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Mutation of *Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase at Trp-110 Affects Stereoselectivity of Aromatic Ketone Reduction

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Alcohol dehydrogenases (ADHs) are enzymes that catalyze the reversible reduction of carbonyl compounds to their corresponding alcohols. We have been studying a thermostable, nicotinamide-adenine dinucleotide phosphate ($NADP^+$)-dependent, secondary ADH from *Thermoanaerobacter ethanolicus* (TeSADH). In the current work, we expanded our library of TeSADH and adopted the site-saturation mutagenesis approach in creating a comprehensive mutant library at W110. We used phenylacetone as a model substrate to study the effectiveness of our library because this substrate showed low enantioselectivity in our previous work when reduced using W110A TeSADH. Five of the newly designed W110 mutants reduced phenylacetone at >99.9% ee, and two of these mutants exhibit an enantiomeric ratio (E-value) of over 100. These five mutants also reduced 1-phenyl-2-butanone and 4-phenyl-2-butanone to their corresponding (*S*)-configured alcohols in >99.9% ee. These new mutants of TeSADH will likely have synthetic utility for reduction of aromatic ketones in the future.

Introduction

The field of biocatalysis essentially includes using whole cell microorganisms and isolated enzymes to carry out synthesis of organic molecules [1]. Enzymes are very effective catalysts in organic synthesis due to their high degree of chemo-, regio-, and stereospecificity. Their reactions are carried out under mild reaction conditions, such as room temperature and pressure, and hence formation of undesired by-products is unlikely. The natural reaction medium for these enzymatic systems is water; this can be considered an advantage because of the recent push for "greener" synthetic approaches. On the other hand, there are some significant disadvantages for enzymatic reactions such as: 1) Most enzymes do not tolerate organic solvents well, and many of the interesting organic substrates are sparingly soluble in water, hence their synthesis via enzymatic methods becomes challenging; 2) Most wild type enzymes have very high substrate specificity (i.e. they have optimal reactivity for substrates that are similar to the natural substrate); and 3) Most enzymes operate under a very narrow range of optimum conditions such as pH, temperature and atmospheric pressure. In the last twenty years, however, advancements in molecular biology techniques have eliminated most of these disadvantages[2].

Alcohol dehydrogenases (ADHs) are a class of nicotinamide-adenine dinucleotide (phosphate) [$NAD(P)^+$]-dependent enzymes which catalyze the reversible oxidation of alcohols to the corresponding ketones or aldehydes. These enzymes are valuable in producing optically active alcohols by reduction of prochiral ketones. Most ADH-catalyzed reactions follow Prelog's rule, in which the *pro-R* hydride is delivered to the *Re*-face of a ketone substrate, thus producing (*S*)-alcohols[3]. Bryant et al. isolated and characterized a secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH), an $NADP^+$-dependent ADH, which has a remarkable tolerance to high temperature and high concentrations of organic solvents[4]. In subsequent years, several mutants of TeSADH were designed, including C295A[5], I86A[6], and W110A TeSADH[7], and those mutants expanded the substrate specificity of TeSADH to accommodate ketones and alcohols that are not substrates for the wild-type enzyme. One of these mutants, W110A TeSADH, which accommodates phenyl-ring-containing substrates in Prelog fashion, is particularly interesting and is the one that has been most extensively studied. An unexpected result was obtained when reduction of phenylacetone was attempted with W110A TeSADH, which gave the corresponding (*S*)-alcohol in low enantioselectivity (37% ee)[8]. This is in contrast not only to what was observed with other similar sized substrates, like 4-phenyl-2-butanone, which are reduced with >99% ee, but also suggested that phenylacetone might fit in the large pocket of the mutant enzyme's active site in an alternative mode, thus lowering the enantioselectivity of its reduction by W110A TeSADH. Because of this intriguing result, we decided to create a library of enzyme mutants at Trp-110, which could expand substrate specificity and increase enantioselectivity. The results of this study are presented herein.

