Site-Directed Mutagenesis Combined with Chemical Modification as a Strategy for Altering the Specificity of the S_1 and S_1' Pockets of Subtilisin *Bacillus lentus*†

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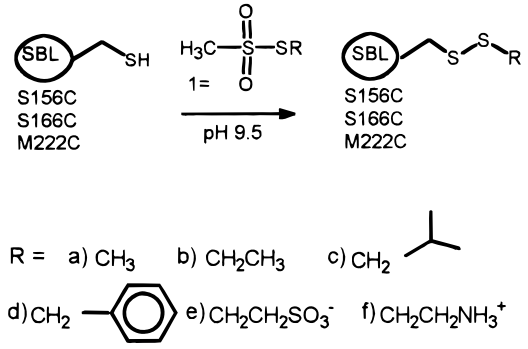
ABSTRACT: By combining site-directed mutagenesis with chemical modification, we have altered the S_1 and S_1' pocket specificity of subtilisin *Bacillus lentus* (SBL) through the incorporation of unnatural amino acid moieties, in the following manner: WT → Cys_{mutant} + H_3CSO_2SR → Cys−SR, where R may be infinitely variable. A paradigm between extent of activity changes and surface exposure of the modified residue has emerged. Modification of M222C, a buried residue in the S_1' pocket of SBL, caused dramatic changes in k_{cat}/K_M, of an up to 122-fold decrease, while modification of S166C, which is located at the bottom of the S_1 pocket and is partially surface exposed, effected more modest activity changes. Introduction of a positive charge at S166C does not alter k_{cat}/K_M, whereas the introduction of a negative charge results in lowered activity, possibly due to electrostatic interference with oxyanion stabilization. Activity is virtually unaltered upon modification of S156C, which is located toward the bottom of the S_1 pocket and surface exposed and whose side chain is solvated. An unexpected structure−activity relationship was revealed for S166C−SR enzymes in that the pattern of activity changes observed with increasing steric size of R was not monotonic. Molecular modeling analysis was used to analyze this unprecedented structure−activity relationship and revealed that the position of the β-carbon of Cys166 modulates binding of the P_1 residue of the AAPF product inhibitor.

Creating enzymes with new catalytic activities, and tailoring the specificity of existing ones to better accommodate unnatural substrates, is an area of considerable current research (*1*−*4*). Site-directed mutagenesis (*5*) and random mutagenesis (*4*) have emerged as practical methods to alter enzyme specificity by natural amino acid replacements. Insights into the electrostatic (*6*−*8*), steric (*9*), and hydrophobic (*10*) factors which govern enzyme−substrate interactions have also been probed by site-directed mutagenesis studies. However, despite recent advances in understanding the intermolecular interactions which determine enzyme structural- and stereospecificity, truly rational tailoring of enzyme specificity remains an elusive goal.

To better understand the factors which control enzyme specificity, and to permit fine-tuning of specificity, biosynthetic methods have recently been developed to incorporate unnatural amino acids into proteins (*11*). Also, unnatural functionalities have been covalently attached to proteins by chemical modification techniques (*12*−*17*).

Recently, the combination of site-directed mutagenesis and chemical modification has been recognized as a powerful tool for the creation of new active-site environments in carboxypeptidase Y (*18*) and subtilisin (*19*), in mechanistic studies of aspartate aminotransferase (*20*) and ribulosebisphosphate carboxylase (*21*), and for the investigation of protein packing in thioredoxin (*22*). We have also begun to exploit this technique for altering substrate specificity (*23*) and for increasing enzyme activity (*24*) of the alkaline subtilisin from *Bacillus lentus* (SBL). In this paper we demonstrate some of the specificity changes which chemical modifications of mutant enzymes can elicit.

