Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 2293–2301

BIOORGANIC & MEDICINAL CHEMISTRY

The Controlled Introduction of Multiple Negative Charge at Single Amino Acid Sites in Subtilisin *Bacillus lentus*

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Received 25 February 1999; accepted 6 April 1999

Abstract—The use of methanethiosulfonates as thiol-specific modifying reagents in the strategy of combined site-directed mutagenesis and chemical modification allows virtually unlimited opportunities for creating new protein surface environments. As a consequence of our interest in electrostatic manipulation as a means of tailoring enzyme activity and specificity, we have adopted this approach for the controlled incorporation of multiple negative charges at single sites in the representative serine protease, subtilisin *Bacillus lentus* (SBL). A series of mono-, di- and triacidic acid methanethiosulfonates were synthesized and used to modify cysteine mutants of SBL at positions 62 in the S_2 site, 156 and 166 in the S_1 site and 217 in the S_1' site. Kinetic parameters for these chemically modified mutant (CMM) enzymes were determined at pH 8.6 under conditions which ensured complete ionization of the unnatural amino acid side-chains introduced. The presence of up to three negative charges in the S_1, S_1' and S_2 subsites of SBL resulted in up to 11-fold lowered activity, possibly due to interference with oxyanion stabilization of the transition state of the hydrolytic reactions catalyzed. Each unit increase in negative charge resulted in a raising of K_M and a reduction of k_{cat}. However, no upper limit was observed for increases in K_M, whereas decreases in k_{cat} reached a limiting value. Comparison with sterically similar but uncharged CMMs revealed that electrostatic effects of negative charges at positions 62, 156 and 217 are detrimental, but are beneficial at position 166. These results indicate that the ground-state binding of SBL to the standard substrate, Suc-AAPF-*p*NA, to SBL is reduced, but without drastic attenuation of catalytic efficiency, and show that SBL tolerates high levels of charge at single sites.

Introduction

The introduction of charge into wild-type (WT) enzymes, through random[1–3] or site-directed[4–16] mutagenesis or chemical modification,[17,18] can be used to broaden substrate specificity or to enhance catalytic activity. The successful tailoring of specificity towards charged substrates has confirmed the validity of exploiting the electrostatic attraction between complementary ions as a viable strategy for improving binding. For example, the introduction of negative charge into the active sites of subtilisin BPN′,[4] aspartate aminotransferase[6] and L-lactate dehydrogenase[11] has expanded their structural specificities towards substrates with positively charged side chains.

Introductions of charge by protein engineering have typically been limited to naturally occurring amino acids such as negatively charged aspartate[4,6,9,11–16] or positively charged arginine[4,7,9] or lysine.[4,5,9,12] Chemical modification allows a greater variety of charged groups to be introduced, but the reactions used for their introduction are often non-specific in nature.[19] For example, the reaction of cyclic anhydrides with trypsin caused indiscriminate modification of lysine residues, the effects of which could only be interpreted in general terms.[17] Furthermore, although ingenious molecular biological techniques have been developed for the introduction of non-natural charged amino acids into proteins,[20–22] they are not yet suitable for large-scale synthesis or routine application. In addition, the use of charged amino acids in such techniques can result in poor levels of incorporation.[20,21]

The use of site-directed mutagenesis combined with chemical modification[23–28] of single sites offers a potential solution to these problems. In this technique cysteine is introduced at preselected positions and its

Key words: Mutagenesis; electrostatic effects; enzymes and enzyme reactions; kinetics.
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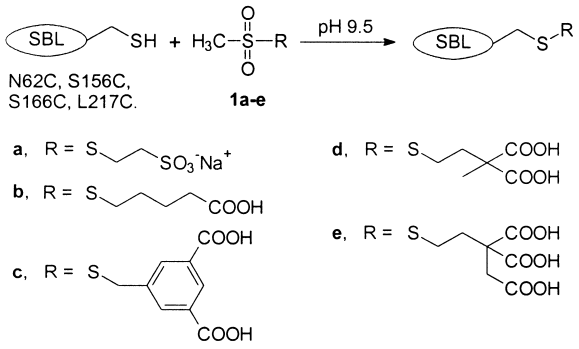
PII: S0968-0896(99)00167-4



Scheme 1.

thiol residue is then reacted with methanethiosulfonate (MTS) reagents (Scheme 1). Methanethiosulfonate reagents react specifically and quantitatively with thiols.[29,30] Recently, using the serine protease subtilisin *Bacillus lentus* (SBL) as our model, we have used this technique to improve enzyme activity[31] and alter specificity.[32,33] SBL is a near-ideal enzyme for evaluating the validity of this strategy since its does not contain a natural cysteine. In the current work we describe the use of this method to introduce locally high charge density into SBL through the incorporation of single residues bearing multiple charges.

Using the X-ray structure of SBL[34] (Fig. 1) as our guide, four sites were chosen for mutation because of their seminal positions in the active site. Two of these, N62 (subtilisin BPN′ numbering) and L217, occupy positions that are equidistant from S221 of the catalytic triad, in the S_2[35] and S1′ pockets, respectively. The other two sites, S156 and S166, are located at the base of the S_1 pocket and their side chains are directed towards SBL's surface and catalytic triad, respectively. The MTS reagents **1a**–**e** were selected to modify these positions.

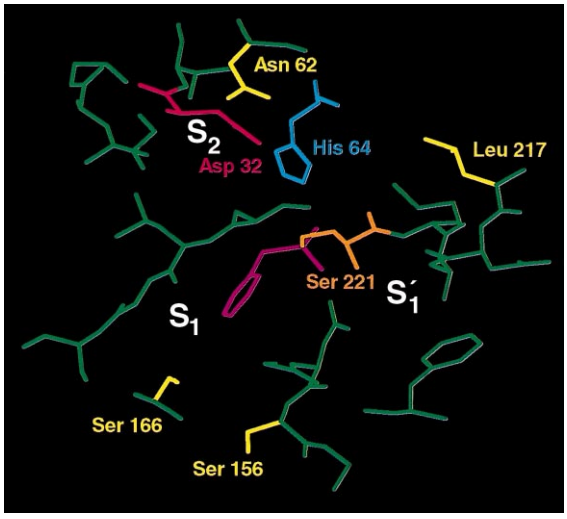


Figure 1. The active site of SBL showing the residues of the catalytic triad, Ser221 (orange), His64 (blue) and Asp32 (red). The irreversible phenylmethylsulfonyl inhibitor (pink) forms a bond to the Oγ atom of Ser221 and its phenyl ring occupies the S1 binding site. The residues chosen for mutation, Asn62 in the S_2 site, Ser156 and Ser166 in the S_1 site and Leu217 in the S_1′′ site are highlighted in yellow.[34]

