A Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase mutant derivative highly active and stereoselective on phenylacetone and benzylacetone

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The secondary alcohol dehydrogenase from Thermoanaerobacter ethanolicus 39E (TeSADH) is highly thermostable and solvent-stable, and it is active on a broad range of substrates. These properties make TeSADH an excellent template to engineer an industrial catalyst for chiral chemical synthesis. (S)-1-Phenyl-2-propanol was our target product because it is a precursor to major pharmaceuticals containing secondary alcohol groups. TeSADH has no detectable activity on this alcohol, but it is highly active on 2-butanol. The structural model we used to plan our mutagenesis strategy was based on the substrate's orientation in a horse liver alcohol dehydrogenase•p-bromobenzyl alcohol•NAD+ ternary complex (PDB entry 1HLD). The W110A TeSADH mutant now uses (S)-1-phenyl-2-propanol, (S)-4-phenyl-2-butanol and the corresponding ketones as substrates. W110A TeSADH's kinetic parameters on these substrates are in the same range as those of TeSADH on 2-butanol, making W110A TeSADH an excellent catalyst. In particular, W110A TeSADH is twice as efficient on benzylacetone as TeSADH is on 2-butanol, and it produces (S)-4-phenyl-2-butanol from benzylacetone with an enantiomeric excess above 99%. W110A TeSADH is optimally active at 87.5°C and remains highly thermostable. W110A TeSADH is active on aryl derivatives of phenylacetone and benzylacetone, making this enzyme a potentially useful catalyst for the chiral synthesis of aryl derivatives of alcohols. As a control in our engineering approach, we used the TbSADH•(S)-2-butanol binary complex (PDB entry 1BXZ) as the template to model a mutation that would make TbSADH active on (S)-1-phenyl-2-propanol. Mutant Y267G TbSADH did not have the substrate specificity predicted in this modeling study. Our results suggest that (S)-2-butanol's orientation in the TbSADH•(S)-2-butanol binary complex does not reflect its orientation in the ternary enzyme–substrate–cofactor complex.

Keywords: (S)-alcohol/benzylacetone/enantioselectivity/phenylacetone/secondary alcohol dehydrogenase

Introduction

Up to 90% of the drugs currently being made are sold as racemic mixtures, but the FDA has been increasingly mandating chiral purity, or evidence to show that the inactive enantiomer is not harmful. Indeed, while generally only one enantiomer is biologically active for the intended purpose of the drug, the other enantiomer can be toxic (Devaux-Basseguy et al., 1997; Burdette et al., 2000; Eriksson et al., 2001). For these reasons, pharmaceutical companies are looking for more efficient ways of producing enantiomerically pure compounds. Much effort has been spent in the last two decades to replace conventional chemical reactions with biological reactions. With their high substrate specificity and their high enantio- and region-selectivities, enzymes can catalyze in one step reactions that would otherwise require costly blocking and deblocking steps. These enzyme properties also lead to fewer by-products by helping minimize undesirable side reactions such as isomerization, racemization, epimerization and rearrangement (Schmid et al., 2001; Patel, 2005).

Chiral alcohols are important building blocks in a variety of high-value chemicals used in the food, fine chemical and pharmaceutical industries (Machielsen et al., 2006). Alcohol dehydrogenases (ADHs) (EC 1.1.1.1 and 1.1.1.2) catalyze the reversible reduction of ketones and aldehydes to alcohols (Burdette et al., 1996). Commercially available ADHs often have one or more shortcomings that prevent their use in industrial applications. Most lack long-term stability, most lack activity in organic solvents and at elevated temperatures and their substrate specificity is often not adapted to a particular application (Hummel, 1997). The secondary ADH (SADH) from the thermophilic bacterium Thermoanaerobacter ethanolicus 39E (TeSADH) has several properties that make it a promising enzyme for chiral alcohol production. It is optimally active near 90°C, thermostable (half-life of 1.7 h at 90°C) (Burdette et al., 2000) and specific for secondary alcohols (Burdette and Zeikus, 1994). TeSADH is also stable in solvents. It retains 90%, 100%, 80% and 68% activity after a 3-h incubation at 50°C in 100% n-dodecane, n-octane, toluene and pyridine, respectively (Keinan et al., 1986; Burdette and Zeikus, 1994; Miriolaie and Nemat-Gorgani, 2002).

The T. ethanolicus adhB gene was cloned and expressed in Escherichia coli (Burdette et al., 1996). TeSADH is a medium chain, zinc-containing, tetrameric ADH composed of identical 40 kDa subunits. This NAD(P)-dependent enzyme contains a single catalytic zinc coordinated by Cys37, His59 and Asp150 (Bogin et al., 1997). TeSADH is commercially available under the name Thermoanaerobacter [formerly Thermoanaerobium (Lee et al., 1993)] brockii SADH (TbSADH) (Sigma, St. Louis, MO). Early sequencing results (Burdette et al., 1996) suggested that TeSADH and TbSADH differed by three residues: Trp91, Pro313 and Gln325 in TeSADH versus Arg91, Arg313 and Arg325 in TbSADH. Recent T. ethanolicus 39E genome sequencing
results (NCBI entry 2P_00779753) and repeated sequencings in our lab (Laivenieks, unpublished results) indicate that TeSADH is identical to TbSADH. Both enzymes have very broad substrate specificities. Examples of successful synthesis with TbSADH have been reported in the literature (Keinan et al., 1986).

