Chemical Modifications at a Single Site Can Induce Significant Shifts in the pH Profiles of a Serine Protease†

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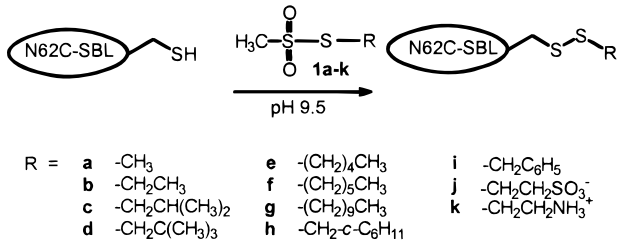
Abstract: Developing the ability to vary the pH−activity profile of an enzyme in a controlled manner has been a long sought-after goal. Such tailoring provides important insights into mechanism and permits optimization of enzyme performance in organic synthesis applications. The most successful approaches to date toward altering pH−activity profiles of enzymes have employed either site-directed mutagenesis or chemical modification to alter enzyme surface charge. We now report that, by combining these two methodologies, dramatic pK_a changes can be induced in the serine protease subtilisin *B. lentus*. In particular, site specific incorporation of unnatural amino acid side chains by the following strategy, WT → $Asn62Cys_{mutant}$ + H_3C-SO_2-S-R → Asn62Cys-S−R, where R may be infinitely variable, has demonstrated that pK_a shifts of up to 0.72 unit are achievable and are accompanied by significant activity enhancements. The most dramatic pK_a shifts are caused by chemical modification with hydrophobic aliphatic moieties. A linear correlation between the hydrophobicity indicator log *P* for the side chain of the modification and the observed pK_a demonstrates that the hydrophobicity in the vicinity of the catalytic triad modulates the pK_a of the catalytic residue His64. Molecular modeling analysis reveals that the side chain R of Asn62Cys-S−R, where R is a hydrophobic modification such as decyl or cyclohexyl, positions itself over the imidazole moiety of His64 in the minimized structure.

Introduction

The ability to vary the pH−activity profile of an enzyme in a controlled manner has been a long sought-after goal.[1] Such tailoring can help to elucidate important insights into mechanism and permits optimization of enzyme performance for use in organic synthesis.[2] Thus far, the most successful approaches to altering pH−activity profiles have used either site-directed mutagenesis[1b−f,h] or chemical modification[1a,g] to alter enzyme surface charge. These studies have established that increasing positive surface charge of the serine proteases destabilizes the protonated imidazolium group of the active site histidine and thus lowers its pK_a, while increasing negative surface charge stabilizes the histidine imidazolium, thereby raising its pK_a.[1] While predictive models to determine the effect of electrostatic modification on His64 pK_a have recently been established,[1h] the introduction of uncharged hydrophobic mutations to alter enzyme pH−activity profiles has not been investigated. In addition, while it has been recognized that the pK_as of amino acid side chains in proteins, particularly those essential for catalysis, are affected by their environment,[3] these values cannot always be predicted.

Scheme 1



Our strategy of chemical modification of site-directed mutant enzymes[4,5] offers a new opportunity for altering surface properties and active site environments, thereby opening up novel possibilities for controlling pH optima. This approach entails the introduction of a cysteine residue at a selected position by site-directed mutagenesis which is then quantitatively and specifically thioalkylated using methanethiosulfonate reagents such as **1a**−**k**, as shown in Scheme 1. This methodology permits the development of more finely controlled structure−activity relationships since virtually any moiety may be linked site-specifically, thus permitting the investigation of the steric and the electrostatic factors which contribute to altered specificity without the limitations imposed by the 20 natural amino acids.

