The Mechanistic Dissection of the Plunge in Enzymatic Activity upon Transition from Water to Anhydrous Solvents

Jennifer L. Schmitke, Charles R. Wescott, and Alexander M. Klibanov*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received November 28, 1995[⊗]

Abstract: Subtilisin Carlsberg dissolved in aqueous solution is several orders of magnitude more active than the enzyme suspended in anhydrous acetonitrile. In order to ascertain why, we employed crystalline subtilisin lightly cross-linked with glutaraldehyde as a catalyst in both aqueous and organic media. The structure of this crystalline enzyme in acetonitrile had been previously found to be virtually identical to that in water, thus ruling out solvent-induced conformational changes as the cause of the enzymatic activity drop. Titration of the competent active centers of subtilisin revealed that k_{cat}/K_M is solely responsible for this activity plunge. Quantitative mechanistic analysis of the 7-order-of-magnitude difference in k_{cat}/K_M values between subtilisin dissolved in water and cross-linked subtilisin crystals suspended in anhydrous acetonitrile allowed accounting for at least 5.6 orders of magnitude. This drastic decline is due to (i) a marked shift in the activity *vs* pH profile of the cross-linked crystalline enzyme compared to its soluble counterpart; (ii) different (far less favorable in acetonitrile than in water) energetics of substrate desolvation; and (iii) very low thermodynamic activity of water in anhydrous acetonitrile resulting in a much more rigid and thus less active enzyme.

Introduction

Enzymes have been found to possess unique properties in nonaqueous media, e.g., the ability to catalyze transformations virtually impossible in aqueous solution.[1] However, a major drawback limiting the utility of enzymes is that their catalytic activity in anhydrous organic solvents is drastically diminished relative to that in water. For example, lyophilized powder of the serine protease subtilisin Carlsberg is some 6 orders of magnitude less active when suspended in acetonitrile than the soluble enzyme is in water.[2] A tempting explanation for this drop in activity is that a conformational change inflicted upon the enzyme by lyophilization and/or subsequent placement in the solvent is responsible.[3] Recently, however, it was demonstrated that this could be no more than a minor part of the explanation because the crystal structure of cross-linked crystals (CLCs) of subtilisin in anhydrous acetonitrile was found to be indistinguishable from that in water,[4,5] and yet the CLCs are still far less catalytically active in acetonitrile than in water.[4]

In the present study, we addressed this issue mechanistically using the aforementioned CLCs of subtilisin as a model system. Since the structure of this crystalline enzyme preparation is the same in acetonitrile and in water,[4,5] the solvent-induced conformational change hypothesis, while viable with the amorphous enzyme, may not be invoked. In addition, because CLCs are highly stable and possess other attractive properties in aqueous and aqueous−organic mixtures,[6] they may turn out to be the future catalyst of choice in both aqueous and nonaqueous enzymology.

Results and Discussion

In order to approach the explanation of the difference in catalytic activity of enzymes in organic solvents *vs* in water, we selected a model system on which to focus our study, namely, the difference in the catalytic activity of soluble subtilisin in aqueous solution at pH 7.8 for the hydrolysis of N-Ac-L-Phe-OEt and that of the CLCs of subtilisin in anhydrous acetonitrile for the transesterification of this ester with propanol. We found that V_{max}/K_M plummets more than 7 orders of magnitude upon transition from the aqueous to organic system,[7] from 2.1 s^{-1} to 4.5 × 10^{-8} s^{-1} (1 mg/mL of enzyme).

Two factors could contribute to the observed reduction in V_{max}/K_M, namely, a reduction in the catalytic efficiency of the enzyme (k_{cat}/K_M) or in the concentration of catalytically competent enzyme ($[E]_0$). Although the conformation of cross-linked crystalline subtilisin suspended in acetonitrile is virtually identical to that in water[4,5] (indicating that subtilisin in the CLCs is native), it is possible that only a small fraction of the active centers in the CLCs is accessible to the substrate because of hindering protein−protein contacts or cross-links within the crystal, resulting in a reduced $[E]_0$. To test for such an effect, the percentage of catalytically competent active centers was measured by the direct titration of Ser 221 of subtilisin's catalytic triad (see Experimental Section for details). The

* To whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1996.

(1) Koskinen, A. M. P.; Klibanov, A. M., Eds. *Enzymatic Reactions in Organic Media*; Blakie Academic & Professional: London, 1996. Santaniello, E.; Ferraboschi, P.; Grisenti, P. *Enzyme Microb. Technol.* **1993**, *15*, 367−382. Faber, K. *Biotransformations in Organic Chemistry*; Springer-Verlag: Berlin, 1992. Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114−120. Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194−211. Chen, C.-S.; Sih, C. J. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 695−707.

(2) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194−3201.

(3) Desai, U. R.; Osterhout, J. J.; Klibanov, A. M. *J. Am. Chem. Soc.* **1994**, *116*, 9420−9422.

(4) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8653−8657.

(5) Fitzpatrick, P. A.; Ringe, D.; Klibanov, A. M. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 675−681.

(6) Lalonde, J. J.; Govardhan, C.; Khalaf, N.; Martinez, A. G.; Visuri, K.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 6845−6852. Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 2732−2737. Sobolov, S. B.; Bartoszko-Malik, A.; Oeschger, T. R.; Montelbano, M. M. *Tetrahedron Lett.* **1994**, *35*, 7751−7754. St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314−7316.

