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Probing the Altered Specificity and Catalytic Properties of Mutant Subtilisin Chemically Modified at Position S156C and S166C in the S_1 Pocket

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Abstract—A series of chemically modified mutants (CMMs) of subtilisin *B. lentus* (SBL) were generated employing the combination of site-directed mutagenesis and chemical modification. This strategy entails the mutation of a selected active site residue to cysteine and its subsequent modification with a methanethiosulfonate reagent CH_3SO_2S-R, where R may be infinitely variable. The present study was undertaken to evaluate the changes in specificity and pH-activity profiles that could be induced by modification of S156C and S166C in the S_1 pocket of SBL with a representative range of side chain modifications, namely R=$-CH_3$, $-CH_2C_6H_5$, $-CH_2CH_2NH_3^+$ and $CH_2CH_2SO_3^-$. The side chain of S156C is surface exposed and well solvated while that of S166C points into the pocket. Kinetic evaluation of the CMMs with suc-AAPF-pNA as substrate showed that the k_{cat}/K_Ms changed very little for the S156C CMMs, but varied by up to 11-fold for the S166C CMMs. pH-Activity profiles were also determined, and showed that a negatively or positively charged side chain modification increased or decreased respectively, the pK_a of the catalytic triad histidine for both modification sites but with more dramatic changes for the interior pointing S166C than for the solvent exposed S156C site. As an additional probe of altered specificity, inhibition of the CMMs by a representative series of 5 boronic acid transition state analogue inhibitors was determined. The K_Is observed ranged from a 3.5-fold improvement over the WT value, to a 12-fold decrease in binding. Overall, greater variability in all the parameters measured, activity, pK_a, and boronic acid binding resulted from modification at the inward pointing 166 site than at the solvent-exposed 156 site.

Introduction

Enzymes are now widely exploited as catalysts in organic synthesis since they permit exquisite control of regio- stereo- and enantio-selectivity under very mild reaction conditions in environmentally benign reaction media.[1–4] This explosion in the applications of enzymes has been accompanied by significant advances in the understanding of enzyme reactivity and the development of enzyme models to predict specificity.[5,6] However, in order to expand the synthetic utility of enzymes even further, additional insights into the precise nature of the factors which control their structural and stereospecificity are an imperative. Furthermore, the creation of enzymes with new catalytic activities, and tailoring the specificity of existing ones to accommodate unnatural substrates, is an area of current intense research since it leads to the development of new biocatalysts and provides an additional test of the validity of our collective understanding of the chemical basis for enzyme specificity.[7–10]

Recently, we have begun to exploit a combined chemical modification site-directed mutagenesis approach to gain insights into the factors which control substrate specificity and to create enzymes with altered or novel selectivity.[11–13] This strategy is outlined in Scheme 1, and entails the introduction of a cysteine residue at a key active site position via site-directed mutagenesis whose side chain is then thioalkylated with an alkyl methanethiosulfonate reagent (**1a–d**) to yield a chemically modified mutant enzyme (CMM).[14–18] Alkyl methanethiosulfonate reagents react chemo-selectively and quantitatively with sulfhydryls under mild reaction conditions, and are routinely used for the chemical modification of protein thiols.[19–21] For example, the combination of site-directed mutagenesis and chemical modification has been recognized as a powerful tool for the creation of new active-site environments,[14,22] in mechanistic studies,[15,17,18] and for cofactor incorporation.[23] The approach has also been applied in studies of ion-channel properties,[24–26] site-directed incorporation of spin-labels,[27,28] to probe receptor binding,[29] and for investigations of membrane spanning proteins.[30,31]

Key words: Amino acids and derivatives; electrostatic effects; enzyme inhibitors; mutagenesis.

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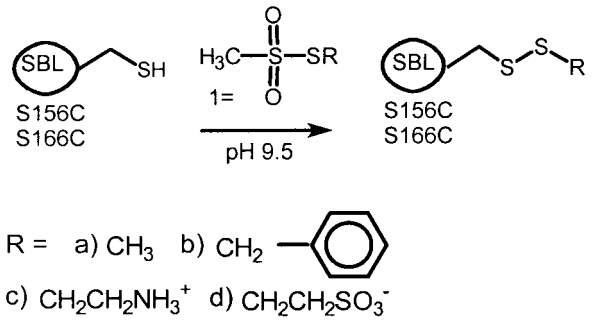


Scheme 1.

