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Effects of Hydrostatic Pressure on Stereospecificity of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter Ethanolicus* Support the Role of Solvation in Enantiospecificity

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Supporting Information

ABSTRACT: The effects of hydrostatic pressure and temperature on the reaction of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* with enantiomers of 2-butanol, 2-pentanol, and 2-hexanol have been measured. For all substrates, increasing pressure favors the *S* enantiomer, whereas increasing temperature favors the *R* enantiomer. Fitting of the pressure and temperature data for 2-hexanol provided apparent $\Delta\Delta S^{\ddagger}$ and $\Delta\Delta V^{\ddagger}$ values of +46 ± 15 J/mol and +(2.0 ± 0.4) × 10^{-2} L/mol, respectively. These results support our previous proposal that desolvation of the enzyme active site plays an important role in stereospecificity.

KEYWORDS: *alcohol dehydrogenase, secondary alcohol, NADP, stereochemistry, temperature, hydrostatic pressure*

The stereochemistry of enzyme-catalyzed reactions is remarkable; however, despite decades of study, the physical bases of enzymatic stereochemistry remain enigmatic. In the case of alcohol dehydrogenases, Prelog envisioned a static model based on steric constraints for stereospecificity of alcohol oxidation.[1] This empirical model, widely referred to as "Prelog's Rule", is useful qualitatively, but is not quantitative in its predictions. Jones expanded this concept to the "diamond-lattice section" model for horse liver alcohol dehydrogenase reactions.[2] These models were based on energetically favorable van der Waals contacts and unfavorable steric effects, which are enthalpic in origin, and did not consider entropic contributions to stereochemistry. We demonstrated that the effects of temperature on the stereospecificity of alcohol oxidation by thermostable secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus* can be quantified, and entropy is a significant contributor to stereochemical discrimination for small secondary alcohols, such as 2-butanol.[3,4] We later suggested that this entropy difference could arise from differential desolvation of the enzyme active site by binding of enantiomeric substrates.[5] In the present work, we tested this desolvation hypothesis by determining the effect of hydrostatic pressure on stereospecificity of SADH. The results support a role of differential desolvation in the enantiomeric discrimination by SADH.

The ratio of reaction rates for two enantiomers is given by the *E* value, which from transition-state theory is exponentially related to the difference in activation free energies, $\Delta\Delta G^{\ddagger}$, for the *R* and *S* enantiomers (eq 1).

$$E = k_R/k_S = \exp(-(\Delta G_R^{\ddagger} - \Delta G_S^{\ddagger})/RT) = \exp(-\Delta\Delta G^{\ddagger}/RT) \quad (1)$$

$$\ln E = -\Delta\Delta G^{\ddagger}/RT \quad (2)$$

$$-RT \ln E = \Delta\Delta G^{\ddagger} \quad (3)$$

In logarithmic form, eq 1 can be written as eq 2, and rearranged to eq 3. We have previously shown that eq 3 can be expanded for the effects of temperature on enantiospecificity with eq 4.[3,4] To allow for the effects of both variable pressure and temperature on stereospecificity,

$$-RT \ln E = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \quad (4)$$

Equation 4 can be further expanded into eq 5. The $\Delta\Delta V^{\ddagger}$ should arise primarily from differences in transition state solvation for the enantiomers, since any pressure-induced changes in the overall protein structure (e. g., arising from compressibility or conformational changes) will be approximately constant for reactions of both enantiomers. We showed

$$-RT \ln E = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} + P\Delta\Delta V^{\ddagger} \quad (5)$$

previously that under constant *P* conditions, it is possible to define a "racemic temperature", T_r, with eq 6, when $E = 1$.[3,4] In this case, because atmospheric pressure is 0.1 MPa, the $P\Delta\Delta V^{\ddagger}$ term is negligible, and eq 5 reduces to eq 4. By analogy, it is

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possible to define a "racemic pressure", P_r, under constant temperature conditions, as given in eq 7.

$$T_r = \Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger} \quad (6)$$

$$P_r = (T\Delta\Delta S^{\ddagger} - \Delta\Delta H^{\ddagger})/\Delta\Delta V^{\ddagger} \quad (7)$$