Results and Discussion

Synthesis of carboxyalkyl methanethiosulfonates 1b–e

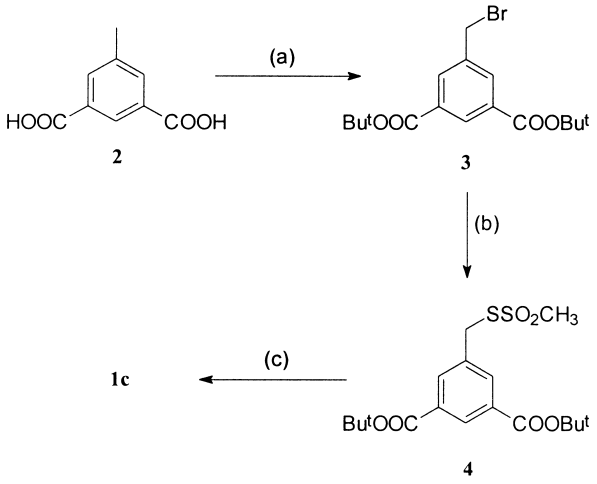
Previous work[29,31] has demonstrated that, of the methods available,[36–41] direct nucleophilic displacement of a primary alkyl bromide by methanethiosulfonate ion provides the most efficient method for the preparation of alkyl methanethiosulfonates. This general method was therefore adopted as the basis for the preparation of all of **1b**–**e**. The aliphatic monocarboxylate MTS **1b**[42] was prepared from 5-bromopentanoic acid and $NaSSO_2CH_3$ in 80% yield.

The aromatic dicarboxylate MTS **1c** was prepared from toluene-3,5-dicarboxylic acid (**2**) via a precursor benzylic bromide **3** as shown in Scheme 2. Treatment of **3** with $NaSSO_2CH_3$ gave the protected aromatic MTS **4** in 60% yield. Hydrolysis of **4** with TFA gave **1c** as a white solid (91% yield, 44% overall yield from **2**).

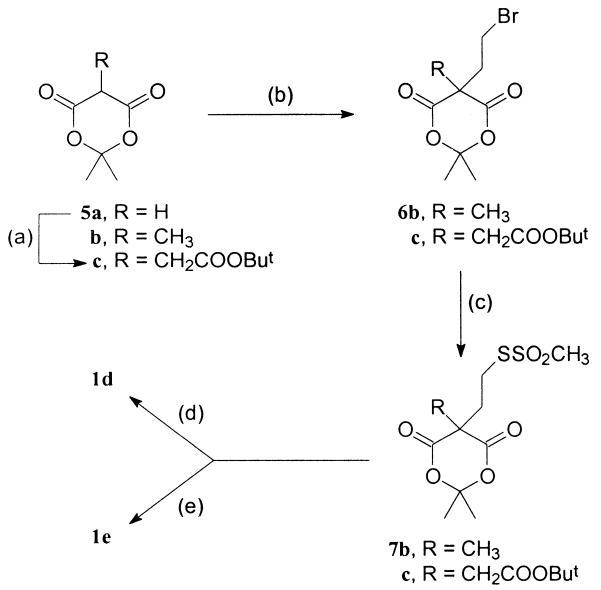
The aliphatic di- and tricarboxylates **1d,e** were prepared from Meldrum's acid (**5a**) using 1,2-dibromoethane to introduce a brominated linker group as shown in Scheme 3. The low pK_a of **5a**[43,44] allowed the use of mildly basic conditions compatible with this choice of linker. For the sake of simplicity, we chose methyl Meldrum's acid (**5b**) as a starting material for **1d** in which one alkylation site is blocked as a methyl group.[45] The synthesis of **1e** utilized this position to introduce a third carboxylate moiety.

Alkylation of **5b** with 1,2-dibromoethane afforded bromide **6b** in 71% yield. Treatment of **6b** with $NaSSO_2CH_3$ in DMF at 50°C led to the displacement of the remaining bromide and resulted in the formation of protected dicarboxylate MTS **7b**. Hydrolysis of **7b** using acidic ion exchange resin allowed the successful formation of the aliphatic diacidic MTS **1d** (79% yield, 37% overall yield from **5b**).

The synthesis of the triacidic reagent **1e** required the construction of a protected tricarboxylate **5c** before



Scheme 2. (a) (i) Im_2CO, DMF, 40°C then DBU, *t*-BuOH, 84%, (ii) NBS, azobis(cyclohexanecarbonitrile), CCl_4, Δ, 96%; (b) $NaSSO_2CH_3$, DMF, 50°C, 60%; (c) CF_3COOH, CH_2Cl_2, 91%.



Scheme 3. (a) K_2CO_3, DMF then $BrCH_2COOBu^t$, 59%; (b) K_2CO_3, DMF then $Br(CH_2)_2Br$, 71% for **6b**, 66% for **6c**; (c) $NaSSO_2CH_3$, DMF, 50°C, 83% for **7b**, 86% for **7c**; (d) Dowex 50W(H^+), *p*-dioxane, H_2O, 79%; (e) CF_3COOD, D_2O, 50°C, 70%.

elaboration. Alkylation of Meldrum's acid (**5a**) with *tert*-butyl bromoacetate allowed the formation of **5c** with moderate selectivity in 59% yield. Elaboration of tricarboxylate **5c** was carried out using 1,2-dibromoethane and then $NaSSO_2CH_3$ in an analogous manner to that used for the synthesis of **1d** and gave protected tricarboxylate MTS **7c** in 57% yield over 2 steps. Complete deprotection of **7c** using CF_3COOD in D_2O was followed by ^1H NMR, and resulted in the formation of target **1e** (70% yield, 23% overall yield from **5a**).

Preparation of chemically modified mutants (CMMs)

MTS reagents **1a–e** were used to modify the chosen SBL cysteine mutants, N62C, S156C, S166C and L217C under conditions described previously.[31–33] These reactions proceeded rapidly and quantitatively, as judged by the monitoring of changes in specific activity and by titration of residual free thiols with Ellman's reagent,[46] respectively. The structure of the charged CMMs was confirmed by ES–MS. Non-reducing native PAGE was used to determine the purity of all the enzymes, which appeared as single bands. Consistent with the introduction of negative charge, each of the CMMs showed retarded mobility in the direction of the cathode relative to WT. The active enzyme concentration of the resulting CMM solutions was determined by active site titration with α-toluenesulfonyl fluoride (PMSF) using a fluoride ion-sensitive electrode.[47]

Kinetic effects of site specific modification

The effects of modification upon SBL were assessed by the determination of k_{cat} and K_M for the hydrolysis of succinyl-AAPF-*p*-nitroanilide (Suc-AAPF-*p*NA). Our usual assay pH of 8.6 ensured complete ionization of the unnatural amino acid side-chains introduced. The kinetic parameters of the 20 CMMs generated are compared with those of WT and unmodified mutants in Table 1 and Figure 2.

