Modulation of Esterase and Amidase Activity of Subtilisin *Bacillus lentus* by Chemical Modification of Cysteine Mutants†

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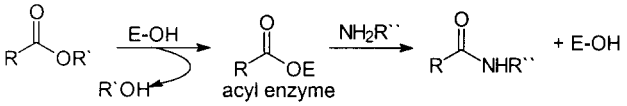
Abstract: For synthetic applications of proteases, such as for peptide coupling, a combination of high esterase and low amidase activities is required. While achieving such specificity has been a long-standing goal, the decreases in amidase activity achieved to date have often also been accompanied by decreases in esterase activity. In the current study, a strategy of combined site-directed mutagenesis and chemical modification was applied to the serine protease subtilisin *Bacillus lentus* (SBL) to improve its esterase-over-amidase specificity. Using the crystal structure of SBL as a guide, the N62, L217, S166, and M222 active site residues were chosen for mutagenesis to cysteine and subsequent modification by alkyl methanethiosulfonate reagents. An initial rapid, combinatorial screen of the chemically modified mutant enzymes (CMMs) generated and, of their parent cysteine mutants, identified 25 promising candidates which were then subjected to detailed kinetic evaluations. Of these CMM and mutant enzymes, 20 exhibited an improvement, of up to 52-fold, in esterase-over-amidase activity compared to the wild type (WT). Furthermore, these increased esterase-to-amidase ratios were not gained at the expense of esterase activity, which was improved up to 3-fold higher than that of the WT in absolute terms. The general success of this approach is evident from the fact that, of the 25 CMMs and cysteine mutants evaluated, 19 displayed higher esterase activity than the WT, whereas only 3 had better than WT amidase activity.

Introduction

Subtilisins are alkaline serine proteases that are finding increasing use in biocatalysis, particularly in resolutions,[1] regioselective acylation of polyfunctional compounds,[2] and peptide coupling,[3] with the latter application being of particular current interest. As shown in Scheme 1, subtilisins can catalyze peptide bond formation starting from an ester substrate, by first forming an acyl enzyme intermediate which then reacts with a primary amine to form the peptide product. This application thus requires high esterase activity to promote acyl enzyme formation and then low amidase activity to minimize hydrolysis of the peptide bond of the desired product. Generally, subtilisins do not meet these requirements, and the improvement of the esterase to amidase selectivities of subtilisins has been a long sought after goal. Previous strategies for lowering the amidase activity include the use of water-miscible organic solvents[3b,4,5] and site-directed mutagenesis.[3c,6] However, while the ratios of esterase-to-amidase activities were improved by these approaches, the absolute esterase activities were lowered concomitantly.[3c] Chemical modification techniques,[7] which permit the incorporation of unnatural amino acid moieties, have also been applied to improve esterase-to-amidase selectivity of subtilisins. For example, chemical conversion of the catalytic triad serine (Ser221) of subtilisin to cysteine[7a−b,3a] or to selenocysteine,[7c] and methylation of the catalytic triad histidine (His57) of chymotrypsin,[7d] effected substantial improvement in esterase-

Scheme 1



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† List of abbreviations: CMMs = chemically modified mutant enzymes; DTNB = 5,5′-dithiobis-2,2′-nitrobenzoic acid; MTS = methanethiosulfonate; suc-AAPF-pNA = succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; suc-AAPF-SBn = succinyl-alanine-alanine-proline-phenylalanine-thiobenzyl ester.

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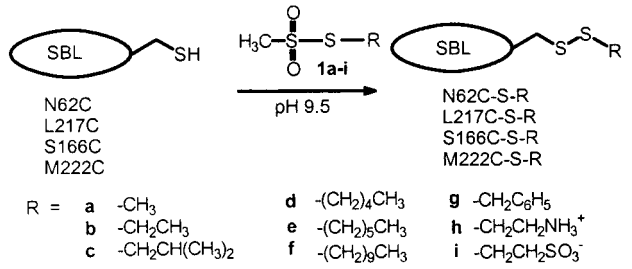
Scheme 2



to-amidase selectivities. Unfortunately however, these modifications were again accompanied by 50−1000-fold decreases in absolute esterase activity.