TbSADH’s 3D structure has been solved by X-ray crystallography in complex with NADP\(^+\) (PDB entry 1YKF) (Korkhin et al., 1998) and in complex with (S)-2-butanol (PDB entry 1BXZ) (Li et al., 1999). TeSADH has been structurally characterized by X-ray crystallography in the Arni lab at the Universidade de Sao Paulo, Ribeirao Preto-SP, Brazil (personal communication). Since the two enzymes are identical, the published TbSADH structures were used for our modeling studies. The TbSADH substrate-binding site is composed of a large pocket and a small pocket (Fig. 1), whose structural and chemical makeup determine the enzyme’s substrate specificity and stereo-specificity (Heiss et al., 2001). The small pocket has a higher affinity for alkyl groups than the larger pocket, and it can accommodate methyl, ethyl, isopropyl, and cyclopropyl groups, whereas anything larger is excluded. The current hypothesis explaining TeSADH’s stereospecificity is that, if the larger of the alkyl groups of a ketone fits into the small alkyl-binding pocket, the enzyme will likely produce an (R)-alcohol. If the larger of the alkyl groups is too large to fit into the small pocket, the large group will be forced into the large alkyl-binding pocket, causing the enzyme to produce an (S)-alcohol (Keinan et al., 1986). This substrate specificity mechanism was demonstrated by mutations S39T and C295A in the TeSADH active site. Mutation S39T decreased the size of the large alkyl-binding pocket. Mutation C295A enlarged the small binding pocket, allowing for longer alkyl chains to fit. Both mutations shifted TeSADH enantioselctivity toward (R)-alcohols (Tripp et al., 1998; Heiss et al., 2001). This specificity mechanism is also illustrated by the fact that TbSADH is unable to use either 4-heptanone (butyl groups on each side of the ketone) or 2-nonanone (pentyl groups on each side of the ketone), whereas both 2-heptanone and 2-nonanone are substrates (Keinan et al., 1986).

We chose TeSADH as our target enzyme to develop a catalyst able to produce 1-phenyl-2-propanol from phenylacetone. One-phenyl-2-propanol is important to the pharmaceutical industry because it is an immediate precursor to amphetamine and amphetamine derivatives (Liese et al., 2000), and no thermostable and solvent-stable ADH is known to be active on this alcohol. TeSADH is naturally inactive on phenylacetone. In this study, we designed a catalytic site point mutation, W110A, that makes TeSADH active on phenylacetone, producing 1-phenyl-2-propanol. We also show that W110A TeSADH is active on benzylacetone, and that it is specific for (S)-4-phenyl-2-butanol and (S)-1-phenyl-2-propanol. Four-phenyl-2-butanol is also important to the pharmaceutical industry because it is used as a precursor to anti-hypertensive agents and spasmyotics (anti-epileptic agents) (Liese et al., 2000).

**Materials and methods**

**Chemicals**

The racemic, (S)-, and (R)-forms of 1-phenyl-2-propanol, racemic 4-phenyl-2-butanol, NaO\(_4\), Na\(_2\)Cr\(_2\)O\(_7\)-2H\(_2\)O and isopropanol acetate were purchased from Aldrich (St. Louis, MO). Benzylacetone was purchased from ACROS Organics (Morris Plains, NJ). *Candida Antarctica* lipase immobilized on acrylic resin was purchased from Sigma (L4777).

**Syntheses of phenylacetone and (S)- and (R)-4-phenyl-2-butanol**

Phenylacetone was synthesized from (rac)-1-phenyl-2-propanol as described (Vondervoot et al., 2002), with the only modification that the reaction was performed at room temperature instead of 4°C. The yield was 33\%. \(^1\)H-NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer at 400 MHz. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.2–7.4 (m, 5H), \(\delta\) 3.7 (s, 2H), \(\delta\) 3.7 (s, 2H), \(\delta\) 3.7 (s, 2H).

(S)-4-phenyl-2-butanol was produced from (rac)-4-phenyl-2-butanol by kinetic resolution as described (Choi et al., 2004), with the exception that the ruthenium catalyst and tert-butoxide were omitted. (S)-4-Phenyl-2-butanol and (R)-1-methyl-3-phenylpropyl acetate were produced in quantitative yields after a 5-day reaction at room temperature. \(\delta\) 7.2–7.4 (m, 5H), \(\delta\) 3.8 (m, 1H), \(\delta\) 2.8 (m, 2H), \(\delta\) 1.80 (m, 3H), \(\delta\) 1.3 (m, 3H). \(\delta\) 1.3 (d, 3H).

