Effects of Substrate Structure and Temperature on the Stereospecificity of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus*

Van T. Pham and Robert S. Phillips*

Contribution from the Departments of Chemistry and Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602. *Received April 13, 1989*

Abstract: Enzymes from thermophilic microorganisms are of considerable interest in biotechnological applications. The temperature dependence of the rates of reaction of the enantiomers of secondary alcohols with a secondary alcohol dehydrogenase (SADH) from a thermophilic bacterium, *Thermoanaerobacter ethanolicus*, has been determined. These results demonstrate a novel temperature-dependent reversal of stereospecificity. At temperatures below 26 °C, (*S*)-2-butanol is a better substrate than (*R*)-2-butanol on the basis of k_{cat}/K_m values; however, above 26 °C, (*R*)-2-butanol is a better substrate than (*S*)-2-butanol. (*S*)-2-Pentanol is the preferred substrate up to 60 °C; however, our data predict that (*R*)-2-pentanol would be preferred above 70 °C. (*S*)-2-Hexanol is predicted to be the preferred enantiomer up to 240 °C. At 50 °C, the k_{cat}/K_m values for oxidation of *S* alcohols obey a linear free energy relationship with respect to increasing chain length, indicating a constant mechanism. However, the *R* alcohols show a convex plot, suggesting that there is a chain-length-dependent change in the rate-determining step. 3-Pentanol, cyclobutanol, and cyclopentanol are also good substrates on the basis of comparison of their k_{cat}/K_m values with those of 2-propanol.

The use of enzymatic systems for asymmetric synthesis has become a successful method in organic chemistry.[1] Prelog and co-workers found that the same carbonyl substrate can be reduced from opposite faces (*re* or *si*) by different microorganisms.[2] Horse liver alcohol dehydrogenase (HLADH) catalyzed oxidation of *meso*-diols occurs with *pro-S* selectivity, and reduction of highly symmetrical *cis*- and *trans*-decalindiones gives complete *pro*-9*R* selectivity to produce the keto alcohols.[3,4] Horse liver alcohol dehydrogenase also has been shown to resolve racemic alicyclic ketones such as 3-methylcyclohexanone to give optically active (*R*)- and (*S*)-3-methylcyclohexanone, respectively.[5] Furthermore, bakers' yeast catalyzes the reduction of keto esters to hydroxy esters in very high optical purity.[6] However, yeast and horse liver alcohol dehydrogenases have low activity for the reduction of acyclic aliphatic ketones, limited thermal stability, and sensitivity to organic cosolvents. For these reasons, dehydrogenases from thermophilic microorganisms are of considerable biotechnological interest.

Recently, Bryant et al.[7] have isolated and characterized two alcohol dehydrogenases from *Thermoanaerobacter ethanolicus*, a thermophilic anaerobic bacterium that ferments a wide range of hexoses and pentoses as well as starch and xylan. Both dehydrogenases are NADP-dependent, contain Zn^{2+}, and are thermostable to at least 70 °C. One of these enzymes appears to be a classical alcohol dehydrogenase; it oxidizes straight- or branched-chain primary alcohols from ethanol to 1-heptanol as well as 1,2-propanediol and ethylene glycol.[7] In contrast, the best substrates for the other alcohol dehydrogenase are 2-propanol and related secondary alcohols.[7] An alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) that is similar to this latter enzyme catalyzes the asymmetric reduction of aliphatic ketones.[8] An interesting substrate-size-induced reversal of stereoselectivity at 37 °C was observed by Keinan and co-workers in their studies of this enzyme.[8] The smaller substrates (2-butanone, 3-methyl-2-butanone, and methyl cyclopropyl ketone) were reduced to *R* alcohols, whereas 2-pentanone and the longer chain ketones provided the *S* alcohols.[8] To evaluate the mechanistic basis of this unusual stereochemical reversal, we have studied the kinetics of the reaction of secondary alcohol dehydrogenase (SADH) from *T. ethanolicus* with the *R* and *S* enantiomers of the simple secondary alcohols 2-butanol, 2-pentanol, 2-hexanol, and 2-heptanol.[9] We have also compared the rates of the reaction of SADH with straight-chain and cyclic alcohols in order to develop an understanding of the structural requirements for optimal activity with the enzyme.

