Altering the Specificity of Subtilisin *Bacillus lentus* Through the Introduction of Positive Charge at Single Amino Acid Sites

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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 recently adopted this approach for the controlled incorporation of multiple negative charges at single sites in the representative serine protease, subtilisin *Bacillus lentus* (SBL). We now describe the use of this strategy to introduce multiple positive charges. A series of mono-, di- and triammonium methanethiosulfonates were synthesized and used to modify cysteine mutants of SBL at positions 62 in the S2 site, 156 and 166 in the S1 site and 217 in the S1’ site. Kinetic parameters for these chemically modified mutants (CMM) enzymes were determined at pH 8.6. The presence of up to three positive charges in the S1, S1’ and S2 subsites of SBL resulted in up to 77-fold lowered activity, possibly due to interference with the histidinium ion formed in the transition state of the hydrolytic reactions catalyzed. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The introduction of charge into wild-type (WT) enzymes, through random1–3 or site-directed4–16 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 a positive charge into the active site of a guanylyl cyclase has recently allowed the complete alteration of its structural specificity towards adenylyl substrates.19

Introductions of charge by protein engineering have typically been limited to naturally occurring amino acids such as negatively charged aspartate4,6,9,11–16 or positively charged arginine4,7,9 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.20

Recently, using the serine protease subtilisin *Bacillus lentus* (SBL) as our model we have demonstrated the use of site-directed mutagenesis combined with chemical modification21–26 of single sites as a solution to these problems and have used this technique to improve enzyme activity,27 alter specificity28,29 and to introduce locally high charge density into SBL through the incorporation of single residues bearing multiple negative charges.30 In this technique cysteine is introduced at preselected positions and its thiol residue is then reacted with methanethiosulfonate (MTS) reagents (Scheme 1). These react specifically and quantitatively with thiols.31,32 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 multiple positive charges and the comparison of the specificity changes elicited with those caused by the introduction of multiple negative charges.30

Using the X-ray structure of SBL33 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 S34 and S1’ pockets, respectively. The other two sites, S156 and S166, are located at the base of the S1 pocket and their side chains are directed towards SBL’s surface and
catalytic triad respectively. The MTS reagents 1a–d were selected to modify these positions.

Results and Discussion

Synthesis of alkylammonium methanethiosulfonates 1b–d

Previous work\textsuperscript{27,30,31} has demonstrated that, of the methods available,\textsuperscript{35–40} 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–d. The singly-charged trimethylammonium MTS 1b\textsuperscript{41} was prepared from 2-bromoethyltrimethylammonium bromide and NaSSO\textsubscript{2}CH\textsubscript{3} in 57% yield.

The doubly-charged MTS reagent 1c was synthesized from the readily available dichloroalcohol 1 (Scheme 2). Initial attempts at constructing an untethered dipositive MTS failed as consequence of the hindered nature of the neopentyl-like system\textsuperscript{42} and therefore the less-hindered tethered system 1c was adopted as a target.

Nitrogen was introduced through the treatment of a solution of 2 with an excess of NaN\textsubscript{3} at 130°C to give diazide 3, which was formed in a high degree of purity and used without further purification. Br(CH\textsubscript{2})\textsubscript{2}OH protected as its tert-butylidemethylsilyl (TBDMS) ether 4\textsuperscript{43,44} was used to introduce an ethyl tether to 2. NaH was used to deprotonate the free alcohol in 2 and the resulting alkoxy anion was alkylated with 3 to give tethered diazide 5 in 55% yield.

Diazide 5 was hydrogenated in the presence of Pd-black in MeOH and the free amine groups thus formed were protected by treatment of diamine 6 with Boc\textsubscript{2}O to give dicarbamate 7 (72% yield over 2 steps from 5). With the amine groups now suitably protected the silyl ether moiety of 7 was selectively deprotected using TBAF to give alcohol 8 in 93% yield. Treatment of 7 with MsCl and then LiBr allowed the formation of the primary bromide group in 10 via the corresponding mesylate 9 in 93% yield. The primary bromide group in 10 was displaced with methanethiosulfonate to give protected diammonium MTS 11 in 87% yield. Gratifyingly, deprotection of the Boc groups in 11 using TFA in DCM allowed the successful preparation of target dipositive MTS reagent 1c in 67% yield (35% overall yield from dichloroalcohol 1).

