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Impact of Expanded Small Alkyl Binding Pocket by Triple Point Mutations on Substrate Specificity of *Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase

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Abbreviations: ADH (Alcohol dehydrogenases), TeSADH (*Thermoanaerobacter ethanolicus* alcohol dehydrogenase)

ABSTRACT

Site-directed mutagenesis was employed to generate five different triple point mutations in the double mutant (C295A/I86A) of *Thermoanaerobacter ethanolicus* alcohol dehydrogenase (TeSADH) by computer-aided modeling with the aim of widening the small alkyl binding pocket. TeSADH engineering enables the enzyme to accept sterically hindered substrates which could not be accepted by the wild-type enzyme. The underline in the mutations highlights the additional point mutation on the double mutant TeSADH introduced in this work. The catalytic efficiency (k_{cat}/K_M) of the M151A/C295A/I86A triple TeSADH mutant for acetophenone increased about 4.8-fold higher than that of the double mutant. A 2.4-fold increase in conversion of 3'-methylacetophenone to (*R*)-1-(3-methylphenyl)-ethanol with a yield of 87% was obtained by using V115A/C295A/I86A mutant in asymmetric reduction. The A85G/C295A/I86A mutant also produced (*R*)-1-(3-methylphenyl)-ethanol (1.7-fold) from 3'-methylacetophenone and (*R*)-1-(3-methoxyphenyl)ethanol (1.2-fold) from 3'-methoxyacetophenone, with improved yield. In terms of thermal stability, the M151A/C295A/I86A and V115A/C295A/I86A mutants significantly increased $\Delta T_{1/2}$ by +6.8ºC and +2.4ºC, respectively, with thermal deactivation constant (k_d) close to the wild-type enzyme. The M151A/C295A/I86A mutant reacts optimally at 70 ºC with almost 4 times more residual activity than the wild-type. Considering broad substrate tolerance and thermal stability together, it would be promising to produce (*R*)-1-(3-methylphenyl)-ethanol from 3'-methylacetophenone by V115A/C295A/I86A, and (*R*)-1-phenylethanol from acetophenone by M151A/C295A/I86A mutant, in large-scale bioreduction processes.

Keywords: Computer-aided modeling, alcohol dehydrogenase, substrate specificity, asymmetric reduction, thermostability

Introduction

The asymmetric reduction of ketones to chiral alcohols has been a method of choice to pharmaceutical and fine chemical companies [1, 2]. Alcohol dehydrogenases (ADHs) are biocatalysts that catalyze the reversible reduction of ketones and aldehydes to alcohols [3, 4]. The advantages of using ADHs as novel catalysts for biotransformation include not only its high chemo-, regio- and enantio-selectivity but also the ability to react in non-aqueous media [5, 6]. Recent comprehensive reviews [5, 7-9] have thoroughly described various ADHs used in the asymmetric reduction of hydrophobic ketones to produce chiral alcohols. Substrate specificity and selectivity is important in pharmaceutical settings to produce enantiopure compounds, however, such catalytic properties are not always attained in the wild-type enzyme [8]. Reduction of some substituted aromatic, cyclic, and aliphatic ketones by ADHs with broad substrate specificity have been reported [10, 11]. Yang *et al.* [12] used plant tissues to reduce prochiral ketones to chiral alcohols with enhanced enantioselectivity.

Thermoanaerobacter ethanolicus alcohol dehydrogenase (TeSADH) has been proven in the past decades as an alternative biocatalyst for producing chiral alcohols with broad substrate tolerance and thermal stability at extreme process conditions [8, 13]. Modeling and experimental studies by Heiss *et al*. [13] identified key residues in the small and large alkyl binding pockets essential for catalysis, hence their substitutions may affect

substrate binding and kinetic parameters. Alanine mutations at Cys-295 [13], Ile-86 [14], and Trp-110 [15, 16] were shown to broaden the TeSADH active site to accommodate some phenyl-ring-containing compounds which are not natural substrates for the wild-type. However, these mutants still lack the ability to accommodate and convert several phenyl-ring-containing ketones with electron donating/withdrawing groups.

By applying site-directed mutagenesis, this study targeted and replaced five key residues in the small binding pocket (Ala-85, Val-115, Met-151, Thr-153, and Val-178) of the double mutant (I86A/C295A) TeSADH enzyme with alanine or glycine to generate five different triple mutants (Fig. 1). Substitutions of high molecular weight residues with low molecular weight ambivalent amino acids by computer-aided modeling widened the active site to accommodate ketones with large alkyl groups to enhance catalysis. We compared the relative activity, catalytic efficiency, and turn-over number of the generated mutants by reacting them with non-natural prochiral derivatives of acetophenone (Fig. 2). We chose the *meta*- and *para*-substituted derivatives of acetophenone (charged and neutral substituents) because most reports have not described thoroughly the effect of such functional groups on kinetic parameters.

To elucidate the correlation between short-time kinetic studies and the enzyme conversion capacity, asymmetric reduction of substrates (**1a**–**6a**) in biphasic media was studied. Additionally, we coupled the reduction process with the mutant's thermal stability by calculating the thermodeactivation constant (k_d) at different temperatures. We herein report that each mutant shows diverse specificity to electron accepting or donating derivatives of acetophenone notwithstanding the structural position of

mutation with remarkable catalytic efficiencies, yield and thermal stabilities. Most mutants show broad substrate tolerance due to their selectivity 'mistakes' making them ideal candidates for chiral alcohol synthesis. Also, the kinetic results give a fair knowledge of the large-scale asymmetric reduction, however, there is no clear correlation between the two.

