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Mutation of Serine-39 to Threonine in Thermostable Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus* Changes Enantiospecificity

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Abstract: The substrate specificity of wild-type and Ser39 → Thr (S39T) secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus* was examined. The S39T mutation increases activity for 2-propanol without any significant effect on $NADP^+$ binding. There is no significant effect of the mutation on the primary and secondary alcohol specificity of SADH. However, an effect on the enantiospecificity of SADH by the S39T mutation is demonstrated. Throughout the temperature range from 15 to 55 °C, wild-type SADH exhibits a preference for (*S*)-2-pentanol. In contrast, a temperature-dependent reversal of enantiospecificity is observed for 2-butanol, with a racemic temperature of 297 K. Throughout the same range of temperatures, S39T SADH exhibits higher enantiospecificity for the (*R*)-enantiomers of both 2-butanol and 2-pentanol. Examination of individual k_{cat}/K_m values for each enantiomer of the chiral alcohols reveals that the effect of the mutation is to decrease (*S*)-2-butanol specificity, and to preferentially enhance (*R*)-2-pentanol specificity relative to (*S*)-2-pentanol. These results are the first step toward expanding the synthetic utility of SADH to allow efficient preparation of a range of (*R*)-alcohols.

Introduction

The application of enzymatic systems in asymmetric synthesis is a subject of continuing interest as a result of the high enantiospecificity characteristic of enzymes.[1] The most widely investigated enzymes for this purpose have been lipases, esterases, and dehydrogenases. Most alcohol dehydrogenases (ADHs) are Zn-containing enzymes utilizing nicotinamide cofactors, which exist in either dimeric or tetrameric form.[2] The classification of an alcohol dehydrogenase as primary or secondary is dependent on the preference of activity for primary or secondary alcohol oxidation. The well-known alcohol dehydrogenases obtained from yeast (YADH) and mammalian liver (LADH) are NAD-dependent dimers which have a marked preference for oxidation of primary alcohols. Unfortunately for synthetic application, YADH and LADH are thermally unstable and have low activity for reduction of acyclic ketones. The

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secondary alcohol dehydrogenases (SADH), which have been isolated from a number of anaerobic bacteria, are Zn-containing $NADP^+$-dependent tetramers that have high activity toward secondary alcohols. The SADH from the anaerobic thermophile, *Thermoanaerobacter ethanolicus*, is stable at temperatures up to 80 °C,[3] and exhibits high activity in the enantioselective reduction of cyclic and acyclic secondary alcohols, as well as ketoesters.[4,5] Because of its thermostability, resistance to organic cosolvents, and reactivity for a wide variety of substrates, SADH is a potentially useful biocatalyst for synthetic applications. The SADH from *Thermoanaerobium brockii* (TBADH) has been applied in several chiral syntheses.[6]

Previously, a temperature-dependent reversal of the enantiospecificity of SADH was documented by Pham and Phillips.[7] (*S*)-2-Butanol is the preferred substrate at temperatures below 26 °C, while (*R*)-2-butanol is preferred at temperatures above 26 °C. Similarly, 2-butanone is reduced to (*R*)-2-butanol by SADH, while ketones with longer alkyl chains give predominantly (*S*)-alcohols. This indicates that SADH has the capability, albeit limited, of (*R*)-alcohol production. If it is possible to enhance this characteristic, then ketone reduction with SADH could become a successful method of producing a wide variety of (*R*)-alcohols. This is significant, since ADHs typically obey Prelog's rule and are hence specific for production of (*S*)-enantiomers of secondary alcohols.[8] Since little is known about the molecular basis for the stereochemical preferences of SADH, the purpose of this work is to initiate the study of SADH active site residues that govern binding and catalysis of enantiomeric substrates and products. We have examined the effect of mutation of Ser39 to threonine on the regiospecificity and enantiospecificity of SADH. The results show that a threonine residue at position 39 of SADH increases the (*R*)-specificity for 2-butanol and 2-pentanol.

Results

Activity of Wild-Type of S39T SADH. The mutation of Ser39 to threonine increases the specific activity of purified SADH from 77.5 units/mg to 118.3 units/mg under standard assay conditions, using 2-propanol. This result is consistent with the report by Sakoda and Imanaka[9] of a Thr → Ser mutation in the active site of *Bacillus stearothermophilus* primary ADH that diminished activity by a comparable amount. To determine if these effects on activity are related to substrate specificity or cofactor specificity, steady state kinetic parameters for $NADP^+$ specificity were measured for each protein (Table 1). Both enzymes exhibit very similar K_m values for $NADP^+$, but k_{cat} and k_{cat}/K_m values are higher for S39T SADH.

