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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 S_2 site, 156 and 166 in the S_1 site and 217 in the S_1' 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 S_1, S_1' and S_2 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.

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 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 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.[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 modification[21–26] of single sites as a solution to these problems and have used this technique to improve enzyme activity,[27] alter specificity[28,29] 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 SBL[33] 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[34] 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

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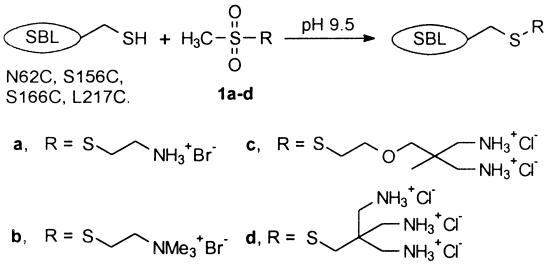
catalytic triad respectively. The MTS reagents **1a–d** were selected to modify these positions.

Results and Discussion

Synthesis of alkylammonium methanethiosulfonates 1b–d

Previous work[27,30,31] has demonstrated that, of the methods available,[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**[41] was prepared from 2-bromoethyltrimethylammonium bromide and $NaSSO_2CH_3$ 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[42] and therefore the less-hindered tethered system **1c** was adopted as a target.



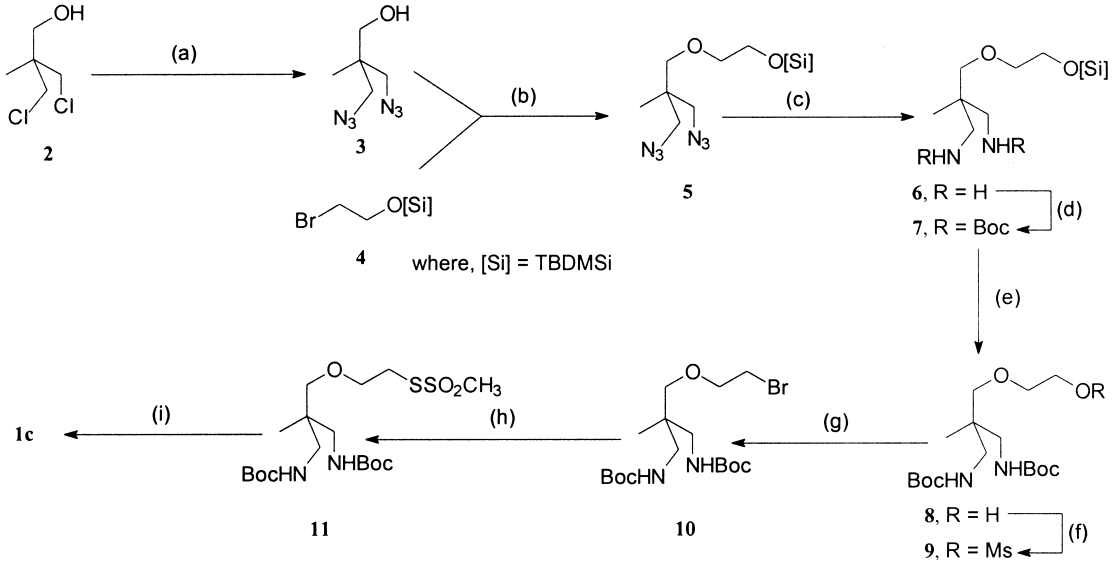
Scheme 1.

Nitrogen was introduced through the treatment of a solution of **2** with an excess of NaN_3 at 130°C to give diazide **3**, which was formed in a high degree of purity and used without further purification. $Br(CH_2)_2OH$ protected as its *tert*-butyldimethylsilyl (TBDMS) ether **4**[43,44] was used to introduce an ethyl tether to **2**. NaH was used to deprotonate the free alcohol in **2** and the resulting alkoxide anion was alkylated with **3** to give tethered diazide **5** in 55% yield.