*To whom correspondence should be addressed at the Department of Chemistry.

Table I. Kinetic Parameters for Achiral and Cyclic Substrates at 50 °C

substrate	k_{cat}/K_m,[a] M^{-1} s^{-1}	k_{cat}, s^{-1}
ethanol	61	6
1-propanol	170	4
2-propanol	4.0×10^4	74
3-pentanol	7.1×10^3	48
cyclobutanol	9.3×10^3	71
cyclopentanol	1.7×10^4	136
cyclohexanol	2.7×10^3	21

[a] k_{cat}/K_m and k_{cat} values for 2-propanol were estimated by assuming a maximum specific activity of 105 units/mg at 50 °C and a subunit molecular mass of 4.2×10^4 kDa.[7] k_{cat}/K_m and k_{cat} values for other alcohols were estimated from values normalized to those for 2-propanol.

Results and Discussion

Substrate Structure. In order to gain a greater insight into the structural features required for catalysis by SADH, we examined the reactivity of a series of achiral and cyclic alcohols, with the results presented in Table I. The presence of two alkyl groups is apparently critical for proper orientation and catalysis, since k_{cat}/K_m is reduced about 600-fold for ethanol relative to 2-

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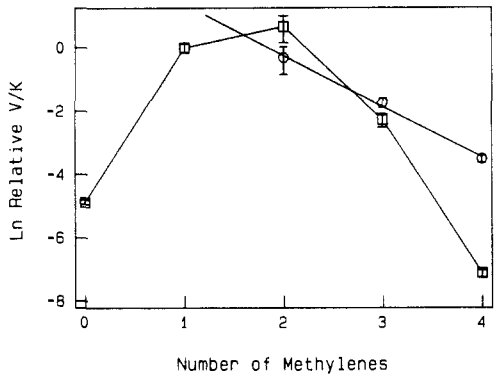

Figure 1. Dependence of substrate reactivity on chain length of methylcarbinols with the general structure CH_3–CHOH–$(CH_2)_n$–H: *S* alcohols, open circles; ethanol, 2-propanol, and *R* alcohols, open squares. k_{cat}/K_m values were normalized to that of 2-propanol. Error bars indicate calculated standard errors.

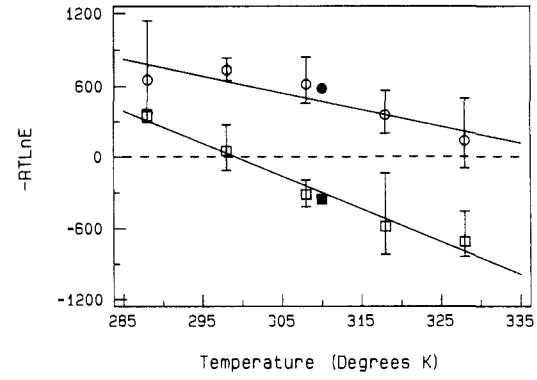

Figure 2. Temperature dependence of free energy of activation differences for 2-butanol and 2-pentanol: 2-butanol, open squares; 2-pentanol, open circles; reduction of 2-butanone, filled square; reduction of 2-pentanone, filled circle. Error bars indicate calculated standard errors.

propanol. This effect can be clearly seen in the plot of ln relative V/K against the chain length for the methylcarbinols, shown in Figure 1. (*R*)-2-Butanol (n = 2) has the optimal substrate structure on the basis of this analysis. 1-Propanol is slightly better than ethanol on the basis of k_{cat}/K_m comparison; however, 1-butanol exhibits much lower activity.[7] Extension of both alkyl groups in 3-pentanol does not significantly affect the reaction, since k_{cat}/K_m is only reduced modestly. The cyclic alcohols cyclobutanol, cyclopentanol, and cyclohexanol are moderate to good substrates on the basis of comparison of k_{cat}/K_m values (Table I). Cyclohexanol has a rather low k_{cat} value compared with those of the other cyclic alcohols examined; however, k_{cat} values for alcohol dehydrogenases are often limited by the rates of release of products.[10] These results are in sharp contrast to those observed with HLADH, where the rates of oxidation of cyclopentanol and cyclobutanol are much lower than that of cyclohexanol.[11] Thus, the differences in ring strain do not seem to be reflected in the reactions of the cyclic alcohols with SADH, whereas they are critical in reactions with HLADH.

