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Enantiomeric Resolution of 2-Aryl Propionic Esters with Hyperthermophilic and Mesophilic Esterases: Contrasting Thermodynamic Mechanisms

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Biocatalytic resolution of chiral molecules has received considerable attention in recent years because of the significant demand for optically pure compounds.[1−3] Along these lines, efforts have been made to alter enzyme specificity for enantiomeric resolution through various approaches, including reaction environment modification and protein engineering.[4−7] Thus far, however, only the use of organic solvents to enhance enzyme enantioselectivity has proven especially useful, although in most cases the general mechanism of action remains unclear.[8] Despite this fact, several important features governing enzyme selectivity have been determined, among which is the importance of catalyst structural rigidity.[8,9] Enzyme rigidity has been implicated in controlling accessibility and positioning of substrates within the active site. As enzymes become more flexible, arising from changes in their reaction environment, these conformational restraints are relaxed and a concomitant loss of enantioselectivity may be observed.[10]

Biocatalysts from hyperthermophilic sources seem to be inherently more rigid at suboptimal temperatures, a characteristic related to their need to function at elevated temperatures.[11,12] As such, these enzymes may represent viable alternatives to their mesophilic counterparts for enzymatic chiral resolutions. Currently, little is known about the potential of hyperthermophilic enzymes for this purpose, with one notable exception being thermostable secondary alcohol dehydrogenases.[13−16] Few reports exist detailing what, if any, differences exist between the mechanisms by which thermophilic and mesophilic enzymes interact with chiral molecules. To further explore these issues, a carboxylesterase from the extreme thermoacidophile *Sulfolobus solfataricus* P1 (Sso EST1)[17] was compared to mesophilic esterases for the resolution of (*R*,*S*)-Naproxen methyl ester[18−21] (Scheme 1).

An enzyme's resolving power for two competing enantiomers is described by the enantiomeric ratio (E),[22] which is usually temperature dependent.[23] This ratio can be related to differences in the free energy of activation ($\Delta\Delta G^{\ddagger}$) of competing enantiomers and subsequently connected to the differences in activation enthalpy ($\Delta\Delta H^{\ddagger}$) and entropy ($\Delta\Delta S^{\ddagger}$).[16] In the absence of enantiomeric discrimination, $\Delta\Delta G^{\ddagger} = 0$, and the racemic temperature, T_r, for a given system can be defined: $\Delta\Delta G^{\ddagger} = 0$, $\Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger} = T_r$,). System temperatures above T_r (entropically controlled) result in an increase of E with temperature, while temperatures below T_r (enthalpically controlled) result in a decrease of E with increasing temperature.[24] Table 1 lists $\Delta\Delta H^{\ddagger}$, $\Delta\Delta S^{\ddagger}$, and T_r for the chiral resolution of a racemic Naproxen methyl ester mixture, as catalyzed by Sso EST1, a lipase from *Candida rugosa* (CRL),[18] and a lipase from *Rhizomucor miehei* (Palatase®),[25] as determined from the corresponding ln E versus $1/T$ plots shown in Figure 1.

Table 1 indicates that the separation of Naproxen enantiomers by the hyperthermophilic and mesophilic enzymes is driven by

Scheme 1. Esterase-Catalyzed Hydrolysis of (*R*,*S*)-Naproxen Methyl Ester

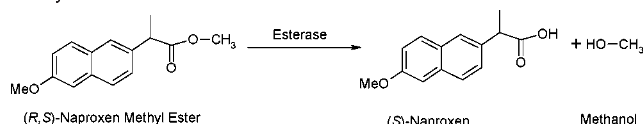

Table 1. $\Delta\Delta H^{\ddagger}$ (kcal/mol), $\Delta\Delta S^{\ddagger}$ (cal/(mol·K)), and T_r (°C) for the Enzyme-Catalyzed Hydrolysis of Racemic Naproxen Methyl Ester[a]

enzyme	selectivity	$\Delta\Delta H^{\ddagger}$	$\Delta\Delta S^{\ddagger}$	T_r
Sso EST1	S (T < T$_r$)	−19.5 ± 0.74	−54.0 ± 2.2	88.1 ± 6.9
CRL	S (T > T_r)	6.8 ± 0.76	30.0 ± 2.4	−46.3 ± 8.9
Palatase	R (T > T_r)	3.7 ± 0.72	13.5 ± 2.4	1.1 ± 0.4

[a] Thermodynamic values were calculated by plotting the ln E versus $1/T$[35,36] (Figure 1) and represent the differences in the fast versus slow reacting enantiomer.