Scheme 1: Chemical Modification of Subtilisin *B. Lentus* (SBL) Cysteine Mutants S156C, S166C and M222C with Methanethiosulfonate Reagents (**1a**−**f**) to Generate Chemically Modified Mutant Enzymes



The strategy involves the introduction of one cysteine residue at a key active site position via site-directed mutagenesis. This is then thioalkylated with an alkyl methanethiosulfonate reagent (**1a**−**f**) to give chemically modified mutant enzymes (CMMs) as illustrated in Scheme 1. Alkyl

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methanethiosulfonate reagents react specifically and quantitatively with thiols and are routinely used for chemical modification of protein thiols (*25*, *26*). The modification can be performed under mild reaction conditions, on a large scale, and is independent of the nature of the R group. The CMM approach avoids the difficulties which can result from nonconservative mutations that produce proteins which are unable to fold properly or that do not undergo the required auto-processing. This potential difficulty is particularly relevant to the subtilisins, which are initially expressed as pre-proenzymes that must undergo autoproteolysis (*27*).

The alkaline serine protease subtilisin *Bacillus lentus* (SBL, EC 3.4.21.14)[1] is a good candidate for evaluating this strategy since it is a well-characterized enzyme and is of synthetic (*3*, *28*) as well as industrial interest (*29*). Furthermore, SBL's high-resolution crystal structure has been solved (*30*), it has been cloned, overexpressed, and purified (*31*), and its kinetic behavior has been well characterized (*32*). Also, studies toward altering its specificity (*33*−*35*) have been reported. Importantly, WT-SBL contains no natural cysteine residues and methanethiosulfonate reagents, therefore, react *only* with the introduced cysteine residue.

Our goal was to alter the specificity of the S_1 and S_1' pockets of SBL. With the crystal structure of SBL as our guide, two residues at the bottom of the S_1 pocket, Ser156, one which is surface exposed, and Ser166, one whose side chain points into the pocket, were chosen for mutagenesis and chemical modification. The buried residue, Met 222,[2] which flanks the S_1 and S_1' pockets (*36*) and is adjacent to the oxyanion hole and catalytic Ser221, was also targeted with the anticipation that its modification should dramatically affect catalytic performance (*23*, *31*).

MATERIALS AND METHODS

Enzyme Purification. Wild-type subtilisin *Bacillus lentus*, and its M222C, S156C, and S166C mutants, were purified by the general method of Stabile et al. (1996). The crude protein concentrates containing PEG (50%) as a stabilizer, which were obtained as previously described (*31*), were purified on a Sephadex G-25 desalting matrix with a pH 5.2 buffer (20 mM sodium acetate, 5 mM $CaCl_2$), to remove small molecular weight contaminants. Pooled fractions from the desalting column were then applied to a strong cation exchange column (SP Sepharose FF) in the HEPES buffer (20 mM HEPES, 2 mM $CaCl_2$, pH 7.8), and SBL was eluted with a one-step gradient of 1−200 mM NaCl−HEPES buffer (20 mM HEPES, 2 mM $CaCl_2$, 200 mM NaCl, pH 7.8). Salt-free enzyme powder was obtained by dialysis (MWCO 12 000−14 000)[3] of the eluent against Millipore purified water and subsequent lyophylization. The purity of the WT and mutant enzymes, denatured by incubation with 0.1 M HCl at 0 °C for 30 min, was determined by SDS−PAGE on 20% homogeneous gels using the Phast System from Pharmacia. For all enzymes used, only one band was visible.

Preparation of Methanethiosulfonate Reagents. Reagent **1a** was purchased from Aldrich Chemical Co. Inc., **1e**,**1f** were purchased from Toronto Research Chemical (2 Brisbane Rd., Toronto, ON, Canada), and all were used as received. Reagents **1b**−**d** were prepared as previously described (*24*).

Site-Specific Chemical Modification. To 25 mg of a SBL mutant in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) at 20 °C was added one of the methane thiosulfonate reagents (**1a**−**f**) (100 μL of a 1 M solution: **1a** in MeOH, **1b** in EtOH, **1c** in EtOH, **1d** in CH_3CN, **1e** in CHES buffer, **1f** in MeOH), in a PEG (10 000) coated polypropylene test tube, and the mixture was agitated in an end-over-end rotator. Blank reactions containing 100 μL of solvent instead of the reagent solution were run in parallel. Each of the modification reactions was monitored spectrophotometrically (ϵ_{410} = 8800 M^{-1} cm^{-1}; *37*) 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 (10 μL) 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 3 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 nondenaturing gradient (8−25%) gels at pH 4.2, run toward the cathode on the Pharmacia Phast-System,[4] and appeared as one single band. Each of the CMMs was analyzed in parallel with its parent cysteine mutant and the WT enzyme.