Regiospecificity of Wild-Type and S39T SADH. To determine whether the S39T mutation has any effect on specificity for primary or secondary alcohol substrates, k_{cat}/K_m values were compared for ethanol, 1-propanol, and 2-propanol (Table 2). Wild-type SADH is observed to have a 370-fold specificity ratio for 2-propanol relative to 1-propanol. For S39T SADH, a 145-fold specificity ratio is seen for 2-propanol relative to 1-propanol, mainly due to the reduction of k_{cat}/K_m for 2-propanol. Additionally, the S39T mutation has the effect of diminishing ethanol specificity. These results show that although there are some effects on primary versus secondary alcohol specificities by this mutation, they are not significant enough to regard Ser39 as a key residue governing the regiospecificity of SADH.

Table 1. Kinetic Parameters for $NADP^+$ for Wild-Type and S39T SADH

parameter	wild-type SADH	S39T SADH
k_{cat} (s^{-1})	49.3 ± 2.7	106.6 ± 6.3
K_m ($\times 10^{-6}$ M)	7.4 ± 0.7	8.9 ± 1.0
k_{cat}/K_m ($\times 10^6$ M^{-1} s^{-1})	6.7 ± 0.7	12.0 ± 1.5

Table 2. k_{cat}/K_m Values for Oxidation of Ethanol, 1-Propanol, and 2-Propanol at 50 °C by Wild-Type and S39T SADH

	k_{cat}/K_m (M^{-1} s^{-1})	
substrate	wild-type	S39T
ethanol	222 ± 36	43 ± 5
1-propanol	273 ± 35	206 ± 25
2-propanol	$(1.0 \times 10^5) \pm (2 \times 10^4)$	$(3.0 \times 10^4) \pm (0.7 \times 10^4)$

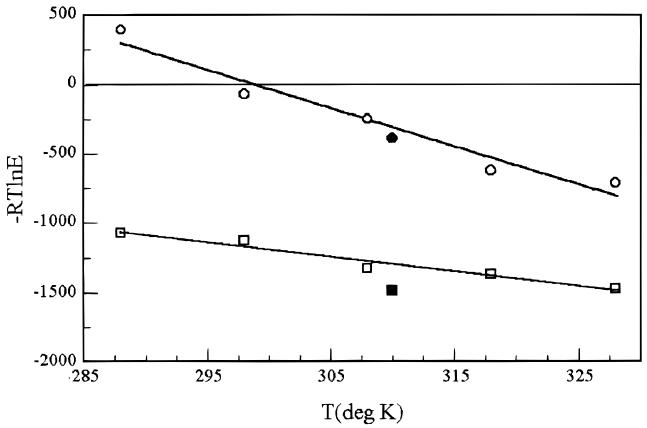

Figure 1. Temperature dependence of enantiospecificity of wild-type and S39T SADH for 2-butanol. Open circles: Wild-type SADH. Open squares: S39T SADH. Filled circle: Data from reduction of 2-butanone by wild-type SADH. Filled square: Data from reduction of 2-butanone by S39T SADH.

Enantiospecificity of Wild-Type and S39T SADH. Values of k_{cat}/K_m were measured by the procedure of Pham et al.[7] for the (*R*)- and (*S*)-enantiomers of 2-butanol and 2-pentanol at temperatures between 288 and 328 K for wild-type and S39T mutant SADH. The recombinant wild-type *T. ethanolicus* SADH exhibits temperature-dependent stereospecificity for 2-butanol identical to the enzyme isolated from *T. ethanolicus* used in previous experiments[7] (Figure 1, open circles). Analysis of the data collected for S39T SADH (Figure 1, open squares) demonstrates that it is more specific for (*R*)-2-butanol than wild-type SADH, and its racemic temperature,[10] T_r, decreases from 297 K for wild-type SADH to 183 K (Table 3). Furthermore,

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(10) The enantiospecificity ratio, $E = (k_{cat}/K_m)_R/(k_{cat}/K_m)_S$, is directly related to the difference in free energy of activation between the (*R*)- and (*S*)-alcohols. $\Delta\Delta G^{\ddagger} = -RT \ln E$, from transition state theory. Separation of $\Delta\Delta G^{\ddagger}$ into entropic and enthalpic components is given by the expression $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$. The racemic temperature,[7] $T_r = \Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger}$, is the temperature at which $\Delta\Delta G^{\ddagger} = 0$, and no discrimination is made in reaction or formation of enantiomers.