As our target enzyme, we chose the alkaline serine protease subtilisin *B. lentus* (SBL, EC 3.4.21.14).[6] SBL has been cloned,

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overexpressed, and purified,[7] its kinetic properties are well characterized,[8] and its crystal structure has been solved.[9] Furthermore, subtilisins are of both synthetic[2] and industrial[10] interest and previous approaches to altering their pH−activity profiles have been reported.[1b−e,h] We have already exploited the strategy illustrated in Scheme 1 to alter substrate specificity[4] and to increase activity for this enzyme.[5] For example, we recently achieved an up to 3-fold higher than wild type (WT) activity enhancement by chemical modification of the N62C cysteine mutant of SBL.[5] In this study, we found that increased alkyl side-chain length caused a monotonic increase in k_{cat}/K_M with the most dramatic effect being exhibited by N62C−SCH_2-*c*-C_6H_{11} (-**h**). Due to the proximity of Cys62 to His64,[5] we hypothesized that these activity enhancements were due in part to the altered pK_a of the catalytic triad residue His64. We undertook the current study to investigate the validity of this hypothesis and to uncover altered pH−activity profiles of SBL which can expand its synthetic utility. We now report that dramatic pK_a changes can be induced by chemical modification of site-directed mutants and that pK_a shifts of up to 0.72 unit are achievable by these two methodologies.

Results and Discussion

Each of the CMMs N62C-**a** to -**k** were prepared and characterized as described previously.[5] Briefly, the N62C−SBL mutant was treated with the methane thiosulfonate reagents (**1a**−**k**) as shown in Scheme 1, with the modification reactions being complete within 30 min. Titration of the CMMs with Ellman's reagent established that reactions were quantitative with the free thiol content of the CMMs being less than 2% in all cases. The CMMs were purified on a disposable Sephadex G-25 desalting column, and analysis of each CMM by electrospray mass spectrometry was consistent (±6 Da) with the calculated mass. The purities of the modified enzymes were assessed by native-PAGE and in all cases only one band was visible. Furthermore, treatment of SBL-WT, in which no cysteine is present, with each of **1a**−**k** resulted in no change in activity. Additionally, β-mercaptoethanol treatment of the CMMs restored activity to that of the parent cysteine mutant, verifying that chemical modification at cysteine is solely responsible for the observed changes in activity, and that the modification is fully reversible. The active enzyme concentration was determined by monitoring fluoride release upon reaction of each CMM with phenylmethylsulfonyl fluoride (PMSF).[11]

Initially, molecular mechanics calculations were performed to probe the molecular basis for the 3-fold activity enhancements observed for N62C−SCH_2-*c*-C_6H_{11} (-**h**). These results, which are depicted in Figure 1a, revealed that in the minimized structure the cyclohexyl moiety is positioned directly over the His64 imidazole of the catalytic triad. In contrast, for N62C-**k**, for which a 1.7-fold decrease in activity was observed,[5] the $-SCH_2CH_2NH_3^+$ side chain is solvated and oriented away from the catalytic triad region in the minimized structure, as shown in Figure 1b. In light of these molecular modeling results, and considering the proximity of Cys62 to His64, we postulated that the more hydrophobic environment of the catalytic triad in