Table 1. Kinetic parameters[a] for modified enzymes

Entry	Enzyme	Pocket	R	Level of charge	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
1	SBL-WT	—	—	—	153 ± 4	0.73 ± 0.05	209 ± 15
2	N62C	S_2	H	0	174 ± 9	1.90 ± 0.20	92 ± 11
3			**a**	1	119 ± 4	0.93 ± 0.07	128 ± 11
4			**b**	1	106 ± 2	1.01 ± 0.05	105 ± 6
5			**c**	2	113 ± 7	1.00 ± 0.10	113 ± 13
6			**d**	2	90 ± 4	1.47 ± 0.14	61 ± 6
7			**e**	3	129 ± 3	1.46 ± 0.06	88 ± 4
8	L217C	S_1'	H	0	41 ± 1	0.80 ± 0.04	51 ± 3
9			**a**	1	89 ± 6	1.80 ± 0.20	50 ± 6
10			**b**	1	54 ± 1	1.03 ± 0.06	52 ± 3
11			**c**	2	69 ± 2	0.81 ± 0.06	85 ± 7
12			**d**	2	63 ± 2	1.65 ± 0.11	38 ± 3
13			**e**	3	55 ± 2	1.48 ± 0.08	37 ± 3
14	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
15			**a**	1	87 ± 2	1.20 ± 0.07	73 ± 5
16			**b**	1	76 ± 1	1.08 ± 0.04	70 ± 3
17			**c**	2	61 ± 1	1.39 ± 0.10	44 ± 3
18			**d**	2	53 ± 1	1.67 ± 0.06	32 ± 1
19			**e**	3	74 ± 2	1.87 ± 0.08	39 ± 2
20	S166C		H	0	42 ± 1	0.50 ± 0.05	84 ± 9
21			**a**	1	25 ± 1	1.34 ± 0.08	19 ± 1
22			**b**	1	48 ± 1[b]	1.52 ± 0.06	31 ± 1[b]
23			**c**	2	47 ± 3	1.60 ± 0.20	29 ± 4
24			**d**	2	67 ± 2	2.26 ± 0.10	30 ± 2
25			**e**	3	76 ± 2	2.46 ± 0.11	31 ± 2

[a] Michaelis–Menten constants were measured at 25°C according to the initial rates method in 0.1 M Tris–HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-*p*NA as the substrate.
[b] Based on total protein concentration.[48]

Position 62 in the S_2 pocket is the most tolerant of mutation and modification (Fig. 2(a)) and mutation to cysteine reduces k_{cat}/K_M by only a factor of 2 (Table 1, Entry 2). Upon modification, activity is partially restored and values of k_{cat}/K_M for N62C-**a,b,c** are raised approximately 1.5-fold relative to N62C (Table 1, entries 3–5). Modifications with aliphatic di- and tricarboxylate MTS reagents **1d,e** elicit further drops in k_{cat}/K_M, with N62C-**d** being 3.5-fold lower than WT. However, despite the increased charge, this k_{cat}/K_M drop is less marked for N62C-**e**.

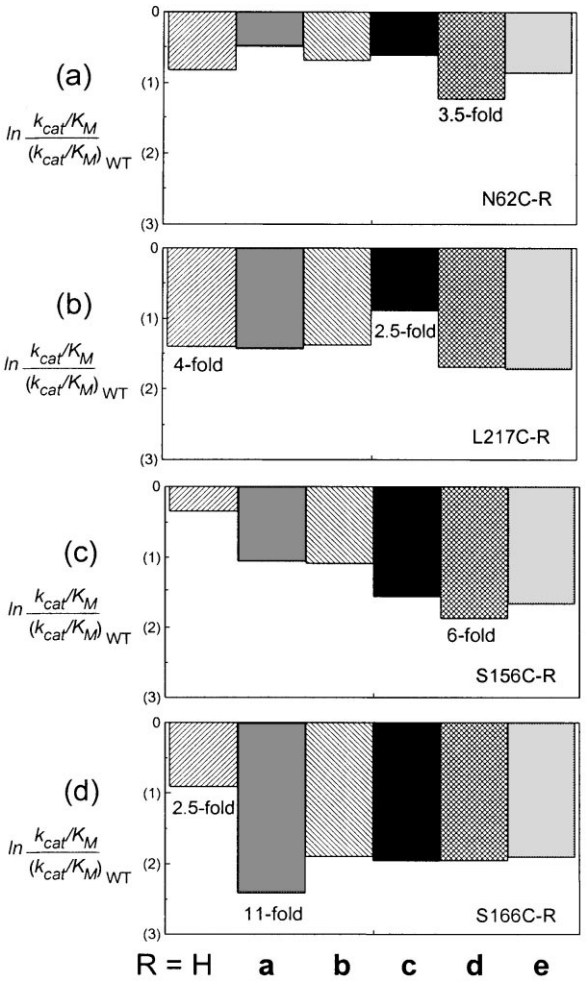


Figure 2. Altered specificity patterns relative to WT as the level of negative charge increases in N62C, L271C, S156C and S166C mutants and CMMs with suc-AAPF-*p*NA as the substrate: (a) The k_{cat}/K_Ms for N62C CMMs alternate at moderately reduced levels, 1.5- to 3.5-fold lower than WT, which are established by the initial mutation to N62C (R=H). (b) L217C CMMs show steady but lower levels of k_{cat}/K_M, 4- to 5.5-fold lower than WT, which are again established by the initial mutation to cysteine. The exception is L217C-**c** which is only 2.5-fold lower than WT, possibly due to favourable binding of substrate to the phenyl ring of the aromatic side chain introduced by modification. (c) From the small reduction caused by mutation to S156C (R=H), k_{cat}/K_Ms decrease monotonically to 6-fold lower than WT for S156C-**d**. The k_{cat}/K_M of S156C-**e** is partially restored. (d) k_{cat}/K_M decreases only 2.5-fold upon mutation to S166C (R=H) but decreases dramatically to 11-fold lower than WT when the negatively charged sulfonatoethyl side chain **a** is introduced. In parallel to N62C and L217C CMMs, k_{cat}/K_M for S166C CMMs does not decrease further to any significant extent as the level of negative charge increases.

The deleterious effect of negative charges in the S_2 pocket upon k_{cat} is apparent in the 1.3-fold decrease observed for N62C-**a** (Table 1, entry 3) relative to WT. However, as the level of negative charge increases, k_{cat} values do not decrease further to any significant extent. In fact, of all the CMMs, the k_{cat} level of N62C-**e** (129 s^{-1}, Table 1, Entry 7) is the highest. In contrast, K_M values increase continually with each additional charge, reaching values for N62C-**d** (Table 1, Entry 6) and N62C-**e** (Table 1, Entry 7) that are 2-fold higher than WT.