(R)-4-phenyl-2-butanol was then produced from (R)-1-methyl-3-phenylpropyl acetate by saponification. The yield was 90%. \(\delta\) 7.2–7.4 (m, 5H), \(\delta\) 3.8 (m, 1H), \(\delta\) 2.7 (m, 2H), \(\delta\) 1.8 (m, 2H), \(\delta\) 1.3 (d, 3H).

**Modeling**

Modeling was done manually in InsightII (Accelrys, San Diego, CA) on a Silicon Graphics Octane 2 computer. The TbSADH•(S)-2-butanol complex (PDB #1BXZ) was superimposed with the TbSADH•NAD\(^+\) complex (PDB #1YKF).
#1YKF) (Korkhin et al., 1998) in InsightII. After superposition, the entire 1BXZ structure, except for the (S)-2-butanol molecule, was removed, and the (S)-2-butanol was merged with the 1YKF structure. The result was a single TbSADH enzyme structure model containing NADP\(^+\), zinc and (S)-2-butanol. The 3D-structure of (S)-1-phenyl-2-propanol was generated with CORINA-Gasteiger Research. This substrate was fitted in the active site of the new TbSADH•(S)-2-butanol•NADP\(^+\) model, with its reactive oxygen superimposed with that of (S)-2-butanol.

In a second modeling approach, we started from the structure of the horse liver ADH (HLADH) co-crystallized with a substrate (i.e. p-bromobenzyl alcohol, BRB) and NAD\(^+\) (PDB #1HLD). The TbSADH•(S)-2-butanol•NADP\(^+\) model was superimposed with the structure of the HLADH•BRB•NAD\(^+\) complex using the conserved catalytic site residues for the alignment. Starting from the (S)-1-phenyl-2-propanol PDB file we had generated in CORINA, we generated the lowest energy conformations of this substrate using Omega (OpenEye Scientific Software, Santa Fe, NM). Individual conformations were then fitted manually into the TbSADH catalytic site, their C–OH bond superimposed with that of BRB in HLADH. All individual conformations of (S)-1-phenyl-2-propanol were manually rotated around their C–OH bond axis to identify orientations that would minimize steric overlap between atoms of the substrate and active site residues. One of the seven (S)-1-phenyl-2-propanol conformations tested (the lowest energy conformation) created steric overlap with a single residue, Trp110, in TeSADH’s catalytic site. All other (S)-1-phenyl-2-propanol conformations created overlaps with more than one residue.

The 3D structures of the mutant enzymes were modeled using the SUPERMODEL (Peitsch, 1995; Guex and Peitsch, 2003) program with the TbSADH•NADP\(^+\) complex as the template. The wild-type enzyme was also modeled as a control to detect any changes that may be modeling artifacts. The models were superimposed with the TbSADH crystal structure to determine how much if any the backbone of the mutant structure deviated from the crystal structure.

**Mutagenesis**

Mutations W110A and Y267G were first tested in crude extracts from 5 ml cultures as (alcohol oxidation), or conversion (ketone reduction) at 340 nm for 1 min in a Varian Cary 300 UV/vis spectrophotometer equipped with a Peltier heating system. In all cases, the enzyme was preincubated

Protein expression and purification

W110A TeSADH was expressed in HB101(DE3) cells. Cultures were grown in 500 ml LB medium (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl) containing 100 mg/l kanamycin. When the culture reached 0.6–1 OD\(_{600}\), W110A TeSADH expression was induced with 1 mM IPTG for 5 h. The cells were spun down (5000 rpm for 10 min) and resuspended in 4 volumes of lysis buffer (50 mM Tris–HCl [pH 8.5], 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) per 1 g of cells. The cells were lysed in a French pressure cell, and the lysate was spun down (10 000 rpm for 10 min) to remove the cell debris. The crude extract was heat-treated at 85°C for 15 min to inactivate non-thermostable proteins, then spun down (10 000 rpm for 10 min) to remove the denatured proteins. The cleared crude extract was loaded on a 5 ml Ni-NTA (Qiagen, Valencia, CA) column. W110A TeSADH was purified using the Gibco BRL procedure for Protein Expression System, pRex-E1 vector (cat. no. 10197-010, Gaithersburg, MD). Protein expression in the fractions was tested by SDS-PAGE (Laemmli, 1970). Protein concentration was quantified using the Biorad Protein Dye with bovine serum albumin as the standard. The purified protein was frozen in 250 µl aliquots at −80°C for months without affecting enzyme activity.

