The Stereospecificity of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus* Is Partially Determined by Active Site Water

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The application of enzymes as chiral catalysts for organic synthesis is a rapidly growing field.[1] However, the physical bases of stereorecognition by enzymes are not yet well understood. We have now obtained evidence suggesting that release of structured water from an enzyme active site upon substrate binding may play a key role in enzymatic stereospecificity. Alcohol dehydrogenases (ADHs) have been used extensively for the asymmetric synthesis of chiral building blocks, because of their high chemoselectivity, enantioselectivity, tolerance of a wide spectrum of functional groups, and their relative ease of use.[1] A number of ADH's active with secondary alcohols have been isolated from thermophilic organisms, the best studied being that from *Thermoanaerobacter brockii* (TBADH).[2] Recently, we reported that the secondary ADH from *Thermoanaerobacter ethanolicus* (SADH) reduces ethynyl ketoesters with high enantioselectivity to yield chiral propargyl alcohols.[3] We performed modeling studies of SADH with isopropyl 4-oxo-5-hexynoate, using the available X-ray coordinates of the highly homologous (96% identity) TBADH.[4] The result of these modeling studies predicted that Cys-295 is located in the "small alkyl group" binding pocket proposed by Keinan et al.[5] The sequence alignment of SADH and related dehydrogenases gleaned from Genbank using BLAST[6] revealed that this cysteine is conserved in the ADHs from *T. brockii*, *T. ethanolicus*, *Clostridium beijerinckii*, and *Alcaligenes euthropus*, but is replaced by threonine in the ADH from *Entamoeba histolytica* and methionine in that from *Mycoplasma pneumoniae*. Thus, we anticipated that the mutation Cys295 → Ala could be readily performed to increase the size of the small alkyl binding pocket without significant loss of activity. C295A SADH was prepared and it was found to have specific activity with 2-propanol comparable to that of wild-type SADH.[7]

We then investigated the effect of the C295A mutation on the stereospecificity of SADH. We have previously studied the temperature dependence of the enantiospecificity of wild-type[8] and S39T[9] SADH with chiral secondary alcohols, and we found that both enzymes show an increase in (*R*)-specificity with increasing temperature. We analyzed the temperature effect on stereospecificity[8,9] according to eq 1 , where *E* is defined as $(k_{cat}/K_m)_R/(k_{cat}/K_m)_S$. The temperature dependence of stereospecificity is not attributable to conformational changes in SADH, since studies with denaturants have shown that the enzyme is rigid throughout the temperature range studied.[10] We determined the values of k_{cat}/K_m at various temperatures between 288 and 328 K for the oxidation of the (*R*)- and (*S*)-enantiomers of 2-butanol, 2-pentanol, and 2-hexanol by C295A SADH (Figure 1). In striking contrast to wild-type and S39T SADH, C295A SADH shows no significant temperature dependence of stereospecificity (Figure 1). The stereospecificity of C295A SADH for 2-hexanol is dramatically reduced (Figure 1, solid line with filled triangles), as expected due to the larger size of the mutated small alkyl binding pocket. With wild-type SADH and 2-hexanol (Figure 1, dashed line with open triangles), it was not possible to obtain good kinetic data for (*R*)-2-hexanol at $T < 308$ K because of the very low activity of this substrate. However, C295A SADH exhibits good activity with (*R*)-2-hexanol at all temperatures examined. The effect of the C295A mutation is less striking for 2-pentanol, although the enantioselectivity shifts toward a very slight preference for (*R*)-2-pentanol (Figure 1, compare the dashed line with the open squares and the solid line with the squares). For 2-butanol, C295A SADH is more specific for the (*R*)-enantiomer than wild-type SADH at low temperature and less so above 306 K (Figure 1, compare the dashed line with the open circles and the solid line with the circles).

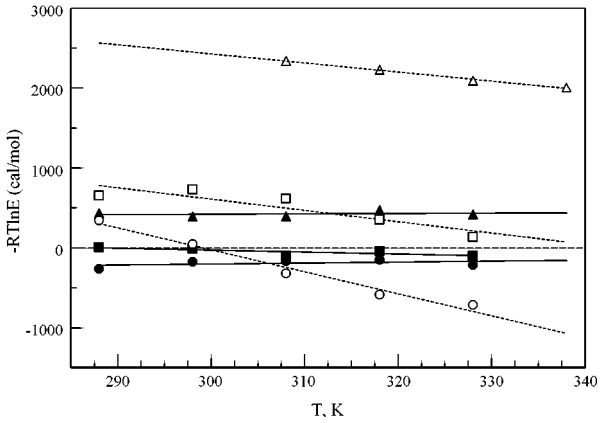

Figure 1. Temperature dependence of enantiospecificity for wild-type (dashed lines and open symbols) and C295A SADH (solid lines and filled symbols) for 2-butanol (circles), 2-pentanol (squares), and 2-hexanol (triangles).

$$-RT \ln E = \Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \qquad (1)$$

Within experimental error, both $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are dramatically reduced for reactions with C295A SADH (Table 1). These