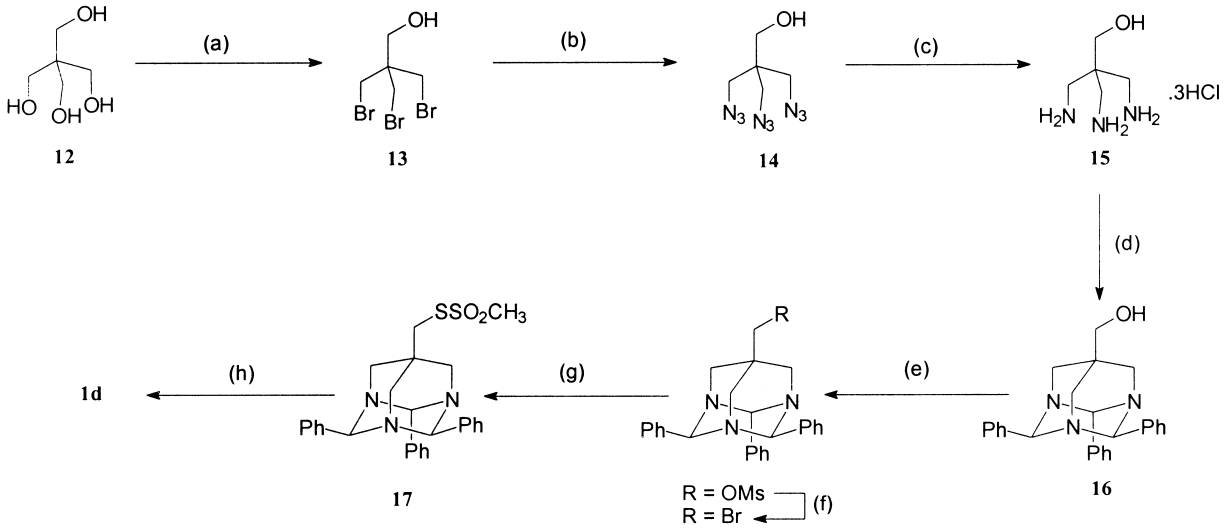
Diazide **5** was hydrogenated in the presence of Pd-black in MeOH and the free amine groups thus formed were protected by treatment of diamine **6** with Boc_2O to give dicarbamate **7** (72% yield over 2 steps from **5**). With the amine groups now suitably protected the silyl ether moiety of **7** 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 **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,[45] conditions which allowed the formation of tribromide **13**[46] in 49% yield.

As for the synthesis of **1c**, NaN_3 was used to introduce the required amine groups in masked form. Compounds



Scheme 2. (a) NaN_3, 130°C, 98%; (b) NaH, Bu_4NI, THF then **4**, 55%; (c) H_2, Pd-black, MeOH; (d) Boc_2O, NaOH(aq)/dioxan, 72% over 2 steps; (e) TBAF, THF, 93%; (f) MsCl, EtN, CH_2Cl_2, 99%; (g) LiBr, acetone, Δ, 93%; (h) $NaSSO_2CH_3$, DMF, 50°C, 87%; (i) CF_3COOH, CH_2Cl_2, then ion exchange chromatography, 67%.



Scheme 3. (a) HBr, AcOH, Δ then HBr, c. H_2SO_4, Δ, 49%; (b) NaN_3, DMF, 100°C; (c) (i) PPH_3, NH_3(aq)/dioxan (ii) HCl (aq), 57% over 3 steps; (d) PhCHO, EtN, MeOH, 99%; (e) MsCl, Et_3N, CH_2Cl_2, 77%; (f) LiBr, acetone, Δ, 78%; (g) $NaSSO_2CH_3$, DMF, 80°C, 65% (d) HCl (aq), 69%.

containing high levels of nitrogen, such as triazide **14** present potential explosion hazards[47] and therefore preparations avoided isolation through immediate reduction of a solution of **14** using the Staudinger reaction.[48,49] 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**[50] (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 Et_3N which allowed the rapid formation of the triazadamantane **16**[47] 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 $NaSSO_2CH_3$ 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 triaazaadamantane 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.[27–29] 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,[51] 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 α toluenesulfonyl fluoride (PMSF) using a fluoride ion-sensitive electrode.[52]

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) 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 S_2 pocket, mutation to cysteine reduces k_{cat}/K_M by 2-fold. The tolerance of the S_2 pocket for multiple charge at a single site that was previously observed for negatively charged CMMs,[30] is also observed here and k_{cat}/K_Ms for N62C-**a–d** are near-identical to that of N62C. The underlying k_{cat}s and K_Ms 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 S_1' 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_Ms of L217C-**a,b** 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_Ms 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**.