Enzyme assays

Activities of the wild-type, Y267G and W110A TeSADHs were first tested in crude extracts from 5 ml cultures as described (Burdette and Zeikus, 1994) with (rac)-2-butanol, (rac)-1-phenyl-2-propanol and (rac)-4-phenyl-2-butanol as substrates. All assays were done in the presence of 50 mM Tris–HCl pH 8.0 and 0.4 mM NADP\(^+\) (alcohol oxidation), or pH 6.5 and 0.4 mM NADPH (ketone reduction). Initial velocity was measured spectrophotometrically at 60°C by following NADPH production (alcohol oxidation), or consumption (ketone reduction) at 340 nm for 1 min in a Varian Cary 300 UV/vis spectrophotometer equipped with a Peltier heating system. In all cases, the enzyme was preincubated
for at least 5 min at 40°C before being added to the assay. The same conditions were used to test activity of the purified W110A TeSADH with (rac)-2-butanol; (rac)-1-phenylethan-1-ol; the (S)- and (R)-4-phenyl-2-butanol; (S)- and (R)-1-phenyl-2-propanol; acetophenone; benzylacetone; phenylacetone and the aryl derivatives (1 mM each) 1-chloro-3-phenyl-2-propanol, 3-chloro-3-methyl-4-phenyl-2-butanone, (2-fluorophenyl) acetone and 1-(4-bromophenyl) acetone. One unit of activity was defined as the amount of enzyme needed to consume or produce 1 µmol of NADPH per minute.

To determine the effect of temperature on activity, enzyme assays were performed at temperatures 5–97°C, with 10 mM (rac)-1-phenyl-2-propanol as the substrate. To determine the kinetic parameters, enzyme assays were performed with (rac)-2-butanol (0.5–500 mM), (S)-1-phenyl-2-propanol (0.05–10.5 mM), (S)-4-phenyl-2-butanol (0.05–10 mM), benzylacetone (0.05–5 mM), and phenylacetone (0.05–7.5 mM) at 60°C for 1 min in 50 mM Tris–HCl pH 8.0 (alcohol oxidation) or pH 6.5 (ketone reduction) in the presence of 0.4 mM NADP(H). At least eight substrate concentrations were used for each data set and each set was performed in triplicate. The $K_m$ and $V_{max}$ values of W110A TeSADH were calculated using the Non-Linear Curve Fit tool of Origin 6.1. The $K_m$ and $V_{max}$ values of W110A TeSADH were calculated using the Non-Linear Curve Fit tool of Origin 6.1. The $K_m$ and $V_{max}$ values of W110A TeSADH were calculated using the Non-Linear Curve Fit tool of Origin 6.1.

**Results and discussion**

It has previously been shown with TbSADH that large substrates, such as 2-decanone, can be reduced to the corresponding alcohol at very low rates. In contrast, TeSADH is not significantly active on substrates containing rings with more than three carbons (Keinan et al., 1986); the bulk of the ring does not fit in the active site. Mutations were decided upon to increase the size of the active site and accommodate the large phenyl ring of 1-phenyl-2-propanol.

**Modeling and mutagenesis**

When we superimposed the TbSADH•(S)-2-butanol and TbSADH•NADP$^+$ complexes, we noticed that the 2-butanol molecule in the TbSADH•(S)-2-butanol complex almost overlapped NADP$^+$‘s nicotinamide ring in the TbSADH•NADP$^+$ complex (Fig. 2). In addition, the catalytic Zn$^{2+}$ moves by more than 1 Å between the two structures. These observations suggest that the positions in which NADP$^+$‘s nicotinamide ring is oriented in the TbSADH•NADP$^+$ complex and/or the position in which 2-butanol is oriented in the TbSADH•(S)-2-butanol complex do not reflect their orientations in an active enzyme–substrate-cofactor ternary complex. In PDB structure 1HLD, HLADH is co-crystallized with a substrate (i.e. p-bromobenzyl alcohol, BRB) and NAD$^+$. When we superimposed this structure with those of the TbSADH•(S)-2-butanol and the TbSADH•NADP$^+$ complexes (1BXZ and 1YKF, respectively; W110A TeSADH solvent stability was tested with the same procedure as the thermostability tests (Burdette et al., 2000) with 30% 2-propanol in the inactivation enzyme solutions. Time points were taken every 30 min for 2 h. Fifty microliters of the 0.2 mg/ml W110A TeSADH (10 µg per assay) in 30% 2-propanol (195 mM final assay concentration) inactivation solution was added to each enzyme assay. All assays were performed in 50 mM Tris–HCl (pH 8.0) and 0.4 mM NADP$^+$. The 2-propanol present in the inactivation solution was used as the substrate.

**Asymmetric reduction of benzylacetone to the corresponding sec-alcohol**

A mixture of 0.3 mmol benzylacetone, 1.31 µmol (0.131 mM final concentration) NADP$^+$ and 0.56 mg W110A TeSADH in 10.0 ml Tris–HCl (pH 6.5)/isopropanol (70:30) was stirred at 50°C for 12 h before being extracted with CH$_2$Cl$_2$. The organic layer was then concentrated under vacuum. The residual compound was purified on a silica gel column. The absolute configuration of the produced alcohol was determined by comparing the sign of the optical rotation with those reported for (S)- and (R)-4-phenyl-2-butanol (Nakamura et al., 1999). The percent conversion and enantiomeric excess (ee) were determined by chiral column GC.