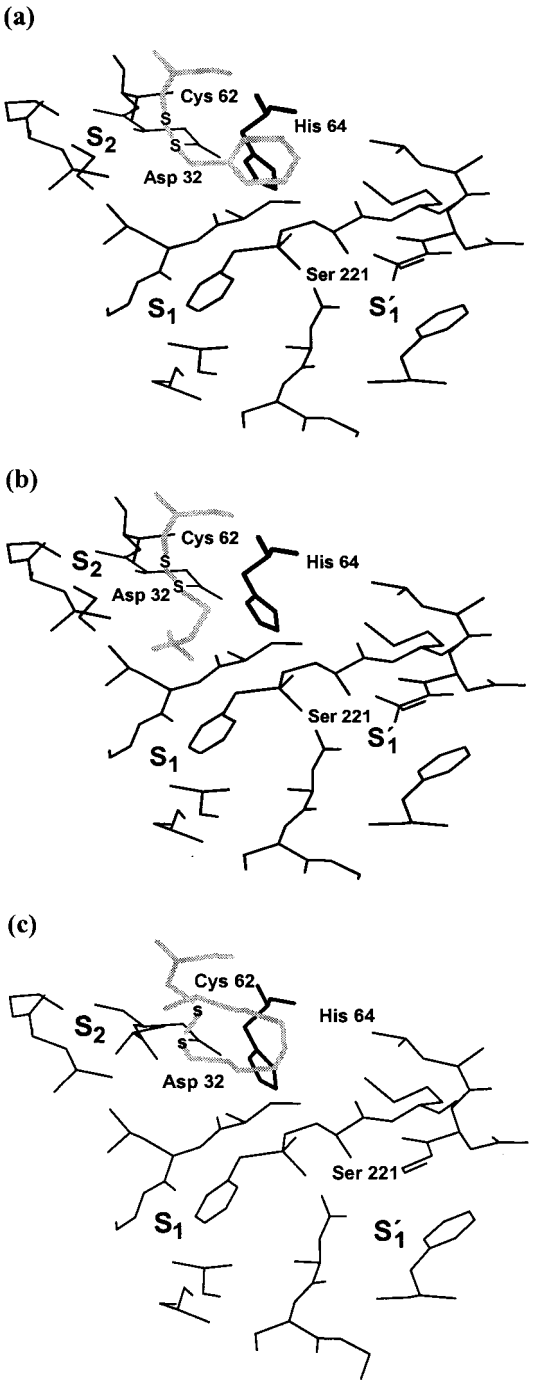

Figure 1. (a) The active site of the N62C-**h** CMM showing the preferred position of the disulfide-linked CH_2-cyclohexyl side chain (in gray) over the top of the imidazole side chain of His64 (heavy black). (b) Active site of the N62C-**k** CMM showing the preferred position of the disulfide-linked ethylamino side chain (in gray) for which the ammonium group points toward the aqueous solvent. (c) Active site of the N62C-**g** CMM showing the preferred position of the disulfide-linked decyl side chain (in gray) which is coiled over the top of the imidazole side chain of His64 (heavy black).

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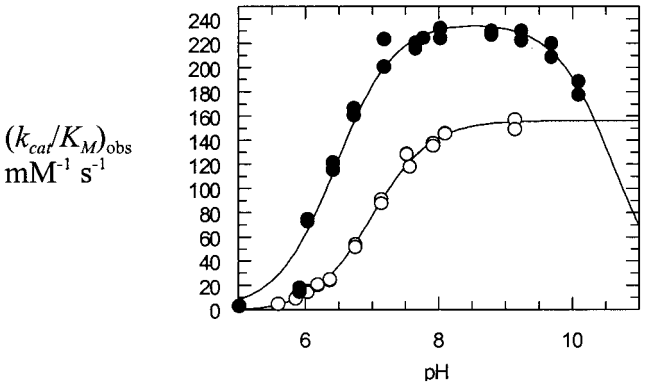

Figure 2. pH−activity profile for WT-SBL (○, pK_a = 7.01 ± 0.02) and the N62C-**h** CMM (•, pK_a = 6.45 ± 0.05).

Table 1. Kinetic and PK_a Data for SBL CMMs

enzyme	pK_a[a]	$(k_{cat}/K_M)_{max}$,[b] s^{-1} $mmol^{-1}$
WT	7.01 ± 0.02	157 ± 2
N62C	6.80 ± 0.03	91 ± 2
N62C−S-**a**	6.70 ± 0.04	105 ± 2
N62C−S-**b**	6.79 ± 0.06	200 ± 5
N62C−S-**c**	6.52 ± 0.04	196 ± 3
N62C−S-**d**	6.59 ± 0.07	228 ± 6
N62C−S-**e**	6.61 ± 0.06	271 ± 6
N62C−S-**f**	6.53 ± 0.07	228 ± 7
N62C−S-**g**	6.29 ± 0.04	256 ± 7
N62C−S-**h**	6.45 ± 0.05	238 ± 5
N62C−S-**i**	6.56 ± 0.08	344 ± 15
N62C−S-**j**	7.03 ± 0.05	125 ± 3
N62C−S-**k**	6.59 ± 0.05	111 ± 2

[a] Determined in duplicate by the method of initial rates at low substrate concentration,[13] with succinyl-AAPF-pNA as the substrate, in 0.02 M ethylenediamine buffer, ionic strength 0.05 M adjusted with KCl at 25 °C. [b] $(k_{cat}/K_M)_{max}$ represents the maximal rate at the pH optimum and was calculated from pH−activity profiles.