The alkaline serine protease subtilisin *Bacillus lentus* (SBL, EC 3.4.21.14)[32] is an ideal vehicle for the CMM strategy since WT-SBL contains no natural cysteines and methanethiosulfonate reagents therefore react *only* with the introduced cysteine residue. Furthermore, the general class of enzymes known as hydrolases is synthetically the most utilized, and the serine proteases, of which SBL is a member, are a subset of this group.[33] In addition SBL has been well characterized,[34–37] has been over-expressed and purified,[38] and its crystal structure is known.[32,39] Also, the validity of the CMM strategy for specificity and stability studies have already been established in studies on subtilisin.[16]

Employing the crystal structure of SBL[39] as our guide, two residues at key locations at the bottom of the S_1 pocket,[40] which is the primary specificity determining cleft in the enzyme, were identified. These were Ser156, which is surface exposed, and the more buried Ser166, whose side chain points into the pocket. Each was subjected to mutagenesis and chemical modification (Scheme 1). The S156C-**a** to -**d** and S166C-**a** to -**d** CMMs were prepared as depicted in Scheme 1 and characterized as described previously.[13] The objective of the current study was to further explore the S_1 pocket specificity changes inducible by the CMM methodology by evaluating changes in the kinetic parameters, in the pH-activity profiles, and in alterations in binding of a series of transition state analogue boronic acid inhibitors (Fig. 1).

Results and Discussion

Preparation, characterization, and kinetic evaluation of CMMs

Each of the chemically modified enzyme mutants (CMMs) was prepared by the general method described previously,[13] which entails reaction of the S166C and S156C mutants of SBL with each of the MTS reagents **1a–1d**, yielding S156C-S-**a** to -**d** and S166C-S-**a** to -**d** as outlined in Scheme 1. Each of the CMMs was fully characterized, with electrospray mass spectrometry (ES-MS), the absence of residual free thiol was established by titration with Ellman's reagent,[41] and by native-polyacrylamide gel electrophoresis (PAGE), all of which demonstrate that the modifications were quantitative and specific for the introduced cysteine.[13]

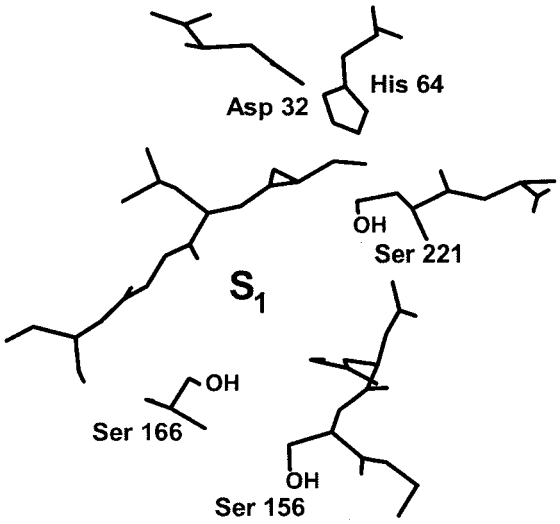


Figure 1. Active site of subtilisin *B. lentus*[39] showing the catalytic triad residues Asp32, His64, and Ser221, and the S_1 pocket residues Ser156 and Ser166 chosen for mutation and modification.

The kinetic constants were evaluated at the pH 8.6 optimum of WT-SBL in Tris-HCl buffer with succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (suc-AAPF-pNA) as the standard substrate. These results are summarized in Table 1, which for comparative purposes also includes data determined previously at pH 7.5 (0.1 $NaHPO_4$ 0.5 M NaCl) under high salt conditions.[13] In all the cases the CMMs were more active under the pH 8.6 conditions. However, the trends in k_{cat}/K_M variations at both pH 8.6 and 7.5 parallel each other very closely.

