# THE SOLVENT DEPENDENCE OF ENZYMATIC SELECTIVITY

by

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B.S., Chemistry
The University of Vermont, 1986

Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY
IN CHEMISTRY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June, 1996



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**ABSTRACT**

The exacting selectivity of enzymatic catalysis is the most valuable characteristic of enzymes to the synthetic chemist. Ironically, this strict specificity also limits the generality of enzymatic synthesis, because enzymes that catalyze the reaction of interest with the desired selectivity are not always available. Nonaqueous enzymology, and the discovery that enzymatic selectivity can be changed by the reaction medium, thus greatly enhances the utility of enzyme-catalyzed syntheses. To further exploit this solvent effect, we seek herein to mechanistically explain the dependence of enzymatic selectivity on the solvent.

The substrate specificity of the serine protease subtilisin Carlsberg in the transesterification reaction of N-Ac-L-Ser-OEt and N-Ac-L-Phe-OEt with propanol has been examined in 20 anhydrous solvents. The serine substrate is strongly favored in some solvents, while the phenylalanine substrate is greatly preferred in others. An equation has been derived on the basis of the thermodynamics of substrate desolvation, which correctly predicts the substrate specificity as a function of the solvent-to-water partition coefficients of the substrates and the substrate specificity of the enzyme-catalyzed hydrolysis of the esters in water. This model is herein demonstrated to be independent of the enzyme and the substrate, so long as the latter is removed from the solvent in the transition state.

Experimentally measured solvent-to-water partition coefficients are nonideal for use in the prediction of the solvent dependence of enzymatic selectivity for several reasons. First, partition coefficients cannot be readily measured for water-miscible solvents. Second, the mutual solubility of aqueous and organic phases influences the measured partition coefficients. Third, the effects of additives to the reaction medium, such as a second substrate, cannot be included. These problems have been overcome by calculating the partition coefficients from the substrate activity coefficients using UNIFAC.

For the case of substrate specificity, the differential free energy of desolvation for two substrates is primarily driven by chemical differences in the substrates. In cases of stereoselectivity (e.g. enantioselectivity, prochiral selectivity, and regioselectivity), however, chemically identical substrates lead to the formation of multiple products. For such identical substrates, desolvation energy differences arise from differences in the solvation of the substrates in the transition states which lead to the various products. Our model has been expanded to account for partial transition state desolvation, which is assessed using molecular modeling based on the crystal structure of the enzyme. Using this methodology, we are able to quantitatively predict the solvent dependence of the enantioselectivity of cross-linked chymotrypsin crystals in the resolution of racemic methyl 2-hydroxy-3-phenylpropionate.

Thesis Supervisor: Dr. Alexander M. Klibanov, Professor of Chemistry

**ACKNOWLEDGMENTS**

I wish to express my gratitude to my thesis advisor, Alexander Klibanov for both the guidance and financial support which has allowed me to pursue this research.

I am also grateful to the past and present members of the Klibanov group, especially Paul Burke, Henry Costantino, Shinichirou Tawaki, Edmond Magner, Jennifer Schmitke, Tao Ke, and Hidetaka Noritomi, for helpful discussions and for their friendship.

Most importantly, I am thankful to my parents, Kermit and Marilyn Wescott, and to Arianne Lyng, for their support, guidance, and patience.

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# I. Background and Significance

## A. Introduction

Prior to the advent of nonaqueous enzymology (Klibanov, 1989; Dordick, 1992; Gupta, 1992), the only means of changing the selectivity of an enzymatic reaction were through protein engineering or enzyme screening. Recently, however, it has become apparent that through prudent choice of the solvent, one can profoundly manipulate, and in some cases reverse, the substrate specificity, enantioselectivity, prochiral selectivity, regiospecificity, and chemoselectivity of enzyme-catalyzed reactions.

Aside from the solvent effect on selectivity, the ability to use enzymes as catalysts in organic solvents with little or no added water offers several important advantages to the synthetic organic chemist (Klibanov, 1990). First, lipophilic substrates which are sparingly soluble in water are quite soluble in organic solvents. Moreover, because enzymes are usually insoluble in organic solvents, the enzyme is recovered from the product solution by simple filtration. Biocatalytic reactors which use nonaqueous solvents remain free of bacterial contamination. Also, enzymes suspended in anhydrous solvents often exhibit extreme thermostability, allowing their use at temperatures as high as 100 °C (Zaks and Klibanov, 1984; Garza-Ramos, *et al.* 1990; Volkin, *et al.*, 1991). Furthermore, since the thermodynamic equilibrium of many reactions is markedly solvent-dependent, a solvent can be selected which favors the formation of the desired products. Finally, some reactions which are essentially impossible to perform in water can be readily catalyzed by enzymes in organic solvents. For instance, the transesterification of a carboxylic ester in aqueous solution is rarely feasible because water is a reactant in the competing hydrolysis reaction; in nonaqueous media, however, hydrolases readily catalyze transesterifications with high yields (Zaks and Klibanov, 1985).

## B. Mechanistic Overview

Enzyme-catalyzed reactions are traditionally conducted in water which is essential to biological processes and to the forces which drive protein folding, such as the hydrophobic effect. Proteins typically fold in such a way that apolar residues are buried in a hydrophobic core, while polar residues tend to reside on the surface, where they are hydrated (Creighton, 1991; Branden and Tooze, 1991). In an anhydrous solvent, there is no hydrophobic effect (Tanford, 1980), and apolar residues are soluble, while charged ones are not. Thus, when placed in organic media, the protein should "want to turn inside out", with the hydrophobic residues solvated and the polar side chains buried in the core. The fact that enzymes retain catalytic activity in organic solvents is, therefore, non-trivial to explain.

The current hypothesis is that when the enzyme is placed in an anhydrous solvent, it is kinetically trapped in the native state, in part due to the low dielectric constant which greatly strengthens electrostatic forces. This "rigid enzyme" hypothesis is used to explain the extreme thermostability of enzymes in organic solvents (Volkin, *et al.*, 1991). In support of this hypothesis, reduced conformational flexibility of α-lytic protease in organic solvents compared to water has been demonstrated through the measurement of $^2$H-tyrosine residue flipping rates by solid-state deuterium NMR (Burke, *et al.*, 1993). For instance, for one of the tyrosine residues, the flipping rate constant in aqueous crystals of the enzyme exceeds $10^7$ $s^{-1}$, while in lyophilized enzyme powder suspended in methyl *tert*-butyl ether it is less than $10^3$ $s^{-1}$. The flipping rates of spin labels attached to another serine protease, α-chymotrypsin, quantified using EPR (Affleck, *et al.*, 1992), are also lower in organic solvents than in water (and depend on the dielectric constant of the solvent), indicating that the enzyme is conformationally restricted in the anhydrous environment.

When subtilisin Carlsberg is inactivated with phenylmethylsulfonyl fluoride, which specifically reacts with the catalytically-crucial serine 221 residue in the active center of

the enzyme (Fahrney and Gold, 1963), catalytic activity is not detected in organic solvents (or in water). This indicates that an intact active center is necessary for catalysis in nonaqueous media (Zaks and Klibanov, 1988). Also, the activity of lyophilized enzyme powder in organic solvents depends on the pH of the aqueous solution from which the enzyme was lyophilized, and the "pH dependence" in organic solvents resembles that in water. The explanation of this effect is that the ionogenic groups of the protein retain their ionization state upon lyophilization and that the same state of ionization is required for catalysis in both water and organic solvents (Zaks and Klibanov, 1988).

Enzymatic transesterifications catalyzed by lipases (Zaks and Klibanov, 1985) and by subtilisin Carlsberg (Zaks and Klibanov, 1988) follow Michaelis-Menten kinetics. Moreover, Hammett analysis of the cleavage of various *para*-substituted phenyl acetates catalyzed by subtilisin Carlsberg in water and in various anhydrous solvents reveals that the structure of the transition state for acylation of the enzyme is independent of the solvent. (Kanerva and Klibanov, 1989) Likewise, kinetic isotope effect studies indicate that the transition state for deacylation of the acyl-enzyme does not change from solvent to solvent. (Adams, *et al.*, 1990)

The solid-state NMR spectrum of α-lytic protease where the critical His residue has been labeled with $^{15}N$ demonstrates that the unique hydrogen bonding network of the catalytic triad of this enzyme is intact in anhydrous acetone and octane (Burke, *et al.*). In another direct assessment of the structure of the active center in organic solvents, α-chymotrypsin has been observed to stabilize a transition state intermediate analog both in dry lyophilized powders and in those suspended in a variety of anhydrous solvents, as evidenced by solid-state $^{13}C$ NMR (Burke, *et al.*, 1992). Finally, the structure of subtilisin Carlsberg crystals in anhydrous acetonitrile, determined to 2.3 Å resolution by X-ray crystallography, has been found to be identical to that in water (Fitzpatrick, *et al.* 1993).

All of these data point to a surprising solvent independence of enzyme structure and mechanism of action. At the same time, as seen below, enzymatic specificities in nonaqueous media are markedly affected by the solvent.

## C. Solvent Dependence of Enzyme Specificity

The specificity of an enzyme for the catalysis of one reaction relative to another is classically defined by the ratio of the specificity constants ($k_{cat}/K_M$) (Fersht, 1985). In order to determine specificity values, however, it is not always necessary to measure the individual $k_{cat}/K_M$ parameters for each reaction. If the substrates for both reactions are present simultaneously in the same reaction mixture, and thus compete for the same population of free enzyme, the ratio of the initial velocities of the competing reactions is equal to that of the $k_{cat}/K_M$ (Wescott and Klibanov, 1993). Additionally, for enantioselectivity, the $k_{cat}/K_M$ ratio can be calculated from the enantiomeric excess (*ee*) and the degree of conversion of the reaction (Chen, *et al.*, 1982).

### 1. Substrate Specificity

The fact that enzyme substrate specificity is a function of the reaction medium is now well documented. Zaks and Klibanov observed a complete reversal in the selectivity of α-chymotrypsin and subtilisin Carlsberg in organic solvents relative to water (Zaks and Klibanov, 1986). In the transesterification of N-Ac-L-Ser-OMe and N-Ac-L-Phe-OEt with propanol, catalyzed by the proteases in octane, the serine derivative is approximately 3 times more reactive than the phenylalanine substrate. In contrast, when their hydrolysis is performed in water, the ratio of $k_{cat}/K_M$ inverts to $2 \cdot 10^{-5}$ for α-chymotrypsin and $10^{-2}$ for subtilisin. Thus, the catalytic power of enzymes can be targeted to different substrates simply by changing the reaction medium. This outcome was attributed to the fact that, in water, substrate binding is driven by the hydrophobic effect; by replacing the water with an

anhydrous solvent, this effect is eliminated, thereby changing the driving force for the binding of enzyme and substrate.

When α-chymotrypsin is immobilized on celite and used to catalyze the methanolysis of N-substituted amino acids in acetonitrile and ethyl acetate, the side chain specificity follows the same trend as in water, but the specificity for the N-protecting group is inverted (Clapés and Adlercreutz, 1991). For the enzymatic peptide bond synthesis in a mixture of dimethyl formamide and acetonitrile, the specificity for the amino acid which acts as the nucleophile is unrelated to that for the identical reaction in water. In the latter, the nucleophile specificity correlates positively with the hydrophobicity of the amino acid side chain as measured by its log P (where P is the partition coefficient between 1-octanol and water (Rekker, 1977)). In the nonaqueous system, however, the selectivity is independent of the log P, but related to the van der Waals volume of the amino acid side chain. Furthermore, the specificity in an organic solvent for nearly all of the amino acids tested is the opposite of that in water (Gololobov, *et al.*, 1992). A similar inversion of nucleophile specificity was observed in the catalysis of peptide synthesis by α-chymotrypsin in acetonitrile; the acyl donor specificity, however, remained parallel to that in water (Nagashima, *et al.*, 1992).

Another example in which substrate specificity in organic media is the reverse of the aqueous specificity was described by Gaertner and Puigserver (Gaertner and Puigserver, 1989). In the aminolysis of N-benzoyl-L-Tyr-OEt or N-benzoyl-L-Lys-OMe in benzene, subtilisin exhibits a 3 fold preference for the lysine substrate. In contrast, in the enzymatic hydrolysis of the same substrates in water, $k_{cat}/K_M$ for the lysine derivative is 34 fold *lower* than that for the tyrosine one. An even larger difference is seen for the same reactions catalyzed by α-chymotrypsin. In benzene, the lysine substrate is slightly favored over the tyrosine ester. In water, however, no enzymatic hydrolysis of the former ester is observed, while $k_{cat}/K_M$ for the enzymatic hydrolysis of the tyrosine ester is 800 $s^{-1}$ $mM^{-1}$.

Not only does the substrate preference of an enzyme change upon transition from aqueous to nonaqueous media, it also varies from one organic solvent to another. Ryu and Dordick report that the selectivity of horseradish peroxidase-catalyzed oxidation of various *para*-substituted phenols correlates with the hydrophobicity of both the solvent and the substrate. The authors explain this dependence as a consequence of partitioning of the substrate from the solvent into the active center of the enzyme (Ryu and Dordick, 1989; Ryu and Dordick, 1992).

It is worth noting that in addition to substrate specificity of enzymes, their inhibitor specificity (Zaks and Klibanov, 1988), as well as the antigen specificity of an antibody (Russell, *et al.*, 1989) are also strongly dependent on the solvent.

## 2. Enantioselectivity

The exquisite enantioselectivity of enzymatic catalysis is the most valuable characteristic of enzymes to the organic chemist (Simon, *et al.*, 1985; Yamada and Shimizu, 1988; Jones, 1986). Ironically, this strict specificity also limits the generality of enzymatic synthesis, because enzymes that catalyze the reaction of interest with the desired stereochemistry are not always available. The expansion of enzymatic catalysis to nonaqueous solvents, and the subsequent discovery that enantioselectivity can be changed and even inverted by the reaction medium, thus greatly enhances the utility of enzyme-catalyzed syntheses.

For example, in organic solvents, but not in water, subtilisin Carlsberg can synthesize peptides containing D-amino acid residues (Margolin, *et al.*, 1987). The enzyme is enabled to incorporate the unnatural D-enantiomers of amino acids into peptides due to the extreme solvent dependence of its enantioselectivity.

Sakurai *et al.* discovered a strong solvent dependence of enantioselectivity in the transesterification of N-Ac-(L or D)-Ala-OEtCl with propanol catalyzed by the same enzyme (Sakurai *et al.*, 1988). It was proposed that binding of the ester to the enzyme

displaces water from the active center. As the solvent hydrophobicity (log P) increases, more energy is required to transfer water from the active center into the solvent. The D-enantiomer binds to the enzyme differently than the L-enantiomer to form a reactive complex and displaces less water from the active center. Hence it is less hindered by the hydrophobic solvents than the L enantiomer, causing $(k_{cat}/K_M)_L$ / $(k_{cat}/K_M)_D$ to drop with increasing log P. A similar correlation between enzyme enantioselectivity and solvent log P has been recently reported for *Candida cylindracea* lipase (Gubicza and Kelemen-Horváth, 1993).

A different solvent effect is observed when subtilisin Carlsberg is used to resolve the chiral alcohol *sec*-phenethanol through transesterification with vinyl butyrate (Fitzpatrick and Klibanov, 1991). In this case, $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$ jumps from 3 in acetonitrile to 61 in dioxane, but the enantioselectivity does not systematically depend on the log P of the solvent. Instead, subtilisin's enantioselectivity toward the two enantiomers of the alcohol correlates with the dielectric constant of the solvent. Furthermore, the rise in enantioselectivity with the dielectric constant is primarily due to the reduction of the reactivity of the R-enantiomer. The authors hypothesized that, in order for the R-enantiomer to react, the phenyl group of the alcohol must fit into a sterically constrained binding pocket. In solvents with a low dielectric constant (e.g., dioxane), the protein is rigid, which exacerbates the steric constraints. As the dielectric constant rises, the protein becomes more flexible. Consequently, binding of the phenyl group is facilitated, thereby increasing the reactivity of R-*sec*-phenethanol relative to the S-enantiomer. The plausibility of the putative binding modes of *sec*-phenethanol was confirmed by molecular modeling studies (Fitzpatrick, *et al.*, 1992).

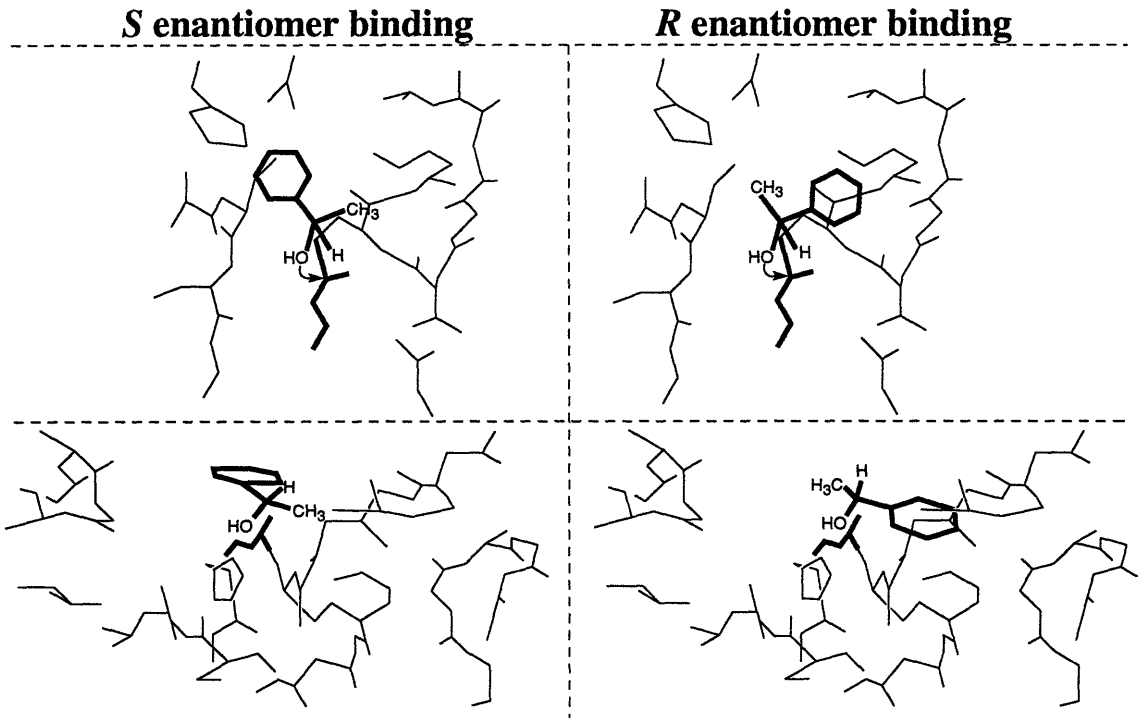



**Figure 1.1.** Computer-generated structures of the relevant portion of the active center of butyryl-subtilisin containing either S (left) or R (right) enantiomers of *sec*-phenethanol bound in a reactive manner. The chiral alcohol molecule and the butyryl moiety of the acyl-enzyme are shown in bold lines. The upper part of the figure depicts a view of an observer looking into the active center from directly above; the lower part provides a perpendicular view of the active center region. The OH group of the alcohol is correctly oriented for a nucleophilic attack (symbolized by an arrow) on the butyryl's carbonyl leading to deacylation (Fitzpatrick, *et al.*, 1992).

Figure 1.1 depicts possible modes of binding between each enantiomer of *sec*-phenethanol and butyryl-subtilisin. As a quantification of the steric constraints, the number of close contacts (intermolecular contacts with interatomic distances of less than 2.5 Å) is 18 for the bound R-*sec*-phenethanol, and only 2 for the bound S-alcohol. Thus, binding of the R-enantiomer is indeed more restrained than that of its S counterpart.

