Temperature-Dependent Enantiospecificity of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus*

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Received October 26, 1988

The stereochemical properties of enzymes are remarkable, and the use of enzymatic systems for asymmetric synthesis[1] has become a successful method in organic chemistry. For example, horse liver alcohol dehydrogenase (HLADH)-catalyzed oxidation of *meso*-diols occurs with *pro-S*-selectivity, and reductions of highly symmetrical decalin diones gives complete *pro-9R* selectivity to produce the ketoalcohols.[2] However, HLADH has low activity for reduction of acyclic ketones and has limited thermal stability. Recently, Bryant et al.[3] have isolated and characterized two alcohol dehydrogenases from the thermophilic bacterium, *Thermoanaerobacter ethanolicus*. These enzymes are NADP-dependent, and they both have high thermostability. However, one of these enzymes has a preference for 2-propanol rather than ethanol.[3] This secondary alcohol dehydrogenase (SADH) is especially interesting, since it exhibits high activity with a wide range of acyclic secondary alcohols and ketones.[3] A similar alcohol dehydrogenase reported from *Thermoanaerobium brockii* catalyzes the asymmetric reduction of aliphatic acyclic ketones.[4] An interesting substrate size-induced reversal of stereoselectivity at 37 °C was observed by Keinan and co-workers in their studies of this latter enzyme.[4] The smaller substrates (2-butanone, 3-methyl-2-butanone, or methyl cyclopropyl ketone) were reduced to (*R*)-alcohols, whereas 2-pentanone and the longer chain ketones provided the (*S*)-alcohols.[4] In order to evaluate the mechanistic basis of this unusual stereochemical reversal, we have studied the kinetics of the reaction of SADH from *T. ethanolicus* with the (*R*)- and (*S*)-enantiomers of the simple secondary alcohols, 2-butanol and 2-pentanol.[5]

The specificity constant of the enzyme with respect to (*R*)- or (*S*)-alcohol substrates is defined as the ratio k_{cat}/K_m.[6] Hence, the enantiospecificity ratio $E = (k_{cat}/K_m)_R/(k_{cat}/K_m)_S$, 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*)-alcohol. 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*)-isomer occurs. The temperature is thus the "racemic temperature", T_r, and will be a constant for a particular alcohol.[7]

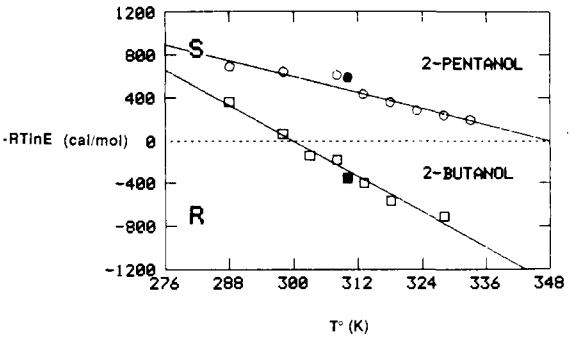

Figure 1. The temperature dependence of free energy of activation differences for 2-butanol and 2-pentanol: 2-butanol, open squares; 2-pentanol, open circles; reduction of 2-butanone, filled square; 2-pentanone, filled circle.

Values of k_{cat}/K_m were determined for the (*R*)- and (*S*)-enantiomers of both 2-butanol and 2-pentanol between 15 °C and 65 °C.[5] Analysis of the data as described above resulted in the unexpected discovery that the enantiospecifity of the reaction of 2-butanol is temperature-dependent, as shown in Figure 1. For 2-butanol, $\Delta\Delta H^{\ddagger} = 8.27$ kcal/mol and $\Delta\Delta S^{\ddagger} = 27.6$ 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 1). For 2-pentanol and 2-butanol, the reversal of stereospecificity, as reported by Keinan et al.,[4] was confirmed by our observations at 37 °C. However, at 15 °C, it is the (*S*)-isomer of both alcohols which is the preferred substrate (Figure 1). The T_r for 2-pentanol is predicted to be 77 °C from the data shown in Figure 1, since $\Delta\Delta H^{\ddagger} = 4.23$ kcal/mol and $\Delta\Delta S^{\ddagger} = 12.1$ cal deg^{-1} mol^{-1}. We have attempted to perform kinetic studies at temperatures above 65 °C, but we were unable to obtain reliable data, possibly due to the instability of the enzyme and NADP at pH 8.9 and temperatures greater than 65 °C.