Materials and Methods

Materials

Acetophenone (**1a**), 3'-methylacetophenone (**2a**), 4'-methylacetophenone (**3a**), 3'-chlorolacetophenone (**4a**), 4'-chloroacetophenone (**5a**), 3'-methoxyacetophenone (**6a**) and $NADP^+$/NADPH were purchased from Sigma-Aldrich. Isopropanol (**7a**) was from J. T. Baker Chemical Company. (*R*)-1-phenyl-1-ethanol, (*R*)-1-(3-chlorophenyl)-ethanol and (*R*)-1-(3-methylphenyl)-ethanol standard alcohols were purchased from Enamine Ltd. All other standard chemicals were purchased from Sigma-Aldrich.

Site-Directed Mutagenesis

The 50 µl reaction mixture consist of 160 µM each of dNTPs, *Pfu*Turbo DNA polymerase (2.5 U/µl, Agilent) and 10 × buffer solution. The forward primer sets are listed in Table 1. Template used is pBluescript II KS (+) with TeSADH gene containing I86A/C295A mutations. The reaction was run in a thermal cycler (Bio-Rad S1000™) for 25 cycles at 95ºC for 1 min, 95ºC for 45 sec, 55 ± 2ºC for 1 min, 68ºC for 6 min and 16 ºC for storage. PCR products were digested with *Dpn* I (New England BioLabs) at

37 ºC for 5 h to thoroughly degrade the parental template.

DNA Sequencing, Protein Expression and Purification

The PCR products (2 µl) were transformed into 200 µl *E. coli* DH5*α* competent cells following the method described in [17] with some modifications. The mixture was incubated at 36.5ºC for 60 min with 120 rpm shaking which was further increased to 180 rpm for 90 min, and then spread out on LB agar plate containing kanamycin and ampicillin. Colonies were randomly selected for plasmid mini-prep and all the mutations were confirmed by DNA sequencing (SolGent Co., Korea). Protein expression and purification was performed as previously reported [18] with some modifications. Mutant TeSADH was expressed in *E. coli* DH5*α* cells in 500 ml LB medium containing 50 µg/ml each of ampicillin and kanamycin. Crude TeSADH extract was loaded on a 5 ml of 50% suspended Reactive Red 120-Agarose, Type 3000-CL (Sigma-Aldrich) affinity chromatography, equilibrated with 0.07 M $NaClO_4$ in buffer A (50 mM Tris-HCl [pH 8.0 at 25ºC], 3 mM DTT and 10 µM $ZnCl_2$). Proteins were eluted with 0.2 M $NaClO_4$ in buffer A. Protein concentration was estimated using Bio-Rad protein assay kit with bovine serum albumin as standard.

Modeling of Mutant TeSADHs

Modeling of mutant TeSADHs was done using automatic Swiss modeling tool [19, 20], and analyzed graphically with PyMol [21] . In all, chain A of template protein [PDB; 1bxz (2.99 Å) [22] was used to generate the ribbon structure (Fig. 1). QMEAN4 and QMEAN Z-score are statistical parameters used to estimate modeling quality of the mutants [23] (supplementary data).

Enzyme Activity Measurement and Determination of Kinetic Parameters

All enzyme assays were performed at 60 ºC in triplicate following $NADP^+$/NADPH production at 340 nm (ϵ_{340} = 6,220 $M^{-1}cm^{-1}$) in a UV/Vis spectrophotometer equipped with TCC-temperature controller (Shimadzu, Japan). The reaction contained 50 mM Tris-HCl buffer (pH 8.9 at 60ºC), 0.5 mM $NADP^+$/NADPH, 4.0 mM substrate, 3.0 mM DTT and 10 µM $ZnCl_2$ in a total of 1 ml. Enzymes (0.52 mg/ml) were pre-incubated at 60ºC for 3 min before the reaction. One unit of activity is defined as the amount of enzyme needed to oxidize or reduce 1 µmol of $NADP^+$/NADPH per minute. The relative activity of mutants with isopropanol were calculated following the formulae, in which DM means the double mutant (C295A/I86A) TeSADH (Table 2):

$$\text{Relative Activity} = \frac{\text{Specific activity of triple mutant}}{\text{Specific activity of DM control}} \times 100\%$$

The K_M values of **1a**, **4a**, **5a**, and **7a** were determined using increasing concentrations from 0.1–32 mM (Table 3 and Table 4). The K_M and V_{max} values were estimated by non-linear fit using OriginPro 9.0. All values represent mean of triplicate reactions ± standard deviation.

Asymmetric Reduction in Biphasic System

Asymmetric reduction of ketones follow methods described previously [14] with some modifications (Fig. 3). The mixture contained **1a**–**6a** (0.21 mmol), NADPH (1 mg), DTT (1 mg), $ZnCl_2$ (10 µM) in 50 mM Tris-HCl buffer solution (300 µl; pH 8.0 at 25ºC), 2-propanol (200 µl) and hexane (500 µl) as solvent. In all, 200 µl (0.52 mg/l) of enzyme were used and reaction mixtures incubated at 50ºC, shaken at 120 rpm for 24 h.

