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

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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 that 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 +6.8°C and +2.4°C, respectively, with thermal deactivation constant (k_d) close to the wild-type enzyme. The M151A/C295A/I86A mutant reacts optimally at 70 °C 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 for 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 their 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 are important in pharmaceutical settings to produce enantiopure compounds, however, such catalytic properties are not

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

Yellow (amino acid to be substituted by site-directed mutagenesis), Red (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.

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 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

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.

catalysis. We compared the relative activity, catalytic efficiency, and turnover 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 provide 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'-chloroacetophenone (4a), 4'-chloroacetophenone

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

Mutations	Forward primer sequences
A85G	5'-GCGTGTGTGTCAC <u>GGT</u> GCTACCCCTGATTG
V115A	5'-TGGAAATTTTCGAATG <u>C</u> AAAAGATGGTGTTTTG
M151A	5'-AGTTATGATTCCTGATG <u>CCG</u> ATGACCACTGGTTTTC
T153A	5'-GATTCCCGATATGATG <u>CCC</u> ACTGGTTTTCACG
V178A	5'-TGGGTATTGGCCCAG <u>C</u> AGGTCTTATGGCAGT

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

(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 consisted 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 the TeSADH gene containing I86A/C295A mutations. The reaction was run in a thermal cycler (Bio-Rad S1000) for 25 cycles at 95°C for 1 min, 95°C for 45 sec, 55 ± 2°C for 1 min, 68°C for 6 min and 16 °C for storage. PCR products were digested with *Dpn* I (New England BioLabs) at 37°C 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* DH5α competent cells following the method described in [17] with some modifications. The mixture was incubated at 36.5°C 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 were performed as previously reported [18] with some modifications. Mutant TeSADH was expressed in *E. coli* DH5α cells in 500 ml LB medium containing 50 µg/ml each of ampicillin and kanamycin. Crude TeSADH extract was loaded on 5 ml of 50% suspended Reactive Red 120-Agarose, Type 3000-CL (Sigma-Aldrich) affinity chromatography, equilibrated with 0.07 M NaClO₄ in buffer A (50 mM Tris-HCl [pH 8.0 at 25°C], 3 mM DTT and 10 µM ZnCl₂). Proteins were eluted with 0.2 M NaClO₄ in buffer A. Protein concentration was estimated using a Bio-Rad Protein Assay kit with bovine serum albumin as standard.

Modeling of Mutant TeSADHs

Modeling of mutant TeSADHs was done using the automatic Swiss-Model 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 the modeling quality of the mutants [23] (supplementary data).

Enzyme Activity Measurement and Determination of Kinetic Parameters

All enzyme assays were performed at 60°C in triplicate following NADP⁺/NADPH production at 340 nm ($\epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$) in a UV/Vis spectrophotometer equipped with TCC-temperature controller (Shimadzu, Japan). The reaction contained 50 mM Tris-HCl buffer (pH 8.9 at 60°C), 0.5 mM NADP⁺/NADPH, 4.0 mM substrate, 3.0 mM DTT and 10 µM ZnCl₂ in a total of 1 ml. Enzymes (0.52 mg/ml) were pre-incubated at 60°C 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 was calculated using the following formula, in which DM means the double mutant (C295A/I86A) TeSADH (Table 2):

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

TeSADH	T _{1/2} (°C) ^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.

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

^bRelative activity of mutants measured at 60°C in 50 mM Tris-HCl buffer (pH 8.9) containing 10 µM ZnCl₂, 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%).

Table 3. Kinetic parameters determined using isopropanol or acetophenone as a 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

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.

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

*Protein concentration is halved.

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

The K_M values of **1a**, **4a**, **5a**, and **7a** were determined using increasing concentrations from 0.1–32 mM (Tables 3 and 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 follows methods described previously [14] with some modifications (Fig. 3). The mixture contained **1a–6a** (0.21 mmol), NADPH (1 mg), DTT (1 mg), ZnCl₂ (10 μM) in 50 mM Tris-HCl buffer solution (300 μl; pH 8.0 at 25°C), 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 were

incubated at 50°C and shaken at 120 rpm for 24 h. The reaction products were extracted using diethyl ether (3 × 2.5 ml), centrifuged at 5,000 ×g for 3 min, and dried over Na₂SO₄ 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 was determined by converting the alcohols to their corresponding acetate derivatives and comparing them with their *R*- or *S*-acetate derivatives of standard samples. The retention times of the acetylated alcohols and their corresponding acetylated standards helped to identify the configuration of the produced alcohols. To confirm the absolute configuration of the produced alcohols, co-injection on a chiral column with a standard alcohol was done.