While all CMMs exhibited lower than WT k_{cat}/K_Ms, all remain viable enzymes (Table 1). The modest activity changes caused by chemical modification of S156C are consistent with the surface exposed nature of this residue. Notably, a 2.9-fold decrease and a 1.7-fold decrease in k_{cat}/K_M was effected for S156C-$CH_2CH_2SO_3^-$ (-**d**) and for S156C-$CH_2CH_2NH_3^+$ (-**c**), respectively, relative to WT. This contrasts the pH 7.5 conditions, for which both of these CMMs had the same k_{cat}/K_M, indicating that some shielding of electrostatic interactions occurs under the high salt, pH 7.5 conditions. More dramatic changes in specificity were induced by modification of the 166 site. In particular, a 10.5-fold decrease in k_{cat}/K_M compared to WT, resulting from a synergistic decrease in k_{cat} and increase in K_M, was observed for the hydrophobic side chain of S166C-$SCH_2C_6H_5$ (-**b**) and is attributed to the unfavorable steric contacts revealed previously by molecular modelling analysis.[13] However, for the negatively charged side chain CMM, S166C-$SCH_2CH_2SO_3^-$ (-**d**) the k_{cat}/K_M was equally (11-fold) reduced. In this case we conclude that the unfavorable steric interactions revealed by molecular modelling[13] are augmented by the destabilizing effect of the repulsion between the negative charge of the sulfonato group with that of the incipient oxyanion of the transition state.[43] Conversely, the introduction of a positive charge in the S_1 pocket, as for S166C-$SCH_2CH_2NH_3^+$ (-**c**), ameliorated the rate reducing effects, with only a 3-fold sterically induced

Table 1. Kinetic constants for CMMs

	k_{cat} s^{-1a}	K_M mM[a]	k_{cat}/K_M s^{-1} mM^{-1a}	pK_a[c]
WT	153 ± 4	0.73 ± 0.05	209 ± 15 (87 ± 10)[b]	7.01 ± 0.02
156C	125 ± 4	0.85 ± 0.06	147 ± 11 (66 ± 9)[b]	n.d.
156C-S-**a**	84 ± 3	0.84 ± 0.06	100 ± 8 (38 ± 4)[b]	n.d.
156C-S-**b**	72 ± 2	0.59 ± 0.05	122 ± 11 (40 ± 4)[b]	7.07 ± 0.07
156C-S-**c**	90 ± 2	0.73 ± 0.04	123 ± 7 (45 ± 4)[b]	6.90 ± 0.07
156C-S-**d**	87 ± 2	1.2 ± 0.07	74 ± 4 (45 ± 4)[b]	7.49 ± 0.06
166C	42 ± 1	0.5 ± 0.05	84 ± 7 (28 ± 3)[b]	n.d.
166C-S-**a**	46 ± 2	0.34 ± 0.05	135 ± 21 (45 ± 6)[b]	n.d.
166C-S-**b**	23.1 ± 0.5	1.17 ± 0.06	20 ± 1 (9 ± 1)[b]	7.00 ± 0.09
166C-S-**c**	50 ± 1	0.68 ± 0.04	74 ± 5 (27 ± 3)[b]	6.85 ± 0.06
166C-S-**d**	25.0 ± 0.7	1.34 ± 0.08	19 ± 1 (5.4 ± 0.05)[b]	7.93 ± 0.07

[a]Michaelis–Menten constants were measured by the initial rates method in pH 8.6 Tris–HCl buffer at 25 °C with suc-AAPF-pNA as the substrate.
[b]k_{cat}/K_M s^{-1} mM^{-1} measured in pH 7.5 phosphate buffer (0.1 $NaHPO_4$ 0.5 M NaCl) at 25 °C with suc-AAPF-pNA as the substrate. Taken from ref 13.
[c]The observed pK_a was calculated from pH-activity profiles of k_{cat}/K_M,[42] measured in 0.02 M ethylene diamine buffer, ionic strength 0.05 M adjusted with KCl 25 °C. n.d. = not determined.

decrease in k_{cat}/K_M relative to WT being manifest, and no change at all relative to the unmodified cysteine parent S166C.