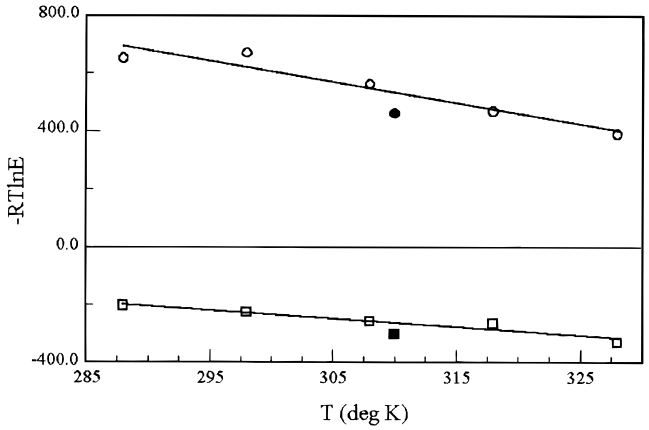

Figure 2. Temperature dependence of enantiospecificity of wild-type and S39T SADH for 2-pentanol. Open circles: Wild-type SADH. Open squares: S39T SADH. Filled circle: Data from reduction of 2-pentanone by wild-type SADH. Filled square: Data from reduction of 2-pentanone by S39T SADH. The units of $-RT$ ln E are cal/mol.

Figure 2 shows that the S39T mutation effects a reversal of enantiospecificity for 2-pentanol, and T_r for 2-pentanol decreases from 384 K for wild-type SADH to 207 K for S39T SADH (Table 3). The filled-in points in both figures are calculated from enantiomeric excesses of alcohols obtained from the corresponding ketone reductions, which were carried out under kinetic control and analyzed for enantiomeric composition by gas chromatography. These product distributions are similar to those predicted by kinetic measurements of alcohol oxidation, as expected.

Both entropic and enthalpic effects associated with the change of enantiospecificity are evident, as shown in Table 3. The values of $\Delta\Delta S^{\ddagger}$ and $\Delta\Delta H^{\ddagger}$ for the reaction of wild-type SADH with 2-butanol are in excellent agreement with our previous studies.[7] However, the values for both parameters are somewhat smaller for the reaction of wild-type SADH with 2-pentanol in the present work. Since the 2-butanol parameters obtained in the present study are in excellent agreement with previous results, these differences are probably not due to differences in protein structure, so it is likely that these new parameters for 2-pentanol are more accurate. For the reaction of 2-butanol with S39T SADH, $\Delta\Delta S^{\ddagger}$ decreases by 17.2 entropy units compared to wild-type SADH, while $\Delta\Delta H^{\ddagger}$ decreases by 6.3 kcal mol^{-1} (Table 3). In the reaction of 2-pentanol, $\Delta\Delta S^{\ddagger}$ decreases by 4.4 entropy units for S39T SADH, and $\Delta\Delta H^{\ddagger}$ decreases by 2.2 kcal mol^{-1} (Table 3).

In evaluation of structural effects on stereochemistry, it is important to differentiate between enhancement of (*R*)-enantiomer specificity and inhibition of (*S*)-enantiomer specificity in changing enantiospecificity. Comparison of individual k_{cat}/K_m values associated with wild-type and S39T SADH for each enantiomer of 2-butanol and 2-pentanol measured at 55 °C reveals that the S39T mutation does not affect enzyme specificity for (*R*)-2-butanol, but rather decreases (*S*)-2-butanol specificity (Table 4). In contrast, S39T SADH shows considerably higher specificity for both (*R*)-2-pentanol and (*S*)-2-pentanol than does wild-type SADH, but the increase is greater for (*R*)-2-pentanol, resulting in a net reversal of enantiospecificity for 2-pentanol from (*S*) to (*R*).