N62C−SCH_2-*c*-C_6H_{11} induced by the cyclohexyl group would cause a decrease in the observed pK_a of His64.[12] Accordingly, pH−activity profiles for WT-SBL, the parent cysteine mutant N62C, and each of the 11 CMMs N62C-**a** to -**k** were evaluated by the method of initial rates.[13] Gratifyingly, a decrease in pK_a for N62C−SCH_2-*c*-C_6H_{11} was observed, as shown in the representative pH−activity profile (Figure 2), which indicates that the His pK_a is shifted toward the more acidic side by 0.56 unit. This pK_a change turned out to be general and other, very significant, pK_a changes were also observed for N62C−S-**a** to -**k** (Table 1).

To evaluate the stabilities of WT-SBL and the CMMs, and to ensure that the enzymes were not irreversibly altered under the pH range of this investigation, each enzyme was incubated in the buffers used in this evaluation and then assayed under the standard conditions. All enzymes were stable under these conditions and their activities were not irreversibly altered. Since the observed pH dependence of k_{cat}/K_M follows the ionization of the free enzyme or free substrate[14] unless there is a pH-dependent change of rate-determining step, and that in the present study the succinyl-AAPF-pNA substrate does not have any groups which ionize within the range of pHs investigated, the pK_a observed in the pH−activity profiles is that of a

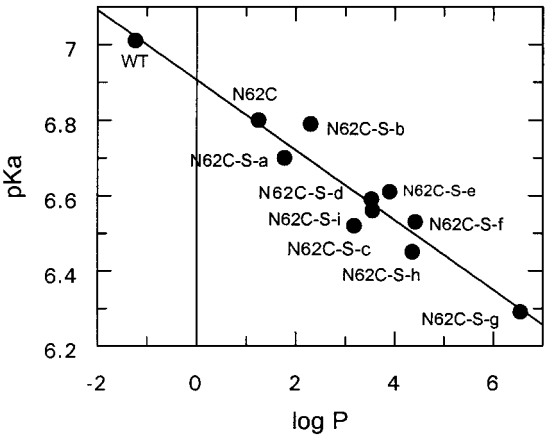

Figure 3. Linear correlation between the apparent pK_a and calculated log *P*.[16]

catalytically important residue in the free enzyme. Previous pH−activity profiles of subtilisins have also indicated an apparent pK_a of ∼7,[1b−e,h] which was ascribed by ^{1}H NMR studies to the ionization of His64.[15]

The most marked pK_a shifts reported in Table 1 are for the −SCH_2-*c*-C_6H_{11} (N62C−S-**h**), −$SCH_2C_6H_5$ (N62C-**i**), and −$SCH_2(CH_2)_8CH_3$ (N62C-**g**) CMMs. In fact, the ΔpK_a of 0.72 observed for N62C−$SCH_2(CH_2)_8CH_3$ (-**g**) is unprecedented for a single uncharged modification. That the decyl group was the largest hydrophobic group of the current series prompted us to ascertain if there was a linear relationship between pK_a and hydrophobicity. The Figure 3 plot of pK_a versus the calculated hydrophobicity index log $P_{(calcd)}$,[16] where log *P* = log (solubility in *n*-octanol/solubility in H_2O), confirms this relationship and validates the hypothesis that the hydrophobicity of the environment around His64 modulates its pK_a.[17] That such dramatic pK_a shifts are induced by the single, uncharged, groups of the current study is truly remarkable, since shifts of these magnitudes have previously been achievable only by multiple electrostatic modifications of serine proteases.[1,2] For example, succinylation of 14 lysine side chains of chymotrypsin to decrease the positive charge on the enzyme surface by 28 units was required to effect a 1.0 unit increase in the pK_a of the active site histidine.[1a] On the other hand, increasing chymotrypsin's positive surface charge by the reaction of 13 surface carboxylates with ethylenediamine was needed to elicit a 0.9 unit pK_a decrease.[1a] Also, by the site-directed mutagenesis approach, a 4 unit increase of net positive surface charge of the related protease, subtilisin BPN′, was needed to induce a 1.0 unit His64 pK_a decrease.[1c] The slight increase in pK_a effected in this study by the introduction of a negative charge, as for N62C−SCH_2-$CH_2SO_3^-$ (N62C-**j**), and the decrease effected by the addition of a positive surface charge, as for N62C−$SCH_2CH_2NH_3^+$ (N62C-**k**) are consistent with previously established models of the effects of altered surface charges.