pH-activity profiles of CMMs

It is often desirable to utilize enzymes under conditions that differ from their optima.[2] Accordingly, in light of our recent demonstration that CMM modifications can induce dramatic shifts in pH-activity profiles,[44] the effect of chemical modifications on the pH-activity profiles of SBL-CMMs were examined further. This approach to altering pH-activity profiles turned out to be quite general in that pK_a changes of up to 0.92 are also observed in the current study. A representative pH-activity profile is illustrated in Figure 2, and the overall pK_a data are summarized in Table 1. In the absence of a change in rate determining step, and with no group in the substrate being ionizable within the pH range of the study, the pH dependence of $(k_{cat}/K_M)_{obs}$ for the serine proteases follows the ionization of the free enzyme.[45–47] The ΔpK_as are observed to vary from −0.16 to +0.92 and are attributed to differences in the ionization of the catalytic His64 in the various CMMs.[48–52] For the uncharged modifications of S156C-$SCH_2C_6H_5$ (-**b**) and S166C-$SCH_2C_6H_5$ (-**b**) the pK_as of 7.07 and 7.00, respectively, are virtually unchanged relative to WT (pK_a = 7.01). This result contrasts our previous observation of dramatic decreases in pK_a induced by the hydrophobic interactions between the phenyl moiety of the N62C-$SCH_2C_6H_5$ CMM, which positions itself within van der Waals contact of the imidazole side chain of the nearby His64.[44] This situation cannot apply here because both the 166 and 156 positions are further removed from His64 and thus no large effect on His64 pK_a was expected. In contrast, introduction of a negative charge, as for S156C-$CH_2CH_2SO_3^-$ (-**d**) and S166C-$CH_2CH_2SO_3^-$ (-**d**), elicited a 0.48 and a 0.92 unit increase in the pK_a of His64, respectively, while introduction of a positive charge as for S156C-$SCH_2CH_2NH_3^+$ (-**e**) and S166C-$SCH_2CH_2NH_3^+$ (-**e**) caused a 0.09 unit and 0.16 unit decrease in the pK_a, respectively. The observed increases in pK_a with increasing negative surface charge, and decreases with increasing positive surface charge, are attributed to stabilization of the imidazolium form of His64 by a negative charge and its destabilization by a positive charge. These results are in accord with previous reports on charge effects[48–52] and also demonstrate that electrostatic interactions are capable of more far reaching influences than hydrophobic or steric ones.[47] The electrostatic effect of modification is more pronounced for the 166 than for the 156 site due to the different environments of the side chains. Since the 156 residue is more surface exposed and thus more readily solvated, the impact of the positively and negatively charged side chains on the active site are reduced. In contrast, the more buried nature of the S166C CMM side chains provides a protected environment whose influence on the enzyme active site residues is not diluted by solvation.

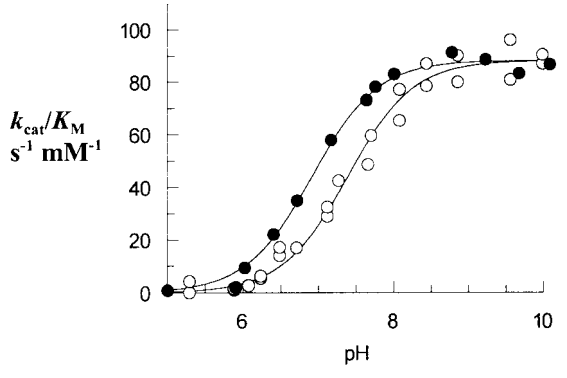


Figure 2. pH-Activity profile for WT-SBL (●, $pK_a = 7.01 \pm 0.02$) and the S156C-S-**d** CMM (○, $pK_a = 7.49 \pm 0.06$).