Recently, several examples have been reported in which enzymatic enantioselectivity is reversed in one organic solvent relative to another (Wu, *et al.*, 1991; Tawaki and Klibanov, 1992; Ueji, *et al.*, 1992). For instance, for the *Aspergillus oryzae* protease-catalyzed transesterification of N-Ac-(L or D)-Phe-OEtCl with propanol, $(k_{cat}/K_M)_L$ / $(k_{cat}/K_M)_D$ is 6.6 in acetonitrile, but inverts to 0.24 in toluene (Tawaki and Klibanov, 1992). When the enantioselectivity is measured over a range of organic solvents, a correlation with their log P is observed. To explain these data, it is assumed that the natural L-phenylalanine substrate has its phenyl group buried in the binding pocket of the enzyme in the productive mode of binding. For the D-enantiomer to form a productive complex with the enzyme, the substrate orients in such a way that the phenyl moiety is exposed to the solvent. In hydrophilic solvents, solvation of the phenyl group is energetically costly, causing the productive binding mode of the L-substrate to be favored over that of the D-enantiomer. As log P of the reaction medium is increased, the solvation of the phenyl group eventually turns favorable and thus binding of the D-substrate becomes preferred.

Over the last few years, there have been numerous other reports on the solvent dependence of enzyme enantioselectivity (Ueji, *et al.*, 1992; Parida and Dordick, 1993; Martins, *et al.*, 1993; Secundo, *et al.*, 1992; Parida and Dordick, 1991; Nakamura, *et al.*, 1991; Bovara, *et al.*, 1991; Kanerva, *et al.*, 1990; Kitaguchi, *et al.*, 1989); however, in most of them, no mechanistic explanation of the observed behavior was offered.

As an alternative to organic solvents, supercritical fluids can be employed as nonaqueous reaction media for biocatalysis (Nakamura, 1990). Because the physical

properties, such as the dielectric constant, of a supercritical fluid depend on the pressure, Russell and coworkers (Kamat, *et al.*, 1993) reasoned that it should be possible to control enzyme selectivity by altering the pressure in such a system. By increasing the pressure of supercritical fluoroform from 950 to 5100 psi, the authors indeed effected a 2-fold increase in the preference of subtilisin Carlsberg and *Aspergillus* protease for the L-enantiomer of N-Ac-Phe-OEt over its D-counterpart in the transesterification with methanol.

### 3. Prochiral Selectivity

The ability of enzymes to selectively catalyze the conversion of a prochiral substrate to a single enantiomer of a chiral product provides a valuable alternative to enantioselective resolutions for the preparation of optically active compounds. Such prochirally selective transformations have been profitably used in organic solvents (Gutman, *et al.*, 1990; Santaniello *et al.*, 1993). As with enantioselectivity, the prochiral selectivity of enzymes has been recently found to be significantly affected by the reaction medium.

Hirose and coworkers have used *Pseudomonas* sp. lipase to catalyze the hydrolysis of achiral bis-(alcoxymethyl)-1,4-dihydro-3,5-pyridine dicarboxylates to produce optically active monoesters (Hirose, *et al.*, 1992). When the reaction is performed in water-saturated isopropanol, the S-product is recovered with an enantiomeric excess of 99%. In contrast, in cyclohexane saturated with water, the prochiral substrate is preferentially converted to the R-enantiomer, with an *ee* of 89%. If *Pseudomonas cepacia* lipase is used instead, only the R-product is produced in both of the aforementioned solvent systems. Therefore the authors have concluded that the solvent effect arises from specific interactions between the solvent and the former lipase.

Terradas *et al.* have examined the monohydrolysis of another prochiral diester, 2-(1-naphthoylamino)trimethylene dibutyrate, catalyzed by *Pseudomonas* sp. lipase in a

variety of organic solvents (Terradas *et al.*, 1993). The observed prochiral selectivity varies greatly depending on the solvent, with $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$ exceeding 30 in nitrobenzene and acetonitrile and dropping to less than 3 in carbon tetrachloride. When the logarithm of the prochiral selectivity is plotted against the solvent's log P, an inverse correlation between the two is observed (Fig. 1.2).

A mechanistic explanation for the solvent effect has been proposed (Terradas *et al.*, 1993) that involves two modes of binding between the prochiral substrate and the lipase active center. The first is stereoselective, in which the naphthoyl group of the substrate is buried in the hydrophobic cleft of the lipase and is thus inaccessible to the solvent. Substrate molecules which bind in this manner form a pro-R acyl-enzyme which deacylates to form the R-product. The second binding mode, in which the naphthoyl moiety remains solvated, is non-stereoselective, where a substrate molecule is converted to the R or S product with equal likelihood. Because solvation of the naphthoyl group is less energetically taxing in solvents of high log P, the non-stereoselective binding mode becomes favored as the solvent becomes more hydrophobic, thus leading to the fall in prochiral selectivity. The importance of a naphthoyl binding site to the solvent dependence of the selectivity is demonstrated by the >10 fold drop in the prochiral selectivity caused by the addition of 1 M 1-naphthol to acetonitrile. Presumably the naphthol competes with the substrate for the binding pocket, thus hindering stereoselective binding.

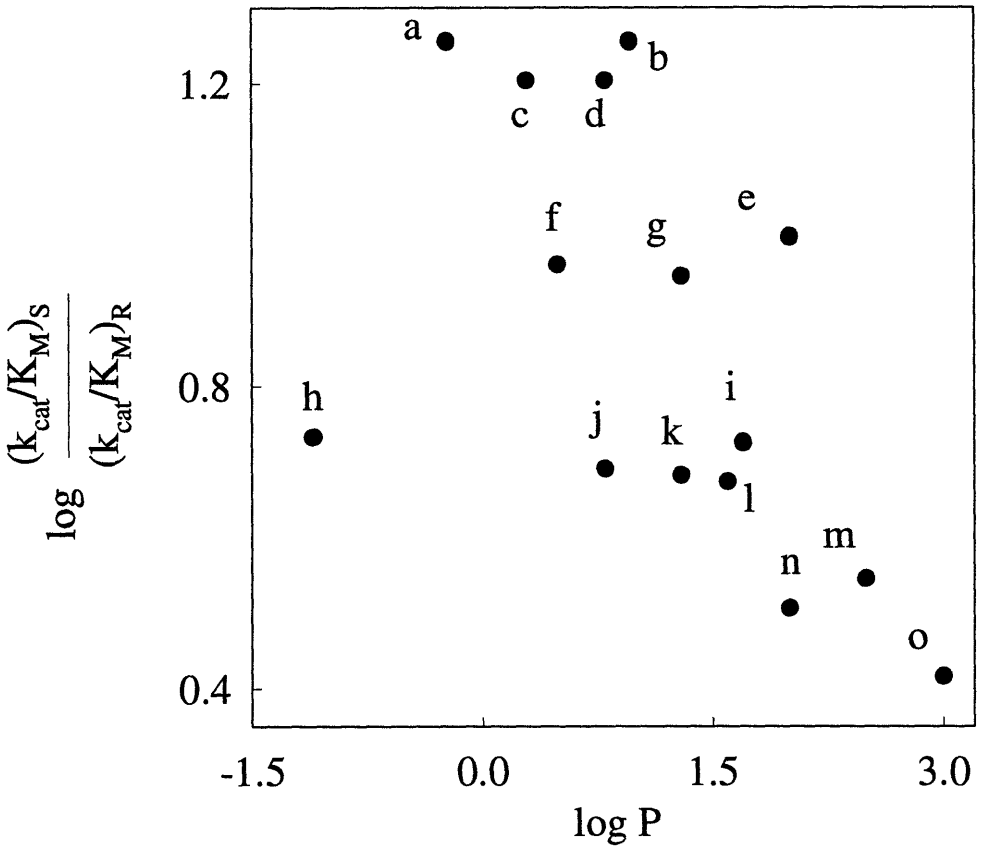



**Figure 1.2.** Prochiral selectivity of *Pseudomonas* sp. lipase in the monohydrolysis of 2-(1-naphthoylamino)trimethylene dibutyrate as a function of the hydrophobicity of the solvent. Solvent hydrophobicity is defined as log P, where P is the partition coefficient of the solvent between 1-octanol and water. Solvents: (a) acetone, (b) cyclohexanone, (c) butanone, (d) 2-pentanone, (e) chloroform, (f) tetrahydrofuran, (g) 2-hexanone, (h) dioxane, (i) *tert*-butyl acetate, (j) *tert*-butanol, (k) *tert*-amyl alcohol, (l) triethylamine, (m) toluene, (n) benzene, and (o) carbon tetrachloride (Terradas, *et al.*, 1993).

## 4. Regioselectivity

The regioselective (i.e., position-selective) modification of polyfunctional compounds is an arduous problem in organic chemistry. The ability of enzymes to catalyze such regioselective transformations has found many synthetic applications (Therisod and Klibanov, 1987; Riva, *et al.*, 1988; Carrea, *et al.*, 1989; Ciuffreda, *et al.*, 1990; Colombo, *et al.*, 1991). The possibility of affecting the regioselectivity of a given enzymatic process by the solvent would offer additional exciting opportunities.

The only study of this sort to date has been undertaken (Rubio, *et al.*, 1991) with *Pseudomonas cepacia* lipase which catalyzed the butanolysis of 1,4-dibutyryloxy-2-octylbenzene in 11 nonaqueous solvents. In toluene, this lipase preferentially deacylates the substrate at the 4 position, with $(k_{cat}/K_M)_4 / (k_{cat}/K_M)_1$ of 2. However, the enzyme can be made to favor deacylation at the alternate, 1 position by switching to acetonitrile, where the regioselectivity inverts to 0.5. The regioselectivity roughly correlates with log P of the solvent (Fig. 1.3).

These results are rationalized in terms of a model based on the partitioning of the substrate's octyl moiety between the solvent and the binding pocket of the lipase. Two binding modes are hypothesized, one leading to the 4-hydroxy product, and the other to the 1-hydroxy compound. The first requires that the octyl group remain solvated, orienting the 4-butyryl moiety toward the active center nucleophile to effect deacylation at the 4 position. Alternatively, the substrate binds to the enzyme in such a way that the octyl group is buried in the lipase's hydrophobic cavity, and butanolysis at the 1 position ensues. In hydrophilic solvents, partitioning of the hydrophobic octyl moiety into the hydrophobic binding site is energetically favored, yielding the 1-phenol. As solvent log P increases, solvation of the octyl group increasingly occurs, leading to the 4-phenol.

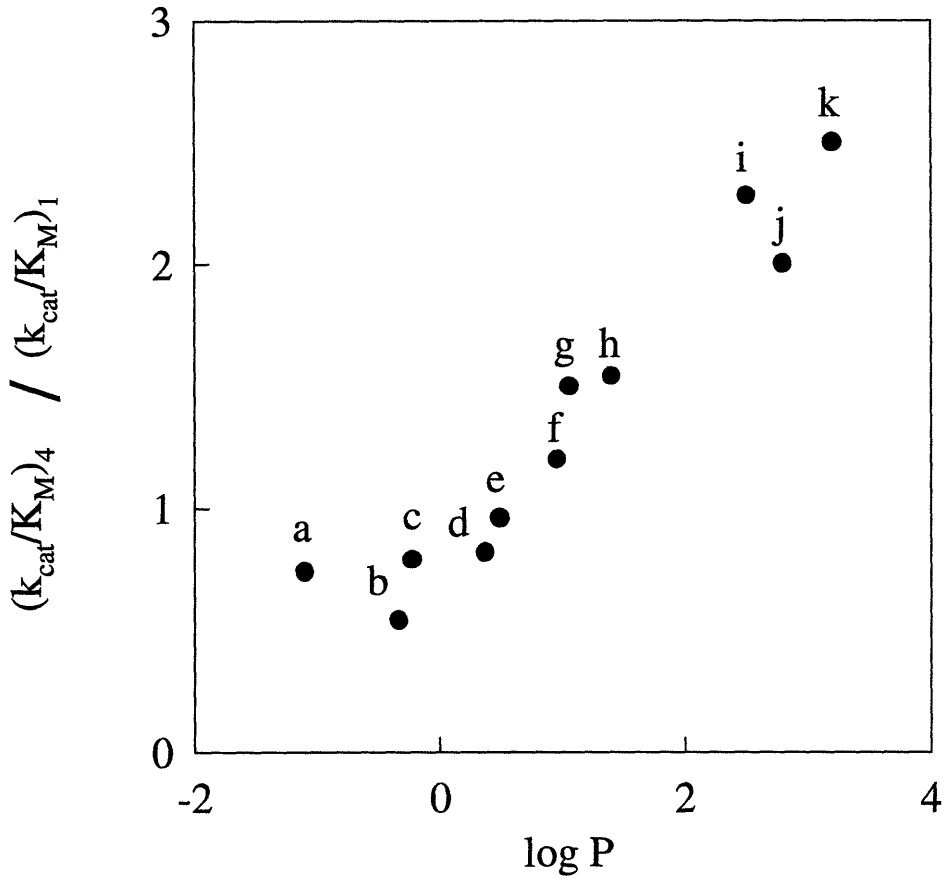



**Figure 1.3.** The dependence of the regioselectivity of *Pseudomonas cepacia* lipase in the transesterification of 1,4-dibutyryloxy-2-octylbenzene with butanol on the hydrophobicity of the solvent. Here, regioselectivity is defined as the ratio of the specificity constants for the formation of either the 1 or the 4 phenol derivative. Log P is the logarithm of the octanol-to-water partition coefficient of the solvent. Solvents: (a) dioxane, (b) acetonitrile, (c) acetone, (d) *tert*-butanol, (e) tetrahydrofuran, (f) cyclohexanone, (g) isopropyl acetate, (h) methyl *tert*-butyl ether, (i) toluene, (j) carbon tetrachloride, and (k) cyclohexane (Rubio, *et al.*, 1991).

## 5. Chemoselectivity

In addition to the problem of regioselective transformations, the task of performing chemoselective (i.e., chemical-function-selective) conversions of heterofunctional molecules is of major preparative significance. Nonaqueous biocatalysis has been used to modify substrates with chemoselectivities distinct from those of the chemical reactions (Chinsky, *et al.*, 1989). Like other manifestations of enzymatic specificity, chemoselectivity is apparently dictated by the solvent.

Tawaki and Klibanov (1993) have examined the hydrolase-catalyzed butyrylation of various substrates containing both amino and hydroxyl groups, and have reported that chemoselectivity in acetonitrile is distinct from that in toluene. For the acylation of N-α-benzoyl-L-lysinol catalyzed by *Pseudomonas* sp. lipoprotein lipase, $(k_{cat}/K_M)_O$ / $(k_{cat}/K_M)_N$ plummets from 21 in dichloroethane to 1.1 in *tert*-butanol. Interestingly, the chemoselectivity of the enzyme in organic solvents follows a trend generally opposite to that of the uncatalyzed reaction and roughly correlates with the hydrogen bonding parameter (Reichardt, 1988) of the solvent: N-acylation is favored in those with a high propensity to engage in hydrogen bonding (Fig. 1.4).

This suggests the following explanation for the solvent dependence. When a nucleophilic group is participating in a hydrogen bond with the solvent, it cannot attack the butyryl-enzyme. Because hydroxyl groups are better hydrogen bond donors than amino groups, their ability to act as nucleophiles is hindered to a greater extent by hydrogen-bond-forming solvents than that of amino groups. This model successfully predicts the solvent dependence of the lipase's chemoselectivity for the butyrylation of 6-amino-1-hexanol. Unlike *Pseudomonas* sp. lipoprotein lipase, the chemoselectivities of *Mucor miehei* and porcine pancreatic lipases exhibit solvent dependencies similar to those of the uncatalyzed reactions.

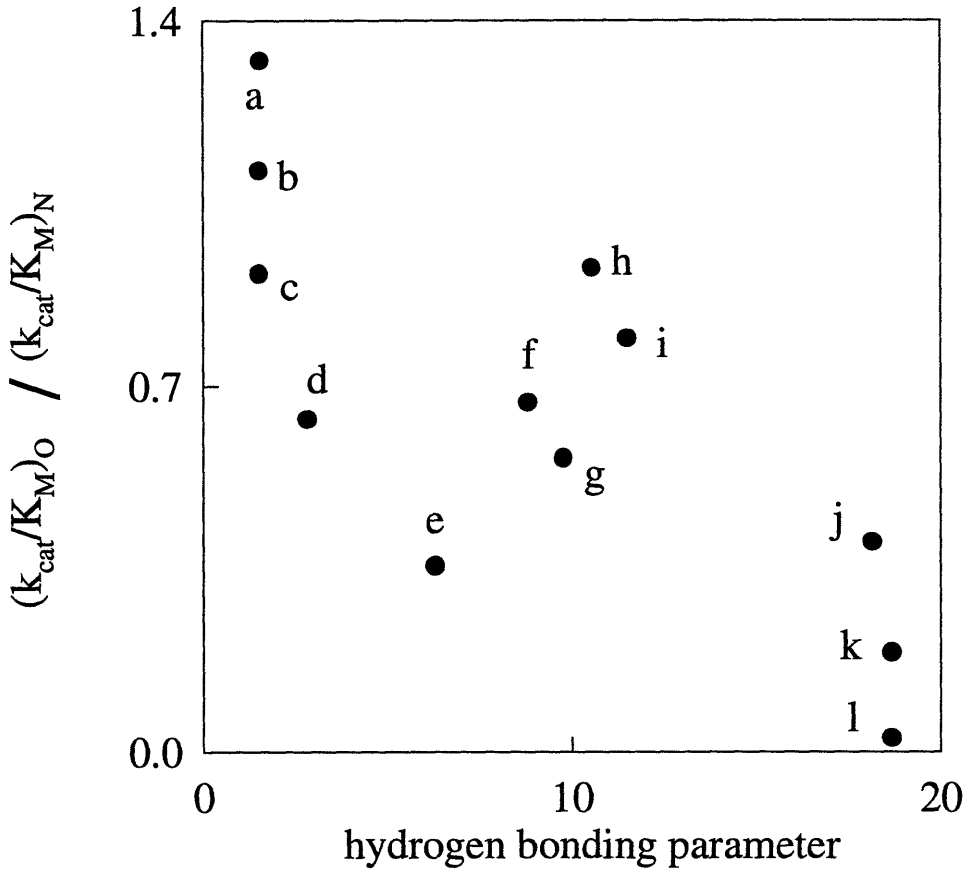



**Figure 1.4.** Dependence of the chemoselectivity of monobutyrylation of N-α-benzoyl-L-lysinol catalyzed by *Pseudomonas* sp. lipoprotein lipase on the hydrogen bonding parameter (Reichardt, 1988) of the solvent. Solvents: (a) 1,2-dichloroethane, (b) chloroform, (c) dichloromethane, (d) nitrobenzene, (e) acetonitrile, (f) *tert*-butyl acetate, (g) dioxane, (h) methyl *tert*-butyl ether, (i) tetrahydrofuran, (j) pyridine, (k) *tert*-amyl alcohol, and (l) *tert*-butanol (Tawaki and Klibanov, 1993).

## D. Project Objective.

While many have observed distinct solvent effects on the various types of enzymatic selectivity, these effects have only been investigated in an empirical manner. It is the goal of this project to develop a mechanistic understanding of the solvent dependence of enzymatic selectivity, and to use this knowledge to develop methods for the quantitative prediction of such dependencies. Such predictive methods should ultimately lead to the ability to rationally control the selectivity of an enzymatic transformation through proper choice of the solvent.

