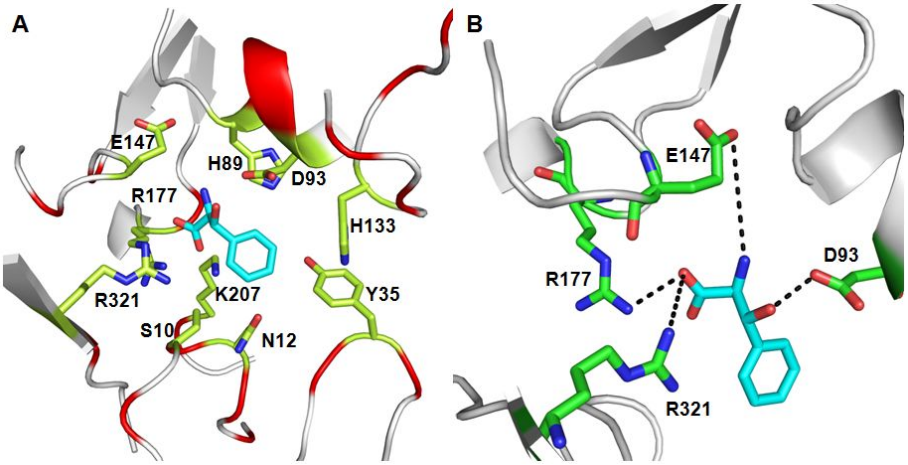
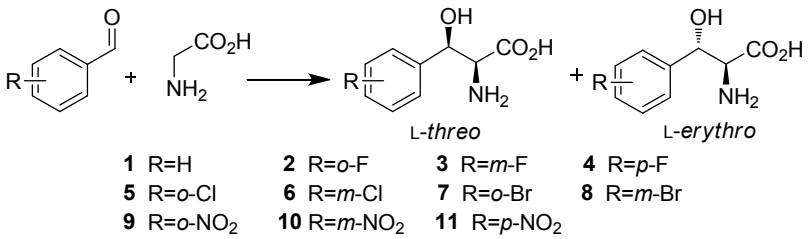


Figure 2. Target amino acid residues chosen for mutagenesis marked in the homology mode of L-PsTA. (A) The initial selected ten amino acid residues (S10, N12, Y35, H89, D93, H133, E147,

R177, K207 and R321) are displayed in lemon. The extended eighteen sites which showed in cartoon are displayed in red. (B) Amino acid residues selection based on interaction with different functional groups of substrate (black dashed lines).

As shown in Figure 2B, the carboxylate and hydroxyl group of the substrate interacts with R177 and R321 or D93, respectively. E147 was close to the amino group of substrate without direct interaction with each other due to the steric hindrance. After the above unsuccessful attempts, that usually work well for other enzymes,[37,39] we envisioned that in order to improve the stereoselectivity of TAs, it might be necessary to tune the interactions of the amino acid residues with the different functional groups of the substrate by simultaneously mutating the amino acid residues which interact with different functional groups of the substrate. To prove this hypothesis, three saturation mutagenesis libraries (D93/E147, E147/R321 and R177/R321) were created and screened by the SVS method using benzaldehyde (**1**) and *o*-fluorobenzaldehyde (**2**) as the aldehyde substrate. Positive mutants from D93/E147 mutant library exhibited improvement or reversal of C_β-stereoselectivity. The wild-type enzyme catalyzed the aldol addition of **2** and glycine to afford the product with 31% de, whereas D93F/E147D, D93H/E147D and D93N/E147D catalyzed the reaction with de of 68%, 69% and -40%, respectively (Table 1). D93S/E147D with lower de was also identified as the variant had higher activity. It has been reported that E90 and D126 of L-TA from *A. jandaei*, which were located close to H85 and H128, respectively, were observed to play important roles in aiding these two histidine residues during the recognition of substrate.[40] In our case, D93 is close to H89 (corresponding to H85), while E147 is not near H133 (corresponding to H128) as shown in the Figure S6. The mutation of D147 may not directly aid H133 but affect the overall microenvironment during the recognition of substrate.