Results and discussion

We used site-saturation mutagenesis at Trp-110 in TeSADH to prepare a library of novel mutant enzymes. We then monitored the effect of alterations in the large pocket of the

active site of TeSADH on the enantioselectivity of the reduction reaction of three phenyl-ring-containing ketones which are not substrates for wild-type TeSADH. We sequenced about 160 colonies for mutations from the site-saturation mutagenesis and isolated nine new mutants. For evalution of activity, the new mutant TeSADHs were initially screened for activity with (*S*)-1-phenyl-2-propanol, since we were particularly interested in mutant enzymes with improved enantiospecificity. Among the isolated mutants, W110R, W110S, and W110F were not active in this screen, and they were not studied further. Of the mutant proteins which we did not obtain, those with polar side chains (W110K, W110T, W110C, W110D, and W110E) were considered unlikely to have activity, since W110R and W110S were inactive in the screen. In addition, other mutants containing amino acids with aromatic rings (W110Y and W110H) are not likely to have activity, since W110F was found to be inactive. Of the remaining mutants not isolated (W110P and W110N), only W110N seems likely to be of interest, since W110Q was found to have low activity in the screen. We purified the six active mutants of TeSADH, and subsequently conducted detailed kinetic studies on the oxidation reactions of (*R*)- and (*S*)-1-phenyl-2-propanol (Table 1). These results showed that four of these mutants (W110I, W110Q, W110V, and W110L) have E values > 80 [$E = (k_{cat}/K_m)_S/(k_{cat}/K_m)_R$]. In contrast, W110G showed an E value of <10, which is expected because this mutation expands the "large pocket" in the active site of W110G TeSADH more than that in the other mutants. Increasing the size of the large pocket may give the bound substrate more binding modes, thus lowering the enantiospecificity of the oxidation reaction of 1-phenyl-2-propanol. Moreover, an unexpectedly low E value was obtained for W110M (E value= 16.3) indicating that mutating Trp-110 with methionine, which is comparable in size to leucine and isoleucine, gave a similar E value as the enantiospecific oxidation of 1-phenyl-2-propanol by W110A. It is possible that branching contributes more to the steric interactions, since valine, leucine and isoleucine have branching at the β- or γ-carbons, while methionine does not. This observation suggests that size of the substrate binding pocket is not the only factor influencing the stereoselectivity of these TeSADH-catalyzed redox reactions. The best mutant TeSADH was found to be W110V, which gave an E value of 134.5, and its k_{cat}/K_m value for (*S*)-1-phenyl-2-propanol is 45,300 $M^{-1}s^{-1}$, a value that is comparable to the specificity constant of a natural substrate[9]. Thus, the improved stereospecificity of these mutants, and W110V in particular, is due to a large increase in the reactivity of the *S*-enantiomer, rather than decreasing reactivity of the *R*-enantiomer. It is clear that small alterations in the active site of TeSADH using site-saturation mutagenesis at a single amino acid residue plays an important role in enhancing the enantiospecificity of the oxidation reaction of 1-phenyl-2-propanol. This conclusion is evident from the significant change in E values of the enantiospecific oxidation of 1-phenyl-2-propanol by different W110 TeSADH mutants in Table 1.

In order to correlate the E values obtained from the kinetic studies to stereoselectivity of alcohols produced from enantioselective reduction of their ketones by TeSADH mutants, we performed the reduction reactions of

Table 1. k_{cat}/K_m values for enantiospecific oxidation of (*S*)- and (*R*)-1-phenyl-2-propanol with mutant TeSADH.

Mutant TeSADH	Enantiomer	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	E = $(k_{cat}/K_m)_S$ / $(k_{cat}/K_m)_R$
W110I	*S*	18.8 ± 1.9	15200 ± 2300	80.3 ± 16.2
	R	0.46± 0.05	188 ± 25	
W110Q	*S*	2.3 ± 0.2	551.4 ± 31.2	80.0 ± 17.5
	R	0.025±0.006	6.9 ± 1.5	
W110M	*S*	4.5 ± 0.4	1990 ± 230	16.3 ± 3.5
	R	0.045 ± 0.003	121.0 ± 22.1	
W110V	*S*	38.6 ± 3.06	45300 ± 4500	134.5 ± 27.7
	R	1.2 ± 0.3	336.5 ± 61.0	
W110L	*S*	0.65 ± 0.03	2510.0 ± 560	104.4 ± 42.1
	R	0.0080 ± 0.00081	24.0 ± 9.0	
W110G	*S*	17.8 ± 3.1	5800 ± 940	9.02 ± 2.6
	R	1.4 ± 0.3	639.0 ± 149.0	
W110A	*S*	31.1 ± 8.1	4935 ± 715	17.4 ± 4.7
	R	0.56 ± 0.09	284 ± 65	