(7) V_{max}/K_M in the two systems can be directly compared as long as they involve the same ester substrate. This is because $(k_{cat}/K_M)_{N\text{-}Ac\text{-}L\text{-}Phe\text{-}OEt}$, the bimolecular rate constant for the reaction of the free enzyme with this ester substrate, is independent of the nucleophile substrate—whether water (in aqueous solution) or an alcohol (in organic solvents).[2]

0002-7863/96/1518-3360$12.00/0

Table 1. Values of $(k_{cat}/K_M)_{N\text{-Ac-L-Phe-OEt}}$ for the Subtilisin-Catalyzed Hydrolysis of N-Ac-L-Phe-OEt or Its Transesterification with Propanol

form of subtilisin[a]	solvent	k_{cat}/K_M[b] (M^{-1} s^{-1})
dissolved	water (pH 7.8)	$(9.1 \pm 1.3) \times 10^4$
CLCs	water (pH 7.8)	$(3.4 \pm 0.5) \times 10^3$
CLCs	octane ($a_w = 1$)	40 ± 20
CLCs	octane ($a_w = 0.006$)	0.93 ± 0.40
CLCs	acetonitrile ($a_w = 0.006$)	$(2.3 \pm 0.5) \times 10^{-2}$
CLCs	acetonitrile ($a_w \leq 0.002$)	$(1.1 \pm 0.4) \times 10^{-2}$

[a] All organic solvents contained 100 mM propanol as the nucleophile. [b] The values of the portion of the CLCs that is active subtilisin, used to calculate $[E]_0$, are $57 \pm 20\%$ in water, $45 \pm 24\%$ in octane, and $33 \pm 14\%$ in acetonitrile. These values were in turn used to calculate k_{cat}/K_M from V_{max}/K_M.

fractions of active enzyme dissolved in aqueous solution and in CLCs suspended in acetonitrile were found to be similar, $63 \pm 5\%$ and $33 \pm 14\%$, respectively, thus ruling out the possibility that the 7-order-of-magnitude drop in activity could be due to a decrease in $[E]_0$. When the $[E]_0$ values determined from the active center titrations are used to convert V_{max}/K_M to k_{cat}/K_M, the diminished activity of CLCs in acetonitrile compared to that of the dissolved enzyme in water is unequivocally attributable to decreased catalytic efficiency of the enzyme (Table 1).

When one compares soluble subtilisin in water to CLCs of subtilisin in acetonitrile, two parameters have been changed simultaneously, namely the solvent and the form of the enzyme. In order to determine the contribution of each factor individually, we examined the behavior of the CLCs relative to the soluble enzyme in water, thus changing only the form of the enzyme, while keeping the solvent the same. The k_{cat}/K_M value of the CLCs in water at pH 7.8 was found to be 1.4 orders of magnitude lower than that of soluble subtilisin (Table 1). Therefore, the first question to be addressed was why this is so.

One plausible explanation is that the reaction catalyzed by the CLCs is slowed by internal diffusion of the substrate within them. To test this, we co-crystallized active and inactivated (with phenylmethylsulfonyl fluoride) subtilisin at various f values (where f is the fraction of the active subtilisin in the CLCs) and studied the dependence of the enzymatic activity on f. If the reaction is limited by internal diffusion, this dependence should be convex.[8] If, however, the reaction is free of diffusional limitations (internal or external), a linear dependence should result.[8] As seen in Figure 1, the dependence of the activity of the CLCs on f is essentially linear, thus ruling out diffusional limitations as the cause of the 27-fold drop in activity of the CLCs in water compared to the soluble enzyme. Note that if the enzymatic activity of the CLCs of subtilisin is not limited by diffusion in water, it certainly will not be limited by diffusion in organic solvents, where the reaction is far slower (Table 1) and diffusion faster (because the solvents' viscosities are lower than water's).[8]

Another tenable reason for the difference in activity of the CLCs compared to soluble subtilisin in water at pH 7.8 is that the activity *vs* pH profiles of the two forms of the enzyme are distinct. Indeed, we found (Figure 2) that the pH dependence of the activity of the CLCs is markedly shifted (by 3.7 pH units) compared to that of the dissolved enzyme.[9] This shift can readily account for the 1.4-order-of-magnitude difference in the k_{cat}/K_M values between the cross-linked crystalline and dissolved subtilisins at pH 7.8. Therefore, the first drop in enzymatic activity that occurs upon transition from soluble subtilisin in aqueous solution at pH 7.8 to the CLCs in dry acetonitrile can be explained by the shift of the activity *vs* pH profile of the CLCs relative to that of the enzyme in solution.