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 and 0.01% TFA as the running buffer and eluted with 80% acetonitrile and 0.01% TFA in a one-step gradient. Electrospray mass spectra were recorded on a PE SCIEX API III biomolecular mass analyzer.

Regeneration of Unmodified Enzyme by Treatment with β-Mercaptoethanol. To a solution of CMM (2.0 mg) in 250 μL of CHES buffer (70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) was added 10 μL of a solution of β-mercaptoethanol (1 M in 95% EtOH). The reaction was monitored by specific activity measurements.

Free Thiol Titration. The free thiol content of M222C and its CMMs was determined by I_2 titration in 0.25 M phosphate buffer, pH 6.9. The uptake of iodine was monitored spectrophotometrically at 355 nm (ϵ_{355} = 13 000 M^{-1} cm^{-1}; *38*). The free thiol content of WT, S156C, S166,C and their CMMs was determined spectrophotometrically by titration with Ellman's reagent (ϵ_{412} = 13 600 M^{-1} cm^{-1}; *39*) in 0.25 M phosphate buffer, pH 8.0.

[1] SBL is identical in sequence to Savinase.

[2] BPN′ numbering.

[3] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ES-MS, electrospray mass spectrometry; EtOH, ethanol; FPLC, fast performance liquid chromatography; MES, 4-morpholineethanesulfonic acid; MMTS, methyl methanethiolsulfonate; MW, molecular weight; MWCO, molecular weight cutoff; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); pNA, *p*-nitroaniline; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

[4] Pharmacia Application File No. 300.

Active Site Titrations. The active enzyme concentration was determined as previously described (*40*) by monitoring fluoride release upon enzyme reaction with α-toluenesulfonyl fluoride (Aldrich Chemical Co. Inc.) 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.

Kinetic Measurements. Michaelis−Menten constants were measured at 25 °C by curve fitting (GraFit 3.03) of the initial rate data determined at eight concentrations (0.125−4.0 mM) of the succinyl−AAPF−pNA substrate in 0.1 M phosphate buffer containing 0.5 M NaCl, 0.005% Tween 80, and 1% DMSO, pH 7.5 ($\epsilon_{410} = 8800\ M^{-1}\ cm^{-1}$).

Molecular Modeling. The X-ray structure of subtilisin *B. lentus* with the peptide inhibitor AAPF bound (Brookhaven database entry 1JEA pending) was used as the starting point for calculations on wild type and CMMs. The enzyme setup was done with Insight II, version 2.3.0.[5] To create initial coordinates for the minimization, hydrogens were added at the pH 7.5 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-terminal carboxyl group. The protonated form of His64 was used in all calculations. The model system was solvated with a 5 Å layer of water molecules. The total number of water molecules in the system was 1143. 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 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, followed by water molecules and the amino acid side chains, and then finally the entire enzyme. The mutated and chemically modified enzymes were generated by modifying the relevant amino acid using the Builder module of Insight. These structures were then minimized in a similar manner. Initially the side chain of the mutated residue and the water molecules were minimized. Then the amino acid side chains within a 10 Å radius of the α-carbon of the mutated residue were minimized while all other residues were constrained, and then all of the atoms within a 10 Å shell were minimized. The AAPF inhibitor was free to move throughout all stages of the minimization.

RESULTS

Each of the S156C, S166C, and M222C SBL mutants was treated with the methanethiosulfonate reagents (**1a**−**f**) as shown in Scheme 1, and modification reactions were monitored by specific activity measurements. In all cases, reactions at the 156 and 166 sites with reagents **1a**−**f** were complete within 30 min, consistent with the surface exposure of the C156 residue and the ready accessibility of the C166 residue. In contrast, for the more buried 222 site, modifications with the larger $-SCH_2C_6H_5$ (**1d**) and $-SCH_2CH(CH_3)_2$ (**1c**) reagents, and with the negatively charged $-SCH_2CH_2SO_3^-$ reagent (**1e**), reactions were significantly slower, requiring reaction times of up to 3 h.