In the present study, we address the goal of increasing the esterase-to-amidase selectivity of subtilisins, without reducing the wild-type (WT) esterase activity, by using a combined site-directed mutagenesis and chemical modification approach. We have previously applied this strategy, which is outlined in Scheme 2, to alter enzyme specificity,[8a,c] to increase amidase activity,[8b] and to alter pH−activity profiles.[8d] The approach entails the introduction of a cysteine residue via site-directed mutagenesis at a key active site position in subtilisin *Bacillus lentus* (SBL), which itself contains no natural cysteine residues. The thiol side chain of the introduced cysteine is then thio-alkylated with an alkyl methanethiosulfonate reagent[9] (**1a**−**i**) to give a chemically modified mutant enzyme (CMM).[8] The power of this approach has been recognized previously, not only for modulating the properties of subtilisin,[10] but also for the creation of novel active site environments,[11−14] as a probe of ion-channel properties[15] for the site-directed incorporation of spin-labels,[16] as a probe of receptor binding,[17] and for investigations of membrane-spanning proteins.[18]

Employing the crystal structure of SBL[19] as a guide, we selected four active site residues for mutagenesis and modification. These residues were N62, L217, S166, and M222, which are located in the S_2, S_1', and S_1 pockets and near the oxyanion stabilization site, respectively (Figure 1). Residues 62 and 217 were chosen because they are close to the active site, and are approximately equidistant from His64 of the catalytic triad. Furthermore, residue 217, which is located at the edge of the S_1' leaving group pocket, was an obvious choice since its esterase-to-amidase modulating influence has already been demonstrated.[6] Position 166 was chosen since it is located in the primary specificity-determining S_1 pocket, and its mutations have been shown to greatly affect selectivity toward the P_1 groups of substrates.[3c,6a,20] Furthermore, and somewhat surprisingly given the remoteness of position 166 from the catalytic triad, its mutations have also been shown to induce altered esterase-to-amidase selectivities.[6a] Finally, position 222 was selected since it is adjacent to the oxyanion stabilization region and points toward the S_1' binding pocket for the leaving group and because its modification has also been shown to change esterase-to-amidase activity.[6b] In fact, with the exception of N62, all sites selected have previously been shown to influence esterase-to-amidase selectivity. In the current study, the esterase and amidase activities of each of the CMMs were evaluated using the suc-AAPF-SBn and suc-AAPF-pNA substrates, respectively.[6]

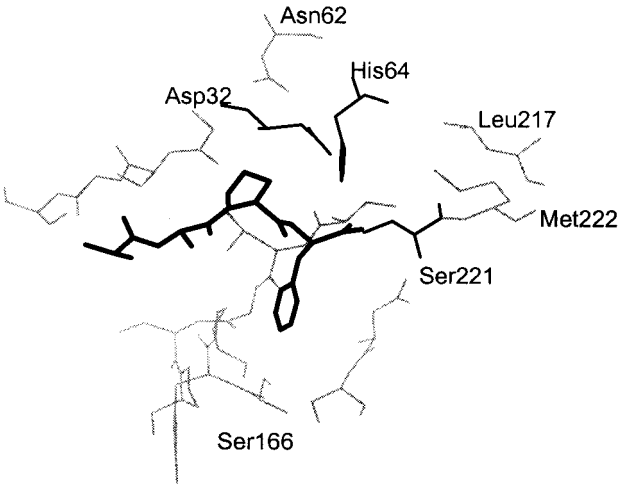


Figure 1. Active site of subtilisin (SBL) with AAPF (bold) bound. The catalytic triad and the four active site residues investigated are shown. Residue 62 is part of the S_2 pocket, residue 217 is at the mouth of the S_1' (leaving group) pocket, residue 166 is at the bottom of the S_1 pocket, and residue 222 is between the S_1 and S_1' pockets.

