Articles

Tailoring Lipase Specificity by Solvent and Substrate Chemistries

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An acyl binding structural model has been developed to explain the observed catalytic efficiencies and enantioselectivities of *Candida rugosa* lipase-catalyzed (trans)esterification reactions involving 2-hydroxy acids and vinyl esters, respectively, and acylation reactions involving both cyclic and acyclic alcohols. A clear minimum was observed for (trans)esterification of six-carbon acyl moieties. Morever, the stereoselectivity of 2-hydroxy acid esterification in a number of hydrophilic and hydrophobic solvents was dependent on the acyl chain length: *S*-isomers of 2-hydroxy acids were acylated for acyl chain lengths of six or fewer, whereas the *R*-isomers were preferentially esterified for acyl chain lengths of eight or more. These results suggest that CRL contains both large and small acyl binding regions or pockets with high catalysis observed for proper fitting substrates into either pocket. CRL is also highly selective and reactive on secondary cyclic alcohols. In particular, the *R* isomers of menthol and *sec*-phenethanol are acylated efficiently by straight-chain vinyl esters. The catalytic efficiency of acylation (i.e., V_{max}/K_m for the secondary alcohol) is strongly dependent on the acyl chain length. Once again, a clear minimum is observed with vinyl caproate (C_6) as acyl donor. This phenomenon may reflect the greater degree of steric hinderance in the acyl enzyme intermediate caused by the caproate group. A mechanistic and thermodynamic rationale was proposed for the effects of solvent and substrate chemistries on CRL catalysis in organic solvents.

Introduction

Lipases catalyze a wide array of reactions in aqueous and organic media.[1,2] However, only in the latter are the scope of reactions particularly diverse and include (trans)-esterifications,[2,3] lactonizations,[4] peptide synthesis,[5] polymerization,[6] and coupled reactions with chemical synthesis (e.g., chemoenzymatic synthesis).[7] The most important theme that unifies these synthetic strategies is the high stereoselectivity inherent in lipase catalysis. The general utility of lipases in organic synthesis involving nonaqueous media, however, is limited by the high variability of catalysis and specificity with respect to solvent and substrate chemistries. For example, the solvent in which a lipase is placed can dramatically affect substrate and enantioselectivity.[8] Moreover, because each synthetic reaction involves two substrates, the alcohol (or other nucleophile) and an acyl donor (either an acid or an ester), substrate specificity may be expected to be a function of the structure and chemistry of both substrates as both bind in close proximity in the enzyme's active site.

Although X-ray crystallographic structural information of lipases is becoming more available,[9] the development of empirical, yet functional, active-site models remains the most common approach used to describe lipase

* To whom correspondence should be addressed.

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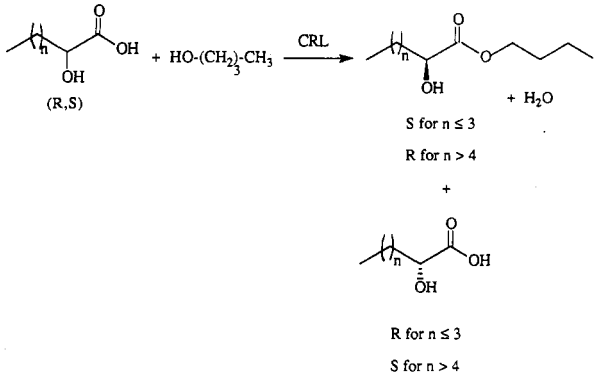
Scheme I. Esterification of 2-Hydroxy Acids



catalysis and specificity.[3a,10] These models typically are used to describe solvent and substrate effects on enantioselectivity for either the alcohol moiety[3a,11] or acyl group.[12] To date, no model adequately predicts the effects of solvent *coupled* with substrate structure on lipase catalysis and specificity.[13]

We were, therefore, motivated to study the combined effects of solvent and substrate on lipase catalysis in nonaqueous media and develop a model that incorporates the salient features of such a study. The lipase from *Candida rugosa* (CRL, formerly known as *C. cylindracea*) was used to catalyze esterification and transesterification reactions involving 2-hydoxy acids and vinyl esters, respectively, and acylation reactions involving both cyclic and acyclic alcohols. It is shown that the solvent hydrophobicity and acyl group chain length control both acyl and alcohol enantioselectivities and catalytic efficiencies.