The reaction products were extracted using diethyl ether (3 × 2.5 ml), centrifuged at 5,000 rpm for 3 min, and dried over Na_2SO_4 for 30 min. The organic layer was vacuum concentrated (Ecospin 3180C, Biotron), and the product/yield estimated by GC (Younglin Instruments; Acme 6000E GC) equipped with a Supelco *β*-Dex 120 chiral capillary column (supplementary data). The absolute configuration of the alcohols produced were determined by converting the alcohols to their corresponding acetate derivatives and comparing them with their *R*- or *S*-acetate derivatives of a standard samples. The retention time of the acetylated alcohols and their corresponding acetylated standards helped to identify the configuration of produced alcohols. To confirm the absolute configuration of the produced alcohols, co-injection on a chiral column with a standard alcohol was done.

Measurement of Thermal Stability and Determination of $T_{1/2}$ and k_d Values of TeSADH

The thermal stability of triple mutants, double mutant, and wild-type TeSADH were determined by measuring the residual enzymatic activity after 60 min of incubation in buffer from 30–90ºC (Fig. 4). $T_{1/2}$ is the temperature at which 50% of the enzymatic activity is lost after 60 min incubation [24, 25] (Table 2). Activity assay follows the standard procedure previously described and modified by using 4 mM isopropanol as substrate. The thermodeactivation constant (k_d) was calculated following the equation, $\ln A_t = \ln A_o - k_d\,(t)$, where A_t is the enzymes activity after incubation, A_o is the initial enzyme activity, and t is the time of incubation which is usually 60 min [26] (Table 5).

Results and Discussion

Relative Activity and Kinetics Studies

Site-directed mutagenesis was used to generate an additional single point mutation in the small alkyl binding pocket of double mutant (I86A/C295A) TeSADH enzyme to obtain five different triple mutants. Point mutations at Val-115, Met-151, Thr-153, and Val-178 to alanine as well as a point mutation of Ala-85 to glycine were confirmed by DNA sequencing. Substitutions of these amino acid residues with smaller alanine or glycine widened the TeSADH active site to accept bulkier aromatic and alkyl ketones. The relative activity of mutants with isopropanol (7a), a model substrate, was assayed to ascertain the effectiveness of how point mutations affected both the wild-type and the double mutant in accommodating the substrate in its active site.

From our results in Table 2, the M151A/C295A/I86A, T153A/C295A/I86A, and V178A/C295A/I86A triple mutants remarkably improved their relative activity for isopropanol catalysis between 41% to 57% higher than the double mutant control. Li *et al*. [22] reported van der Waals interactions of the carbon C1 atom of *sec*-butanol with atoms in Ala-85, Trp-110, and Leu-294 residues, hence, the necessity of these residues for *sec*-butanol catalysis. Interestingly, a substitution of Ala-85 with glycine reduced the reactivity of the A85G/C295A/I86A mutant by 45% with isopropanol. Also, the reactivity of V115A/C295A/I86A mutant reduced drastically by 85% in isopropanol catalysis signifying the importance of Val-115 residue in the wild-type. These initial findings suggest that triple point mutations in the small binding pocket of double mutant TeSADH alter the enzymes relative activity to isopropanol catalysis. These results also

act as a predictive assay to evaluate the effectiveness of Ala-85, Val-115, Met-151, Thr-153, and Val-178 for catalysis in a hydrophilic environment. In view of this, the kinetic parameters of the mutants in different organic media (polar (**7a**), and non-polar (**1a**, **4a**, and **5a**)) were studied to show how each triple point mutation alters the mutants binding affinity, turnover number, and catalytic efficiency.

As shown in Table 3, the V178A/C295A/I86A triple mutant had the lowest binding affinity (K_M = 20.2 ± 6.3 mM) with A85G/C295A/I86A having the highest turnover number of 88.1 ± 6.3 s^{-1} for isopropanol. The wild-type TeSADH shows much better values in binding affinity (K_M = 0.069 ± 0.014 mM; 60–290 times) and catalytic efficiency (3.4 × 10^5 $M^{-1}s^{-1}$; 35–240 times). Conversely, the triple mutants M151A/C295A/I86A, V178A/C295A/I86A, and T153A/C295A/I86A exhibited about 1.2 times higher relative activity with isopropanol compared to the wild-type enzyme (Table 2). The possible explanation to this inconsistency is that kinetics studies at various substrate concentrations (Table 3) give a true representation of the kinetic behavior of the enzyme rather than measurement of the relative activity at a definite substrate concentration (4mM) as in Table 2. From the kinetic point of view, the T153A/C295A/I86A and V178A/C295A/I86A triple mutants showed similar binding affinity (K_M), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_M) to 3'-chloroacetophenone (**4a**) (Table 4). Furthermore, a similar kinetic pattern is observed between both mutants due to their similar catalytic efficiency to acetophenone (**1a**) and 4'-chloroacetophenone (**5a**) (Table 3 and Table 4).