Results

The N62C, L217C, S166C, and M222C mutants of SBL were prepared and purified, and the introduced $-CH_2SH$ side chain was specifically and quantitatively chemically modified with the methanethiosulfonate reagents **1a**−**i**, as described previously.[8] The purities of the CMMs generated were established by native polyacrylamide gel electrophoresis (PAGE), which showed only one band in each case, thereby demonstrating that the CMMs were pure and that dimerization had not occurred. Mass analyses of the CMMs by electrospray mass spectrometry were consistent (±6 Da) with the calculated masses for single-site modifications.[8b,c] Titration of the N62C, S166C, and L217C CMMs with Ellman's reagent (DTNB) showed a residual thiol content of less than 2% in all cases, confirming that the MTS reactions were virtually quantitative.[21] The residual free thiol

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Table 1. Kinetic Constants of Chemically Modified Mutants for Amidase and Esterase Activities

	amidase[a]			esterase[b]		
enzyme	k_{cat} (s^{-1})[c]	K_M (mM)[c]	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})[c]	K_M (mM)[c]	k_{cat}/K_M (s^{-1} mM^{-1})
WT	153 ± 4	0.73 ± 0.05	209 ± 15	1940 ± 180	0.54 ± 0.07	3560 ± 540[d]
N62C	163 ± 8	1.9 ± 0.2	86 ± 10	2370 ± 90	0.54 ± 0.06	4380 ± 510
N62C−S−**a**	73 ± 2	0.55 ± 0.04	133 ± 10	3130 ± 90	0.31 ± 0.03	10100 ± 1000
N62C−S−**b**	97 ± 2	0.55 ± 0.04	177 ± 13	2220 ± 110	0.2 ± 0.04	11100 ± 2300
N62C−S−**c**	139 ± 4	0.75 ± 0.06	185 ± 16	2180 ± 80	0.25 ± 0.04	8700 ± 1430
N62C−S−**e**	146 ± 7	0.63 ± 0.08	230 ± 30	2330 ± 150	0.26 ± 0.06	8970 ± 2150
N62C−S−**f**	124 ± 4	0.36 ± 0.04	344 ± 40	1000 ± 47	0.39 ± 0.06	2570 ± 410
N62C−S−**g**	121 ± 3	0.34 ± 0.03	355 ± 33	1840 ± 110	0.29 ± 0.06	6330 ± 1360
N62C−S−**h**	96 ± 5	1.0 ± 0.1	98 ± 11	2660 ± 80	0.48 ± 0.04	5540 ± 490
N62C−S−**i**	111 ± 4	0.93 ± 0.07	120 ± 10	3190 ± 110	0.61 ± 0.06	5230 ± 540
L217C	38 ± 1	0.80 ± 0.04	48 ± 3	3160 ± 120	0.57 ± 0.06	5540 ± 620
L217C−S−**a**	47 ± 2	0.62 ± 0.07	76 ± 9	2520 ± 120	0.56 ± 0.07	4500 ± 600
L217C−S−**c**	93 ± 2	0.61 ± 0.03	152 ± 8	2450 ± 70	0.31 ± 0.03	7900 ± 800
L217C−S−**d**	87 ± 3	0.52 ± 0.05	167 ± 17	2280 ± 80	0.39 ± 0.04	5840 ± 640
L217C−S−**f**	120 ± 3	0.54 ± 0.03	223 ± 13	1840 ± 100	0.50 ± 0.08	3690 ± 620
L217C−S−**h**	36 ± 1	0.64 ± 0.06	56 ± 6	3070 ± 90	0.41 ± 0.04	7490 ± 760
L217C−S−**i**	83 ± 6	1.8 ± 0.2	47 ± 6	5060 ±130	1.0 ± 0.1	5060 ± 520
S166C	42 ± 1	0.50 ± 0.05	84 ± 9	600 ± 70	1.7 ± 0.4	350 ± 90
S166C−S−**a**	46 ± 2	0.34 ± 0.05	135 ± 20	2320 ± 50	0.38 ± 0.03	6100 ± 500
S166C−S−**g**	23 ± 0.5	1.2 ± 0.1	20 ± 1	1530 ± 110	0.31 ± 0.08	4900 ± 1300
S166C−S−**h**	50 ± 1	0.68 ± 0.04	74 ± 5	1350 ± 50	0.61 ± 0.07	2200 ± 270
S166C−S−**i**	25 ± 1	1.3 ± 0.1	19 ± 1	1950 ± 90	1.9 ± 0.2	1030 ± 120
M222C	61 ± 2	0.81 ± 0.07	75 ± 6	3080 ± 140	0.58 ± 0.07	5300 ± 680
M222C−S−**a**	56 ± 2	0.91 ± 0.07	62 ± 6	2090 ± 120	1.3 ± 0.2	1610 ± 270
M222C−S−**h**	5.0 ± 0.2	0.91 ± 0.08	5.6 ± 0.9	1970 ± 140	0.4 ± 0.1	4920 ± 1280