Results and Discussion

In our previous study, we showed that the structure of the acyl moiety and the hydrophobicity of the organic solvent control CRL catalysis.[8] Straight-chain 2-hydroxy acids and hydrophobic solvents promote high enantioselectivity in esterification reactions with primary alcohols. Conversely, bulky acyl moieties and hydrophilic solvents lower both the catalytic efficiency (e.g., V_{max}/K_m) and enantioselectivity of CRL-catalyzed esterification of 2-hydroxy acids. Intrigued by these results, we examined in the present work the esterification of a series of 2-hydroxy acids (ranging from C_3 to C_{10}) with *n*-butanol catalyzed by CRL in several organic solvents ranging from hydrophobic cyclohexane to hydrophilic diethylether (Scheme I). As expected, CRL catalysis is optimal in hydrophobic solvents. Furthermore, in all solvents tested, the V_{max}/K_m is minimum for 2-hydroxycaproic acid (C_6) and is relatively high for lactic acid, 2-hydroxybutyric acid (C_4), and 2-hydroxycaprylic acid (C_8) (Figure 1). Nearly identical results were obtained when *n*-butanol is replaced with *n*-octanol suggesting that this effect is dependent on the acyl chain length and solvent, only. Functionally similar observations were made by Holmberg et al. for the

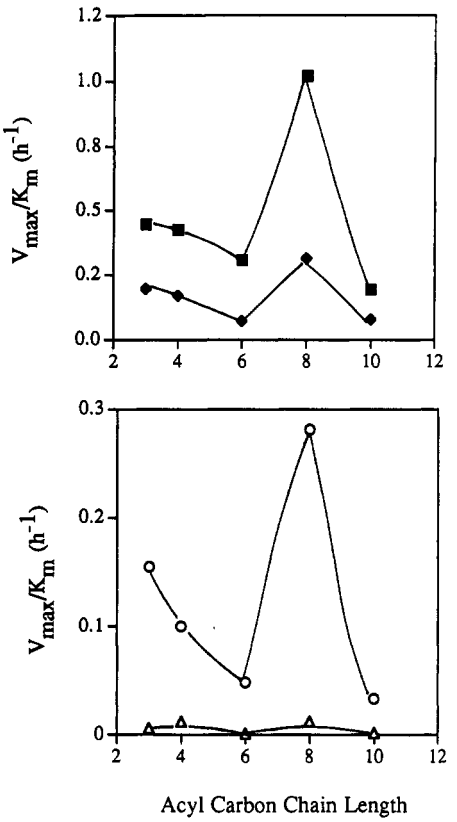


Figure 1. Dependence of the V_{max}/K_m of CRL on the combined effects of 2-hydroxy acid acyl chain length and the nature of the organic solvent: ■, cyclohexane; ◆, toluene; ○, dipropyl ether; △, diethyl ether. Conditions: 50 mg/mL of CRL, 5–25 mM 2-hydroxy acid, 60 mM *n*-butanol, shaken at 250 rpm at 25 °C.

esterification of straight-chain carboxylic acids with primary alcohols in organic solvents.[14]

Development of Acyl Binding Model. A plausible explanation for the acyl dependence depicted in Figure 1 is the existence of two acyl binding "pockets" or regions in CRL's active site: one small in which 2-hydroxy acids with carbon chain lengths less than six bind well and one large in which 2-hydroxy acids with carbon chain lengths greater than six bind well. This hypothesis can be tested in two different ways. First, it is expected that this phenomenon depends on the chain length of acyl moiety and is independent of the type of reaction catalyzed. Indeed, (trans)esterifications of vinyl esters of different acyl chain lengths with *n*-butanol in various hydrophobic solvents show strikingly similar results as those obtained with 2-hydroxy acid esterifications (e.g., (trans)esterification of *n*-butanol with a straight-chain C_6 substrate shows a clear minimum) (Figure 2).[15] Second, the existence of two binding regions must result in two different orientations of 2-hydroxy acids in CRL's active site that can result in favorable catalysis. These orientations must be nonequivalent and provide for a different 2-hydroxy acid optical isomer that is reactive. In fact, this enantiose-

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(15) It should be noted that $(V_{max}/K_m)_{acyl}$ represents the rate of interaction of the free enzyme with the free ester or acid substrate.[16] In lipase catalysis, therefore, it depends on the acylation step and is independent of the reaction of the acyl enzyme with alcohols. Thus, V_{max}/K_m values for 2-hydroxy acid esterification and vinyl ester transesterification measure acylation steps in both reactions.

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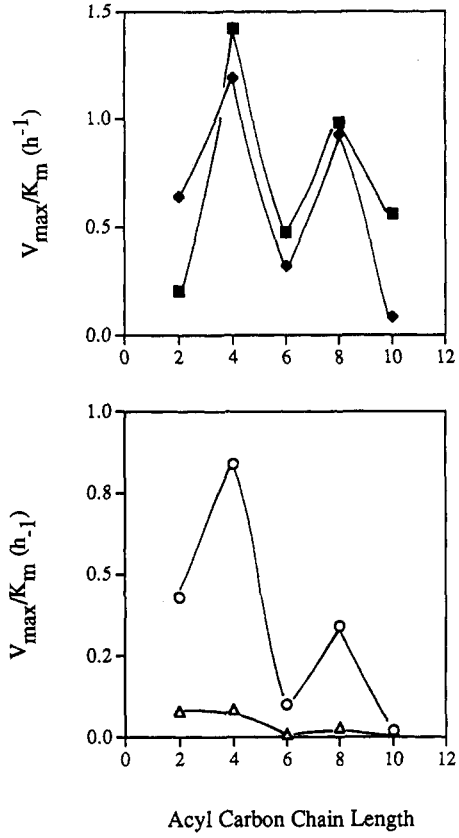

Figure 2. Dependence of the V_{max}/K_m of CRL on the combined effects of vinyl ester acyl chain length and the nature of the organic solvents: ■, isooctane; ◆, cyclohexane; ○, dipropyl ether; △, diethyl ether. Conditions: 50 mg/mL of CRL, 5–25 mM vinyl ester, 60 mM *n*-butanol, shaken at 250 rpm at 25 °C.