phenylacetone, 1-phenyl-2-butanone, and 4-phenyl-2-butanone by using the different mutants, and compare the % ee of the alcohols obtained, as shown in Table 2. These reduction reactions were conducted in Tris-HCl buffer solution containing 2-propanol (5%, v/v) as a cosolvent and a cosubstrate for cofactor recycling. We are delighted to report that five of the new mutants (W110I, W110Q, W110M, W110V, and W110L) reduced phenylacetone quantitatively to (*S*)-1-phenyl-2-propanol in >99.9% ee. When W110G was used, the same reduction reaction proceeded in 79% ee, in good agreement with the E value in Table 1, since E = 9 corresponds to 80% ee. Although W110A and W110G have similar E values to W110M, however, the reduction reaction of phenylacetone by the latter mutant gave (*S*)-1-phenyl-2-propanol in >99.9% ee. In a previous report[8], W110A

TeSADH-catalyzed reduction reaction of phenylacetone gave the corresponding (*S*)-alcohol in only 37% ee when Tris-HCl buffer solution containing 2-propanol (30% v/v) was used as the reaction medium. However, this lower stereoselectivity in that experiment may be the result of the recently discovered racemization of alcohols by W110A TeSADH.[10] When the same reaction was conducted in reaction medium containing 5% 2-propanol (i.e. the same conditions for the current study), (*S*)-alcohol was produced in 84.1% ee (Table 2, entry 7).

Table 2. Asymmetric reductions of phenylacetone, 1-phenyl-2-butanone, and 4-phenyl-2-butanone by mutant TeSADH.[a]

Entry	Mutant TeSADH	Phenylacetone		1-phenyl-2-butanone		4-phenyl-2-butanone	
		Conv (%)[b]	ee (%)[c]	Conv (%)[b]	ee (%)[c]	Conv (%)[b]	ee (%)[c]
1	W110I	>99.9	>99.9	99.4	>99.9	99.1	>99.9
2	W110Q	>99.9	>99.9	83.5	>99.9	99.1	>99.9
3	W110M	>99.9	>99.9	97.3	>99.9	99.3	>99.9
4	W110V	>99.9	>99.9	99.2	>99.9	99.1	>99.9
5	W110L	>99.9	>99.9	98.9	>99.9	99.2	>99.9
6	W110G	>99.9	79	95.8	91.6	99.1	70.5
7	W110A	>99.9	84.1	-	-	99d	>99[d]

Conditions: [a]Tris-HCl buffer solution (50 mM, pH 8.0), $NADP^+$ (1.0 mg), mutant TeSADH (0.35 mg) and ketone (0.04 mmol) with 0.5 mL 2-propanol in a total volume of 10 mL. [b] % conversion was determined by GC. [c] % ee was determined by a GC equipped with a chiral stationary phase for the corresponding acetate derivatives. [d] From reference 8.

Reduction of 1-phenyl-2-butanone gave the corresponding (*S*)-alcohol in high yields and >99.9% ee with all newly developed mutants, except W110G which gave slightly lower enantioselectivity (91.6% ee) than the other five mutants (Table 2). Similar results were obtained when 4-phenyl-2-butanone was reduced by the different mutants. It is notable that W110G TeSADH-catalyzed reduction of 1-phenyl-2-butanone gave the corresponding (*S*)-alcohol in higher enantioselectivity than when phenylacetone or 4-phenyl-2-butanone were used, as shown in entry 6 of Table 2. This may be explained by the greater ability of 1-phenyl-2-butanone to fit within the active site of W110G in the Prelog orientation (i.e. the ethyl group in the small pocket and the benzyl moiety in the large pocket) than phenylacetone and 4-phenyl-2-butanone, which can encounter more selectivity mistakes. This Prelog fit is encouraged by the higher affinity of the small pocket of the active site of TeSADH for the ethyl group in comparison with the methyl group present in the two other substrates. This result is consistent with previous reports for *Thermoanaerobium brockii* ADH and TeSADH.[11, 12]

When the W110V TeSADH-catalyzed reduction of phenylacetone was conducted at 36 mM concentration using Tris-HCl buffer solution containing 2-propanol (30%, v/v), the corresponding (*S*)-alcohol was produced in 79% conversion and >99.9% ee (Table 2, Scheme 1). The lower yield under these conditions may be due to product inhibition. W110A TeSADH-catalyzed reduction of phenylacetone, in a previous report, resulted in the corresponding (*S*)-alcohol in 95%