Free thiol titration of the S156C and S166C CMMs with Ellman's reagent established that reactions were quantitative. The sterically more demanding M222C CMMs were titrated with the smaller I_2 titrant due to the inaccessibility of Ellman's reagent to this site. In all cases, the free thiol content of the CMM's was less than 2%.[6] Mass analysis of the CMMs by electrospray mass spectrometry was consistent (±6 Da) with the calculated mass. The purity of the modified enzymes was assessed by native-PAGE and in all cases only one band was visible. CMMs S156C−S-**a to -d**, S166C−S-**a to -d** and M222C−S-**a to -d** could not be distinguished from the parent cysteine mutant nor the WT enzyme on native-PAGE. However, the negatively charged CMMs derived from reactions with **1e**, S156C$-SCH_2CH_2SO_3^-$, S166C$-SCH_2CH_2SO_3^-$, and M222C$-SCH_2CH_2SO_3^-$ displayed retarded mobility, while the positively charged S156C$-SCH_2CH_2NH_3^+$, S166C$-SCH_2CH_2NH_3^+$, and M222C$-SCH_2CH_2NH_3^+$ CMMs derived from reactions with **1f** displayed greater mobility relative to wild type.

That modification of cysteine is wholly responsible for altered activity was established by demonstrating that treatment of SBL-WT with each of **1a**−**f** resulted in no change in activity or of molecular weight. Furthermore, treatment of S166C−S-**a** to-**f** , S156C−S-**a** to-**f**, M222C−S-**a**, and of M222C−S-**f** with β-mercaptoethanol restored activity to that of the parent cysteine mutant, verifying that chemical modification at cysteine was solely responsible for the observed changes in activity and is fully reversible by this treatment. The activities of M222C$-SCH_2CH_3$ (−S-**b)**, M222C$-SCH_2CH(CH_3)_2$ (−S-**c**), M222C$-SCH_2C_6H_5$ (−S-**d)** and M222C$-SCH_2CH_2SO_3^-$ (−S-**e**) could not be restored to that of the unmodified mutant M222C in this way, due to the inaccessibility of β-mercaptoethanol to the more sterically congested 222. This inability to restore activity parallels the slow chemical modification rates of M222C when exposed to **1c**, **1d**, and **1e**.

Kinetic constants for each of the CMMs were determined with the suc−AAPF−pNA substrate and are shown in Table 1; K_Ms are virtually unaltered, and consequently, k_{cat}/K_M changes are mainly reflective of k_{cat} variations.

DISCUSSION

Once characterized, the specificity of each of the CMMs was evaluated with succinyl−AAPF−pNA as the standard reference substrate. Chemical modifications at each of the three sites resulted in very different activity patterns, as indicated in Table 1. Chemical modification of the S156C mutant effected only very small changes in k_{cat}/K_M. In contrast, chemical modification of the S166C and M222C mutants resulted in more dramatic changes in activity.

Mutation of Ser156 to cysteine and modification with **1a**−**f** results in somewhat decreased k_{cat}/K_Ms. As illustrated in Figure 1 chemical modification of S156C resulted in small

[5] Biosym Technologies, Inc., San Diego, CA.

[6] For the M222C-SBn CMM (3d) only 61% modification was accomplished as determined by I_2 titration for free thiol. This results in overestimating the kinetic constants. However, since this is a very deleterious modification, no further attempt was made to accomplish 100% modification.