Discussion

The X-ray crystal structure of an SADH from *T. brockii* was solved in 1995,[11] but the coordinates (1YKF.PDB) were released from the Protein Data Bank only very recently. Consequently, our selection of active site residues for mutation studies of SADH until now has been based on predicted sequence homologies with other functionally similar alcohol dehydrogenases from mammalian sources that have known structures. Sequence alignments of SADH with mammalian ADHs show about 30% identity, especially in the regions around the Zn binding site and the NADP binding site.[12]

The mutation chosen for this study is Ser39 → Thr, which was chosen because it permits a change in the steric environment of the active site, without disrupting an essential proton relay system in which the Ser39 hydroxyl group is expected to participate. Since the disruption of this H-bonding network in ADH prohibits a proton uptake or release mechanism directly associated with alcohol reduction or oxidation, respectively, the substituting residue must also have a side-chain hydroxyl group to preserve enzyme activity. Mutation of the homologous Ser48 to alanine in human βADH results in an inactive protein.[13] Indeed, threonine residues in the corresponding active site positions of other ADHs have been shown to perform identical roles in facilitating enzyme function.[14] The second rationale supporting this mutation is that mammalian ADHs with threonine residues participating in the aforementioned H-bonding network were predicted by computer-simulated active site models to exhibit greater (*R*)-preference for secondary alcohols than those with corresponding serine residues.[15] However, there are other changes in active site residues for these enzymes besides the serine/threonine, which could contribute partially to the observed differences in stereospecificity. The proximity of Ser39 to the active site zinc and $NADP^+$ in SADH can be readily seen from Figure 3.

As predicted for mammalian ADHs, the S39T mutant SADH is more (*R*)-specific than wild-type SADH, implicating this serine/threonine as an important residue contributing to the enantiospecificity of SADH as well as mammalian ADHs. The decrease in $\Delta\Delta H^{\ddagger}$ for S39T compared to wild-type SADH is indicative of more favorable binding energy of the (*R*)-alcohols with the S39T mutant, possibly resulting from van der Waals' contact between the methyl group of the threonine side chain and the alkyl chain of the substrate. The value of $\Delta\Delta S^{\ddagger}$ decreases for the reaction of S39T compared to wild-type SADH, which could be accounted for by the release of fewer water molecules for S39T to reach the transition state. Furthermore, the additional methyl group of threonine in the active site may restrict the freedom of rotation of other active site residues. These results demonstrate the utility and sensitivity of our temperature-dependence method[7] for analysis of effects of mutations on stereochemistry of enzymatic reactions.

This appears to be the first reported demonstration of a single mutation resulting in significant effects on the enantiospecificity of an enzyme, while simultaneously augmenting its activity.

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Table 3. Entropic and Enthalpic Components of the Difference of Free Energy of Activation between (*R*)- and (*S*)-Enantiomers of 2-Butanol and 2-Pentanol

	2-butanol		2-pentanol	
parameter	wild-type	S39T	wild-type	S39T
$\Delta\Delta S^{\ddagger}$ (cal deg^{-1} mol^{-1})	27.6 ± 3.4 [27.9 ± 2.5][a]	10.4 ± 1.3	7.3 ± 1.2 [17.3 ± 3.3][a]	2.9 ± 0.5
$\delta\Delta\Delta S^{\ddagger}$ (WT-S40T)	17.2		4.4	
$\Delta\Delta H^{\ddagger}$ (kcal mol^{-1})	8.2 ± 1.0 [8.37 ± 0.73][a]	1.9 ± 0.4	2.8 ± 0.4 [5.88 ± 0.99][a]	0.6 ± 0.15
$\delta\Delta\Delta H^{\ddagger}$ (WT-S40T)	6.3		2.2	
T_r (K)	297 ± 51 [300 ± 38][a]	183 ± 45	384 ± 84 [340 ± 86][a]	207 ± 63

[a] From Pham and Phillips (ref 6b).