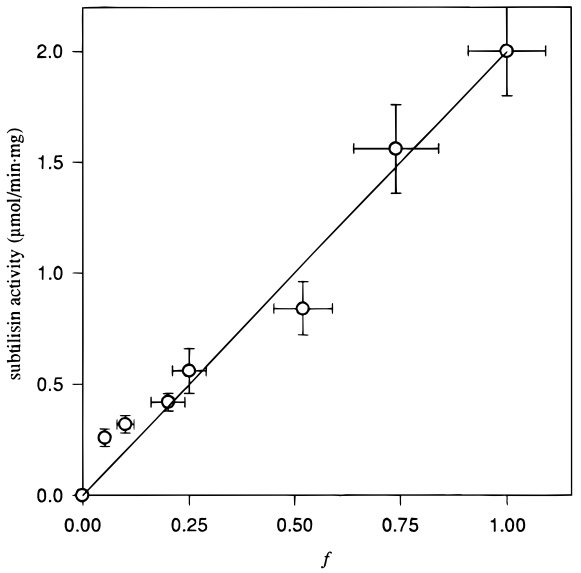


Figure 1. Dependence of the catalytic activity of CLCs of subtilisin in the hydrolysis of N-Ac-L-Phe-OEt in water (pH 7.8) on the fraction (f) of the active subtilisin in the CLCs. The value of f was manipulated by co-crystallizing mixtures having different ratios of active subtilisin and PMSF-inactivated subtilisin. For conditions, see the Experimental Section.

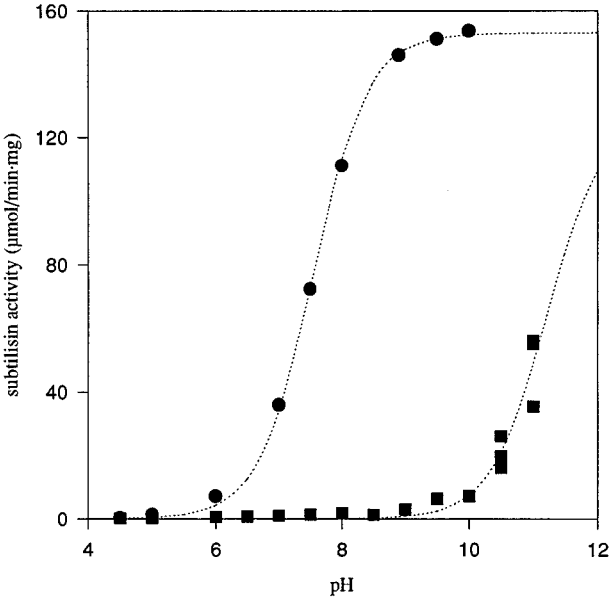


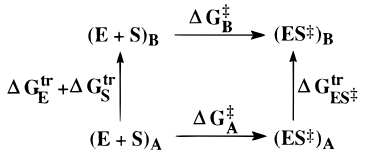
Figure 2. The pH dependence of the initial rate (v) of the enzymatic hydrolysis of N-Ac-L-Phe-OEt in water for dissolved subtilisin (●) and the CLCs of subtilisin (■). The data were fitted to the theoretical ionization curve (- - -) by nonlinear regression, where $v = (A \times 10^{(pH-pK_a)})/(1 + 10^{(pH-pK_a)})$, where A is a constant. For conditions, see the Experimental Section.

Having accounted for 1.4 orders of magnitude, there is still a 5.6-order-of-magnitude decrease in activity to be rationalized that transpires upon moving the CLCs from water to anhydrous acetonitrile. Two factors, which have previously been shown

(8) Boudart, M.; Burwell, R. L. *Techniques in Chemistry*, 3rd ed.; Wiley: New York, 1973; Vol. 6, Chapter 12. Crank, J. *The Mathematics of Diffusion*; Oxford University Press: London, 1957; Chapter 5.

(9) Although the exact cause of the shift in the activity *vs* pH profile is not known, at least one possible explanation seems likely. In the crystalline state, the subtilisin molecules are held in a close proximity and fixed orientation to each other (packed in the crystal lattice). The ensuing intermolecular electrostatic interactions could affect the pK_as of the active center's catalytic residues, leading to the observed effect.

Scheme 1



to influence the kinetics of enzymes suspended in organic solvents, are likely to be responsible. The first is the difference between the desolvation free energies of the substrate, N-Ac-L-Phe-OEt, in acetonitrile and water. Because the binding energy of a substrate is utilized for enzyme catalysis,[10] and this binding of a substrate molecule to an enzyme active center requires the desolvation of the substrate, k_{cat}/K_M depends on the free energy of substrate desolvation.[11] The second likely contributor is the dehydration of the CLCs, i.e., their exposure to a much lower thermodynamic water activity (a_w). For lyophilized powders of enzymes in general,[12] and of subtilisin in particular,[13] increasing the a_w in organic solvents leads to enhanced enzymatic activity, presumably because water acts as a molecular lubricant,[14] increasing the conformational mobility of the enzyme. We endeavored to isolate these two factors and elucidate their individual contributions to the decrease in catalytic efficiency of the CLCs upon transition from water to dry acetonitrile.

The two effects can be segregated using the following four-staged stepladder (Table 1). Because in anhydrous acetonitrile the a_w is less than 0.002 but not known exactly, the first move is to acetonitrile at a still low but defined a_w (step 1). Salt hydrate pairs can be used in organic solvents (where they are insoluble) to buffer the a_w to a constant value.[15] We selected the pair $BaBr_2 \cdot 1H_2O/BaBr_2$ which buffers the a_w to 0.006. The next transition is from acetonitrile with $a_w = 0.006$ to octane with the same a_w to isolate the contribution of the substrate desolvation energy difference (step 2); the introduction of this intermediate solvent is necessary because it is impossible to attain the same water activity, $a_w = 1$, in acetonitrile (infinitely miscible with water) and in water. The subsequent shift from octane with $a_w = 0.006$ to that with $a_w = 1$ addresses the water activity effect alone (step 3). The final move from octane with $a_w = 1$ to water should again indicate the role of substrate desolvation in these solvents (step 4).