Table 1: Kinetic Parameters[a] for SBL CMMs

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
WT[b]	48 ± 2	0.55 ± 0.06	87 ± 10
S156C	43 ± 2	0.65 ± 0.08	66 ± 9
S156C−S-**a**	30 ± 1	0.79 ± 0.08	38 ± 4
S156C−S-**b**	23.4 ± 0.5	0.68 ± 0.04	34 ± 2
S156C−S-**c**	24.2 ± 0.6	0.60 ± 0.04	40 ± 3
S156C−S-**d**	21.8 ± 0.7	0.54 ± 0.05	40 ± 4
S156C−S-**e**	31.6 ± 0.9	0.78 ± 0.06	40 ± 3
S156C−S-**f**	39 ± 1	0.86 ± 0.08	45 ± 4
S166C	14.2 ± 0.5	0.51 ± 0.05	28 ± 3
S166C−S-**a**	17.4 ± 0.7	0.39 ± 0.05	45 ± 6
S166C−S-**b**	11.8 ± 0.3	0.92 ± 0.05	12.8 ± 0.5
S166C−S-**c**	31 ± 1	0.72 ± 0.07	47 ± 4
S166C−S-**d**	6.9 ± 0.4	0.74 ± 0.09	9 ± 1
S166C−S-**e**	3.8 ± 0.1	0.70 ± 0.06	5.4 ± 0.5
S166C−S-**f**	16.3 ± 0.5	0.60 ± 0.06	27 ± 3
M222C[b]	17.3 ± 0.4	0.77 ± 0.05	22 ± 2
M222C−S-**a**[b]	33.3 ± 0.8	0.68 ± 0.04	49 ± 3
M222C−S-**b**	7.2 ± 0.2	0.63 ± 0.05	11 ± 1
M222C−S-**c**	0.49 ± 0.02	0.69 ± 0.07	0.71± 0.08
M222C−S-**d**[c]	6.9 ± 0.1	0.67 ± 0.04	10.3 ± 0.6
M222C−S-**e**[b]	1.64 ± 0.05	0.55 ± 0.05	3.0 ± 0.3
M222C−S-**f**[b]	1.04 ± 0.03	0.61 ± 0.05	1.7 ± 0.1

[a] Michaelis−Menten constants were measured at 25 °C according to the initial rates method in 0.1 M phosphate buffer containing 0.5 M NaCl, at pH 7.5, and 1% DMSO, with succinyl−AAPF−pNA as the substrate. [b] Berglund, et al. (1996). [c] Only 61% modified, 39% M222C.

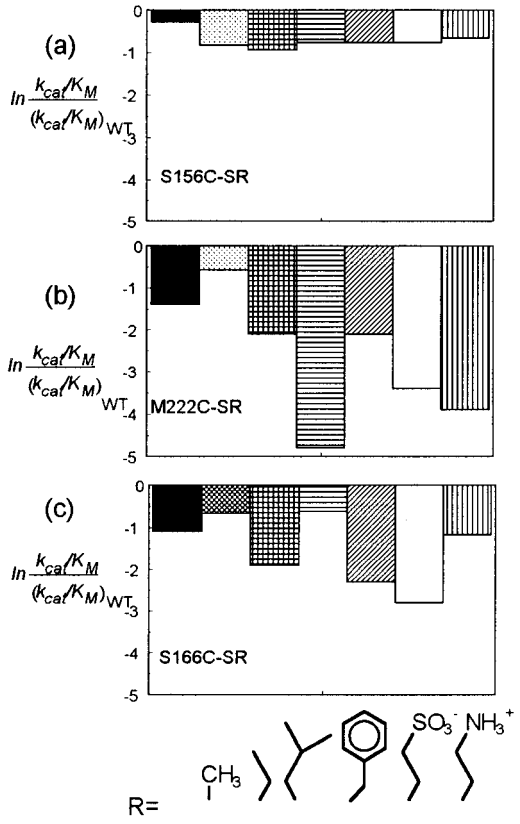

FIGURE 1: Altered specificity patterns for S156C, S166C, and M222C CMMs: $\ln[k_{cat}/K_M/(k_{cat}/K_M)_{WT}]$ measured at 25 °C in 0.1 M phosphate buffer containing 0.5 M NaCl at pH 7.5, and 1% DMSO, with succinyl−AAPF−pNA as the substrate.

