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pH Control of the Catalytic Activity of Cross-Linked Enzyme Crystals in Organic Solvents

Kui Xu and Alexander M. Klibanov*

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

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Abstract: Lyophilized subtilisin Carlsberg exhibits "pH memory" in organic solvents, i.e., its catalytic activity in such media is profoundly affected by the pH of the aqueous solution from which the enzyme was lyophilized. In contrast, cross-linked crystalline subtilisin displays no appreciable pH memory. This disparate behavior is rationalized in terms of the different protonation states of the two enzyme preparations in organic solvents. Since cross-linked crystals (CLCs) of enzymes in organic solvents offer performance and mechanistic advantages over their lyophilized counterparts, the opportunities for pH maximizing their catalytic activity in such media are explored. It is found that subtilisin CLCs in pentanone can be activated more than 100-fold by adding a "buffer" consisting of a mixture of a suitable acid and its sodium salt (conjugate base). The maximal activation is achieved (i) when the pK_a value of the buffer acid in water lies in the appropriate range, (ii) at an optimal acid/base composition of the buffer mixture, and (iii) when the buffer mixture is present in a sufficient concentration.

Introduction

Cross-linked crystals (CLCs) of enzymes, due to their robustness and other superior performance characteristics, are attractive biocatalysts for synthetic applications.[1] Enzyme CLCs have been explored in aqueous solutions[1] and recently also in organic solvents.[1c,f,2] With respect to the latter, an additional advantage of using crystalline enzymes is that their conformation even in anhydrous media appears to be nearly the same as in water,[3] thus allowing meaningful structure-activity correlations.[2] Such analysis may not be possible for the conventional, lyophilized (or other amorphous) enzyme preparations suspended in organic solvents[4] because of the observed conformational changes in enzymes upon lyophilization (and other modes of dehydration).[5]

While dependence on the pH of the reaction medium is one of the basic characteristics of enzyme catalysis,[6] the concept of

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(1) (a) St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314. (b) Sobolov, S. B.; Bartoszko-Malik, A.; Oeschger, T. R.; Montelbano, M. M. *Tetrahedron Lett.* **1994**, *35*, 7751. (c) Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 2732. (d) Lalonde, J. J.; Govardhan, C.; Khalaf, N.; Martinez, A. G.; Visuri, K.; Margolin, A. L. *J. Am. Chem. Soc.* **1995,** *117***,** 6845. (e) Sobolov, S. B.; Leonida, M. D.; Bartoszko-Malik, A.; Voivodov, K. I.; McKinney, F.; Kim, J.; Fry, A. J. *J. Org. Chem.* **1996**, *61*, 2125. (f) Khalaf, N.; Govardhan, C. P.; Lalonde, J. J.; Persichetti, R. A.; Wang, Y.-F.; Margolin, A. L. *J. Am. Chem. Soc.* **1996**, *118*, 5494.

(2) (a) Schmitke, J. L.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 3360. (b) Ke, T.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 3366.

(3) (a) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8653. (b) Fitzpatrick, P. A.; Ringe, D.; Klibanov, A. M. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 675. (c) Yennawar, N. H.; Yennawar, H. P.; Farber, G. K. *Biochemistry* **1994**, *33*, 7326. (d) Yennawar, H. P.; Yennawar, N. H.; Farber, G. K. *J. Am. Chem. Soc.* **1995**, *117*, 577.

(4) (a) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141. (b) Chen, C.-S.; Sih, C. J. *Angew. Chem., Int. Ed. Engl.* **1989,** *28*, 695. (c) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194. (d) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114. (e) Gupta, M. N. *Eur. J. Biochem.* **1992**, *203*, 25. (f) Faber, K.; Riva, S. *Synthesis* **1992**, 895. (g) Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178. (h) Koskinen, A. M. P., Klibanov, A. M., Eds. *Enzymatic Reactions in Organic Media*; Blackie: London, 1996.

(5) (a) Dong, A.; Prestrelski, S. J.; Allison, S. D.; Carpenter, J. F. *J. Pharm. Sci.* **1995**, *84*, 415. (b) Griebenow, K.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10969.

