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Protein and Solvent Engineering of Subtilisin BPN′ in Nearly Anhydrous Organic Media

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Abstract: The combined effects of protein and solvent engineering have been studied using subtilisin BPN′ as a model protease. The effects of site-specific mutations in the active site of subtilisin BPN′ on the reactivity and substrate specificity of the enzyme are strongly dependent on the polarity of the substrate, active-site mutation, and solvent. In going from a polar solvent such as acetone to a nonpolar solvent such as hexane, subtilisin BPN′ catalysis is activated dramatically (up to 178-fold) by employing a polar active-site mutation ($Gly_{166} \rightarrow Asn$). This activation is proposed to be due to significant transition-state stabilization afforded by the polar mutation on subtilisin catalysis. Analysis of the individual kinetic and binding constants for subtilisin indicates that the polar mutation in the S_1 binding site of the enzyme results in improved catalysis over the wild-type solely because of increased enzyme–substrate interaction (decreased $(K_m)_{true}$). Water also effects the kinetics of subtilisin catalysis. In dry tetrahydrofuran, acylation is rate limiting. Addition of small concentrations of water to the organic solvent (<2% v/v) results in both an increased rate constant for acylation and a decreased $(K_m)_{true}$. At 2% (v/v) added water and above, subtilisin reverts to a deacylation rate-limiting reaction on its ester substrates. These results suggest that water and polar mutations activate enzyme catalysis in nearly anhydrous solvents, albeit by different mechanisms, and further increase our understanding of the nature of polarity on enzyme function. From a practical standpoint, it is concluded that the effectiveness of protein engineering is strongly dependent on the solvent conditions.

The use of protein engineering to alter the substrate specificity of enzymes is well-documented.[1] Polar, charged, and hydrophobic mutations can alter the interaction between the active site of an enzyme and its substrates and thereby change both the reactivity and selectivity of the enzyme. Often the reaction specificity catalyzed by the enzyme is changed dramatically. For proteases, as an example, amidase activity has been nearly completely replaced by esterase activity.[2] Recently, several studies have employed protein-engineered enzymes in organic media such that dramatic improvements have been made in catalytic function and alterations made in substrate specificity.[3]

Protein engineering of enzymes for use in organic solvents serves two purposes. First, changes in the structure and chemistry of a given enzyme can be studied with respect to observed functional changes in catalysis and specificity. This, in turn, can provide clues as to the nature of enzyme function in environments vastly distinct from aqueous solutions. Second, enzyme function in organic media can be tailored to meet a desired catalytic outcome. This latter goal has been the subject of numerous recent studies. For example, Arnold and co-workers have used random mutagenesis to improve subtilisin catalysis in DMF/water mixtures.[3b,c] Enzymes with multiple mutations show dramatically improved

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activities in *both* aqueous buffer and DMF/water mixtures of up to 85% (v/v) organic solvent. Thus, enzymes that are engineered to function better in water also show improved catalysis in hydrated organic solvents. The use of anhydrous organic solvents, however, can produce strikingly different results. In our previous work, we showed that several active-site mutants of subtilisin BPN′ which are *less* active than the wild-type in aqueous solutions become *more* active in anhydrous or partially hydrated (i.e., <1% v/v added water) organic solvents.[4] Hence, in nearly anhydrous organic solvents, the function of protein-engineered enzymes can be far different from that in substantially hydrated organic solvents.

The organic solvent itself can also induce dramatic changes in enzyme activity and specificity.[5] Thus, in nonaqueous media, enzyme specificity can be altered, and in fact inverted, by simply changing the physiocochemical properties of the reaction medium (e.g., the polarity, hydrophobicity, and degree of hydration of the organic solvent).[6] It is logical, therefore, that dramatic changes in the reactivity and substrate specificity of an enzyme can be made by combining protein engineering with solvent variation (i.e., solvent engineering) in a rational approach. In the present work, we address the following question: How does the effectiveness of protein engineering change with the nature of the organic solvent and solvent hydration? It is shown that the combined effects of protein and solvent engineering, particularly with respect to solvent, substrate, and active-site polarities, and solvent hydration dramatically govern enzyme function in nearly anhydrous organic solvents.

Results and Discussion

Our experimental strategy was to select a reaction system that accentuates differences in substrate, solvent, and enzyme chemistries, primarily with respect to polarities. In this manner, the effects of protein engineering on subtilisin in various anhydrous organic solvents can be evaluated. To that end, we examined the transesterification of N-Ac-L-Ser ethyl ester (ASEE) and N-Ac-L-Phe ethyl ester (APEE) with *n*-propanol in acetone and hexane.

Table I. Catalytic Efficiencies of Subtilisin BPN′ and Its Protein-Engineered Variants in Acetone and Hexane[a]

enzyme	k_{cat}/K_m (M^{-1} s^{-1}) acetone	k_{cat}/K_m (M^{-1} s^{-1}) hexane	hexane/acetone[b]	(hexane/acetone)$_{normalized}$[c]
		ASEE as Substrate		
wild-type	0.76	0.98	1.29	1.00
G166N[d]	0.025	4.46	178	138
M222F[e]	0.16	1.61	10.3	7.95
		APEE as Substrate		
wild-type	1.00	0.34	0.34	1.00
G166N[d]	0.64	2.10	3.28	9.77
M222F[e]	0.93	1.29	1.39	4.13