## II. Prediction of Substrate Specificity using Solvent-to-Water Partition Coefficients of the Substrates

### A. Introduction

Exquisite substrate specificity is a hallmark of enzymatic catalysis (Fersht, 1985). To what extent is this phenomenon due to the enzyme itself, as opposed to the aqueous reaction medium? Non-aqueous enzymology (Klibanov, 1989) has allowed this question to be addressed experimentally (Zaks and Klibanov, 1986; Gaertner and Puigserver, 1989; Clapés and Adlercreutz, 1991; Gololobov, *et al.*, 1992; Ryu and Dordick, 1992). It has been found that substrate preference of the proteases α-chymotrypsin and subtilisin markedly changes when water is replaced with a non-aqueous solvent as the reaction medium (Zaks and Klibanov, 1986). In the present work, we have observed a dramatic shift, and indeed inversion, of subtilisin's substrate specificity upon transition from one anhydrous solvent to another; this dependence is rationalized quantitatively in terms of a simple, enzyme-independent, physico-chemical concept.

### B. Results and Discussion

We kinetically examined the transesterification between N-acetyl-L-(amino acid) ethyl esters and 1-propanol catalyzed by subtilisin Carlsberg in 20 anhydrous solvents. Table 2.1 depicts substrate specificity of subtilisin, defined as the ratio of the specificity constants (Fersht, 1985) $k_{cat}/K_M$, for the two esters of different hydrophobicities, Phe and Ser, as a function of the solvent.

**Table 2.1.** Substrate Specificity in the Transesterification of N-Ac-L-Ser-OEt and N-Ac-L-Phe-OEt with 1-Propanol Catalyzed by Subtilisin Carlsberg in Various Anhydrous Organic Solvents. See Methods for details.

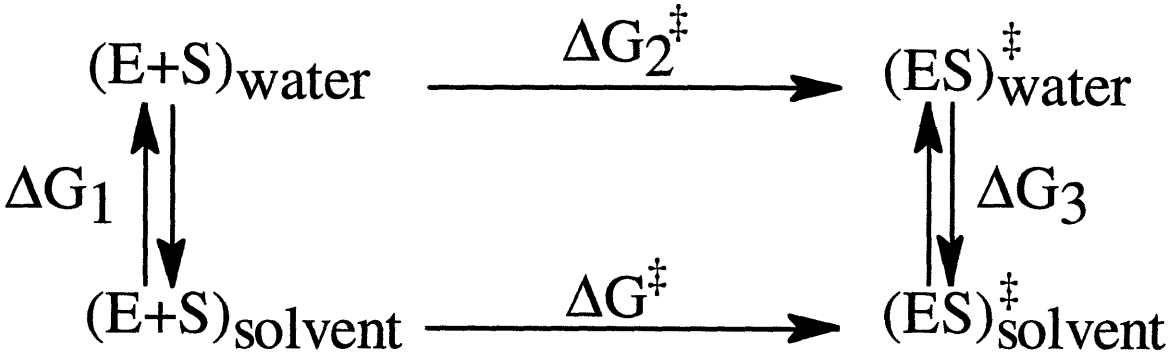
| Solvent | $(k_{cat}/K_M)_{Ser}/(k_{cat}/K_M)_{Phe}$ |
|---|---|
| dichloromethane | 8.2 |
| chloroform | 5.5 |
| toluene | 4.8 |
| benzene | 4.4 |
| N,N-dimethylformamide | 4.3 |
| *tert*-butyl acetate | 3.7 |
| N-methylacetamide | 3.4 |
| diethyl ether | 3.2 |
| carbon tetrachloride | 3.2 |
| ethyl acetate | 2.6 |
| *tert*-butyl methyl ether | 2.5 |
| octane | 2.5 |
| isopropyl acetate | 2.2 |
| acetonitrile | 1.7 |
| dioxane | 1.2 |
| acetone | 1.1 |
| pyridine | 0.53 |
| *tert*-amyl alcohol | 0.27 |
| *tert*-butyl alcohol | 0.19 |
| *tert*-butyl amine | 0.12 |

It is seen that a solvent variation results in up to a 68-fold change in substrate specificity. Furthermore, while subtilisin strongly prefers the serine substrate in solvents at the top of the table, the phenylalanine substrate is greatly favored in those at the bottom (with all other conditions being identical).

Substrate specificity of enzymes arises from their utilization of the free energy of binding with substrates for catalysis (Fersht, 1985). This binding energy consists of several components, the first of which, the free energy of the desolvation of the substrate molecule, is solvent-dependent. This dependence must be different for such dissimilar substrates as the Phe and Ser esters, thereby suggesting that substrate specificity should depend on the solvent.

To quantify this dependence and explain the data in Table 2.1, consider a thermodynamic diagram in Scheme 2.1.

**Scheme 2.1**




The lower horizontal arrow represents enzyme (E) and substrate (S) reacting in an organic solvent to form a transition state ($ES^{\ddagger}$). Another, and thermodynamically equivalent, path exists leading to the same event. It involves partitioning of the enzyme and the substrate

from the solvent into water, formation of the transition state in water, and its subsequent partitioning from water into the solvent.

Expressing $\Delta G^{\ddagger}$ as the sum of the energetic terms of the alternate path yields:

$$\Delta G^{\ddagger} = \Delta G_1 + \Delta G^{\ddagger}_2 + \Delta G_3 \qquad (2.1)$$

$\Delta G^{\ddagger}$ is related to $k_{cat}/K_M$ in the solvent (Fersht, 1985):

$$\Delta G^{\ddagger} = -RT \ln\left[\left(\frac{k_{cat}}{K_M}\right)_{solvent}\left(\frac{h}{\kappa T}\right)\right] \qquad (2.2)$$

where h is the Planck constant, $\kappa$ is the Boltzmann constant, and T is the absolute temperature. Likewise,

$$\Delta G^{\ddagger}_2 = -RT \ln\left[\left(\frac{k_{cat}}{K_M}\right)_{water}\left(\frac{h}{\kappa T}\right)\right] \qquad (2.3)$$

$\Delta G_1$ depends on the solvent-to-water partition coefficients of the substrate (P) and the enzyme ($P_E$):

$$\Delta G_1 = RT \ln P + RT \ln P_E \qquad (2.4)$$

$\Delta G_3$ is the free energy of transfer of $ES^{\ddagger}$ from water into the solvent. Substituting equations 2.2, 2.3, and 2.4 into equation 2.1, and dividing by -RT, one obtains

$$\ln\left[\left(\frac{k_{cat}}{K_M}\right)_{solvent}\left(\frac{h}{\kappa T}\right)\right] = -\ln P - \ln P_E + \ln\left[\left(\frac{k_{cat}}{K_M}\right)_{water}\left(\frac{h}{\kappa T}\right)\right] - \frac{\Delta G_3}{RT} \qquad (2.5)$$

Equation 2.5 can be written for each of the substrates N-Ac-L-Ser-OEt and N-Ac-L-Phe-OEt (denoted herein as Ser and Phe, respectively). Subtracting the Phe equation from the Ser equation produces:

$$\ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Ser}_{solvent}\left(\frac{h}{\kappa T}\right)\right]-\ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Phe}_{solvent}\left(\frac{h}{\kappa T}\right)\right]=-\ln P_{Ser}+\ln P_{Phe}-\ln P_E+$$

$$+\ln P_E+\ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Ser}_{water}\left(\frac{h}{\kappa T}\right)\right]-\ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Phe}_{water}\left(\frac{h}{\kappa T}\right)\right]+\frac{\Delta G_{3,Phe}-\Delta G_{3,Ser}}{RT} \qquad (2.6)$$

Since $P_E$ is independent of the substrate, these terms cancel each other out. Because subtilisin is 100 times larger than either substrate (thus dominating the partitioning characteristics of the complex) and the transition state is shielded from the solvent (Kanerva and Klibanov, 1989), $\Delta G_{3,\,Ser} = \Delta G_{3,\,Phe}$. Thus, these terms also cancel each other out. Consequently, rearranging equation 2.6 yields:

$$\log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{solvent}=\log\frac{P_{Phe}}{P_{Ser}}+\log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{water} \qquad (2.7)$$

Equation 2.7 predicts that a double logarithmic plot of substrate specificity in any solvent *vs.* the P ratio for the substrates will yield a straight line, with a slope of one, whose intercept with the ordinate should equal the substrate specificity in water. Therefore we experimentally measured $P_{Phe}$ and $P_{Ser}$ (Table 2.2), and the resultant plot is shown in Figure 2.1.

**Table 2.2.** Solvent-To-Water Partition Coefficients of N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt.[a]

| Solvent | $P_{Phe}$[b] | $P_{Ser}$[b] | |
|---|---|---|---|
| | | measured by gas chromatography | measured by scintillation counting |
| *tert*-butyl alcohol | 23 | 1.9 | 1.1 |
| *tert*-amyl alcohol | 23 | 1.4 | 0.81 |
| isopropyl acetate | 21 | 0.13 | 0.071 |
| ethyl acetate | 25 | 0.24 | 0.15 |
| *tert*-butyl methyl ether | 6.0 | 0.045 | 0.033 |
| diethyl ether | 4.0 | 0.035 | 0.023 |
| carbon tetrachloride | 4.4 | 0.017 | 0.013 |
| *tert*-butyl acetate | 23 | 0.065 | 0.039 |
| toluene | 7.1 | 0.015 | 0.019 |
| benzene | 15 | 0.016 | 0.025 |
| chloroform | 190 | 0.19 | 0.083 |
| dichloromethane | 130 | 0.065 | 0.070 |

[a] Experimental conditions are reported in the Experimental section. [b] $P_{Phe}$ and $P_{Ser}$ are the solvent-to-water partition coefficients of N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt, respectively. Partition coefficients were measured by gas chromatography. In most instances, the numbers depicted are the mean values of two independent measurements. Additionally, an independent determination of $P_{Ser}$ was performed by scintillation counting.

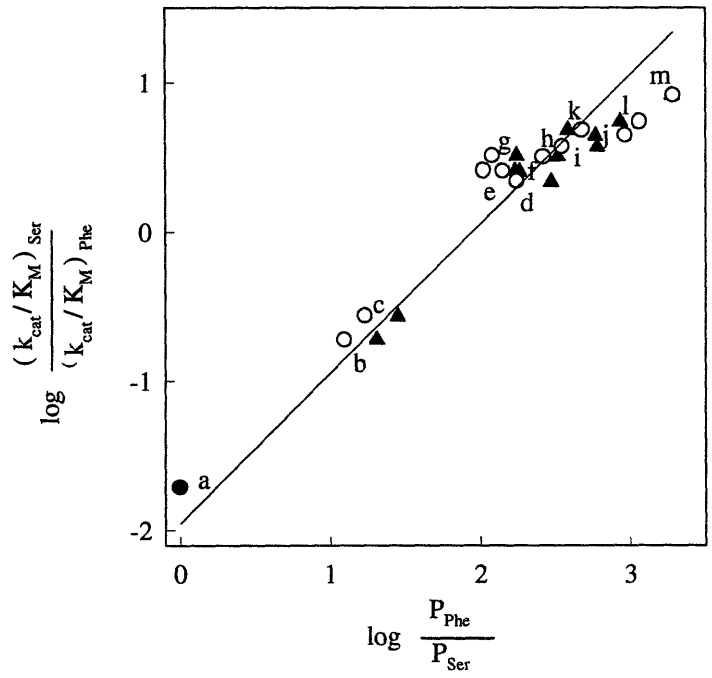



**Figure 2.1.** Dependence of substrate specificity of subtilisin Carlsberg in water and in anhydrous organic solvents on the ratio of the solvent-to-water partition coefficients of N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt. Solvents: a - water (closed circle), b - *tert*-butyl alcohol, c - *tert*-amyl alcohol, d - isopropyl acetate, e - ethyl acetate, f - *tert*-butyl methyl ether, g - diethyl ether, h - carbon tetrachloride, i - *tert*-butyl acetate, j - toluene, k - benzene, l - chloroform, and m - dichloromethane. The partition coefficients were determined by two independent methods (see the Experimental section): the equilibrium substrate concentrations in each phase of biphasic aqueous-organic mixtures were measured by gas chromatography (open circles), or, alternatively, the equilibrium concentrations of the tritiated serine substrate in such mixtures were measured by scintillation counting (triangles).

One can see a general agreement between the straight line (with the tangent of unity) and the experimental points. The substrate specificity in water derived from the intercept is $1.1 \cdot 10^{-2}$, as compared to the experimentally determined $1.9 \cdot 10^{-2}$. This discrepancy is probably due to the fact that kinetic measurements (Table 2.1) were conducted in the presence of 1 M propanol as the nucleophile. While the propanol certainly influenced the solvation energies of the substrates, it was necessarily omitted from the partitioning experiments, where it would have partitioned from the solvent to the aqueous phase.

The model presented, while mechanistic and predictive, is essentially independent of the enzyme because the contribution of subtilisin-substrate binding is accounted for by the substrate specificity in water. Thus this model, which is based on a general thermodynamic analysis of enzyme action in organic solvents, should be applicable to any enzyme/substrate pair as long as the substrates are inaccessible to the solvent in the transition state.

## C. Materials and Methods

**Enzyme.** Subtilisin Carlsberg (serine protease from Bacillus licheniformis, EC 3.1.1.3) was purchased from Sigma Chemical Co. The enzyme was prepared by lyophilization from a 5 $mg \cdot mL^{-1}$ solution in 20 mM aqueous potassium phosphate buffer (pH 7.8). Lyophilized enzyme powder was stored over anhydrous $CaSO_4$ in an evacuated desiccator at $4^{o}C$.

**Chemicals and solvents.** N-Ac-L-Phe-OEt and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. All solvents used in this study were of the highest

purity commercially available and were dried prior to use to a water content below 0.01% by shaking with 3-Å molecular sieves (Linde).

**N-Ac-L-Ser-OEt** was synthesized as follows: 1.7 g (10 mmol) of L-Ser-OEt·HCl (Sigma) was added to 40 mL of dry ethyl acetate. The suspension was stirred at room temperature while 2.8 mL (20 mmol) of triethylamine was added. After cooling the mixture to $0^{o}$C, 0.71 mL (10 mmol) of acetyl chloride dissolved in 40 mL of ethyl acetate was added dropwise to the suspension. Precipitated triethylamine•HCl was removed by filtration and the solvent by rotary evaporation. The resultant product was an oil with $[\alpha]_D^{25} = +12.8^{o}$. $^1$H NMR ($CDCl_3$, $CHCl_3$ as an internal standard) $\delta$ 6.58 (1H, d, J = 6.0 Hz), 4.66 (1H, dt, J = 3.6, 3.6 Hz), 4.27 (2H, q, J = 7.2 Hz), 3.96 (2H, s), 2.90 (1H, s), 2.08 (3H, s), 1.31 (3H, t, J = 7.2 Hz).

**[$^3$H]N-Ac-L-Ser-OEt** was synthesized as follows: 1 mCi of [$^3$H]L-Ser (318 mCi per mg, Amersham Corp.) was added to 1.7 g (10 mmol) of L-Ser-OEt•HCl in 50 mL of dry ethanol. Anhydrous HCl (Aldrich Chemical Co.) was bubbled through the stirred mixture until it began to reflux. The reaction was continued for 1 hr, and then the solvent was removed by rotary evaporation. The resulting [$^3$H]L-Ser-OEt•HCl was dried under vacuum overnight. The subsequent amino group acylation of the [$^3$H]ester was accomplished as described above.

**Measurement of Partition Coefficients by Gas Chromatography.** Two mL of an aqueous solution containing 10 mM N-Ac-Phe-OEt and 10 mM N-Ac-Ser-OEt were added to 2 mL of each water-immiscible organic solvent in a 7-mL glass scintillation vial. In the case of *tert*-butyl alcohol, the aqueous phase contained 1 M NaCl to impart

immiscibility with the alcohol. After the vials were shaken for 24 hr at 30°C, the phases were separated by centrifugation. The concentration of each substrate was measured in both the aqueous and the organic phases by gas chromatography. The partition coefficient for a given substrate is defined as the ratio of its equilibrium concentration in the organic phase to that in the aqueous phase.

**Measurement of Partition Coefficients by Scintillation Counting.** The partition coefficients for [$H^3$]N-Ac-L-Ser-OEt were measured as outlined above, except the concentrations in both phases were measured by liquid scintillation counting as follows. One mL of each phase was placed in a 20-mL scintillation vial. The solvent was evaporated under vacuum and replaced by 8 mL of scintillation cocktail. Count rates were converted to concentrations using a calibration curve.

**Kinetic measurements.** The $k_{cat}/K_M$ values in water were measured potentiometrically (via pH stat) in the subtilisin-catalyzed hydrolysis of the esters (1 to 10 mM esters; 2 and 200 nM enzyme for the Phe and Ser substrates, respectively; pH 7.8; 30°C; 100 mM KCl). Kinetic data was fitted to the Michaelis-Menten equation using the non-linear curve fitting function of SigmaPlot (Jandel Scientific).

The $k_{cat}/K_M$ values in organic solvents were determined as follows. Both ester substrates (10 mM of each) were placed in the same vial with 5 $mg \cdot mL^{-1}$ lyophilized enzyme powder and 1 M 1-propanol. The suspension was shaken at 30°C and 300 rpm. Periodically, a 2-μL sample was withdrawn and assayed by gas chromatography.

**Diffusion Limitation Assay.** To ensure that enzymatic transesterification rates in

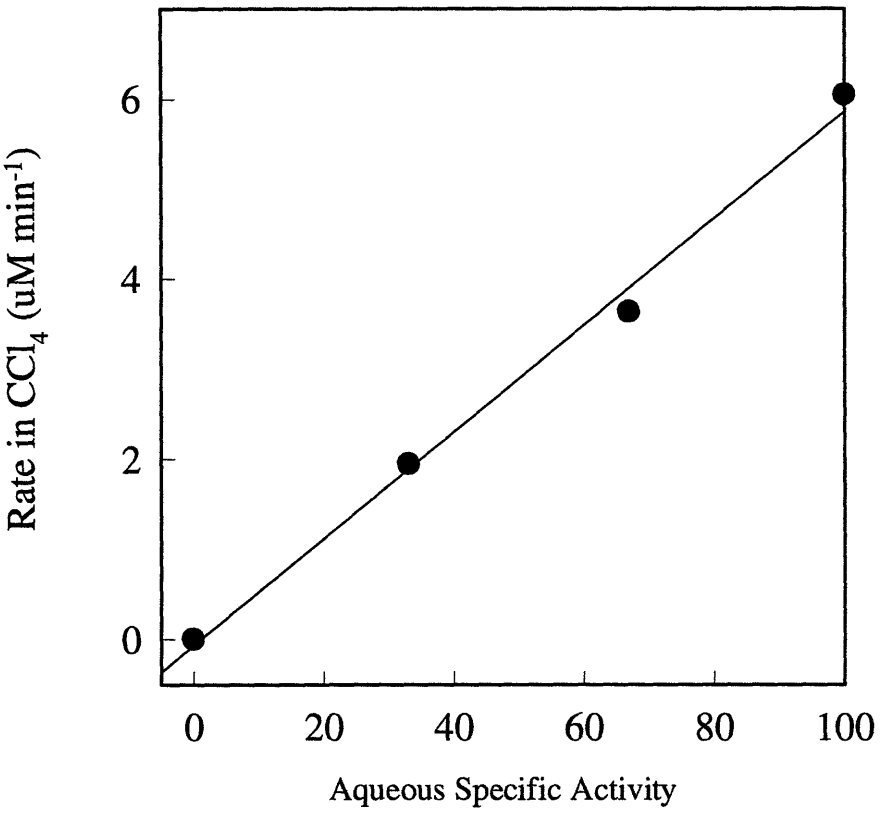


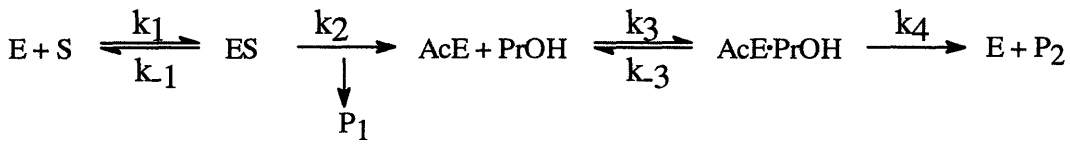

**Figure 2.2.** Dependence of the catalytic activity of subtilisin Carlsberg in several anhydrous organic solvents on the specific activity of the enzyme in water. Data are shown for the enzymatic transesterification (at 5 mg·mL$^{-1}$ of subtilisin) between N-Ac-L-Ser-OEt and 1-propanol in carbon tetrachloride. The linear dependency observed proves that the enzymatic reaction rateis not limited by internal or external mass transfer of the substrate. Enzyme preparations of varying specific activities were produced by inactivating a portion of the enzyme with PMSF.

organic solvents were not limited by internal or external mass transfer, as has been recently suggested in the literature (Kamat, *et al.*, 1992), the reaction rate for each substrate in several organic solvents was shown to be directly proportional to the specific activity of the enzyme (Boudart and Burwell, 1973) (e.g., Fig. 2.2 depicts representative data for the Ser substrate in three different anhydrous solvents).