We selected the S39T mutant of *T. ethanolicus* SADH to study the effects of pressure on enantiospecificity, since it has higher activity and lower stereospecificity for *S* enantiomers of simple secondary alcohols than wild-type SADH.[6] In these experiments, we determined the specific activity of the enzyme with enantiomers of 2-butanol, 2-pentanol, and 2-hexanol at various temperatures and pressures. We found that the specific activity measurements showed *R*/*S* ratios for 2-butanol and 2-pentanol at atmospheric pressure similar to those obtained previously by complete kinetic analysis, with the *R* enantiomer the favored substrate.[6] The enantiomeric ratios were ~2 for 2-butanol and near 1 (i. e., racemic) for 2-pentanol, whereas for 2-hexanol, the reaction favored the *S* enantiomer, with an enantiomeric ratio near 0.25 at atmospheric pressure. Larger effects of pressure were noted on the reaction of 2-hexanol, so we then proceeded to obtain a set of data with both enantiomers of 2-hexanol at a wider range of temperatures and pressures. The rate data were collected in triplicate at 4 temperatures (293, 298, 318, 325 K) and pressures (0.1, 50, 100, 137.5 MPa) in a Cary 14 UV−vis spectrophotometer equipped with a high pressure cell obtained from ISS (Champaign-Urbana, IL). The reactions were contained in 1.2 mL quartz bottles capped with Teflon tubing. The pressure was applied with a manual pump from High Pressure Equipment Co. (Erie, PA) using spectroscopic grade ethanol as the pressurizing fluid.

Fitting the pressure and temperature data for SADH oxidation of the 2-hexanol enantiomers to eq 5 provides apparent parameters, $\Delta\Delta H^{\ddagger}$, $\Delta\Delta S^{\ddagger}$, and $\Delta\Delta V^{\ddagger}$ for the reaction, which are given in Table 1.[7] The 3-D fit of the data is shown in Figure 1. $\Delta\Delta H^{\ddagger}$ has a positive value, indicating that steric effects and van der Waals forces inherently favor the *S* alcohol. The value of $\Delta\Delta S^{\ddagger}$ is positive, as we previously observed for 2-butanol and 2-pentanol,[6] thus favoring the reaction of (*R*)-2-hexanol as temperature increases. The value of $\Delta\Delta V^{\ddagger}$ is also positive, thereby making the reaction of (*R*)-2-hexanol less favorable as pressure increases. Thus, we observe *E* for 2-hexanol decreasing from 0.25 at 298 K and atmospheric pressure to 0.08 at 137.5 MPa. The racemic temperature for 2-hexanol can be calculated from the parameters in Table 1 and eq 6 to be 380 K at 0.1 MPa (atmospheric pressure). At 298 K, the racemic pressure is estimated to be −189.6 MPa, so it is not physically possible. However, at temperatures above the racemic temperature, real values of the racemic pressure are possible; for example, at 385 K, the racemic pressure is calculated from eq 7 to be 10.5 MPa.

Table 1. Apparent Thermodynamic Parameters for Reaction of 2-Hexanol with SADH

$\Delta\Delta H^{\ddagger}$	+17.5 ± 4.6 kJ/mol
$\Delta\Delta S^{\ddagger}$	+46 ± 15 J/mol K
$\Delta\Delta V^{\ddagger}$	+(2.0 ± 0.4) × 10^{-2} L/mol

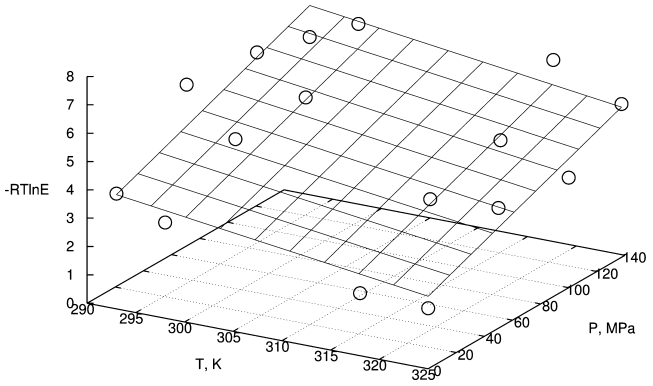

Figure 1. 3-D plot of $-RT$ ln E for 2-hexanol as a function of temperature and pressure. The data were fit to eq 5, and the plot was created with Gnuplot (http://www.gnuplot.info/). The grid is calculated using the parameters given in Table 1. The circles are the experimental points.