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pH is foreign to organic solvents.[7] Instead, it has been discovered[8] that lyophilized enzymes have a "pH memory" in organic media which stems from the retention by their ionogenic groups of the ionization state they had in the last aqueous solution prior to dehydration (e.g., lyophilization). In order to preserve the aforementioned structural integrity of enzymes in CLCs, lyophilization is to be avoided.[2] Rather, the CLCs have to be separated from aqueous solution by centrifugation or filtration followed by repeated washing with a water-miscible organic solvent (to remove the interstitial water) before placement in the organic solvent of interest.[3a,b] The question arises whether this recovery procedure retains the pH memory of enzymes and, if not, how the enzymatic activity of CLCs in organic solvents can be pH-manipulated.

In the present study, we have found that washing (water-filled) CLCs with such solvents as acetonitrile indeed destroys the enzyme pH memory. However, the enzymatic activity of CLCs in organic media can be markedly pH-controlled and -maximized by adding appropriate buffer pairs of acids and their conjugate bases.

Results and Discussion

As a model for this investigation we selected the serine protease subtilisin Carlsberg which has been a workhorse of nonaqueous enzymology,[4] including studies with CLCs.[2] The catalytic activity of subtilisin in organic solvents was measured on the basis of the initial rate of the enzymatic transesterification between N-Ac-L-Phe-OEt and propanol.[8b] Figure 1A depicts the dependence of the *lyophilized* subtilisin activity in anhydrous acetonitrile on the pH of the aqueous solution from which the enzyme was lyophilized. A dramatic effect is seen; for example, upon changing this pH from 5 to 11, the subtilisin activity *in the same reaction mixture in acetonitrile* rose 324 times. Similar dependences were reported in the literature for lyophilized[9] and dried immobilized[10] subtilisin in different organic solvents;[11] these observations, as well as Figure 1A, are the manifestation of the pH memory of lyophilized subtilisin in nonaqueous media.

We next examined whether *cross-linked crystalline* subtilisin would display pH memory under the same conditions as in Figure 1A. To this end, subtilisin CLCs were soaked in aqueous buffered solutions of the pH ranging from 5 to 11; each CLC sample was then separated from solution by centrifugation and decantation. To 10 mg of the crystals obtained from different pHs was added 1 mL of acetonitrile, and the suspension was vortexed, centrifuged, and decanted; this procedure was repeated thrice. Curve a in Figure 1B shows the dependence of the activity of subtilisin CLCs on the pH of the soaking solution. One can see that the enzymatic reaction rate varies less than 2-fold and with no discernible trend, as opposed to more than 2 orders of magnitude for the lyophilized enzyme in Figure 1A. Likewise, no pH memory of subtilisin CLCs was observed in another, unrelated solvent, 3-pentanone, whether anhydrous or hydrous (curves b and c, respectively, in Figure 1B).

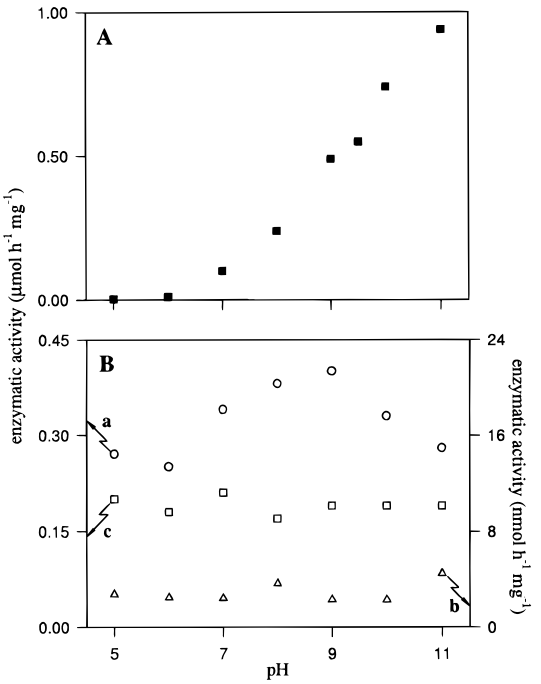


Figure 1. Dependence of the catalytic activity of subtilisin Carlsberg suspended in organic solvents on the pH of the aqueous solution from which the enzyme was obtained: (A) lyophilized subtilisin in anhydrous acetonitrile and (B) subtilisin CLCs in anhydrous acetonitrile (a), anhydrous 3-pentanone (b), and hydrous ($a_w = 0.85$) 3-pentanone (c). Note that a and c refer to the left axis, while b refers to the right axis. The enzymatic activity was measured as the initial rate of the subtilisin-catalyzed transesterification between N-Ac-L-Phe-OEt and propanol at 30 °C. For other conditions, see the Experimental Section.