Table 1. Synthesis of β-hydroxy-α-amino acids employing L-PsTA and its mutants[a]



Substrate	WT	D93F/E147D	D93H/E147D	D93N/E147D
1	55[b], 19[c] (*threo*)	42, 12 (*erythro*)	62, 30 (*threo*)	36, 74 (*erythro*)
2	55, 31 (*threo*)	80, 68 (*threo*)	84, 69 (*threo*)	40, 40 (*erythro*)
3	47, 36 (*threo*)	54, 20 (*threo*)	43, 40 (*threo*)	65, 59 (*erythro*)
4	54, 36 (*threo*)	84, 18 (*threo*)	84, 26 (*threo*)	61, 43 (*erythro*)
5	75, 48 (*threo*)	34, 39 (*threo*)	91, 71 (*threo*)	43, 59 (*erythro*)
6	64, 26 (*threo*)	73, 25 (*threo*)	91, 26 (*threo*)	33, 49 (*erythro*)
7	62, 33 (*threo*)	46, 64 (*threo*)	81, 68 (*threo*)	38, 49 (*erythro*)
8	54, 59 (*threo*)	64, 9 (*erythro*)	66, 9 (*threo*)	30, 44 (*erythro*)
9	50, 33 (*threo*)	60, 36 (*threo*)	62, 45 (*threo*)	13, 24 (*erythro*)
10	43, 41 (*threo*)	62, 40 (*threo*)	63, 38 (*threo*)	41, 38 (*threo*)
11	42, 28 (*threo*)	45, 47 (*threo*)	29, 55 (*threo*)	46, 37 (*threo*)

[a]The reactions were carried out as follows: aldehyde (100 mM), glycine (1 M), PLP (10 μM), 10% DMSO (v/v) and purified mutants (1 mg/mL) were mixed in Tris-HCl buffer (100 mM, pH 7.5). The reactions were performed at 25°C for 12h. [b]Yield in percent. [c]de value in percent.

A series of aromatic aldehyde substrates was investigated with purified wild-type enzyme or mutants (Figure S7). High stereoselectivity at β-carbon for *threo* enantiomer products was retained with D93H/E147D, especially up to nearly 70% de towards benzaldehydes with an *ortho*-halogen substitution on the phenyl ring (Table 1 and Figures S8-12). Surprisingly, D93N/E147D dramatically inverted the C_β-stereoselectivity in preference for the *erythro*

enantiomer product, especially for benzaldehyde or benzaldehyde with 3′-fluoro- and 2′-chloro-substituent (Table 1 and Figures S8-12). Benzaldehyde with nitro substitution on the phenyl ring had little effect on C_β-stereoselectivity. It is interesting that the difference of a single amino acid at 93 results in such reversal of C_β-stereoselectivity for a TA. Although this has been observed for other enzymes,[41-43] this is the first example for TAs. In addition, this study also represents the rare example of improvement of C_β-stereoselectivity catalyzed by an engineered L-TA at high conversion reported so far.

Although all the obtained mutants share one single point mutation E147D, the mutant E147D itself has lower activity. That is why this variant was not screened by SVS assay of the site-saturation mutant library. As Asp93 had remarkable effect on the activity and stereoselectivity, Asp93 was substituted with other amino acids on the basis of E147D, but these mutants were not better than the obtained mutants. These results also demonstrate the viability of the SVS method.

In order to gain the molecular insights for the alteration of stereoselectivity, the structural models of mutant D93H/E147D and D93N/E147D were constructed based on the known crystal structure (PDB: 1V72), and L-β-phenylserine and L-β-*o*-fluorophenylserine were docked into the substrate-binding site of wild-type and mutant enzymes. The previous studies have shown that the water molecules interacted with the phosphate of PLP and a water molecule interact with the hydroxyl group of threonine and the phosphate of PLP in the crystal structure of *E. coli* L-TA complex with L-threonine.[34] Since L-β-phenylserine is bigger and more hydrophobic than L-threonine, water molecules were omitted in our docking studies. The lowest-energy docking conformations of enzyme-substrate complexes were selected by using the score functions of flexible docking program. In these conformations, H89 forms π-π stacking interaction with PLP-

cofactor and the distances between the amino group of substrate and aldehyde group of cofactor PLP are in the range (2.4-4.2 Å) for the formation of the imine intermediate.