Scheme 1. Enantioselective reduction of phenylacetone using W110V TeSADH.

conversion and 37% ee.[8] As described above, five of the designed mutants accept phenylacetone, 1-phenyl-2-butanone, and 4-phenyl-2-butanone, as well as their corresponding alcohols, with high enantioselectivities and enantiospecificities, respectively. In order to understand the molecular basis for these changes in substrate specificity and stereochemistry, we conducted docking studies for the mutants with phenylacetone and compared that with wild-type TeSADH. As a representative example, phenylacetone was docked with the structure of W110V *Thermoanaerobium brockii* SADH[‡] created in silico using Autodock Vina, and we

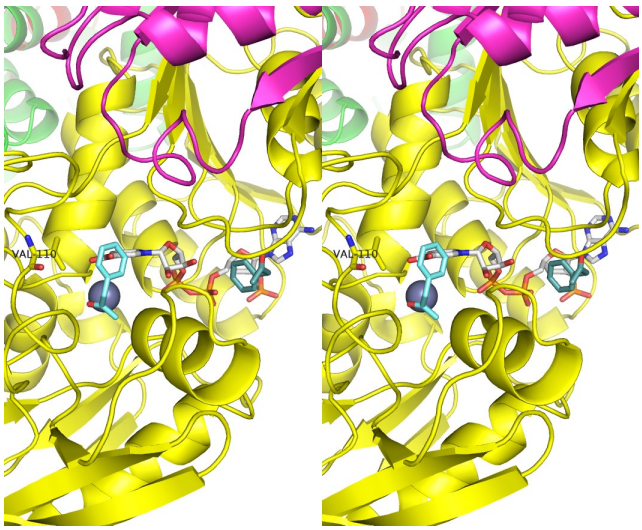

Figure 1. Crossed-eye stereo view of docking phenylacetone to W110V TeSADH using Autodock Vina on the holoenzyme form of *Thermoanaerobacter brockii* ADH (PDB: 1YKF). The phenylacetone, shown in cyan, on the left is the lowest energy pose for W110V SADH. The phenylacetone molecule on the right side of the structure is the lowest energy pose for wild-type TeSADH overlaid on the W110V structure. The gray sphere is the catalytic zinc. The CPK stick structure is the NADP$^+$.

found that the lowest energy pose of phenylacetone docks within the active site, close to residue 110, and the carbonyl oxygen is about 5 Å away from the catalytic Zn^{2+}, as shown in Figure 1 (molecule in cyan on the left side of the figure); however, for wild-type enzyme, phenylacetone is unable to enter the active site, probably because the large size of Trp-110 does not allow it to go in, and it is blocked in the mouth of the active site (Figure 1, molecule in cyan on the right side of the figure). This is consistent with the results obtained in this report, as well as previous ones,[7,8] as it is known that phenylacetone is not a substrate for wild-type TeSADH, whereas it is a substrate for the other W110 TeSADH mutants described herein.

Conclusions

In this paper, we used site-saturation mutagenesis to create a library of mutants of TeSADH at residue W110, and we were able to successfully obtain six very interesting mutants. Five of them gave >99.9% ee in the enantioselective reductions of phenylacetone, 1-phenyl-2-butanone, and 4-phenyl-2-butanone, which represent remarkable improvement over the previously reported mutant, W110A TeSADH, which reduced phenylacetone to (*S*)-1-phenyl-2-propanol in only 37% ee. One of the new mutants, W110V TeSADH, shows extremely high E and k_{cat}/K_m values of 134.5 and 45300 $M^{-1}s^{-1}$, respectively, for (*S*)-1-phenyl-2-propanol. This new mutant TeSADH library provides new insights in understanding the enzyme's stereospecificity, and accompanied by further optimization of TeSADH by mutations at other amino acid residues will make this enzyme a more attractive catalyst for chiral aromatic alcohol synthesis.

Materials and Methods

General

Commercial grade solvents were used as obtained from the suppliers. 1-Phenyl-2-butanone, 4-phenyl-2-butanone, and $NADP^+$ were used as purchased from commercial suppliers. All buffer solutions were adjusted at room temperature. Capillary GC measurements were performed on a GC equipped with a flame ionization detector and either an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 µm film thickness) or a Supelco β-Dex 120 chiral column (30 m, 0.25 mm [i.d.], 0.25 µm film thickness) using He as the carrier gas. Enzyme kinetic studies were performed on a Varian Cary 1E UV-Vis spectrophotometer equipped with a 6x6 Peltier temperature controlled cell block.