The effects of mutation at position 217 in the S_1' pocket (Fig. 2(b)) are intrinsically more dramatic than at all three other sites since the value of k_{cat}/K_M for L217C is 4-fold lower than WT. The introduction of a single negative charge only affects k_{cat}/K_M a little and leads to near-identical k_{cat}/K_M values for L217C-**a,b** (Table 1, Entries 9,10). As negative charge increases further, two opposite trends are observed, with the k_{cat}/K_M value for aromatic L217C-**c** being raised 1.6-fold relative to L217C-**a,b,** while those for aliphatic L217C-**d,e** are reduced by 1.4-fold.

These slight k_{cat}/K_M changes seen at position 217 are the result of larger, but counteracting changes in each of k_{cat} and K_M. For example, while L217C-**a** has the highest value of k_{cat}, it also has the highest K_M value (both 2.2-fold higher than L217C). As at position 62, when the level of negative charge increases, from L217C-**a** to L217C-**e**, k_{cat} values decrease only slightly and remain 1.3- to 1.7-fold higher than L217C (Table 1, entries 10–13). K_M values increase unevenly to 2-fold higher than WT. Interestingly, the underlying cause of the out-of-line k_{cat}/K_M of L217C-**c** is an unusually low K_M (0.81 mM, entry 11), which may be a consequence of complementary aromatic interactions between the substrate and the phenyl ring of side chain **c**.

The effects of mutation and modification at positions 156 and 166 in the S_1 pocket are shown in Figure 2(c) and (d). Mutation at position 156 to cysteine causes a 1.4-fold drop in k_{cat}/K_M (S156C, Table 1, entry 14). From S156C-**a** to S156C-**d** k_{cat}/K_M decreases monotonically to a value that is 6-fold lower than WT. The additional negative charge present in S156C-**e** partially restores this value, to only 5.4-fold lower than WT.

Mutation and modification at position 166 leads to the least active negatively charged CMMs, with k_{cat}/K_Ms 6 to 11-fold lower than WT. This partly reflects the intrinsically lower k_{cat}/K_M value of the unmodified mutant S166C, which is already 2.5-fold lower than WT. However, the presence of the sulfonatoethyl side chain in S166C-**a** causes a dramatic drop to a value that is 11-fold lower than WT. k_{cat}/K_M is increased 1.5-fold for S166C-**b** and remains steady as the level of negative charge increases from S166C-**c** to S166C-**e**.

The k_{cat} values for S156C and S166C CMMs are similar to those found for L217C CMMs, typically 2 to

2.5-fold lower than WT. As at positions 62 and 217, the detrimental effect of a single negative charge on k_{cat} is not amplified by the introduction of additional negative charges. In fact, k_{cat} values for S166C CMMs increase steadily from 6-fold lower than WT for S166C-**a** (Table 1, entry 21) to 2-fold lower than WT for S166C-**e** (Table 1, entry 25).

The K_M values for both S156C and S166C CMMs increase steadily with increasing negative charge and are largest for S166C-**e** (K_M 2.46 mM, 3.5-fold higher than WT, Table 1, entry 25). Consistent with its surface-exposed nature these effects are less pronounced at position 156, with K_M increasing to only 2.5-fold higher than WT for S156C-**e** (Table 1, entry 19).

Kinetic effects of negative charge

To separate the contribution of electrostatics from steric effects, a comparison of these charged CMMs with those containing sterically similar uncharged side chains[49] was made. For example, N62C-**a** was compared with N62C-*S*-ethyl, N62C-**b,d,e** were compared with N62C-*S*-*n*-pentyl and N62C-**c** was compared with N62C-*S*-benzyl. Figure 3 illustrates the results of introducing charge to these near-isosteric systems. This provides an estimate of the effect of negative charge upon the kinetics of SBL when corrected for underlying steric and hydrophobic effects.

Two differing trends emerge from Figure 3. At positions 62, 217 and 156, the electrostatic contribution of each of side chains **a**–**e** is detrimental to k_{cat}/K_M. The reductions caused are similar for each side chain, vary little from site to site and increase with the level of negative charge introduced. These reduced k_{cat}/K_M values resulting from the introduction of negative charge are consistent with earlier findings.[12,31–33] Such effects may be attributed, in part, to destabilization of the tetrahedral oxyanion intermediate that is formed in the rate limiting step of catalysis.[50]

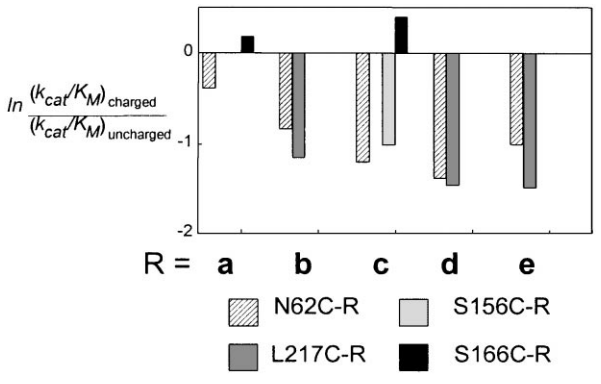


Figure 3. The effects of introducing negative charge to CMMs: ln (k_{cat}/K_M), with suc-AAPF-*p*NA as the substrate, of the negatively charged N62C, L217C and S156C CMMs decreases relative to that of near-isosteric uncharged CMMs as the level of negative charge increases (from side chain **a** to **e**). In contrast, this value for the corresponding S166C CMMs increases with increasing negative charge.

In contrast, the introduction of negative charge at position 166 partially restores some of the activity lost through the introduction of near-isosteric uncharged side chains. Therefore, the drastically lowered k_{cat}/K_Ms of CMMs S166C-**a**–**e** relative to WT are, in fact, a result of steric or hydrophobic effects. Mutation analysis of subtilisin BPN′ has shown that k_{cat}/K_M decreases dramatically when the optimal binding volume of the S_1-pocket is exceeded.[51] The effect of introducing even small groups at position 166 of SBL[52] is to fill the S_1-pocket and this dramatically decreases k_{cat}/K_M. For example, uncharged CMM S166C-*S*-ethyl has a k_{cat}/K_M 13.5-fold lower than WT.[49] Molecular mechanics analysis of S166C CMMs has shown that charged side chains introduced at position 166 may orientate themselves towards external solvent.[33] This serves to reduce the volume of the S_1 pocket that is occupied by the side chain. The existence of such an orientation for S166C-**a**–**e**, which is lacking in uncharged CMM counterparts, might, in part, explain the beneficial effects of introducing charge. As a result, charged CMM S166C-*S*-EtSO3$^-$, side chain **a**, has a k_{cat}/K_M only 11-fold lower than WT.