**Stability assays**

Enzyme kinetic stability was tested as described (Burdette et al., 2000) at 85°C and 90°C for W110A TeSADH and 90°C for TeSADH. Activity assays on the heat-treated enzymes were performed with 5 mM (rac)-4-phenyl-2-butanol (W110A TeSADH) and 5 mM (rac)-2-butanol (TeSADH). Fifty microliters of the 0.2 mg/ml W110A TeSADH inactivation solution (10 µg enzyme) or 30 µl of the wild-type inactivation solution (6 µg enzyme) was added to each activity assay. Inactivation curves were performed in triplicate and fit using the Non-Linear Curve Fit tool of Origin 6.1.

![Fig. 2. Modeling of the TbSADH•(S)-2-butanol and the TbSADH•NADP$^+$ binary complexes into a single, TbSADH•(S)-2-butanol•NADP$^+$ model. Green: TeSADH residues; red: NADP$^+$; blue: 2-butanol and orange: Zn$^{2+}$. The two structures were superimposed in InsightII using the heavy atoms of catalytic site residues C37, S39, H59, E60, Asp150, L294 and C295. 1 and 2 denote the positions of the catalytic Zn$^{2+}$ in the TbSADH•NADP$^+$ and TbSADH•(S)-2-butanol binary complexes, respectively;](image-url)
Fig. 3A, we could see (i) that the orientation of (S)-2-butanol in TbSADH’s catalytic site did not correspond to that of BRB in HLADH and (ii) that NADP⁺’s nicotinamide ring in the TbSADH•NADP⁺ complex is rotated by almost 90° in comparison to NAD⁺’s nicotinamide ring in the HLADH•BRB•NAD⁺ ternary complex.

Because HLADH is a primary ADH that is only very poorly active on secondary alcohols, it is unclear how well the orientation of the substrate in TeSADH should match that of BRB in HLADH. For this reason, we decided to adopt two modeling and mutagenic strategies: one based on the orientation of (S)-2-butanol in a TbSADH•(S)-2-butanol•NADP⁺ ternary model (construction described in ‘Materials and methods’) and one based on the orientation of BRB in the HLADH•BRB•NAD⁺ ternary complex.

(S)-1-Phenyl-2-propanol was fitted manually into the TbSADH•(S)-2-butanol•NADP⁺ model with the reactive hydroxyl group superimposed with that of (S)-2-butanol. As seen in Fig. 4, the phenyl ring of (S)-1-phenyl-2-propanol is in close proximity (1.69 Å) with the Cβ of Y267. This steric clash potentially excludes (S)-1-phenyl-2-propanol from being a substrate for TeSADH. To remove this steric overlap, we constructed the Y267G mutant to increase the depth of the large pocket enough to accommodate the phenyl ring of (S)-1-phenyl-2-propanol.

In our second modeling approach, all seven Omega-generated conformations of (S)-1-phenyl-2-propanol were manually fitted into the TbSADH catalytic site, with their C–OH bond superimposed with that of BRB in HLADH. (S)-1-phenyl-2-propanol individual conformations were manually rotated around their C–OH bond axis to identify orientations that would minimize steric overlap between atoms of the substrate and active site residues. Two orientations of one (S)-1-phenyl-2-propanol conformation tested (the lowest energy conformation) created steric overlap with a single residue, Trp110, in TeSADH’s catalytic site (Fig. 3B). All other (S)-1-phenyl-2-propanol conformations created overlaps with more than one residue (not shown). We constructed the W110A mutant to remove the steric overlap between (S)-1-phenyl-2-propanol and Trp110 (Fig. 3C).

The Y267G and W110A mutations were individually modeled into TbSADH using SWISS-MODEL to predict changes in the mutant structure in comparison to the TbSADH crystal structure. Wild-type TbSADH was also modeled to check for possible artifacts in the modeling process. In both mutant 3D-models, the backbones of the mutated residues, Y267G and W110A, remained unchanged with the same (φ,ψ) angles as in the wild-type crystal structure. In both mutant models, the catalytic zinc shifted by 0.78 Å, and the side-chains of zinc-binding residues, His59, Cys37 and Asp150, were slightly reoriented. Because these changes also occurred in the wild-type (control) model they were discounted as modeling artifacts.

Mutations W110A and Y267G were introduced into T. ethanolicus adhB by SDM. The mutant TeSADHs were expressed in E. coli as fusion proteins with a C-terminal His₆ tag. Both mutant enzymes were abundantly expressed as soluble proteins. They were stable during a 15-min heat
treatment at 85°C suggesting that they were properly folded despite the active site mutations.

