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How does active site water affect enzymatic stereorecognition?

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Abstract

The *E* value for ketone reduction catalyzed by a secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus* is temperature dependent, due to a relatively large activation entropy difference, $\Delta\Delta S^{\ddagger}$, which favors the formation (or reaction) of the (*R*)-enantiomer of 2-butanol and 2-pentanol. In contrast, the activation enthalpy, $\Delta\Delta H^{\ddagger}$, favors the formation of the (*S*)-enantiomer of 2-butanol and 2-pentanol. C295A mutant SADH shows very large reductions in $\Delta\Delta S^{\ddagger}$ compared to wild-type SADH, in addition to the expected reductions in $\Delta\Delta H^{\ddagger}$. The decrease in $\Delta\Delta H^{\ddagger}$ can be readily explained on the basis of steric interaction and van der Waals contacts of the enantiomeric substrates in the active site. The comparison of $\Delta\Delta S^{\ddagger}$ of the wild-type and C295A mutant SADH reduces the possible entropy contributions to those associated with the Cys-295 sulfhydryl group. Examination of the crystal structure shows an ordered water molecule in the small pocket, located at a distance of 4.1 Å from the sulfur atom of Cys-295. Thus, the stereospecificity of SADH may be at least partly determined by the selective expulsion of this bound water from the small alkyl binding pocket upon binding of large substituents in the small alkyl pocket. The data obtained with wild-type, S39T and C295A SADH with NADP, SNADP and APADP show a reasonable enthalpy–entropy compensation relationship, suggesting a contribution of solvation. Our results suggest that selective release of bound solvent from the active site upon binding of enantiomeric substrates may contribute to the differential activation entropy, $\Delta\Delta S^{\ddagger}$.

Keywords: Stereoselectivity; Activation enthalpy; Activation entropy; Alcohol dehydrogenase; Enthalpy–entropy compensation

1. Introduction

The application of enzymes as chiral catalysts for organic synthesis is a rapidly growing field, due to the high stereoselectivity and regioselectivity of enzymatic reactions. However, the physical basis of stereorecognition by enzymes is not yet well understood. For reductions of ketones catalyzed by alcohol dehydrogenases, it was found by Prelog that the (*S*)-alcohol is often the product [1]. This is the basis of the well-known "Prelog's Rule". This rule assumes that the enzyme has two hydrophobic sites, or "pockets" which can accommodate alkyl groups of different size (Scheme 1). If one of the pockets is small and the other is large, then stereoselectivity would be observed based on the steric restraints of the active site. This concept was expanded by Jones into the "Diamond lattice model", which was quite successful in prediction of the outcome of ketone reductions by horse liver alcohol dehydrogenase [2]. Keinan et al. proposed a model similar to Prelog's for the reduction of

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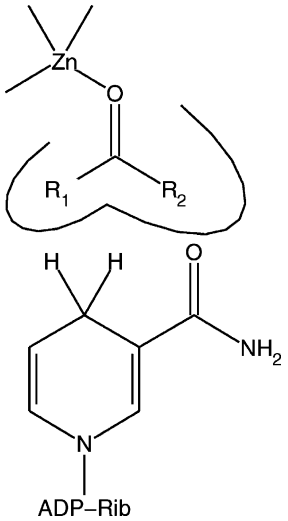

Scheme 1.

ketones by a secondary alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) [3]. However, these models are based solely on the steric demand and van der Waals forces of substrate binding, which are contained in the activation enthalpy, and the possible role of activation entropy in the stereoselectivity of alcohol dehydrogenases was not previously considered.