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(7) PCR-based gene mutagenesis was performed using plasmid pADHB25-kan as a template. A primer (KA4N) was synthesized complementary to the noncoding strand that included a *Kpn*I restriction site, the native *adh*B ribosome binding site, and the *adh*B translation initiation codon. A second primer (KA4C) that included the complement to the *adh*B termination codon and the *Apa*I restriction site was synthesized complementary to the coding strand. Complementary 33-mer primers that contained the mutated bases were used together with the two KA4 end primers to amplify the 5′ and 3′ ends of the *adh*B gene. Cells were grown and the enzyme was purified by affinity chromatography on Red-Agarose as previously described,[9] which gave C295A SADH with a specific activity of 43 U/mg protein.
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Table 1. Differential Eyring Parameters for C295A and Wild-type SADH with Secondary Alcohols

	C295A SADH		wild-type SADH		S39T SADH	
substrate	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/°C mol)	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/°C mol)	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/°C mol)
2-butanol	-0.6 ± 0.5	-1.2 ± 1.5	8.2 ± 1.0[a]	27.6 ± 3.4[a]	1.9 ± 0.4[a]	10.4 ± 1.3[a]
2-pentanol	0.7 ± 0.3	2.4 ± 1.2	2.8 ± 0.4[a]	7.3 ± 1.2[a]	0.60 ± 0.15[a]	2.9 ± 0.5[a]
2-hexanol	0.3 ± 0.4	-0.5 ± 1.1	5.8 ± 0.2	11.4 ± 0.7	-	-

[a]From ref 9.

changes imply a role of Cys-295 in $\Delta\Delta H^{\ddagger}$. The values of $\Delta\Delta H^{\ddagger}$ for reactions of wild-type SADH are positive (Table 1), indicating that the (*S*)-enantiomers of secondary alcohols have the best steric fit in the active site.[8] In this regard, it should be noted that the crystal structure of apoTBADH crystallized in the presence of racemic 2-butanol shows that (*S*)-2-butanol is preferentially bound.[11] The reduction in $\Delta\Delta H^{\ddagger}$ for these substrates observed with C295A SADH implies that there is unfavorable interaction between the larger alkyl group of (*R*)-alcohols bound in the small pocket and the Cys-295 sulfhydryl. This strain is evidently relieved by the alanine mutation, which removes the bulky S atom from the small alkyl-binding pocket. This result confirms our prediction that Cys-295 is in the small alkyl-binding pocket. Introducing steric bulk in the "large pocket", as we have done previously with the S39T mutation of SADH, can also reduce $\Delta\Delta H^{\ddagger}$ (Table 1).

The $\Delta\Delta S^{\ddagger}$ values for wild-type SADH are also positive, demonstrating that the binding of (*R*)-alcohols has a more favorable associated entropy.[8,9] The loss of this entropy gain in the reactions of C295A SADH implies a role of Cys-295 in $\Delta\Delta S^{\ddagger}$ as well. Assignment of activation entropy to a specific molecular process is generally fraught with difficulty, due to the large number of possible contributions from translational, rotational, and vibrational motions of enzyme and substrate. However, the comparison of wild-type and C295A mutant SADH reduces the possible contributions to those associated with the Cys-295 sulfhydryl group. There could be a rotational entropy loss of the Cys-295 sulfhydryl upon binding of an alkyl group in the small alkyl-binding pocket. It would be expected that binding of a larger group in the small pocket would result in greater restriction of the freedom of motion of the sulfhydryl group, and thus result in a net entropy loss. However, the binding of (*R*)-alcohols places the larger alkyl group in the small alkyl binding pocket, yet paradoxically results in an observed increase in the activation entropy. Alternatively, the SH of Cys-295 may interact with bound ordered water molecules, which could be selectively expelled by the binding of a large, but not a small, alkyl group in the small alkyl-binding pocket. Water activity has been shown to affect the enantioselectivity of ketone reduction by TBADH in hexane.[12] Expulsion of a tightly bound, structured water molecule from an enzyme-active site into the bulk solvent would result in an entropy increase of about 10 cal/ °C mol,[13] comparable to the values of $\Delta\Delta S^{\ddagger}$ seen in wild-type SADH (Table 1). Indeed, examination of the crystal structure of TBADH shows just such an ordered water molecule, HOH5, in the small pocket, located at a distance of 4.06 Å from the sulfur atom of Cys-295 and 3.54 Å from the β-carbon of Ile-86. This bound water molecule is also observed in the structure of the apoTBADH with bound (*S*)-2-butanol,[11] as expected, since it should be expelled only upon binding of (*R*)-alcohols. Hence, the most likely explanation for the $\Delta\Delta S^{\ddagger}$ values in Table 1 is that this bound water is displaced by binding of (*R*)-alcohols. The mutation of Cys-295 to alanine may either eliminate this structured water from the active site or allow it to become disordered, thereby eliminating the $\Delta\Delta S^{\ddagger}$ observed in the reaction with chiral secondary alcohols. *Thus, the stereospecificity of SADH may be at least partly determined by the selective expulsion of bound water from the small alkyl binding pocket upon binding of (R)-alcohols*. To our knowledge, this is the first experimental data implicating a direct role of active site water in the stereospecificity of enzymatic reactions.

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