Furthermore, there appears to be a correlation between the magnitude of the change in pK_a [$\Delta pK_{a\,(WT-CMM)}$] and increasing alkyl side chain size. This is illustrated in Figure 4 by comparison of the monotonic increase of ΔpK_a for the series

(12) Such a pK_a decrease is analogous to the substantially lowered pK_a of Lys in the active site of aceto-acetate decarboxylase as compared with its pK_a in solution. Schmidt, D. E.; Westheimer, F. H. *Biochemistry* **1971**, *10*, 1249−1253.

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(17) In view of this, it is interesting to speculate that the Trp96Leu, Trp96Tyr, and Trp96Phe mutants of Barnase, whose 0.62, 0.11, and 0.38 unit decreases in the pK_a of His18,[1g] respectively, were attributed the loss of favorable aromatic interactions with histidine may well be due to a hydrophobic effect analogous to those discussed above.

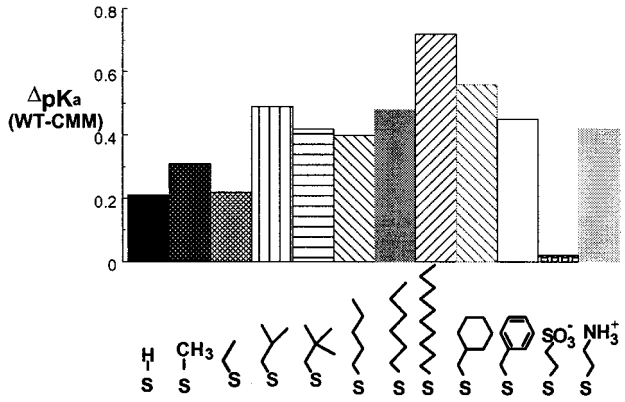

Figure 4. ΔpK_as of N62C and CMMs **1a**−**k**, determined under the Figure 2 conditions.

N62C−SCH_3, N62C−SCH_2CH_3, N62C−$SCH_2(CH_2)_3CH_3$, N62C−$SCH_2(CH_2)_4CH_3$ and N62C−$SCH_2(CH_2)_8CH_3$. While, a Brønsted plot[18] of pK_a versus log $(k_{cat}/K_M)_{max}$ s^{-1} mM^{-1} for a serine protease has not been reported since, nevertheless, to a first approximation it appeared that increased activities for these CMMs corresponded to decreased His64 pK_a values, such a plot was constructed to see if it would be informative. However, no correlation was evident despite the fact that general acid−base catalysis is exploited by the serine proteases.[19,20] This is consistent with the overall catalytic rate being affected by factors other than His64 acidity, such as local conformational changes, or electrostatic, hydrophobic and steric interactions

All of the above results support our original hypothesis that the lower dielectric constant environment induced by hydrophobic modifications stabilize the unprotonated form of His64, and thus renders its conjugate acid a more effective general acid.[21] Since the decyl modified enzyme N62C-**g** caused the greatest pK_a change, the modified crystal structure of this enzyme was subjected to molecular modeling. As shown in Figure 1c, the modeled decyl moiety adopts a coiled structure such that it is positioned over the His64 imidazole in a manner similar to that of the cyclohexyl modified N62C-**h,** apparently generating an even more hydrophobic environment. From these additional data, the bases of the rate enhancements observed previously for CMMs N62C-**b** to -**i**,[5] which reflect the monotonic ΔpK_a changes, can now be attributed, at least in part, to shifts in their pH−activity profiles and in particular to lowered His64 pK_a's. NMR titration studies are planned to confirm the His64 pK_a's of these CMMs.