For the synthesis of triply-charged MTS 1d (Scheme 3), pentaerythritol (12) was chosen as a cheap and readily available starting material. In order to introduce three amine groups and one methanethiosulfonate it was necessary to differentiate three of the four alcohol groups found in 12. This was readily achieved through the treatment of 12 with concentrated HBr in the presence of refluxing strong acid,\textsuperscript{45} conditions which allowed the formation of tribromide 13 in 49% yield.

As for the synthesis of 1c, NaN\textsubscript{3} was used to introduce the required amine groups in masked form. Compounds

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{scheme.png}
\caption{(a) NaN\textsubscript{3}, 130°C, 98%; (b) NaH, Bu\textsubscript{4}NI, THF then 4, 55%; (c) H\textsubscript{2}, Pd-black, MeOH; (d) Boc\textsubscript{2}O, NaOH(aq)/dioxan, 72% over 2 steps; (e) TBAF, THF, 93%; (f) MsCl, Et\textsubscript{3}N, CH\textsubscript{2}Cl\textsubscript{2}, 99%; (g) LiBr, acetone, (h) NaSSO\textsubscript{2}CH\textsubscript{3}, DMF, 50°C, 87%; (i) CF\textsubscript{3}COOH, CH\textsubscript{2}Cl\textsubscript{2}, then ion exchange chromatography, 67%.
\end{scheme}
containing high levels of nitrogen, such as triazide 14 present potential explosion hazards and therefore preparations avoided isolation through immediate reduction of a solution of 14 using the Staudinger reaction. The iminophosphorane product was hydrolyzed upon acidic work up to the corresponding triaminoalcohol which was isolated and purified through crystallization as its trihydrochloride salt 15 (28% over 4 steps from 12).

Functionalization of the remaining alcohol group in 15 required protection of the three introduced amino groups. This was achieved through the condensation of 15 with PhCHO in the presence of Et3N which allowed the rapid formation of the triazadamantane 16 in an excellent 99% yield. The free alcohol group in 16 was converted to a methanethiosulfonate group in an analogous manner to that used for the synthesis of 1c. Therefore, base-catalyzed mesylation of 16 and subsequent treatment with LiBr and then NaSSO2CH3 gave protected triamino MTS 17 in 39% yield over 3 steps. Treatment of an ethereal solution of 17 with dilute HCl allowed cleavage of the triazaadamantane protecting group and crystallization of the target triammonium MTS reagent as its trihydrochloride salt 1d (69% yield, 7% overall yield from 12).

Preparation of chemically modified mutants (CMMs)

MTS reagents 1a–d were used to modify the chosen SBL cysteine mutants, N62C, S156C, S166C and L217C under conditions described previously. 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, 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 positive charge, each of the CMMs showed increased 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 p-toluenesulfonyl fluoride (PMSF) using a fluoride ion-sensitive electrode.

Kinetic effects of site specific modification

The effects of modification upon SBL were assessed by the determination of $k_{cat}/K_M$ for the hydrolysis of succinyl-AAPF-p-nitroanilide (Suc-AAPF-pNA) at pH of 8.6. The kinetic parameters of the 16 CMMs generated are compared with those of WT and unmodified mutants in Table 1 and Fig. 1.

At position 62, in the S2 pocket, mutation to cysteine reduces $k_{cat}/K_M$ by 2-fold. The tolerance of the S2 pocket for multiple charge at a single site that was previously observed for negatively charged CMMs, is also observed here and $k_{cat}/K_M$s for N62C-a–d are near-identical to that of N62C. The underlying $k_{cat}$s and $K_M$s of N62C-a–d are also near-identical to each other, although 2-fold lower than N62C. The lowered $k_{cat}$s of N62C-a–d indicate that introduced positive charges destabilize the transition state of catalysis, possibly due to formation of the histidinium form of His64, although this destabilization does not increase further with increasing charge. At position 217, in the S10 pocket, mutation to cysteine causes a 4-fold decrease in $k_{cat}/K_M$ and demonstrates that mutation at this site is intrinsically more dramatic. Subsequent modification with singly positively charged MTS reagents 1a,b alters $k_{cat}/K_M$ little and the $k_{cat}/K_M$s of L217C-a,d are only 1.2-fold greater than L217C. In stark contrast, the introduction of two and three positive charges dramatically decreases $k_{cat}/K_M$. In fact, the $k_{cat}/K_M$s of L217C-c,d are 77- and 27-fold lower than WT, respectively. These decreases are the result of both decreased $k_{cat}$s that are up to 26-fold lower than WT for L217-c and increased $K_M$s that are up to 4-fold greater than WT for L217C-d.