[a] Conditions are as follows: 1.0 mg/mL of enzyme suspended in anhydrous acetone or hexane containing 1 M *n*-propanol and ASEE (N-Ac-L-Ser ethyl ester) or APEE (N-Ac-L-Phe ethyl ester) (ranging from 1 to 150 mM), shaken at 250 rpm and 30 °C. All other conditions are described in detail in the Experimental Section. Kinetic data were obtained from triplicate sets of initial rate measurements with a standard error less than 5% of the reported values in all cases. The kinetic data were converted from V_{max}/K_m to k_{cat}/K_m by normalizing the concentrations of active enzyme used. This was done via titration of the active sites by *N-trans*-cinnamoylimidazole as previously described.[22] In aqueous buffer, all enzymic active sites were accessible to the solvent and 54, 37, and 46% of the wild-type, G166N, and M222F powders, respectively, were active enzyme. In dry acetone and hexane, ca. 15% of the active enzymes were accessible to the solvent.[4] Enzymes preinactivated by either heat treatment (i.e., boiling in water for 30 min) or with the active-site serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) were completely inactive in all cases. [b] Defined as $(k_{cat}/K_m)_{hexane}/(k_{cat}/K_m)_{acetone}$. [c] Ratio of catalytic efficiency divided by the ratio for the wild-type. [d] Glycine to asparagine mutation at the 166 position. [e] Methionine to phenylalanine mutation at the 222 position.

The substrates and solvents represent extremes in polarities with APEE being relatively nonpolar compared to ASEE and hexane being relatively nonpolar compared to acetone. Values of catalytic efficiency (k_{cat}/K_m) were measured for the wild-type BPN′ and two mutants, a polar $Gly_{166} \rightarrow Asn$ (G166N) and a nonpolar $Met_{222} \rightarrow Phe$ (M222F). The former is a mutation in the back of the S_1 acyl-binding pocket of the enzyme, whereas the latter is a mutation in the S_1' leaving group site of the enzyme, adjacent to the active-site Ser_{221}. Previous investigations showed that these mutations, while often deleterious in aqueous solutions, resulted in *improved* catalysis in organic solvents.[3a,4]

Effects of Substrate and Solvent on the Efficiency of Protein Engineering in Organic Media

Table I depicts the catalytic efficiencies of the wild-type and mutant enzymes on both substrates in acetone and hexane. Both mutants provide for higher values of k_{cat}/K_m than the wild-type on both substrates in hexane but lower values of k_{cat}/K_m than the wild-type in acetone. Thus, the nature of the organic solvent can affect the catalytic efficiency of protein-engineered variants. Closer inspection of Table I, however, reveals that preferential activation of subtilisin on specific substrates occur via protein engineering. For ASEE, the G166N mutant is 178-fold more active in hexane than in acetone, whereas the wild-type and M222F mutant are 1.3- and 10.3-fold more active, respectively. Also shown in Table I are the normalized enhancements in enzyme activity in going from acetone to hexane. Because enzyme function is dependent upon the nature of the organic solvents,[5] the normalization takes into account the enhanced activity of the wild-type in going from acetone to hexane. It is now easier to see that, while the M222F mutant provides for only 8-fold activation in hexane as compared to acetone, the G166N mutation provides for nearly 140-fold activation. Hence, subtilisin catalysis on a polar substrate in a nonpolar solvent is activated by a polar mutation.

The increased value of $(k_{cat}/K_m)_{hexane}/(k_{cat}/K_m)_{acetone}$ for G166N on ASEE as compared to the wild-type is due to both

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activation of the G166N mutant relative to the wild-type in hexane and deactivation in acetone. Because catalysis of subtilisin is being studied in two different solvents, it is important to distinguish between intrinsic solvent effects on the enzyme and thermodynamic effects of the solvent on the reaction. For example, the value of k_{cat}/K_m is proportional to the difference in free energy between the transition state and ground state of an enzymatic reaction for a given substrate in a given solvent according to eq 1,[7] where h is Planck's constant, k_B is Boltzmann's constant, and

$$\Delta G^{\ddagger} = -RT \ln \frac{(k_{cat}/K_m)h}{k_B T} \quad (1)$$

T is the absolute temperature. The difference in transition-state free energies for a given enzymic reaction between hexane and acetone is given by eq 2. However, this value is comprised of both

$$\Delta\Delta G^{\ddagger} = -RT \ln \frac{(k_{cat}/K_m)_{hexane}}{(k_{cat}/K_m)_{acetone}} \quad (2)$$

an intrinsic transition-state free energy term ($\Delta\Delta G_{ES}^{\ddagger}$) as well as substrate and enzyme ground-state free energy terms ($\Delta\Delta G_S$ and $\Delta\Delta G_E$, respectively) as given by eq 3. The substrate ground-

$$\Delta\Delta G_{ES}^{\ddagger} = \Delta\Delta G^{\ddagger} + \Delta\Delta G_S + \Delta\Delta G_E \quad (3)$$

state free energy can be determined by calculating the Gibbs excess free energy of the substrate in solution according to eq 4,[8] where γ is the activity coefficient of the substrate in a given

$$\Delta G^E = \Delta G_S = RT \ln(\gamma/\gamma^\circ) \quad (4)$$

organic solvent (in our case hexane or acetone, each containing 1 M *n*-propanol) and γ° is the activity coefficient in an arbitrary standard solution. The difference in ground states of a given substrate between hexane and acetone is given by eq 5. The ground

$$\Delta\Delta G_S = RT \ln \frac{\gamma_{hexane}}{\gamma_{acetone}} \quad (5)$$

state of the enzyme is assumed to be indifferent to the solvent employed as the enzyme is not in solution. Thus, $\Delta\Delta G_E$ is assumed to be near zero. The intrinsic solvent effect on the enzymic transition state ($\Delta\Delta G_{ES}^{\ddagger}$) can then be calculated according to eq 3 and can be used to provide further insight into the observed effects of solvent and protein engineering on enzymatic catalysis in the absence of ground-state effects which are independent of enzyme function.