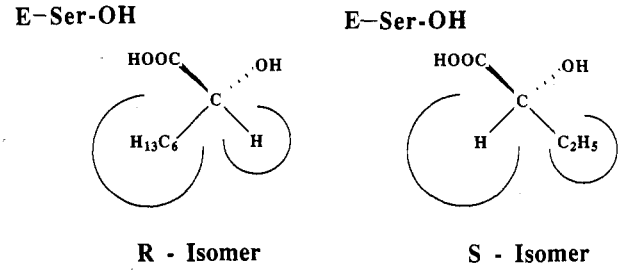

Figure 3. Proposed acyl binding model for CRL. The (*E*)-Ser-OH represents the active-site nucleophile that reacts with the free acid moiety to form the acyl enzyme intermediate.

lectivity "switch" is vividly observed for 2-hydroxycaprylic and 2-hydroxycapric (C_{10}) acids when compared to their smaller counterparts: the *S*-isomer is favored for chain lengths of C_3 to C_6, while the *R*-isomer is favored for chain lengths of C_8 and C_{10}. The high degree of enantiomeric excess for all straight-chain 2-hydroxy acids (all >90% in toluene, data not shown) suggests that the enantioselective "switch" is highly efficient. These data support a simple and empirical active-site model that represents the approximate size and shape of the acyl-binding region of CRL (Figure 3). The large acyl binding region is shown at the left side of the schematic with the serine hydroxyl in the center. The acyl moiety must be properly placed to undergo nucleophilic attack by the active-site serine hydroxyl to form the characteristic acyl enzyme intermediate. For large (>C_6) 2-hydroxy acids, the most favorable binding conformation has the 2-hydroxyl moiety facing the opposite direction as that for smaller acid chain lengths. The reactivity of a 2-hydroxy acid properly

Table I. Solvent Effect on CRL's Substrate Specificity

		obsd specificities		intrinsic specificities	
solvent	log P^a	ratio of C_8/C_6[b]	ratio of C_8/C_4[c]	ratio of C_8/C_6[d]	ratio of C_8/C_4[d]
cyclohexane	3.2	3.3	2.4	6.2	9.6
toluene	2.5	4.4	1.9	7.5	9.0
dipropyl ether	1.9	5.9	2.8	9.8	8.8
diethyl ether	0.85	12.0	1.0	21.3	3.6

[a] Defined as the logarithm of the partition coefficient of the solvent between 1-octanol and water (Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81). [b] Defined as the ratios of V_{max}/K_m for 2-hydroxycaprylic acid to 2-hydroxycaproic acid. [c] Defined as the ratios of V_{max}/K_m for 2-hydroxycaprylic acid to 2-hydroxybutyric acid. [d] Calculated from $\Delta\Delta G_{ES}^{\dagger} = -RT \ln [(V_{max}/K_m)_{substrate1}/(V_{max}/K_m)_{substrate2}]$.

positioned in the active site of the lipase results in greater transition-state stabilization and, thereby, higher values of V_{max}/K_m. The size of the acyl binding pockets can be estimated from the observed catalytic efficiencies of CRL catalysis. The smaller pocket is optimal for 2-hydroxybutyric acid and must have a volume equivalent to that occupied by a C_2H_5 alkyl chain. The larger pocket is optimal for 2-hydroxycaprylic acid and must have a volume equivalent to a C_6H_{13} alkyl chain. Hence, the volumes of the acyl binding pockets are estimated to be 39.8 and 104.5 Å^3, respectively, as determined by using the Sybyl molecular graphics package.

Solvent Effects on the Observed vs Intrinsic Acyl Substrate Specificities. The model depicted in Figure 3 can be used to help elucidate the combined effects of substrate and solvent properties on lipase catalysis. The values of V_{max}/K_m for CRL catalysis is greatest in hydrophobic solvents, and this is consistent for all substrates tested as shown in Figures 1 and 2. Furthermore, the solvent affects the observed substrate specificity of CRL catalysis. This is depicted in Table I for comparison between 2-hydroxycaprylic and 2-hydroxycaproic acids. In cyclohexane, the V_{max}/K_m of 2-hydroxycaprylic acid is 3.3-fold higher than for 2-hydroxycaproic acid. This ratio increases to 12 in diethyl ether. The major difference between these substrates is that the 2-hydroxycaprylic acid is postulated to bind well to the enzyme, whereas the 2-hydroxycaproic acid binds poorly to the enzyme.[17] It is important to note that little observed solvent dependence exists between 2-hydroxycaprylic and 2-hydroxybutyric acids, both of which are postulated to bind well to CRL (Table I), albeit to opposite binding pockets. These results suggest that 2-hydroxy acids which fit well in the active site of CRL are more reactive in hydrophilic solvents than 2-hydroxy acids which do not fit well.