Site-saturation mutagenesis

The codon NNK (N=GATC and K=GT) was used at W110 site to get all the possible mutations using site saturation mutagenesis based on the Quikchange (Stratagene) protocol. This degenerate primer design approach gives 32 codons, and theoretically gives access to all 20 amino acids in a single reaction. Primer design was done using broad guidelines from Liu and Naismith as well as Zheng et al.[13,14] Partially overlapping primer design was used in this case, and their guidelines stated that there should be at least 8-18 overlapping bases, targeted mutation in both forward and reverse primers, and at-least one G or C should be at each of the termini.

Primers used:
GGCAGGCNNKAAATTTTCGAATGTAAAAGATGGTGTTT TTG

GAAAATTTMNNGCCTGCCAGCATTCCACCGGAGTGCT GGTG.

The PCR reaction was done in a 50 µL tube containing 1 µL of template (2-10 ng), 1 µM of primer pair, 200 µM of dNTP and 3 units of Pfu Turbo DNA polymerase. The PCR cycle was initiated by incubation at 95 ºC for 5 min to denature the template DNA, followed by 16 amplification cycles, each of 95 ºC for 1 min, 52 ºC for 1 min, and 68 ºC for 24 min, followed by incubation at 68 ºC for 1 h. The PCR reaction was then digested by addition of 1 µL of Dpn I restriction enzyme (10 U/µL) with incubation for 1 h at 37 ºC to digest the entire parental methylated supercoiled DNA. After going onto the further steps, we realized that we were having very high background of wild-type mutants, and that was attributed to incomplete DNA digestion. In order to minimize the WT background, we ended up digesting the PCR mixture 4 times using the above procedure, and then we were able to bring down the WT background to more acceptable 40-50% from 95%. Supercompetent DH5α *E. coli* cells were prepared as reported by Inoue *et al.* with the modification that we used SOC media instead of SOB media.[15] These cells were transformed with 1 µL of the mutagenesis reaction and spread on LB-ampicillin agar plates.

Screening of colonies

A colony from the agar plate prepared in the earlier step was picked using a 10-µL micropipette tip and directly transferred into a 10-mL tube containing LB media with ampicillin (100 mg/L). This was grown overnight and about 1.0 mL of this grown culture was kept as glycerol stock (50:50 v/v) and stored at -78 ºC. Plasmid prep was subsequently performed on the remaining amount of culture using either a Qiagen plasmid miniprep kit or an Invitrogen kit. The resulting plasmids were then sequenced.

Enzyme Purification

Cell cultures from each of the positive hits were taken and grown in a 10-mL LB media overnight at 37 ºC with shaking at 180 rpm. This was subsequently used as a preculture inoculum, and was transferred to 400 mL LB media, where the cells were grown overnight at 37 ºC with shaking at 180 rpm. Cells were collected by centrifugation at 4000 rpm for 15 min. The wet cells were resuspended in 10 mL of 50 mM Tris-HCl buffer solution at pH 8.0 (buffer A). The cells were then lysed by sonication at 0 ºC in three 3-min intervals. The cell debris was removed by centrifugation at 4000 rpm for 90 min. The resulting supernatant was incubated at 70 °C for 15 min and centrifuged at 4000 rpm for 90 min to remove the thermally denatured protein impurities. The crude supernatant solution of TeSADH was applied to a 15 mL Red Agarose column that had been regenerated, pre-equilibrated and washed with buffer A. Impurities were eluted with buffer A containing $NaClO_4$ (10 mM), followed by elution of TeSADH fractions with buffer A containing of $NaClO_4$ (0.2 M). The eluted TeSADH solution was then concentrated by centrifugal ultrafiltration. The resulting enzyme showed one major band on polyacrylamide gel electrophoresis. This enzyme was stored at -78 ºC for several months without loss of activity, and was used in the subsequent kinetic studies and enantioselective reduction reactions. The protein concentration was determined by the method of Bradford.[16] The Red Agarose column was regenerated by washing at a flow rate of 1 mL/min with two column volumes each of guanidine.HCl (4M) solution, distilled deionized water, NaCl solution (4.0 M) lastly, and buffer A.