Table 4. k_{cat}/K_m Values for Oxidation of Enantiomers of 2-Butanol and 2-Pentanol at 55 °C for Wild-Type and S39T SADH

	k_{cat}/K_m (M^{-1} s^{-1})	
substrate	wild-type SADH	S39T SADH
(*R*)-2-butanol	$(3.1 \times 10^5) \pm (0.3 \times 10^5)$	$(2.8 \times 10^5) \pm (0.5 \times 10^5)$
(*S*)-2-butanol	$(1.1 \times 10^5) \pm (0.2 \times 10^5)$	$(0.29 \times 10^5) \pm (0.07 \times 10^5)$
(*R*)-2-pentanol	$(0.87 \times 10^5) \pm (0.08 \times 10^5)$	$(3.5 \times 10^5) \pm (0.4 \times 10^5)$
(*S*)-2-pentanol	$(1.3 \times 10^5) \pm (0.2 \times 10^5)$	$(2.1 \times 10^5) \pm (0.3 \times 10^5)$

Using the parameters in Table 4, the predicted enantiomeric purity of (*R*)-2-butanol produced by reduction of 2-butanone with S39T SADH at 55 °C is 81% ee, which is significantly greater than that for wild-type SADH, 47% ee. For 2-pentanol, S39T SADH at 55 °C gives (*R*)-2-pentanol in 25% ee, while wild-type gives (*S*)-2-pentanol in 20% ee. The Ser39 → Thr mutation thus broadens the preparative scope of SADH to include production of (*R*)-enantiomers of both 2-pentanol and 2-butanol. This mutation represents the first step toward the ultimate goal of tailoring SADH to produce (*R*)-alcohols of greater synthetic utility in high enantiomeric purity from ketone substrates. This may be achieved by further mutations of the active site of SADH. Alternatively, the (*R*)-specificity of SADH may be enhanced by manipulation of physical variables. For example, we have shown that SADH gives increasing (*R*)-enantiospecificity for these smaller substrates at higher temperatures.[7] In addition, we demonstrated that coenzyme analogs increase the (*R*)-specificity of SADH.[16] Lower pH values were also demonstrated to favor (*R*)-2-butanol.[17] Recently, Simpson and Cowan have reported cosolvent effects on the enantiospecificity of a similar SADH,[18] in which solvent systems such as acetonitrile/H_2O (40:60 v/v), DMF/H_2O (20:80 v/v), and DMSO/H_2O (20:80 v/v) were found to increase (*R*)-specificity without significant decrease of activity. In conjunction with these temperature, coenzyme, pH, and cosolvent effects, the S39T mutation could expand the synthetic utility of SADH to include highly enantiospecific production of (*R*)-alcohols.

Experimental Section

Materials. Ethanol, 1-propanol, 2-propanol, (*R*)- and (*S*)-2-butanol, (*R*)- and (*S*)-2-pentanol, dichloromethane, acetyl chloride, and pyridine were purchased from Aldrich Chemical Co. Ammonium sulfate and sodium sulfate were obtained from Baker Chemical Co. Tris·HCl and Red A agarose were purchased from Sigma Chemical Co. $NADP^+$, dithiothreitol (DTT), kanamycin, ampicillin, and tryptone were obtained from the United States Biochemical Corp. Yeast extract was purchased from Difco Laboratories.

Methods and Instruments. Enzyme assays and kinetic experiments were performed with a Varian Cary 1E UV−visible spectrophotometer equipped with a Peltier thermoelectric temperature-controlled 12-cell holder. Kinetic data were analyzed with use of the Hyper hyperbolic nonlinear regression program developed by J. S. Easterby. Gas chromatography was performed on a Varian 3300 equipped with a Supelco Beta-Dex120 cyclodextrin capillary column.

Growth of Cells. *Escherichia coli* DH5α containing the recombinant *adhB* gene coding for wild-type or mutant SADH was grown aerobically in rich complex medium containing 20 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 25 μg/L kanamycin, and 100 μg/L ampicillin at 37 °C. Cells were collected by centrifugation at 10000 × g for 15 min.