The data presented in Figures 1 and 2 provide us with the observed acyl substrate specificity of CRL. However, such information does not accurately reflect the intrinsic solvent effect on the transition state of the enzymic reaction due to differences in the ground states of acyl substrates in different organic solvents. Such an intrinsic substrate

(17) Poor solubilities of 2-hydroxy acids in the organic solvents employed did not allow us to reach saturation kinetics. Hence, only values of V_{max}/K_m could be determined, and the use of the term "binding" is in the context of being proportional to transition-state stabilization of the hydroxy acid substrate in CRL's active site. In fact, and as a representative example, this was shown to be a good assumption for the transesterification of vinyl caproate and vinyl butyrate with *n*-butanol in dipropyl ether. The high solubilities of these esters in this solvent enabled us to reach saturation kinetics and obtain values of K_m for the C_6 and C_4 vinyl esters to be 69 ± 15 and 27 ± 8 mM, respectively. Thus, the C_4 acyl group does indeed bind better to CRL than the C_6 acyl group, consistent with the higher transition-state stabilization of vinyl butyrate than vinyl caproate.

Table II. Calculated Values for 2-Hydroxy Acid Activity Coefficients in Several Organic Solvents[a]

	substrate				
solvent	C_3	C_4	C_6	C_8	C_{10}
cyclohexane	547	343	160	85	49
toluene	102	67	34	20	13
dipropyl ether	39	26	14	8.3	5.4
diethyl ether	21	13	6.7	3.8	2.4

[a] Calculated via the UNIFAC model (Smith, J. M.; Van Ness, H. C. *Introduction to Chemical Engineering Thermodynamics*, 4th ed.; McGraw-Hill: New York, 1987; pp 676–683).

specificity is fundamentally important as it represents the effects of solvent and substrate chemistries on the transition states of acyl enzyme intermediates independent of solvent effects on substrate ground states.[18] Hence, intrinsic substrate specificities represent the true measure of an enzyme's ability to stabilize a transition state of one substrate in comparison to another. At this point, therefore, it is instructive to pose the following question: How does the organic solvent affect the intrinsic transition-state stabilization of CRL?

In a given solvent, the observed substrate specificity of enzymatic catalysis is represented by the ratios of V_{max}/K_m for the different substrates as described in eq 1 (for comparison between two substrates) where $\Delta\Delta G_t^{\dagger}$ is the difference in the activation energies of the reaction (proportional to values of V_{max}/K_m). Differences between the observed and intrinsic substrate specificities arise from difference in the ground states of substrates in various organic solvents which can be calculated from the activity coefficients of the acyl substrates in different organic media.[19] For example, the differential ground-state free energies ($\Delta\Delta G_S$) between two substrates is depicted in eq 2.

$$\Delta\Delta G_{T}^{\dagger}{}_{(substrate1-substrate2)} = -RT \ln [(V_{max}/K_m)_{substrate1}/(V_{max}/K_m)_{substrate2}] \quad (1)$$

$$\Delta\Delta G_{S(substrate1-substrate2)} = -RT \ln [\gamma_{substrate2}/\gamma_{substrate1}] \quad (2)$$

Table II summarizes the activity coefficients of the 2-hydroxy acids studied in this work in several organic solvents. In all cases, the activity coefficients decrease as the solvent becomes more hydrophilic, indicative of the increased solubility of the hydroxy acids in these solvents. The effect of solvent on activity coefficient is significant. For example, the activity coefficients uniformly decrease by ca. 25-fold upon decreasing the hydrophobicity of the solvent (as calculated from log P values) over 100-fold. Furthermore, in any given solvent, the activity coefficients are lower for larger, more hydrophobic 2-hydroxy acids, once again, consistent with the increased solubilities of these 2-hydroxyacids in organic solvents.