2. Discussion

We have been studying the effects of physical variables on the stereoselectivity of alcohol oxidation and ketone reduction by the thermostable secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus* [4,5]. Under kinetic control, the enantiomeric ratio, E, is equivalent to the ratio of the rate constants (k_{cat}/K_m) of formation (or reaction) of the enantiomers (Eq. (1)) [6]. The differential free energy of activation, $\Delta\Delta G^{\ddagger}$, is given by Eq. (2). The temperature dependence of $\Delta\Delta G^{\ddagger}$ can be given by the thermodynamic relationship in Eq. (3). Combining Eqs. (2) and (3), one can write Eq. (4). Thus, a

$$E = \frac{R}{S} = \frac{(k_{cat}/K_m)_R}{(k_{cat}/K_m)_S} \quad (1)$$

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

$$\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \quad (3)$$

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

$$T_r = \frac{\Delta\Delta H^{\ddagger}}{\Delta\Delta S^{\ddagger}} \quad (5)$$

plot of $-RT\ln E$ versus T for the reaction of a pair of enantiomers is predicted to be linear, with a slope of $\Delta\Delta S^{\ddagger}$ and an intercept of $\Delta\Delta H^{\ddagger}$ [4,5]. If there is no enantiospecificity in a reaction, $E = 1$, and hence $\Delta\Delta G^{\ddagger} = 0$. Eq. (3) then can be rewritten as Eq. (5), where T_r is the "racemic temperature", at which there is no stereochemical discrimination in the reaction. We found that there is a significant temperature dependence of the E value for ketone reduction, due to a relatively large activation entropy difference, $\Delta\Delta S^{\ddagger}$, between the (*R*)- and (*S*)-enantiomers, which favors the formation (or reaction) of the (*R*)-enantiomer of 2-butanol and 2-pentanol [4,5]. In contrast, the activation enthalpy, $\Delta\Delta H^{\ddagger}$, favors the formation of the (*S*)-enantiomer of 2-butanol and 2-pentanol [4,5]. This dichotomy between activation enthalpy and activation entropy results in an inversion of the stereochemical preference for 2-butanol at around 300 K, the "racemic temperature". We found that a similar temperature dependence is seen in the reaction in the presence of the cofactor analogs, thionicotinamide adenine dinucleotide phosphate (SNADP) and acetyl adenine dinucleotide phosphate (APADP) [7], although the (*R*)-alcohol is more preferred, due to a reduction in $\Delta\Delta H^{\ddagger}$. More recently, we found that the S39T mutant SADH has higher (*R*)-selectivity, mainly due to a decrease in $\Delta\Delta H^{\ddagger}$, with less effect on $\Delta\Delta S^{\ddagger}$ [8]. Based on modeling of substrate into the crystal structure of the highly homologous TBADH, we prepared C295A mutant SADH, and we found that the mutation affected the stereoselectivity of reduction of some ketones, as expected if Cys-295 forms part of the small alkyl group binding pocket [9]. Surprisingly, C295A SADH shows very large decreases in $\Delta\Delta S^{\ddagger}$ compared to wild-type SADH (Table 1), in addition to the expected decreases in $\Delta\Delta H^{\ddagger}$ [10]. The decrease in $\Delta\Delta H^{\ddagger}$ for 2-hexanol seen in C295A SADH shows that the small binding pocket in the mutant can easily accommodate a larger alkyl group, up to *n*-butyl, than wild-type SADH. Thus, $\Delta\Delta H^{\ddagger}$ can be readily explained on the basis of steric interaction and van der Waals

Table 1
Activation parameters for wild-type and mutant SADH[a]

Substrate	Wild-type SADH		S39T SADH		C295A SADH	
	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/° mol)	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/° mol)	$\Delta\Delta H^{\ddagger}$ (kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/° mol)
2-Butanol	8.2	27.6	1.9	10.9	−0.6	−1.2
2-Pentanol	2.8	7.3	0.6	2.9	0.7	2.4
2-Hexanol	5.8	11.4	–	–	0.3	−0.5

[a] Data from [10].

contacts of the enantiomeric substrates in the active site.