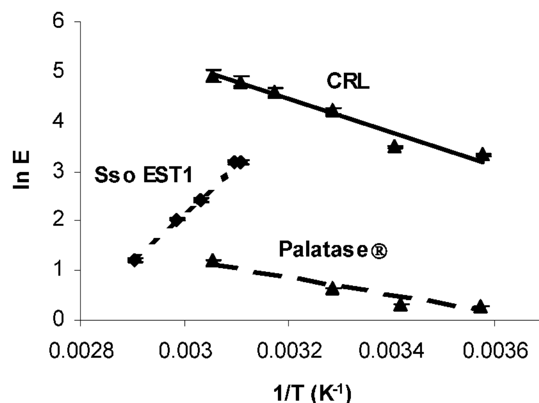

Figure 1. ln E versus $1/T$ (K^{-1}) for the enzymatic hydrolysis of Naproxen methyl ester by Sso EST1, CRL, and Palatase®. $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.[37]

contrasting thermodynamic features.[26] Sso EST1-catalyzed hydrolysis is predominantly enthalpically controlled. This indicates that the (*S*)-methyl ester is more tightly bound to the enzyme in the transition state, presumably due to favorable van der Waals interactions and hydrogen bonding between the (*S*) enantiomer and the amino acid residues lining the substrate binding pocket.[24,27,28] CRL- and Palatase®-catalyzed hydrolyses are both predominantly under entropic control. This may arise from a number of factors associated with the preferred enantiomer,[27] such as favorable interactions with the solvent and an increase in conformational entropy of the ligand.[29] The predominant entropic contribution, however, is likely connected to an increase in rotational motion of the preferred substrate in relation to the residues within the enzyme's substrate binding pocket.[24]

The contrast in controlling thermodynamic features between the hyperthermophilic and mesophilic enzymes is not likely the result

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of environmental factors, as the reaction conditions used between the experiments were identical. The significant differences relate to the thermostability of these enzymes, as both CRL and Palatase® have optimal temperatures of activity of approximately 40 °C,[30,31] while Sso EST1 has been reported as being extremely thermostable with an optimal temperature of greater than 95 °C.[17] Consequently, these enzymes, over the temperature range investigated, will be in different conformational states.[32−34] Sso EST1, at temperatures well below 70 °C, will be in a conformationally rigid state, thus mitigating the possibility of an increase in rotational motion of one enantiomer in the substrate binding pocket. Therefore, at these temperatures, the reaction proceeds under enthlapic control and is based more on steric and electrostatic interactions between the preferred enantiomer and the enzyme. Both CRL and Palatase® approach and surpass their thermal optima over the temperature range investigated and, thus, their structural states have an increased level of plasticity. Therefore, differences in entropic interactions with the preferred enantiomer are more likely and, as a result, govern selectivity.

In summary, the thermodynamic strategy by which enzymes discriminate between enantiomers is based on either enthalpic or entropic interactions, or a combination of both. Changes in an enzyme's conformational flexibility with temperature, at least in this case, play a major role in the thermodynamic features governing substrate selectivity.

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(21) Racemic Naproxen methyl ester was synthesized according to existing techniques.[30,38] Essentially, 30 g of (*R*,*S*)-Naproxen was added to 100 mL of methanol in a round-bottomed flask. Concentrated sulfuric acid, which was used as a catalyst, was then added and the mixture was brought to reflux for 5 h. The residual methanol was then removed via vacuum evaporation and the remaining material was then washed three times with both 1 M $NaHCO_3$ and pico-pure water to remove any unreacted acid, methanol, or catalyst. The material, (*R*,*S*)-Naproxen methyl ester, was then dried and used for subsequent enzymatic reactions.
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(26) Enzymatic reactions were carried out in 1.0 mL reaction volumes with 0.235 mg of Sso EST1 and 45 mg of CRL, or 24 mg of Palatase and 25 mg of racemic Naproxen methyl ester, suspended in 0.2 M sodium phosphate buffer pH 7.9 and energetically stirred for a 16 h.[39] The reaction material was then centrifuged for 15 min at $16000 \times g$ and supernatant removed. The remaining pellet was resuspended in 1.0 mL of 0.2 M sodium phosphate buffer at pH 7.9 and the suspension was centrifuged at $16000 \times g$ for 15 min. The supernatant was then combined with the previous supernatant, sterile filtered with a 0.45 mm syringe filter (Acrodisc GHP, Pall Gelman Laboratory, Ann Arbor, MI), and acidified with concentrated HCl to precipitate the acid. The resulting *R*,*S*-Naproxen acid was then isolated by centrifugation ($16000 \times g$, 15 min) and analyzed by HPLC. The functional temperature range used for the experiments was 48.5−70 and 4−55 °C for Sso EST1 and CRL and Palatase, respectively. Temperatures greater than higher 70 °C could not be evaluated because of substrate lability. In addition, both CRL and Palatase were not evaluated at temperatures greater than 55 °C because of poor enzyme stability.
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(37) The extent of conversion (c), percent enantiomeric excess of product (ee_p %), and the enantiomeric ratio (E) were calculated according to established methods.[22] The concentration of (*R*) and (*S*) Naproxen was determined by HPLC (Waters Breeze System, Franklin, MA), using the (*S*,*S*)-Whelk-O-1 chiral column derived from 1-(3,5-dinitrobenamido)tetrahydrophenanthrene covalently bound to 5 μm of silica (Regis Technologies, Morton Grove, IL). The mobile phase used was a mixture of 80% methanol/20% water/0.1% acetic acid (v/v) at a flow rate of 1 mL/min with 254 nm being used as the wavelength of detection. Retention times for (*R*) and (*S*) Naproxen were 4.1 and 4.9 min, respectively.
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