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	103 ± 5	1.00 ± 0.10	103 ± 11
4			**b**	1	73 ± 2	0.86 ± 0.05	85 ± 5
5			**c**	2	92 ± 3	1.06 ± 0.07	87 ± 6
6			**d**	3	98 ± 3	1.17 ± 0.08	84 ± 6
7	L217C	S_1'	H	0	41 ± 1	0.80 ± 0.04	51 ± 3
8			**a**	1	38 ± 1	0.64 ± 0.06	59 ± 6
9			**b**	1	43 ± 1	0.69 ± 0.03	62 ± 3
10			**c**	2	8.0 ± 0.2	2.94 ± 0.28	2.7 ± 0.3
11			**d**	3	23 ± 3	2.90 ± 0.16	7.8 ± 1.2
12	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
13			**a**	1	90 ± 2	0.73 ± 0.04	123 ± 7
14			**b**	1	68 ± 2	0.74 ± 0.04	92 ± 5
15			**c**	2	64 ± 1	0.76 ± 0.04	85 ± 5
16			**d**	3	46 ± 1	0.81 ± 0.05	57 ± 4
17	S166C		H	0	42 ± 1	0.50 ± 0.05	84 ± 9
18			**a**	1	50 ± 1	0.68 ± 0.04	74 ± 5
19			**b**	1	33 ± 2	1.42 ± 0.13	23 ± 2
20			**c**	2	55 ± 2	1.27 ± 0.10	43 ± 4
21			**d**	3	9.3 ± 2	1.16 ± 0.05	8.0 ± 0.4

[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.

At position 156, in the S_1 pocket, mutation to cysteine decreases k_{cat}/K_M 1.4-fold. From S156C to S156C-**a** to S156C-**d** 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 S_1 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 S166C-**b** is a dramatic 3-fold lower than that of S166C-**a** and 9-fold lower than WT. This difference is largely a result of decreased substrate binding and the K_M of S166C-$(CH_2)_2NMe_3^+$ (-**b**) is 2-fold greater than that of S166C-$S(CH_2)_2NH_3^+$ (-**a**). This may be attributed to the added steric bulk of peralkylated side chain -$S(CH_2)_2NMe_3^+$ (-**b**) as compared with the unalkylated side chain -$S(CH_2)_2NH_3^+$ (-**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 S_1 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 S166C-**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_Ms at position 166 are largely due to decreases in k_{cat} alone. In fact, from S166C-**b** to -**d** the K_M decreases monotonically to only 1.6-fold greater than WT.