Kinetic resolution of (*RS*)-1-phenyl-2-propanol

Enantiomerically pure (*R*)- and (*S*)-1-phenyl-2-propanol were obtained via *Candida antarctica* lipase B (CALB, Novozyme 435)-catalyzed kinetic resolution of their racemic alcohol with isopropenyl acetate as the acyl donor. The reaction was carried out using (*RS*)-1-phenyl-2-propanol (1.0 g, 7.3 mmol), CALB (80 mg), isopropenyl acetate (1.5 eq), and toluene (12 mL). The reaction mixture was stirred at room temperature and monitored on a chiral GC; and when the kinetic resolution was complete, the reaction was quenched by filtering out the immobilized CALB, and (*S*)-1-phenyl-2-propanol and the corresponding ester of (*R*)-1-phenyl-2-propanol were separated using column chromatography. The (*R*)-ester was subsequently hydrolyzed using KOH (4 eq) in 1:1 water/methanol solution to get (*R*)-1-phenyl-2-propanol. The kinetic resolution was repeated in order to get both *R* and *S* isomers with >95% ee, and these were subsequently used as substrates for all the kinetic experiments.

Synthesis of phenylacetone

Phenylacetone was synthesized using the Dakin-West synthesis procedure using 1-methylimidazole as modified by Tran and Bicker.[16] The reaction was conducted using phenylacetic acid (5.0 g), and was run overnight, then quenched by adding water (10 mL) to hydrolyze acetic anhydride. It was extracted with ethyl acetate (3 × 50 mL), and the extracts were then washed with sodium bicarbonate (2 × 50 mL) followed by water (2 x 50 mL). The extract was then dried over sodium sulfate, filtered, and concentrated under vacuum. About 50% conversion of phenylacetic acid to phenylacetone was obtained. A short path vacuum distillation with a small packed column was conducted to purify phenylacetone prior to use in the reactions.

Determination of kinetic parameters for oxidation of (*R*)- and (*S*)-1-phenyl-2 propanol with mutants of TeSADH

Reaction rates were determined in cuvettes containing $NADP^+$ (0.4 mM), Tris-HCl buffer solution (50 mM, pH 8.9), enantiopure 1-phenyl-2-propanol (0.33 – 4 mM) at 50 ºC in a total volume of 0.6 mL. The cuvettes were incubated for at least 15 min in the spectrophotometer to allow temperature equilibration before a TeSADH mutant enzyme was added to initiate the oxidation reaction. The reaction rates were measured by following the production of NADPH spectrophotometrically at 340 nm ($\Delta\varepsilon$= 6220 M^{-1} cm^{-1}). Values of k_{cat} and k_{cat}/K_m were calculated using a molecular weight for TeSADH of 36.5 kDa. The reported values for each enantiomer were the result of at least three repetitions.

General procedure for reduction of ketones by mutant TeSADH

The reduction reactions were conducted by mixing Tris-HCl buffer solution (9.5 mL, 50 mM, pH 8.0), $NADP^+$ (1.0 mg), 2-propanol (0.5 mL), mutant TeSADH (0.35 mg), and ketone substrate (0.04 mmol) in a round-bottomed flask equipped with a magnetic stirrer. The flask was tightly stoppered and the reaction mixture was stirred at 50 ºC for 6 h. It was then extracted with methylene chloride, dried over sodium sulfate and then concentrated in vacuo. The residue was acetylated with acetic anhydride (one drop) and pyridine (three drops) for one hour. The resulting product was diluted with chloroform, and then analyzed by GC equipped with a chiral column to calculate % conversion and % ee.

Docking experiments

The docking experiments were performed with Autodock Vina using the Autodock plugin for Pymol.[17] The wild-type holoenzyme structure (1YKF.pdb) was used to make the mutations of Trp-110. The phenylacetone was created with ChemDraw-12, minimized, and saved as a pdb file. The grid was centered on the nicotinamide ring of the $NADP^+$ in the structure. Phenylacetone was docked to wild-type, W110V, and W110I TeSADH. Ten poses were saved from each docking, and the lowest energy poses were examined. The position of the lowest energy pose for W110V and W110I were very similar, about 5Å from the zinc, while the lowest

energy pose for wild-type TeSADH was not located in the active site.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/
‡ The amino acid sequences of SADH from *T. ethanolicus* and *T. brockii* are identical.

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