Enzyme preparations of various specific activities were prepared by mixing a 5 $mg \cdot mL^{-1}$ solution of catalytically active subtilisin Carlsberg in 20 mM phosphate buffer (pH 7.8) with varying proportions of an identical solution made with PMSF-inactivated subtilisin Carlsberg. The solutions were lyophilized immediately after mixing. The specific activity of each preparation in water was determined by measuring the initial rates of enzymatic hydrolysis of N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide in 20 mM phosphate buffer (pH 7.8).

Inactivated subtilisin was prepared as follows (Fahrney and Gold, 1973): 2.5 mg of PMSF was dissolved in 0.4 mL of 1-propanol and added to 20 mL of 20 mM aqueous phosphate buffer (pH 7.8). Subtilisin (100 mg) was dissolved in the resulting solution, and allowed to react for 4 hr. Excess PMSF was removed by dialysis against 20 mM phosphate buffer (pH 7.8).

**Kinetic calculations.** The subtilisin-catalyzed transesterification of an N-acetyl amino acid ethyl ester with 1-propanol follows the ping-pong, bi-bi mechanism: (Zaks and Klibanov, 1988)




(2.8)

where AcE is the acyl-enzyme, and ES and AcE·PrOH are the Michaelis complexes for the free enzyme and for the acyl-enzyme, respectively. The initial velocity of the reaction ($v_i$) is $d[P_1]/dt = k_2[ES]$. Using the steady-state assumption, one obtains:

$$v_i = \frac{k_2[E][S]}{K_{M,S}} \tag{2.9}$$

where $K_{M,S} = (k_{-1} + k_2)/k_1$ and $k_2 = k_{cat}$. For the two substrates, Ser and Phe, the ratio of the initial rates is:

$$\frac{v_{Ser}}{v_{Phe}} = \frac{k_{2,Ser}[E][Ser]/K_{M,Ser}}{k_{2,Phe}[E][Phe]/K_{M,Phe}} \tag{2.10}$$

If the two substrates compete for the same populations of free enzyme (i.e., are present in the same reaction mixture), [E] is identical in the numerator and denominator of equation 2.10. If the substrate concentrations are made equal, they will cancel each other out. Then:

$$\frac{v_{Ser}}{v_{Phe}} = \frac{(k_2/K_M)_{Ser}}{(k_2/K_M)_{Phe}} \tag{2.11}$$

If the ping-pong kinetic scheme is solved with respect to $[E]_o$ instead of [E], then the expressions for $k_{cat}$ and $K_{M,S}$ are given by the following formulas:

$k_{cat} = k_2k_4/(k_2 + k_4)$ and $K_{M,S} = k_4(k_{-1} + k_2)/k_1(k_2 + k_4)$. Note that although these expressions are distinct from those in equation 2.9, the expression for $k_{cat}/K_{M,S}$ is the same.

## III. Prediction of Substrate Specificity using UNIFAC

### A. Introduction

Discovery that substrate specificity of enzymes is markedly dependent on the solvent (Zaks and Klibanov, 1986; Gaertner and Puigserver, 1989; Clapés and Adlercreutz, 1991; Gololobov, *et al.*, 1992; Ryu and Dordick, 1992; Wescott and Klibanov, 1993a) has prompted a search for a mechanistic explanation of this phenomenon. Recently, we have succeeded (Wescott and Klibanov, 1993a) in rationalizing the observed solvent effect on the transesterification reactions of N-Ac-L-Ser-OEt (Ser) and N-Ac-L-Phe-OEt (Phe) with 1-propanol catalyzed by the protease subtilisin Carlsberg. A thermodynamic model has been elaborated which explains a strong preference of the enzyme for Ser in some anhydrous solvents and for Phe in others on the basis of substrate solvation/desolvation differences. Furthermore, a mathematical equation has been derived which relates the substrate specificity in an organic solvent to that in water and to the solvent-to-water partition coefficients (P) of the substrates:

$$\log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{solvent} = \log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{water} + \log\frac{P_{Phe}}{P_{Ser}} \qquad (3.1)$$

where $k_{cat}$ and $K_M$ are the turnover number and the Michaelis constant for the ester substrate, respectively, in the medium indicated. When we measured and plotted the substrate specificities of subtilisin *vs.* the experimentally determined ratio of the partition coefficients, the expected linear dependence indeed ensued (Wescott and Klibanov, 1993a).

A fundamental limitation of this approach, however, is that it can be used only with water-immiscible solvents because direct measurements of partition coefficients between water and water-miscible solvents are not feasible. In addition, even the data obtained for water-immiscible solvents are quite imperfect. First, due to mixing of the solvent and

water during the measurement of partition coefficients, the actual two phases in contact are unavoidably water-saturated solvent and solvent-saturated water, instead of the anhydrous solvent and pure aqueous solution in which the substrate specificities are measured. Second, 1 M propanol is present in the nonaqueous reaction medium as a nucleophile during the specificity measurements. The partition coefficients, however, cannot be determined in its presence because the propanol will partition into the aqueous phase. Finally, the measurement of partition coefficients is rather laborious and time-consuming.

In the present work, we eliminate the aforementioned problems and broaden the utility of our approach by using a computer program to calculate the P ratio of the substrates for different solvents.

## B. Results and Discussion

It can be shown[1] that

$$\frac{P_{Phe}}{P_{Ser}} = \left(\frac{\gamma_{Ser}}{\gamma_{Phe}}\right)_{solvent} \times \left(\frac{\gamma_{Phe}}{\gamma_{Ser}}\right)_{water} \qquad (3.2)$$

where $\gamma$ is the thermodynamic activity coefficient of the substrate indicated. The $\gamma$ values for a given molecule in a solvent can be calculated on the basis of the Van der Waals

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[1] The partition coefficient of Phe ($P_{Phe}$) is defined by the expression: $P_{Phe} = [Phe]_{solvent} / [Phe]_{water}$ where the brackets represent the molar concentration in the indicated phase at equilibrium. The thermodynamic activity (a) is related to the activity coefficient ($\gamma$) and the mole fraction (x) by: $a_{Phe} = \gamma_{Phe}\, x_{Phe}$. Since at equilibrium $a_{Phe}$ is the same in both phases: $(\gamma_{Phe})_{water} / (\gamma_{Phe})_{solvent} = (x_{Phe})_{solvent} / (x_{Phe})_{water}$. For dilute solutions, $n_{Phe} << n_{solvent}$, where n is the number of moles of the designated component, and thus: $(x_{Phe})_{solvent} \approx n_{Phe} / n_{solvent} = [Phe]_{solvent} / [solvent]$. Likewise, $(x_{Phe})_{water} \approx [Phe]_{water} / [water]$. Substitution of these expressions for x into the expression for the $\gamma$ ratio yields: $(\gamma_{Phe})_{water} / (\gamma_{Phe})_{solvent} = ([Phe]_{solvent} / [Phe]_{water})\,([water] / [solvent])$. Solving this equation for $[Phe]_{solvent} / [Phe]_{water}$ and substituting it into the expression for $P_{Phe}$ yields: $P_{Phe} = \{(\gamma_{Phe})_{water} / (\gamma_{Phe})_{solvent})\}\,([solvent] / [water])$. When the same procedure is repeated for N-Ac-L-Ser-OEt, and the ratio of $P_{Phe} / P_{Ser}$ is expressed, the concentrations of solvent and water cancel out, yielding equation (2).

volumes and surface areas of the constituent groups of that molecule and of those of the solvent and empirically determined interaction parameters between these groups (Fredenslund *et al.*, 1977). Such calculations can be carried out using the UNIFAC group contribution algorithm (Fredenslund *et al.*, 1977; Steen, *et al.*, 1979; Rasmussen and Fredenslund, 1982; Macedo, *et al.*, 1983; Teigs, *et al.*, 1987; Hansen, *et al.*, 1991). Unfortunately, insufficient UNIFAC interaction parameters are available in the literature to calculate the activity coefficients for N-Ac-Phe-OEt and N-Ac-Ser-OEt. However, log P of a molecule is an additive function of its component groups(Rekker, 1977). Thus, when calculating the P ratios for two similar molecules, the contributions of identical groups which exist in both will cancel out. Because our two substrates differ only in that the hydroxyl group in Ser is replaced by a phenyl group in Phe, $P_{Phe}/P_{Ser} = P_{tol}/P_{MeOH}$, where $P_{tol}$ and $P_{MeOH}$ are the corresponding partition coefficients for toluene and methanol, respectively. Consequently, we have written a computer program which implements UNIFAC to calculate $\gamma_{tol}$ and $\gamma_{MeOH}$ in organic solvents containing 1 M propanol; the $\gamma$ ratio in water has been determined experimentally.[2] Then equation 3.2 was employed to calculate the $P_{Phe}/P_{Ser}$ ratios.

Table 3.1 contains the calculated $\gamma$ values and the resultant P ratios of the substrates for 8 water-miscible, as well as 11 water-immiscible, solvents. According to equation 3.1, a double-logarithmic plot of substrate specificity *vs.* $P_{Phe}/P_{Ser}$ should yield a straight line with a slope of unity and an intercept equal to the logarithm of the substrate specificity in water (-1.7) (Wescott and Klibanov, 1993a). When such a plot is produced using the calculated values of $P_{Phe}/P_{Ser}$ (Figure 3.1), linear regression yields a slope of 0.89 and an intercept of -1.7, with a correlation coefficient of 0.96.

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[2] Since 10 mM toluene is insoluble in water under our conditions, we could not use UNIFAC to calculate $(\gamma_{tol})_{water}$. Instead, we rewrote equation (2) for methanol and toluene and used it to calculate $(\gamma_{tol}/\gamma_{MeOH})_{water}$ from the experimentally determined values of the P ratios and the calculated values of the $\gamma$ ratios in water-immiscible solvents. The average of the resultant values was $88 \pm 17$.

**Table 3.1.** Calculated Activity Coefficients for Methanol and Toluene and Calculated Partition Coefficient Ratios for N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt[a]

| solvent | $\gamma_{MeOH}$ | $\gamma_{tol}$ | $\gamma_{MeOH}/\gamma_{tol}$ | $P_{Phe}/P_{Ser}$[b] |
|---|---|---|---|---|
| water | *c* | *c* | 0.011[c] | 1 |
| *water-miscible* | | | | |
| *tert*-butanol | 1.1 | 3.5 | 0.32 | 28 |
| acetonitrile | 2.1 | 3.8 | 0.55 | 48 |
| dioxane | 2.0 | 3.2 | 0.63 | 56 |
| pyridine | 1.1 | 1.7 | 0.64 | 56 |
| acetone | 2.3 | 1.8 | 1.3 | 120 |
| 2-butanone | 2.4 | 1.3 | 1.8 | 160 |
| methyl acetate | 2.8 | 1.6 | 1.8 | 160 |
| tetrahydrofuran | 2.9 | 1.5 | 2.0 | 170 |
| *water-immiscible* | | | | |
| *tert*-amyl alcohol | 1.1 | 2.9 | 0.39 | 34 |
| ethyl acetate | 2.8 | 1.4 | 2.1 | 180 |
| isopropyl acetate | 2.9 | 1.2 | 2.4 | 210 |
| *tert*-butyl acetate | 2.9 | 1.2 | 2.4 | 210 |
| diethyl ether | 3.8 | 1.3 | 2.8 | 250 |
| chloroform | 4.1 | 0.74 | 5.5 | 480 |
| octane | 7.9 | 1.4 | 5.6 | 490 |
| dichloromethane | 4.7 | 0.76 | 6.2 | 540 |
| toluene | 6.4 | 1.0 | 6.2 | 550 |
| benzene | 7.2 | 0.97 | 7.5 | 660 |
| carbon tetrachloride | 8.5 | 0.99 | 8.6 | 750 |

[a] Activity coefficients ($\gamma$) for 10 mM each methanol (MeOH) and toluene (tol) in the indicated organic solvents containing 1 M 1-propanol were calculated using the UNIFAC group-contribution method. [b] Partition coefficient ratios were calculated from activity coefficients as described in footnote 1. Note that the calculated partition coefficient ratios are different from those measured experimentally (Wescott and Klibanov, 1993a). This is because the latter involve partitioning between water-saturated solvents and solvent-saturated water and do not include 1 M propanol. In contrast, the calculated values were for the pure phases where the organic phase contained 1 M propanol. [c] Because $\gamma_{tol}$ could not be calculated in water, $\gamma_{MeOH}/\gamma_{tol}$ in water was calculated as described in footnote 2.

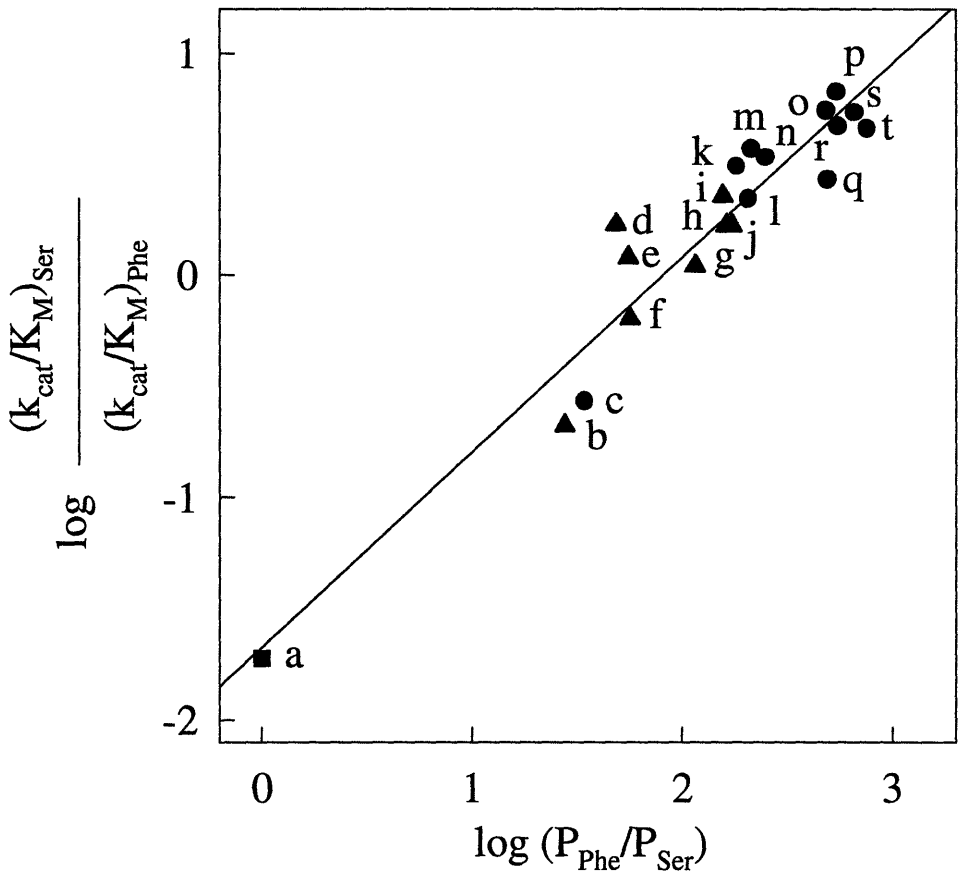



**Figure 3.1.** Dependence of the substrate specificity of subtilisin Carlsberg in water-miscible (triangles) and water-immiscible (circles) solvents on the ratio of the calculated solvent-to-water partition coefficients of N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt. Solvents: (a) water, (b) *tert*-butanol, (c) *tert*-amyl alcohol, (d) acetonitrile, (e) dioxane, (f) pyridine, (g) acetone, (h) 2-butanone, (i) methyl acetate, (j) tetrahydrofuran, (k) ethyl acetate, (l) isopropyl acetate, (m) *tert*-butyl acetate, (n) diethyl ether, (o) chloroform, (p) dichloromethane, (q) octane, (r) toluene, (s) benzene, and (t) carbon tetrachloride. The $k_{cat}/K_M$ values were measured as previously described (Wescott and Klibanov, 1993a).

The model on which equation 3.1 is based assumes that the substrates are fully removed from the solvent in the transition state (Wescott and Klibanov, 1993a). If one or both of the substrates are partially solvated in the transition state, then only a fraction of the free energy of solvation of the substrates is available to influence the enzymatic specificity. This may be the reason why the observed slope (0.88, Fig. 3.1) is slightly less than the expected value of 1.

In principle, our approach is independent of the enzyme (Wescott and Klibanov, 1993a) and thus should be of general significance. This fact, combined with the ability to calculate the P ratios of substrates by computer, allows the quantitative prediction of enzymatic specificity for various enzyme/substrate systems in any solvent, given the substrate specificity in a single reference solvent (e.g., in water, as herein).

## C. Materials and Methods

**Activity coefficient calculation.** All activity coefficients were calculated using the UNIFAC method (Fredenslund *et al.*, 1977; Steen, *et al.*, 1979; Rasmussen and Fredenslund, 1982; Macedo, *et al.*, 1983; Teigs, *et al.*, 1987; Hansen, *et al.*, 1991). Because UNIFAC is a group contribution method, it allows the estimation of activity coefficients in systems for which there is no experimental data by assessing the individual contribution of each group which makes up the system. Use of this method requires three types of parameters for each group in the system: the group's surface area, the volume of the group, and empirical parameters which reflect the free energy of interaction between a given group and every other group in the system (calculated via multiple linear regression of vapor-liquid equilibria data). See Methods in Chapter V for more details.

## IV. The Solvent Dependence of the Substrate Specificity of α-Chymotrypsin and Subtilisin Carlsberg toward Unnatural Substrates

### A. Introduction

In Chapters Two and Three, a methodology is developed for the prediction of the solvent dependence of enzymatic substrate specificity based on the thermodynamics of substrate desolvation. This methodology, based on equation 4.1, is validated in the

$$\log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{solvent} = \log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{water} + \log\frac{P_{Phe}}{P_{Ser}} \qquad (4.1)$$

examination of a single reaction system, namely the selective enzymatic transesterification of either N-Ac-L-Phe-OEt or N-Ac-L-Ser-OEt with propanol. Because the only specific assumption regarding the substrate made in the derivation of equation 4.1 is that it must be desolvated in the transition state (Wescott and Klibanov, 1993a), the abovementioned methodology should be applicable to any substrate that is so desolvated. Additionally, it has been observed that, while enzymes may be highly selective toward substrates derived from natural compounds, their selectivity toward compounds such as pharmaceuticals is often less than satisfactory (Lalonde, *et al.*, 1995). To test the generality of our predictive model to other substrates, and to address the question of its applicability to unnatural substrates, we herein examine the selectivity of subtilisin Carlsberg toward two unnatural substrates (methyl 2-hydroxyethanoate (**1**) and 2,2,2-trichloroethyl phenylacetate (**2**)) in their transesterification with propanol.