The intrinsic effect of the solvent on the transition-state stabilization of subtilisin BPN' and its protein-engineered variants is summarized in Table II and schematized in Figure 1. The enhanced catalytic efficiency for the wild-type in going from acetone to hexane ($\Delta\Delta G^{\ddagger} = -0.15$ kcal/mol) is more than negated by the ground-state destabilization of ASEE in hexane as compared to acetone ($\Delta\Delta G_S = 1.90$ kcal/mol). The intrinsic effect in going from acetone to hexane, then, is to *destabilize* the transition state of the enzymic reaction ($\Delta\Delta G_{ES}^{\ddagger}$) by 1.75 kcal/mol. This is surprising as nonpolar (as well as hydrophobic) organic solvents are generally described as being more capable of supporting enzymatic catalysis than polar solvents.[5] Clearly, though, subtilisin BPN' intrinsically prefers a polar solvent such as acetone as the reaction medium in contrast to a nonpolar solvent such as hexane. The G166N polar mutant must also be evaluated with respect to intrinsic transition-state stabilization. Table II shows that the G166N mutant *stabilizes* the intrinsic transition state of subtilisin BPN' in hexane by 1.22 kcal/mol as compared to acetone.

Why is the wild-type subtilisin BPN's transition state intrinsically more stable in acetone than in hexane and why should the G166N mutant stabilize the transition state in hexane relative to acetone? We may speculate that the polarity of the transition-state environment is critical and may help to answer these questions. Because subtilisin catalyzes the transesterification of ASEE via a charged and highly polar transition state, it is reasonable to expect that a polar environment would act to stabilize the transition state. Thus, acetone, being substantially more polar than hexane, is able to stabilize the transition state of wild-type subtilisin BPN' by 1.75 kcal/mol. Moreover, replacing a relatively nonpolar amino acid residue with a polar one in the vicinity of

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Table II. Differences in Free Energies between Hexane and Acetone for Subtilisin BPN' and Its Protein-Engineered Variants

enzyme	$\Delta\Delta G_S$[a] (kcal/mol)	$\Delta\Delta G^{\dagger}$[b] (kcal/mol)	$\Delta\Delta G_{ES}^{\dagger}$[c] (kcal/mol)
	ASEE as Substrate		
wild-type	1.90	−0.15	1.75
G166N	1.90	−3.12	−1.22
M222F	1.90	−1.40	0.50
	APEE as Substrate		
wild-type	2.34	0.66	3.00
G166N	2.34	−0.72	1.62
M222F	2.34	−0.20	2.04

[a] $\Delta\Delta G_S = \Delta\Delta G^E = \Delta G^E_{hexane} - \Delta G^E_{acetone} = RT[\ln \gamma_{hexane} - \ln \gamma_{acetone}]$. The ground state of the enzyme ($\Delta\Delta G_E$) is assumed to be near zero as the enzyme is insoluble in both acetone and hexane. [b] $\Delta\Delta G^{\dagger} = -RT \ln[(k_{cat}/K_m)_{hexane}/(k_{cat}/K_m)_{acetone}]$. [c] $\Delta\Delta G_{ES}^{\dagger} = \Delta\Delta G_S + \Delta\Delta G^{\dagger}$. The ratios of activity coefficients in hexane/acetone were measured in triplicate as described in the Experimental Section with a standard error less than 10%. These ratios were as follows: APEE, $\gamma^{\infty}_{hexane}/\gamma^{\infty}_{acetone} = 48.9$; ASEE, $\gamma^{\infty}_{hexane}/\gamma^{\infty}_{acetone} = 23.4$.

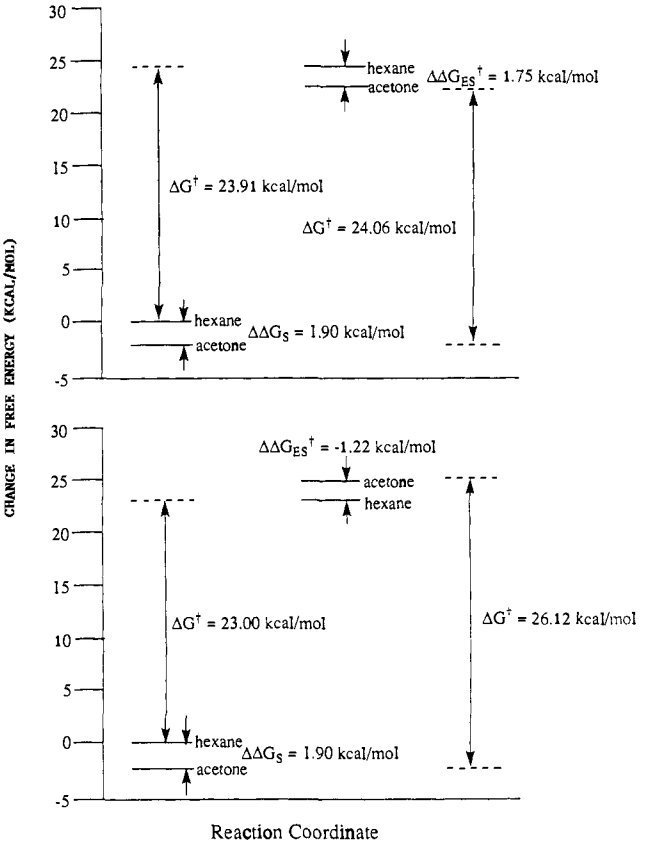

Figure 1. Free energy diagrams for subtilisin BPN' catalyzed transesterification of ASEE with *n*-propanol (comparison between hexane and acetone): (A) wild-type; (B) G166N mutant.