Activation entropy is a complex term which can contain contributions from restricted motion of the substrate and enzyme side chains in the transition state, as well as contributions from desolvation of substrate and enzyme. In the comparison of rates of reaction of enantiomers, the desolvation of the free substrate and free enzyme cancels, leaving only the desolvation and the freedom of motion differences between the enzyme and enantiomeric substrate complexes to contribute to $\Delta\Delta S^{\ddagger}$. The separation of these entropic contributions is impossible to achieve in the absence of additional information. However, the comparison of $\Delta\Delta S^{\ddagger}$ of the wild-type and C295A mutant SADH in Table 1 reduces the possible entropy 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. 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. Indeed, examination of the crystal structure of TBADH [11] shows just such an ordered water molecule, HOH5, in the small pocket, located at a distance of 4.1 Å from the sulfur atom of Cys-295 and 3.5 Å from the β-carbon of Ile-86 (Fig. 1). This water is also present in the structure of a related mesophilic secondary alcohol dehydrogenase from *Clostridium beijerinckii* [11]. Release of this structured water from the active site would be effected by binding of (*R*)-alcohols, which place a large alkyl substituent in the small binding pocket, but not by the binding of (*S*)-alcohols, which place a methyl group in the small pocket. Thus, the stereospecificity of SADH may be at least partly determined by the selective expulsion of this bound water from the small alkyl binding pocket upon binding of large substituents in the small alkyl pocket. The data obtained with wild-type, S39T and C295A SADH with NADP, SNADP and APADP show a reasonable enthalpy–entropy compensation relationship (Fig. 2). The presence of an enthalpy–entropy compensation relationship in a reaction has been interpreted as evidence for a contribution from solvation [12,13]. However, it is difficult to determine if such relationships are physically meaningful or are statistical artifacts [14], especially when the range of temperature measurements is limited, as is the case with enzymatic reactions.

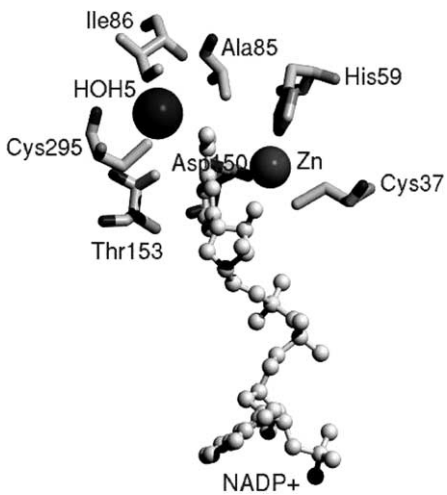

Fig. 1. Active site of alcohol dehydrogenase from *T. brockii*, showing the position of the bound water in the small alkyl group binding pocket. Coordinates from 1YKF in the Protein Data Bank.