changes in activity relative to WT as indicated by the ratio of $\ln[(k_{cat}/K_M)/(k_{cat}/K_M)_{WT}]$. They are in accord with introduced groups being able to take up positions away from the active site as a result of the surface exposed nature of the S156C residue. Even when the enzyme's surface charge was altered, as for S156C−$SCH_2CH_2SO_3^-$ (−S-**e**) and S156C−$SCH_2CH_2NH_3^+$ (−S-**f**), activity remained unaffected. This result contrasts previous observations for the related enzyme subtilisin BPN′, for which increasing positive charge on the enzyme surface destabilizes the imidazolium form of the active site histidine and stabilizes the negatively charged oxyanion resulting in altered activity (*41*). The absence of such an electrostatic effect for SBL indicates a significant difference in specificity between the S_1 pockets of SBL compared to subtilisin Carlsberg or subtilisin BPN′ (*30*). This may be due the 4 amino acid deletion in SBL relative to subtilisin BPN′ for which mutation of residue 156 significantly affects P_1 binding (*8*) and enzyme activity (*42*). While these data were determined under conditions of high ionic strength, which may mask electrostatic effects (*7*), k_{cat}/K_M determinations at lower ionic strength did not affect k_{cat}/K_M either (*43*). This suggests that the $-SCH_2CH_2NH_3^+$ group of S156C-**f** and $-SCH_2CH_2SO_3^-$ group of S156C-**e** are extensively solvated.

Although mutation of Met222 to Cys caused a decrease in k_{cat}/K_M, modification of M222C with **1a** restored activity to 50% of WT, which represents a 2-fold activity increase. This is in accord with the similarity between the $-SCH_3$-modified cysteine side chain of M222C−S-**a** and the side chain of methionine in SBL-WT, with the only difference being the replacement of the methylene group of the methionine side chain by a sulfur atom in M222C−S-**a**. Chemical modification of M222C to generate the M222C−SCH_2CH_3 (−S-**b**), M222C−$SCH_2CH(CH_3)_2$ (−S-**c**), and M222C−$SCH_2C_6H_5$ (−S-**d**) CMMs effected monotonic decreases in enzyme activity, as illustrated in Figure 1b. This points to the steric limitations of this site for accommodating bulky side chains. The observed activity decrease of M222C−$SCH_2CH_2SO_3^-$ (−S-**e**) compared to WT is attributed to the destabilizing effect of a negative charge on the incipient oxyanion (*41*). Surprisingly M222C−$SCH_2CH_2NH_3^+$ (−S-**f**) also effected a 3-fold activity decrease. This is presumably due to the beneficial effects of the partially charged groups being overwhelmed by the deleterious steric effects of the ethylamino group. These results parallel the activity decreases observed for subtilisin BPN′ upon mutation of Met222 to any of the 19 natural amino acids (*44*) and are consistent with the highly conserved nature of this residue among the subtilisins (*30*). While chemical modifications of M222C generally resulted in decreased k_{cat}/K_Ms and virtually unaltered K_Ms, the CMMs M222C−SCH_3 (−S-**a**), M222C−$SCH_2CH_2SO_3^-$ (−S-**e**), and M222C−$SCH_2CH_2NH_3^+$ (−S-**f**) showed up to 13-fold improvements in the binding of sterically demanding boronic acid transition-state analogue inhibitors (*23*). The data indicate that while ground-state binding is unaffected by modification, transition state binding is improved.

The most interesting and unexpected results are manifest for the S166C CMMs. An intriguing structure−activity relationship (SAR) of alternating increases and decreases in k_{cat}/K_M upon modification of S166C with methanethiosulfonate reagents of increasing steric volume, specifically **1a** ($-SCH_3$), **1b** ($-SCH_2CH_3$), **1c** ($-SCH_2CH(CH_3)_2$), and **1d** ($-SCH_2C_6H_5$), is observed. The SAR illustrated in Figure 1c is unprecedented and quite different from what would have been predicted from previous site-directed mutagenesis studies of residue 166 in the related enzyme, subtilisin BPN′