the transition state appears to compensate for the loss in solvent polarity upon going from acetone to hexane. Hence, the G166N mutant stabilizes the enzymic transition state in hexane as compared to the wild-type enzyme. The M222F mutation also stabilizes the transition state in going from acetone to hexane albeit not to the same extent as for the G166N mutant. Thus, while the nonpolar mutant would be expected to destabilize the transition state of subtilisin BPN′ in hexane relative to acetone, its stabilizing effect appears to be the result of a more efficient loss of the ethoxyl leaving group from the tetrahedral intermediate.[1f,9]

The transesterification of APEE is much less affected by the solvent than the transesterification of ASEE (Table I). As is the case with ASEE, the activity of the G166N mutant in hexane is greater than that of the wild-type, although the activation with G166N for APEE is less striking than for ASEE (in fact, the transition state of APEE is destabilized in hexane relative to acetone with the polar mutant). The intrinsic solvent effect on APEE for subtilisin BPN′ and its protein-engineered variants is given in Table II. For the wild-type, the intrinsic transition state of the enzymic reaction for APEE is dissimilar to that for ASEE (compare values of $\Delta\Delta G_{ES}^{\ddagger}$ for APEE and ASEE). This suggests that the effect of the organic solvent on the transition state of the wild-type is strongly dependent on the nature of the substrate. There is also a dramatic substrate dependence on the transition state for the G166N mutant in different solvents. The polar mutant dramatically stabilizes the transition state of the polar ASEE as compared to that of the nonpolar APEE. This is consistent with the aforementioned hypothesis that a polar transition state is stabilized by a polar environment. The transition-state stabilization due to the G166N mutation must be somewhat local to the S_1 binding site. One possibility is that an enhanced interaction (perhaps hydrogen bonding) between asparagine's amide and the hydroxyl of ASEE takes place and stabilizes the enzymic transition state (see below). Such a polar interaction is not possible with APEE, and this is evident in the destabilization of the intrinsic transition state for APEE with the G166N mutant.

General Solvent Effect on Protein-Engineered Variants of Subtilisin

The activation of subtilisin catalysis using a polar mutant in a nonpolar solvent was found to be general over a range of different solvent polarities. Figure 2 depicts the effect of solvent polarity (as portrayed as the inverse of the solvent dielectric constant)[10] on the improvement of the enzyme function for the polar active-site mutation as compared to the wild-type ($\Delta\Delta G^{\ddagger}_{Mutation}$, in this case defined according to eq 6). The polar mutation is deleterious

$$\Delta\Delta G^{\ddagger}_{Mutation} = -RT \ln \frac{(k_{cat}/K_m)_{G166N}}{(k_{cat}/K_m)_{WT}} \qquad (6)$$

for catalysis in polar solvents (e.g., water with a $1/\epsilon = 0.0125$) while activating in nonpolar solvents such as hexane (e.g., $1/\epsilon = 0.45$). Because $\Delta\Delta G^{\ddagger}_{Mutation}$ for a given substrate is depicted, changes in substrate ground state are negated and the intrinsic transition-state stabilization due to a polar mutation in the active site is shown. The slope for the ASEE substrate is ca. 2.5-fold steeper than that for the APEE substrate, indicating that the G166N mutation has a more pronounced effect on the stabilization of the more polar transition state of ASEE as compared to APEE, as previously described. Importantly, $\Delta\Delta G^{\ddagger}_{Mutation}$ does not correlate well with solvent hydrophobicity. For example, isopropyl ether, propyl ether, and 2-heptanone have nearly identical log P values (1.9, 1.9, and 1.8, respectively), far different dielectric constants (3.89, 3.30, and 11.90, respectively), and very different values for $\Delta\Delta G^{\ddagger}_{Mutation}$ (−0.60, −1.08, and 0.11 kcal/mol, respectively) for APEE as the substrate. These results demonstrate that solvent polarity dominates the nature of the solvent interaction with the active site of subtilisin BPN′. It should be noted that hydrophobicity, while not having a direct effect on the transition state of subtilisin, does strongly affect the stabilization of substrate ground states. The effect of active-site mutations on subtilisin catalysis is, therefore, both substrate and solvent dependent with polarity and ground-state stabilization providing significant physicochemical driving forces for the observed kinetics.

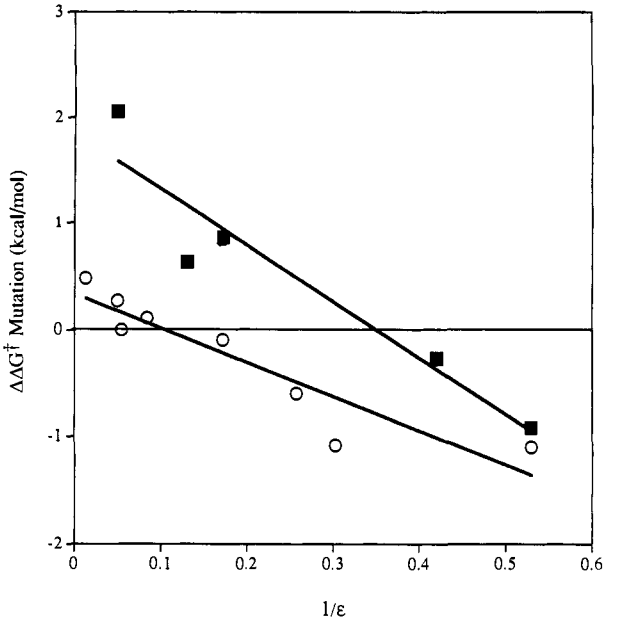

Figure 2. Dependence of the intrinsic transition-state stabilization of subtilisin BPN′ by a polar active-site mutation ($\Delta\Delta G^{\ddagger}_{Mutation}$) on the polarity of the organic reaction medium. The substrates were APEE (○) and ASEE (■). The solvents from left to right were (i) with ASEE as the substrate, acetone, THF, *tert*-amyl alcohol, dipropyl ether, and hexane; and (ii) with APEE as substrate, water (hydrolysis of APEE only), acetone, cyclohexanone, 3-heptanone, 2-heptanone, *tert*-amyl alcohol, diisopropyl ether, and hexane.