Stereochemistry. The specificity constant of an enzyme for its substrates is defined as the ratio k_{cat}/K_m.[12] Hence, the enantiospecificity ratio E is defined as the ratio of k_{cat}/K_m values for the *R* and *S* alcohols, and from transition-state theory, $-RT \ln E = \Delta\Delta G^{\ddagger}$, where $\Delta\Delta G^{\ddagger}$ is the difference in free energy of activation between the *R* and *S* alcohols. The temperature dependence of the activation free energy is given by the expression $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$. When $\Delta\Delta G^{\ddagger} = 0$, $T_r = \Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger}$ and no discrimination of the enzyme between the *R* and *S* isomers occurs. The temperature is thus the "racemic temperature", T_r, and will be a constant for a particular alcohol.[13]

Values of k_{cat}/K_m were determined at pH 8.9 for the *R* and *S* enantiomers of a series of secondary alcohols between 15 and 65 °C.[14] As expected, k_{cat}/K_m values for both enantiomers of a given alcohol increase with increasing temperature; however, the slopes are not identical. We attempted to perform kinetic studies at temperatures above 65 °C but failed to obtain reliable results.[15] Analysis of the data as described above resulted in the unexpected discovery that the enantiospecificity of the reaction of 2-butanol shows a temperature-dependent reversal, as shown in Figure 2. For 2-butanol, $\Delta\Delta H^{\ddagger} = 8.37 \pm 0.73$ kcal/mol and $\Delta\Delta S^{\ddagger} = 27.9 \pm 2.5$ cal deg^{-1} mol^{-1}; thus, the T_r for 2-butanol is 26 °C. Accordingly, (*S*)-2-butanol is the preferred substrate below 26 °C, while at temperatures above 26 °C, (*R*)-2-butanol is preferred (see Figure 2). For 2-pentanol and 2-butanol, the reversal of stereochemistry, as reported by Keinan et al.,[8] was confirmed by our observations at 37 °C. However, at 15 °C, it is the *S* isomer of both alcohols that is the preferred substrate (Figure 2). The T_r for 2-pentanol is predicted to be 70 °C from the data shown in Figure 2, since $\Delta\Delta H^{\ddagger} = 5.88 \pm 0.99$ kcal/mol and $\Delta\Delta S^{\ddagger} = 17.3 \pm 3.3$ cal deg^{-1} mol^{-1}. For 2-hexanol, T_r was estimated to be 240 °C from similar measurements; in fact, (*R*)-2-hexanol exhibits no measurable substrate activity at temperatures below 40 °C. We were not able to calculate T_r for 2-heptanol since the *R* enantiomer did not exhibit measurable activity with the enzyme, even at 60 °C. For 2-octanol, we could not perform kinetic measurements due to its limited solubility in aqueous solution.

Since the transition state in the direction of oxidation of the alcohol and of ketone reduction must be identical, we expect that the graph in Figure 2 will also predict the product distribution (under kinetic control) for reduction of 2-butanone and 2-pentanone. Thus, we would expect to isolate (*R*)-2-butanol if the temperature of the reaction is above 26 °C. In contrast, if the temperature is less than 26 °C, the *S* isomer should result. We carried out the reduction of 2-butanone and 2-pentanone at 37 °C, and after analysis of stereochemical purity by gas chromatography, we found that (*R*)-2-butanol and (*S*)-2-pentanol are formed in 28% and 44% ee, respectively, as the data in Figure 2 predict (see filled square and filled circle in Figure 2). In reactions of 2-hexanone and 2-heptanone, the *S* alcohols were isolated in 96% and 99% ee. These results are supported by the results of Keinan et al.;[8] they found that the stereochemical purity of (*S*)-2-pentanol diminished as the temperature of the reaction mixture for 2-pentanone reduction by TBADH was increased. However, our data predict that the stereochemical purity of (*R*)-2-butanol from 2-butanone reduction will *increase* with increasing reaction temperature above 26 °C (see Figure 2).