In previous studies, two conserved histidine residues, H83 and H126, or H85 and H128 were shown to interact with the hydroxyl group of L-threonine or L-*allo*-threonine.[34,40] The interaction of the two histidine residues with the hydroxyl group of the substrate was proposed to activate the hydroxyl group and facilitate the reaction. Although the sizes of aromatic compounds are quite different from the threonine, we thought that the proposed reaction mechanism might apply in our case, and H89 and H133 played similar roles in the catalytic reaction. The docking studies confirmed that the substrate bindings of L-*threo*-phenylserine, L-threonine and L-*allo*-threonine in the catalytic center of wild-type L-PsTA were similar (Figure S13). Therefore, we compared the distance between imidazole of H133 or H89 and hydroxyl of substrate in lowest-energy docking conformations of enzyme-substrate complexes with the experimentally observed activity. The similar distances between the hydroxyl of *threo*- or *erythro*- isomers and H133 indicate that the stereoselective control of the wild-type enzyme at β-carbon may be poor (Figure 3A-B and Figure S14). This is in agreement with the experimental data. In variant D93H/E147D, the amino acid residues H93 and D147 interact with hydroxyl and amino group of the substrate, respectively, resulting in the π-π stacking interaction between H133 and substrate benzene ring (Figure 3C-D). For L-*threo*-phenylserine, the distance between imidazole of H133 and hydroxyl of substrate is shortened to 4.6 Å compared to 5.5 Å in wild-type enzyme (Figure 3C). The corresponding distance is further shortened to 3.6 Å for L-*threo*-*o*-fluorophenylserine as the hydrogen bond is formed between fluorine atom at 2-position of phenyl ring and hydrogen atom on imidazole of His89 (Figure 3D). As such, D93H/E147D exhibits higher C_β-stereoselectivity than the wild-type enzyme, especially for the substrate **2**.

It was particularly worthy noting that D93N/E147D showed a greatly increased preference for *erythro* isomers. The hydroxyl and amino group of *erythro* isomer of substrate **1** are directed to the amide side chain of N93, and the phenyl group forms a CH-π interaction with the benzene ring of Y312 (Figure 3E). As such, the distance between hydroxyl group of L-*erythro*-phenylserine and imidazole of H133 is shortened from 7.3 Å (wild-type enzyme, Figure S14) to 3.9 Å (Figure 3E). This is consistent with the high de (74%) for *erythro* isomer. For L-*erythro*-*o*-fluorophenylserine, the *ortho*-fluoro group forms a hydrogen bond with imidazole of H89, weakening the CH-π interaction between the phenyl group of the substrate and the benzene ring of Y312. The distance between the hydroxyl group of substrate and the imidazole of H133 is thus increased to 5.1 Å (Figure 3F), leading to a lower de than the substrate **1**. The docking results also show the shortened corresponding distances between the imidazole of H89 and the hydroxyl of substrate for the mutants D93H/E147D and D93N/E147D.

After being mutated to Asp, D147 shows interaction with the α-amino group of L-β-phenylserine or L-β-fluorophenylserine in either mutants, the distance between hydroxyl group of D147 and α-amino group of substrate are in the range (2.3-2.8 Å) compared to 6.6 Å in the wild-type enzyme. Therefore, mutations at D93 and E147 affect the interactions of the substrate with the amino acid residues in the catalytic site, thus resulting in the change of the distances between the hydroxyl group of substrate and the imidazoles of H133 and H89, which may play an important role in regulating the stereoselectivity at β-carbon by tuning the deprotonation of the hydroxyl group in the retro-aldol reaction or the protonation of the aldehyde group in the aldol addition.