As shown in Table 3 and Table 4, a high catalytic efficiency of 9.3 × 10^3 $M^{-1}s^{-1}$ to acetophenone (**1a**) and 5.6 × 10^3 $M^{-1}s^{-1}$ to 3'-chloroacetophenone (**4a**) by the M151A/C295A/I86A triple mutant is an indication of the mutant's broad selectivity for

acetophenone and the *meta*-electron withdrawing substituent, 3'-chloroacetophenone. A high binding affinity of M151A/C295A/I86A to both acetophenone (K_M = 0.3 ± 0.11 mM) and 3'-chloroacetophenone (K_M = 0.31 ± 0.05 mM) might be responsible for the 4.8-fold increase in mutants catalytic efficiency compared to the double mutant control. From Table 3 and Table 4, a binding affinity of the triple mutant's range between 0.31 mM and 1.3 mM for 3'-chloroacetophenone (**4a**) and 0.75 mM and 8.3 mM for 4'-chloroacetophenone (**5a**). Converse to the high binding affinity of mutants with 3'-chloroacetophenone (**4a**) reaction, the *para*- chlorinated substituent, 4'-chloroacetophenone, have a low binding affinity which could be attributed to steric effects. Similarly, apart from V115A/C295A/I86A, all the other triple mutants together with the wild-type had a lower catalytic efficiency to 4'-chloroacetophenone (**5a**). The inductive effect of the electron withdrawing group, chlorine, promotes electron delocalization in the *meta* position to cause reduction. It was previously found that strongly electron donating *para*-substituents on acetophenone suppresses electron delocalization by resonance or induction effect to cause enzymatic reduction to occur [27]. The mutants showed diverse K_M and k_{cat}/K_M values with substrates signifying different effects of each mutation in the double mutant TeSADH binding pocket.

Conversion of Acetophenone Derivatives by Asymmetric Reduction

Asymmetric reduction of triple mutants, double mutant, and wild-type were carried out in biphasic media to further investigate the activity and kinetics data. The reduction of aromatic hydrophobic ketones (**1a**–**6a**) were done using hexane as solvent and water-miscible isopropanol as co-substrate for NADPH regeneration. Asymmetric reduction of substrates catalyzed by the double mutant enzyme gave productivity which are

consistent with our kinetics data in the order of **4a** > **1a** > **6a** > **2a** > **5a** (Fig. 3). The V115A mutation widened the small binding pocket in the V115A/C295A/I86A triple mutant to significantly convert 87% of the *meta*-methyl electron donating substrate, 3'-methylacetophenone (**2a**), to (*R*)-1-(3-methylphenyl)-ethanol (2.4-fold increase to the double mutant) with percent enantiomeric excess (% ee) > 99%. High % *ee* values were observed from our chromatographs (supplementary data) since all the product peaks were in either *R* or *S* configuration. It is important to note that the V115A mutation introduced in the double mutant did not alter the stereospecificity of the triple mutant in producing alcohols with *R* enantio preference. The V115A/C295A/I86A mutant also showed broad substrate specificity by producing 63% of (*R*)-1-(3-chlorophenyl)ethanol from **4a**, 48% of (*R*)-1-phenylethanol from **1a**, and 36% of (*R*)-1-(3-methoxyphenyl)ethanol from **6a**. The A85G/C295A/I86A triple mutation in the small binding pocket enlarged it to accommodate **1a**, **2a**, **4a**, and **6a** and converts these substrates more than 50% to their corresponding chiral alcohols. The A85G/C295A/I86A triple mutant asymmetrically produced 58% of (*R*)-1-(3-methoxyphenyl)ethanol from 3'-methoxyacetophenone (**6a**) compared to 49% yield by the double mutants. The oxygen atom in 3'-methoxyacetophenone due to its high electronegativity difference may account for the preferential increase in yield by the triple mutant.

This broad substrate specificity of the double and triple mutants make the enzyme a good candidate for dynamic-kinetic resolution [28]. The T153A/C295A/I86A triple mutant only showed broad specificity to acetophenone (**1a**) and 3'-chloroacetophenone (**4a**) with ≥ 65% yield in (*R*)-1-phenylethanol and (*R*)-1-(3-chlorophenyl)ethanol, respectively. The V178A/C295A/I86A triple mutant produced ≤ 12% of both (*R*)-1-

phenylethanol and (*R*)-1-(3-chlorophenyl)ethanol. Though, T153A/C295A/I86A and V178A/C295A/I86A mutants have similar K_M and k_{cat}/K_M values to acetophenone and 3'-chloroacetophenone, the mutants have different convertibility in asymmetric reduction.

The triple point mutants A85G/C295A/I86A, V115A/C295A/I86A, and T153A/C295A/I86A have unfavorable kinetic outcomes, nonetheless, their asymmetric reduction surprisingly gave higher yield of (*R*)-1-(3-methoxyphenyl)ethanol from **6a**, (*R*)-1-(3-methylphenyl)-ethanol from **2a**, and (*R*)-1-phenylethanol from **1a**, respectively, compared to the double mutant (Fig. 3). The results indicate that the double and triple mutants have a broad substrate specificity to acetophenone (**1a**), 3'-chloroacetophenone (**4a**), 3'-methylacetophenone (**2a**), and 3'-methoxyacetophenone (**6a**). The broader substrate tolerance of mutant enzymes (A85G/C295A/I86A, V115A/C295A/I86A, and T153A/C295A/I86A) to sterically demanding electron withdrawing/donating substituents of acetophenone is important for producing optically active and enantiomeric pure alcohols. None of the triple mutants generated, nor the double mutant show any substantive preference for 4'-chloro- and 4'-methyl-acetophenone which may be attributed to steric effects of the ketones in the active site. It is worth mentioning that the wild-type could not produce alcohols more than 2% from acetophenone derivatives, despite proving reactive for some of them in the kinetic studies and activity assay.