Scheme 3. (a) HBr, AcOH, Δ then HBr, c. H2SO4, Δ, 49%; (b) NaN3, DMF, 100°C; (c) (i) PPH3, NH3(aq)/dioxan (ii) HCl (aq), 57% over 3 steps; (d) PhCHO, Et3N, MeOH, 99%; (e) MsCl, Et3N, CH2Cl2, 77%; (f) LiBr, acetone, Δ, 78%; (g) NaSSO2CH3, DMF, 80°C, 65% (d) HCl (aq), 69%.
At position 156, in the S1 pocket, mutation to cysteine decreases $k_{cat}/K_M$ 1.4-fold. From 156C to $156C^-$ to $156C^{--}$ $k_{cat}/K_M$ decreases monotonically to 3.6-fold lower than WT as the level of positive charge increases. This gradual tailoring of the specificity of SBL away from hydrophobic substrate Suc-AAPF-$p$NA even at surface-exposed position 156 is consistent with a parallel trend for multiply negatively charged CMMs.30

At position 166, in the S1 pocket, mutation of the internally-oriented side chain to cysteine decreases $k_{cat}/K_M$ 3-fold. Subsequent modification with singly positively charged MTS reagents $1a,b$ decreases $k_{cat}/K_M$ further. In spite of the identical level of positive charge introduced the $k_{cat}/K_M$ of 166C-$b$ is a dramatic 3-fold lower than that of 166C-$a$ and 9-fold lower than WT. This difference is largely a result of decreased substrate binding and the $K_M$ of 166C-(CH$_2$)$_2$NMe$_3^+$($b$) is 2-fold greater than that of 166C-S(CH$_2$)$_2$NH$_3^+$(a). This may be attributed to the added steric bulk of peralkylated side chain -S(CH$_2$)$_2$NMe$_3^+$(b) as compared with the unalkylated side chain -S(CH$_2$)$_2$NH$_3^+$(a). Modification with doubly positively charged MTS reagent $1c$ partially restores $k_{cat}/K_M$ to only 4-fold lower than WT. This exception to the general decreases in activity with increased positive charge may be a consequence of the added flexibility of tethered side chain c. This may allow the orientation of this side chain out of the S1 pocket and towards external solvent in a manner analogous to that suggested by molecular modeling analysis for other charged CMMs.29 In contrast, the $k_{cat}/K_M$ of 166C-$d$, which bears a triply positively charged side chain, is severely lowered to 26-fold lower than WT. In contrast to the trend observed at position 217, the general decreases in $k_{cat}/K_M$ at position 166 are largely due to decreases in $k_{cat}$ alone. In fact, from 166C-$b$ to $d$ the $K_M$ decreases monotonically to only 1.6-fold greater than WT.

At position 156, in the S1 pocket, mutation to cysteine decreases $k_{cat}/K_M$ 1.4-fold. From 156C to 156C-$a$ to 156C-$b$ $k_{cat}/K_M$ decreases monotonically to 3.6-fold lower than WT as the level of positive charge increases. This gradual tailoring of the specificity of SBL away from hydrophobic substrate Suc-AAPF-$p$NA even at surface-exposed position 156 is consistent with a parallel trend for multiply negatively charged CMMs.30