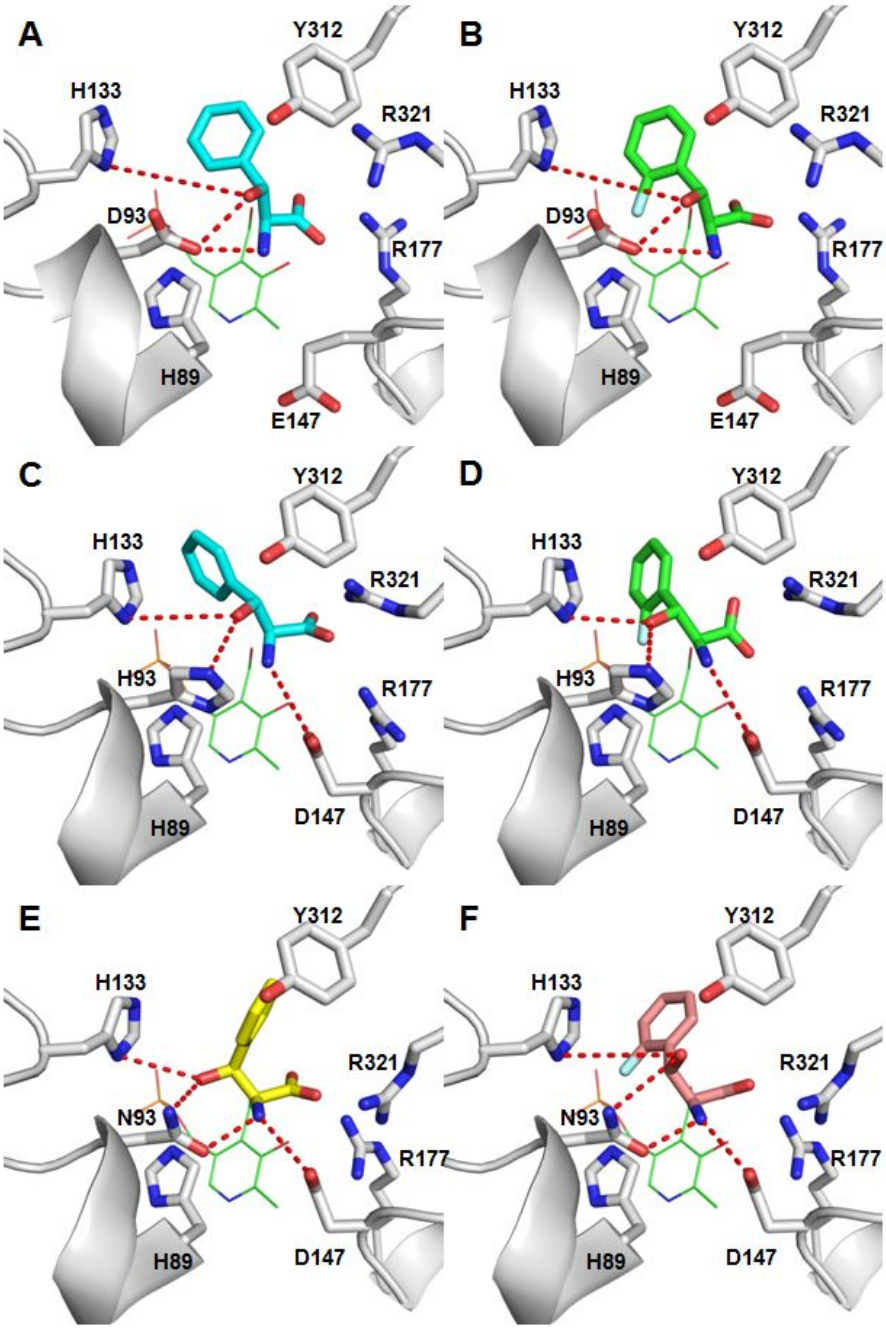


Figure 3. Flexible docking into the active sites of wild-type and mutant enzymes. (A) and (B) Catalytically active conformations of wild-type enzyme complexed with L-*threo*-phenylserine and L-*threo*-*o*-fluorophenylserine, respectively. (C) and (D) Catalytically active conformations of mutant D93H/E147D complexed with L-*threo*-phenylserine (cyan) and L-*threo*-*o*-fluorophenylserine (green), respectively. (E) and (F) Catalytically active conformations of

mutant D93N/E147D complexed with L-*erythro*-phenylserine (yellow) and L-*erythro*-*o*-fluorophenylserine (salmon), respectively. PLP is shown as green in lines.

CONCLUSION

In summary, a SVS method was developed for measuring the conversion and stereoselectivity of TA-catalyzed aldol condensation reaction by detecting the consumption of aldehyde and the generation of keto acid from one stereoisomer of the aldol condensation product. The optimized SVS method provide a simple and rapid strategy suitable for HTS in the discovery of TAs with desired synthetic activity and stereoselectivity. By using this method, mutants of L-PsTA with improved or inverted stereoselectivity towards aromatic aldehydes were obtained. The commonly used site-saturation mutagenesis of the amino acid residues lining the substrate-binding site and neighbouring residue grouping failed to generate benefit mutant, suggesting that the stereoselective control mechanism of TAs is unique from other enzymes and the interactions of the substrate with enzyme should be manipulated as a whole to achieve the desired stereoselectivity. By simultaneously mutating the amino acid residues which interact with the amino and hydroxyl groups of the substrate, we successfully obtained mutants with improved or inverted C_β-stereoselectivity towards aromatic aldehydes. The molecular modelling studies suggested that the mutations tuned the distances between hydroxyl group of the substrate and imidazole groups of H133 and H89 by altering the interactions of the substrate with the amino acid residues in the catalytic site, thus resulting in the changes of the C_β-stereoselectivity. This study not only provides a new SVS method for identifying beneficial stereoselective mutant TAs, but also acquires mutant enzymes with improved or inverted C_β-stereoselectivity, offering some hints for the direction of further enzyme engineering to address the challenge of stereochemical

control at the β-carbon in the TA-catalyzed aldol condensation reaction. To fully understand the molecular basis for the substrate-binding and stereoselectivity control mechanisms, and ultimately address the C_β-stereoselectivity problem, more studies such as the crystal structure determination of wild-type L-PsTA and mutant enzymes are needed, and this is being pursued in our laboratory.

ASSOCIATED CONTENT

Supporting Information

The experimental details, source of L-threonine aldolases, primers used for mutagenesis, purification of enzymes and mutants, docking analysis, and the HPLC spectra of the aldol addition reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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