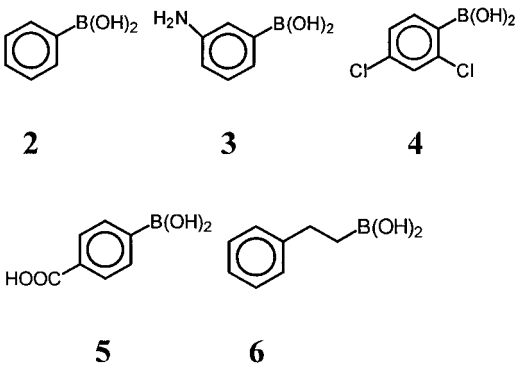
Boronic acid inhibition of CMMs

As a further probe of the S_1 pocket specificity changes induced by SBL-CMMs the altered binding of the representative structural range of transition state analogue boronic acid inhibitors[38,53–58] (Formulae 2–6) was evaluated. The observed K_Is revealed significant specificity changes from WT, as summarized in Table 2.

Table 2. Inhibition constants[a] for CMMs

Enzyme/Inhibitor	**2** K_I mM	**3** K_I mM	**4** K_I mM	**5** K_I mM	**6** K_I mM
WT	1.3 ± 0.1	1.3 ± 0.1	0.15 ± 0.01	8.4 ± 0.6	1.07 ± 0.08
156C	0.94 ± 0.07	1.09 ± 0.08	0.18 ± 0.01	7.1 ± 0.6	1.08 ± 0.08
156C-S-**a**	0.79 ± 0.06	1.4 ± 0.1	0.18 ± 0.01	5.4 ± 0.4	1.13 ± 0.08
156C-S-**b**	1.16 ± 0.09	1.19 ± 0.09	0.22 ± 0.02	2.4 ± 0.2	1.3 ± 0.1
156C-S-**c**	2.3 ± 0.1	1.8 ± 0.1	0.15 ± 0.01	8.1 ± 0.5	1.26 ± 0.07
156C-S-**d**	1.6 ± 0.1	2 ± 0.1	0.26 ± 0.02	6 ± 0.4	1.60 ± 0.1
166C	0.38 ± 0.04	0.42 ± 0.04	0.18 ± 0.2	9.0 ± 0.9	1.9 ± 0.2
166C-S-**a**	3.8 ± 0.6	0.42 ± 0.06	0.22 ± 0.03	4.6 ± 0.7	2.6 ± 0.5
166C-S-**b**	2.7 ± 0.2	4.3 ± 0.3	1.8 ± 0.1	65 ± 4	9.7 ± 0.5
166C-S-**c**	0.57 ± 0.04	0.78 ± 0.05	0.3 ± 0.02	4.52 ± 0.03	1.4 ± 0.1
166C-S-**d**	0.96 ± 0.06	2.3 ± 0.2	0.72 ± 0.05	18 ± 1	3.7 ± 0.2

[a]Inhibition constants were determined in pH 8.6 Tris–HCl buffer at 25 °C with suc-AAPF-pNA as the substrate by the method of Waley.[59]



These binding differences are more clearly revealed in Figure 3. The influence of the surface exposed 156 site is further reinforced since, as illustrated in Figure 3(a), despite the widely varying structures of the boronic acids (**2–6**) evaluated only small changes in K_Is were observed. The greatest of these was a 3.5-fold improvement in the binding of 4-carboxyphenyl boronic acid (**5**) to S156C-$SCH_2C_6H_5$ (-**b**). This indicated that some electrostatic interaction between the carboxylate group of **5** and the S_1 pocket of S156C-$SCH_2C_6H_5$ was operating.

As illustrated in Figure 3(b), more dramatic changes in boronic acid binding were observed for the S166C CMMs, most notably for S166C-$SCH_2C_6H_5$ (-**b**). For this CMM, poorer binding of each of the boronic acids **2–6** was observed, with the largest effect being a 12-fold increase over WT in the K_I of 2,4-dichlorophenyl boronic acid (**4**) with S166-$SCH_2C_6H_5$ (-**b**). As expected, phenethyl boronic acid (**6**), the inhibitor with the largest P_1 group of the series, binds best to WT-SBL, which has the biggest S_1 pocket. That the K_I of 4-carboxyphenyl boronic acid (**5**) with S166C-$SCH_2CH_2NH_3^+$ (-**c**) is 2-fold lower than for WT is attributed to the improved interaction between the positively charged ammonium side chain of S166C-S-**c** and the negatively charged carboxylate moiety of **5**. In further support of this interpretation, 4-carboxyphenyl boronic acid (**5**), with its negatively charged carboxylate group, binds more poorly to S166C-$SCH_2CH_2SO_3^-$ (-**c**) than to WT, due to electrostatic repulsion.