Why is it that lyophilized subtilisin exhibits pH memory (Figure 1A), while its cross-linked crystalline counterpart does not (Figure 1B)? We propose the following explanation. During lyophilization, the enzyme is exposed to the original aqueous medium, albeit now frozen. Therefore, the protonation state of its ionogenic groups, including those controlling the enzymatic activity, should be conserved throughout the drying process. In the lyophilized enzyme, the pK_a values of the ionogenic groups must differ from those in water because the surrounding medium, vacuum or air, is distinct. Nevertheless, the protonation states remain unchanged because protons cannot be transferred through vacuum or (dry) air. When the lyophilized enzyme is suspended in an aprotic solvent, such as acetonitrile, the protonation status of the ionogenic groups still does not change for the same reason. These ionogenic groups kinetically trapped in the "aqueous" protonation states give rise to pH memory of enzymes in organic solvents.[8]

The situation with CLCs is quite different in that following their removal from a soaking aqueous solution, the crystals are still filled with interstitial water. When the CLCs are subsequently placed in acetonitrile (during washing), the solvent diffuses into the crystals while the interstitial water diffuses out. As the intracrystalline medium becomes increasingly enriched in acetonitrile, the pK_a values of the enzyme ionogenic groups change. Since this occurs while there is still mobile interstitial water present, the protonation states adjust accordingly, for they are not kinetically trapped as in the case of the lyophilized enzyme. Presumably this gradual depletion of interstitial water in CLCs allows for the attainment of the protonation state dictated mainly by the composition of the eventual medium. Since the latter is the same, acetonitrile, regardless of the pH

(6) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985; Chapter 5.

(7) Bates, R. G. *Determination of pH. Theory and Practice*, 2nd ed.; Wiley: New York, 1964; Chapters 7 and 8.

(8) (a) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192. (b) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194.

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

(10) Blackwood, A. D.; Curran, L. J.; Moore, B. D.; Halling, P. J. *Biochim. Biophys. Acta* **1994**, *1206*, 161. An organic-phase buffer is an appropriate mixture of a Brønsted−Lowry acid and its conjugate base which can control the protonation state of other ionogenic compounds present in the same organic solvent.

(11) Yang et al.[9] found that the pK_a values determined from the dependences like the one in Figure 1A are a function of the solvent and also the water content in a given solvent, with the pK_a decreasing when the activity of water increases.

of the soaking solution, one would expect similar enzymatic activities, i.e., the absence of pH memory with CLCs, which is what was indeed observed (Figure 1B).

The hypothesis outlined above predicts that if lyophilized subtilisin is treated the same way as the CLCs, its memory should drastically decline. To test this prediction, we lyophilized the enzyme from two aqueous solutions, pH 5 and 11. Then 10 mg of each lyophilized powder was wetted with 25 μL of the buffered aqueous solution of pH 5 and 11, respectively, followed by addition of 1 mL of acetonitrile and subsequent washing with the latter as for the CLCs. When the catalytic activity of the resultant enzyme preparations was measured in acetonitrile, it was found that the activity of the pH 5 sample jumped 7.6-fold and that of the pH 11 sample dropped 12-fold compared to their corresponding nonwetted counterparts. Consequently, the difference in the enzymatic activities between the pH 11 and 5 samples plummeted from 324-fold (Figure 1A) to some 3-fold, thus indeed reflecting a severe diminution of its pH memory.[12]

Since subtilisin CLCs prepared in a way which preserves their structural integrity[3a,b] lack pH memory (Figure 1B), an alternative mode of pH maximizing their activity had to be designed that would readjust the protonation state of the CLCs *after* their dispersion in organic solvents. To this end, we adopted the organic-phase buffer approach.[10] Just like in aqueous solution the protonation state of enzyme ionogenic groups, and hence enzymatic activity, can be controlled by adding a buffer, i.e., an appropriate mixture of an acid and its conjugate base, the same can be accomplished in organic solvents by using organic-soluble buffers.[13] The first such organic buffer we chose was a mixture of phenylboronic acid (**1**) and sodium phenylboronate.