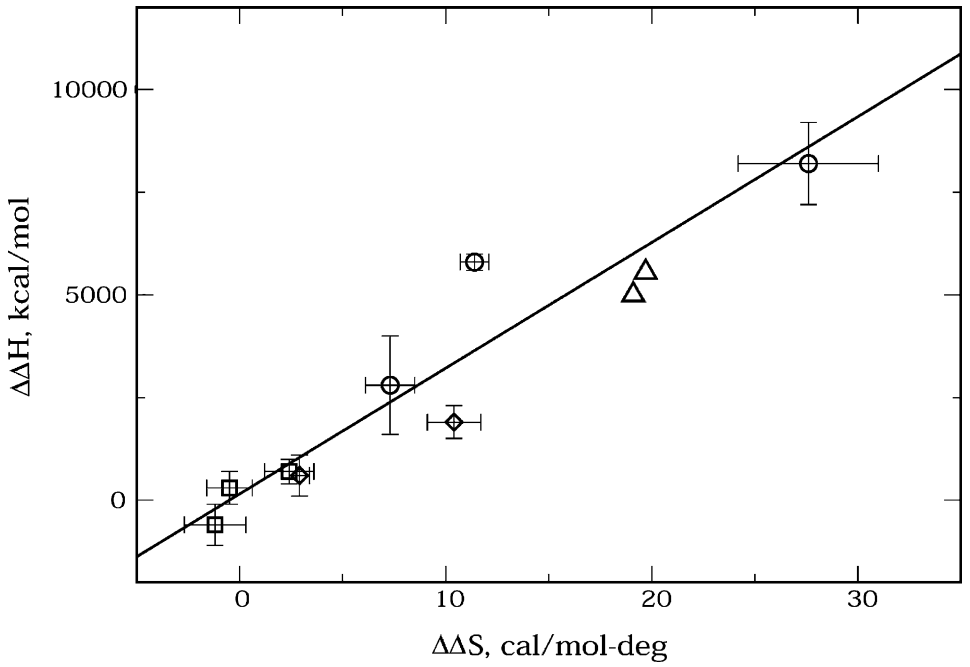

Fig. 2. Enthalpy–entropy correlation plot of data from wild-type, S39T and C295A SADH. (○) Wild-type SADH with 2-butanol, 2-pentanol and 2-hexanol; (◇) S39T SADH with 2-butanol and 2-pentanol; (□) C295A SADH with 2-butanol, 2-pentanol and 2-hexanol; (△) wild-type SADH with 2-butanol and SNADP or APADP.

The effect of water activity on the stereoselectivity of ketone reduction by TBADH has been studied by Jönsson et al. [15]. The enzymatic reduction reaction is extremely slow in hexane in the absence of water, primarily due to very unfavorable activation entropy. The decreased flexibility of the enzyme in anhydrous organic solvents may be responsible for the large negative activation entropy. As the water activity increases from 0.53 to 0.97, the rate increases 16-fold. The authors measured the E value for 2-pentanol obtained from 2-pentanone reduction at various water activities, and it was found that the enantioselectivity decreases significantly at water activity of 0.53. However, $\Delta\Delta H^{\ddagger}$ decreases at low water activity, while $\Delta\Delta S^{\ddagger}$ is relatively constant in this range of water activity. Hence, the main contribution to the change in E with water activity in organic solvents appears to be enthalpic in origin.

Do changes in active site solvation similar to those discussed above affect the stereochemistry of lipase reactions? The dependence of stereoselectivity on reaction temperature has also been observed in lipase reactions. Sakai et al. found that the optimal temperature for preparative lipase-catalysed resolution of some alcohols is −40 °C [16,17]. The temperature dependence of E for these reactions shows a linear Arrhenius-type plot down to −40 °C, indicating a significant entropic contribution to stereospecificity. However, these data do not give a significant enthalpy–entropy correlation relationship.

Overbeeke et al. examined the effects of temperature on a number of lipase-catalysed resolutions in water and hexane, and they found that entropy is as important as enthalpy in the stereospecificity of the reactions [18]. A reasonable enthalpy–entropy correlation relationship could be made for a wide range of substrates. However, the correlation was concluded to be a statistical artifact, due to the limited practical range of measurement of temperature and E values. However, a valid enthalpy–entropy correlation relationship, which is consistent with differential desolvation, was observed for only a small series of homologous substrates.

In another study, Overbeeke et al. investigated the effect of binary mixtures of solvents on lipase enantiospecificity at various temperatures [19]. In some cases, an unexpected bell-shaped relationship between E and % solvent was observed. Furthermore, the enthalpy–entropy compensation plots give non-linear, "hairpin" plots. This can be explained as the result of a solvent-dependent equilibrium between two different states of the enzyme, with different enantiospecificity. Thus, desolvation of the active site did not appear to play a major role in enantiospecificity for these reactions in binary solvent mixtures.

3. Conclusion

Entropy plays a significant role in the stereoselectivity (or stereospecificity) of enzymatic reactions. Our results suggest that selective release of bound solvent from the active site upon binding of enantiomeric substrates may contribute to the differential activation entropy, $\Delta\Delta S^{\ddagger}$.

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