Combined Effects of Protein Engineering and Solvent Hydration on Enzyme Function

In addition to a polar mutation, the addition of water can increase the polarity of the active site and improve enzyme function.[4,11] What are the similarities between water addition and active-site polar mutations? This question can provide insight into the nature of active-site polarity on subtilisin BPN′ catalysis. Subtilisin offers a unique opportunity to dissect the combined effects of protein engineering and solvent hydration on enzyme function. The reaction mechanism of subtilisin catalysis is well-known,[12] and it is possible to study the effect of enzyme hydration on the fundamental steps in subtilisin catalysis.

Subtilisin catalyzes the facile hydrolysis ($k_{cat}/K_m = 2.84 \times 10^3$ M^{-1} s^{-1}) of APEE in aqueous buffer.[4] The rate-limiting step of the reaction is *deacylation*[13] with $k_{cat} = k_3 = 116$ s^{-1} and $(K_m)_{apparent}$ = 41 mM. In anhydrous THF (a solvent which has been used in previous studies involving subtilisin BPN′, water addition, and catalytic efficiency), the transesterification of the same substrate with 1 M *n*-propanol proceeds slowly ($k_{cat}/K_m = 9.5 \times 10^{-2}$ M^{-1}

(9) The 222 position lies in the S_1' binding region of subtilisin BPN′. As such, it is adjacent to the ethoxyl moiety of ASEE or APEE and not the amino acid substrate side chain.

(10) The inverse of the dielectric constant is directly proportional to electrostatic interactions via Coulomb's law.

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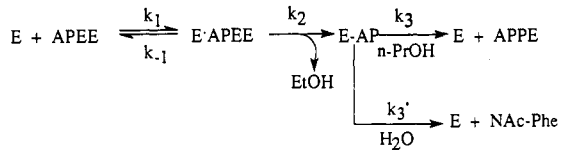
Scheme I. Simplified Kinetic Mechanism for Subtilisin BPN' Catalysis[a]



[a] The following nomenclature is used: APEE, N-Ac-L-Phe ethyl ester; E·APEE, Michaelis complex; E–AP, acyl enzyme intermediate with AP indicating the N-Ac-L-Phe moiety; EtOH, ethanol leaving group; *n*-PrOH, *n*-propanol nucleophile; APPE, N-Ac-L-Phe propyl ester; NAc-Phe, N-Ac-L-Phe free acid.

s^{-1}), albeit considerably faster than the nonenzymatic reaction.[14] The determination of individual rate constants can be performed in several ways in organic media. Chatterjee and Russell[15] used a competitive nucleophile approach tailored after the work of Bender et al.[16] in which a second alcohol is added to the reaction mixture and competitive inhibition of the desired nucleophile's attack on the acyl enzyme intermediate is measured. We have chosen an alternative method to measure individual rate constants in an organic medium that is supplemented with small concentrations of water. Scheme I depicts the effect of water on the reaction sequence of subtilisin. Water competes with *n*-propanol for the acyl enzyme and shifts the reaction away from transesterification and toward hydrolysis. Equation 7 represents a simplified kinetic expression for transesterification only.

$$v_{\text{transesterification}} = \frac{(k_{cat})_{\text{apparent}}[E_0][\text{APEE}]}{(K_m)_{\text{apparent}} + [\text{APEE}]} \quad (7)$$

Using such a kinetic expression, the values of $(k_{cat})_{\text{apparent}}$ and $(K_m)_{\text{apparent}}$ can be shown to be as follows.

$$(k_{cat})_{\text{apparent}} = \frac{k_2k_3[\text{PrOH}]}{k_2 + k_3[\text{PrOH}] + k_3'[H_2O]} \quad (8)$$

$$(K_m)_{\text{apparent}} = \frac{(K_m)_{\text{true}}\{k_3[\text{PrOH}] + k_3'[H_2O]\}}{k_2 + k_3[\text{PrOH}] + k_3'[H_2O]} \quad (9)$$

$$(K_m)_{\text{true}} = \frac{k_{-1} + k_2}{k_1} \quad (10)$$

In the absence of added water, THF contains ca. 3 mM measurable water (ca. 0.005% v/v), enough to result in intersections of the Lineweaver–Burk plots of $1/v$ vs $1/[\text{APEE}]$ for different initial *n*-propanol concentrations ranging from 10 to 200 mM (Figure 3). Hence, even in nearly anhydrous THF, the water content is high enough to act as a competitive nucleophile in transesterification, and this competition is increasingly significant at lower concentrations of *n*-propanol. Competition by water results in an intersection of the lines at different *n*-propanol concentrations. As shown in Figure 3, the *y*-value for this intersection point corresponds to $-1/k_3'[H_2O]$ and the *x*-value for this intersection point corresponds to $-(k_2 + k_3'[H_2O])/(K_m)_{\text{true}}k_3'[H_2O]$ (see supplementary material). A secondary replot of the *y*-intercepts of the primary plot ($1/k_{cat}$ values) vs $1/[n\text{-propanol}]$ provides the true value of $1/k_2$ as the *y*-intercept (Figure 4A). Finally, a secondary replot of $(K_m/k_{cat})_{\text{apparent}}$ vs $1/[n\text{-propanol}]$ provides the value of $(K_m/k_{cat})_{\text{true}}$ with the slope equal to $(K_m)_{\text{true}}k_3'[H_2O]/k_2k_3$ (Figure 4B). Values of k_3 and $(K_m)_{\text{true}}$ are then calculated with the available information.