To our knowledge, this is the first report of significant changes in the pH−activity profiles of a serine proteases induced by chemical modifications which do not alter enzyme surface charge. It is clear that inducing pK_a changes by controlled hydrophobic modifications represents a new strategy with great potential, and that chemical modification of site-directed cysteine mutants provides a powerful and versatile tool in this regard. Furthermore, the CMM approach described should be generally applicable to tailoring the pH−activity profiles of other synthetically useful hydrolases.

Experimental Section

Enzyme Purification. Wild-type subtilisin *Bacillus lentus* and its N62C mutant were prepared as previously described[6] and purified by desalting on a Sephadex G-25 matrix followed by strong cation exchange chromatography on SP Sepharose FF. The purity of WT- and N62C−SBL, denatured by incubation with 0.1 M HCl at 0 °C for 30 min, was determined by SDS−PAGE and in each case, only one band was visible.

Preparation of Methanethiosulfonate reagents. Reagent **1a** was purchased from Aldrich Chemical Co., Inc., **1j** and **1k** from Toronto Research Chemical (2 Brisbane Rd., Toronto, ON), and all were used as received. Reagents **1b**−**i** were prepared as previously described.[5]

Site-Specific Chemical Modification. As previously reported,[4,5] to N62C−SBL (25 mg, 0.95 μmol) in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) at 20 °C was added a 100-fold molar excess of each of the methanethiosulfonate reagents **1a**−**k** (100 μL of a 1.0 M solution, 100 μM: **1a**−**d** in EtOH, **1e**−**i** in CH_3CN, **1j** in CHES buffer, **1k** in MeOH) in a PEG (10 000) coated polypropylene test tube, and the mixture agitated in an end-over-end rotator. Blank reactions were run in parallel. Each of the modification reactions was monitored spectrophotometrically (ϵ_{410} = 8800 M^{-1} cm^{-1})[22] on a Perkin-Elmer Lambda 2 spectrophotometer, by specific activity measurements. After the reaction was quenched by dilution in MES buffer (5 mM MES, 2 mM $CaCl_2$, pH 6.5) at 0 °C, the specific activity of the CMM, was determined in buffer containing: 0.1 M TRIS pH 8.6, 0.005% Tween 80, and 1% DMSO, with the succinyl-AAPF-pNA substrate (1 mg/mL) (purchased from Bachem Bioscience Inc.) at 25 °C. The reaction was terminated when the addition of a further 100 μL of methanethiosulfonate solution effected no further change in specific activity, generally in 30 min to 1 h. The reaction solution was purified on a disposable desalting column (Pharmacia Biotech PD-10, Sephadex G-25 M) preequilibrated with MES buffer. The CMM was eluted with MES buffer (3.5 mL), dialyzed against Millipore water (3 × 1 L) at 4 °C, and subsequently lyophilized. Modified enzymes were analyzed by native gradient (8−25%) gels at pH 4.2, run toward the cathode on the Pharmacia Phast-System, and appeared as one single band.

Electrospray Mass Spectrometry. Prior to ES-MS analysis, CMMs were purified by FPLC (BioRad, Biologic System) on a Source 15 RPC matrix (17−0727−20 from Pharmacia) with 5% acetonitrile, 0.01% TFA as the running buffer, and eluted with 80% acetonitrile, 0.01% TFA in a one step gradient. Electrospray mass spectra were recorded on a PE SCIEX API III Biomolecular Mass Analyzer. In all cases the experimentally determined and theoretically calculated masses were found to agree within 6 Da.[5]

Free Thiol Titration. The free thiol content of N62C CMMs was determined spectrophotometrically by titration with Ellman's reagent[23] (ϵ_{412} = 13 600 M^{-1} cm^{-1}) in phosphate buffer 0.25 M, pH 8.0.

Active Site Titration. The active enzyme concentration was determined as previously described[11] by monitoring fluoride ion release upon enzyme reaction with the irreversible inhibitor, α-toluenesulfonyl fluoride (Aldrich Chemical Co.) as measured by a fluoride ion sensitive electrode (Orion Research 96−09). The active enzyme concentration determined in this way was used to calculate kinetic parameters for each CMM.