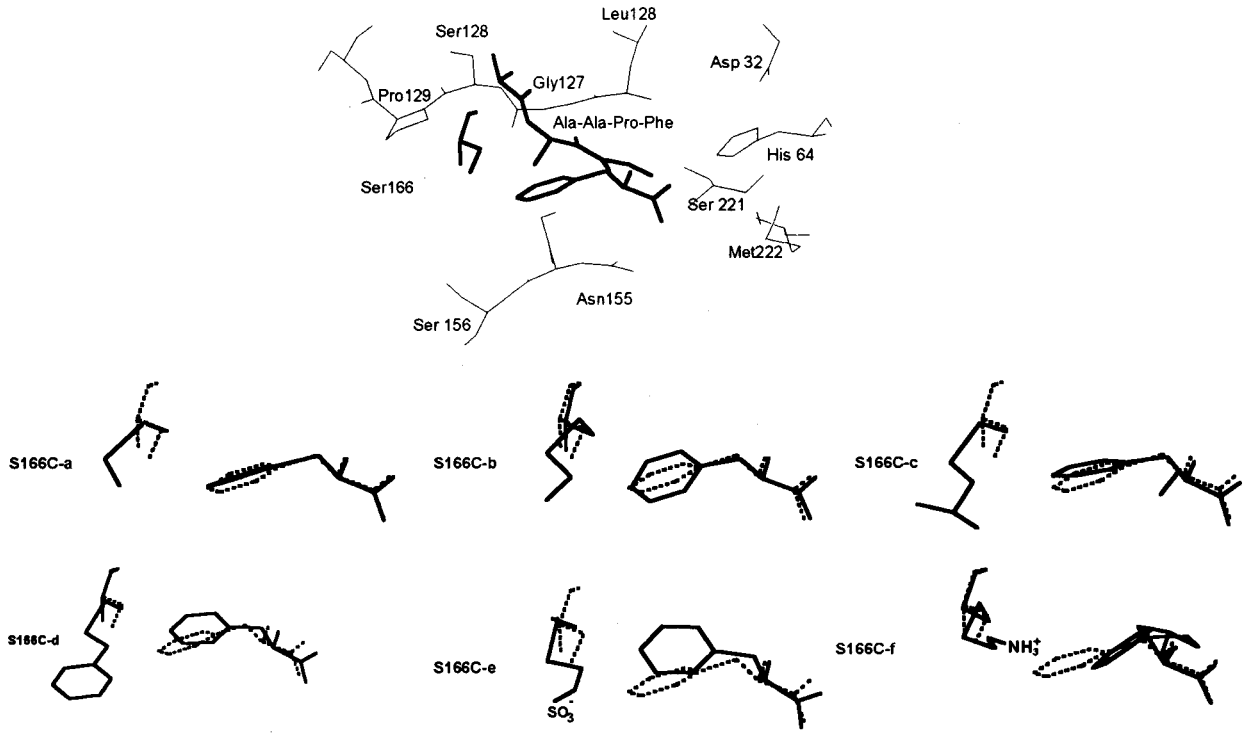

FIGURE 2: Molecular mechanics analysis: Top panel shows the active site of WT-SBL (thin line) with the product inhibitor Ala-Ala-Pro-Phe (thick line) bound, highlighting the residues which comprise the S_1 pocket, 155−156, 166, 126−129, and 222, and the catalytic triad residues, 32, 64, and 221. Oxyanion stabilization is provided by the amide backbone hydrogens of Ser222 and Asn155. Lower panels show the position of the S166C−SR (thick line) side chain and the altered binding of the Phe residue for each of the S166C−S-**a** to -**f** CMMs (thick line) with respect to WT (- - -).

(*9*, *10*). The molecular basis for this novel activity pattern was analyzed by molecular modeling of the peptidyl product inhibitor AAPF bound to SBL-CMMs.[7]