The aforementioned thermodynamic effect, which arises from the difference in the substrate desolvation energy between two solvents and the resulting difference in reactivity toward the enzyme, is depicted by the cycle in Scheme 1.[11] The lower horizontal arrow represents the enzyme (E) reacting with the substrate (S) in solvent A to form the transition state ($ES^‡$). In a hypothetical, equivalent path, E and S separately partition from solvent A into solvent B, and the transition state is formed there and is transferred from solvent B back into A. In the cycle, $\Delta G^‡_A$ and $\Delta G^‡_B$ represent the free energies of activation in solvents A and B, respectively, and ΔG^{tr}_E, ΔG^{tr}_S, and $\Delta G^{tr}_{ES^‡}$ are the free energies of transfer of the enzyme, substrate, and transition state, respectively, from A to B. Expressing $\Delta G^‡_A$ as the sum of the energetic terms of this alternative path produces

$$\Delta G^‡_A = \Delta G^{tr}_E + \Delta G^{tr}_S + \Delta G^‡_B - \Delta G^{tr}_{ES^‡} \quad (1)$$

Assuming that the entire substrate is removed from the solvent in the transition state, the solvation of E and $ES^‡$ is essentially identical, provided that there is no substrate-induced conformational change in the enzyme[16] and that desolvation of the active center can be neglected. Thus, $\Delta G^{tr}_E \approx \Delta G^{tr}_{ES^‡}$. (If the assumption does not hold, then the effect described below will be less.)

Canceling the equal terms yields

$$\Delta G^‡_A = \Delta G^‡_B + \Delta G^{tr}_S \quad (2)$$

$\Delta G^‡_A$ is related to $(k_{cat}/K_M)_A$ by[10]

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

where h, κ, and R are Planck's, the Boltzmann, and the gas constants, respectively, and T is the absolute temperature. The analogous expression can be written for solvent B. ΔG^{tr}_S can in turn be expressed in terms of the thermodynamic activity coefficient of the substrate[17] (γ):

$$\Delta G^{tr}_S = RT \ln(\gamma_B/\gamma_A) \quad (4)$$

Substitution of eqs 3 and 4 into eq 2 yields:

$$\frac{(k_{cat}/K_M)_A}{(k_{cat}/K_M)_B} = \frac{\gamma_A}{\gamma_B} \quad (5)$$

This predictive model, culminating in eq 5, was tested in step 2 of the ladder. In this step, when the situation in octane with $a_w = 0.006$ is compared to acetonitrile with $a_w = 0.006$, the experimentally obtained k_{cat}/K_M ratio of 40 ± 19 (Table 1) was similar to the value predicted by the activity coefficient ratio of N-Ac-L-Phe-OEt in these two solvents, 63 ± 6 (Table 2). These data support the thermodynamic model that predicts changes in k_{cat}/K_M based on variations in the substrate desolvation energetics.

Table 2. Thermodynamic Activity Coefficients γ of N-Ac-L-Phe-OEt in Various Solvent Systems

solvent[a]	$\gamma_{\text{N-Ac-L-Phe-OEt}}$[b]
water (pH 7.8)	$(2.6 \pm 0.2) \times 10^3$
hydrous octane ($a_w = 1$)	$(8.2 \pm 0.2) \times 10^2$
octane ($a_w = 0.006$)	$(8.2 \pm 0.3) \times 10^2$
acetonitrile ($a_w = 0.006$)	13 ± 1
acetonitrile ($a_w \leq 0.002$)	13 ± 1

[a] All organic solvents contained 100 mM propanol. [b] Calculated from the solubility of the ester in the appropriate solvent system (see Experimental Section).

(10) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985; Chapter 12.

(11) Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 1629−1631.

(12) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017−8021. Halling, P. J. *Trends Biotechnol.* **1989**, *7*, 50−52. Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178−206.

(13) Yang, Z.; Zacherl, D.; Russell, A. J. *J. Am. Chem. Soc.* **1993**, *115*, 12251−12257.

(14) Poole, P. L.; Finney, J. L. *Int. J. Biol. Macromol.* **1983**, *5*, 308−310.

(15) Halling, P. J. *Biotechnol. Tech.* **1992**, *6*, 271−276.

(16) Serine proteases do not act via an induced-fit mechanism.[10] The X-ray crystal structures of subtilisin Carlsberg in its native state and that inhibited by eglin *c* are indistinguishable.[4]

(17) The free energy of a solute dissolved in a solvent is described by the equation $G = G° + RT \ln(x\gamma)$, where x and γ are the solute mole fraction and activity coefficient, respectively. Because the solvent is the primary variable in our work, the standard state is chosen as the pure liquid solute. Thus $G°$ is independent of the solvent, and the free energy of transfer of the solute from solvent A to solvent B (ΔG^{tr}_S) is $RT \ln(x_B\gamma_B/x_A\gamma_A)$. If the transfer is made at constant mole fraction (i.e., the volumes of the solvents are directly proportional to their molar volumes), this simplifies to $\Delta G^{tr}_S = RT \ln(\gamma_B/\gamma_A)$. For dilute solutions, the concentration dependence of the activity coefficient can be disregarded.[26] Thus, the variation in x which occurs in our experiments (e.g., due to concentration variation in the measurement of k_{cat}/K_M or differences in the molar volumes of the solvent) is neglected.