Support for the hypothesis of an electrostatic interaction between the carboxylate group of **5** and the S_1 pocket of S156C-$SCH_2C_6H_5$ was sought by molecular modelling. Molecular modelling revealed an additional hydrogen bond between the carboxylate of *para*-carboxyphenyl boronic acid (**5**) and the Asn155 amide backbone N−H of S156C-S-**b**, which is not present in the WT (Fig. 4). This additional hydrogen bond appears to have been gained at the expense of a His64 $HN_{\varepsilon 3}$ hydrogen bond with a boronic acid hydroxyl oxygen which is present in the minimized structure of the WT.

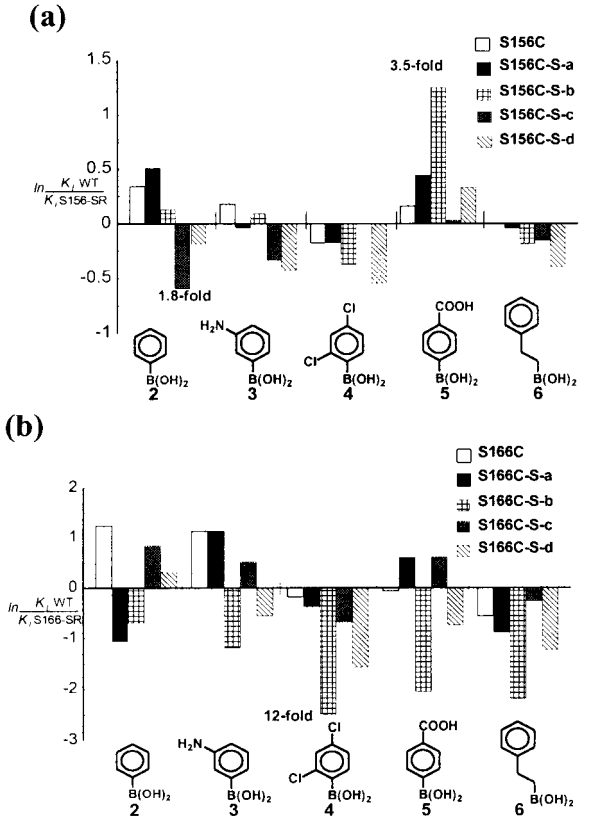


Figure 3. Relative binding of boronic acids inhibitors **2–6** to (a) S156C CMMs and (b) S166C CMMs compared to WT. Stronger binding of the boronic acids to the WT is shown as a bar below the X-axis and stronger binding of the boronic acids to the CMM as a bar above the X-axis.