The derivation of equation 4.1 is not only unconstrained by the substrates, it is also independent of the enzyme. In this chapter, the applicability of equation 4.1 to other

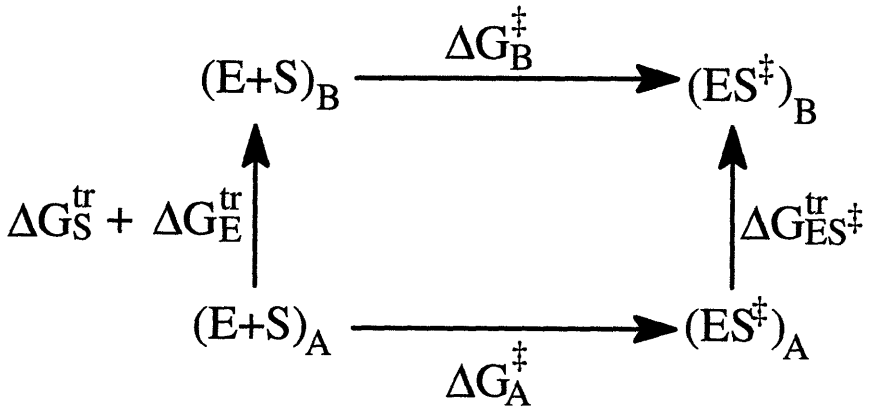
enzymes will also be tested by catalyzing the abovementioned propanolysis of **1** and **2** with α-chymotrypsin.

Because our original experimentally measurable parameter related to the free energy of desolvation of the substrate is the substrate's solvent-to-water partition coefficient, equation 4.1 is derived in terms of this quantity. Our subsequent ability to calculate the activity coefficients of the substrates in the various solvents employed as the reaction medium (Wescott and Klibanov, 1993b), however, has made the use of the partition coefficient unnecessary. Consequently, a variant of equation 4.1, which directly relates the substrate selectivity to the activity coefficients of the substrates, is derived below, and will be used throughout this chapter.

**B. Theory.**

The solvent dependence of enzymatic substrate selectivity can be directly related to the activity coefficients of the substrates through consideration of Scheme 4.1 (which is a modified version of Scheme 2.1 in Chapter 2).

**Scheme 4.1**



The lower horizontal arrow represents the enzyme (E) and substrate (S) reacting in solvent A to form the transition state ($ES^{\ddagger}$) in solvent A. Another, thermodynamically equivalent, path exists which leads to the formation of the same transition state solvated in solvent A. The enzyme and substrate are separately transferred to solvent B, where they react to form the transition state, which is subsequently transferred back to solvent A. Because the two alternative paths are thermodynamically equivalent,[3]

$$\Delta G_A^{\ddagger} = \Delta G_S^{tr} + \Delta G_E^{tr} + \Delta G_B^{\ddagger} - \Delta G_{ES^{\ddagger}}^{tr} \tag{4.2}$$

If the substrate is completely desolvated in the transition state, E and $ES^{\ddagger}$ will have identical solvent-accessible surface areas. Consequently, $\Delta G_E^{tr}$ and $\Delta G_{ES^{\ddagger}}^{tr}$ will be identical, and will cancel out of equation 4.2 to yield:

$$\Delta G_A^{\ddagger} = \Delta G_S^{tr} + \Delta G_B^{\ddagger} \tag{4.3}$$

The activation energy terms (the $\Delta G^{\ddagger}$ terms) are related to $k_{cat}/K_M$ in their solvents (A or B) by (Fersht, 1985):

$$\Delta G_{solvent}^{\ddagger} = -RT \ln\left[\left(\frac{k_{cat}}{K_M}\right)_{solvent}\left(\frac{h}{\kappa T}\right)\right] \tag{4.4}$$

The free energy of transfer terms (the $\Delta G^{tr}$ terms) are functions of the solute activity coefficients in solvents A and B. This becomes apparent when one considers that the free energy of a solute dissolved in a solvent is related to the solute mole fraction (x) and solute activity coefficient ($\gamma$) by:

---

[3] Each of the steps of the cycle in Scheme 1 is reversible. Single, rather than double, arrows are used in the scheme solely to illustrate the directionality in the definition of the energetic terms.

$$G = G^\circ + RT \ln (x\gamma) \tag{4.5}$$

Because the solvent is the primary variable in our work, the standard state is chosen as the pure liquid solute (i.e., Raoult's law activity coefficients are used). Thus $G^\circ$ is independent of the solvent, and the free energy of transfer of the solute from solvent A to B is described by:

$$\Delta G^{tr} = RT \ln(x_B\gamma_B / x_A\gamma_A) \tag{4.6}.$$

where x and $\gamma$ are the solute mole fraction and activity coefficient, respectively, in the solvent indicated by the subscript. Substitution of equations 4.4 and 4.6 into equation 4.3, and subsequent simplification yields:

$$(k_{cat}/K_M)_A = (\gamma_A/\gamma_B)(x_A/x_B)(k_{cat}/K_M)_B \tag{4.7}$$

For dilute solutions, $x_{solute} \approx n_{solute} / n_{solvent}$ = [solute] $V_M$, where $V_M$ is the molar volume of the solvent. For solvents A and B, $x_A / x_B$ = [solute] $V_{MA}$ / [solute] $V_{MB}$. If the solute concentration is kept the same in both solvents (i.e., its transfer from A to B is done at a constant molar concentration), [solute] cancels out, yielding: $x_A / x_B = V_{MA} / V_{MB}$, i.e., the ratio of the molar volumes of the solvents. Thus, the mole fraction ratio is independent of the substrate, and cancels out when equation 4.7 is expressed for two substrates, **1** and **2**, and the ratio is taken to solve for the log of the substrate specificity in solvent A:

$$\log\left[\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)_2}\right]_A = \log\left(\frac{\gamma_1}{\gamma_2}\right)_A + \log\left[\frac{\gamma_2(k_{cat}/K_M)_1}{\gamma_1(k_{cat}/K_M)_2}\right]_B \tag{4.8}$$

If solvent B is chosen as a fixed reference solvent (note that this can be chosen as any reaction medium and is unrelated to the standard state of the activity coefficients), the final term in equation 4.8 will be a constant:

$$\log\left[\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)_2}\right]_A = \log\left(\frac{\gamma_1}{\gamma_2}\right)_A + \textit{constant} \qquad (4.9)$$

Equation 4.9 relates the substrate specificity in any solvent A to the ratio of the substrate activity coefficients in the solvent. Note that although equation 4.9 differs from equation 4.1, the two equations are based on the same theory, i.e., that the solvent controls enzymatic substrate specificity through the differential free energy of desolvation of the substrates in the transition state. Equation 4.9 will be used throughout this chapter because it relates substrate specificity directly to the activity coefficients of the substrates, which can be calculated using UNIFAC (Fredenslund, *et al.*, 1977).

## C. Results and Discussion

To ascertain the generality of our model with respect to different substrates, we examined the subtilisin catalyzed propanolysis of **1** and **2** in a variety of organic solvents. Note that, unlike the amino acid ethyl esters used in the previous studies (Wescott and Klibanov, 1993a and 1993b), substrates **1** and **2** differ from each other not only in their acyl moieties, but also in their leaving groups.

The activity coefficient ratio of the substrates ($\gamma_1/\gamma_2$), calculated for a range of organic solvents, is reported in the fourth column of Table 4.1. It is evident from Table 4.1

Table 4.1. Thermodynamic activity coefficients and enzymatic substrate specificities for substrates **1** and **2** in various organic solvents.

| solvent | $\gamma_1$ [a] | $\gamma_2$ [a] | $\gamma_1/\gamma_2$ [a] | $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ [b] |
|---|---|---|---|---|
| *subtilisin* | | | | |
| propanol | 1.84 | 10.3 | 0.179 | 1.31 |
| *tert*-butanol | 2.05 | 9.76 | 0.210 | 1.08 |
| *tert*-amyl alcohol | 2.21 | 8.50 | 0.260 | 1.16 |
| acetonitrile | 0.815 | 1.54 | 0.529 | 5.35 |
| propionitrile | 1.20 | 0.782 | 1.53 | 4.53 |
| acetone | 1.59 | 0.471 | 3.38 | 10.3 |
| octane | 19.3 | 3.76 | 5.13 | 16.5 |
| hexane | 19.7 | 3.76 | 5.13 | 18.3 |
| chloroform | 1.55 | 0.191 | 8.12 | 20.5 |
| cyclohexane | 22.7 | 2.49 | 9.12 | 28.0 |
| dichloromethane | 2.15 | 0.175 | 12.3 | 38.4 |
| carbontetrachloride | 10.5 | 0.844 | 12.4 | 27.2 |
| benzene | 6.09 | 0.427 | 14.3 | 37.1 |
| *chymotrypsin* | | | | |
| *tert*-amyl OH | 1.95 | 8.33 | 0.234 | 0.022 |
| acetonitrile | 0.868 | 1.23 | 0.706 | 0.090 |
| propionitrile | 1.22 | 0.727 | 1.68 | 0.530 |
| ethyl acetate | 2.49 | 0.603 | 4.13 | 1.05 |
| hexane | 56 | 6.47 | 8.66 | 45.7 |
| carbontetrachloride | 21 | 1.52 | 13.8 | 8.72 |
| benzene | 9.37 | 0.611 | 15.3 | 31.3 |

[a] Activity coefficients and coefficient ratios were calculated for the reaction conditions used in the measurement of $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$. See Materials and Methods for the specific reaction conditions. [b] See Materials and Methods for details on the measurement of $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$.

that $\gamma_1/\gamma_2$ changes by a factor of 80 when the solvent is changed from propanol to benzene. As predicted by equation 4.9 a similar change in substrate specificity (here defined as $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ ) of subtilisin (reported in column 5 of Table 4.1) ensues.

Equation 4.9 further predicts that a double logarithmic plot of subtilisin's substrate selectivity vs. $\gamma_1/\gamma_2$ will follow a linear dependence, with unity slope. Such a plot is presented in Figure 4.1. Linear regression reveals a slope of 0.78 , and an $R^2$ value of 0.96. While the experimental data adhere to the linear dependence dictated by equation 4.9, the observed slope is slightly lower than that predicted by theory. The primary assumption made in the derivation of equation 4.9 is that the substrate is completely shielded from the solvent by the enzyme in the transition state. If, however, the substrate is partially solvated in the transition state, only a fraction of the free energy of desolvation will be available to influence the substrate specificity. Preliminary molecular modeling studies indicate that the transition states are indeed approximately 30% solvated. This may explain the slight deviation of the observed slope from unity. Overall, the experimental data correlate well with the theoretical predictions, thus it can be concluded that the model is generally applicable to various substrates, so long as they are substantially desolvated in the enzyme-bound transition state.

In addition to being able to explain the solvent dependence of selectivity for different types of substrates, our methodology should also be valid when different enzymes are employed. As a test of this enzyme independence, the propanolysis of substrates **1** and **2** has been observed once again, this time catalyzed by α-chymotrypsin. The substrate specificity for the reaction, measured in a variety of organic solvents, is

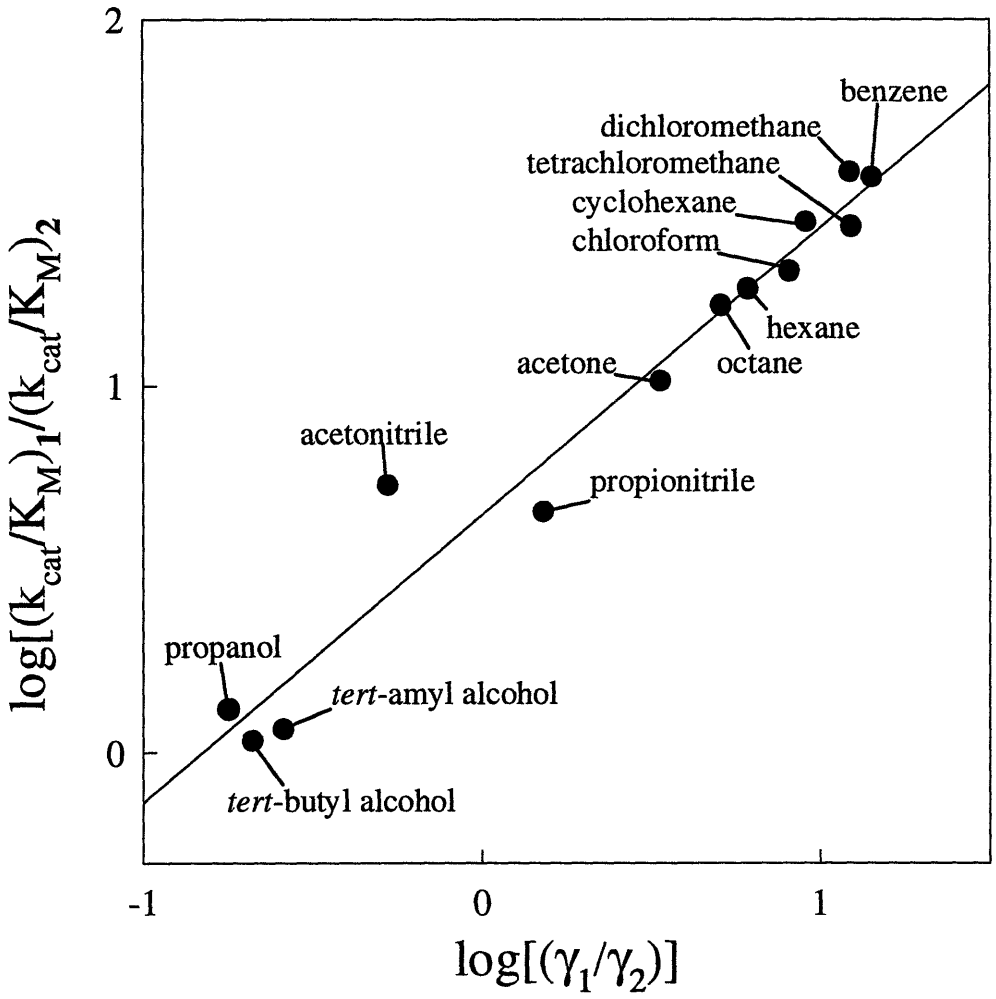



**Figure 4.1.** Dependence of subtilisin Carlsberg substrate specificity for unnatural substrates on the ratio of the substrate activity coefficients. $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ ratios were measured as described in Materials and Methods. Activity coefficient ratios were calculated as explained in the footnote to Table 4.1.

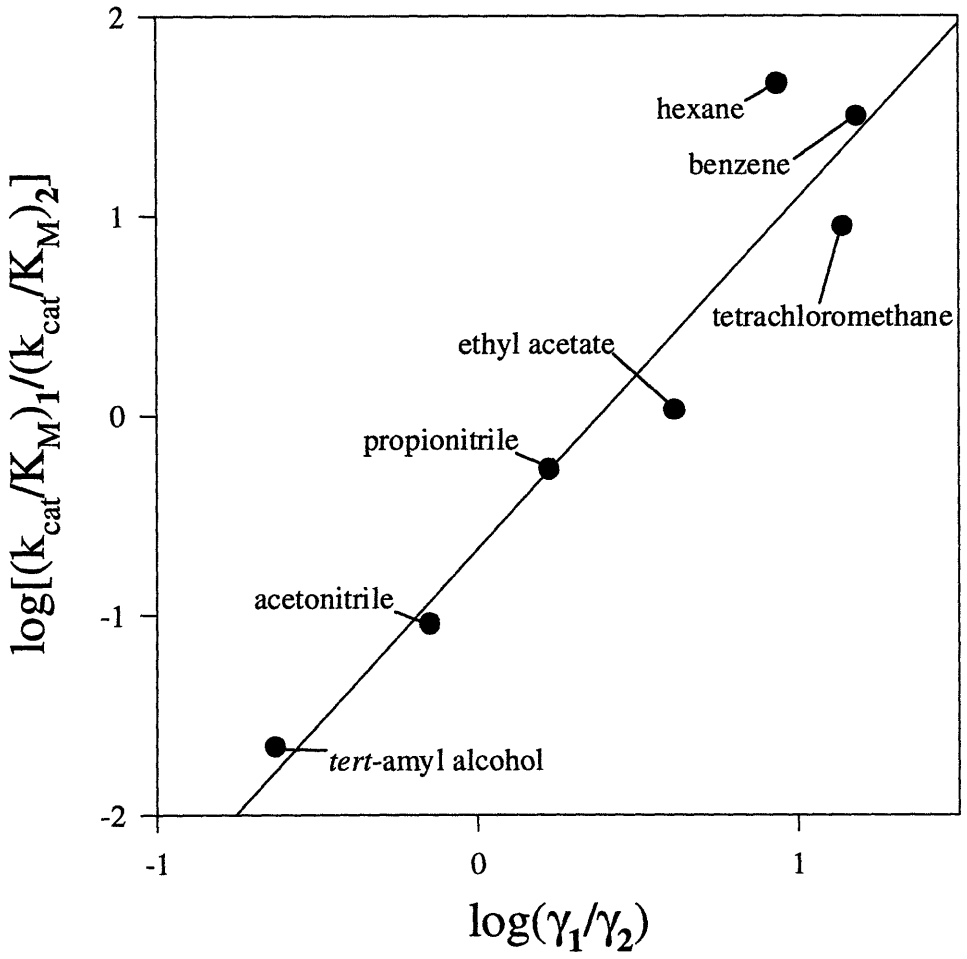



**Figure 4.2.** Dependence of α-chymotrypsin substrate specificity for unnatural substrates on the ratio of the substrate activity coefficients. $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ ratios were measured as described in Materials and Methods. Activity coefficient ratios were calculated as explained in the footnote to Table 4.1.

reported in the last column of Table 4.1. A double logarithmic plot of the selectivity vs. the activity coefficient ratio (Figure 4.2) demonstrates the linear dependence predicted by equation 4.9 ($R^2$=0.92), demonstrating that the methodology is able to qualitatively describe the solvent dependence of substrate selectivity for enzymes in general.

Although equation 4.9 qualitatively fits the data in figure 4.2, the slope of the plot is 1.8, rather than the predicted slope of one. Slopes of less than 1 can easily be explained by the current level of analysis as a result of incomplete desolvation of the substrate in the transition state. Slopes exceeding unity, however, cannot be understood using the present model. This apparent shortcoming has prompted a more rigorous examination of the effects of partial transition state solvation (detailed in the following chapter), which not only allows the explanation of the observed slope of Figure 4.2, but also extends our methodology to the prediction of the solvent dependence of a more biotechnologically important class of enzymatic selectivity, namely stereoselectivity, on the basis of partial solvation of the substrate in the transition state.

## D. Materials and Methods.

**Chemicals and solvents** were purchased from Aldrich Chemical Co. The organic solvents were of the highest purity available from that vendor (analytical grade or better) and were dried prior to use to a water content below 0.01% (as determined by the Karl Fischer titration (Laitinen and Harris, 1975)) by shaking with Linde's 3-Å molecular sieves.

**Enzymes.** Subtilisin Carlsberg (serine protease from Bacillus licheniformis, EC 3.1.1.3) and α-chymotrypsin (EC 3.4.21.1) were purchased from Sigma Chemical Co. The enzymes were prepared by lyophilization from a 5 $mg \cdot mL^{-1}$ solution in 20 mM aqueous potassium phosphate buffer (pH 7.8). Lyophilized enzyme powders were stored over anhydrous $CaSO_4$ in an evacuated desiccator at $4^oC$.

**Kinetic measurements.** $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ ratios (determined from the initial velocity ratios for each substrate as described previously (Wescott and Klibanov, 1993a)) for subtilisin in organic solvents were measured as follows. Both ester substrates were placed in the same vial with 5 $mg \cdot mL^{-1}$ lyophilized enzyme powder and 1 M 1-propanol. The millimolar concentrations of **1** and **2**, respectively, were: propanol, 100 and 100; *tert*-butanol, 100 and 100; *tert*-amyl alcohol, 100 and 100; acetonitrile, 100 and 100; propionitrile, 100 and 100; acetone, 50 and 200; octane, 50 and 200; hexane, 50 and 200; chloroform, 50 and 200; cyclohexane, 50 and 200; dichloromethane, 50 and 200; carbontetrachloride, 50 and 200; benzene, 50 and 200. The suspension was shaken at $30^oC$ and 300 rpm. Periodically, a 2-μL sample was withdrawn and assayed by gas chromatography.