In addition to describing the solvent dependence of the acylation step of subtilisin-catalyzed transesterifications, Scheme 1 is equally applicable to the deacylation step. The model predicts that a difference in the desolvation energy of the nucleophile substrate (as opposed to the ester substrate) between two solvents will also result in a k_{cat}/K_M difference. In agreement with this, when a small activity coefficient ratio was calculated, e.g., for propanol in acetonitrile *vs* in *tert*-amyl alcohol (2.7), the corresponding experimentally determined k_{cat}/K_M ratio was also small (2.2). When a larger difference in $(k_{cat}/K_M)_{nucleophile}$ was predicted by the activity coefficient ratio, as is the case for 2-methoxyethanol in cumene *vs* in acetonitrile (14), the experimental ratio of the specificity constants was also higher (7.6).[18]

On the basis of all these data, one can conclude that the difference in the desolvation energy of N-Ac-L-Phe-OEt in water compared to dry acetonitrile can account, as indicated by $\gamma_{water}/\gamma_{acetonitrile}$ (Table 2), for 2.3 orders of magnitude of the difference in k_{cat}/K_M of the CLCs in these solvents. Hence only 3.3 orders of magnitude out of the original 7 are now left to explain.

As discussed earlier, the water activity may have a profound effect on the catalytic efficiency of the CLCs in organic solvents. Indeed, as seen in step 3 of the ladder (Table 1), k_{cat}/K_M of the CLCs in octane at $a_w = 1$ is 43-fold higher than that in the same solvent at $a_w = 0.006$. In addition, a doubling in the activity of the CLCs is observed in step 1, i.e., in acetonitrile with $a_w = 0.006$ compared to this solvent with $a_w < 0.002$. As mentioned above, one of the likely explanations for the increase in enzymatic activity with a_w is that water acts as a molecular lubricant and affords a higher conformational flexibility of the enzyme resulting in enhanced catalysis. Another possibility[19] is that the added water increases the dielectric constant (polarity) of the solvent and thus in the enzyme active center, with an ensuing stabilization of the charged transition state and hence an elevation in k_{cat}/K_M.

To distinguish between these two possibilities, we investigated the effects of two selected additives on k_{cat}/K_M in octane. The first, formamide, is a good molecular lubricant due to its high propensity to form hydrogen bonds[20] and the second, *N*-methylacetamide, while a poorer molecular lubricant (because it can form fewer hydrogen bonds), has a higher dielectric constant than formamide (191 *vs* 111).[21] If the flexibility hypothesis is correct, formamide should increase the observed catalytic activity in octane to a greater extent than *N*-methylacetamide. If the polarity hypothesis is correct, the opposite will be the case.[22] While the addition of formamide (at $a = 1$) raises k_{cat}/K_M by a factor of 13 (from 0.93 ± 0.40 to 12 ± 5 M^{-1} s^{-1}), no enhancement occurs when *N*-methylacetamide is added to the same activity—$k_{cat}/K_M = 0.79 \pm 0.30$ M^{-1} s^{-1}. (The value of $\gamma_{N\text{-Ac-L-Phe-OEt}}$ in octane is unaffected by these additives.) These data support the hypothesis that an elevated a_w leads to a greater conformational mobility of the enzyme that translates into a higher k_{cat}/K_M. Therefore, the depressed enzymatic flexibility of the CLCs in dry acetonitrile ($a_w < 0.002$) can account for 1.9 orders of magnitude of activity lost between water and acetonitrile. (This value is obtained by adding the effects of going from $a_w < 0.002$ to $a_w = 0.006$ (0.28, step 1) and from $a_w = 0.006$ to $a_w = 1$ (1.6, step 3) in Table 1.)

Thus only 1.4 out of the original 7 orders of magnitude are now left unexplained. This gap is seen in Table 1 as the difference between CLCs in water and CLCs in octane with $a_w = 1$, where the relative substrate desolvation energies (Table 2) predict only a 3.2-fold drop in catalytic efficiency as opposed to the 85-fold observed. The exact origin of this disparity is still unclear. One possibility is that the activity *vs* pH profile in organic solvents is responsible, i.e., the protonation state of the catalytic triad's histidine is different for CLCs suspended in organic solvents than it is in water at pH 7.8. In fact, we did find the ratio of $(k_{cat}/K_M)_{N\text{-Ac-L-Phe-OEt}}$ for CLCs at pH 7.8 *vs* pH 4.5 in water to be 34-fold; whereas the $(k_{cat}/K_M)_{N\text{-Ac-L-Phe-OEt}}$ ratio in octane for CLCs that were removed from aqueous buffers of pH 7.8 *vs* pH 4.5 is only 7-fold. Alternatively, the solvent binding in the active center could compete with the substrate and thus inhibit subtilisin in organic solvents. Four organic solvent molecules bind in subtilisin's active center region in the CLCs in acetonitrile.[4,5] Also, in the γ-chymotrypsin crystal structure in hexane (a solvent homologous to octane), two hexane molecules bind in the vicinity of the active center.[23]

