Solvent Variation Inverts Substrate Specificity of an Enzyme

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Abstract: 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 1-propanol was examined in 20 anhydrous solvents. The serine substrate was strongly favored in some solvents, while the phenylalanine substrate was greatly preferred in others. A thermodynamic model was derived which correctly predicted 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 independent of the enzyme and the substrate, so long as the latter is removed from the solvent in the transition state.

Exquisite substrate specificity is a hallmark of enzymatic catalysis.[1] To what extent is this phenomenon due to the enzyme itself, as opposed to the aqueous reaction medium? Nonaqueous enzymology[2] has allowed this question to be addressed experimentally.[3,4] It has been found that substrate preference of the proteases α-chymotrypsin and subtilisin markedly changes when water is replaced with a nonaqueous solvent as the reaction medium.[3] In the present work, we have observed a dramatic shift, and indeed an 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, physicochemical concept.

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 I depicts the substrate specificity of subtilisin, defined as the ratio of the specificity constants[5] k_{cat}/K_M, for the two esters of opposite hydrophobicities, Phe and Ser, as a function of the solvent. 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.[5] 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 I, consider the thermodynamic diagram in Scheme I. The lower horizontal arrow represents enzyme (E) and substrate (S) reacting in an organic solvent to form a transition state (ES‡). Another, 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 subsequent partitioning of the transition state from water into the solvent.

Expressing $\Delta G^‡$ as the sum of the energetic terms of the alternate path yields

$$\Delta G^‡ = \Delta G_1 + \Delta G^‡_2 + \Delta G_3 \quad (1)$$

$\Delta G^‡$ is related to k_{cat}/K_M in the solvent:

$$\Delta G^‡ = -RT \ln \left[\left(\frac{k_{cat}}{K_M} \right)_{solvent} \left(\frac{h}{\kappa T} \right) \right] \quad (2)$$

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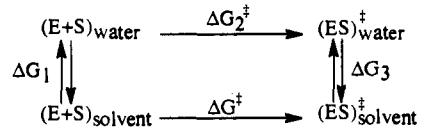
Table I. Substrate Specificity in the Transesterification of *N*-Ac-L-Ser-OEt and *N*-Ac-L-Phe-OEt with 1-Propanol Catalyzed by Subtilisin Carlsberg in Various Anhydrous Organic Solvents[a]

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

[a] See the Experimental Section for details.

Scheme I



where h is the Planck constant, κ is the Boltzmann constant, and T is the absolute temperature. Likewise,

$$\Delta G^‡_2 = -RT \ln \left[\left(\frac{k_{cat}}{K_M} \right)_{water} \left(\frac{h}{\kappa T} \right) \right] \quad (3)$$

ΔG_1 depends on the solvent-to-water partition coefficients of the substrate (P) and the enzyme (P_E):

$$\Delta G_1 = RT \ln P + RT \ln P_E \quad (4)$$

ΔG_3 is the free energy of transfer of ES‡ from water into the solvent. Substituting eqs 2, 3, and 4 into eq 1 and dividing by $-RT$, one obtains

$$\ln \left[\left(\frac{k_{cat}}{K_M} \right)_{solvent} \left(\frac{h}{\kappa T} \right) \right] = -\ln P - \ln P_E + \ln \left[\left(\frac{k_{cat}}{K_M} \right)_{water} \left(\frac{h}{\kappa T} \right) \right] - \frac{\Delta G_3}{RT} \quad (5)$$

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Table II. 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	P_{Ser}[b] 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.

Equation 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

$$\ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Ser}_{solvent}\left(\frac{h}{\kappa T}\right)\right] - \ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Phe}_{solvent}\left(\frac{h}{\kappa T}\right)\right] = -\ln P_{Ser} + \ln P_{Phe} - \ln P_E + \ln P_E + \ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Ser}_{water}\left(\frac{h}{\kappa T}\right)\right] - \ln\left[\left(\frac{k_{cat}}{K_M}\right)^{Phe}_{water}\left(\frac{h}{\kappa T}\right)\right] + \frac{\Delta G_{3,Phe} - \Delta G_{3,Ser}}{RT} \quad (6)$$

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,[6] $\Delta G_{3,Ser} = \Delta G_{3,Phe}$. Thus, these terms also cancel each other out. Consequently, rearranging eq 6 yields

$$\log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{solvent} = \log\frac{P_{Phe}}{P_{Ser}} + \log\left[\frac{(k_{cat}/K_M)_{Ser}}{(k_{cat}/K_M)_{Phe}}\right]_{water} \quad (7)$$

Equation 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 1, whose intercept with the ordinate should equal the substrate specificity in water. Therefore, we experimentally measured P_{Phe} and P_{Ser} (Table II), and the resultant plot is shown in Figure 1. 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 1.1×10^{-2}, compared to the experimentally determined 1.9×10^{-2}. This discrepancy is probably due to the fact that kinetics measurements (Table I) 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.