The intrinsic transition-state stabilization ($\Delta\Delta G_{ES}^{\dagger}$) is represented by the sum of the activation energies of catalytic efficiencies ($\Delta\Delta G_T^{\dagger}$) and ground states ($\Delta\Delta G_S$). This is mathematically addressed in eq 3.

$$(\Delta\Delta G_{ES}^{\dagger})_{substrate1-substrate2} = -RT \ln [(V_{max}/K_m)_{substrate1}/(V_{max}/K_m)_{substrate2}] - RT \ln [\gamma_{substrate2}/\gamma_{substrate1}] \quad (3)$$

The intrinsic effect of solvent hydrophobicity on the transition-state stabilization of CRL is shown in Figure 4 for all 2-hydroxy acids studied ranging from lactic to 2-hydroxycapric acids. The standard state is chosen to be 2-hydroxycaproic acid, and increased transition-state stabilization of a substrate compared to 2-hydroxycaproic acid is shown as a positive value in the figures. In cyclohexane, 2-hydroxycaproic acid is actually an intrinsically better substrate than either lactic or 2-hydroxybutyric acids (transition state stabilization of 0.51 and 0.26 kcal/mol, respectively). As the solvent becomes more hydrophilic, however, 2-hydroxycaproic acid becomes progressively poorer with respect to all 2-hydroxy acid substrates (up to 1.81 kcal/mol less transition state stabilized with respect to 2-hydroxycaprylic acid in diethyl ether), more in line with the observed substrate specificities (Figure 1). In all solvents tested, 2-hydroxycaprylic acid is the most favorable substrate intrinsically (compare Figures 1 and 4). A representative comparison between the intrinsic and observed substrate specificities of CRL catalysis is given in Table I. The intrinsic specificity ratio of 2-hydroxycaprylic acid to 2-hydroxycaproic acid increases (and, hence, the former becomes a better substrate relative to the latter as the solvent becomes more hydrophilic) in a manner similar to that for the observed specificity ratio. Conversely, 2-hydroxycaprylic acid becomes a progressively poorer substrate relative to 2-hydroxybutyric acid as the solvent becomes more hydrophilic. For example, the transition-state stabilization of 2-hydroxycaprylic acid relative to 2-hydroxybutyric acid drops from 1.34 to 0.75 kcal/mol in cyclohexane and diethyl ether, respectively.

These results are consistent with the hypothesis (schematically represented by Figure 3) that CRL prefers to bind acyl moieties of specific sizes and to orient them in a proper way for catalysis to ensue. The dependence of substrate specificity on solvent hydrophobicity reflects the solvent effect on the transition state of CRL catalysis. CRL is inherently less active in hydrophilic solvents as compared to hydrophobic ones, and this must be due to the direct effect of the solvent on enzyme structure. A plausible hypothesis that has been proposed to account for lower enzyme activity in more hydrophilic solvents is that the stripping of water from the enzyme in such solvents is more severe than in more hydrophobic solvents.[20] The loss of enzyme-bound water affects the structure and dynamics of the active-site[21] and results in poor values for V_{max}/K_m. For example, it has been hypothesized that active sites of enzymes become more rigid as the water content of the enzyme decreases.[22] On the basis of this hypothesis, one can envision a scenario in which the loss of water from the vicinity of CRL's active site in a hydrophilic solvent such as diethyl ether may affect the larger acyl binding pocket to a greater extent than the smaller pocket and result in improved interaction of the 2-hydroxybutyric acid relative to 2-hydroxycaprylic acid.

(18) The formation of the acyl enzyme is governed by the catalytic efficiency (or specificity) constant, V_{max}/K_m, which is proportional to k_2/K_s where k_2 is the rate constant of acylation.

(19) Activity coefficients are inversely proportional to the mole fraction of solute in the solvent as described by the following expression: $\gamma_i = a_i/x_i$, where a_i is the thermodynamic activity of solute i (Smith, J. M., Van Ness, H. C. *Chemical Engineering Thermodynamics*, 3rd ed.; McGraw-Hill: New York, 1975; p 266).

(20) This has been determined directly by measuring the loss of tritiated water bound to an enzyme in hydrophilic and hydrophobic organic media (Gorman, L. S.; Dordick, J. S. *Biotechnol. Bioeng.* **1992**, *39*, 392).

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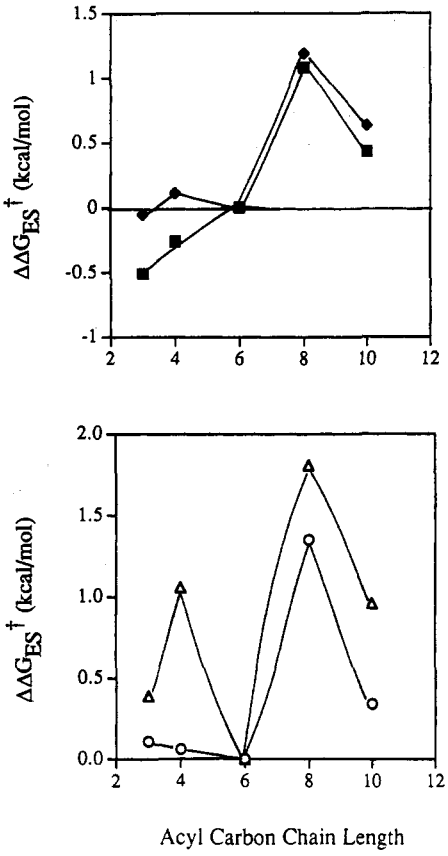

Figure 4. Solvent and acyl chain length effect on the intrinsic transition-state stabilization of 2-hydroxy acids. 2-Hydroxycaproic acid is the standard-state substrate and is given a zero value of $\Delta\Delta G_{ES}^{\dagger}$: ■, cyclohexane; ●, toluene; ○, dipropyl ether; △, diethyl ether.