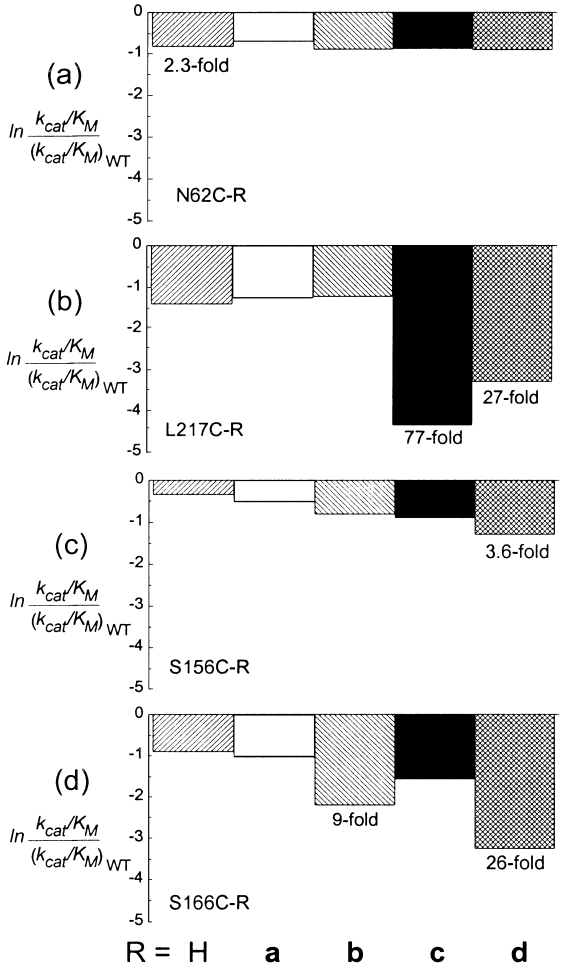


Figure 1. Altered specificity patterns for N62C, L271C, S156C and S166C Cmms: variations in ln (k_{cat}/K_M), with suc-AAPF-*p*NA as the substrate, for cysteine mutants and positively charged CMMs relative to WT.

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)[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 were able to fully modify 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. 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 *Bacillus lentus* (SBL) were generated, and WT and mutant enzymes purified as described previously.[28,29] $NaSSO_2CH_3$[31] (mp 269–269.5°C (dec.) [lit.,[31] mp 272–273.5°C]) and $Br(CH_2)_2OSiBu^tMe_2$[43] 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. 2-Aminoethyl methanethiosulfonate hydrobromide (**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, 60Å, 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 or Unity 400 NMR spectrometers at the frequencies indicated. 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. Solvents were removed in vacuo.

2-(Trimethylammonium)ethyl methanethiosulfonate bromide (1b).

A solution of 2-bromoethyltrimethylammonium bromide (1.25 g, 5.06 mmol) and $NaSSO_2CH_3$ (0.75 g, 5.6 mmol) in MeOH (10 mL) was heated under N_2 under reflux. After 50 h the resulting solution was cooled to −18°C. The white solid formed was filtered from the mixture and recrystallized from EtOH to give **1b** (796 mg, 57%) a white crystalline solid; mp 157.5–158.5°C (EtOH) [lit.,[41] 160°C (EtOH)]; ^{1}H NMR (D_2O, 200 MHz) δ 3.14 (s, 9H, $N(CH_3)_3$), 3.54 (s, 3H, CH_3SO_2), 3.57–3.64 (m, 2H, H-1), 3.67–3.77 (m, 2H, H-2).

5,5-Bis(aminomethyl)-3-oxo-hexyl methanethiosulfonate dihydrochloride (1c). NaN_3 (10.78 g, 166 mol) 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_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 ($MgSO_4$), 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_3) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (s, 3H, H-3), 1.76 (br s, 1H, OH), 3.32 (s, 4H, -CH_2N-), 3.47 (s, 2H, H-1); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.3 (C-3), 41.1 (C-2), 55.7 (-CH_2N-), 66.2 (C-1); HRMS *m*/*z* (FAB+) found 171.0970 ($M+H^+$); $C_5H_{11}N_6O$ requires 171.0994

NaH (480 mg, 80% dispersion, 16 mmol) was added to a solution of **3** (2.10 g, 12.4 mmol) and Bu_4NI (228 mg, 0.62 mmol) in THF (50 mL) under N_2. After 20 min 2-bromo-1-*O*-*tert*-butyldimethylsilylethanol (4.6 g, 19.2 mmol) was added dropwise. After 22 h, the solvent was reduced and the residue partitioned between ether (150 mL) and water (40 mL). The aqueous layer was reextracted with ether (50 mL×2). The organic fractions were combined, washed with brine (40 mL), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:19) to give 5,5-bis(azidomethyl)-1-*O*-*tert*-butyldimethylsilyl-3-oxo-hexan-1-ol (**5**) (2.24 g, 55%) as a colorless oil; IR (film) 2100 (N_3) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.05 (s, 6H, $Si(CH_3)_2$), 0.88 (s, 9H, $SiC(CH_3)_3$), 0.94 (s, 3H, H-6), 3.24 (d, 2H, $J_{H,H'}$ = 11.9 Hz, -CHH'N-×2), 3.26 (s, 2H, H-4), 3.28 (d, 2H, $J_{H,H'}$ = 12.1 Hz, -$\overline{C}$H$\overline{H}$'N-×2), 3.47–3.50 (m, 2H, H-2), 3.72–3.74 (m, 2H, $\overline{H}$-1); ^{13}C NMR ($CDCl_3$, 100 MHz) δ -5.3 ($Si(CH_3)_2$), 18.1 ($SiC(CH_3)$), 18.4 (C-6), 25.9 ($C(\underline{C}H_3)_3$), 41.2 (C-5), 55.8 (-CH_2N-), 62.6, 73.0, 73.7 (C-1, C-2, C-4); MS *m*/*z* (FAB+): 351 ($M+Na^+$, 3), 329 ($M+H^+$, 45).