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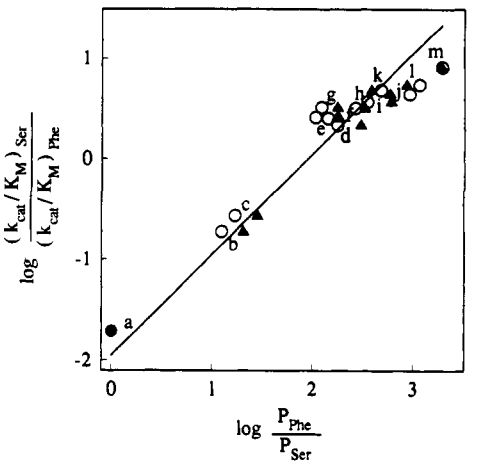

Figure 1. Dependence of the substrate specificity of subtilisin Carlsberg in water and in anhydrous organic solvents on the ratio of the solvent-to-water partition coefficients of *N*-Ac-L-Phe-OEt and *N*-Ac-L-Ser-OEt. Solvents: (a) water (●), (b) *tert*-butyl alcohol, (c) *tert*-amyl alcohol, (d) isopropyl 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 (○), or alternatively, the equilibrium concentrations of the tritiated serine substrate in such mixtures were measured by scintillation counting (▲).

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.

Experimental Section

Enzyme. Subtilisin Carlsberg (serine protease from *Bacillus licheniformis*, EC 3.4.21.14) was purchased from Sigma Chemical Co. The enzyme was prepared by lyophilization from a 5 mg·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 °C.

Chemicals and Solvents. *N*-Ac-L-Phe-OEt and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma.

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 the mixture was cooled to 0 °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 was removed by rotary evaporation. The resultant product was an oil with $[\alpha]^{25}_D = +12.8°$. ^{1}H NMR ($CDCl_3$, $CHCl_3$ as an internal standard): δ 6.58 (1 H, d, J = 6.0 Hz), 4.66 (1 H, dt, J = 3.6, 3.6 Hz), 4.27 (2 H, q, J = 7.2 Hz), 3.96 (2 H, s), 2.90 (1 H, s), 2.08 (3 H, s), 1.31 (3 H, 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·mg^{-1}; 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 h, 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.

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).

Measurement of Partition Coefficients by Gas Chromatography. Two milliliters of an aqueous solution containing 10 mM *N*-Ac-L-Phe-OEt and 10 mM *N*-Ac-L-Ser-OEt was 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 h at 30 °C, the phases were separated by centrifugation. The concentration of each

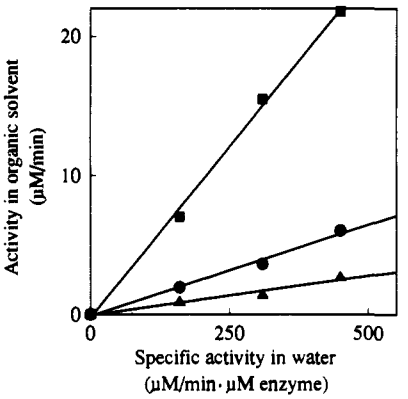

Figure 2. Dependence of the catalytic activity of subtilisin Carlsberg in several anhydrous organic solvents on the specific activity of the enzyme in water. Data are shown for the enzymatic transesterification (at 5 mg·mL^{-1} of subtilisin) between *N*-Ac-L-Ser-OEt and 1-propanol in dodecane (■), carbon tetrachloride (●), and acetonitrile (▲). The linear dependencies observed prove that the enzymatic reaction rates in these organic solvents are 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 (see the Experimental Section).

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 milliliter 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.

Kinetics Measurements. The k_{cat}/K_M values in water were measured potentiometrically in the subtilisin-catalyzed hydrolysis of the esters (1–10 mM esters; 2 and 200 nM enzyme for the Phe and Ser substrates, respectively; pH 7.8; 30 °C; 100 mM KCl). Kinetics data was fitted to the Michaelis–Menten equation using the nonlinear 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·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 organic solvents were not limited by internal or external mass transfer, as has been recently suggested in the literature,[7] the reaction rate for each substrate in several organic solvents was shown to be directly proportional to the specific activity of the enzyme[8] (e.g., Figure 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·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:[9] 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 h. Excess PMSF was removed by dialysis against 20 mM phosphate buffer (pH 7.8).

Kinetics Calculations. The subtilisin-catalyzed transesterification of an *N*-acetyl amino acid ethyl ester with 1-propanol follows the ping-pong, bi–bi mechanism,[10]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} AcE + PrOH \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$$

$$\downarrow P_1$$

$$AcE\cdot PrOH \xrightarrow{k_4} E + P_2 \quad (8)$$

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

$$v_i = \frac{k_2[E][S]}{K_{M,S}} \quad (9)$$

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

$$\frac{v_{Ser}}{v_{Phe}} = \frac{k_{2,Ser}[E][Ser]/K_{M,Ser}}{k_{2,Phe}[E][Phe]/K_{M,Phe}} \quad (10)$$

If the two substrates compete for the same population of free enzyme (i.e., are present in the same reaction mixture), [E] is identical in the numerator and denominator of eq 10. If the substrate concentrations are made equal, they will cancel each other out. Then

$$\frac{v_{Ser}}{v_{Phe}} = \frac{(k_2/K_M)_{Ser}}{(k_2/K_M)_{Phe}} \quad (11)$$

If the ping-pong kinetic scheme is solved with respect to $[E]_0$ instead of [E], then the expressions for k_{cat} and $K_{M,S}$ are given by the following formulas:[11] $k_{cat} = k_2k_4/(k_2 + k_4)$ 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 eq 9, the expression for $k_{cat}/K_{M,S}$ *is the same.*

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