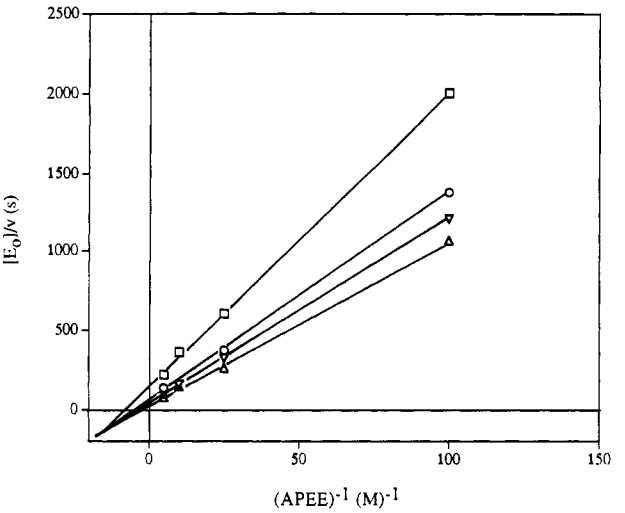


Figure 3. Lineweaver–Burk plots for the transesterification of APEE with different *n*-propanol concentrations in "dry" THF catalyzed by wild-type subtilisin BPN'. The *n*-propanol concentrations were 10 mM (□), 40 mM (○), 100 mM (▽), and 200 mM (△). The concentration of water was 0.005% v/v, as measured by the Karl Fischer technique. This was enough water to deflect parallel lines and indicates that water acts as a competitive nucleophile. Note that the lines intersect at a point in the double-negative quadrant. This is further described in the supplementary materials. The *x*-axis of intersection represents the value of $-(k_2 + k_3'[H_2O])/(K_m)_{\text{true}}k_3'[H_2O]$, and the *y*-axis intersection represents the value of $-1/k_3'[H_2O]$. The concentrations of enzyme and APEE, and all other experimental details, are as described in the Experimental Section.

The transesterification reaction in tetrahydrofuran (THF) is limited by the *acylation* rate with $(K_m)_{\text{true}} = 460$ mM, $k_2 = 0.044$ s^{-1}, and $k_3 = 0.71$ M^{-1} s^{-1} (with 1 M *n*-propanol the pseudo-first-order value of k_3 is 0.71 s^{-1}, which is ca. 16-fold higher than that for k_2). In fact, k_3 is not significantly different from its value for hydrolysis (2.09 M^{-1} s^{-1} in aqueous solution taking into account the presence of 55.5 M water as the nucleophile). Because $k_3[\text{PrOH}] > k_2$, the value of $(K_m)_{\text{apparent}}$ approximates $(K_m)_{\text{true}}$.[7] The value of $(K_m)_{\text{true}}$ in THF is similar to the value obtained by Chatterjee and Russell[15] using N-Ac-L-Phe-OMe as the substrate. Thus, the reaction in THF is characterized by a slow acylation step and relatively weak substrate binding (making a first approximation that $(K_m)_{\text{true}}$ is of the same order of magnitude as K_s, the true enzyme–substrate dissociation constant) as compared to the reaction in aqueous buffer. Much of this must be due to the low level of enzyme hydration in anhydrous THF and the direct effect of dehydration on subtilisin's catalytic power. It was of interest, therefore, to study the effect of solvent hydration on subtilisin kinetics.

Addition of water to THF does increase the catalytic efficiency of subtilisin such that, at 5% (v/v) added water, the value of $(k_{cat}/K_m)_{\text{true}} = 1.12$ M^{-1} s^{-1}. The rate-determining step switches from acylation to deacylation between 0.5 and 2% (v/v) added water and is primarily due to an increase in k_2. Thus, as water is added to subtilisin in THF, the enzyme becomes a better acylating catalyst. This is similar to our previous results with phenylmethanesulfonyl fluoride-induced (PMSF-induced) inactivation of subtilisin BPN' in THF with increasing water concentrations.[4] In the absence of added water, PMSF-induced inactivation is slow but rapidly increases in the presence of ca. 1% (v/v) added water. This was shown by electron paramagnetic resonance spectroscopy to be correlated to a dramatic increase in the local polarity of the enzyme's active site. The data presented

(14) The enzyme preinactivated by boiling in water for 30 min or by the active-site serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) showed no observed activity. Furthermore, use of the $Ser_{221} \rightarrow$ Ala active-site mutant (where the nucleophilic serine is replaced by a catalytically inert alanine) also gave no enzyme activity. Thus, the reactions studied in this work were truly of enzymatic origin.