Conclusions

In summary, we have devised short and efficient synthetic routes to three novel multiply charged methanethiosulfonates, **1c, d** and **e**. Such compounds, as well as being of interest in our approach to the controlled tailoring of enzyme activity, may prove useful in the study of ion channels. The use of MTS reagents in techniques such as the substituted-cysteine accessibility method (SCAM)[53–55] has allowed aspects of membrane ion channel topology and conformation to be determined. In particular the use of charged MTS reagents has given an invaluable insight into ion specificity[56] and mechanism of action.[57–61]

Using our established methodology, we selectively modified the cysteine thiols of SBL mutants, N62C, S156C, S166C, and L217C, with these reagents. Without exception, mutation and modification at all four sites led to reduced catalytic efficiency in the hydrolysis of Suc-AAPF-*p*NA. However these reductions do not exceed 11-fold relative to WT and the lowest k_{cat} values determined were only 6-fold reduced. This reduced efficiency is manifested largely through decreased binding interactions, i.e. decreased K_M values, that increase with the level of charge introduced. In contrast, k_{cat} values corresponding to the introduction of multiple charge are similar to, if not higher than, those for single charge. Comparison with near-isosteric uncharged CMMs revealed that electrostatic effects are important at positions 62, 217 and 156. However at position 166 steric effects dominate and the introduction of negative charge is, in fact, beneficial.

The hydrolysis of different substrates, including those containing basic residues, and pH-activity profiles, are being investigated. The pK_a effects and specificity consequences will be presented in due course.

Experimental

Mutants of subtilisin *Bacillus lentus* (SBL) were generated, and WT and mutant enzymes purified as described previously.[32,33] $NaSSO_2CH_3$ (mp 269–269.5°C (dec.) (lit.,[29] mp 272–273.5°C)) and toluene-3,5-dicarboxylic acid (**2**)[62] (mp 294.5–296 °C (water) (lit.,[62] mp 287–288°C)) were prepared according to literature methods. DMF was distilled under N_2 from CaH_2 and stored over molecular sieves under N_2 before use. CCl_4 was fractionally distilled before use. Sulfonatoethyl methanethiosulfonate (**1a**) was purchased from Toronto Research Chemicals (2 Brisbane Rd., Toronto, ON, Canada). All other chemicals were used as received from Sigma-Aldrich or Baker. All flash chromatography was performed using silica gel (Whatman, 60 Å, 230–400 mesh). Melting points were determined using an Electrothermal IA9000 series digital melting point apparatus and are uncorrected. IR spectra were recorded on Bomem MB or Perkin–Elmer FTIR Spectrum 1000 spectrophotometers. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 200 NMR spectrometer at 200 and 50.3 MHz, respectively. ES–MS data were acquired using a PE SCIEX API III Biomolecular mass spectrometer. All other MS and HRMS data, were acquired using Micromass 70–250S or Micromass ZAB-SE mass spectrometers according to the ionization methods indicated. Microanalyses were performed by Canadian Microanalytical Service Ltd. (Delta, B.C., V4G 1G7, Canada). Solvents were removed in vacuo.

4-Carboxybutyl methanethiosulfonate (1b).[42] A solution of 5-bromopentanoic acid (1.238 g, 6.84 mmol) and $NaSSO_2CH_3$ (0.916 g, 6.84 mmol) in DMF (6 mL) was heated at 70°C under N_2. After 2 h the solution was cooled, water (15 mL) added and the resulting mixture extracted with ether (30 mL × 3). The organic fractions were combined, washed with brine, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (ether:CH_2Cl_2:AcOH, 40:120:1) to give **1b** (1.167 g, 80%) as a white solid; mp 61–62.5°C [lit.,[42] mp 69–71°C]; IR (KBr) 1703 cm^{-1} (C=O), 1311, 1125 cm^{-1} (S–SO_2); ^{1}H NMR ($CDCl_3$) δ 1.70–2.00 (m, 4H, H–2, H–3), 2.43 (t, J = 6.9 Hz, 2H, H-4), 3.20 (t, J = 6.8 Hz, 2H, H-1), 3.34 (s, 3H, SSO_2CH_3), 8.82 (br s, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ 23.4, 28.9, 33.1, 35.9 ($(CH_2)_4$) , 50.7 (SSO_2CH_3), 178.7 (COOH); MS m/z (EI$^+$): 213 ($M+H^+$, 2), 195 ($M+H^+$-H_2O, 11), 133 (50), 115 (M^+-CH_3SO_2–H_2O, 100%); HRMS m/z (EI+): Found 213.0251 ($M+H^+$); $C_6H_{13}O_4S_2$ requires 213.0255.

3,5-Dicarboxybenzyl methanethiosulfonate (1c). 1,1′-Carbonyldiimidazole (6.67 g, 0.0411 mol) was added to a solution of toluene-3,5-dicarboxylic acid (**2**) (3.364 g, 0.0187 mol) in DMF (30 mL) and the resulting mixture stirred at 40°C under N_2. After 1.5 h DBU (6.15 mL, 0.041 mol) and *t*-BuOH (7.7 mL, 0.0822 mol) were added. After 24 h the solution was cooled, ether (150 mL) added and the mixture acidified (HCl (aq), 1.5 M). The ethereal layer was separated and the aqueous layer further extracted (ether, 150 mL). The organic fractions were combined, washed with water and 10% K_2CO_3 (aq), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:50) to afford a colorless oil which solidified upon standing to give di-*tert*-butyl toluene-3,5-dicarboxylate (4.58 g, 84%) as a white solid; mp 86.5–87.5°C (hexane); IR (film) 1712 cm^{-1} (C=O), 1606, 1476 cm^{-1} (Ar C=C); ^{1}H NMR ($CDCl_3$) δ 1.60 (s, 18H, $C(CH_3)_3$), 2.43 (s, 3H, CH_3), 7.95 (br s, 2H, H-2, H-6), 8.38 (br s, 1H, H-4); ^{13}C NMR ($CDCl_3$) δ 21.4 (CH_3), 28.2 ($C(\underline{C}H_3)_3$), 81.4 ($C(\underline{C}H_3)_3$), 127.7, 132.1, 133.7, 138.1 (Ar), 165.2 (COO).