For the α-chymotrypsin-catalyzed reactions, $(k_{cat}/K_M)_1/(k_{cat}/K_M)_2$ ratios (determined from the initial velocity ratios for each substrate as described previously (Wescott and Klibanov, 1993a)) in organic solvents were measured as follows. Both ester substrates were placed in the same vial with 20 $mg \cdot mL^{-1}$ lyophilized enzyme powder and 100 mM 1-propanol. The millimolar concentrations of **1** and **2**, respectively, were: *tert*-amyl OH, 100 and 10; acetonitrile, 10 and 10; propionitrile, 10 and 10; ethyl acetate, 10

and 10; hexane, 10 and 100; carbontetrachloride, 10 and 100; benzene, 10 and 100. The suspension was shaken at 45°C and 300 rpm. Periodically, a 10-μL sample was withdrawn and subjected to precolumn derivatization with a 1:1 mixture of trimethylchlorosilane and pyridine, followed by analysis of 1-μL of the derivatized sample by gas chromatography. The lower ester and alcohol concentrations used for the chymotrypsin-catalyzed reactions were necessary to enable the use of the precolumn derivatization method. Precolumn derivatization, higher enzyme concentrations, and higher reaction temperature (relative to those conditions used for subtilisin), were needed to overcome the lower activity of chymotrypsin (relative to subtilisin).

**1** was synthesized by refluxing 0.5 g of glycolic acid in 25 mL of anhydrous methanol containing 5 drops of concentrated $H_2SO_4$ for 12 h. The reaction mixture was subsequently concentrated by rotary evaporation, dissolved in 50 mL of diethyl ether, and then washed with five 10-mL aliquots of 5% $NaHCO_3$ and with 10 mL of deionized water. The crude product was recovered from the organic phase by rotary evaporation and subsequently purified by vacuum distillation.

**2** was synthesized by refluxing 0.5 g of phenylacetic acid in 35 mL of 2,2,2-trichloroethanol containing 5 drops of concentrated $H_2SO_4$ for 24 h. The reaction mixture was processed as for **1**, except the product was purified by preparative TLC rather than by vacuum distillation.

**Propyl glycolate**, used to calibrate the gas chromatograph, was synthesized as was **1**, except 50 mL of anhydrous propanol was used instead of methanol.

**Propyl phenylacetate**, used to calibrate the gas chromatograph, was produced by refluxing 0.5 g of phenylacetic acid in 30 mL of anhydrous propanol containing 5 drops of concentrated $H_2SO_4$ for 12 hours. Workup of the reaction mixture followed the procedure used for **1**. The crude product was subsequently purified by vacuum distillation.

## V. The Solvent Dependence of Enzymatic Stereoselectivity

### A. Introduction

One of the most profound revelations arisen from nonaqueous enzymology (Klibanov, 1989; Chen and Sih, 1989; Dordick, 1989; Klibanov, 1990; Gupta, 1992; Faber and Riva, 1992; Halling, 1994; Koskinen and Klibanov, 1996) is the discovery that the specificity of an enzyme strongly depends on the solvent (Wescott and Klibanov, 1994; Carrea *et al.*, 1995). Of all the types of enzyme specificity found to be controlled by the solvent — enantioselectivity, prochiral selectivity, substrate specificity, regioselectivity, and chemoselectivity — the first two are particularly important for synthetic applications (Simon *et al.*, 1985; Yamada and Shimizu, 1988; Jones, 1986; Faber, 1992; Poppe and Novak, 1992; Sheldon, 1993; Margolin, 1993; Wong and Whitesides, 1994; Roberts, *et al.*, 1995; Drauz and Waldman, 1995). Indeed, if generalized and understood, solvent control of enzymatic stereoselectivity should enhance the utility of biocatalysis in organic chemistry by allowing the rational manipulation of the stereochemical outcome of asymmetric transformations simply by altering the reaction medium. The ultimate challenge in this regard is to learn how to predict enzyme selectivity as a function of the solvent.

As a first step toward this goal, we have recently elaborated a thermodynamic model which explains the substrate specificity of the protease subtilisin Carlsberg in organic solvents on the basis of solvent-to-water partition coefficients of the substrates (Wescott and Klibanov, 1993a). These partition coefficients can be either measured

experimentally or calculated using the UNIFAC computer algorithm (Wescott and Klibanov, 1993b). An explicit assumption of our analysis is that the substrates are fully desolvated, i.e., inaccessible to the solvent, in the enzyme-bound transition state. This assumption precludes the extension of the proposed model to enantioselectivity, since the partition coefficients for different enantiomers of the same compound are identical. Likewise, prochiral, regio-, and chemo- selectivities cannot be analyzed either, because in all these instances the same substrate molecule (just different parts of it) reacts with the enzyme.

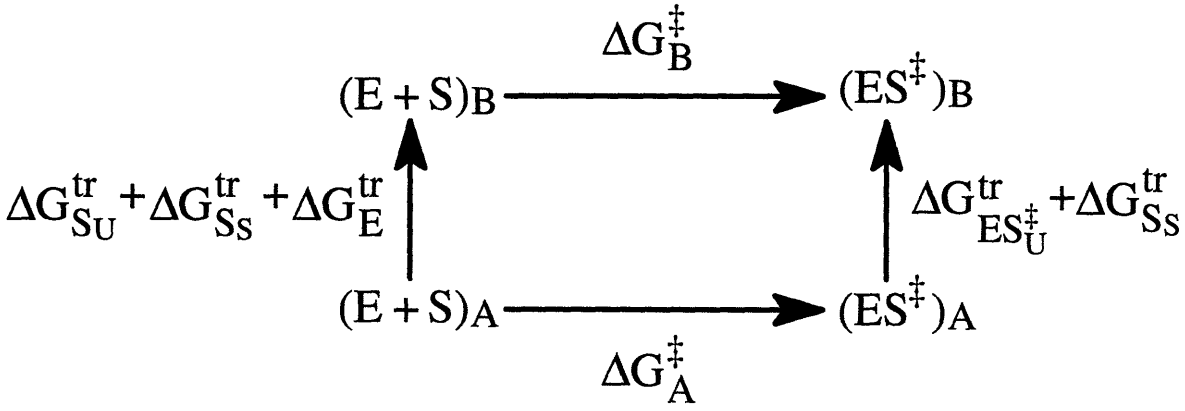
In the present study, we further develop and broaden our thermodynamic treatment to eliminate the aforementioned limitations. The resultant model, tested herein with enantioselectivity, takes into account variations in substrate desolvation in the transition states for *R* and *S* substrates. Thermodynamic activity coefficients of the desolvated portions of the substrate, calculated using computer-generated, transition-state structures and UNIFAC, correctly predict the solvent dependence of enantioselectivity of crystalline chymotrypsin.

## B. Theory

The solvent may influence enzymatic selectivity through several distinct mechanisms. For instance, it could change the enzyme conformation and thus affect the selectivity of the reaction by altering enzyme-substrate interactions (Wu *et al.*, 1991; Ueji, *et al.*, 1992). In the present work, this mechanism is selected against through the use of crystalline enzyme, for which conformations have been shown to be essentially unaffected

by replacement of water by organic solvents as the medium (Fitzpatrick, *et al.*, 1993; Fitzpatrick, *et al.*, 1994; Yennawar, *et al.*, 1994; Yennawar, *et al.*, 1995). Alternatively, solvent molecules could bind within the enzyme active site and block the normal binding mode of the substrate (Nakamura *et al.*, 1991; Secundo, *et al.*, 1992).[4] While these two possible mechanisms do not necessarily influence selectivity, a third, driven by the energetics of substrate solvation, must do so, regardless of the presence of other mechanisms. Indeed, it has been demonstrated that the energetics of substrate solvation is the dominant means by which the solvent influences the substrate specificity of subtilisin (Wescott and Klibanov, 1993a,b).

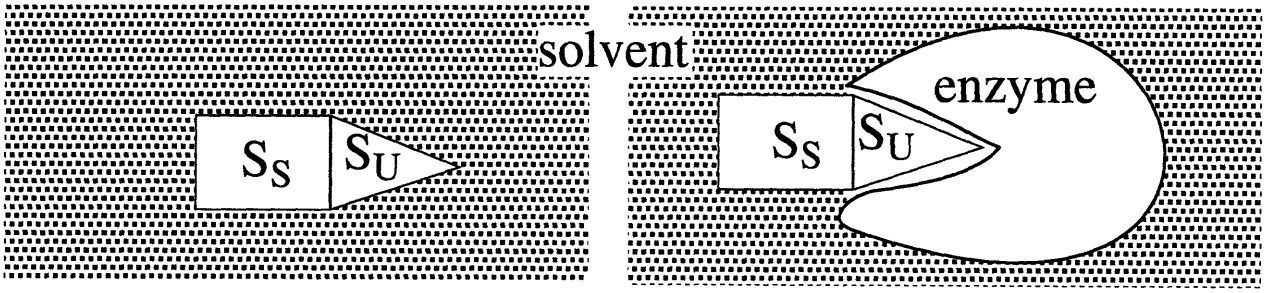
**Scheme 5.1**




[4] It is likely (Fitzpatrick, *et al.*, 1993; Fitzpatrick, *et al.*, 1994; Yennawar, *et al.*, 1994; Yennawar, *et al.*, 1995) that the active center of the enzyme is occupied by at least a few solvent molecules when a substrate molecule is not bound. These bound solvent molecules would be in a dynamic equilibrium between the enzyme active center and the bulk solvent, described by a binding constant (effectively an inhibition constant, $K_I$). If $1/(K_I V_M) << [S]/K_M$ (where $V_M$ is the molar volume of the solvent), the bound solvent would exert little effect on the catalytic properties of the system. If, on the other hand, the opposite were true, the solvent would act as an effective inhibitor. If such a tightly bound solvent molecule affects each substrate binding mode equally, it would not influence the prochiral selectivity. If the solvent molecule is tightly bound in such a way that it only hinders one substrate binding mode, the prochiral selectivity would be affected in a manner that could not be predicted by the treatment used in the present work.

The contribution of solvation energies to the solvent dependence of enzyme kinetics is demonstrated by the thermodynamic cycle in Scheme 5.1 (a modification of Scheme 5.1 in Chapter IV). The lower horizontal arrow represents the enzyme (E) and the substrate (S) reacting in solvent A to form the transition state $(ES^{\ddagger})_A$ with an activation free energy of $\Delta G^{\ddagger}_A$. This transition state spontaneously decomposes to ultimately form the free enzyme and products. An alternative, hypothetical path exists leading to the same transition state complex. In this path, the substrate and enzyme are separately transferred from solvent A to solvent B, where they react to form the transition state $(ES^{\ddagger})_B$, which is subsequently transferred back to solvent A. For thermodynamic purposes, the substrate can be represented as the sum of two portions (see Scheme 5.2), that which is solvated in the transition state ($S_S$) and that which is enveloped by the enzyme and is thus unsolvated in the transition state ($S_U$).

**Scheme 5.2**




solvation of free substrate ($S_S + S_U = S$)

solvation of enzyme-substrate transition state complex

Similarly, the transition state is regarded as the sum of $S_S$ and another portion which includes only the enzyme and the unsolvated substrate moiety in the transition state ($ES_U^{\ddagger}$). $\Delta G_A^{\ddagger}$ can be expressed as the sum of the energetic terms of the alternative path:[5]

$$\Delta G_A^{\ddagger} = \Delta G_B^{\ddagger} + \Delta G_E^{tr} + \Delta G_{S_S}^{tr} + \Delta G_{S_U}^{tr} - \Delta G_{ES_U^{\ddagger}}^{tr} - \Delta G_{S_S}^{tr} \qquad (5.1)$$

where $\Delta G_B^{\ddagger}$ is the activation free energy for the reaction in solvent B, and $\Delta G^{tr}$ is the free energy of transfer of the moiety indicated in the subscript from solvent A to B. Assuming that the solvated surfaces of E and $ES_U^{\ddagger}$ for low-molecular-weight substrates are identical,[6] $\Delta G_E^{tr} = \Delta G_{ES_U^{\ddagger}}^{tr}$. Consequently, equation 5.1 can be simplified to:

$$\Delta G_A^{\ddagger} = \Delta G_B^{\ddagger} + \Delta G_{S_U}^{tr} \qquad (5.2)$$

$\Delta G^{\ddagger}$ is related to $k_{cat}/K_M$ as (Fersht, 1985):

$$\Delta G^{\ddagger} = -RT \ln \left[ \left( \frac{k_{cat}}{K_M} \right) \left( \frac{h}{\kappa T} \right) \right] \qquad (5.3)$$

where h, $\kappa$, R, $k_{cat}$, $K_M$ and T are the Planck, Boltzmann, gas, catalytic, and Michaelis constants and temperature, respectively. $\Delta G^{tr}$ can be expressed in terms of thermodynamic activity coefficients as RT ln ($x_B\gamma_B/x_A\gamma_A$), where $\gamma$ and x are the activity

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[5] Each of the steps of the cycle in Scheme 1 is reversible. Single, rather than double, arrows are used in the scheme solely to illustrate the directionality in the definition of the energetic terms.

[6] Because the conformations of serine proteases are not altered in the transition state (Fersht, 1985), the solvent-accessible surface of the enzyme is assumed to be the same in ground and transition states. The only difference between E and $ES_U^{\ddagger}$ is the addition of $S_U$, which is unsolvated and thus does not contribute to the solvated surface of the complex.

coefficient and mole fraction, respectively, of the solute in the indicated solvent (see Chapter IV). If $\gamma'$ is defined as the activity coefficient of the *unsolvated* substrate moiety ($S_U$), then:

$$\Delta G^{tr}_{S_U} = RT \ln\left(x_B \gamma'_B / x_A \gamma'_A\right) \tag{5.4}$$

Substituting equations 5.3 and 5.4 into 5.2 yields:

$$\left(k_{cat}/K_M\right)_A = \left(\gamma'_A/\gamma'_B\right)\left(x_A/x_B\right)\left(k_{cat}/K_M\right)_B \tag{5.5}$$

Note that for dilute solutions $x_A/x_B$ depends only on the molar volume of the solvents when the transfer is done at constant molar concentration (see Chapter IV). The mole fraction ratio is thus the same for any substrate and cancels out when equation 5.5 is expressed for two substrates, I and II, and solved for the logarithm of the selectivity in solvent A:

$$\log\left[\frac{\left(k_{cat}/K_M\right)_I}{\left(k_{cat}/K_M\right)_{II}}\right]_A = \log\left(\frac{\gamma'_I}{\gamma'_{II}}\right)_A + \log\left[\frac{\gamma'_{II}\left(k_{cat}/K_M\right)_I}{\gamma'_I\left(k_{cat}/K_M\right)_{II}}\right]_B \tag{5.6}$$

Equation 5.6 expresses the general relationship between enzymatic selectivity and solvent-transition-state interactions. In the present work, we specifically test equation 5.6 with respect to enantioselectivity. While chemically identical substrates lead to both the *R* and *S* products, the reactions proceed through conformationally distinct transition states for the production of each enantiomer. Thus, differences between $\gamma'_I$ and $\gamma'_{II}$ arise from variations in transition state solvation, not from chemical differences between two substrates. The parameters for substrates I and II in equation 5.6 can therefore be replaced with those for the *R* and *S* reaction pathways to describe enantioselectivity. Also,

if B is fixed as a reference solvent, the final term in equation 5.6 will be a constant. Consequently, one arrives at the following equation describing the solvent dependence of the enantioselectivity of an enzyme in terms of a $\gamma'$ ratio:

$$\log\left[\frac{(k_{cat}/K_M)_S}{(k_{cat}/K_M)_R}\right] = \log\left(\frac{\gamma'_S}{\gamma'_R}\right) + \textit{constant} \qquad (5.7)$$

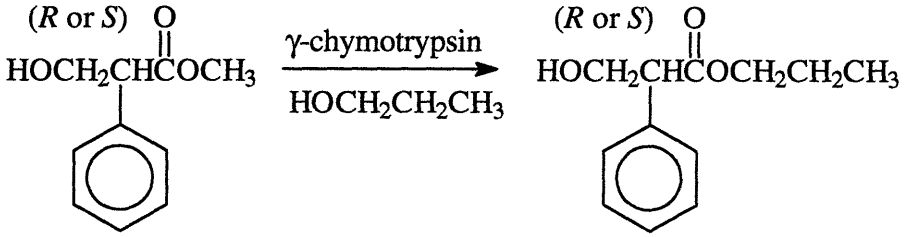
Unlike the situation for substrate specificity, where solvent-dependent variation in the activity coefficient ratio for the two substrates is primarily driven by chemical differences between the substrates, $\gamma'$ for enantioselectivity differs for each enantiomer only due to differences in transition state solvation. In this work, we calculate $\gamma'$ for both the *R* and *S* transition states using a three-step procedure. First, the desolvated portion of the substrate in the transition state is determined using molecular modeling based on the crystal structure of the enzyme. Second, this desolvated moiety is expressed in terms of distinct chemical groups to yield a model compound which approximates the portion of the substrate removed from the solvent in the transition state. Finally, the thermodynamic activity coefficient of this model compound is calculated using UNIFAC and then equated to $\gamma'$. According to equation 5.7, knowing only $\gamma'_R$ and $\gamma'_S$ for a series of solvents, it should be possible to predict the solvent dependence of enantioselectivity.

## C. Results and Discussion.

As an initial test of the ability of the model described above to predict the solvent dependence of enantioselectivity, we examined the transesterification of racemic **3** with propanol catalyzed by cross-linked crystals (St. Clair and Navia, 1992; Sobolov, *et al.*,

1994; Persichetti, *et al.*, 1995; Lalonde, *et al.*, 1995; Sobolov, *et al.*, 1996; Schmitke, *et al.*, 1996) (CLCs) of γ-chymotrypsin (Scheme 5.3).

**Scheme 5.3**




**1**

Various esters of the acid moiety of **3** (3-hydroxy-2-phenylpropionic acid, **4**) are potent anticholinergics, including atropine, hyoscyamine, and scopolamine (Reynolds, 1982). While most synthetic methods produce racemates of these drugs, only the *S*-antipodes are pharmaceutically active (Reynolds, 1982). γ-Chymotrypsin CLCs are employed as the catalyst herein because the crystalline form of the enzyme has been found to retain its native conformation in organic solvents (Yennawar, *et al.*, 1994; Yennawar, *et al.*, 1995), thus allowing the use of structure-based molecular modeling.