At position 166, in the S1 pocket, mutation of the internally-oriented side chain to cysteine decreases $k_{cat}/K_M$ 3-fold. Subsequent modification with singly positively charged MTS reagents $1a,b$ decreases $k_{cat}/K_M$ further. In spite of the identical level of positive charge introduced the $k_{cat}/K_M$ of 166C-$b$ is a dramatic 3-fold lower than that of 166C-$a$ and 9-fold lower than WT. This difference is largely a result of decreased substrate binding and the $K_M$ of 166C-(CH$_2$)$_2$NMe$_3^+$($b$) is 2-fold greater than that of 166C-S(CH$_2$)$_2$NH$_3^+$(a). This may be attributed to the added steric bulk of peralkylated side chain -S(CH$_2$)$_2$NMe$_3^+$(b) as compared with the unalkylated side chain -S(CH$_2$)$_2$NH$_3^+$(a). Modification with doubly positively charged MTS reagent $1c$ partially restores $k_{cat}/K_M$ to only 4-fold lower than WT. This exception to the general decreases in activity with increased positive charge may be a consequence of the added flexibility of tethered side chain c. This may allow the orientation of this side chain out of the S1 pocket and towards external solvent in a manner analogous to that suggested by molecular modeling analysis for other charged CMMs.29 In contrast, the $k_{cat}/K_M$ of 166C-$d$, which bears a triply positively charged side chain, is severely lowered to 26-fold lower than WT. In contrast to the trend observed at position 217, the general decreases in $k_{cat}/K_M$ at position 166 are largely due to decreases in $k_{cat}$ alone. In fact, from 166C-$b$ to $d$ the $K_M$ decreases monotonically to only 1.6-fold greater than WT.

### Table 1. Kinetic parameters for modified enzymes

<table>
<thead>
<tr>
<th>Entry</th>
<th>Enzyme</th>
<th>Pocket</th>
<th>$R$</th>
<th>Level of charge</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
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<td>SBL-WT</td>
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<td>–</td>
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<td>0.73 ± 0.05</td>
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<td>S$_2$</td>
<td>H</td>
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<td>1.90 ± 0.20</td>
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<td>1.00 ± 0.10</td>
<td>103 ± 11</td>
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<td></td>
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<td>0.86 ± 0.05</td>
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<td>5</td>
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<td>1.06 ± 0.07</td>
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<tr>
<td>6</td>
<td></td>
<td></td>
<td>d</td>
<td>3</td>
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<td>1.17 ± 0.08</td>
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<td>S$_1'$</td>
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<td>0.80 ± 0.04</td>
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<td>S156C</td>
<td>S$_1$</td>
<td>H</td>
<td>0</td>
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<td>0.85 ± 0.06</td>
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<td>0.73 ± 0.04</td>
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<td></td>
<td>d</td>
<td>3</td>
<td>46 ± 1</td>
<td>0.81 ± 0.05</td>
<td>57 ± 4</td>
</tr>
</tbody>
</table>

* 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.
Conclusions

In summary, we have devised short and efficient synthetic routes to two novel multiply charged methanethiosulfonates, 1c and d. 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)\textsuperscript{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\textsuperscript{56} and mechanism of action.\textsuperscript{57–61} Using our established methodology, we were able to fully modify the cysteine thiois 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-pNA. The application of these CMMs to the hydrolysis of different substrates, including those containing negatively-charged residues, are being investigated and the specificity consequences will be presented in due course.

Experimental

Mutants of subtilisin \textit{Bacillus lento}s (SBL) were generated, and WT and mutant enzymes purified as described previously.\textsuperscript{28,29} NaSSO\textsubscript{2}CH\textsubscript{3} (31 mp 269–269.5 °C (dec.) \textsuperscript{[lit.\textsuperscript{31} mp 272–273.5 °C]) and Br(CH\textsubscript{2})\textsubscript{2}OSiButMe\textsubscript{2}43 were prepared according to literature methods. DMF was distilled under N\textsubscript{2} from CaH\textsubscript{2} and stored over MgSO\textsubscript{4}, filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:2) to give 2-bromo-1-(methoxymethyl)-propan-1-ol (1b) (796 mg, 57%) a white crystalline solid; mp 157.5–158.5 °C (EtOH) \textsuperscript{[lit.,\textsuperscript{41} 160 °C]}