Pd-black (70 mg) was added to a solution of **5** (1 g, 3.05 mmol) in MeOH (27 mL) under N_2. The resulting suspension was thoroughly degassed and H_2 introduced. After 18 h the suspension was degassed, N_2 introduced, filtered through Celite (MeOH as eluant) and the solvent removed to give crude 5,5-bis(aminomethyl)-1-*O*-*tert*-butyldimethylsilyl-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. Boc_2O (1.4 g, 6.41 mmol) was added and after 30 min the resulting mixture warmed to room temperature. After 3 h the reaction mixture was acidified to pH 4 with 1 M $KHSO_4$ (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. $NaHCO_3$ (aq, 100 mL), brine (100 mL), dried ($MgSO_4$), 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*-butoxycarbonyl-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 ($CDCl_3$, 400 MHz) δ −0.02 (s, 6H, $Si(CH_3)_2$), 0.75 (s, 3H, H-6), 0.81 (s, 9H, $SiC(CH_3)_3$), 1.34 (s, 18H, $OC(CH_3)_3\times2$), 2.83 (dd, 2H, $J_{H,NH}=5.3$ Hz, $J_{H,H'}=$ 14.0 Hz, -CHH N-×2), 3.00 (dd, 2H, $J_{H',NH}=7.9$ Hz, $J_{H,H'}=14.1$ Hz, -CHH'N-×2), 3.16 (s, 2H, H-4), 3.39 (t, 2H, $J=4.9$ Hz, H-2), 3.65 (t, 2H, $J=4.9$ Hz, H-1), 5.30 (br t, 2H, $J=6.8$ Hz, NH×2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 5.4 ($Si(CH_3)_2$), 18.2 ($SiC(CH_3)$), 18.8 (C-6), 25.7 ($SiC(CH_3)_3$), 28.3 ($OC(CH_3)_3\times2$), 40.2 (C-5), 44.4 (-CH_2N-), 62.3, 72.8, 76.3 (C-1, C-2, C-4), 78.7 ($OC(CH_3)_3\times2$), 156.7 (-NH(CO)O-×2); MS *m/z* (FAB +): 351 ($M+Na^+$, 3), 329 ($M+H^+$, 45).