Examination of the values of k_{cat}/K_m for the alcohols reveals another interesting point.[16] There is about a 3-fold decrease in

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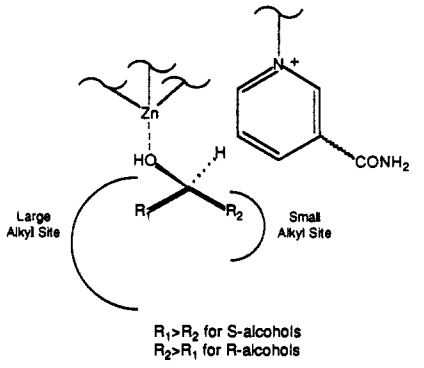
(13) The racemic temperature, T_r, is analogous to the isokinetic temperature known from linear free energy relationships. See, for example: Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*; 3rd ed.; Harper and Row: New York, 1987; pp 158–159.
(14) These experiments were performed with constant 1 mM NADP. Since K_m for NADP may vary with temperature, it is important that NADP is saturating at all temperatures. Measured values of K_m for NADP with (*R*)-2-butanol vary from 4 μM at 25 °C to 40 μM at 55 °C. Other alcohols gave similar values; thus, 1 mM NADP is sufficient to saturate SADH under all of our experimental conditions.
(15) Both the enzyme and NADP are apparently unstable above 65 °C at pH 8.9, since no difference was observed between reactions initiated by adding enzyme and by adding NADP to reaction mixtures. However, at pH values around 7, which are not optimal for enzymatic activity, we were able to obtain kinetic data up to 80 °C. Since the interpretation of kinetic data of alcohol dehydrogenases is likely to be complicated by ionization effects at pH 7 (see ref 10), we have only used pH 8.9 in our studies. SADH is stable for long periods (≫ 12 h) at 70 °C if the pH of the solution is close to 7.

Chart I



k_{cat}/K_m with each methylene added on the carbon chain of the *S* alcohols. A plot of ln (k_{cat}/K_m) at 50 °C for each enantiomer of the alcohols versus the number of methylene groups in the carbon chain is displayed in Figure 1. We obtain a reasonable linear relationship for the *S* alcohols from (*S*)-2-butanol to (*S*)-2-hexanol (open circles, Figure 1). This observation implies that the reaction mechanism for the reactions of the *S* alcohols studied in the experiments is constant. The slope of this line should be $-\Delta\Delta G^{\ddagger}_n/RT$, where $\Delta\Delta G^{\ddagger}_n$ is the change in free energy of activation per methylene group.[17] The slope of the graph, −1.73, indicates that addition of a methylene group results in an increased free energy of activation of 1.11 ± 0.06 kcal/mol at 50 °C. In contrast, we observe a much steeper decline in activity for the *R* alcohols (open squares, Figure 1). The data for the *R* alcohols does not give a good linear fit and is more likely to be convex. This implies a change in the rate-determining step in the reaction of the *R* alcohols with increasing chain length, with the change occurring between (*R*)-2-pentanol and (*R*)-2-hexanol.