5,5-Bis(aminomethyl)-3-oxo-hexyl methanethiosulfonate dihydrochloride (1c). Na\textsubscript{2}C\textsubscript{2}H\textsubscript{3}O\textsubscript{2} (10.78 g, 166 mmol) was added to a solution of 2,2-bis(chloromethyl)-propan-1-ol (2) (1.98 g, 12.6 mmol) in DMF (88 mL) under N\textsubscript{2} and the resulting suspension warmed to 130 °C. After 6 h the solution was cooled, poured into water (600 mL) and extracted with ether (100 mL then 5×50 mL). The organic fractions were combined, dried (MgSO\textsubscript{4}), filtered and the solvent removed to give 2,2-bis(azidomethyl)-propan-1-ol (3) (2.1 g, 98%) as a yellow oil; IR (film) 3380 (O-H), 2102 (N-H) cm\textsuperscript{-1}.\textsuperscript{3}H NMR (CDCl\textsubscript{3}, 400 MHz) \(\delta\) 0.93 (s, 3H, H-3), 1.76 (br s, 1H, OH), 3.32 (s, 4H, -CH\textsubscript{2}N-), 3.47 (s, 2H, H-1); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100 MHz) \(\delta\) 18.3 (C-3), 41.1 (C-2), 55.7 (-CH\textsubscript{2}N-), 66.2 (C-1); HRMS m/z (FAB+) found 171.0970 (M + H\textsuperscript{+}).

\(\text{C}_3\text{H}_7\text{N}_6\text{O}\) requires 171.0994

Pd-black (70 mg) was added to a solution of 5 (1 g, 3.05 mmol) in MeOH (27 mL) under N\textsubscript{2}. The resulting suspension was thoroughly degassed and H\textsubscript{2} introduced. After 18 h the suspension was degassed, N\textsubscript{2} introduced, filtered through Celite (MeOH as eluant) and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:2) to give 5,5-bis(azidomethyl)-1-O-tert-butylidimethylsilyl-3-oxo-hexan-1-ol (6) (800 mg, 2.90 mmol). This was dissolved in dioxan: 1 M aq NaOH (2:1, 20 mL) and the resulting solution cooled to 0 °C. After 3 h the reaction mixture was acidified to pH 4 with 1 M H\textsubscript{2}SO\textsubscript{4} (aq) and partitioned between EtOAc (300 mL) and water (100 mL). The aqueous layer was further extracted with EtOAc (200 mL). The organic fractions were combined, washed with sat. NaHCO\textsubscript{3} (aq, 100 mL), brine (100 mL), dried (MgSO\textsubscript{4}), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane,
1:9 to 1:3) to give 5,5-bis(aminomethyl)-1-O-tert-butyldimethylsilyl-N,N′-di-tert-butoxy carbonyl-3-oxo-hexan-1-ol (7). (1.04 g, 72% over 2 steps) as a colorless oil; IR (film) 3360 (NH), 1701, (amide i), 1508 (amide ii) cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ -0.02 (s, 6H, Si(CH₃)₃), 0.75 (s, 3H, H-6), 0.81 (s, 9H, Si(CH₃)₃), 1.34 (s, 18H, OC(CH₃)₃×2), 2.83 (dd, 2H, JHH,N = 5.3 Hz, JHH = 14.0 Hz, CHH-N×2), 3.00 (dd, 2H, JHH,N = 7.9 Hz, JHH = 14.1 Hz, CHH'-N×2), 3.16 (s, 2H, H-4), 3.39 (t, 2H, JH,H' = 4.9 Hz, H-2), 3.65 (t, 2H, JH,H' = 4.9 Hz, H-1), 5.30 (br t, 2H, JH,H' = 6.8 Hz, NH×2); ¹³C NMR (CDCl₃, 100 MHz) δ 5.4 (Si(CH₃)₃), 18.2 (Si(CH₃)₂), 28.3 (OC(CH₃)₃), 40.2 (C-5), 44.4 (C-6), 62.3, 72.8, 76.3 (C-1, C-2, C-4), 78.7 (OC(CH₃)₃×2), 156.7 (CHH'-N×2) cm⁻¹; MS m/z (FAB+): 385 (M+Na+, 45), 457 (M+H+, 10%) .