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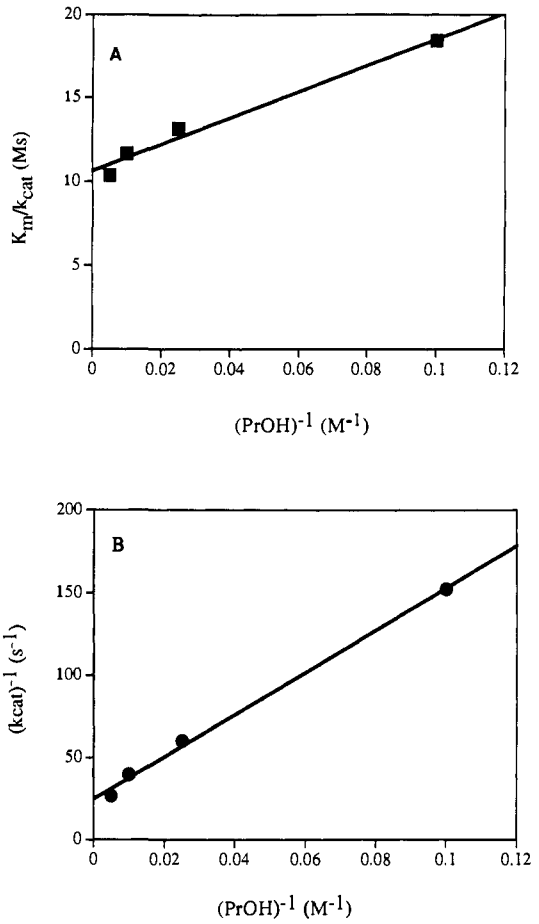

Figure 4. Secondary replots for wild-type subtilisin BPN′ in "dry" THF. (A) Calculation of $(k_2/K_m)_{true}$ from the intersection of (K_m/k_{cat}) vs $1/[n\text{-PrOH}]$. The slope is equal to $(K_m)_{true}k_3'[H_2O]/k_2k_3$. (B) Calculation of k_2 from intersection of $(1/k_{cat})_{apparent}$ vs $1/[n\text{-PrOH}]$.

Table III. Individual Kinetic Constants for Subtilisin BPN′ and Its Polar Mutant, G166N, in Dry THF and THF Supplemented with Water[a]

enzyme	$[H_2O]$ (% v/v)	k_2 (s^{-1})	k_3 (M^{-1} s^{-1})	$\frac{k_3[PrOH]}{k_2}$[b]	$(K_m)_{apparent}$ (M)
wild-type	0[c]	0.044	0.71	16.1	0.462[d]
	0.5	0.12	0.36	3.0	0.153[d]
	2.0	0.24	0.15	0.63	0.243
	5.0	3.14	0.42	0.13	2.800
G166N	0[b]	0.042	1.04	24.8	0.132[d]
	0.5	0.19	0.50	2.63	0.370[d]
	2.0	0.23	0.19	0.83	0.198
	5.0	0.55	0.47	0.86	0.377

[a] Conditions as described in Table I, except that *n*-propanol concentrations ranged from 10 mM to 1 M. The kinetic data were obtained from triplicate measurements for each APEE and *n*-propanol concentration. The values depicted in this table have standard errors of no greater than 15%. [b] Data obtained for PrOH concentration of 1 M. [c] Dry THF contained ca. 0.005% v/v water as determined by the Karl Fischer technique. [d] These values are equivalent to $(K_m)_{true}$ as $k_3[PrOH] > k_2$.

in this paper are also consistent with a rapid activation of enzyme function due to an increase in the local polarity in the enzyme's active site in the presence of added water.

Table III depicts the values of k_2, k_3, and $(K_m)_{apparent}$ for wild-type and G166N in dry THF and in the presence of added water. Water addition, up to 0.5% v/v, results in a lower value of $(K_m)_{apparent}$ than in dry THF. The values of $(K_m)_{apparent}$ approximate $(K_m)_{true}$ in these cases as $k_3[PrOH] > k_2$. Hence, small concentrations of water improve the interaction of the enzyme with the substrate. This interaction is expected to take place mainly in the S_1 subsite of subtilisin BPN′ which binds the APEE substrate. Interestingly, the polar mutation to G166N in dry THF results in a similar drop in $(K_m)_{true}$ as for the wild-type in 0–0.5% v/v water added to THF. The value of k_2, however, is not affected by the polar mutation in dry THF. This implies that the nature of the transition-state stabilization of the G166N mutant is due to enhanced interaction of the enzyme with the substrate in dry organic solvents, and this can be mimicked by adding water. Addition of water, however, goes beyond increasing the enzyme–substrate interaction as the rate constant of acylation also increases. Importantly, the values of k_3 do not fluctuate significantly as a function of added water concentration, a finding that is consistent with the similar values of k_3 in dry THF and aqueous solution. Nor are the values of k_3 for the polar mutant significantly different than those for the wild-type. This is not surprising because the 166 position is distant from the S_1' site where deacylation takes place.

In THF supplemented with 5% (v/v) water, the wild-type is nearly as active as the polar mutant. The catalytic efficiency of the wild-type is increased 12-fold in going from 0 to 5% (v/v) added water in THF, while that of the G166N mutant increases only 4.5-fold. This increased activity for the wild-type relative to the G166N mutant (Table III) is primarily due to a dramatically increased value of k_2 as compared to that for the active-site mutant.[17] Thus, water affects the polar mutant differently than the wild-type. The diminished advantage of the polar G166N mutant over that of the wild-type as water is added to THF can be explained as follows: The increased polarity of the G166N mutation enhances the polarity of the active site, particularly in the S_1 pocket, and this enhances the stabilization of the polar transition state mainly through binding interactions. In the presence of added water, however, this advantage is negated, presumably by polar water molecules penetrating into the active site of the enzyme.[18] Water addition results in both an increase in the rate constant of acylation and a decrease in $(K_m)_{true}$. For the G166N mutant in dry THF, on the other hand, $(K_m)_{true}$ is decreased but the rate constant of acylation is unchanged. Thus, hydration affects enzyme function is a manner that is more detailed than that of increased polarity alone. It has been speculated that water acts as a plasticizer to improve enzyme function by increasing the flexibility of the enzymic transition state.[11] The increased rate constant of acylation is consistent with such an increase in active-site flexibility. Alternatively, it has been suggested that water can increase the proton conductivity within an enzyme and that this is critical for the efficiency of general acid–base catalysis.[19] Serine proteases, such as subtilisin, utilize general acid–base catalysis during catalysis. It has been suggested that proton transfer to the catalytic histidine is rate-limiting during acylation.[2a] Addition of water would increase this proton-transfer rate and increase the value of k_2. Water, therefore, may play multiple roles in the enzyme's active site, either directly or indirectly.