This is consistent with the observation by Holmberg et al.[14] in aqueous solutions that the hydrolysis of butyric acid esters is optimal over a range of straight-chain fatty acid ester substrates from C_2 to C_{12}. In any event, the solvent effect on the transition state of CRL is expected to be exaggerated with 2-hydroxy acids that do not fit well into the active site of the enzyme.

Alcohol Selectivity. Alcohols are cosubstrates in (trans)esterification reactions and are, therefore, integral components of catalysis. As with the acyl substrate, the alcohol must bind to a specific region in the site of the lipase and in the proper orientation for catalysis to ensue. Thus, the nature of the organic solvent must affect the alcohol specificity of the enzyme for the alcohol. Likewise, the nature of the cosubstrate (in this case the acyl donor) must also affect catalysis. For example, the (trans)esterification reaction formally involves reaction of an alcohol with an acyl enzyme whose structure must affect the acyl transfer (deacylation) step. This is qualitatively depicted in Scheme II.

This hypothesis was tested initially by examining the values of V_{max}/K_m and enantiospecificity of CRL catalysis as a function of both the organic solvent and vinyl ester chain length. In isooctane, CRL catalyzes the facile transesterification of 2-butanol with vinyl butyrate and vinyl caprylate. However, no clear preference for the *R* or *S* isomer is observed, and *E* values are close to unity. Changing the solvent to the more hydrophilic acetonitrile dramatically lowers the V_{max}/K_m of CRL catalysis, yet again, no clear enantiomeric preference is observed. Similar results were obtained with 2-octanol. The results

Scheme II. CRL-Catalyzed Acylation of Secondary Alcohols

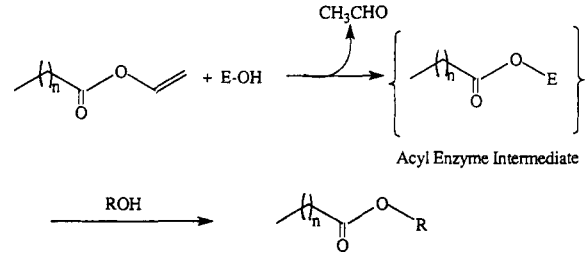

Table III. Values of V_{max}/K_m and Enantiospecificities for CRL-Catalyzed Transesterification of Vinyl Esters with Secondary Alcohols in Various Organic Solvents[a]

solvent	alcohol	ester[b]	*S* isomer[c]	*R* isomer[d]	*E*[e]
isooctane	2-butanol	C_4	720	890	1.25
		C_8	910	1157	1.27
	2-octanol	C_4	692	678	0.98
		C_8	708	590	0.84
toluene	2-butanol	C_4	510	680	1.33
		C_8	550	620	1.12
	2-octanol	C_4	290	370	1.28
		C_8	184	504	2.78
acetonitrile	2-butanol	C_4	1.44	1.32	0.92
		C_8	13	12	0.92
	2-octanol	C_4	0.52	0.72	1.38
		C_8	0.92	1.0	1.08

[a] Conditions: 50 mg/mL CRL, 5–25 mM *sec*-alcohol, 60 mM vinyl ester, shaken at 250 rpm at 25 °C. [b] Straight-chain acyl moieties. [c] $(V_{max}/K_m)_S \times 10^3\ h^{-1}$. [d] $(V_{max}/K_m)_R \times 10^3\ h^{-1}$. [e] Defined as the ratio of $(V_{max}/K_m)_R/(V_{max}/K_m)_S$.

are summarized in Table III. Apparently, little discrimination in the transition state of the deacylation reaction exists between *R* and *S* isomers of secondary alcohols. The solvent does, however, dominate the overall V_{max}/K_m of CRL, as previously discussed, such that in hydrophilic solvents, the enzyme is poorly active. Similar findings with acylic alcohols have been observed by Rabiller et al.[23]

As opposed to acylic alcohols, CRL is known to be highly reactive and enantioselective toward cyclic alcohols.[24] A well-established active-site structural model has been developed to account for alcohol specificity which is similar to that proposed to account for differences in acyl chain length specificity (Figure 3).[3a] The values of V_{max}/K_m of CRL catalysis for (*R*)-menthol are of the same order of magnitude as that obtained with (*R*)- or (*S*)-2-butanol and 2-octanol; however, the (*S*)-menthol is far less reactive (Table IV). CRL, therefore, cannot orient the (*S*)-menthol properly in its active site, and the enzyme shows high enantiospecificity toward menthol. Interestingly, a striking dependence on the acyl chain length is observed for the V_{max}/K_m of the (*R*)-menthol. Furthermore, in general, the greater the V_{max}/K_m of (*R*)-menthol, the greater the enantiospecificity (Table IV), consistent with a single isomer (*R*)-menthol) being the predominant reactive species. The acyl chain length dependence that is summarized in Table IV was nearly identical to that observed in the (trans)esterification reactions depicted in Figures 1 and 2 for interaction of the free enzyme with acyl substrate; a clear minimum in V_{max}/K_m for (*R*)-menthol transesterification is observed with vinyl caproate as acyl