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 1 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. On the contrary, 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 optical purity,[8] we found that (*R*)-2-butanol and (*S*)-2-pentanol are formed in 28% and 44% ee, respectively, as the data in Figure 1 predict. The results from Keinan et al.[4] support our observations; they found that the optical purity of (*S*)-2-pentanol diminished as the temperature of the reaction mixture for 2-pentanone reduction was increased.

What do these data imply about the molecular basis of the enzymatic enantiospecificity? For both 2-butanol and 2-pentanol (and presumably for other secondary alcohols), the enthalpy of activation is lower for the reactions of the (*S*)-enantiomers. In contrast, the entropy of activation is more favorable for the (*R*)-enantiomers. It is this dichotomy between enthalpy and entropy which results in the observed temperature dependence. To our knowledge, this is the first demonstration of temperature-dependent enantiospecificity in an enzymatic reaction. However, we believe this phenomenon is not unique to our system. Indeed, 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.[9] We believe that other examples of temperature-dependent stereospecificity (or stereoselectivity) will emerge as more reactions are examined over suitable temperature ranges. From

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(5) Reaction conditions: temperature 15-60 °C using 1.25 mM NADP or 1.00 mM NADP, 100 mM Tris HCl (pH adjusted to 8.9 at the desired temperature). The enzyme was purified by a modification of the procedure of Bryant et al.[3] Rates were measured spectrophotometrically by the absorbance of NADPH at 340 nm ($\Delta\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}). The data were analyzed by fitting to the Michaelis-Menten equation by using a nonlinear least-squares kinetics program, ENZFITTER, from Elsevier Biosoft. The values of k_{cat} and K_m of each enantiomer of the alcohol were calculated at each temperature. (*R*)-2-Butanol and (*S*)-2-butanol were obtained from Aldrich Chemical Co., while (*R*)-2-pentanol and (*S*)-2-pentanol were obtained from Fairfield Chemical Co.

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(7) The racemic temperature, T_r, is analogous to the isokinetic temperature known from linear free energy relationships. See, e.g.: Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 3rd ed.; Harper and Row: 1987; pp 158-159.

(8) Optical purities for the isolated alcohols were determined by GC analysis of the *N*-trifluoroacetyl-L-prolyl esters on a Chirasil-Val (Alltech) capillary column.

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a practical standpoint, our results demonstrate that reaction temperature is a critical variable in asymmetric reactions catalyzed by alcohol dehydrogenase[9] and possibly other enzymes,[10] and not only the optical purity but also the preferred stereochemical configuration of products may be altered by running reactions at different temperatures.

Acknowledgment. This work was supported by a Biotechnology Grant from the University of Georgia Research Foundation and by Project DE-FG09-86ER13614 from the U.S. Department of Energy. We also thank Nancy Bond for supplying a culture of *Thermoanaerobacter ethanolicus* and for assistance in growth of the organism.

(10) A similar reversal of stereoselectivity with increasing chain length has been observed in the reaction of pig liver esterase with dialkyldimethylmalonates, 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 reversal would be observed in the case of pig liver esterase, since the reported reactions were performed only at 25 °C.

Gallane at Last! Synthesis and Properties of Binary Gallium Hydride

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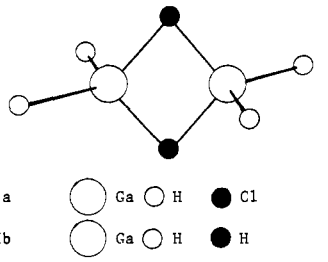
Received October 12, 1988

The uncoordinated binary hydride of gallium is terra incognita beyond the vapor-phase transients GaH[1a] and GaH_3.[1b] This has not been for want of exploration. As early as 1941 Wiberg et al. laid claim to the synthesis of the free hydride via two routes.[2] Neither stood the test of subsequent re-examination,[3a] but Greenwood and Wallbridge[3b] presented analytical and spectroscopic evidence for displacement reaction 1. More recent studies[4,5]

$$Me_3N{\cdot}GaH_3 + BF_3 \xrightarrow{-15\ °C} \underset{\text{viscous liquid}}{\tfrac{1}{x}[GaH_3]_x} + Me_3N{\cdot}BF_3 \quad (1)$$

disclose, however, that the predominant pathway entails not displacement but *halide–hydride exchange*. Here we outline the synthesis of gallane and preliminary details of its characterization.