Conclusions

Using a systematic approach to explain the 7-order-of-magnitude drop in catalytic activity of CLCs of subtilisin in anhydrous acetonitrile relative to the enzyme dissolved in aqueous solution, we have been able to account for 5.6 of those by the following: (i) a shift in the activity *vs* pH profile of the CLCs in water relative to the aqueous enzyme solution; (ii) the unfavorable desolvation energy of the substrate in the organic solvent relative to water; and (iii) the diminished conformational flexibility of the CLCs in the solvent due to dehydration. Consequently, this study has provided the rationale for avoiding much of this activity loss by optimizing the pH of the solution from which the CLCs are collected, judiciously selecting the organic solvent to minimize the unfavorable substrate desolvation energetics, and maintaining a high water activity in the system.

Experimental Section

Cross-Linked Enzyme Crystals (CLCs) of Subtilisin. Subtilisin Carlsberg (serine protease from *Bacillus licheniformis*, E.C. 3.4.21.14) was purchased from Sigma Chemical Co. Crystals were grown at 30 °C from an aqueous 330 mM cacodylate buffer, pH 5.6, saturated with Na_2SO_4.[24] The crystals were cross-linked with a 1.5% glutaraldehyde solution (pH 7.5, 30 mM cacodylate buffer, 13% Na_2SO_4), followed by washing with the buffer (containing no glutaraldehyde) and distilled water, and stored in a 20 mM phosphate buffer, pH 7.8, at 10 °C.[4,5] The average dimensions of the needle-like CLCs were (100 ± 40) μm $\times$ (15 ± 5) μm $\times$ (15 ± 5) μm. Note that the size of the CLCs did not change upon transition from water to acetonitrile and octane, as evidenced by microscopic examination. Furthermore, the unit cell dimensions of the subtilisin CLCs are essentially the same in acetonitrile as in water.[4,5]

Substrates, Products, and Solvents. N-Ac-L-Phe-OEt, *N-trans*-cinnamoylimidazole, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. The organic solvents, including alcohols, utilized were reagent grade and were dried over 3-Å molecular (Linde) sieves prior to use to a water content below 0.01%. Anhydrous $BaBr_2$ was purchased from Aldrich Chemical Co. The mixture of $BaBr_2$·

(18) The water activity was not buffered in the solvents in which $(k_{cat}/K_M)_{nucleophile}$ was determined.

(19) Xu, Z.; Affleck, R.; Wangikar, P.; Suzawa, V.; Dordick, J. S.; Clark, D. S. *Biotechnol. Bioeng.* **1994**, *43*, 515−520.

(20) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272−9273. Ray, A. *Nature* **1971**, *231*, 313−315.

(21) Reichardt, C. *Solvents and Solvent Effects in Organic Chemistry*, 2nd ed.; VCH: New York, 1988, Appendix Table A-1.

(22) The dielectric constants for these solvent systems are 2.0, 2.1, 2.2, and 2.6 for pure octane, octane saturated with water, octane saturated with formamide, and octane saturated with *N*-methylacetamide, respectively. These values were calculated as described by: Onsager, L. *J. Am. Chem. Soc.* **1936**, *58*, 1486−1493.

(23) Yennawar, N. H.; Yennawar, H. P.; Farber, K. *Biochemistry* **1994**, *33*, 7326−7336.

(24) Niedhart, D. J.; Petsko, G. A. *Protein Eng.* **1988**, *2*, 271−276.

$1H_2O/BaBr_2$ was prepared by placing the anhydrous salt (5 g/16.8 mmol) over pure water (75.8 mg/4.2 mmol) in a sealed vessel saturated with water vapor.

One of the product esters, *N*-acetyl-L-phenylalanine 2-methoxyethyl ester (N-Ac-L-Phe-OEtOMe), used for GC calibration, was synthesized as follows. To a mixture of 2 g of *N*-Ac-L-Phe (10 mmol) in 45 mL of benzene were added 4.5 mL of 2-methoxyethanol (60 mmol) and 4 drops of 95% H_2SO_4. The reaction mixture was refluxed for 3 h and then transferred to a separatory funnel containing 100 mL of ether, where it was washed 5 times with 50 mL of a 5% aqueous solution of $NaHCO_3$. The ether phase was dried over anhydrous $MgSO_4$ and concentrated by rotary evaporation; the product ester crystallized out of the solution. The purity (99%) of the product N-Ac-L-Phe-OEtOMe was verified by GC. *N*-Acetyl-L-phenylalanine propyl ester was similarly synthesized from the free acid and propanol, and its purity (99%) was likewise confirmed.

Phenylmethylsulfonic acid (PMSOH) was synthesized from PMSF. One gram of the latter (6 mmol) was dissolved in 10 mL of propanol, to which 10 mL of 5 M NaOH (50 mmol) was added. The reaction mixture was refluxed overnight. The solution was then cooled and acidified with H_2SO_4, and the resultant PMSOH was extracted into 50 mL of ether. The ether phase was washed thrice with 50 mL of a 10% solution of H_2SO_4, dried over anhydrous $MgSO_4$, and subjected to rotary evaporation. The resultant solid PMSOH was 99% pure, as verified by HPLC.