We emphasize that, though the kinetic data does not always correlate with the thermodynamic hydrogen transfer asymmetric reduction [29]; this analysis is vital to explain bioreduction activity assay and kinetic parameters like K_M and V_{max}. The decrease in hydrophobicity by alanine mutation, recycling of NADPH, and accessibility

of substrates to the enzymes active site may account for the difference between the kinetic and asymmetric reduction values. The use of hexane as solvent and 2-propanol as co-substrate in this enzymatic biotransformation gave higher conversions as reported by Musa *et al.* [16]. However, other media systems may enhance the convertibility and enantioselectivity of substrates by mutant enzymes (supplementary data contain GC chromatographs and modeled TeSADH mutants). The improved catalytic efficiency, binding affinity, and remarkable productivity of mutants to substrates demonstrate that mutations in the small alkyl binding pocket affect catalysis.

Thermal Stability Studies

To ascertain how the mutated amino acid residues altered the geometrical stability and the thermal stability of TeSADH, the wild-type, double mutant, and triple mutants were monitored by estimating residual activity at which 50% enzymatic activity is lost ($T_{1/2}$) (Table 2). This was done by incubating enzymes for 60 min at increasing temperatures from 30–90ºC. As expected, the $T_{1/2}$ of the wild-type was 86.5ºC [30] confirming the tolerance of the 352-amino acid tetramer to extremely high temperatures. We herein report that, the double point mutation, C295A/I86A, in TeSADH reduced the $T_{1/2}$ to 73.1ºC, and optimally reacts with isopropanol at 50ºC (Fig. 4). The temperature coefficient (Q_{10}) of all mutants, double mutant, and wild-type increased exponentially from 0.8–2.2 at 30ºC to 50ºC (data not shown), and does not depend on enzyme concentration [30].

From Table 2, for A85G/C295A/I86A, V178A/C295A/I86A, and T153A/C295A/I86A mutants, an additional single point mutation in the small alkyl binding pocket of the double mutant did not only alter its catalysis but also lowered

321 $\Delta T_{1/2}$ by –8ºC, –4.6ºC, and –0.6ºC, respectively. The M151A/C295A/I86A and
322 V115A/C295A/I86A mutants significantly increased $\Delta T_{1/2}$ by +6.8ºC and +2.4ºC,
323 respectively, to restore the double mutant's lost thermal stability. As expected, a
324 substitution of Ala-85 with glycine drastically increased conformational entropy of
325 unfolding the A85G/C295A/I86A 3-dimensional structure [31], thereby reducing the
326 $T_{1/2}$ to 65.1ºC. Also, we can deduce that the branched Cβ of Thr-153 and Val-178
327 contributes in maintaining the geometrical stability of TeSADH. A substitution of Val-
328 178 with alanine in V178A/C295A/I86A triple mutant enzyme may reduce the
329 hydrophobic packing of enzyme thereby deteriorating the thermal stability.

330 From Fig. 4, the M151A/C295A/I86A triple mutant reacts optimally at 70 ºC (with
331 almost 4 times more residual activity than the wild-type) showing enhancement of the
332 mutant's thermal stability in organic media. The thermal stability and high productivity
333 of mutant enzymes make them ideal candidates for industrial usage. From the data in
334 Table 5, the thermodeactivation constant (k_d) increases in response to increasing
335 temperatures with the double mutant deactivating from 60 to 90ºC, however, the
336 M151A/C295A/I86A and V115A/C295A/I86A triple mutants showed significant
337 deactivation from 80 to 90ºC. Substitutions of M-115 and Val-151 with alanine
338 improved the $T_{1/2}$ of the mutants which may be attributed to alanine's ability to interact
339 properly with neighboring residues to form hydrophobic bonds to stabilize the enzyme
340 [32]. We have shown that substitutions at key positions in the TeSADH small binding
341 pocket with alanine or glycine affect catalysis, stability, or both. Previous studies
342 reported improvement of ADH enzyme stability by proline substitutions [24, 25, 33],
343 however, we propose the restoration of the double mutant TeSADH stability by alanine
344 substitution, hence, the need to research more into other residues for TeSADH

345 stabilization.

346 In this study, an additional single point mutation at five key residues in the small
347 binding pocket (Ala-85, Val-115, Met-151, Thr-153, and Val-178) expanded the small
348 alkyl binding pocket even more in the active site of the double mutant (C295A/I86A)
349 TeSADH to accommodate aromatic hydrophobic ketones with electron donating or
350 electron withdrawing substituents. The mutants show diverse binding and catalytic
351 efficiencies to substrates from the kinetic study. Coupling the kinetic studies with the
352 hydrogen transfer asymmetric reduction in biphasic media suggest some similarity
353 between the two, however, no direct correlation exists. The A85G/C295A/I86A,
354 V115A/C295A/I86A, and T153A/C295A/I86A triple mutants showed broad substrate
355 specificity and productivity to acetophenone, 3'-methylacetophenone, and 3'-
356 chloroacetophenone to produce enantiopure alcohols. Mutants showed little or no
357 preference for the *para*- substituted derivatives, but most importantly, mutants reacted
358 moderately to more than one substrate explaining their broad substrate tolerance. From
359 the mutant's thermal stability studies, alanine mutations at some key positions in the
360 small pocket restored the lost thermal stability of the double mutant and make it similar
361 to the wild-type. The generated triple mutants may be useful in dynamic-kinetic
362 resolution reactions for producing chiral alcohols.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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466 **Table 1.** List of forward primers used for site-directed mutagenesis.