Of our earlier attempts to prepare gallane,[5] only the interaction of $GaCl_3$ with a tetrahydrogallate gave any encouragement, the solid mixture yielding at ambient temperatures small amounts of a volatile, thermally unstable product, but this could never be freed entirely from chloride. Altogether superior to $GaCl_3$ as a precursor is monochlorogallane, $[H_2GaCl]_2$ **Ia**, a compound conveniently synthesized by the reaction of $GaCl_3$ with an excess of Me_3SiH.[6] Not only is reduction already two-thirds complete,



but **Ia** is also a liquid even at quite low temperatures and therefore susceptible to more efficient mixing with the hydride ion source ($MGaH_4$). We find that **Ia** reacts in vacuo with freshly prepared $LiGaH_4$ at −30 °C to give not only substantial quantities of elemental gallium and hydrogen but also a volatile product, shown to be gallane, typically in amounts of 4–40 mg and yields of ca. 5% based on eq 2. Operations were carried out at a pressure of

$$\tfrac{1}{2}[H_2GaCl]_2 + LiGaH_4 \xrightarrow[\text{neat reagents or } n\text{-octane}]{-30\ °C} \tfrac{1}{x}[GaH_3]_x + LiGaH_3Cl \quad (2)$$

$<10^{-4}$ mmHg in an all-glass apparatus which had been preconditioned by heating under continuous pumping, with short distillation paths and the maintenance of all glassware to which the gallane had access at temperatures <−20 °C. Gallane condenses as a white solid which melts at ca. −50 °C and has a vapor pressure at −63 °C of ca. 1 mmHg.

(a) Elemental analysis confirmed that the compound contained no chlorine, only gallium and hydrogen. The reaction with an excess of anhydrous HCl at −95 °C resulted in the quantitative formation of $GaCl_3$ and H_2 in accordance with eq 3.

$$\tfrac{1}{x}[GaH_3]_x + 3HCl \rightarrow GaCl_3 + 3H_2 \quad (3)$$

(b) IR Spectrum. A film of the annealed solid compound at 77 K displayed an IR spectrum resembling that of the condensate formed by the vapors derived from the reaction of an excess of $NaGaH_4$ with $GaCl_3$,[5] with three main absorptions at 1978 (s), 1705 (s, br), and 550 cm^{-1} (s, br), which shifted to 1422, 1200, and 400 cm^{-1}, respectively, for the perdeuterated compound. Very different spectra were exhibited by the vapor (Figure 1) or by solid matrices formed by codepositing the vapor with an excess of Ar, Kr, or N_2 at ca. 20 K. Here the pattern and energies of the absorptions—with two distinct features near 2000 cm^{-1} attributable to $\nu(Ga-H_{term})$ modes and two others at 1200–1300 cm^{-1} attributable to $\nu(Ga-H_{bridge})$ modes—advocate the molecule Ga_2H_6 with a diborane-like structure **Ib**. Of the six bands clearly discernible in the vapor spectrum three (at 1976, 1200, and 671 cm^{-1}) had the P–R doublet structure characteristic of the parallel-type bands of just such a pseudo-linear molecule. The average P–R branch separation at 10.3 cm^{-1} implies[7] then a rough value of 260 pm for the Ga···Ga distance, in excellent agreement with the corresponding distance of 261 pm in the related molecule $Me_2Ga(\mu\text{-}H)_2GaMe_2$, as determined by electron diffraction.[8] The identification of Ga_2H_6 receives support not only from obvious parallels with the spectra of **Ia**[6] and $Me_2Ga(\mu\text{-}H)_2GaMe_2$[8] but also from the energy shifts induced by perdeuteration of the product (see Table I). Annealing an Ar matrix containing the gallane at temperatures up to ca. 35 K caused the decay of the bands associated with Ga_2H_6 and the appearance and growth of a spectrum resembling that of the solid gallane. Facile aggregation of Ga_2H_6 molecules in the solid phase appears then to give an oligomer with a change in the mode of hydrogen bridging but retaining terminal Ga–H bonds (cf. α-AlH_3[9]).

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