Subtilisin CLCs were prepared as outlined above and also washed with pentanone, subsequently used as the reaction medium.[13] The enzymatic activity of the CLCs was then examined as a function of the buffer mixture composition. It was found that **1** alone had a relatively minor effect—the initial rate of the subtilisin-catalyzed transesterification in pentanone increased less than 50% upon the addition of 10 mM acid. However, when the sodium phenylboronate fraction in the mixture was raised, the enzymatic reaction accelerated dramatically to reach a 110-fold activation at a 4:1 base/**1** ratio (Figure 2, curve a).

That the catalytic activity of subtilisin CLCs in pentanone is markedly dependent on the conjugate base mole fraction suggests that the latter parameter is analogous to the pH in aqueous solution. Subtilisin is catalytically active when the histidine (His) residue of its catalytic triad is deprotonated.[14] Presumably this deprotonation is completed at an 80% sodium phenylboronate mole fraction in the buffer mixture when the CLCs exhibit maximal activity. A one-third drop in the enzymatic activity upon further increase in the sodium salt content is probably because the base deprotonates some other ionogenic group required for the enzyme action in the protonated form (e.g., an NH_3^+ group involved in a salt bridge). (Subtilisin inhibition by high concentrations of the base is another possibility.) Note that the more than a 100-fold subtilisin activation observed at the optimal buffer composition (curve a in Figure 2) indicates that without buffer at least 99% of the enzyme in the CLCs is in the protonated His, i.e., catalytically inactive, form.

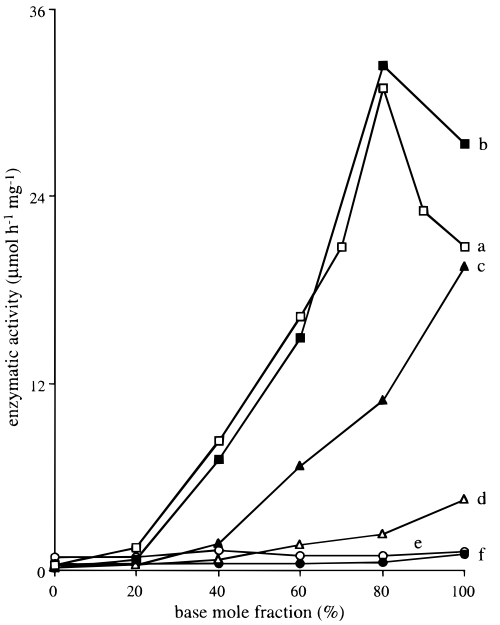
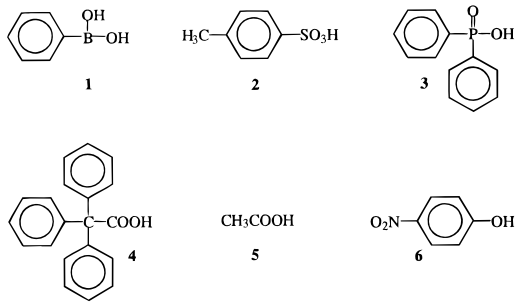


Figure 2. Dependence of the enzymatic activity of subtilisin CLCs in hydrous (a_w = 0.85) 3-pentanone on the composion of various pairs of acids and their conjugate bases (sodium salts) added to the enzyme suspension: (a) **1** (□), (b) **6** (■), (c) **4** (▲), (d) **5** (△), (e) **2** (○), and (f) **3** (●). The total concentration of each buffer pair was 10 μmol in 1 mL. The enzymatic activity was measured as the initial rate of the subtilisin-catalyzed transesterification between N-Ac-L-Phe-OEt and propanol at 30 °C. For other conditions, see the Experimental Section.

To test the generality of the buffer activation phenomenon, we examined the effect of several other acid/conjugate base pairs on the catalytic activity of subtilisin CLCs in pentanone. The acids were *p*-toluenesulfonic acid (**2**), diphenylphosphinic acid (**3**), triphenylacetic acid (**4**), acetic acid (**5**), and *p*-nitrophenol (**6**); in all cases the corresponding sodium salts were used as conjugate bases. The acids **1**−**6** were selected to cover a wide pK_a range in water (from −0.53 to 8.84) because their acidity



was expected to be related to the buffer properties of the pairs in organic media. Analysis of the crystal structure of subtilisin reveals that all these acids and their conjugate bases should have ready access to the catalytic triad's His residue.