Kinetics. pH−activity profiles for the subtilisin CMMs were constructed by monitoring product release via its colorometric absorbance at 410 nm versus time at 25 °C (ϵ = 8800 cm^{-1}

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M^{-1}).[22] k_{cat}/K_M values were determined in duplicate in 0.02 M ethylenediamine buffer, ionic strength 0.05 M adjusted with KCl, [S] = 2.083−12.5 × 10^{-5} M of succinyl-AAPF-pNA. [E] = 1−50 × 10^{-7} M at 25 °C. pK_as were calculated using *GraFit* version 3.0 curve fit, single pK_a or bell-shaped double pK_a, with the minimum set to zero. Specifically, into a 1.5-mL polystyrene cuvette was added 980 μL of buffer (0.02 M ethylenediamine) and 10 μL of substrate (2.083−12.5 × 10^{-3} M in DMSO). The solution was incubated in a cell holder at 25 °C, before the absorbance reading was set to zero. Then 10 μL of enzyme solution (1 × 10^{-5} to 5 ×10^{-6} M) was added to initiate the reaction. After an 8 s delay, absorbance versus time measurements were recorded on a Perkin-Elmer lambda 2 spectrophotometer. k_{cat}/K_M values were calculated employing the low substrate approximation, where the Michaelis−Menten equation reduces to:[13,24] $v = k_{cat}/K_M\ [E]_0[S]$ when $[S] \ll K_M$.

Evaluation of Enzyme Stability in pH Range. To ensure that the enzymes were not irreversibly altered by pH changes, the CMMs were incubated in ethylenediamine buffers at the same pH as the assay conditions at room temperature (22 °C) for 1 min. These were then diluted (100-fold) into 0.1 M Tris, pH 8.6 containing 0.005% Tween 80 and enzyme specific activity was determined. All CMMs were found to be stable under these conditions.

Calculation of Log *P*. Calculations used ACD/log *P*[16,25] based on the side-chain structure of the amino acid or modified amino acid. For example, the model structure for WT (Asn62) is $CH_3C(O)NH_2$, whereas the model structure for N62C−S-**b** is $CH_3-S-S-CH_2CH_3$. The plot of pK_a versus log P gave a slope of −0.093 ± 0.009, an intercept of 6.91 ± 0.03, and a correlation coefficient of −0.96.

Molecular Modeling. X-ray structure of subtilisin *Bacillus lentus* (Brookhaven PDB entry 1JEA)[9b] was used as the starting point for calculations on the wild type and chemically modified mutant enzymes. The enzyme setup was performed with Insight II, Version 2.3.0.[26] To create initial coordinates for the minimization, hydrogens were added at the pH used for kinetic measurements. This protonated all Lys and Arg residues and the N-terminus and deprotonated all Glu and Asp residues and the C-terminus. The model system with the phenylmethylsulfonate ester of Ser221 bound in the S_1 pocket was solvated with a 5-Å layer of water molecules giving a total number of water molecules of 1174 in this system. The overall charge of the enzyme−inhibitor complex resulting from this setup was +3 for the WT enzyme. Energy simulations were performed with the DISCOVER program, Version 2.9.5 on a Silicon Graphics Iris Indigo computer, using the consistent valence force field function (CVFF). A nonbonded cutoff distance of 18 Å with a switching distance of 2 Å was employed. The nonbonded pair list was updated every 20 cycles and a dielectric constant of 1 was used in all calculations. The WT enzyme was minimized in stages, with initially only the water molecules being allowed to move, then the water molecules and the amino acid side chains, and then the entire enzyme. The mutated and chemically modified enzymes were generated using the Builder module of Insight. These structures were then minimized in a similar manner. Specifically, the side chain of the mutated residue and the water molecules were minimized. The amino acid side chains within a 10-Å radius of the α-carbon of the mutated residue were minimized while all other enzyme residues were constrained, then all of the atoms were minimized.

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Supporting Information Available: Calculated log *P* values (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(24) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman, New York, 1985; pp 105−111.

(25) N62C-**j** and N62C-**k** are omitted since the software package is unable to calculate log *P* for these structures.

(26) Biosym Technologies, Inc. San Diego, CA.