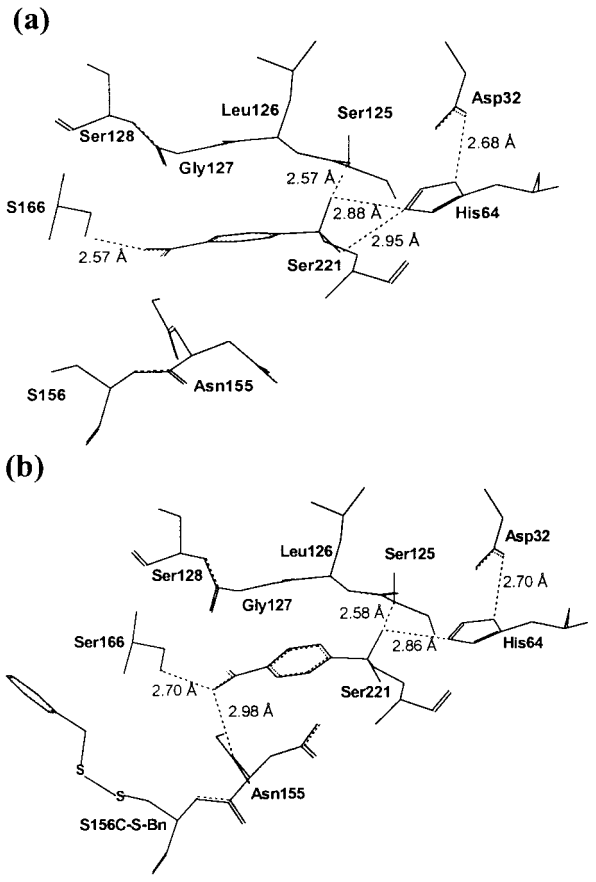


Figure 4. (a) Minimized structure of the covalent complex of *para*-carboxyphenyl boronic acid (**5**) covalently linked to the Ser221 Oγ oxygen of WT-SBL showing H-bonds between: Asp32 carboxylate and His64 $HN_{\delta 2}$ (2.68 Å), His64 $HN_{\varepsilon 3}$ and boronic acid hydroxyl oxygens (2.95 and 2.88 Å), Ser125 carbonyl oxygen and boronic acid hydroxyl oxygen (2.57 Å), Ser166 Oγ hydroxyl and carboxylate of **5** (2.57 Å). (b) Minimized structure of the covalent complex of *para*-carboxyphenyl boronic acid (**5**) covalently linked to the Ser221 Oγ oxygen of S156C-S-**b** showing H-bonds between: Asp32 carboxylate and His64 $HN_{\delta 2}$ (2.70 Å), His64 $HN_{\varepsilon 3}$ and boronic acid hydroxyl oxygen (2.86 Å), Ser125 carbonyl oxygen and boronic acid hydroxyl oxygen (2.58 Å), Ser166 Oγ hydroxyl and carboxylate of **5** (2.70 Å), Asn155 backbone NH and carboxylate of **5** (2.98 Å). Molecular modelling confirms the presence of a favorable interaction between the carboxylate of *para*-carboxyphenyl boronic acid (**5**) and the S_1 pocket of S156C-S-**b**. However, the additional hydrogen bond between the Asn155 backbone NH and carboxylate of **5** was gained at the expense of a His64 $HN_{\varepsilon 3}$ hydrogen bond with the boronic acid hydroxyl oxygen which was present in the minimized structure of the WT.

These results demonstrate that using a screen of different specificity and catalytic property approaches in combination, such as kinetic, pH-activity profiles etc., provide complementary insights into the effect of site-directed chemical modifications of SBL, which taken together are more powerful synergistic tools than independently.

Experimental

Preparation of methanethiosulfonate regents

Reagent **1a** was purchased from Aldrich Chemical Co. Inc., **1c** and **1d** from Toronto Research Chemicals (2 Brisbane Rd. Toronto, ON), and all were used as received. Reagent **1b** was prepared as previously described via the nucleophilic displacement of benzyl bromide by sodium methanethiosulfonate.[12,19,60]

Boronic acids

Boronic acids, **2** and **3** were purchased from Aldrich Chemical Co. Inc., **4** from Lancaster Synthesis Inc. (Windham, NH) and used as received. Boronic acids **5** and **6** were obtained as previously described.[38,55]

Preparation and characterization of CMMs

The chemically modified mutants of SBL were prepared and purified as described previously.[13] Briefly each of S156C or S166C (25 mg) was exposed to each of the methanethiosulfonate reagents **1a–1d** in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) at 20 °C. These were purified by ion exchange chromatography (Pharmacia Biotech PD-10, Sephadex G-25 M). The free thiol content of each of the CMMs was determined by titration with Ellman's[41] reagent and established that the free thiol content of all CMMs was less than 2%, demonstrating completeness of reaction. The purity of each of the CMMs was analyzed by non-denaturing gradient (8–25%) gels run at pH 4.2 run towards the cathode, on the Pharmacia Phast-System® and each appeared as a single band.[13] In addition, ES-MS spectral analysis of the CMMs revealed that their determined masses were found to be in agreement (±6 Da) of the calculated.[13] The active enzyme concentration was determined as previously described[61] 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.