Curves b−f in Figure 2 depict the catalytic activity of subtilisin CLCs in pentanone as a function of the buffer mixture

(12) We also attempted a complimentary test, whereby subtilisin CLCs were *lyophilized* from 20 mM aqueous phosphate buffer solutions of pH 5 and 11 to determine whether this procedure would give rise to the pH memory. However, it was found that both resultant preparations were at least 1 order of magnitude less active than the original CLCs, presumably due to a collapse of the crystal and a consequent blockage of enzyme molecules in its interior. These observations made this particular test equivocal.

(13) Blackwood et at.[10] employed triisooctylamine and its hydrochloride and triphenylacetic acid and its sodium salt to affect enzymatic activities of immobilized and dried subtilisin and lipase in 3-pentanone. The latter was used as a solvent in most of the present study as well to make data comparison possible. It is seen in Figure 1B that the catalytic activity of subtilisin CLCs in hydrous (a_w = 0.85) pentanone is much greater than in its anhydrous counterpart (as is the case in other solvents[2a]). The thermodynamic water activity of 0.85 was maintained in the rest of this work.

(14) For a review, see: Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5.

Table 1. Maximal Activations of Subtilisin CLCs in Hydrous 3-Pentanone Afforded by Pairs of Acids **1**−**6** and Their Sodium Salts (Conjugate Bases)[a]

acid	pK_a in water	max enzyme activation obsd	salt solubility in 3-pentanone[b] (MM)
2	−0.53[15]	5	between 5 and 10 mM
3	1.72[16]	4	>10
4	3.96[17]	69	>10
5	4.76[17]	16	<2.5
6	7.15[17]	115	>10
1	8.84[18]	110	>10

[a] Maximal activation is defined as a ratio of the highest enzymatic activity in the presence of a given buffer pair to the activity in the absence of buffer. For experimental conditions, see the legend to Figure 2. [b] Acids **1**−**6** were completely soluble in pentanone at 10 mM and 30 °C.

composition for acids **2**−**6**. Inspection of the figure leads to several important conclusions. It is seen that some buffers (those corresponding to acids **4** and **6**, in addition to **1**) afford dramatic enzyme activations, whereas others (corresponding to acids **2**, **3**, and **5**) afford only modest ones. This statement can be quantified using the data in Table 1 where the maximal activation effect is given for each buffer. One can see that the magnitude of the maximal activation roughly correlates with the pK_a values of the acid—stronger acids (and hence weaker conjugate bases) generally afforded far lower activation effects, less than 1 order of magnitude, than weaker acids (and hence stronger conjugate bases), up to 2 orders of magnitude. This is presumably because the base must be sufficiently strong to deprotonate the subtilisin's His residue.

It should be pointed out that none of the acids alone had a major impact on subtilisin CLCs activity. This is consistent with the notion that without additives the active center's His is protonated and thus cannot be protonated further by the acids. Figure 2 also reveals that the strongest bases (Na salts of **1** and **6**) alone afforded a lower activation effect than their 4:1 (mol/mol) mixtures with the conjugate acids. In contrast, the weaker bases (Na salts of **4** and **5**) alone provided a greater activation than their mixtures with the corresponding conjugate acids; presumably in these instances the bases were unable to cause the aforementioned deleterious deprotonation of some other (than His) enzyme ionogenic group.

A notable exception from the activation vs acid's pK_a trend mentioned earlier is **5**—although its pK_a in water is higher than that of **4**, the enzymatic rate enhancement by the sodium salt is some 4 times lower, 16- compared to 69-fold (Table 1 and Figure 2). We thought that this discrepancy might be due to a low solubility of **5**'s conjugate base—while all the acids presented in Table 1 are soluble in pentanone at 10 mM, not all the sodium salts are. In particular, sodium acetate's solubility is less than one-fourth of that, whereas the more hydrophobic sodium salt of **4** completely dissolves in the hydrous pentanone at 10 mM. One would expect that only a dissolved base would be able to strip protons off the active center's His, and the concentration of dissolved sodium acetate apparently is too low for that.

This rationale was confirmed in two independent experiments. First, we found that mixtures of monobasic and dibasic sodium phosphates, virtually insoluble in pentanone, afforded no effect on subtilisin CLCs activity in this solvent despite the acid's suitable (compared to those in Table 1) pK_a value of 7.20 in water.[19] Second, we found that when the concentration of a 1:4 (mol/mol) mixture of **1** and its sodium salt in hydrous pentanone was lowered from its original 10 mM (Figure 2 and Table 1) to 3.0 and 1.0 mM, the activating effect also dropped from 110- to 9- and 1.5-fold, respectively. These data indicate that the base must be present at a sufficiently high concentration to activate the enzyme.