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 N_2. 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 EtOAc (100 mL×2). The organic fractions were combined, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc: hexane, 1:1) to give 5,5-bis(aminomethyl)-*N,N'*-di-*tert*-butoxycarbonyl-3-oxo-hexan-1-ol (**8**) (735 mg, 93%) as a colorless oil; IR (film) 3355 (OH, NH), 1700 (amide I), 1520 (amide II) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.80 (s, 3H, H-6), 1.39 (s, 18H, $OC(CH_3)_3\times2$), 2.93 (dd, 2H, $J_{H,NH}=5.6$ Hz, $J_{H,H'}=14.0$ Hz, -CHH'N-×2), 3.01 (dd, 2H, $J_{H',NH}=8.0$ Hz, $J_{H,H'}=$ 13.9×Hz, -CHH'N-×2), 3.14 (s, 2H, H-4), 3.29 (s, 1H, OH), 3.47 (t, 2H, $J=4.5$ Hz, H-2), 3.65 (t, 2H, $J=4.3$ Hz, H-1), 5.30 (br s, 2H, NH×2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.8 (C-6), 28.3 ($OC(CH_3)_3\times2$), 40.3 (C-5), 44.0 (-CH_2N-), 61.3, 72.7, 74.7 (C-1, C-2, C-4), 79.2 ($OC(CH_3)_3\times2$), 156.7 (-NH(CO)O-×2); MS *m/z* (FAB +): 385 ($M+Na^+$, 45), 363 ($M+H^+$, 95%). MsCl (0.24 mL, 3.1 mmol) was added dropwise to a solution of **8** (735 mg, 2.03 mmol) and Et_3N (0.57 mL, 4.09 mmol) in DCM (5 mL) under N_2 at 0°C. After 1 h the solution was warmed to room temperature. After a further 16 h the solution was diluted with DCM (100 mL), washed with sat. $NaHCO_3$ (aq, 30 mL), water (30 mL), brine (30 mL), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (MeOH:$CHCl_3$, 1:25) to give 5,5-bis(aminomethyl)-*N,N'*-di-*tert*-butoxycarbonyl-1-*O*-methanesulfonyl-3-oxo-hexan-1-ol (**9**) (880 mg, 99%) as a colorless oil; IR (film) 3360 (NH), 1700 (amide I), 1520 (amide II) 1362, 1173 ($O-SO_2$) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.83 (s, 3H, H-6), 1.41 (s, 18H, $OC(CH_3)_3\times2$), 2.94 (dd, 2H, $J_{H,NH}=5.9$ Hz, $J_{H,H'}=$ 14.2 Hz, -CHH N-×2), 3.05 (s, 3H, CH_3SO_2), 3.06 (dd, 2H, $J_{H',NH}=7.7$ Hz, $J_{H,H'}=13.9$ Hz, -CHH N-×2), 3.24 (s, 2H, H-4), 3.65–3.67 (m, 2H, H-2), 4.33–4.35 (m, 2H, H-1), 5.27 (br t, 2H, $J=6.4$ Hz, NH×2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.8 (C-6), 28.4 ($OC(CH_3)_3\times2$), 37.7 (CH_3SO_2), 40.5 (C-5), 44.1 (-CH2N-), 68.6, 69.2, 76.3 (C-1, C-2, C-4), 79.2 ($OC(CH_3)_3\times2$), 156.8 (-NH(CO)O-×2); MS *m/z* (FAB +): 441 ($M+H^+$, 10%).

LiBr (860 mg, 9.89 mmol) was added to a solution of **9** (245 mg, 0.62 mmol) in acetone (25 mL) under N_2 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 ($MgSO_4$), 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.

$NaSSO_2CH_3$ (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 N_2. After 20 h TLC (EtOAc:hexane, 1:1) showed the conversion of starting material (R_f 0.8) to a major product (R_f 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) 1320, 1167 ($S-SO_2$) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.83 (s, 3H, H-6), 1.42 (s, 18H, $OC(CH_3)_3\times2$), 2.95 (dd, 2H, $J_{H,NH}=6.1$ Hz, $J_{H,H'}=14.2$ Hz, -CHH'N-×2), 3.05 (dd, 2H, $J_{H',NH}=7.2$ Hz, $J_{H,H'}=14.2$ Hz, -CHH'N-×2), 3.24 (s, 2H, H-4), 3.34 (s, 3H, CH_3SO_2), 3.35 (t, 2H, $J=4.9$ Hz, H-2), 3.71 (t, 2H, $J=4.9$ Hz, H-1), 5.20 (br s, 2H, NH×2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.7 (C-6), 28.4 ($OC(CH_3)_3\times2$), 36.2 (C-1), 40.5 (C-5), 44.2 (-CH_2N-), 50.6 (CH_3SO_2), 69.6, 76.2 (C-2, C-4), 79.2 ($OC(CH_3)_3\times2$), 156.7 (-NH(CO)O-×2); 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 N_2 and TFA (20 mL) was added. After 1 h the solvent was removed. The residue was purified by ion exchange chromatography (Dowex 50W(H^+), 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 (D_2O, 400 MHz) δ 0.91 (s, 3H, H-6), 2.89 (d, 2H, $J_{H,H'}=13.5$ Hz, -CHH' N-×2), 2.99 (d, 2H, $J_{H,H'}=$ 13.2 Hz, -CHH N-×2), 3.28 (t, 2H, $J=5.6$ Hz, H-1), 3.31 (s, 3H, CH_3SO_2), 3.42 (s, 2H, H-4), 3.63 (t, 2H, $J=5.9$ Hz, H-2); ^{13}C NMR (D_2O, 100 MHz) δ 17.8 (C-6), 36.4, 37.1, 45.2 (C-1, C-5, -CH_2N-), 50.6 (CH_3SO_2), 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 257.1000 ($M+H^+$); $C_8H_{21}N_2O_3S_2$ requires 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. H_2SO_4 (23 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 $CHCl_3$ (50 mL), washed with water (20 mL), dried (anhyd. K_2CO_3), 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 (CCl_4)]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.70 (br s, 1H, OH), 3.55 (s, 6H, -CH_2Br), 3.75 (d, 2H, *J* 6 Hz, -CH_2OH).