(23) Rabiller, C. G.; Konigsberger, K.; Faber, K.; Griengl, H. *Tetrahedron* **1990**, *46*, 4231.
(24) (a) Klempier, N.; Faber, K.; Griengl, H. *Synthesis* **1989**, *3*, 933. (b) Koshino, S.; Sonomoto, K.; Tanaka, A.; Fukui, S. *J. Biotechnol.* **1985**, *2*, 47.

Table IV. Values of V_{max}/K_m and Enantiospecificity for CRL-Catalyzed Transesterification of Vinyl Esters with Menthol in Various Solvents[a]

solvent	ester[b]	S isomer[c]	R isomer[d]	E[e]
isooctane	C_2	3.02	156	52.63
	C_4	37.8	830	22.2
	C_6	38	440	11.4
	C_8	7.8	635	83.3
	C_{10}	4.69	325	71.4
cyclohexane	C_2	2.58	101	40.0
	C_4	19	560	29.4
	C_6	8.26	120	14.4
	C_8	6.5	488	75.3
	C_{10}	4.16	280	67.3
toluene	C_2	4.48	78	17.5
	C_4	48.0	319	6.67
	C_6	4.26	84	11.1
	C_8	16	285	17.8
	C_{10}	3.5	235	14.9
isopropyl ether	C_2	4.26	65	15.3
	C_4	24	178	7.41
	C_6	3.24	46	14.19
	C_8	21	230	10.98
	C_{10}	14.8	191	12.9

[a] Conditions: 50 mg/mL of CRL, 5–25 mM menthol, 60 mM vinyl ester, shaken at 250 rpm at 25 °C. [b] Straight-chain acyl moieties. [c] $(V_{max}/K_m)_S \times 10^3$ h^{-1}. [d] $(V_{max}/K_m)_R \times 10^3$ h^{-1}. [e] Defined as the ratio of $(V_{max}/K_m)_R/(V_{max}/K_m)_S$.

donor. For example, in cyclohexane the V_{max}/K_m values for the acylation of (*R*)-menthol are over 4-fold higher with vinyl butyrate and vinyl caprylate as acyl donors compared to vinyl caproate. Using the acyl binding model depicted in Figure 3, this phenomenon may simply reflect the greater degree of steric hinderance (and reduced transition-state stabilization) in the acyl enzyme intermediate caused by the caproate group. As opposed to the acyl enzymes of butyrate and caprylate whose alkyl side chains fit snugly into the small and large acyl pockets, respectively, the alkyl side chain of caproate does not fit well into either the small or large pocket and can interfere with the interaction of the resulting acyl enzyme with menthol. Hence, the V_{max}/K_m of (*R*)-menthol is affected by the acyl chain length of the vinyl ester cosubstrate in a manner proportional to the values of V_{max}/K_m for the interaction of *free* enzyme with vinyl ester substrate.[25] The incorrect isomer ((*S*)-menthol) must bind in a different orientation than that of the (*R*)-isomer and, therefore, is not greatly affected by the acyl chain length. In fact, the entire range of V_{max}/K_m values for (*S*)-menthol (ca. 2.6–48 h^{-1}) is much smaller than the range for (*R*)-menthol.

The most intriguing aspect of alcohol specificity is the reaction rate dependence on the acyl chain length. From a practical standpoint this phenomenon can be advantageously applied to tailor acylation efficiencies and enantioselectivities by changing solvent and substrate chemistries. Moreover, this effect is not specific to menthol. For example, acylation of the (*R*)-isomer of the unrelated *sec*-phenethanol in isocotane with vinyl butyrate, vinyl caproate, and vinyl caprylate resulted in values of V_{max}/K_m of 2.38, 0.35, and 1.35 h^{-1}, respectively. The (*S*)-isomers were consistently on the order of 5-fold less reactive. Once again, a clear minimum is observed for the C_6 ester donor, consistent with the hypothesis that a structurally contrained acyl enzyme intermediate is formed with this acyl chain length.

(25) It should be noted that acyl chain length dependence on alcohol enantioselectivity has been observed with CRL[2d] and *Mucor miehei* lipase for a variety of cyclic alcohols (Sonnet, P. E. *J. Org. Chem.* 1987, *52*, 3477).