Mutations	Forward primer sequences
A85G	5'-GCGTTGTTGTGCCAG**GT**GCTACCCCTGATTG
V115A	5'-TGGAAATTTTCGAAT**GCA**AAAGATGGTGTTTTTG
M151A	5'-AGTTATGATTCCCGAT**GCG**ATGACCACTGGTTTTC
T153A	5'-GATTCCCGATATGATG**GCC**ACTGGTTTTCACG
V178A	5'-TGGGTATTGGCCCA**GCA**GGTCTTATGGCAGT

467

468 Each additional mutation was introduced on the double mutant (C295A/I86A) TeSADH
469 using the forward mutagenic primer and the reverse mutagenic primer as its reverse
470 complement. The mutated nucleotides are bold and the codons are underlined.

Table 2. Residual and relative activity of mutants with isopropanol in organic medium.

TeSADH	$T_{1/2}$ (ºC) [a]	% Relative activity [b]
WT	86.5	128 ± 1.0
DM	**73.1**	**100** ± 0.6
A85G	65.1	55.1 ± 0.3
V115A	75.5	14.7 ± 0.2
M151A	79.9	141 ± 1.0
T153A	72.5	157 ± 0.5
V178A	68.5	148 ± 0.4

WT and DM mean the wild-type and the double mutant (C295A/I86A) TeSADH, respectively. Each additional mutation was introduced on the double mutant (C295A /I86A) TeSADH.

[a] $T_{1/2}$ is the temperature at which 50% enzymatic activity is lost after 60 min incubation in the range of 30 – 90ºC. Values are obtained from interpolation between residual enzymatic activity versus temperature (standard deviation = ± 2ºC).

[b] Relative activity of mutants measured at 60ºC in 50 mM Tris-HCl buffer (pH 8.9) containing 10 µM $ZnCl_2$, 4 mM isopropanol, 0.5 mM $NADP^+$ and 3 mM DTT. Each value represents mean of triplicate enzymatic activity ± standard deviation. The activity of the double mutant with isopropanol is set as baseline (100%).

490 **Table 3.** Kinetic parameters determined using isopropanol or acetophenone as a
491 substrate.

Mutant	Isopropanol			Acetophenone		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
WT	0.069 ± 0.014	23.6 ± 0.8	343,100 ± 70,800	0.30 ± 0.13	0.50 ± 0.05	170 ± 70
DM	**0.12 ± 0.05**	3.6 ± 0.4	29,600 ± 12,500	1.3 ± 0.1	2.4 ± 0.2	1,940 ± 260
A85G*	9.2 ± 1.7	88.1 ± 6.3	9,540 ± 1,890	ND	ND	ND
V115A*	ND	ND	ND	0.91 ± 0.26	3.0 ± 0.2	3,300 ± 990
M151A	4.4 ± 0.8	30.4 ± 2.9	6,840 ± 1,450	0.30 ± 0.11	2.8 ± 0.2	9,290 ± 3,510
T153A	5.7 ± 1.7	12.6 ± 1.5	2,230 ± 720	1.8 ± 0.5	1.9 ± 0.2	1,040 ± 290
V178A	20.2 ± 6.3	28.8 ± 4.6	1,430 ± 500	1.1 ± 0.4	1.1 ± 0.1	1,050 ± 380

492

493 WT and DM mean the wild-type and the double mutant (C295A/I86A) TeSADH,
494 respectively. Each additional mutation was introduced on the double mutant
495 (C295A/I86A) TeSADH.

496 K_M (mM) is the substrate concentration at $1/2V_{max}$. k_{cat} is the turnover number in
497 minutes and k_{cat}/K_M is the catalytic efficiency ($M^{-1}s^{-1}$) of the enzyme. All values
498 represent mean of triplicate reactions ± standard deviation from 0.1 – 32 mM substrate
499 concentration. ND values are not determined due to absorbance inconsistencies.

500 * Protein concentration is halved

501

Table 4. Kinetic parameters calculation using 3'-chloroacetophenone or 4'-chloroacetophenone as a substrate.

	3'-Chloroacetophenone			4'-Chloroacetopheone		
Mutant	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
WT	0.80 ± 0.12	0.29 ± 0.15	370 ± 200	3.1 ± 0.9	2.8 ± 0.1	700 ± 210
DM	**0.30 ± 0.07**	1.6 ± 0.04	5,290 ± 1,230	0.33 ± 0.002	8.6 ± 1.7	26,000 ± 5,100
V115A*	1.2 ± 0.6	2.6 ± 0.3	2,200 ± 1,240	1.1 ± 0.4	3.8 ± 0.3	3,580 ± 1,450
M151A	0.31 ± 0.05	1.7 ± 0.04	5,600 ± 920	6.6 ± 1.0	1.3 ± 0.1	200 ± 30
T153A	1.3 ± 0.4	1.5 ± 0.1	1,110 ± 380	8.3 ± 1.3	1.4 ± 0.1	170 ± 30
V178A	1.3 ± 0.4	1.5 ± 0.1	1,190 ± 340	4.6 ± 0.8	0.75 ± 0.04	160 ± 30

WT and DM mean the wild-type and the double mutant (I86A/C295A) TeSADH, respectively. Each additional mutation was introduced on the double mutant (C295A/I86A) TeSADH.

K_M (mM) is the substrate concentration at 1/2Vmax. k_{cat} is the turnover number in minutes and k_{cat}/K_M is the catalytic efficiency ($M^{-1}s^{-1}$) of the enzyme. All values represent mean of triplicate reactions ± standard deviation from 0.1 – 32 mM substrate concentration.

* Protein concentration is halved

The A85G/C295A/I86A triple mutant did not produce consistent values to plot non-linear fit by using 3'-chloroacetophenone and 4'-chloroacetophenone as substrates.