A solution of TBAF in THF (1M, 3.7 mL, 3.7 mmol) was added dropwise to a solution of 7 (1.04 g, 2.18 mmol) in THF (17 mL) under N₂. After 3 h, the solvent was removed. The residue was dissolved in EtOAc (200 mL) and washed with water (100 mL×2). The aqueous layer was reextracted with ether (50 mL). The organic fractions were combined, dried (MgSO₄), filtered and the solvent removed. The residue was purified by flash chromatography to give tert-butoxycarbonyl-3-oxo-hexane (10) (769 mg, 93%) as a yellow oil which was used directly in the next step.

LiBr (860 mg, 9.89 mmol) was added to a solution of 9 (245 mg, 0.62 mmol) in acetone (25 mL) under N₂ and heated under reflux. After 8 h the reaction mixture was cooled and the solvent removed. The residue was partitioned between ether (150 mL) and water (50 mL). The aqueous layer was reextracted with ether (50 mL×2). The organic fractions were combined, dried (MgSO₄), filtered and the solvent removed to give 5,5-bis(aminomethyl)-1-bromo-N,N′-di-tert-butoxycarbonyl-3-oxo-hexane (10) (769 mg, 93%) as a yellow oil which was used directly in the next step.

NaSSO₂CH₃ (315 mg, 2.35 mmol) was added to a solution of 10 (769 mg, 1.81 mmol) in DMF (30 mL) and the resulting solution warmed to 50°C under N₂. After 20 h TLC (EtOAc/hexane, 1:1) showed the conversion of starting material (R₉ 0.8) to a major product (R₉ 0.35). The solvent was removed and the residue purified by flash chromatography to give EtOAc/hexane, 1:1) 5,5-bis(aminomethyl)-N,N′-di-tert-butoxycarbonyl-3-oxo-hexyl methanethiosulfonate (11) (720 mg, 87%) as a colorless oil; IR (film) 3385 (NH), 1700 (amide i), 1508 (amide ii) cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 0.83 (s, 3H, H-6), 1.42 (s, 18H, OC(CH₃)₃×2), 2.95 (dd, 2H, JHH,N = 6.1 Hz, JHH,N = 14.2 Hz, CHH-N×2), 3.05 (dd, 2H, JHH,N = 7.2 Hz, JHH,N = 14.2 Hz, CHH'-N×2), 3.24 (s, 2H, H-4), 3.34 (s, 3H, CH₃SO₂), 3.35 (t, 2H, JH,H' = 4.9 Hz, H-2), 3.71 (t, 2H, JH,H' = 4.9 Hz, H-1), 5.20 (br s, 2H, NH×2); ¹³C NMR (CDCl₃, 100 MHz) δ 18.8 (C-6), 28.4 (OC(CH₃)₃×2), 36.2 (C-1), 40.5 (C-5), 44.2 (CH₂N-), 50.6 (CH₃SO₂), 69.6, 76.2 (C-2, C-4), 79.2 (OC(CH₃)₃×2), 156.7 (CHH'-N×2) cm⁻¹; MS m/z (FAB+): 479 (M+Na⁺, 8), 457 (M+H⁺, 10%).

Compound 11 (720 mg, 1.58 mmol) was dissolved in DCM (20 mL) under N₂ and TFA (20 mL) was added. After 1 h the solvent was removed. The residue was purified by ion exchange chromatography (Dowex 50W×8, 4×3 cm, eluant aq HCl, concave gradient 0.5–2.5 M) to give 1c as a white foam (348 mg, 67%). ¹H NMR (D₂O, 400 MHz) δ 0.91 (s, 3H, H-6), 2.89 (d, 2H, JHH,N = 13.5 Hz, CHH'-N×2), 2.99 (d, 2H, JHH,N = 13.2 Hz, CHH-N×2), 3.28 (t, 2H, JH,H' = 5.6 Hz, H-1), 3.31 (s, 3H, CH₃SO₂), 3.42 (s, 2H, H-4), 3.63 (t, 2H, JH,H' = 5.9 Hz, H-2); ¹³C NMR (D₂O, 100 MHz) δ 17.8 (C-6), 36.4, 37.1, 45.2 (C-1, C-5, -CH₂N), 50.6 (CH₃SO₂), 70.1, 75.4 (C-2, C-4), MS m/z (FAB+): 289 (M+Na⁺, 15), 257 (M+H⁺, 65%). HRMS m/z (FAB+). Found 257.0994.