**Activity and substrate specificity**

The activity of Y267G TeSADH was tested on heat-treated crude extracts with (rac)-2-butanol, (rac)-1-phenyl-2-propanol and (rac)-4-phenyl-2-butanol as substrates. Results were compared to those of heat-treated crude extracts of TeSADH. TeSADH’s and Y267G TeSADH’s relative concentrations in heat-treated crude extracts were estimated by SDS-PAGE (not shown). Y267G TeSADH showed a slight decrease in specific activity on (rac)-2-butanol (~33 U/mg protein) when compared to the wild-type enzyme (~41 U/mg protein). Y267G TeSADH had no activity on (rac)-1-phenyl-2-propanol or (rac)-4-phenyl-2-butanol, and was not further studied.

Activity assays with heat-treated crude extracts suggested that W110A TeSADH is much less active on 2-butanol than TeSADH is, and that it is inactive on 1-phenylethan-1-ol and acetophenone. In contrast, W110A TeSADH showed significant activity on (rac)-1-phenyl-2-propanol, phenylacetone and (rac)-4-phenyl-2-butanol, substrates on which wild-type TeSADH showed no activity. W110A TeSADH also showed significant activity with benzylacetone, on which wild-type TeSADH showed only slight activity (Table I). W110A TeSADH was purified to homogeneity and characterized. Initial activity assays with purified W110A TeSADH agreed with previous assays on crude extracts (Table I): W110A TeSADH showed 10-fold lower activity on (rac)-2-butanol than TeSADH did; it showed almost no activity on (rac)-1-phenylethan-1-ol and acetophenone; and it showed high activity levels on phenylacetone, benzylacetone, (S)-1-phenyl-2-propano and (S)-4-phenyl-2-butanol (Table I). Further assays showed that W110A TeSADH is not active on (R)-1-phenyl-2-propano and (R)-4-phenyl-2-butanol.

Table II highlights W110A TeSADH’s kinetic parameters. W110A TeSADH’s $V_{\text{max}}$ on (rac)-2-butanol only decreased by about 20%, whereas its affinity for (rac)-2-butanol decreased by more than two orders of magnitude. The low affinity for 2-butanol explains why we detected low specific activity for W110A TeSADH on 10 mM (rac)-2-butanol (Table I). In contrast, W110A TeSADH’s kinetic parameters on (S)-1-phenyl-2-propano, phenylacetone, (S)-4-phenyl-2-butanol and benzylacetone are of the same order of magnitude as those of TeSADH on (rac)-2-butanol, making W110A TeSADH an excellent catalyst on these substrates. It is interesting to note that W110A TeSADH’s $V_{\text{max}}$ and $K_m$ values on benzylacetone are higher than those of TeSADH on (rac)-2-butanol, making W110A TeSADH twice as efficient on benzylacetone as TeSADH is on (rac)-2-butanol.

**Effect of pH on enzyme activity**

Alcohol oxidation and ketone reduction assays were performed at different pH values in citrate and Tris buffers at overlapping pHs to determine how pH affects W110A TeSADH activity. Results show that the optimum pH was 4.9 for ketone reduction and 8.8 for alcohol oxidation (Fig. 5). These results show a significant difference from the TeSADH optimum pH of 6.5 for ketone reduction and a slight difference from 9.0 for alcohol oxidation (Burdette and Zeikus, 1994).

### Table I. Specific activities of wild-type TeSADH and W110A TeSADH on multiple substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TeSADH (U/mg protein)</th>
<th>W110A TeSADH (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Butanol (10 mM)</td>
<td>3.65</td>
<td>45.27</td>
</tr>
<tr>
<td>(rac)-1-Phenylethan-1-ol (10 mM)</td>
<td>0.24</td>
<td>0.15</td>
</tr>
<tr>
<td>Acetophenone (7.5 mM)</td>
<td>0.94</td>
<td>0.90</td>
</tr>
<tr>
<td>(S)-1-Phenyl-2-propano (7.5 mM)</td>
<td>14.62</td>
<td>0.14</td>
</tr>
<tr>
<td>(R)-1-Phenyl-2-propano (7.5 mM)</td>
<td>0.51</td>
<td>0.14</td>
</tr>
<tr>
<td>Phenylacetone (7.5 mM)</td>
<td>37.61</td>
<td>1.02</td>
</tr>
<tr>
<td>Phenylacetone (10 mM)</td>
<td>26.83</td>
<td>0.29</td>
</tr>
<tr>
<td>Benzylacetone (7.5 mM)</td>
<td>34.58</td>
<td>5.94</td>
</tr>
</tbody>
</table>

*Enzyme assays were performed at 60°C for 1 min in 50 mM Tris–HCl pH 6.5 (reduction of ketones) or pH 8.0 (oxidation of alcohols) with 0.4 mM NADP(H).

*Substrate concentrations are indicated in parentheses.