NaN_3 (15.7 g, 241.5 mmol) was added to a solution of **13** (6.6 g, 20.3 mmol) in DMF (120 mL) under N_2 and the resulting mixture warmed to 100°C. After 28 h the solution formed was cooled, poured into water (1 L) and extracted with Et_2O (250 mL then 75 mL×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), PPh_3 (26.6 g, 101.4 mmol) and NH_3 (aq, 30%, 100 mL) were added with stirring. After 19 h the solvent was removed, the residue suspended in $CHCl_3$ (400 mL) and extracted with HCl (aq, 2.5 M, 75 mL×5). The aqueous fractions were combined, washed with $CHCl_3$ (20 mL×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), Et_2O (20 mL×5) and dried under vacuum to give 2,2-bis (aminomethyl)-3-amino-propan-1-ol trihydrochloride (**15**) (2.81 g, 57%) as a white crystalline solid; mp 295–298°C (dec.) [lit.,[50] mp 298°C (dec.)]; ^{1}H NMR (200 MHz, D_2O) δ 3.28 (s, 6H, -CH_2N-), 3.83 (s, 2H, -CH_2O-).

Et_3N (3.3 mL, 23.6 mmol) was added to a suspension of **15** (1.63 g, 6.7 mmol) in MeOH (13 mL) under N_2. PhCHO (2.1 mL, 20.8 mmol) was added to the resulting solution and heated under reflux. After 20 min the solution was cooled and the solvent removed. The residue was slurried with water (10 mL) and filtered to give 7-(hydroxymethyl)-2,4,6-triphenyl-1,3,5-triazaadamantane (**16**) (2.65 g, 99%) as a white solid; R_f 0.5 (EtOAc:hexane, 1:3); mp 91–93°C [lit.,[47] mp 92–95°C]; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.93 (s, 4H, -CH_2N-, -CH_2O-), 2.97 (s, 1H, OH), 3.20 (d, *J* = 12.9 Hz, 2H, -CH_2N-), 3.50 (d, *J* = 13.2 Hz, 2H, -CH_2N-), 5.42 (s, 2H, PhCH_{ax}), 5.64 (s,1H, PhCH_{eq}), 7.23–7.83 (m, 15H, Ar).

MsCl (0.78 mL, 10.1 mmol) was added dropwise to a solution of **16** (2.65 g, 6.63 mmol) and Et_3N (1.9 mL, 13.6 mmol) in CH_2Cl_2 (15 mL) under N_2 at 0°C. After 1 h the reaction mixture was warmed to room temperature. After 21 h the resulting solution was diluted with CH_2Cl_2 (150 mL), washed with $NaHCO_3$ (aq, sat., 30 mL), water (30 mL) and brine (30 mL), dried ($MgSO_4$), filtered 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 methanesulfonate (2.44 g, 77%) as a white solid; R_f 0.5 (EtOAc:hexane, 1:3); mp 167–170°C (dec.); IR (KBr) 1440 cm^{-1} (Ar), 1348, 1173 cm^{-1} (O-SO_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.85 (s, 3H, CH_3SO_2-), 2.96 (s, 2H, -CH2N-), 3.30 (d, *J* = 12.7 Hz, 2H, -CH_2N-), 3.50 (s, 2H, -CH_2O-), 3.54 (d, *J* = 13.2 Hz, 2H, -CH_2N-), 5.45 (s, 2H, PhCH_{ax}), 5.64 (s,1H, PhCH_{eq}), 7.33–7.80 (m, 15H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.3 (C-7), 37.1 (CH_3SO_2-), 45.9, 54.6, 73.5 (-CH_2O-, -CH_2N-) 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$^+$); $C_{27}H_{30}N_3O_3S$ 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 N_2 and heated under reflux. After 22 h TLC (EtOAc: hexane, 1:3) showed the loss of starting material (R_f 0.5) and the formation of a major product (R_f 0.8). The reaction mixture was cooled and the solvent removed. The residue was partitioned between Et_2O (200 mL) and brine (50 mL), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 2:23) to give 7-(bromomethyl)-2,4,6-triphenyl-1,3,5-triazaadamantane (1.82 mg, 78%) as a yellow oil; R_f 0.8 (EtOAc:hexane, 1:3), which was used directly in the next step.