In closing, this study demonstrates that although subtilisin CLCs are devoid of the pH memory in organic solvents, their activity can be increased 100-fold by adding to the reaction medium judiciously selected acid/base buffer mixtures. Parameters affecting the choice of the buffer—the pK_a of the acid in water, the acid/base ratio, and the solubility and concentration of the buffer in the solvent—have been ascertained, and mechanistic insights into this phenomenon have been obtained. This pH maximization contributes a powerful extra tool to the means of enhancing subtilisin CLCs activity in organic solvents which heretofore included increasing the thermodynamic activity of water (a_w),[2a] optimizing the energetics of substrate desolvation,[2a] and adding denaturing organic cosolvents to increase the conformation flexibility of the enzyme.[20] Thus it may be possible to use enzyme CLCs, with the ensuing ability to make structure-based predictions of the solvent control of enzymatic stereoselectivity,[2b] without sacrificing much enzyme activity stemming from the use of nonaqueous, instead of aqueous, reaction media.[2a]

Experimental Section

Materials. Subtilisin Carlsberg (serine protease from *Bacillus licheniformis*, EC 3.4.21.14) of a specific activity of 14 units/mg and N-Ac-L-Phe-OEt were purchased from Sigma Chemical Co. **3** was from Fluka Chemie AG. Sodium acetate, **1**, **2** monohydrate,[21] **4**−**6**, NaH_2PO_4, and 3-pentanone were all from Aldrich Chemical Co. *p*-Nitrophenol sodium salt dihydrate[21] was from Eastman Kodak Co. Acetonitrile and Na_2HPO_4 were from Mallinckrodt Chemical, and $Na_2HPO_4 \cdot 12H_2O$ was from Janssen Chimica. All chemical reagents were of analytical grade or purer. Solvents were of HPLC grade and were predried over 3-Å molecular sieves (Linde) when necessary.

Preparation of Buffer Salts. Sodium *p*-toluenesulfonate was prepared by adding NaOH to an aqueous solution of **2** in an 1:1 (mol/mol) ratio followed by lyophilization. **1**, **3**, and **4** were converted to their conjugate bases by stirring with 0.95 mol equiv of NaOH in water. The unreacted acids were separated by filtration, and lyophilization of the filtrates afforded the desired salts.

Preparation of the Enzyme. Subtilisin was dissolved (5 mg/mL) in 20 mM aqueous phosphate buffer at different pHs and lyophilized for 48 h in a Labconco freeze-drier (−50 °C, 5−10 μmHg). The water contents of the lyophilized subtilisin, measured by the Fischer potentiometric titration[22] using a Mettler DL18 autotitrator, were 2.7−4.3% (w/w), depending on the pH. Subtilisin crystals were grown and cross-linked as described previously[2a] and then washed five times with 30 mM aqueous cacodylate buffer (pH 7.5, containing 13% Na_2SO_4) and five times with deionized water. The average size of the CLCs was 100 × 15 × 15 μm.[2a] In the study of the pH memory, the CLCs of subtilisin were collected by centrifugation, soaked in 20 mM aqueous phosphate buffer overnight at different pHs, centrifuged, washed three times with acetonitrile (1 mL/10 mg of the CLCs), and collected by suction filtration. In the study on buffering effects of acid/base pairs, the procedure was the same (except for the phosphate buffer soaking) plus washing three times with pentanone. The water contents of the

(15) Ochiai, K.; Seto, K. *J. Nucl. Sci. Technol.* **1971**, *8*, 330.
(16) Krasovec, F. *Croat. Chem. Acta* **1965**, *37*, 107.
(17) Dean, J. A., Ed. *Lange's Handbook of Chemistry*, 14th ed.; McGraw-Hill: New York, 1992; Chapter 8.
(18) Edwards, J. O.; Sederstrom, R. J. *J. Phys. Chem.* **1961**, *65*, 862.
(19) Lide, D. R., Ed. *CRC Handbook of Chemistry and Physics*, 76th ed.; CRC Press: New York, 1994; pp 8−44.
(20) Almarsson, Ö.; Klibanov, A. M. *Biotechnol. Bioeng.* **1996**, *49*, 87.
(21) Acids and their sodium salts containing bound water were used without dehydration. The small amounts of water introduced by the buffering species should not affect the initial rates since the water activity was independently controlled by salt hydrates (see below).
(22) Laitinen, H. A.; Harris, W. E. *Chemical Analysis*, 2nd ed.; McGraw-Hill: New York, 1975; p 361.