These results support our previous proposal that desolvation plays a key role in the stereochemical discrimination of simple secondary alcohols by SADH. In our model, the positive entropy difference results from the expulsion of a water molecule(s) which occupies the "small pocket" when the larger alkyl group of the R-alcohol is bound in it (Figure 2).[5] It is interesting in this regard that the magnitude of $\Delta\Delta V^{\ddagger}$ in Table 1, 0.02 L, is approximately the volume of 1 mol of water. Furthermore, the value of $\Delta\Delta S^{\ddagger}$ in Table 1 is remarkably similar in magnitude to the ΔS of −37 J/mol K for a single water molecule bound to the substrate binding pocket of *Candida antarctica* lipase B,[8] as well as for water bound to hydrated salts.[9] In Table 1, the sign of $\Delta\Delta S^{\ddagger}$ is positive because the water is released by the *R* enantiomer, as shown in Figure 2, and *E* was defined in eq 1 as k_R/k_S. A general rule of thumb in thermodynamics is that ΔS and ΔV are correlated in sign. Simply put, a system of particles with more degrees of freedom (larger ΔS) is likely to occupy a larger volume than a system of identical particles with fewer degrees of freedom. Thus, a water molecule released from the enzyme active site upon substrate binding has a larger volume (or lower density) in the bulk solution than in the bound state; however, this larger system volume is opposed by increasing hydrostatic pressure, making the reaction of the *R* alcohol less favorable as the pressure increases.

Although it is widely recognized that solvation influences ligand binding to enzymes,[10,11] there are remarkably only a few previous examples of hydrostatic pressure effects on enzyme stereochemistry. Kahlow et al. studied the effects of pressure on *Candida rugosa* lipase-catalyzed esterification of menthol in chloroform at pressures up to 10 MPa and found the *E* value to decrease dramatically with pressure.[12] Molecular dynamics simulations suggested that changes in active site solvation were responsible. Liese et al. reported the effects of pressures up to 250 MPa on the stereochemistry of the acyloin condensation of benzaldehyde and acetaldehdye catalyzed by benzoylformate decarboxylase.[13,14] Increasing pressure was found to favor the formation of the *R* product; however, this was accompanied by a large reversible decrease in activity. These authors also suggested that changes in enzyme solvation due to the pressure could be the cause of the observed effects; however, none of these previous papers analyzed the effects of both temperature and pressure quantitatively using transition-state theory. To our knowledge, our results are the first measurement of the apparent activation volume difference, $\Delta\Delta V^{\ddagger}$, for an enantiospecific enzymatic reaction. In conclusion, our results

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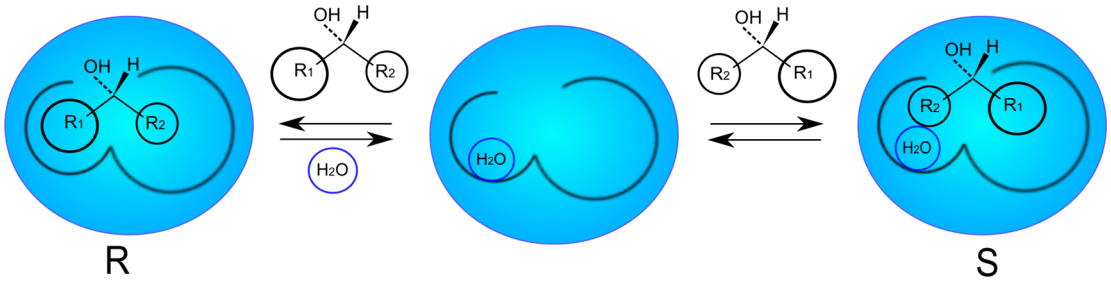

Figure 2. Model for differential desolvation of SADH by enantiomeric substrates. Binding of *R* substrates places the larger substituent in the small pocket, which displays a water; binding of *S* substrates places the small substituent in the small pocket, leaving the water bound.

provide additional support for the role of solvation in enzymatic stereospecificity.

■ ASSOCIATED CONTENT

Supporting Information

The specific activity of S39T SADH with 2-butanol, 2-pentanol, and 2-hexanol at different temperatures and pressures is given in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Both authors contributed equally.

Notes

The authors declare no competing financial interest.

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