$NaSSO_2CH_3$ (440 mg, 3.28 mmol) was added to a solution of this bromide (1.1 g, 2.38 mmol) in DMF (35 mL) under N_2 and the resulting solution heated to 80°C. After 96 h TLC (EtOAc:hexane, 1:3) showed the loss of starting material (R_f 0.8) and the formation of a major product (R_f 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) 1451 cm^{-1} (Ar), 1323, 1132 cm^{-1} (S-SO_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.63 (s, 2H, -CH_2S-), 2.98 (s, 2H, -CH_2N-), 3.18 (s, 3H, CH_3SO_2-), 3.24 (d, 2H, *J* = 13.2 Hz, -CH_2N-), 3.51 (d, 2H, *J* = 13.2 Hz, -CH_2N-), 5.44 (s, 2H, PhCH_{ax}), 5.63 (s, 1H, PhCH_{eq}), 7.22–7.83 (m, 15H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.4 (C-7), 43.0, 48.7, 56.6 (-CH_2O-, -CH_2N-), 50.3 (CH_3SO_2-), 75.1, 82.7 (PhCH-), 126.5, 126.6, 127.5, 127.6, 128.9, 129.0, 139.1, 139.3 (Ar); HRMS *m*/*z* (FAB+). Found 492.1768 (M + H$^+$); $C_{27}H_{30}N_3O_2S_2$ requires 492.1779.

Conc. HCl (aq, 50 drops) was added to a solution of **17** (759 mg, 1.54 mmol) in EtOH:Et_2O (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 Et_2O 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} (NH_3^+), 1284, 1123 cm^{-1} (S-SO_2); ^{1}H NMR (400 MHz, D_2O) δ 3.17 (s, 6H, -CH_2N-), 3.38 (s, 3H, CH_3SO_2-), 3.44 (s, 2H, -CH_2S-); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.0 (C-2), 40.7, 40.8 (-CH_2S-, -CH_2N-), 50.6 (CH_3SO_2-); HRMS *m*/*z* (FAB+): Found 228.0844 (M + H$^+$); $C_6H_{18}N_3O_2S_2$ 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 $CaCl_2$, 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

80, and 1% DMSO, with succinyl-AAPF-pNA (1 mg/mL) as substrate at 25°C, $\varepsilon_{410} = 8800\ M^{-1}\ cm^{-1}$)[62] 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[51] ($\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$) in phosphate buffer 0.25 M, pH 8. In all cases no free thiol was detected. Modified enzymes were analyzed by 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 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-**a**[27] calculated 26763, found 26764; S156C-**a**[29] calculated 26790, found 26791; S166C-**a**[29] calculated 26790, found 26784; L217C-**a**[27] calculated 26764, found 26764; N62C-**b** calculated 26805, found 26808; S156C-**b** calculated 26832, found 26835; S166C-**b** calculated 26832, found 26835; L217C-**b** calculated 26806, found 26808; N62C-**c** calculated 26863, found 26863; S156C-**c** calculated 26890, found 26892; S166C-**c** calculated 26890, found 26899; L217C-**c** calculated 26864, found 26869; N62C-**d** calculated 26834, found 26835; S156C-**d** calculated 26861, found 26866; S166C-**d** calculated 26861, found 26862; L217C-**d** 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 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.

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 ($\varepsilon_{410} = 8880\ M^{-1}\ cm^{-1}$).[62]

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