In conclusion, protein engineering is affected by the nature of the solvent and the level of solvent hydration. This study has provided insights into the effect of active-site polarity on enzyme function and highlights the difference between polarity changes due to active-site mutation and active-site hydration. Furthermore, this work provides more evidence that enzyme hydration is a complex phenomenon. From a practical perspective, these findings have important implications for the rational design of enzyme function in dehydrated environments. Numerous factors

(17) The high value of $(K_m)_{apparent}$ at higher water concentrations is due to the large contribution of k_2 to the expression given in eq 10 as the water content increases.

(18) This phenomenon could also occur if water displaces solvent molecules that are in the active site in nearly anhydrous organic media. The presence of organic solvent in the active site of subtilisin Carlsberg has been shown by X-ray crystallography (Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, in press).

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must be taken into account in tailoring enzyme function to nonaqueous media, not the least of which are the polarities of solvent, substrate, and active-site residues.

Experimental Section

Enzyme. Subtilisin BPN' (from *Bacillus amyloliquefaciens*) wild-type and active-site mutants were obtained from Genencor International (South San Francisco, CA). The mutants were constructed as previously described.[1a,20] Prior to use, the enzymes were prepared by lyophilization from a 5 mg/mL solution in 20 mM phosphate buffer, pH 7.8. Lyophilized enzyme powder was stored over $CaSO_4$ in a desicator at −20 °C. The entire desicator was warmed to room temperature before the contents were exposed to air.

Chemicals and Solvents. N-Ac-L-Phe-OEt and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. All solvents were the highest purity commercially available and were dried prior to use by shaking with 3-Å molecular sieves (Linde) for 24 h. The dried solvents had a water content of below at least 0.005% (v/v) as determined by Karl Fischer titration. N-Ac-L-Ser-OEt was synthesized from L-Ser-OEt·HCl (Sigma) and acetyl chloride (Aldrich) by a published procedure.[21]

Enzyme Kinetics. Enzyme reactions were performed in 4-mL vials containing 1 mL of the reaction mixture and 1 mg of enzyme. The reactions were shaken at 250 rpm at 30 °C. For reactions in anhydrous acetone and hexane with ASEE and APEE, the *n*-propanol concentration was kept fixed at 1 M and the esters were varied from 1 to 150 mM. Values of k_{cat}/K_m were determined by nonlinear regression. The formation of propyl esters was followed by gas chromatography (25-m HP-1, 530-μm fused silica gum capillary column (Hewlett-Packard), N_2 as the carrier gas (30 mL/min), and injector and detector port temperatures of 250 °C). Each enzyme was titrated in aqueous solution prior to use to ascertain the fraction of active sites that were indeed active. This was done with *N*-trans-cinnamoylimidazole and resulted in a determination that 54% of the wild-type was active and 36% of the G166N and M222F mutants were active. Active-site titration with *N*-trans-cinnamoylimidazole[22] in organic media indicated a constant fraction of active centers of subtilisin BPN' in both hexane and acetone (ca. 15 ± 4%).[11]

Values of $(k_{cat}/K_m)_{true}$, k_2, k_3, and $(K_m)_{true}$ were determined from primary and secondary plots as described in the text and the supplementary material. All values were calculated graphically and confirmed using nonlinear regression. Activity coefficients could not be accurately determined by using UNIFAC parameters.[23] Such parameters are inherently inaccurate for molecules with numerous closely-spaced polar groups, as are present on both ASEE and APEE, because such groups will interact with one another.[24] Therefore, the ratios of activity coefficients for ASEE and APEE in hexane/acetone (i.e., $\gamma_{hexane}/\gamma_{acetone}$) were measured experimentally via TLC using silica gel plates and elution systems of acetone/1 M *n*-propanol and hexane/1 M *n*-propanol. Measurements of this kind resulted in standard errors of less than 10% based on triplicate experiments. In such a system the silica gel plates act as a separate phase. Thus, eq 11 holds where C^s and C^m represent the

$$\ln \frac{(1 - R_f)}{R_f} = \ln \frac{C^s}{C^m} = \ln K \propto \ln \frac{\gamma^{m\infty}}{\gamma^{s\infty}} \tag{11}$$

concentration of ASEE or APEE in the stationary and mobile phases, respectively.[25] The ratio of activity coefficients between hexane and acetone for a given substrate is now given in eq 12, where γ^s is independent

$$\left(\frac{\gamma^{m\infty}}{\gamma^{s\infty}}\right)_{hexane} \Big/ \left(\frac{\gamma^{m\infty}}{\gamma^{s\infty}}\right)_{acetone} = \frac{\gamma^{\infty}_{hexane}}{\gamma^{\infty}_{acetone}} \tag{12}$$

of the solvent because the same substrate and silica plate is employed in the measurements and the only difference is the elution solvent.

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Supplementary Material Available: Text describing the method for determining individual rate and binding constants from kinetic data (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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