[a] Substrate: suc-AAPF-pNA. [b]Substrate: sucAAPF−SBn. [c]Determined by the method of initial rates; [d]Mean standard of three (esterase) experiments.

content for the more sterically hindered M222C CMMs, which did not react with Ellman's reagent, was determined with I_2.[22] The M222C CMMs contained ≤2% free thiol, except for M222C−S−$CH_2CH_2SO_3^-$ (**i**), which contained 3% residual thiol groups. The concentration of active enzyme was determined by active site titration with phenylmethanesulfonyl fluoride (PMSF).[23] All of the CMMs were 60−80% active by weight, except for M222C−S−$CH_2CH_2SO_3^-$ (**i**), which contained only 4% active enzyme and was therefore not investigated further.

Initially, a rapid screen on microtiter plates[24] was used to generate estimates of k_{cat}/K_M for amidase and esterase for the enzymes outlined in Scheme 2. Of 36 CMMs and 4 cysteine mutants screened, 25 enzymes were chosen for further kinetic analyses. These included all the promising esterases, as well as a few mutants with severely damaged esterase activity for comparison. The results of the kinetic analyses with suc-AAPF-pNA[6] and suc-AAPF-SBn[6,25] as standard amide and ester substrates, respectively, are presented in Table 1. It is noteworthy that a previous study compared k_{cat}/K_M for thio and oxo esters and demonstrated that they effect similar activity patterns.[6a]

Discussion

The broad applicability of the chemical modification approach for achieving the goal of improved esterase-to-amidase selectivity without reducing absolute esterase activity is evident from the Table 1 data since of 25 CMMs and cysteine mutants evaluated, fully 20 displayed improved esterase-to-amidase selectivity. Furthermore, 19 displayed esterase activity that was higher than that of the WT.

Of the N62 CMMs, all except N62C−S−$(CH_2)_9CH_3$ (**f**) exhibited improved esterase activity relative to the WT. Even the N62 mutation to Cys itself created a better esterase and poorer amidase than the WT. Chemical modification of N62C enhanced the absolute esterase activity still further, to ∼3-fold greater than that of the WT for N62C−S−CH_3 (**a**) and N62C−S−CH_2CH_3 (**b**). In fact, N62C−S−CH_2CH_3 (**b**) with its k_{cat}/K_M of 11 100 ± 2300 s^{-1} mM^{-1} has the highest absolute esterase activity of all the CMMs investigated. However, the larger R groups of N62C−S−$CH_2CH(CH_3)_2$ (**c**) to N62C−S−$CH_2C_6H_5$ (**g**) caused decreases in k_{cat} and k_{cat}/K_M for esterase catalysis, and steady increases in both k_{cat} and k_{cat}/K_M for amidase. Consequently, the ratio of k_{cat}/K_M for esterase-to-amidase activity decreased 10-fold as the chain length of R increased from N62C−S−CH_3 (**a**) to N62C−S−$(CH_2)_9CH_3$ (**f**) (Figure 2a). The positively and negatively charged CMMs, N62C−S−$CH_2CH_2NH_3^+$ (**h**) and N62C−S−$CH_2CH_2SO_3^-$ (**i**), respectively, both exhibited higher esterase activity and lower amidase activity than the WT, with the improvement in the esterase-to-amidase ratio being ∼3-fold regardless of the sign of the charge introduced. In addition, the larger R groups of N62C−S−$CH_2CH(CH_3)_2$ to N62C−S−$CH_2C_6H_5$ (**c** to **g**) elicited reduced K_M values for both ester and amide substrates. Clearly, hydrophobic interactions at the 62 site are beneficial to binding.