Active Center Titration. The percentage of the catalytically competent subtilisin molecules (used to calculate $[E]_0$) of the soluble enzyme preparation was determined by titration with *N-trans*-cinnamoylimidazole.[25] The concentration of the catalytically competent subtilisin in the CLCs which is accessible to the substrate in water and in acetonitrile was determined by titration of the active centers with the irreversible serine protease inhibitor PMSF (to avoid multiple turnovers due to long titration times) in two independent experiments, each done in triplicate. CLCs (20 mg/mL) were placed in 2 mL of either water (pH 5.0; 10 mM acetate buffer) or acetonitrile, both containing 1 mM PMSF, and the suspension was shaken at 30 °C and 300 rpm. The disappearance of PMSF, as well as any PMSOH produced by spontaneous hydrolysis, was monitored by HPLC. The validity of this titration method was verified with a subtilisin solution of an independently determined $[E]_0$.

Kinetic Measurements. The k_{cat}/K_M values in water were measured potentiometrically for the subtilisin-catalyzed hydrolysis of N-Ac-L-Phe-OEt (1−12 mM ester; 79 μg/L and 29 mg/L dissolved and suspended CLCs of subtilisin, respectively; pH 7.8; 30 °C; 100 mM KCl). Initial rate data were fitted to the Michaelis-Menten equation using the nonlinear curve-fitting function of SigmaPlot (Jandel Scientific). The pH dependence of the soluble subtilisin (1.3−17 μg/L) and of the CLCs (15−190 mg/L) was also measured potentiometrically for the same reaction (1.0 mM ester substrate).

In anhydrous organic solvents, the ester and alcohol k_{cat}/K_M values were determined in the following manner. The CLCs of subtilisin (5 mg) were recovered from the pH 7.8 phosphate buffer and washed with anhydrous acetonitrile (2 × 1 mL) and then by the organic solvent in which the kinetics were studied (3 × 1 mL). For $(k_{cat}/K_M)_{\text{N-Ac-L-Phe-OEt}}$ the appropriate solvent containing a solution of the ester (0.6−6 mM in octane; 10−100 mM in acetonitrile) and 100 mM propanol was prepared immediately before kinetic measurements. In the determination of $(k_{cat}/K_M)_{\text{propanol}}$, the solutions of 100 mM N-Ac-L-Phe-OEt and propanol (2.5−100 mM) in *tert*-amyl alcohol and acetonitrile were prepared immediately before use, as was the case for the measurement of $(k_{cat}/K_M)_{\text{2-methoxyethanol}}$ where the solution was comprised of N-Ac-L-Phe-OEt (100 mM in acetonitrile; 50 mM in cumene) and alcohol (50 mM in acetonitrile; 2.5−100 mM in cumene). One milliliter of the appropriate solution was then added to 1 mg of the CLCs. The reaction mixture was shaken at 30 °C and 300 rpm. Periodically, a 1-μL sample was withdrawn and assayed by GC. All k_{cat}/K_M values, except that for 2-methoxyethanol in acetonitrile, were determined by fitting the kinetic data as described above. The value of $(k_{cat}/K_M)_{\text{2-methoxyethanol}}$ in acetonitrile was calculated via the initial rate (v) ratios of 2-methoxyethanol and propanol and the k_{cat}/K_M value of propanol, where $(k_{cat}/K_M)_{\text{2-methoxyethanol}} = (v_{\text{2-methoxyethanol}}/v_{\text{propanol}})(k_{cat}/K_M)_{\text{propanol}}$ since both nucleophiles were present in equal concentrations.[11]

The measurement of $(k_{cat}/K_M)_{\text{N-Ac-L-Phe-OEt}}$ in octane and acetonitrile where the a_w was buffered to 0.006 was carried out as follows. The solvent containing the ester (0.6−6 mM in octane; 10−100 mM in acetonitrile) and 100 mM propanol was added to 100 mg of the mixture of $BaBr_2 \cdot 1H_2O/BaBr_2$; the suspension was equilibrated by shaking at 30 °C and 300 rpm for 90 min. One milligram of the CLCs was added, and the resulting mixture was shaken and the reaction monitored as described above. Again the kinetic data were fitted using nonlinear regression to obtain k_{cat}/K_M.

The $(k_{cat}/K_M)_{\text{N-Ac-L-Phe-OEt}}$ values in octane with $a_w = 0.006$ and $a_{\text{additive}} = 1$ (where the additive was formamide or N-methylacetamide) were determined as follows. The ester (0.6−6 mM) and 100 mM propanol were dissolved in octane containing 100 mg/mL of $BaBr_2 \cdot 1H_2O/BaBr_2$ at 30 °C. The additive (containing 100 mM propanol) was added until the appearance of a second phase. The octane phase (1 mL) was then added to 1 mg of the CLCs. Kinetic measurements and k_{cat}/K_M calculations were performed as above.