Purification of Secondary Alcohol Dehydrogenase. The wet cells were resuspended (0.5 g cells per mL of buffer) in 50 mM Tris·HCl at pH 8.0 containing 5 mM DTT (buffer A) according to the procedure of Burdette et al.[12] The cells were then lysed by sonication at 0 °C in three 3-min intervals. Cell debris was removed by centrifugation at 15000 × g for 45 min. The resulting supernatant was incubated at 70 °C for 15 min and centrifuged at 25000 × g for 30 min to remove the thermally denatured impurities. Ammonium sulfate was added to the supernatant to 30% and stirred for 30 min to remove further impurities, which were discarded after centrifugation at 25000 × g for 30 min. The ammonium sulfate concentration of the supernatant was increased to 70%, and after being stirred for 1 h, the mixture was spun down at 15000 × g for 20 min. SADH was found in the pellet, which was dissolved in a minimum amount of buffer A and then dialyzed against buffer A in three 4-L increments. The dialyzed solution of SADH was applied to a 50 mL Red A agarose column that had been pre-equilibrated and washed with buffer A. Impurities were eluted with buffer A containing 0.07 M $NaClO_4$, followed by elution of SADH fractions with buffer A containing 0.2 M $NaClO_4$. The SADH-containing fractions were pooled and concentrated by ammonium sulfate precipitation followed by dissolving the protein in a minimum amount of buffer A. The purified SADH solutions can be stored at −77 °C for several months without loss of activity, and were used in the subsequent kinetics studies.

Secondary Alcohol Dehydrogenase Assay. SADH was assayed spectrophotometrically at 50 °C by following the production of NADPH ($\Delta\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1}) in a solution containing 200 mM 2-propanol as the substrate and 1.25 mM NADP in 100 mM Tris·HCl buffer (pH 8.9).[7] One unit of activity is the amount of SADH that reduces or oxidizes 1 μmol of NADP or NADPH per minute, respectively.

Kinetic Experiments. For temperature-dependence experiments, the cuvettes contained 1.00 mM $NADP^+$, 0.025−200 mM of alcohol, and 50 mM Tris·HCl buffer at pH 8.9, in a total volume of 0.6 mL. Measurements were made at temperatures from 15 to 65 °C. Since Tris has a high-temperature coefficient, the pH of the buffer stock solutions was adjusted to 8.9 at each temperature. The cuvettes containing the solutions were pre-incubated at each temperature in the cell block of the spectrophotometer prior to initiation of reaction by addition of enzyme solution. The reaction rates were measured by following the production of NADPH spectrophotometrically at 340 nm. Values of k_{cat} and K_m were calculated for each enantiomer of alcohol at each temperature from at least three repetitions. Comparison of

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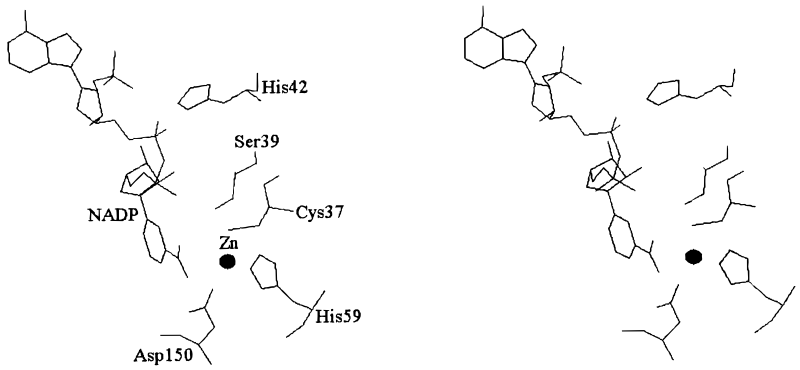

Figure 3. Crossed stereoview of the active site of *T. brockii* SADH (1YKF.PDB) showing the position of Ser39.

primary versus secondary alcohol oxidations was done in the same fashion at 50 °C. Determination of values of k_{cat} and K_m for $NADP^+$ was also performed this way at 50 °C, except that 2-propanol was held at a constant concentration of 100 mM, while $NADP^+$ concentrations were varied from 5×10^{-6} to 4×10^{-4} M.

Ketone Reductions with SADH. Reaction mixtures were prepared in buffer A, containing 1% (v/v) of the substrate ketone and 20% (v/v) 2-propanol. Upon addition of the enzyme solution, the reactions were incubated at 50 °C in a constant-temperature bath. The reaction progress was followed by GC, and the reactions were terminated at 50% ketone conversion. The mixtures were saturated with ammonium sulfate and extracted with dichloromethane, and the extracts were dried over sodium sulfate. The crude alcohol product was stirred under nitrogen with pyridine and acetyl chloride, both in excess of the total amount of alcohol present. After extractive workup and drying over sodium sulfate, the mixtures were analyzed by GC for the chiral acetate esters. The cyclodextrin capillary column gives better resolution of the enantiomers of acetates of acyclic chiral secondary alcohols than it does of the underivatized alcohols.

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