Determination of kinetic constants

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 suc-AAPF-pNA (Bachem, California Inc.) substrate in pH 8.6, 0.1 M Tris-HCl containing 0.005% Tween 80, and 1% DMSO, ($\varepsilon_{410} = 8800\ M^{-1}\,cm^{-1}$),[62] $[E]_f = 6.33\times10^{-9}$ to 1.7×1^{-7}, as described previously.[13]

pH-activity profiles

pH-activity profiles for the subtilisin CMMs were constructed by monitoring product release via its colorometric absorbance at 410 versus time at 25 °C ($\varepsilon = 8800\ cm^{-1}\,M^{-1}$)[62] as described previously.[6] k_{cat}/K_M values were determined in duplicate in 0.02 M ethylenediamine buffer, ionic strength 0.05 M adjusted with KCl, $[S]_f = 4.2-12.5\times10^{-5}$ M of suc-AAPF-pNA, $[E]_f = 2.53$ to 21.7×10^{-8} M at 25 °C. pK_a's 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 ($4.2–12.5\times10^{-3}$ 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 ($2.53–21.7\times10^{-6}$ M in pH 5.8, 20 mM MES, 1 mM $CaCl_2$) 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: $v = k_{cat}/K_M[E]_o[S]$ when $[S] \ll K_M$.[44]

Determination of boronic acid inhibition constants

Boronic acid K_Is were determined in duplicate by the method of Waley.[59] The progress curve without inhibitor was determined, from an assay mixture containing 980 μL of buffer (0.1 M Tris–HCl, 0.005% Tween 80) and 10 μL of suc-AAPF-pNA substrate (25 mM in DMSO). This mixture was incubated in a water jacketed cell for 5 min at 25 °C. The absorbance reading was set to zero prior to initiating the reaction by addition of 10 μL of enzyme solution (3.2×10^{-6} to 1.1×10^{-4} M in pH 5.8, 20 mM MES, 1 mM $CaCl_2$). The final volume of the assay mixture was 1 mL. The progress curve with inhibitor was determined, similarly but with 10 to 980 μL of the boronic acid inhibitor solution (1×10^{-3} to 0.1 M, in 0.1 M Tris–HCl, 0.005% Tween 80) added to a final volume of 1 mL. Absorbance versus time measurements were recorded on a Perkin–Elmer lambda 2 spectrophotometer and points for calculation were taken at 15, 18, 21, 24, 27, 30, 33, 33 and 36% substrate conversion as described previously.[63]

Molecular modelling analysis

Energy simulations were performed with the Discover program version 2.9.5[64] on a Silicon Graphics Indigo computer, using the consistent valence force field function (CVFF). The X-ray structure of subtilisin *B. lentus* with the peptide inhibitor AAPF bound was used as the starting point for calculations.[39] To create initial coordinates for the minimization, hydrogens were added at the pH 8.6 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 His 64 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 +4 for the WT enzyme. A non-bonded cutoff distance of 18 Å with a switching distance of 2 Å was employed. The non-bonded 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, until the maximum derivative of 0.1 kcal mol^{-1} Å^{-1} was reached. The S156C-S-**b** enzyme was generated by modifying the relevant amino acid using the Builder module of Insight.[65] This structure was then minimized in a similar manner. Initially the side-chain of the mutated residue and the water molecules were minimized, followed by water molecules and the amino acid side chains, and then finally the entire enzyme. The AAPF inhibitor was free to move throughout all stages of the minimization. The AAPF inhibitor was deleted and then the minimized enzyme structure was used as the starting point for calculation of the boronic acid–enzyme complex. In the calculation of the boronic acid–enzyme complex, a tetrahedral carbon atom was used to mimic the boron atom since, as yet, no force field parameters are available for boron. The boron atom equivalent of the inhibitor was covalently bound to the Oγ of Ser221. The inhibitor was docked such that its phenyl moiety was directed into the S_1 pocket, one of the hydroxyl groups was directed to Asn155 of the oxyanion hole and the other toward His64. This structure was then minimized as above.

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