What do these data imply about the molecular basis of the enzymatic enantiospecificity? For 2-butanol, 2-pentanol, and 2-hexanol (and presumably for other secondary alcohols), the enthalpy of activation is lower for the reactions of the *S* enantiomers, which suggests that the *S* enantiomers have the best van der Waals contacts with the enzyme active site. Our results can be summarized by the model shown in Chart I. There appear to be a large alkyl-binding pocket and a small alkyl-binding pocket in the enzyme active site. The small pocket can readily accommodate methyl and ethyl groups, but *n*-propyl is apparently the limit, resulting in the convex plot in Figure 1 for the *R* alcohols. The large pocket can readily accept groups as large as *n*-butyl, since the reaction of the *S* alcohols gives a linear free energy relationship through (*S*)-2-hexanol (Figure 1). A similar model was proposed by Keinan et al.[8] in their studies with TBADH. Horse liver alcohol dehydrogenase reacts slowly with acyclic secondary alcohols but shows a preference for the *S* enantiomers of 2-butanol and 2-pentanol.[18] The X-ray structure of HLADH shows the bulky side chain of phenylalanine-93 projecting into the substrate-binding pocket on the *pro-R* side, restricting binding of the *R* alcohols.[19] The amino acid sequence of TBADH has been recently reported;[20] in TBADH, the corresponding residue is isoleucine, which could allow more room for binding of the alkyl substituent of an *R* alcohol. Our data demonstrate that the entropy of activation is more favorable (i.e., more positive) for the reaction of the *R* enantiomers. The activation entropy is more difficult to evaluate in molecular terms; it could be due to expulsion of more ordered water molecules upon substrate binding, less restricted rotation of the substrate, greater flexibility of the enzyme–substrate–NADP complex, or a combination of all of these factors. However, it is this dichotomy between enthalpy and entropy that results in the observed temperature-dependent reversal of stereospecificity.

To our knowledge, the data presented in this report represent the first demonstration of temperature-dependent enantiospecificity in an enzymatic reaction. However, we believe this phenomenon is not unique to our system. As mentioned previously, Keinan et al. found a decrease in the stereoselectivity of 2-pentanone reduction by TBADH at higher temperatures.[8] In addition, a recent report demonstrates that the diastereoselectivity of the HLADH-catalyzed reduction of 3-cyano-4,4-dimethylcyclohexanone is diminished at 45 °C compared with that observed at 5 °C.[21] Temperature-dependent reversals of elution order have recently been reported in gas chromatography on chiral phases.[22] Furthermore, Pracejas and Tille observed temperature-dependent reversals of stereoselectivity in the reaction of ketenes with optically active amines.[23] We believe that other examples of temperature-dependent stereospecificity (or stereoselectivity) will emerge as more chemical and enzymatic reactions are examined over suitable temperature ranges. From a practical standpoint, our results demonstrate that reaction temperature is a critical variable in asymmetric reactions catalyzed by alcohol dehydrogenase, and possibly other enzymes,[24] and that not only the stereochemical purity but also the preferred stereochemical configuration of products may be altered by running a reaction at different temperatures. Further experiments are in progress to gain a better understanding of the mechanistic basis of chiral recognition by SADH and other enzymes.

Experimental Section

Materials. (*R*)- and (*S*)-2-butanol, 2-propanol, 1-butanol, cyclobutanol, cyclopentanol, and cyclohexanol were obtained from Aldrich Chemical Co. (*R*)- and (*S*)-2-pentanol and (*R*)- and (*S*)-2-hexanol were purchased from Fairfield Chemical Co. (*R*)- and (*S*)-2-heptanol were obtained from Chemical Dynamics Co. Tris·HCl, Red A agarose, and octyl-Sepharose CL-4B were purchased from Sigma Chemical Co. NADP and NADPH were obtained from United States Biochemical Corp. Dithiothreitol was purchased from Research Organics, Inc. Hydroxyapatite was obtained from Bio-Rad.

Methods and Instruments. Kinetic experiments were performed on a Gilford Response UV–vis spectrophotometer equipped with a six-cell holder and electronic temperature control. Kinetic data were analyzed by using the nonlinear least-squares program ENZFITTER, obtained from Elsevier BioSoft, and by using the Fortran programs of Cleland, as adapted for PC-compatible computers by C. B. Grissom. Gas chromatography was performed on a Varian 3300 equipped with a Chirasil-Val 25-m capillary column (Alltech).