NBS (0.521 g, 2.93 mmol) and 1,1′-azobis(cyclohexanecarbonitrile) (30 mg, 0.12 mmol) were added to solution of this diester (0.712 g, 2.44 mmol) in CCl_4 (10 mL) and heated under reflux under N_2. After 3 h a second portion of initiator (30 mg, 0.12 mmol) was added. After a further 3 h the reaction solution was cooled and filtered. The filtrate was washed with sat. $NaHCO_3$ (aq), dried ($MgSO_4$), filtered and the solvent removed. The residue was partially purified by flash chromatography (EtOAc:hexane, 1:50) to afford crude 3,5-di(*tert*-butoxycarbo)benzylbromide (**3**) (0.872 g, 96%). A solution of **3** (0.872 g, 2.35 mmol) and $NaSSO_2CH_3$ (0.327 g, 2.44 mmol) in DMF (1 mL) was heated at 50°C under N_2. After 1 h the reaction solution was cooled, diluted with water (5 mL) and extracted with ether (15 mL × 3). The combined extracts were washed with brine, dried ($MgSO_4$) and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:8) to give 3,5-di(*tert*-butoxycarbo)benzyl methanethiosulfonate (**4**) (0.570 g, 60%) as a colorless oil; IR (film) 1717 cm^{-1} (C=O), 1604, 1477, 1456 cm^{-1} (Ar C=C), 1328, 1135 cm^{-1} (S–SO_2); ^{1}H NMR ($CDCl_3$) δ 1.59 (s, 18H, $C(CH_3)_3$), 3.07 (s, 3H, SO_2CH_3), 4.43 (s, 2H, CH_2), 8.13 (s, 2H, H-2, H-6), 8.48 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$) δ 28.2 ($C(\underline{C}H_3)_3$), 40.0 (CH_2), 51.3 (SO_2CH_3), 82.1 ($\underline{C}(CH_3)_3$), 130.2, 133.2, 133.5, 135.7 (Ar), 164.3 (COO).

A solution of **4** (0.941 g, 2.30 mmol) in CF_3COOH:CH_2Cl_2 (1:1 v/v, 10 mL) was stirred at room temperature for 3 h, during which time a white precipitate was formed. The solvents were removed and the residue triturated with CH_2Cl_2 (5 mL). The resulting mixture was filtered, and the residue washed with CH_2Cl_2 and dried under vacuum to give **1c** (0.611 g, 91% from **4**) as a white solid; mp 199.5–200 (°C (dec.); IR (KBr) 1716, 1693 cm^{-1} (C=O), 1605, 1461 cm^{-1} (Ar C=C) , 1319, 1128 cm^{-1} (S-SO_2); ^{1}H NMR (acetone-d_6) δ 3.29 (s, 3H, SO_2CH_3), 4.69 (s, 2H, CH_2), 8.36 (d, J = 1.4 Hz, 2H, H-2, H-6), 8.61 (t, J = 1.7 Hz, 1H, H-4); ^{13}C NMR (acetone-d_6) δ 40.0 (CH_2), 51.2 (SO_2CH_3), 130.8, 132.5, 135.2, 138.5 (Ar), 166.3 (COOH); MS m/z (EI+): 290 (M^+, 2), 273 (M^+-OH, 4), 210 (M^+-CH_3SO_2H, 100), 179 (M^+-SSO_2CH_3, 5); HRMS m/z (FAB+): Found 290.9987 ($M+H^+$), $C_{10}H_{11}O_6S_2$ requires 290.9998.

3,3-Dicarboxybutyl methanethiosulfonate (1d). Anhydrous K_2CO_3 (1.67 g, 12.0 mmol) was added to a solution of methyl Meldrum's acid (**5b**) (1 g, 6.33 mmol) in

DMF (33 mL) under N_2 and stirred vigorously. After 1 h the supernatant liquid was added dropwise to a solution of 1,2-dibromoethane (1.9 mL, 22.2 mmol) in DMF (11 mL) under N_2. After 89 h TLC (EtOAc:hexane, 1:3) indicated conversion of starting material (R_f 0.3) to a major product (R_f 0.5). The reaction mixture was added to water (100 mL) and extracted with ether (100 mL × 3). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:4) to give 5-(2′-bromoethyl)-2,2,5-trimethyl-1,3-dioxocyclohexa-4,6-dione (**6b**) (1.183 g, 71%) as a white solid; mp 84–85°C (ether:hexane); IR (KBr) 1738, 1784 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.66 (s, 3H, CH_3), 1.76, 1.78 (s × 2, 3H × 2, $C(CH_3)_2$), 2.61 (t, J=8 Hz, 2H, H-1′), 3.32 (t, J=8 Hz, 2H, H-2′); ^{13}C NMR ($CDCl_3$) δ 25.2, 26.6, 29.1, 30.1, 42.4 (CH_3, $C(\underline{C}H_3)_2$, C-1′, C-2′), 49.4 (C-5), 106.0 ($\underline{C}(CH_3)_2$), 169.5 (C-4, C-6); MS m/z (EI+): 249, 251 (M^+-CH_3, 5), 206, 208 (M^+-$OC(CH_3)_2$, 14), 162, 164 (M+-$C(O)OCO(CH_3)_2$, 42), 69 (M+-$C(O)OCO(CH_3)_2$-CH_2Br, 100%).

$NaSSO_2CH_3$ (776 mg, 5.80 mmol) was added to a solution of **6b** (1.18 g, 4.46 mmol) in DMF (40 mL) under N_2 and the resulting solution warmed to 50°C. After 29 h the reaction solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 3:7 to give 2-(2′,2′,5′-trimethyl-1,3-dioxocyclohexa-4,6-dionyl)ethyl methanethiosulfonate (**7b**) (1.10 g, 83%) as a cloudy oil; IR (film) 1737, 1771 cm^{-1} (C=O), 1300, 1133 cm^{-1} (S–SO_2); ^1H NMR ($CDCl_3$) δ 1.68 (s, 3H, CH_3), 1.78, 1.79 (s×2, 3H×2, $C(CH_3)_2$), 2.47–2.55 (m, 2H, H-2), 3.08–3.16 (m, 2H, H-1), 3.34 (s, 3H, SSO_2CH_3); ^{13}C NMR $CDCl_3$ δ 24.9, 29.3, 30.0, 31.9, 49.2 (CH_3, $C(\underline{C}H_3)_2$, C-1, C-2), 49.2 (C-5′), 51.2 (SSO_2CH_3), 106.1 ($\underline{C}(CH_3)_2$), 169.5 (C-4′, C-6′); MS m/z (EI+): 281 (M^+-CH_3, 1), 269 (2), 239 (M^+-C_3H_5O, 3), 159 (100), 141 (56), 113 (96), 103 (23), 87 (78), 69 (M^+-$C(O)OCO(CH_3)_2$-$CH_2SSO_2CH_3$, 79%).