All of the L217C CMMs generated also exhibited improved esterase k_{cat}/K_M values compared to the WT. At this site, mutation to Cys alone again generated a superior catalyst having 1.5-fold better esterase activity and 4-fold poorer amidase activity than the WT. However, its modification to L217C−S−CH_3 (**a**) caused a decrease in both esterase k_{cat} and k_{cat}/K_M compared to L217C itself. L217C−S−$CH_2CH_2(CH_3)_2$ (**c**) is the most active 217 esterase and exhibits a k_{cat}/K_M of 7900 ± 800.

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(25) It was recognized that the cysteine thiol of the unmodified cysteine mutants N62C, L217C, S166C, and M222C could react with DTNB, which is used in the kinetic assay to detect the thiol benzyl hydrolysis product of the esterase reaction. This possibility was discounted by studying the rates of reaction of DTNB with N62C, S166C, L217C, and β-mercaptoethanol as a model for a nonhindered thiol, which established that these did not react at a rate sufficient to interfere with the assay at the concentrations used.

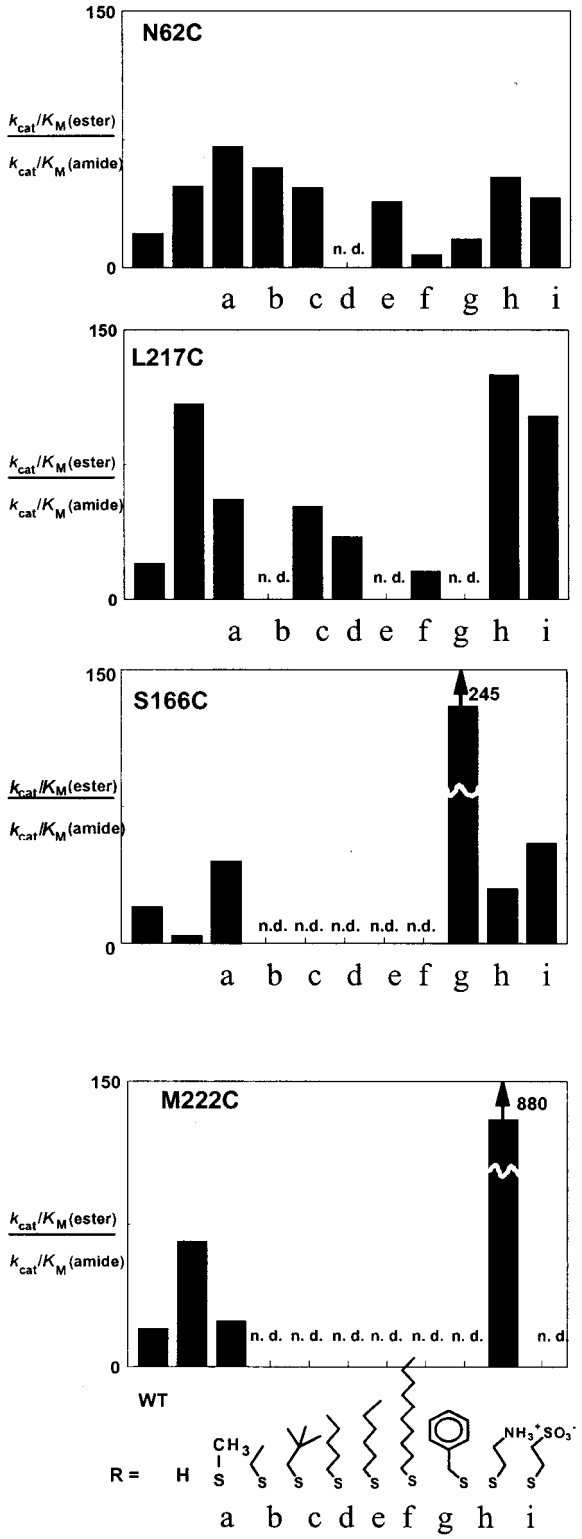

Figure 2. Ratio of k_{cat}/K_M constants for esterase to amidase. Esterase and amidase activities were determined with sucAAPF−SBn and sucAAPF-pNA substrates, respectively. All chemically modified mutants had the structure enzyme−CH_2−S−R, where the structure of the various R groups investigated is shown. In the N62C family, the straight-chain alkyl group was hexyl (**e**), and in the L217C family it was pentyl (**d**); n.d. = not determined. For comparison, the ratio for the WT enzyme was 17.