As shown in Figure 2, molecular modeling analysis of the S166C CMMs reveals that the observed k_{cat}/K_M changes correlate with altered binding of the P_1 benzyl side chain of the AAPF product inhibitor. While the phenyl ring of the P_1 Phe residue is positioned well into the S_1 pocket of WT-SBL, it is slightly distorted out of the pocket for S166C−SCH_3 (−S-**a**). For S166C−SCH_2CH_3 (−S-**b**) the phenyl binding is further distorted but is less so for S166C−SCH_2-$CH(CH_3)_2$ (−S-**c**). Molecular modeling also revealed a very significant repositioning of the phenyl ring out of the S_1 pocket of S166C−$SCH_2C_6H_5$ (−S-**d**), consistent with the lowered k_{cat}/K_M for this CMM. Excitingly, in all cases the extent of binding distortion of the P_1 benzyl side chain in the S_1 pocket of the CMMs modeled correlates with the changes in k_{cat}/K_M. The molecular modeling results also suggest that, for the S166C CMMs, disrupted phenyl binding may be due to changes in the preferred position of the β-carbon ($-^{\beta}\mathbf{CH_2}$SR) of Cys166. As illustrated in Figure 2, for S166C−SCH_2CH_3 (−S-**b**) the β-carbon of Cys166 points further into the S_1 pocket than it does for the S166C−SCH_3 (−S-**a**), S166C−$SCH_2CH(CH_3)_2$ (−S-**c**), or WT enzymes and, thus, pushes the P_1 Phe residue out of the S_1 pocket. However, for S166C−$SCH_2C_6H_5$ (−S-**d**), which also displays lower activity, and for which molecular modeling indicates significantly distorted Phe binding, the position of the β-carbon of Cys166 is not altered. It is not immediately evident how the local reorientation of the modified side chain of S166C−SCH_2CH_3 (−S-**b**) may serve to relieve strain induced elsewhere in the S_1 pocket.

The positively charged $-SCH_2CH_2NH_3^+$ side chain of S166C−S-**f** effected no change in k_{cat}/K_M relative to WT, while the negatively charged $-SCH_2CH_2SO_3^-$ moiety of S166C−S-**e** caused a 5-fold decrease. A positive charge is expected to stabilize the unprotonated form of His64 as well as provide oxyanion stabilization, while a negative charge destabilizes these interactions (41). Thus, factors such as nonoptimal positioning of the positive charge within the enzyme active site and masking of a favorable electrostatic interaction by a unfavorable steric one are clearly operating to negate the beneficial influence of the positive charge of the $-SCH_2CH_2NH_3^+$ side chain of S166C−S-**f**. A dramatic structural change is seen for S166C−S-**f**, in which the $-SCH_2CH_2NH_3^+$ side chain becomes directed into the S_1 pocket and causes the phenyl ring of phenylalanine to be displaced into the upper region of the pocket. This unexpected orientation of the ethylammonium moiety into the S_1 pocket is attributed to a potential hydrogen bond between its ammonium hydrogen and the α-carbonyl of Gly 127 (N−O distance 3.9 Å in the minimized structure). While the conformation of the $-SCH_2CH_2NH_3^+$ side chain of S166C−S-**f** is unusual, it parallels the conformational flexibility of the side chain of Lys166 evident from the disordered electron density map observed in the X-ray structure of the G166K mutant of subtilisin BPN′ (*45*). Furthermore, molecular modeling analysis shows that while the $-SCH_2CH_2SO_3^-$ side chain of S166C−S-**e** does not

[7] While the substrate employed for kinetic analysis is succinylated, we did not include this moiety in molecular modeling since its orientation was not reported in the X-ray structure of SBL, suggesting high mobility in the crystal.

orient itself directly into the S_1 pocket, it does cause significant disruption of inhibitor binding.

The fact that AAPF binding is correlated to the trends in K_{cat}/K_M while K_M is virtually unaltered suggests that the product inhibitor mimics transition state binding and not ground-state binding of the succinyl−AAPF−pNA substrate. These modifications change enzyme turnover to a greater extent than substrate binding.

These results demonstrate that the combination of site-directed mutagenesis and site-specific chemical modification can alter the specificity of the S_1 and S_1' pockets of subtilisin *B. lentus* in unusual ways, and we are pursuing this aspect further. Also, molecular modeling has again proven to be a powerful tool for analyzing the molecular basis for the activity changes induced by chemical modification and suggest that activity changes are correlated to altered binding of the P_1 moiety of AAPF in the S_1 pocket. It has also become apparent that the degree of activity changes which may be engendered upon modification is correlated with the extent of surface exposure of the residue modified.

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SUPPORTING INFORMATION AVAILABLE

A table of ES-MS data for all CMMs (1 page). Ordering information is given on any current masthead page.

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