2,2′-Bis(aminomethyl)-3-aminopropyl methanethiosulfonate trihydrochloride (1d)

Pentaerythritol (12) (12.8 g, 94 mmol) was dissolved in glacial AcOH:40% HBr (aq) (1:5 v/v, 60 mL) and heated under reflux. After 24 h 40% HBr (aq) (50 mL) and c. H₂SO₄ (25 mL) were added and the resulting solution heated under reflux. After a further 24 h the reaction mixture was cooled. The lower liquid layer from the resulting mixture was separated and dissolved in CHCl₃ (50 mL), washed with water (20 mL), dried (anhyd. K₂CO₃), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc/hexane,
1:9) to give 2,2-bis(bromomethyl)-3-bromo-propan-1-ol (13) (15.1 g, 49%) as a white crystalline solid; mp 67–69°C [lit., 46 mp 68–69.5°C (CCl4)]; 1H NMR (200 MHz, CDCl3) δ 1.70 (br s, 1H, OH), 3.55 (s, 6H, -CH2Br), 3.75 (d, 2H, J 6 Hz, -CH2OH).

NaN3 (15.7 g, 241.5 mmol) was added to a solution of 13 (6.6 g, 20.3 mmol) in DMF (120 mL) under N2 and the resulting mixture warmed to 100°C. After 28 h the solution formed was cooled, poured into water (1 L) and extracted with EtO (250 mL) then 75 mL x 4. The organic fractions were combined, dried (MgSO4), filtered and the volume of solvent reduced to 100 mL. p-Dioxan (200 mL) was added and the volume of solvent reduced again to 100 mL. p-Dioxan (250 mL), PPh3 (26.6 g, 101.4 mmol) and NH3 (aq, 30%, 100 mL) were added with stirring. After 19 h the solvent was removed, the residue suspended in CHCl3 (400 mL) and extracted with HCl (aq, 2.5 M, 75 mL x 5). The aqueous fractions were combined, washed with CHCl3 (20 mL x 4) and concentrated to a volume of 50 mL. c. HCl (aq, 10 mL) was added and the solution cooled to 4°C. The white solid that crystallized from solution was filtered, washed with cold c. HCl (aq, 3 mL), EtOH (3 mL), Et2O (20 mL x 5) and dried under vacuum to give 2,2-bis (aminomethyl)-3-amino-propan-1-ol trihydrochloride (3.75 (d, 2H, J 6 Hz, -CH2OH).

NMR (400 MHz, CDCl3) δ 2.85 (s, 3H, CH3SO2-), 2.96 (m, 15H, Ar); 13C NMR (100 MHz, CDCl3) δ 26.3 (C-7), 37.1 (CH2SO2-), 45.9, 54.6, 73.5 (-CH2O-, -CH2N-) 75.3, 82.8 (PhCH-), 126.5, 126.6, 127.4, 127.6, 128.8, 129.0, 139.1, 139.4 (Ar); HRMS m/z (FAB+). Found 476.1996 (M + H+); C26H30N2O2S requires 476.2008.

LiBr (3.2 g, 36.8 mmol) was added to a solution of this mesylate (2.44 g, 5.1 mmol) in dry acetone (30 mL) under N2 and heated under reflux. After 22 h TLC (EtOAc:hexane, 1:3) showed the loss of starting material (Rf 0.5) and the formation of a major product (Rf 0.8). The reaction mixture was cooled and the solvent removed. The residue was partitioned between Et2O (200 mL) and brine (50 mL), dried (MgSO4), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 2:3) to give 7-(bromomethyl)-2,4,6-triphenyl-1,3,5-triazaadamantane (1.82 mg, 78%) as a yellow oil; Rf 0.8 (EtOAc:hexane, 1:3), which was used directly in the next step.