Growth of Bacterium. *T. ethanolicus* (ATCC 31550) was grown in a 20-L carboy fermentor at 55 °C with a sealed rubber stopper in a 0.8% (w/v) glucose medium from a 10% inoculum.[7] We attempted to grow *T. ethanolicus* in a 400-L New Brunswick fermentor at 60 °C according to the procedure described by Bryant[7] and co-workers, but we were unable to obtain cells with high activity of SADH. The yields of wet cells from the 20-L carboy were typically from 25 to 33 g; these cells could

(16) k_{cat}/K_m is an apparent kinetic constant that includes contributions from both binding and chemical processes. Thus, the activation enthalpies and entropies that we obtain may reflect binding and/or catalytic steps, which cannot be distinguished without further experiments. For the purposes of the discussion herein, we will consider the reaction in terms of a single virtual transition state. Since the reaction requires NADP as a cosubstrate, the comparison of the k_{cat}/K_m values is only valid if the kinetic mechanism is constant (e.g., ordered or random) throughout the range of substrates. Our preliminary results have shown that k_{cat}/K_m for NADP is independent of the alcohol substrate, which suggests that an ordered mechanism holds for this dehydrogenase.

(17) From Eyring transition-state theory, $(k_{cat}/K_m)_n = (kT/h)e^{-\Delta G^{\ddagger}_n/RT}$ for a series of homologous substrates, where *n* is the number of methylenes in the chain. In logarithmic form, $\ln (k_{cat}/K_m)_n = -\Delta G^{\ddagger}_n/RT + \ln (kT/h)$, and differentiation with respect to *n* gives $d \ln (k_{cat}/K_m)_n/dn = -d(\Delta G^{\ddagger}_n/RT)/dn$. Thus, the slope of a plot of $\ln (k_{cat}/K_m)_n$ against *n* will be equal to $-\Delta\Delta G^{\ddagger}_n/RT$.

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(24) A similar reversal of stereoselectivity with increasing chain length has been observed in the reaction of pig liver esterase with dialkyl dimethylmalonates. See: Bjorkling, F.; Boutelje, J.; Gatenbeck, S.; Hult, K.; Norin, T.; Szmulik, P. *Tetrahedron* **1985**, *41*, 1347. It is not clear if a similar temperature-dependent stereochemical reversal would be observed in the case of pig liver esterase, since the reported reactions were performed only at 25 °C.

be kept frozen for several months at −89 °C without loss of activity.

Alcohol Dehydrogenase Assays. The two alcohol dehydrogenases were assayed spectrophotometrically at 50 °C with 2-propanol (for secondary alcohol dehydrogenase) and 1-butanol (for primary alcohol dehydrogenase) as the substrates by following the reduction of NADP ($\Delta\epsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). The assay mixture contained 200 mM alcohol and 1.25 mM NADP in 100 mM Tris·HCl buffer (pH = 8.9). One unit of activity is the amount of alcohol dehydrogenase that reduces or oxidizes 1 μmol of NADP or NADPH per minute, respectively.

Crude Cell Extract Preparation. Wet cells or thawed frozen cells (105 g) were suspended in 3 volumes of 20 mM Tris·HCl (pH = 7.6) buffer and were sonified three times (each time for 1.5 min) with cooling in an external ice bath. The suspension was then centrifuged at 18 000 rpm for 1 h. The two alcohol dehydrogenases were found in the supernatant. Generally, the crude cell extract contained between 95% and 70% of the secondary alcohol dehydrogenase activity and between 5% and 30% of the primary alcohol dehydrogenase activity. The crude cell extract could be kept frozen for several months at −89 °C without loss of activity. The total activity of SADH for 350 mL of extract was 20 000 units.

Separation of Primary and Secondary Alcohol Dehydrogenases. The purification was performed at 4 °C. The two dehydrogenase activities were separated on a Red A agarose column (40 × 2.5 cm) previously equilibrated with 5 volumes of 20 mM Tris·HCl (pH = 7.6) containing 5 mM pyruvate, 0.5 mM NADP, 0.1 mM $MgCl_2$, and 2 mM dithiothreitol.[7] The enzyme solution was recycled through the column until all of the enzyme activity remained on the column. After application of the enzyme, the column was washed with 10 mM Tris·HCl containing the components of the equilibrating buffer. The primary alcohol dehydrogenase eluted in the void volume and wash. The secondary alcohol dehydrogenase retained on the column was subsequently eluted with 100 mM Tris·HCl buffer containing 0.5 M NaCl plus the components of the equilibrating buffer.