### Table II. Kinetic parameters of W110A TeSADH on multiple substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$ (µmol/min mg)</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}/K_m$ (ml/min mg)</th>
<th>TeSADH</th>
<th>W110A TeSADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(rac)-2-Butanol</td>
<td>37.0</td>
<td>0.51</td>
<td>0.072</td>
<td>29.2 ± 3.6</td>
<td>80.3 ± 18</td>
</tr>
<tr>
<td>(S)-1-Phenyl-2-propano</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>16.2 ± 2.2</td>
<td>0.75 ± 0.1</td>
</tr>
<tr>
<td>(S)-4-Phenyl-2-butanol</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>33.5 ± 3.4</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>Phenylacetone</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>44.9 ± 2.8</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Phenylacetone</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46.7 ± 2.5</td>
<td>0.86 ± 0.07</td>
</tr>
</tbody>
</table>

*Enzyme assays were performed at 60°C for 1 min in 50 mM Tris–HCl pH 6.5 (reduction of ketones) or pH 8.0 (oxidation of alcohols), with 0.4 mM NADP(H).
W110A TeSADH’s enantioselectivity

GC analysis on a chiral column combined with optical rotation measurements showed a 99% conversion in the asymmetric reduction of benzylacetone with W110A TeSADH. The alcohol produced had the (S) configuration, and it was produced with an ee above 99%. This result shows the applicable value of this enzyme for reducing or eliminating enantiomer separation steps. GC and optical rotation of products from phenylacetone reduction by W110A TeSADH showed a 95% conversion, but only a 37% ee of (S)-1-phenyl-2-propanol. Because this product has a low ee, separation steps would still be required to produce a pure enantiomer, reducing the value of this reaction.

Effects of temperature on W110A TeSADH activity and stability

Assays at temperatures ranging 5–97°C showed that W110A TeSADH activity increased from 5 to 87.5°C, with the enzyme showing maximal activity at 87.5°C, 2.5°C below the temperature for TeSADH maximum activity (90°C). Above 87.5°C, the activity dropped sharply, suggesting that this enzyme starts inactivating above this temperature (Fig. 6). This small decrease in optimum temperature suggests that W110A TeSADH is destabilized in comparison to TeSADH, but that this destabilization is only marginal. W110A TeSADH kinetic inactivation data confirmed this result (Fig. 7). Burdette et al. (2000) initially described TeSADH’s kinetic inactivation as being a one-step mechanism \( (R^2 = 0.9654) \) (Fig. 7 inset). In our hands, though, TeSADH inactivation at 90°C could not be fitted with a simple exponential decay function, but instead was best fitted by the sum of two exponentials, suggesting that W110A TeSADH goes through a two-stage decay process. This change in enzyme property may be due to an extra mutation (A168D) that was found both in the TeSADH that we used as our control and in W110A TeSADH, but not reported to be present in the TeSADH initially tested (Burdette et al., 1996). This Ala168 is a buried residue, and the extra bulk of the Asp side chain may cause a slight destabilization. W110A TeSADH was less stable than TeSADH at 90°C (W110A TeSADH and TeSADH lost 94% and 71% activity, respectively, after 70 min at 90°C), but W110A TeSADH was more stable at 85°C than TeSADH at 90°C. These results confirm that, although mutation W110A destabilizes TeSADH, the mutant enzyme remains highly thermostable.

W110A TeSADH activity on aryl derivatives of phenylacetone and benzylacetone

To determine the potential usefulness of W110A TeSADH in industrial syntheses, W110A TeSADH activity was tested on commercially available aryl derivatives of 1-phenyl-2-propanol, phenylacetone and benzylacetone. W110A TeSADH showed significant levels of activity on 3-chloro-3-methyl-4-phenyl-2-butanone, (2-fluorophenyl) acetone and 1-(4-bromophenyl) acetone, but showed no detectable activity on 1-chloro-3-phenyl-2-propanol (Table III). Because only a small amount of 1-chloro-3-phenyl-2-propanol could be purchased from Sigma-Aldrich’s Rare Chemical Library, the enantiomeric composition of this alcohol is unknown. It is not excluded that this alcohol is provided mostly in the (R) form. It could be one possible reason why W110A TeSADH is inactive on this substrate. Other ketones with side-chains containing phenol rings have been tested as substrates for W110A TeSADH. W110A TeSADH was able to convert 97% or more of 1-phenyl-1,3-butadione, phenoxy-2-propanone and 1-(4-methoxyphenyl)-2-propanone to the corresponding (S)-alcohol with greater than 99% ee (Musa et al., in press).
Effect of solvent on W110A TeSADH stability

Most recently developed cofactor recycling systems for the enzymatic production of chiral alcohols are based on a two-enzyme approach in which one ADH performs the desired ketone reduction and a second enzyme—often formate dehydrogenase—recycles the cofactor. With their usually broad substrate specificity, ADHs also allow a one-enzyme approach, in which the same ADH performs the desired ketone reduction and recycles the cofactor by oxidizing a cosubstrate. To shift the reaction equilibrium toward the production of the desired alcohol and to increase the substrate and product solubilities, the enzyme should be able to withstand high cosubstrate concentrations (Edegger et al., 2006). For example, an SADH from Rhodococcus ruber was recently isolated that shows activity at 30°C in the presence of 80% 2-propanol or 50% acetone (Kosjek et al., 2004). Because reaction products were analyzed only after a 12-h incubation, though, it is unclear how stable this enzyme is in the presence of high solvent concentrations.