Finally, the determination of k_{cat}/K_M of N-Ac-L-Phe-OEt in "hydrous" octane ($a_w = 1$) was performed as follows. The ester (0.6−6 mM) and 100 mM propanol were dissolved in 2 mL of octane, and the solution was allowed to equilibrate with water (containing propanol) through the vapor phase at 30 °C until the activity of water reached unity (3 days). One milliliter of the resulting octane solution was withdrawn and added to 1 mg of CLCs. The initial rate of the reaction shaken at 30 °C and 300 rpm was monitored by GC, and the k_{cat}/K_M value was obtained by nonlinear curve fitting of the kinetic data.

Diffusion Limitation Assay. To test whether the hydrolysis of N-Ac-L-Phe-OEt catalyzed by the CLCs in water at pH 7.8 was limited by diffusion, active and PMSF-inactivated subtilisin were co-crystallized at various *f* values. The dependence of the activity of the resulting partially inactive crystals as a function of *f* was found to be linear (Figure 1).

(a) Inactivation of Subtilisin. To a 58-mL subtilisin solution (10 mg/mL, pH 7.8, 20 mM phosphate buffer) was added 2.0 mL of a PMSF solution (43 mM; 6-fold molar excess) in propanol. The mixture was incubated for 4 h at room temperature. The remaining activity of subtilisin, assayed by measuring the initial rate of the enzymatic hydrolysis of 1.0 mM N-Ac-L-Phe-OEt in water (pH 7.8, 100 mM KCl, 30 °C), was found to be less than 0.1%. The excess PMSF was then removed by centrifugation/ultrafiltration (10 °C), followed by a 15-fold dilution with deionized water (thrice); the PMSF-inactivated subtilisin solution was then concentrated to 15 mg/mL.

(b) Co-crystallization of Active and PMSF-Inactivated Subtilisin. Varying volumes of solutions of the PMSF-inactivated and active subtilisins were mixed to achieve a range of values of *f* from 0.05 to 0.75. The crystals were then grown in the same manner as described above. To verify that the active and inactive subtilisin had co-crystallized, i.e., a single crystal was comprised of both active and inactive subtilisin, a single crystal was removed from a given batch of crystals and dissolved in aqueous solution (0.63 mg/L, pH 7.8, 100 mM KCl, 30 °C), and its activity was then measured in the hydrolysis of 1.0 mM N-Ac-L-Phe-OEt. The activity obtained was compared to that of crystals of completely active subtilisin ($f = 1$), and the *f* value of the given batch of crystals was calculated.

(c) Activity *vs f* Studies. The crystals of inactive/active subtilisin, of a given *f*, were cross-linked as described above. The activity of the CLCs (0.05 mg/mL) was measured in the hydrolysis of 1.0 mM N-Ac-L-Phe-OEt in water (pH 7.8, 100 mM KCl, 30 °C) and plotted against *f* (Figure 1).

Activity Coefficient Calculations. The activity coefficient of N-Ac-L-Phe-OEt was obtained from its solubility in the appropriate solvent,[26] and those of the nucleophiles propanol and 2-methoxyethanol in organic solvents were calculated using the UNIFAC algorithm.[27]

(a) Determining the Solubility (*S*). In anhydrous solvents, an excess of N-Ac-L-Phe-OEt was placed in the solvent containing 100 mM propanol. In hydrous solvents, where the water activity was buffered to 0.006, the excess of ester was placed in 1 mL of solvent containing 100 mM propanol and 100 mg of the $BaBr_2 \cdot 1H_2O/BaBr_2$ mixture. For *S* in water, the ester was placed in a 100 mM KCl solution,

(25) Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930−2935.

pH 7.8. For the octane systems with $a_w = 0.006$ and $a_{additive} = 1$, an excess of the ester was added to the octane (100 mM propanol, 100 mg/mL of $BaBr_2 \cdot 1H_2O/BaBr_2$, 30 °C) and the additive was added until its solubility was reached. The resulting suspensions were shaken at 30 °C and 300 rpm. In octane with $a_w = 1$, the ester was placed in a vial containing octane (and 100 mM propanol) in contact through the vapor phase with water containing propanol. The system was allowed to equilibrate at 30 °C until the water activity reached unity. Periodically, the ester concentration in each system was assayed by GC; the final concentration was denoted *S*.

JA9539958

(26) The relationship between solubility and activity coefficient can be described as follows. If the standard state is the pure liquid solute, the activity of the solute is 1, n is the number of moles of solute in solution, n_S is the number of moles of solvent in solution, γ is the solution phase activity coefficient of the solute, and x is the solution phase mole fraction of the solute, then the solute will dissolve in the solvent until $\gamma x = 1$. If the solute is sparingly soluble, then $x = n/n_S$, where $n = SV$ (S is the molar solubility of the solute and V is the system volume) and $n_S = V/V_M$, where V_M is the molar volume of the solvent. Then $x = SV_M$ and $\gamma = 1/SV_M$. The value of γ, which is a function of solute concentration, was calculated at the concentration equaling *S*. At low mole fractions, where solute−solute interactions are negligible, γ becomes constant with respect to concentration. This is true for both Henry's and Raoult's law activity coefficients.

(27) Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 10362−10363.

(28) This work was financially supported by NIH Grant No. GM39794. C.R.W. is an NIH Biotechnology Predoctoral Trainee.