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

Mutant	3'-Chloroacetophenone			4'-Chloroacetophenone		
	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 $1/2V_{max}$, k_{cat} is the turnover number in minutes and k_{cat}/K_M is the catalytic efficiency (M⁻¹s⁻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.

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.

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

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 30–90°C using a UV-Vis spectrophotometer equipped with temperature controller. WT and DM mean the wild-type and the double mutant (C295A/I86A) TeSADH, respectively. Wild-type (open square), C295A/I86A (open circle), A85G/C295A/I86A (open triangle; up), V115A/C295A/I86A (open triangle; down), M151A/C295A/I86A (open diamond), T153A/C295A/I86A (filled circle), and V178A/C295A/I86A (filled square).

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

Temp (°C)	Deactivation Rate Constant ($k_d \times 10^2$)						
	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	10

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.

enzymatic activity after 60 min of incubation in buffer from 30–90°C (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, $\ln A_t = \ln A_0 - k_d (t)$, where A_t is the enzyme activity after incubation, A_0 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

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 ($K_M = 20.2 \pm 6.3$ 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 ($K_M = 0.069 \pm 0.014$ mM; 60–290 times) and catalytic efficiency (3.4×10^5 M⁻¹s⁻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 for 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 (4 mM) 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⁻¹s⁻¹ to acetophenone (**1a**) and 5.6×10^3 M⁻¹s⁻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 ($K_M = 0.3 \pm 0.11$ mM) and 3'-chloroacetophenone ($K_M = 0.31 \pm 0.05$ mM) might be responsible for the 4.8-fold increase in mutant 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, has 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**) was 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 was 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 makes 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 $\geq 65\%$ yield in (*R*)-1-phenylethanol and (*R*)-1-(3-chlorophenyl)-ethanol, respectively. The V178A/C295A/I86A triple mutant produced $\leq 12\%$ 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 do 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 30–90°C. As expected, the $T_{1/2}$ of the wild type was 86.5°C [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 73.1°C, and optimally reacts with isopropanol at 50°C (Fig. 4). The temperature coefficient (Q_{10}) of all mutants, double mutant, and wild type increased exponentially from 0.8–2.2 at 30°C to 50°C (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 $\Delta T_{1/2}$ by -8°C , -4.6°C , and -0.6°C , respectively. The M151A/C295A/I86A and V115A/C295A/I86A mutants significantly increased $\Delta T_{1/2}$ by $+6.8^\circ\text{C}$ and $+2.4^\circ\text{C}$, respectively, to restore the double mutant's lost thermal stability. As expected, a substitution of Ala-85 with glycine drastically increased conformational entropy of unfolding the A85G/C295A/I86A 3-dimensional structure [31], thereby reducing the $T_{1/2}$ to 65.1°C. Also, we can deduce that the branched C β of Thr-153 and Val-178 contributes in maintaining the geometrical stability of TeSADH. A substitution of Val-178 with alanine in V178A/C295A/I86A triple mutant enzyme may reduce the hydrophobic packing of enzyme thereby deteriorating the thermal stability.

From Fig. 4, the M151A/C295A/I86A triple mutant reacts optimally at 70 °C (with almost 4 times more residual activity than the wild type) showing enhancement of the mutant's thermal stability in organic media. The thermal

stability and high productivity of mutant enzymes make them ideal candidates for industrial usage. From the data in Table 5, the thermodeactivation constant (k_d) increases in response to increasing temperatures with the double mutant deactivating from 60 to 90°C, however, the M151A/C295A/I86A and V115A/C295A/I86A triple mutants showed significant deactivation from 80 to 90°C. Substitutions of M-115 and Val-151 with alanine improved the $T_{1/2}$ of the mutants which may be attributed to alanine's ability to interact properly with neighboring residues to form hydrophobic bonds to stabilize the enzyme [32]. We have shown that substitutions at key positions in the TeSADH small binding pocket with alanine or glycine affect catalysis, stability, or both. Previous studies reported improvement of ADH enzyme stability by proline substitutions [24, 25, 33], however, we propose the restoration of the double mutant TeSADH stability by alanine substitution, hence, the need to research more into other residues for TeSADH stabilization.

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