While all of the 217 CMMs exhibited greater than WT esterase activity, further increases in the chain length of R from **d** to **f** caused further decreases in k_{cat} and k_{cat}/K_M. This is in contrast to the trend observed for amidase k_{cat} and k_{cat}/K_M, values for the same CMMs.[8b] As a result, all of the L217C CMMs except L216C−S−$(CH_2)_9CH_3$ (**f**) have higher than WT esterase-to-amidase selectivity (Figure 2b). The positively charged L217C−S−$CH_2CH_2NH_3^+$ (**h**) and negatively charged L217C−S−$CH_2CH_2SO_3^-$ (**i**) CMMs also displayed higher than WT esterase activities, with L217C−S−$CH_2CH_2SO_3^-$ having a 2.6-fold higher than WT esterase k_{cat}. Furthermore, 5060 s^{-1} is the highest esterase k_{cat} of all the CMMs in the present study. The L217C−S−$CH_2CH_2NH_3^+$ (**h**) has a $(k_{cat}/K_M)_{ester}/(k_{cat}/K_M)_{amide}$ ratio of 134, compared to 17 for the WT. Interestingly, the correlations between decreased esterase and increased amidase activities with increasing chain length of R, and improved esterase and decreased amidase activities for charged modifications, parallel each other for both the L217C and N62C CMMs. These equivalent trends are consistent with residues 217 and 62 being equidistant from His64 of the catalytic triad (Figure 1).

Modification of the S166C residue of the S_1 pocket, which is quite remote from the catalytic triad and from the S_1' leaving group site of both the ester and amide substrates, exerted surprisingly large effects on esterase-to-amidase selectivity. The S166C mutant itself, with a k_{cat}/K_M of 350 s^{-1} mM^{-1}, had the lowest esterase activity of all the CMMs evaluated. However, it also had somewhat decreased amidase activity,[26] giving an esterase-to-amidase selectivity ratio of 4, compared to 17 for the WT. In contrast, modification of S166C to generate S166C−S−CH_3 (**a**) increased esterase-to-amidase selectivity to 45, a ∼3-fold improvement relative to the WT. The large hydrophobic benzyl group of S166C−S−$CH_2C_6H_5$ (**g**) increased esterase-to-amidase selectivity still further to 245, which is 14-fold higher than that of the WT, while the charged hydrophilic groups of S166C−S−$CH_2CH_2NH_3^+$ (**h**) and S166C−S−$CH_2CH_2SO_3^-$ induced little improvement in the esterase-to-amidase ratio. That the esterase K_M decreased, while the amidase K_M increased significantly, relative to the WT for the S166C−S−$CH_2C_6H_5$ (**g**) CMM implies long-range interactions between its S_1 and S_1' pockets and different rate-determining steps. These results complement those previously observed for the more hydrophilic G166N and G166S mutants of subtilisin BPN′, both of which effected improved esterase and esterase-to-amidase activity relative to the WT.[6a]

At the Met222 site, both M222C−S−**h** and M222C exhibited an improved esterase k_{cat}/K_M of up to 1.5, while all of M222C−S−**a**, M222C−S−**h**, and M222C displayed up to 37-fold reduced amidase activity. The esterase-to-amidase activity of the cysteine parent, M222C, with its 4-fold improvement, is itself significantly higher than that of the WT. The M222C mutant has a S_1' leaving group site that is less sterically congested than that of the WT. This may enhance the rate of acyl enzyme hydrolysis, which is often the rate-determining step for ester substrates. Interestingly, M222C−S−CH_3 (**a**), which differs from the WT only in the replacement of one of the methionine side-chain methylenes (CH_2) by sulfur, had the same k_{cat} as the WT, but an increased K_M. At this site, the most improved CMM is M222C−S−$CH_2CH_2NH_3^+$ (**h**), which