Dowex 50W(H^+) resin (2.53 g) was added to a suspension of **7b** (1.08 g, 3.65 mmol) in *p*-dioxan (3.5 mL) and distilled water (35 mL) and stirred at room temperature. After 68 h the reaction mixture was filtered and the solvent removed. The resulting solid was recrystallized from water:acetone:ethyl acetate to give **1d** (738 mg, 79%) as a white solid; mp 109–111°C; IR (KBr) 1706 cm^{-1} (C=O), 1317, 1133 cm^{-1} (S-SO_2); ^1H NMR (D_2O) δ 1.43 (s, 3H, H-4), 2.25–2.33 (m, 2H, H-2), 3.16–3.24 (m, 2H, H-1), 3.45 (s, 3H, SSO_2CH_3); ^{13}C NMR (D_2O) δ 20.3 (C-4), 32.1, 36.4 (C-1, C-2), 50.5 (SSO_2CH_3), 54.3 (C-3), 176.0 (COOH); MS m/z (EI+): 256 (M^+, 6), 132 (M+H^+-$CH_2SSO_2CH_3$, 40), 116 (59), 87 (100%); HRMS m/z (CI-): Found 254.9996 ([M-H]-); $C_7H_{11}O_6S_2$ requires 254.9997.

3,3,4-Tricarboxybutyl methanethiosulfonate (1e). Anhydrous K_2CO_3 (1.2 g, 8.68 mmol) was added to a solution of Meldrum's acid (**5a**) (1 g, 6.94 mmol) in DMF (20 mL) under N_2 and stirred vigorously. After 2 h the supernatant liquid was added dropwise over a period of 1 h 30 min to a solution of *tert*-butylbromoacetate (1.14 mL, 7.63 mmol) in DMF (5 mL) under N_2. After a further 52 h TLC (acetone:toluene, 1:9) indicated the conversion of starting material (R_f 0.45) to major (R_f 0.5) and minor (R_f 0.8) products. The reaction mixture was added to water (100 mL) and extracted with ether (100 mL × 3). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 3:17 to 1:3) to give 5,5-di(*tert*-butoxycarbo)methyl-2,2-dimethyl-1,3-dioxocyclohexa-4,6-dione (412 mg, 16%); mp 103–105°C (ether:hexane); ^1H NMR $CDCl_3$ δ 1.41 (s, 18H, $C(CH_3)_3$×2), 1.92 (s, 6H, $C(CH_3)_2$), 2.97 (s, 4H, $C\underline{H}_2COOBu^t$×2); ^{13}C NMR $CDCl_3$ δ 28.5 ($C(\underline{C}H_3)_3$), 29.2, 44.1 ($\underline{C}H_2COOBu^t$, $C(\underline{C}H_3)_2$) 47.2 (C-5), 83.1 ($\underline{C}(CH_3)_3$), 108.5 ($\underline{C}(CH_3)_2$), 168.0, 168.9 (C-4, C-6, $\underline{C}OOBu^t$); and a mixture of **5a** and 5-(*tert*-butoxycarbo)methyl-2,2-dimethyl-1,3-dioxocyclohexa-4,6-dione (**5c**). This mixture was purified by repeated crystallization from ether:hexane to give **5c** (1.05 g, 59%) as a white solid; mp 124–126°C (ether:hexane); IR (KBr) 1772, 1755, 1712 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H, $C(CH_3)_3$), 1.80 (s, 6H, $C(CH_3)_2$), 3.09 (d, J 4Hz, 2H, $C\underline{H}_2COOBu^t$), 3.70 (t, J 4Hz, 1H, H-5); ^{13}C NMR $CDCl_3$ δ 18.7, 28.8, 33.0, 43.4 ($\underline{C}H_2COOBu^t$, $C(\underline{C}H_3)_2$, C-5), 28.5 ($C(\underline{C}H_3)_3$), 82.8 ($\underline{C}(CH_3)_3$), 105.6 ($\underline{C}(CH_3)_2$), 165.6, 169.6 (C-4, C-6, $\underline{C}OOBu^t$); MS m/z (CI-): 257 ($[M-H]^-$, 100), 200 (8), 159 (25) 143 (32%).

Anhydrous K_2CO_3 (300 mg, 2.17 mmol) was added to a solution of **5c** (400 mg, 1.55 mmol) in DMF (10 mL) under N_2 and stirred vigorously. After 1 h the supernatant liquid was added dropwise to a solution of 1,2-dibromoethane (0.7 mL, 8.06 mmol) in DMF (3 mL) under N_2 at 50°C. After 70 h, TLC (EtOAc:hexane, 1:9) indicated the conversion of starting material (R_f 0.1) to a major product (R_f 0.3). The reaction mixture was cooled, added to distilled water (50 mL) and extracted with ether (50 mL × 3). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:9) to give 5-(2′-bromoethyl)-5-(*tert*-butoxycarbo)methyl-2,2-dimethyl-1,3-dioxocyclohexa-4,6-dione (**6c**) (372 mg, 66%) as a white solid; mp 120–123°C (ether:hexane); IR (KBr) 1773, 1731 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.40 (s, 9H, $C(CH_3)_3$), 1.80, 1.93 (s x 2, 3H × 2, $C(CH_3)_2$), 2.41 (t, J=8 Hz, 2H, H-1′), 3.11 (s, 2H, $C\underline{H}COOBu^t$), 3.32 (t, J=8 Hz, 2H, H-2′); ^{13}C NMR ($\underline{C}DCl_3$) δ 25.2, 29.3, 29.9, 41.0, 41.4 ($\underline{C}H_2COOBu^t$, $C(\underline{C}H_3)_2$, C-1′, C-2′), 28.5 ($C(\underline{C}H_3)_3$), 51.2 (C-5), 83.2 ($\underline{C}(CH_3)_3$), 107.8 ($\underline{C}(CH_3)_2$), 167.8, 170.2 (C-4, C-6, $\underline{C}OOBu^t$); MS m/z (CI-): 287 (2), 257 (M-$(CH_2)_2Br$, 100), 142 (15) 79, 81 (Br, 91%).