Table 5. Effect of temperature on the deactivation of TeSADH mutants.

Temp	Deactivation Rate Constant ($k_d \times 10^2$)						
(ºC)	WT	DM	A85G	V115A	M151A	T153A	V178A
60	–	0.23	–	–	–	–	–
70	–	0.59	6.3	–	–	0.56	1.5
80	–	4.1	8.2	4.7	1.6	7.4	10
90	8.2	9.5	11	8.2	7.6	7.6	12

WT and DM mean the wild-type and the double mutant (C295A/I86A) TeSADH, respectively. Each additional mutation was introduced on the double mutant (C295A/ I86A) TeSADH.

Figure legends

Fig. 1. Ribbon drawing of double mutant (I86A/C295A) TeSADH.

Yellow color (amino acid to be substituted by site-directed mutagenesis), Red color (β-sheet), Cyan (α-helix) and Purple (loop). All mutant TeSADHs were analyzed with PyMol (PyMol Molecular Graphics Systems) using PDB file 1bxz as template.

Fig. 2. Chemical structures of substrates used for activity, kinetics, and reduction studies.

(**1a**) acetophenone, (**2a**) 3'-methylacetophenone, (**3a**) 4'-methylacetophenone, (**4a**) 3'-chloroacetophenone, (**5a**) 4'-chloroacetophenone, (**6a**) 3'-methoxyacetophenone and (**7a**) isopropanol.

Fig. 3. Percentage yield/conversion of selected mutants in the biphasic asymmetric reduction system using acetophenone and its derivatives as a substrate.

Acetophenone (**1a**, ■); 3'-methylacetophenone (**2a**, ▤); 3'-chloroacetophenone (**4a**, ▬), 4'-chloroacetophenone (**5a**, ▨), or 3'-methoxyacetophenone (**6a**, ▩). DM means the double mutant (C295A/I86A) TeSADH.

Fig. 4. Effect of mutations on the thermal stability of TeSADH.

Thermal stability was determined by measuring the residual enzymatic activity after 60 min of incubation from 30 – 90ºC using a UV-Vis spectrophotometer equipped with temperature controller. WT and DM mean the wild-type and the double mutant (C295A/I86A) TeSADH, respectively.

551 Wild-type (open square), C295A/I86A (open circle), A85G/C295A/I86A (open triangle;
552 up), V115A/C295A/I86A (open triangle; down), M151A/C295A/I86A (open diamond),
553 T153A/C295A/I86A (filled circle), and V178A/C295A/I86A (filled square).
554

Fig. 1.

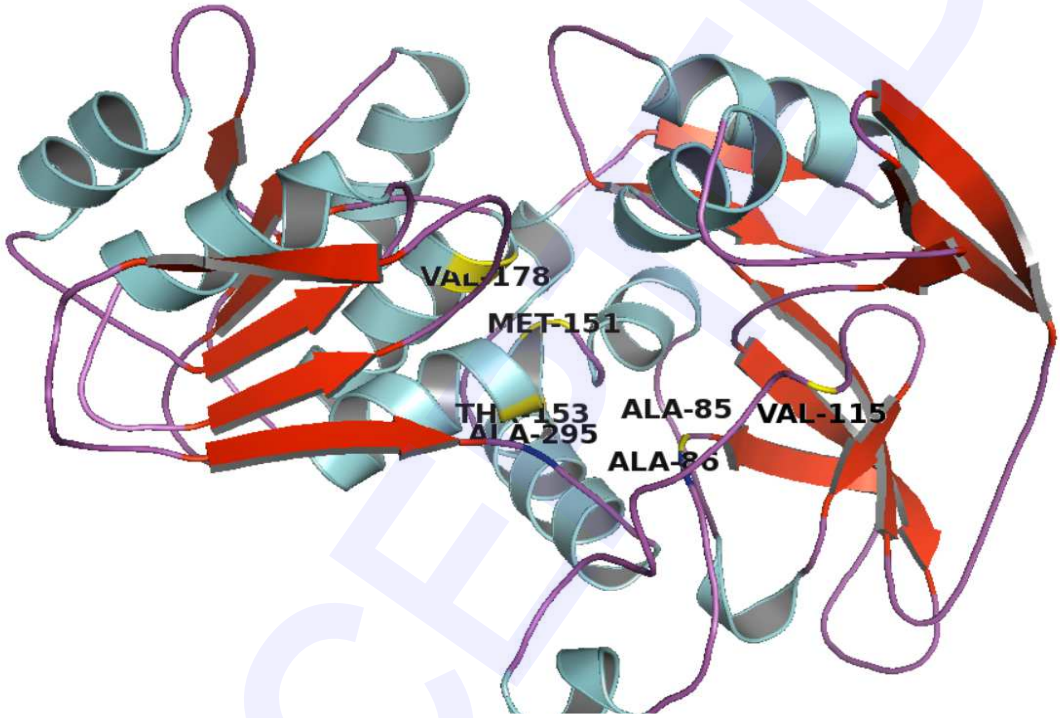

Fig. 2.