Purification of Secondary Alcohol Dehydrogenase. (a) Octyl-Sepharose Chromatography. The secondary alcohol dehydrogenase eluted from the Red A agarose column was concentrated to 8 mL by ultrafiltration on a DM10 membrane and applied to an octyl-Sepharose CL-4B column equilibrated with 10 mM Tris·HCl buffer containing 5 mM pyruvate, 0.5 mM NADP, 1 mM $MgCl_2$, 0.5 M $(NH_4)_2SO_4$, and 2 mM dithiothreitol. The enzyme eluted with the same solvent. Fractions of 5 mL were collected, and those containing 50 units or more of 2-propanol-dependent alcohol dehydrogenase were pooled and concentrated.

(b) Hydroxyapatite Chromatography. The concentrated pooled fractions from the octyl-Sepharose CL-4B column were dialyzed with 10 mM Tris·HCl buffer containing 5 mM pyruvate, 0.05 mM NADP, 1 mM $MgCl_2$, and 2 mM dithiothreitol and then were applied to a hydroxyapatite column (8 × 1.5 cm) equilibrated with 10 mM Tris·HCl buffer containing 5 mM pyruvate, 0.05 mM NADP, 0.1 mM $MgCl_2$, and 2 mM dithiothreitol. The column was washed with this buffer. The secondary alcohol dehydrogenase was then eluted by using a gradient between 50 mM and 0.5 M potassium phosphate buffer (pH = 7.0) containing 2 mM dithiothreitol, 5 mM potassium pyruvate, 0.05 mM NADP, and 0.1 mM $MgCl_2$. The pooled active fractions were concentrated to 20 mL by ultrafiltration in an Amicon cell over a PM30 membrane. This purified enzyme was used in the kinetic studies and could be stored at −89 °C for several months without losing its activity. The specific activity of the purified enzyme was 50–60 units/mg of protein at 50 °C. By comparison with the specific activity for the homogeneous enzyme, about 105 units/mg at 50 °C[7] and by polyacrylamide gel electrophoresis, we estimate that the enzyme is greater than 50% pure. The protein concentration of the purified enzyme was determined according to the procedure of Bradford,[25] as supplied by Pierce, with bovine serum albumin as a standard.

Kinetics Experiments. Cuvettes contained the following components: 1.00 mM NADP, 10–160 μL of 0.2 M alcohol (in the case of butanol and pentanol) or of 0.02 M alcohol (in the case of hexanol), and 100 mM Tris·HCl buffer (pH = 8.9), in a final volume of 0.6 mL. Because of the high temperature coefficient of Tris, the pH of the Tris·HCl buffer was adjusted to 8.9 at each temperature. The cuvettes were preincubated at the appropriate temperature from 15 to 80 °C before the reaction was started by addition of enzyme solution. The rates were measured spectrophotometrically by the increase in absorbance of NADPH at 340 nm. The rate at each temperature was taken on the basis of the average of several separate experiments. The values of k_{cat} and K_m for each enantiomer of the alcohol were calculated at each temperature. The rates of oxidation of achiral and cyclic alcohols were also measured spectrophotometrically at 50 °C by the increase in absorbance of NADPH at 340 nm.

Reduction of Ketones with SADH. Reaction mixtures contained 1% (v/v) of the ketone substrate, 10%–30% (v/v) 2-propanol, 50 mM Tris·HCl, pH 8.0, 0.05 mM NADP, and 3 mM 2-mercaptoethanol. Those solutions were pumped through a jacketed column containing SADH immobilized on polyacrylamide–oxirane beads (Sigma) kept at 37 °C by circulating water from a constant temperature bath. The column effluents were saturated with $(NH_4)_2SO_4$ and extracted with ether. After being dried over $MgSO_4$ and evaporated, the crude mixtures were distilled and the product alcohols analyzed for optical purity by gas chromatography.

Determination of Optical Purity of the Alcohols. A sample of alcohol was mixed under N_2 atmosphere with 1 equiv of dry triethylamine and 0.1 M (*S*)-*N*-trifluoroacetylprolyl chloride in CH_2Cl_2 (Aldrich) at room temperature for 5 min. The resulting solution was then analyzed by gas chromatography on a Chirasil-Val capillary column.

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