NaSSO2CH3 (440 mg, 3.28 mmol) was added to a solution of this bromide (1.1 g, 2.38 mmol) in DMF (35 mL) under N2 and the resulting solution heated to 80°C. After 96 h TLC (EtOAc:hexane, 1:3) showed the loss of starting material (Rf 0.8) and the formation of a major product (Rf 0.2). The solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:3) to give (2,4,6-triphenyl-1,3,5-triazaadamantanyl)methyl methanethiosulfonate (17) (759 mg, 65%) as a colorless oil; IR (film) 1541 cm⁻¹ (Ar), 1323, 1132 cm⁻¹ (S-SO2); 1H NMR (400 MHz, CDCl3) δ 2.63 (2H, CH2), 2.98 (2H, -CH2N-), 3.18 (3H, -CH2O-), 3.19 (3H, -CH2N-), 3.24 (2H, J = 13.2 Hz, -CH2N-), 3.51 (2H, J = 13.2 Hz, -CH2N-), 4.54 (2H, Ph2CH), 5.63 (1H, PhCH=CH), 7.22–7.83 (m, 15H, Ar); 13C NMR (100 MHz, CDCl3) δ 26.6 (C-7), 42.0, 43.0, 48.2, 56.3 (CH2O, -CH2N), 50.2 (CH3SO2), 156.4 (C-16). HRMS m/z (FAB+). Found 492.1768 (M + H+); C26H30N2O2S requires 492.1779.

Conc. HCl (aq, 50 drops) was added to a solution of 17 (759 mg, 1.54 mmol) in EtOH:Et2O (5:1 v/v, 24 mL) and the resulting solution cooled to 4°C. After 1 h, the white solid that crystallized from solution was filtered, washed with cold EtOH and Et2O and dried under vacuum to give 1d (358 mg, 69%) as a fine white powder; mp 199–204°C (dec.); IR (KBr) 2890, 1605, 1513 cm⁻¹ (NH2), 1284, 1123 cm⁻¹ (S-SO2); 1H NMR (400 MHz, CD2O) δ 3.17 (s, 3H, -CH2N-), 3.38 (3H, CH3SO2-), 3.44 (2H, J = 13.2 Hz, -CH2S-); 13C NMR (100 MHz, CD2O) δ 37.0 (C-2), 40.7, 40.8 (-CH2S-, -CH2N-), 50.6 (CH3SO2-); HRMS m/z (FAB+). Found 228.0844 (M + H+); C6H18N3O2S2 requires 228.0840.

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 CaCl2, pH 9.5) at 20°C was added each of the methanethiosulfonate reagents (100 μL of a 0.2 M solution 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 20).
80, and 1% DMSO, with succinyl-AAPF-pNA (1 mg/mL) as substrate at 25°C, ε410 = 8800 M⁻¹ cm⁻¹ on a Perkin–Elmer Lambda 2 spectrophotometer. The reaction was terminated when the addition of a further 100 μL of methanethiosulphonate solve 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 CaCl₂, pH 6.5). The CMM was eluted with this buffer (3.5 mL), dialyzed against MES buffer (10 mM MES, 1 mM CaCl₂ 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 51 (ε412 = 13600 M⁻¹ cm⁻¹) in phosphate buffer 0.25 M, pH 8. In all cases no free thiol was detected. Modified enzymes were analyzed by non-denaturing gradient (8–25%) gels at pH 4.2, run towards the cathode, on the Pharmacia Phastsystem and appeared as a single band. Each of the CMMs showed increased 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- calculated 26861, found 26862; S156C- calculated 26832, found 26835; S166C- calculated 26864, found 26869; L217C- calculated 26890, found 26899; L217C-calculated 26864, found 26869; N62C-calculated 26834, found 26835; S156C-calculated 26861, found 26866; S166C-calculated 26861, found 26862; L217C-calculated 26835, found 26837.

Active site titrations. The active enzyme concentration was determined as previously described 52 by monitoring fluoride release upon enzyme reaction with x-toluene-sulfonyl 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 kcat values for each CMM.

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 mM) of succinyl-AAPF-pNA substrate in 0.1 M Tris–HCl buffer containing 0.005% Tween 80, 1% DMSO, pH 8.6 (ε410 = 8880 M⁻¹ cm⁻¹). 62

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References and notes

33. The coordinates of SBL have been deposited at the Protein Databank at Brookhaven under the code 1JEA.
42. Although untethered 2,2-bis(aminomethyl)-1-bromo-N,N'-di-tert-butoxycarbonyl-propane was successfully prepared, all attempts at introducing methanethiosulfonate ion under a range of conditions failed.