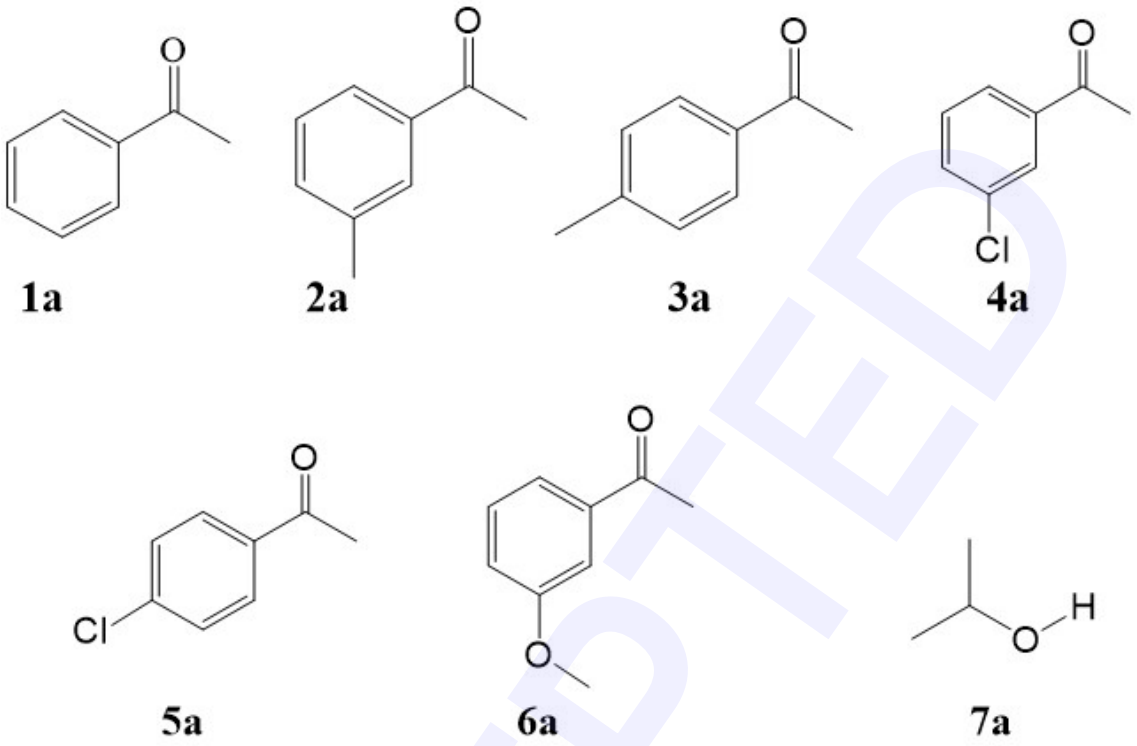

Fig. 3.

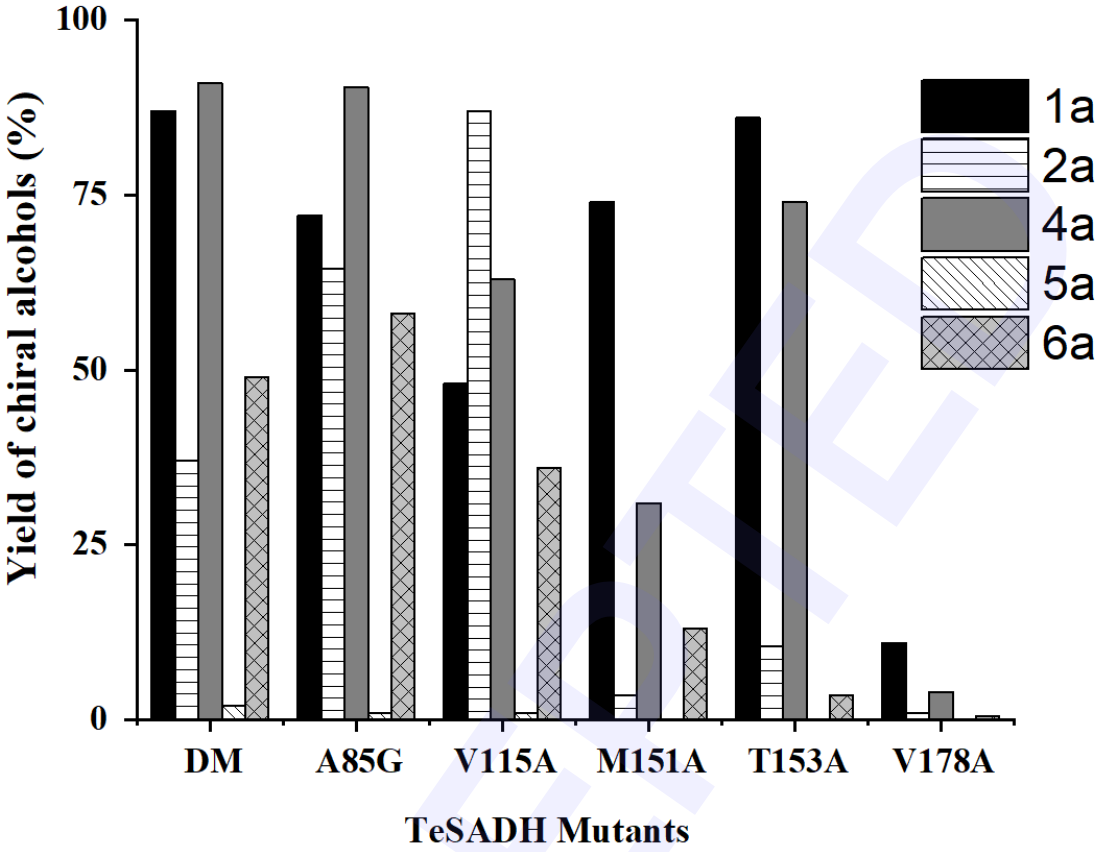

Fig. 4.