Here, we opted instead to measure enzyme stability by measuring the remaining activity after incubation in the presence of solvent. W110A TeSADH solvent stability was tested in 30% 2-propanol at four different temperatures. The 2-propanol present in the inactivation solution was used as the substrate to measure residual activity (195 mM final assay concentration). Almost no decrease in W110A TeSADH activity was observed after incubations in the presence of 30% 2-propanol at up to 50°C. However, W110A TeSADH lost 80% activity after 120 min incubation at 60°C (Fig. 9). These results are very similar to those observed with the wild-type TeSADH (Fig. 8). These results indicate that, for long reaction times in the presence of solvents, W110A TeSADH would be best used at temperatures of 50°C or below. These solvent stability results are highly encouraging. Being able to operate at or below 50°C in the presence of 30% 2-propanol would allow for (i) higher concentrations of substrates that are only moderately soluble in water and (ii) for cofactor recycling using wild-type TeSADH as the coupling enzyme and 2-propanol as the recycling substrate.

Validation of our modeling approach

The facts that W110A TeSADH is active on (S)-1-phenyl-2-propanol and that Y267G TeSADH is not suggest that (S)-2-butanol’s orientation in the TbSADH•(S)-2-butanol binary complex (PDB #1BZX) differs from its orientation in an active ternary enzyme–substrate–cofactor complex. Even though HLADH is a primary ADH and it shows only limited activity on secondary alcohols, the orientation of BRB in the HLADH•BRB•NAD+ ternary complex seems to be an excellent indication of how the reactive oxygen (and the corresponding C–OH bond) in secondary alcohols should be positioned in TeSADH’s catalytic site to yield an active ternary complex.

Table III. Activity of W110A TeSADH on (1 mM) aryl derivatives

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Derivative structure</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Phenyl-2-propanol</td>
<td><img src="1-Phenyl-2-propanol" alt="Image" /></td>
<td>13.5 ± 2.0</td>
</tr>
<tr>
<td>1-Chloro-3-phenyl-2-propanol</td>
<td><img src="1-Chloro-3-phenyl-2-propanol" alt="Image" /></td>
<td>ND</td>
</tr>
<tr>
<td>Benzylacetone</td>
<td><img src="Benzylacetone" alt="Image" /></td>
<td>37.6 ± 4.2</td>
</tr>
<tr>
<td>3-Chloro-3-methyl-4-phenyl-2-butanone</td>
<td><img src="3-Chloro-3-methyl-4-phenyl-2-butanone" alt="Image" /></td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Phenylacetone</td>
<td><img src="Phenylacetone" alt="Image" /></td>
<td>39.8 ± 1.2</td>
</tr>
<tr>
<td>(2-Fluorophenyl) acetone</td>
<td><img src="2-Fluorophenyl" alt="Image" /> acetone)</td>
<td>14.8 ± 0.2</td>
</tr>
<tr>
<td>1-(4-Bromophenyl) acetone</td>
<td>![Image](1-(4-Bromophenyl) acetone)</td>
<td>43.8 ± 1.9</td>
</tr>
</tbody>
</table>

ND: not detectable.

Enzyme assays were performed at 60°C for 1 min in 50 mM Tris–HCl pH 6.5 (reduction of ketones) or pH 8.0 (oxidation of alcohols), with 0.4 mM NADP(H).

Substrates of W110A TeSADH that are the substructures for the aryl derivatives.

Conclusions

The W110A mutation significantly changed the substrate specificity of TeSADH to include a variety of
phenyl-substituted alcohols and ketones. W110A TeSADH is active on benzylacetone, phenylacetone, (S)-1-phenyl-2-propanol and (S)-4-phenyl-2-butanol; it shows almost no activity on the corresponding (R)-alcohols and it produces (S)-4-phenyl-2-butanol at greater than 99% ee. W110A TeSADH is now active on aryl derivatives of phenylacetone and benzylacetone. Its activity and enantiomeric specificity make W110A TeSADH a potentially useful catalyst for chiral synthesis of aryl derivatives of alcohols.

Acknowledgements
This work was supported by NSF Award MCB-0445750 and by a grant from the Michigan Economic Development Corporation through its Michigan Technology Tri-Corridor Program.

References

Received July 30, 2006; revised November 7, 2006; accepted November 13, 2006
Edited by Anthony Wilkinson