$NaSSO_2CH_3$ (143 mg, 1.07 mmol) was added to a solution of **6c** (301 mg, 0.82 mmol) in DMF (20 mL) under N_2 and the resulting solution warmed to 50°C. After 29 h the reaction solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:3) and crystallized from ether to give 2-(5′-(*tert*-butoxycarbo)methyl-2′,2′-dimethyl-1,3-dioxocyclohexa-4,6-dionyl)ethyl methanethiosulfonate (**7c**) (280 mg, 86%) as a colorless crystalline solid; mp 103–105°C (ether:hexane); IR (KBr) 1772, 1738,

1717 cm^{-1} (C=O) 1314, 1129 cm^{-1} (S–SO_2); ^1H NMR $CDCl_3$ δ 1.41 (s, 9H, $C(CH_3)_3$), 1.83, 1.93 (s×2, 3H×2, $C(CH_3)_2$), 2.33–2.41 (m, 2H, H-2), 3.10–3.18 (m, 2H, H-1), 3.13 (s, 2H, CH_2COOBu^t), 3.32 (s, 3H, SSO_2CH_3); ^{13}C NMR ($CDCl_3$) δ 28.0 ($C(CH_3)_3$), 28.9, 29.2, 30.7, 37.9, 40.2 (CH_2COOBu^t, $C(CH_3)_2$, C-1, C-2), 50.0 (C-5′), 50.6 (SSO_2CH_3), 82.8 ($C(CH_3)_3$), 107.3 ($C(CH_3)_2$), 167.2, 169.7 (C-4′, C-6′, $COOBu^t$); MS *m*/*z* (CI-): 395 ($[M-H]^-$, 1), 381 (M^--CH_3, 2), 281 (M^--H-CH_2COOBu^t, 5), 257 (M^--$(CH_2)_2$ SSO_2CH_3, 100), 215 (45), 158 (37%).

A solution of **7c** (138 mg, 0.35 mmol) in CF_3COOD:D_2O (7:3, 2 mL) was heated to 50°C. After 32 h, ^1H NMR spectroscopy showed the conversion of starting material to a single product. The solution was cooled and the solvent removed. The residue was purified by flash chromatography (butan-1-ol:AcOH:water, 4:1:1) and ion exchange chromatography (Amberlyst A21, 30% v/v CF_3COOH (aq) as eluent) to give **1e** (73 mg, 70%) as an amorphous solid; IR (KBr) 1706 cm^{-1} (C=O) 1310, 1127 cm^{-1} (S-SO_2); ^1H NMR (D_2O) δ 2.25–2.34 (m, 2H, H-2), 3.01 (s, 2H, H-4), 3.12–3.20 (m, 2H, H-1), 3.45 (s, 3H, SSO_2CH_3); ^{13}C NMR (D_2O) δ 34.3, 37.7, 41.1 (C-1, C-2, C-4), 52.9 (SSO_2CH_3), 58.0 (C-3), 177.0, 177.1 (COOH, CH_2COOH); MS *m*/*z* (FAB-): 299 ($[MH]^-$, 42), 221 (21), 183 (40), 111 (49), 91 (100%). Anal. calcd for $C_8H_{12}O_8S_2$: C, 32.00; H, 4.03%; found: C, 31.84; H 3.91%;

Site-specific chemical modification. To approximately 25 mg of each of the SBL mutants in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) at 20°C was added each of the methanethiosulfonate reagents (100 μL of a 0.2 M solution: **1b** in CH_3CN:H_2O (1:9), **1a,c,d,e** in water), in a PEG(MW 10,000)-coated polypropylene test tube and mixed using an end-over-end rotator. The progress of modification was followed using specific activity measurement, monitored spectrophotometrically (10 μL aliquots in 0.1 M Tris–HCl buffer, pH 8.6, 0.005% Tween 80, and 1% DMSO, with succinyl-AAPF-*p*NA (1 mg/mL) as substrate at 25°C, $\varepsilon_{410} = 8800\ M^{-1}\ cm^{-1}$)[63] on a Perkin–Elmer Lambda 2 spectrophotometer. The reaction was terminated when the addition of a further 100 μL of methanethiosulfonate solution gave no further change in specific activity, typically after 2 to 3 h. The reaction solution was purified on a disposable desalting column (Pharmacia Biotech PD-10, Sephadex G-25 M) pre-equilibrated with MES buffer (5 mM MES, 2 mM $CaCl_2$, pH 6.5). The CMM was eluted with this buffer (3.5 mL), dialyzed against MES buffer (10 mM MES, 1 mM $CaCl_2$ pH 5.8, 1 L ×3) at 4°C and subsequently flash frozen and stored at −18°C. The free thiol content of all CMMs, was determined spectrophotometrically by titration with Ellman's reagent ($\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$) in phosphate buffer 0.25 M, pH 8.0. In all cases no free thiol was detected. Modified enzymes were analyzed by nondenaturing gradient (8–25%) gels at pH 4.2, run towards the cathode, on the Pharmacia Phast-system and appeared as a single band. Each of the CMMs showed reduced mobility relative to wild-type. 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. MS *m*/*z* (ES–MS): N62C-**a** calculated 26,826, found 26,828; S156C-**a**[33] calculated 26,853, found 26,859; S166C-**a**[33] calculated 26,853, found 26,851; L217C-**a**[31] calculated 26,827, found 26,828; N62C-**b** calculated 26,819, found 26,820; S156C-**b** calculated 26,846, found 26,846; S166C-**b** calculated 26,846, found 26,846; L217C-**b** calculated 26,820, found 26,820; N62C-**c** calculated 26,897, found 26,896; S156C-**c** calculated 26,924, found 26,928; S166C-**c** calculated 26,924, found 26,928; L217C-**c** calculated 26,898, found 26,904; N62C-**d** calculated 26,863, found 26,870; S156C-**d** calculated 26,890, found 26,892; S166C-**d** calculated 26,890, found 26,894; L217C-**d** calculated 26,864, found 26,866; N62C-**e** calculated 26,907, found 26,909; S156C-**e** calculated 26,934, found 26,939; S166C-**e** calculated 26,934, found 26,939; L217C-**e** calculated 26,908, found 26911.

Active site titrations. The active enzyme concentration was determined as previously described[47] by monitoring fluoride ion release upon enzyme reaction with α-toluenesulfonyl fluoride (PMSF) as measured by a fluoride ion sensitive electrode (Orion Research 96-09). The active enzyme concentration determined in this way was used to calculate k_{cat} values for each CMM except in the case of S166C-**c** for which total protein concentration as determined by absorbance at 280 nm ($\varepsilon_{280} = 23000\ M^{-1}\ cm^{-1}$)[64] was used.[48]

Kinetic measurements. Michaelis–Menten constants were measured at 25(±0.2)°C by curve fitting (GraFit® 3.03) of the initial rate data determined at eight or nine concentrations (0.125–4.0 mM) of succinyl-AAPF-*p*NA substrate in 0.1 M Tris–HCl buffer containing 0.005% Tween 80, 1% DMSO, pH 8.6 ($\varepsilon_{410} = 8800\ M^{-1}\ cm^{-1}$).[63]

Acknowledgements

Generous funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Genencor International Inc. who also provided SBL-WT, -N62C, -S156C, -S166C and -L217C enzymes. We also acknowledge the award of a scholarship from NSERC (to G.D.). We thank Drs. Chris Murray, Christian Paech, Thomas Graycar, and Colin Mitchinson for helpful discussions.

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