Because enzyme CLCs (as well as nearly all other enzyme preparations) are insoluble in organic solvents, the transesterification in Scheme 5.3 is catalyzed in a heterogeneous system and thus is susceptible to rate limitation by diffusion of the substrate into the solid catalyst particle. To ensure that the initial velocities measured reflect the true kinetic constants of the enzyme, and not the mass transfer rates of the substrate through the crystals, enzymatic activity was examined as a function of the loading of the biocatalyst particles (Boudart and Burwell, 1973; Wescott and Klibanov, 1993a;

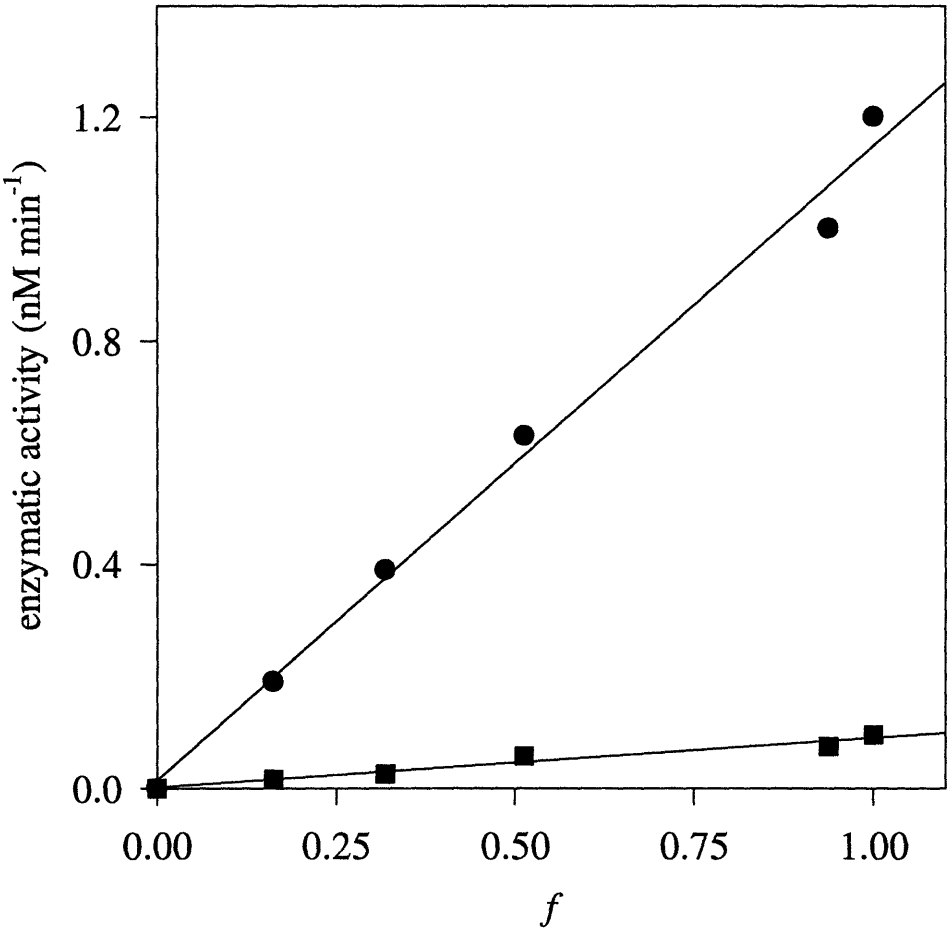



**Figure 5.1.** Dependence of the activity of γ-chymotrypsin cross-linked crystals on the fraction of active enzyme in the crystal (*f*). Activity is measured as the rate of enzymatic transesterification of the *S*- (●) or *R*- (■) enantiomers of **3** with propanol in cyclohexane. *f* is controlled by co-crystallizing native and inactivated (by diisopropyl fluorophosphate) γ-chymotrypsin in varying proportions (see Methods for details).

Schmitke, *et al.*, 1996). To this end, active γ-chymotrypsin was co-crystallized with varying amounts of this enzyme inactivated with diisopropyl fluorophosphate. In the absence of diffusional limitations, a plot of catalytic activity vs. the fraction of active enzyme in the crystal should yield a straight line which passes through the origin (Boudart and Burwell, 1973). If, however, the mass transfer contribution to the reaction rate is not negligible, a convex dependence should be observed (Boudart and Burwell, 1973), because incremental increases in the enzyme loading do not produce similar amplifications in the catalytic efficiency of the CLC. Such a plot (Fig. 5.1) for the transesterification of both enantiomers of **3** in cyclohexane reveals a linear dependence between catalytic activity and the fraction of active enzyme in the crystal, thus ruling out the possibility that the measured reactions are affected by mass transfer of the substrate.

To explore the effect of the solvent on the kinetic resolution of **3**, the enantioselectivity of γ-chymotrypsin CLCs for the reaction depicted in Scheme 5.3 was measured in a variety of organic solvents. The enantiomeric excess (*ee*) at 5% conversion was subsequently calculated(Chen, *et al.*, 1982) to quantify the efficiency of the resolution in each solvent (Table 5.1). Inspection of Table 5.1 reveals that the enantioselectivity, expressed as $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$, can be forced to span a 20-fold range simply by switching from one organic solvent to another under otherwise identical conditions. Perhaps even more striking is the fact that the enantioselectivity can actually be reversed through the choice of the solvent. For instance, in cyclohexane the enzyme preferentially transesterifies the *S*-enantiomer of **3**, while in acetone the *R*-antipode is preferred.

**Table 5.1.** Solvent dependence of the enantioselectivity of γ-chymotrypsin cross-linked crystals for the transesterification of **3** with propanol.

| solvent | $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$ [a] | product *ee*, % [b] (preferred enantiomer) |
|---|---|---|
| cyclohexane | 13 | 85 (*S*) |
| octane | 8.8 | 79 (*S*) |
| hexane | 8.0 | 77 (*S*) |
| toluene | 5.6 | 69 (*S*) |
| isopropyl acetate | 2.4 | 40 (*S*) |
| tetrahydrofuran | 1.8 | 28 (*S*) |
| *tert*-butyl acetate | 1.5 | 20 (*S*) |
| *tert*-butyl alcohol | 0.91 | 4.6 (*R*) |
| *tert*-amyl alcohol | 0.80 | 11 (*R*) |
| dioxane | 0.74 | 15 (*R*) |
| propanol | 0.73 | 15 (*R*) |
| acetone | 0.64 | 21 (*R*) |

[a] See Methods for details on the measurement of $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$. [b] Enantiomeric excesses (*ee*) were calculated from the enantioselectivities for a 5% conversion as described by Chen, *et al.*, 1982.

According to equation 5.7, knowing only the ratio of $\gamma'_R$ and $\gamma'_S$ for a series of solvents, it should be possible to explain the observed solvent dependence of enantioselectivity. Unlike the situation for substrate specificity, where solvent-dependent variation in the activity coefficient ratio for the two substrates is primarily driven by chemical differences between them (Wescott and Klibanov, 1993a,b), $\gamma'$ for enantioselectivity differs for substrate enantiomer only due to differences in transition state solvation. In this work, we calculate $\gamma'$ for both the *R* and *S* transition states using a three-step procedure. First, the desolvated portion of each enantiomer of the substrate in the transition state is determined using molecular modeling based on the crystal structure of the enzyme. Second, this desolvated moiety is approximated in terms of individual UNIFAC groups. Finally, the thermodynamic activity coefficient of this desolvated fragment is calculated using the UNIFAC group contribution method and then equated to $\gamma'$.

As a first step in the calculation of $\gamma'$, molecular models have been constructed for the *S* and *R* transition states for the acylation of γ-chymotrypsin by **3** (see Methods for details). Examination of the *S* transition state model (Fig. 5.2A) reveals that the hydroxyl group of the substrate is buried in chymotrypsin's S1 binding pocket, while the phenyl group extends away from the enzyme toward the solvent. Figure 5.2B depicts the opposite situation for the *R* transition state: the aryl moiety is buried in the active center of the

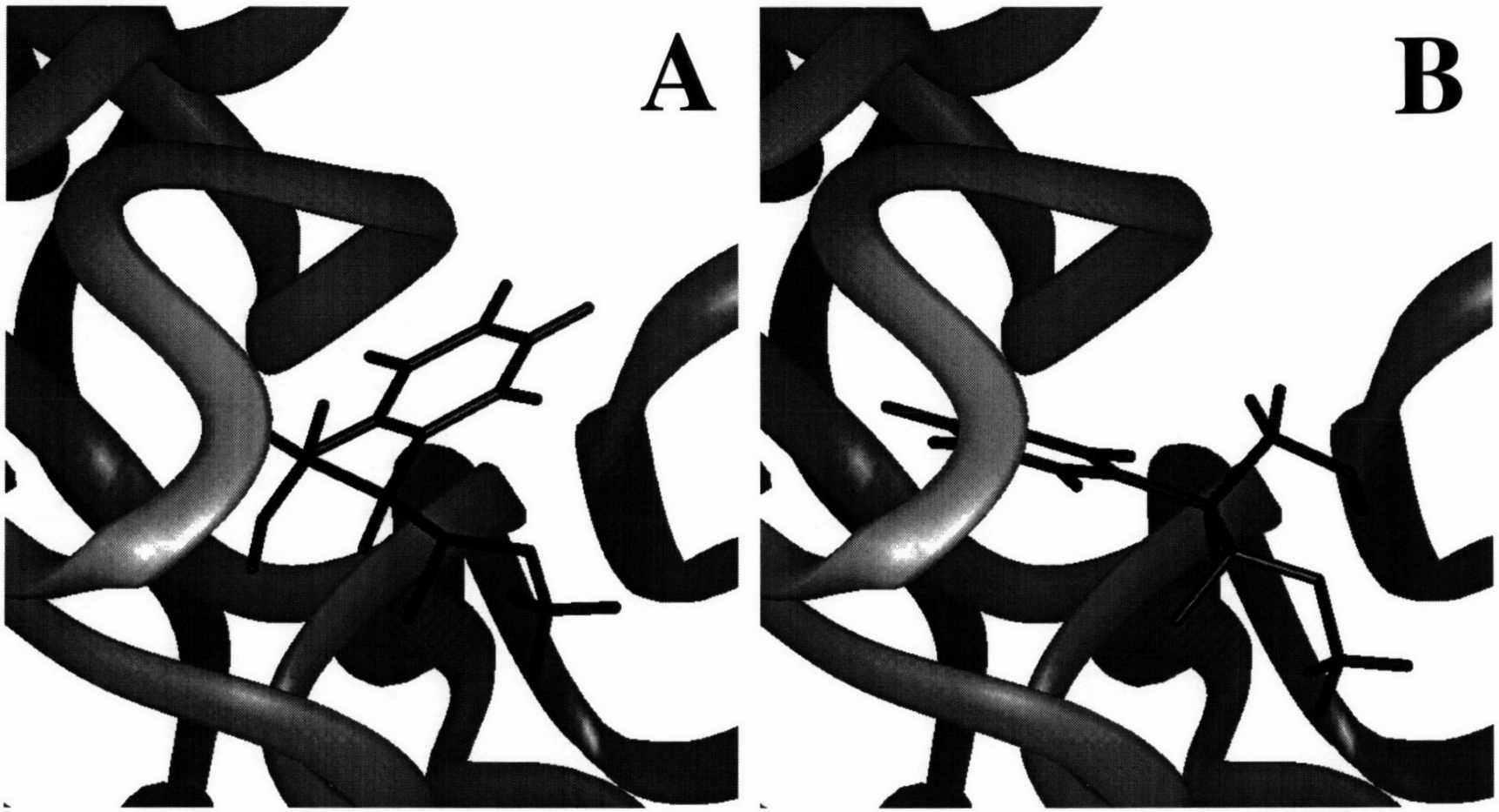



**Figure 5.2.** Molecular models of *S*- (A) or *R*- (B) **3** in the transition state for acylation of γ-chymotrypsin. See Methods for details on creation of the models.

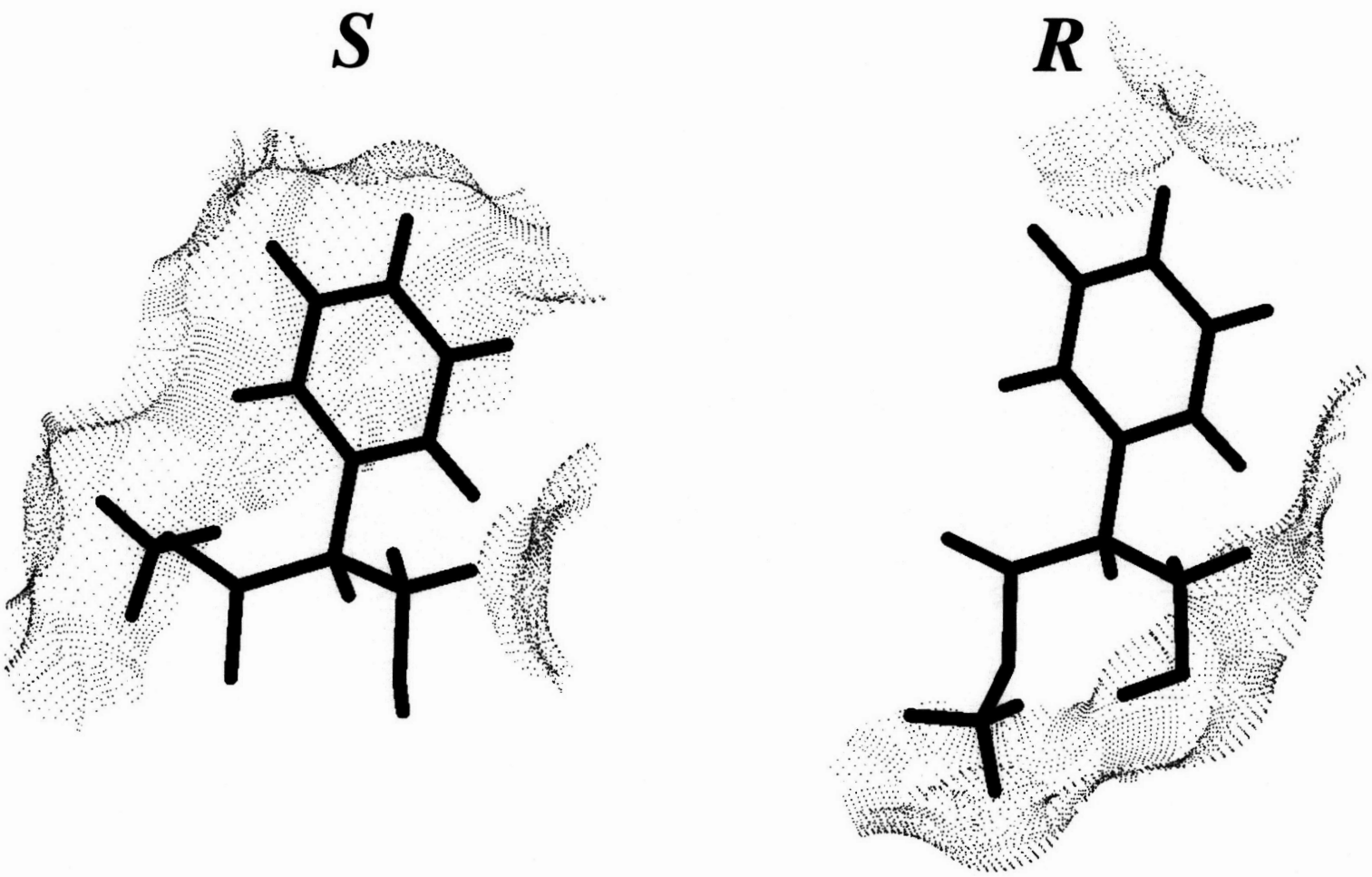



**Figure 5.3.** Solvent-accessible surface areas of **3** in the *S* (left) and *R* (right) transition states with γ-chymotrypsin. See Methods for details.

enzyme, while the hydroxyl group is oriented toward the solvent. The solvated surface areas for the transition states, calculated using the method of Connolly (Connolly, 1983), are displayed as dot surfaces in Figure 5.3. One can see that, for example, in the *S* transition state the hydroxyl group is desolvated, while the phenyl group is not. In contrast, the surface for the *R* transition state indicates the inverse desolvation pattern for these two groups.

With the desolvated portions of the transition states ascertained, the next step in the calculation of $\gamma'$ is the construction of molecular fragments, based on UNIFAC groups (Fredenslund, *et al.*, 1977), which approximate the desolvated portions of the substrates in the transition states. To this end, the enantiomers of **3** have been modeled in terms of the smallest possible UNIFAC groups, and the percent of desolvation for each such group is tabulated in Table 5.2. Groups are then included in the molecular fragment for a given substrate enantiomer if they are at least 50% desolvated. Groups desolvated to a lesser extent are considered solvated and thus not part of the desolvated substrate moiety. According to these rules, the desolvated portion of the *S* transition state is represented by one hydroxyl group, one aryl carbon, two aryl methine groups, 1 carbonyl group, and 1 aliphatic methine group. Similarly, the corresponding *R* molecular fragment consists of one aryl carbon, three aryl methine groups, one carbonyl group, and one aliphatic methine group.

Finally, the activity coefficients for the *S* and *R* model fragments are calculated using UNIFAC and equated with the activity coefficients of the desolvated portions of the corresponding transition states, $\gamma'_S$ and $\gamma'_R$, respectively.

**Table 5.2.** Percent of desolvation of component groups for *S*- or *R*- enantiomers of the transition states for the acylation of γ-chymotrypsin by **3**.

| group [a] | desolvation (%) [b] | |
|---|---|---|
| | *S* | *R* |
| hydroxyl | 100 | 11 |
| aryl C 1 | 100 | 100 |
| aryl CH 2 | 86 | 35 |
| aryl CH 3 | 0 | 100 |
| aryl CH 4 | 11 | 54 |
| aryl CH 5 | 42 | 33 |
| aryl CH 6 | 67 | 100 |
| carbonyl | 100 | 100 |
| methylene | 49 | 36 |
| methine | 100 | 100 |
| methyl | 13 | 35 |

[a] The aryl units make up the phenyl group of **3**. [b] The percent of desolvation of a group is calculated as $[1 - (A_B/A_F)] \times 100\%$, where $A_B$ is the solvent-accessible surface area of the substrate group in the enzyme-bound transition state (Fig. 5.3), and $A_F$ is the solvent-accessible surface area of the same substrate group in the free transition state.