CLCs collected from acetonitrile and 3-pentanone were determined to be[22] 3.4 and 3.5% (w/w), respectively.

Kinetic Measurements. Transesterification reactions between N-Ac-L-Phe-OEt (100 mM) and propanol (1.0 M), catalyzed by lyophilized subtilisin or the CLCs of subtilisin, were carried out in organic solvents. In all enzymatic reactions, 1 mL of the reaction mixture was placed in a 4-mL screw-cap vial and shaken in an incubator shaker at 30 °C and 250 rpm. Samples (0.5 μL) were periodically withdrawn and analyzed with a Hewlett-Packard 5890A gas chromatograph using an HP-5 capillary column coated with 5% phenylsilicone/95% methylsilicone gum (10 m × 0.53 mm × 2.65 μm film thickness). The initial rates were determined by measuring the increase in the concentration of the product (N-Ac-L-Phe-OPr) up to a 5% conversion. The transesterification rates in the absence of enzyme were negligible compared to the enzymatic ones in the same solvents.

Enzymatic Transesterification in Organic Solvents. In lyophilized-subtilisin-catalyzed transesterification reactions, 1 mL of the substrate solution containing 100 mM N-Ac-L-Ph-OEt and 1.0 M propanol in acetonitrile was mixed with the lyophilized enzyme powder (typically 1 mg). The reaction mixture was shaken at 30 °C, and the initial rates were measured as described above. In the case of subtilisin CLCs (approximately 1 mg, collected from 20 mM aqueous phosphate buffer at different pHs), they were added to 1 mL of an organic solvent (acetonitrile or pentanone) containing the same substrate mixture as mentioned above. Raising the thermodynamic activity of water (a_w) has been shown to increase the catalytic activity of enzymes (including subtilisin), both amorphous[23] and cross-linked crystalline.[2] To achieve this activation in the present study, we used $Na_2HPO_4 \cdot 12H_2O$ which, in equilibrium with the heptahydrate at 30 °C, affords $a_w = 0.85$.[24] We added 0.2 g of $Na_2HPO_4 \cdot 12H_2O$ to 1 mL of pentanone[25] when hydration was desired, and the initial rates were determined as described above. In the study of the buffering effects of acid/base pairs, 10 μmol total of the appropriate buffer mixture (an acid and its Na salt) was added to 1 mL of the aforementioned reaction mixture.

Solubility Analyses. Pentanone containing 10 mM organic bases (sodium salts of **1** or **6**), 1.0 M propanol, and 0.2 g/mL $Na_2HPO_4 \cdot 12H_2O$ was shaken at 30 °C in an incubator for 1 h. Then 1 mL of the clear supernatant was withdrawn, and the solvent was removed by blowing purified N_2 gas over the mixture. The residue was acidified by dissolving in 4 or 8 mL of acetonitrile containing 1% trifluoroacetic acid, and 10-μL samples were analyzed by HPLC using a Waters C_{18} reverse-phase column (3.9 × 150 mm) equilibrated with a 50:50 (v/v) water (containing 0.1% CF_3CO_2H):acetonitrile mixture at a flow rate of 0.2 mL/min. The concentrations were determined by measuring the UV absorbance at 218 (**1**) and 227 (**6**) nm. The calibration of the UV absorbance was carried out with the standard acids at the same wavelengths. For other salts, solubilities were estimated by visual inspection of increasingly diluted mixtures.

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(23) Kuhl, P.; Halling, P. J.; Jakubke, H.-D. *Tetrahetron Lett.* **1990**, *31*, 5213. Kuhl, P.; Halling, P. J. *Biochim. Biophys. Acta* **1991**, *1078*, 326. Kvittingen, L.; Sjursnes, B.; Anthonsen, T.; Halling, P. J. *Tetrahedron* **1992**, *48*, 2793.

(24) Halling, P. J. *Biotechnol. Tech.* **1992**, *6*, 271. Yang, Z.; Robb, D. A. *Biotechnol. Tech.* **1993**, *7*, 37.

(25) We determined that at this concentration of $Na_2HPO_4 \cdot 12H_2O$ the dependence of the enzymatic activity of subtilisin CLCs on the salt concentration (at the 2:3 (mol/mol) ratio of **4** to its conjugate base) leveled off.