Conclusions

The nature of the organic solvent and chain length of the acyl moiety control the V_{max}/K_m and enantiospecificity of CRL catalysis both for the acyl donor and acyl acceptor. A simple structural model describes the optimal chain length and srtereoisomer of the acyl moiety that promotes highly active and enantioselective reactions. The model also describes the effect of acyl chain length and stereoisomer on the values of V_{max}/K_m for cyclic alcohol transesterification. For example, transesterification of (*R*)-menthol with acyl donors is substantially more favorable than transesterification of (*S*)-menthol. The poorer the acyl donor, the lower the V_{max}/K_m of transesterification, presumably because of increased steric hindrance in the acyl enzyme intermediate. This steric hindrance is expected to be significantly greater if an unfavorable optical isomer of an acyl moiety were used in menthol acylation. This was further tested using (*R*)- and (*S*)-lactic acid, respectively, as acyl donors. The V_{max}/K_m of (*R*)-menthol esterification in dipropyl ether dropped from 0.02 h^{-1} to 0 upon switching from the highly favored (*S*)-lactic acid to the unfavorable (*R*)-isomer. It should be noted that esterification of menthol with a free acid (such as lactic acid) is inherently slower than transesterification with an activated vinyl ester. Hence, menthol acylation rates between lactic acid and a vinyl ester of a straight-chain acyl group are not directly comparable; however, the ratio of reactivity between the two lactic acid isomers and (*R*)-menthol shows that the expected poor fit of the (*R*)-lactic acid diminishes the V_{max}/K_m of menthol acylation. Interestingly, the (*S*)-menthol was completely inactive with either the (*R*)- or (*S*)-lactic acid; therefore, the use of lactic acid as an acyl donor provides for perfect enantiospecificity in menthol acylation and this may be of interest for stereospecific biotransformations.

Experimental Section

Materials and Methods. *C. rugosa* lipase (CRL) was purchased from Sigma Chemical Co. All substrates were obtained commerically from Aldrich (Milwaukee, WI) and Tokoyo Kasei (Portland, OR). All solvents were of the highest purity commercially available and were dried prior to use over 4-Å molecular sieves (Linde) to a water content of <0.01% (v/v) as determined by Karl-Fischer titration.

Enzyme Kinetics. Catalytic efficiencies for esterification/transesterification reactions with racemic 2-hydroxy acids and vinyl esters, respectively, were measured with 50 mg/mL of CRL suspended in 4 mL of an organic solvent containing 60 mM 1-butanol. The acyl moiety concentration was varied from 5 to 25 mM. The suspensions were magnetically stirred at 250 rpm at 25 °C. Periodically, aliquots were removed, and the initial rates of reaction were followed gas chromatographically (Hewlett-Packard 5890A model) with an HP-1 capillary column (25 m) consisting of 530-μm fused silica gum, N_2 carrier gas (30 mL/min), and injector and detector port temperatures of 250 °C. Values of V_{max}/K_m for secondary alcohols were determined using 50 mg/mL of CRL suspended in 4 mL of an organic solvent containing 60 mM of a vinyl ester and 5–25 mM of the secondary alcohol. In this case, individual optical isomers were employed. Initial rates were measured via gas chromatography as previously described. A nonlinear regression algorithm was used for determination of all V_{max}/K_m values.

Optical Purity Determinations. The optical purities of the butyl esters of 2-hydroxyl acids (from C_3 to C_6) were determined as described in our previous work.[8] For C_8 and C_{10} 2-hydroxy acids, semipreparative-scale syntheses of the butyl esters were commenced. To that end, 100 mg/mL of CRL was suspended in a solution of 0.1 M racemic 2-hydroxycaprylic or 2-hydroxycapric acids and 0.6 M 1-butanol in 0.2 L of toluene. The reaction

was allowed to proceed until 40% conversion of the acid was obtained. The reaction mixtures were terminated by filtering off the enzyme, and the liquid phases were washed three times with 0.3 M $NaHCO_3$ solution. The organic phases were dried and evaporated to yield the pure ester. Isolated amounts for 2-hydroxycaprylic and 2-hydroxycapric acids were 1.3 g (38% isolated yield based on racemic mixture) and 1.2 g (30% isolated yield based on racemic mixture), respectively. The optical rotations of the butyl esters of 2-hydroxycaprylic and 2-hydroxycapric acids were not available. Thus, the esters were chemically hydrolyzed (0.1 M KOH) to their corresponding acids. The optical rotation of the reacted *R* acids were compared with the literature values. For 2-hydroxycaprylic acid we observed $[\alpha]^{25}_D$ +8.3 (*c* 1, EtOH) (lit.[26] $[\alpha]^{25}_D$ *R* isomer +9.1) and for 2-hydroxycapric acid we observed $[\alpha]^{25}_D$ −1.4 (*c* 1, EtOH) (lit.[27] $[\alpha]^{25}_D$ *R* isomer −1.6). Calculation of acyl binding pocket volumes were performed using Sybyl (Tripos Associates, Inc.). Ethyl and hexyl radicals were energy minimized prior to calculation of the volumes. Grid spacings were set at 0.1 Å. The activity coefficients (γ) were determined using the UNIFAC method.[19]

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