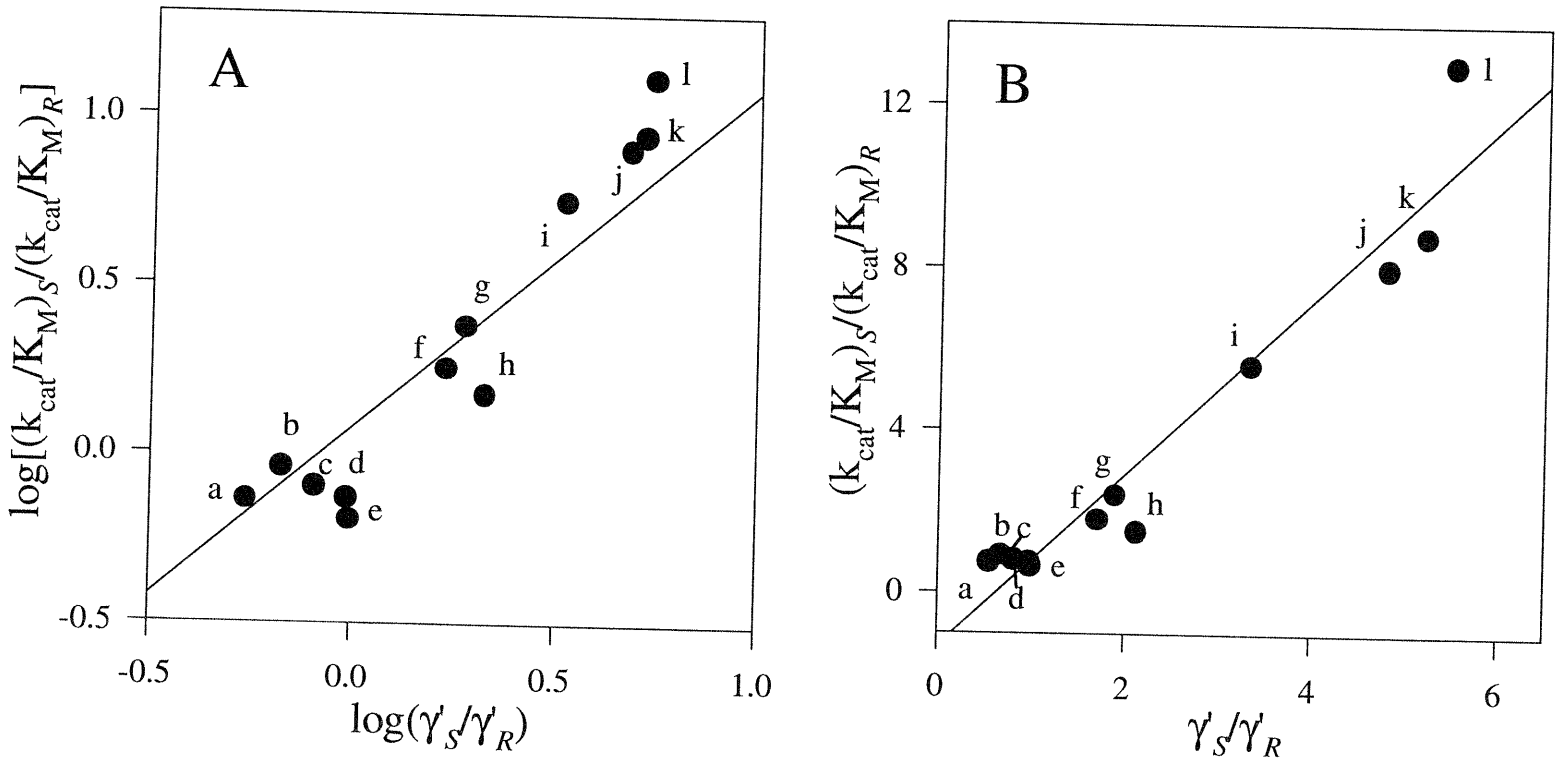

A
B
log[(kcat/KM)S/(kcat/KM)R]
log(γ'S/γ'R)
(kcat/KM)S/(kcat/KM)R
γ'S/γ'R
a
b
c
d
e
f
g
h
i
j
k
l
1.0
0.5
0.0
-0.5
12
8
4
0
2
6

**Figure 5.4.** Dependence of the enantioselectivity of the transesterification in Scheme 5.3 catalyzed by cross-linked crystals of γ-chymotrypsin on the activity coefficient ratio for the desolvated portions of the substrates in the enzyme-bound transition states. (A) Double logarithmic plot with a least-squares fit to a linear dependence with the slope of unity, used to assess predictive ability of equation 5.7 (correlation coefficient is 0.87). (B) Linear plot with linear regression (correlation coefficient is 0.93). Solvents: a - propanol, b - *tert*-butyl alcohol, c - *tert*-amyl alcohol, d - dioxane, e - acetone, f - tetrahydrofuran, g - isopropyl acetate, h - *tert*-butyl acetate, i - toluene, j - hexane, k - octane, l - cyclohexane. See Methods for experimental details.

Equation 5.7 predicts that a double logarithmic plot of enantioselectivity vs. the $\gamma'$ ratio should be linear, with a slope of unity. Such a plot, presented in Fig. 5.4A, does indeed follow the expected dependence: a least squares fit to a linear model with a slope of one yields a correlation coefficient of 0.87. Furthermore, when the data are plotted in linear coordinates (Fig. 5.4B), linear regression yields a correlation coefficient of 0.93. Thus equation 5.7 correctly predicts the solvent dependence of enzymatic enantioselectivity in a nearly quantitative fashion.

The methodology employed above is surprisingly effective despite the fact that, while most substrate groups are in reality only partially desolvated in the transition state (Table 5.2), for the construction of the model fragments all groups are approximated as either 100% or 0% desolvated. Why does this approach work even though a continuous range of desolvated surface areas is approximated by a simple "all-or-nothing" model? To answer this question, we have examined how the method of treatment of partially desolvated groups (herein for simplicity defined as groups which are 20% to 80% desolvated) affects the predictive performance of our model. One extreme alternative to the partially desolvated groups is to include all of them in the model fragments. When these model fragments are used to calculate the activity coefficients of the desolvated portions of the *S* and *R* transition states, and the data are plotted as in Fig. 5.4A, the resultant correlation coefficient (0.89) has been found to be virtually unaffected. A second extreme recourse to the treatment of the partially desolvated groups is to exclude all of them from the model fragments. Once again, fitting these data to a linear dependence with

**Table 5.3.** Solvent dependence of the activity coefficients of component groups of the model fragments for the enantiomers of **3**.

| solvent | activity coefficients [a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | hydroxyl | aryl C | aryl CH | carbonyl | methyl | methylene | methine[b] |
| cyclohexane | 9.3 | 0.35 | 0.37 | 4.1 | 0.56 | 0.42 | 0.34 |
| octane | 6.8 | 0.28 | 0.28 | 3.3 | 0.43 | 0.33 | 0.27 |
| hexane | 7.7 | 0.35 | 0.35 | 3.8 | 0.51 | 0.40 | 0.33 |
| toluene | 5.4 | 0.34 | 0.35 | 1.7 | 0.63 | 0.45 | 0.35 |
| isopropyl acetate | 3.0 | 0.37 | 0.35 | 1.6 | 0.65 | 0.48 | 0.38 |
| tetrahydrofuran | 3.6 | 0.51 | 0.46 | 1.7 | 0.76 | 0.60 | 0.51 |
| *tert*-butyl acetate | 3.0 | 0.33 | 0.31 | 1.6 | 0.56 | 0.42 | 0.34 |
| *tert*-butyl alcohol | 1.3 | 0.43 | 0.42 | 2.5 | 0.71 | 0.54 | 0.43 |
| *tert*-amyl alcohol | 1.4 | 0.37 | 0.37 | 2.3 | 0.61 | 0.46 | 0.37 |
| dioxane | 2.1 | 0.55 | 0.47 | 1.2 | 0.90 | 0.69 | 0.56 |
| propanol | 1.3 | 0.51 | 0.50 | 2.7 | 0.86 | 0.64 | 0.51 |
| acetone | 2.5 | 0.56 | 0.54 | 1.6 | 1.1 | 0.77 | 0.59 |

[a] Activity coefficients were calculated using UNIFAC (see Methods for details). The aryl units make up the phenyl group of **3**. [b] Aliphatic.

a slope of unity yields an unchanged correlation coefficient of 0.87. These results lead to the conclusion that, for the reaction in Scheme 5.3, the solvent dependence of the $\gamma'$ ratio is quite insensitive to those moieties that happen to be partially desolvated and is determined only by those that happen to be essentially desolvated in the transition state (Table 5.2).

In order to rationalize why the groups partially desolvated in our case do not appreciably affect the solvent dependence of the $\gamma'$ ratio, we have calculated the activity coefficients of all the individual component groups. As seen in Table 5.3, the activity coefficients of the partially desolvated groups vary relatively little throughout the series of solvents tested (0.26, 0.67, and 0.44 for the aryl CH, methyl, and methylene groups, respectively). In contrast, two of the groups for which the extent of desolvation is clearly defined, namely the hydroxyl and carbonyl groups, exhibit much larger respective activity coefficient changes of 8.0 and 2.9. Thus the solvent dependence of the $\gamma'$ ratio is dominated by the interaction of the hydroxyl and carbonyl groups with the organic solvents.[7] Consistent with this conclusion is the finding that $\gamma'$ ratios calculated by either including the hydroxyl in, or excluding it from, both transition states produces a correlation coefficient below 0.1 when the data are plotted as in Fig. 5.4A. To generalize, different types of groups impact the free energy of desolvation of the transition states to varying extents. Therefore, in choosing a biocatalyst for the resolution of a chiral

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[7] It should be cautioned that these comparisons are qualitative and indicative of general trends only. This is because the standard states of the individual group activity coefficients are different, and the contribution of each group to the overall activity coefficient of the substrate model is affected by inter-group interaction parameters. (Fredenslund, *et al.*,1977)

compound, one should seek an enzyme with an active center that maximizes the difference in desolvation of the "impactful" groups (such as OH) between the two enantiomers. This optimization of differential desolvation of the impactful groups can be performed at the expense the nonimpactful groups without consequence.

An insight into the nature of the solvent-solute interactions can also be gained from Table 5.3. The activity coefficients for most of the groups in the table are below unity, indicating thermodynamic stabilization of most groups by the solvent. The hydroxyl and carbonyl groups, however, consistently feature activity coefficients greater than unity, i.e., these groups are destabilized by the solvents. Solvent control of the $\gamma'$ ratio (and thereby the enantioselectivity) for this system is thus exercised through thermodynamically unfavorable interactions between the solvents and the hydroxyl and carbonyl groups of the substrate.

Because a given substrate molecule can be represented by several different combinations of groups, it is important to assess the effect of approximating the substrate using different types of fragments. To this end, substrate **3** has been modeled using a series of incrementally larger groups, and the ability of each member of the series to predict the solvent dependence of enantioselectivity has been assessed via the ensuing correlation coefficient. One can see in Table 5.4 that as the size of the groups increases, the ability to approximate the desolvated portion of the substrate in the transition state deteriorates. This results in increasing error in the calculation of the $\gamma'$ ratio, which

**Table 5.4.** Correlation coefficients ($R^2$) for representations of the transition state of **3** using successively larger groups.

| group | desolvation (%) [a] | | $R^2$ [b] |
|---|---|---|---|
| | *S* | *R* | |
| *representation 1* | | | 0.87 |
| see Table 5.2 | | | |
| *representation 2* | | | 0.80 |
| hydroxyl | 100 | 11 | |
| phenyl | 38 | 74 | |
| methyl | 49 | 36 | |
| methyl acetate | 54 | 59 | |
| *representation 3* | | | 0.78 |
| methanol | 70 | 26 | |
| phenyl | 38 | 74 | |
| methyl acetate | 54 | 59 | |
| *representation 4* | | | 0.68 |
| ethanol | 73 | 36 | |
| phenyl | 38 | 74 | |
| methyl formate | 50 | 56 | |
| *representation 5* | | | 0.00 |
| 2-phenylethanol | 46 | 62 | |
| methyl formate | 50 | 56 | |

[a] The percent of desolvation of a group is calculated as described in footnote *b* to Table 5.2. [b] To assess the performance of each representation of the substrate molecule in the prediction of enantioselectivity, a double logarithmic plot of the latter vs. the activity coefficient ratio of the desolvated substrate moiety in the enzyme-bound transition state (calculated for the indicated representation of the substrate) was fit to a linear dependence with a slope of unity (as predicted by equation 5.7), and the resultant correlation coefficient was calculated. By fixing the slope to 1, both random and systematic errors (due to deviation of the slope from unity) are reflected in the correlation coefficient.

erodes and ultimately destroys the predictive power of equation 5.7. Therefore, to optimize the performance of this methodology, one should model the substrate in terms of the smallest possible fragments for the determination of $\gamma'$.

## D. Concluding Remarks

When crystalline enzymes are used as asymmetric catalysts in anhydrous organic solvents, the solvent dependence of enzymatic enantioselectivity can be attributed primarily to changes in the relative solvation energies for the *R* and *S* binding modes of the substrate in the transition state. This work presents a quantitative model which satisfactorily predicts the solvent effect on enantioselectivity solely on the basis of these solvation energies. Thus other factors not considered by the model, e.g. the effect of the solvent on the enzyme or displacement of bound solvent molecules from the active site by the substrate, are deemed relatively unimportant. Because no specific assumptions are made regarding the enzyme or the substrate in the derivation of the equation, the model should be generally applicable. Indeed, we have successfully employed this methodology to predict the solvent dependence of the prochiral selectivity of both crystalline γ-chymotrypsin and subtilisin Carlsberg (Ke, *et al.*, 1996).

## E. Materials and Methods.

**Enzymes.** Native and diisopropyl-fluorophosphate-inactivated α-chymotrypsins (EC 3.4.21.1) were purchased from Sigma Chemical Co. γ-Chymotrypsin crystals were created from the α- form of the enzyme, following the method of Stoddard *et al.*, 1990.

Partially inactivated γ-chymotrypsin crystals (used in the diffusional limitation experiments) were grown from mother liquors containing varying ratios of the diisopropyl-fluorophosphate-inactivated enzyme. Co-crystallization of the native and inhibited forms of the enzyme was confirmed by measuring the activity of single crystals dissolved in water. For use in organic solvents, crystals were cross-linked and prepared for catalysis as described in Ke, *et al.*, 1996.

**Chemicals and solvents** were purchased from Aldrich Chemical Co. The organic solvents were of the highest purity available from that vendor (analytical grade or better) and were dried prior to use to a water content below 0.01% (as determined by the Karl Fischer titration) by shaking with Linde's 3-Å molecular sieves.

**3** was synthesized by refluxing 1 g of **4** in 25 mL of anhydrous methanol containing 5 drops of concentrated $H_2SO_4$ for 12 h. The reaction mixture was subsequently concentrated by rotary evaporation, dissolved in 50 mL of diethyl ether, and then washed with five 10-mL aliquots of 5% $NaHCO_3$ and with 10 mL of deionized water. The crude product was recovered from the organic phase by rotary evaporation and subsequently purified by vacuum distillation. $^1$H NMR ($CDCl_3$) δ 7.3-7.4 (5 H, m), δ 4.1-4.2 (1 H, m), δ 3.8-3.9 (2 H, m), δ 3.7 (3 H, s), δ 2.2 (1 H, s).

**Propyl 3-hydroxy-2-phenylpropionate**, a racemic mixture used to calibrate the HPLC instrument, was synthesized from **4** following the procedure used for **3**, except the methanol was substituted with 50 mL of propanol. $^1$H NMR ($CDCl_3$) δ 7.3-7.4 (5 H, m), δ 4.1-4.2 (1 H, m), δ 4.1 (2 H, t, J = 6.0 Hz), δ 3.8-3.9 (2 H, m), δ 1.8 (1 H, s), δ 1.6-1.7 (2 H, m), δ 0.8-0.9 (3 H, t, J = 7.7 Hz).

**Propyl (*S*)-3-hydroxy-2-phenylpropionate**, used to assign the *S*-product HPLC peak, was synthesized by refluxing 0.5 g of scopolamine·HBr in 10 mL of anhydrous propanol containing 5 drops of concentrated $H_2SO_4$ for 3 days. The crude product was recovered as in the synthesis of **3** and purified by TLC. $^1$H NMR ($CDCl_3$) δ 7.3-7.4 (5 H, m), δ 4.1-4.2 (1 H, m), δ 4.1 (2 H, t, J = 6.0 Hz), δ 3.8-3.9 (2 H, m), δ 2.1 (1 H, s), δ 1.6-1.7 (2 H, m), δ 0.8-0.9 (3 H, t, J = 7.7 Hz).

**Kinetic measurements.** One milliliter of solvent containing 100 mM racemic **3** and 100 mM propanol was added to 10 mg of cross-linked γ-chymotrypsin crystals. Then 0.2% (v/v) water was added to the suspension to enhance the rate of enzymatic transesterification; in the presence of the dissolved substrates, this added water was soluble in each of the solvents used. The hydrolysis product **4** was not detected during any of the reactions studied. Note that any competing hydrolysis would merely reduce the concentration of the acyl-enzyme available for reaction with propanol, equally reducing the rate of production of both enantiomers of the propyl ester product, and thus leaving the enantioselectivity unaffected. The suspensions were shaken at 45 °C and 300 rpm. Periodically, a 10-μL sample was withdrawn and assayed by chiral HPLC. Because the transesterifications which lead to the *R* and *S* products take place in the same reaction mixture and the substrate enantiomers compete for the same population of free enzyme, the ratio of initial velocities of the reactions is equal to $(k_{cat}/K_M)_S$ / $(k_{cat}/K_M)_R$. (Wescott and Klibanov, 1993a).

Chiral HPLC separations were performed using a Chiralcel OD-H column and a mobile phase of 95:5 (v/v) hexane:2-propanol. A flow rate of 0.5 mL/min separated the *R*

and *S* enantiomers of **3** with retention times of 17 and 19 min, respectively. The products were quantified using a UV absorbance detector at 220 nm.

**Activity coefficient calculation.** All activity coefficients were calculated using the UNIFAC method (Fredenslund *et al.*, 1977; Steen, *et al.*, 1979; Rasmussen and Fredenslund, 1982; Macedo, *et al.*, 1983; Teigs, *et al.*, 1987; Hansen, *et al.*, 1991). Because UNIFAC is a group contribution method, it allows the estimation of activity coefficients in systems for which there is no experimental data by assessing the individual contribution of each group which makes up the system. Use of this method requires three types of parameters for each group in the system: the group's surface area, the volume of the group, and empirically determined parameters which reflect the free energy of interaction between a given group and every other group in the system.

As a test of the accuracy of the UNIFAC calculations, we compared some activity coefficients derived from published vapor-liquid equilibrium (VLE) data to those calculated using UNIFAC. The types of systems for which such data are available are quite limited, but we were able to find VLE data for two compounds (3-methylphenol and 2-methyl-1-propanol) which represent some of the functional groups present in our model molecules in the most nonideal solvent observed in the present work, cyclohexane (Gmehling, *et al.*, 1982). Interpreting the VLE data using the Wilson equation of state, for 298 K and a mole fraction of 0.001, the activity coefficients for 3-methylphenol and 2-methyl-1-propanol are 47 and 29, respectively. Under identical conditions, UNIFAC predicts an activity coefficient of 31 for 3-methylphenol, and 21 for 2-methyl-1-propanol. While the individual activity coefficients predicted by UNIFAC are underestimated by

about 30%, the activity coefficient ratio (the quantity used in our work) is estimated to within 6%.

Activity coefficient calculations include the effects of 100 mM propanol and 0.2% (v/v) water.

**Structural modeling.** Molecular models were produced using the crystal structure of γ-chymotrypsin in hexane (Brookhaven data bank entry 1GMC) (Yennawar, *et al.*, 1994; Yennawar, *et al.*, 1995). Because the transition state for the acylation of a serine protease is structurally similar to the corresponding tetrahedral intermediate for the reaction (Warshel, *et al.*, 1989), transition states were modeled as the tetrahedral intermediates for the reactions. Such models were produced using a two-step procedure. First, potential binding modes of the chiral products were generated by performing molecular dynamics simulations, followed by energy minimization. The carbonyl oxygen of the product was tethered to the oxyanion binding site using a harmonic potential with a force constant selected to allow widely different conformations to be explored, while preventing the product from diffusing too far from the enzyme. Second, each product binding mode thus identified was used as a template for creating an initial model of the tetrahedral intermediate. The low-energy conformation of each of these starting models was found using molecular dynamics simulations and energy minimizations. The lowest-energy conformer of the tetrahedral intermediate was selected as the model of the transition state.

The first step (the product binding mode search) is necessary because the covalently bound tetrahedral intermediate is sufficiently sterically constrained that

molecular dynamics simulations do not sample highly different conformations separated by large energetic barriers.

Molecular modeling and dynamics simulations were performed with the Insight II and Discover programs as follows: The initial structures were energy-minimized using the steepest descent method for 50 iterations, followed by conjugate gradient minimization until the maximum derivative was less than 0.001 kcal/Å. The minimized structure was then subjected to 40 ps of molecular dynamics at 900 K with steps of 1 fs. After each simulated ps, the atomic coordinates were saved, resulting in 40 independent structures with different conformations. The resulting structures were then minimized as outlined above, except the minimization proceeded until the maximum derivative was less than 0.0001 kcal/Å. During all minimizations and molecular dynamics simulations, only the atoms of the substrate and those of the catalytic triad's serine were allowed to move, and a cutoff distance of 11 Å was used with the CVFF force field provided with the Discover program. Because solvent molecules and counterions were not included in the simulations, all protein residues were modeled in their un-ionized forms. Of the 40 minimized structures, the lowest energy conformer was selected, and the solvent-accessible surface area was calculated using the Connolly algorithm, as implemented in the Insight software package.

In support of the validity of the structural modeling methods described above, we were able to use this procedure to correctly predict the conformation of *N*-acetyl-L-phenylalanine trifluoromethyl ketone in its hemiketal complex with chymotrypsin (Brady, *et al.*, 1990).

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