(26) Apart from having the lowest k_{cat} for esterase, S166C had a significantly higher K_M for esterase than the WT and was one of few mutants for which K_M (esterase) > K_M (amidase). When enzyme acylation is rate-limiting, as is the case for many amide hydrolyses, $K_M = K_S$, where K_S is the equilibrium constant for the formation of the enzyme−substrate complex. When deacylation is rate-limiting, as is the case for many ester hydrolyses, $K_M = K_S(k_3/(k_2 + k_3)$, where k_2 is the rate constant of acylation and k_3 the rate constant of deacylation. Generally, K_M (esterase) < K_M (amidase) for analogous ester and amide substrates, and in the few cases where this inequality does not hold, the rate-limiting step for ester hydroysis may have changed.

exhibits an esterase-to-amidase selectivity of 879, compared to 17 for the WT. This 52-fold improvement in the esterase-to-amidase ratio of the series arises largely from a 31-fold lowered amidase k_{cat}, but with the WT level of esterase k_{cat} being retained. This result is consistent with the observation that the M222K mutant of subtilisin BPN′ caused improved esterase activity and severely decreased amidase activity,[27] thus generating an enzyme with greatly improved esterase-to-amidase specificity.[6b]

With 19 of 25 CMMs evaluated achieving the goal of better than WT esterase-to-amidase selectivity without diminishing the absolute esterase rate, the CMM approach is clearly broadly applicable. However, it is not yet possible to identify reasons for the activity increases and decreases. Because the balance between different factors that influence esterase-to-amidase selectivity is difficult to predict, we chose systematic chemical modification and a rapid combinatorial-like screen as our initial approach to identify potential candidates for both high esterase activities and improved esterase-to-amidase ratios. The screen was particularly useful in detecting an unexpected amplification in esterase-to-amidase specificity in the 166 family of CMMs. Overall, esterase-to-amidase specificity varied from 4-fold lower than that of the WT for S166C to 52-fold higher than that of the WT for M222C−S−$CH_2CH_2NH_3^+$. At least one member from each of the four families of mutants studied met both criteria of excellent esterase activity and high esterase-to-amidase selectivity, with N62C−S−**a** being 3-fold, L217C−S−**h** and −**i** 6−8-fold, S166C−S−**g** 14-fold, and M222C−S−**h** 52-fold improved in terms of esterase-to-amidase ratio relative to the WT enzyme. With up to 880-fold esterase-to-amidase selectivity achievable by the CMM approach, the potential of chemically modified mutant subtilisins for peptide synthesis has been expanded considerably, and their application in this regard is now being investigated.

Experimental Section

Materials. Suc-AAPF-pNA and suc-AAPF-SBn were both from Bachem Inc. (California), Ellman's reagent (5,5′-dithiobis-2,2′-nitrobenzoic acid, DTNB) and phenylmethanesulfonyl fluoride (PMSF) were from Sigma-Aldrich Inc. (Milwaukee). Sources and syntheses of MTS reagents were as described previously.[8b] Buffers, 2-[*N*-cyclohexylamino]-ethanesulfonic acid (CHES), 4-morpholineethanesulfonic acid (MES), and tris(hydroxymethyl)aminomethane (Tris), were from Aldrich. Wild-type SBL and cysteine mutants N62C, S166C, L217C, and M222C were provided by Genencor International and purified as described previously.[28]

Chemical Modification. Chemical modification with alkyl MTS reagents was carried out as described previously.[8b,c] Briefly, to a solution (5−10 mg/mL, 3.5 mL) of the cysteine mutant in 70 mM CHES, 5 mM MES, and 2 mM $CaCl_2$, pH 9.5, was added in two portions over 30 min 200 μL of a 1 M solution of MTS reagent in a suitable solvent. Reaction mixtures were kept at 20 °C with continuous end-over-end mixing. Reactions were monitored by following the specific activity with suc-AAPF-pNA and by tests for residual free thiol with Ellman's reagent. Once the reaction was completed, the reaction mixture was loaded onto a Sephadex PD-10 G25 column with 5 mM MES and 2 mM $CaCl_2$, pH 6.5. The protein fraction was dialyzed against 1 mM $CaCl_2$, and the dialysate was lyophilized.

Characterization of CMMs. The molecular mass of each CMM was determined by electrospray ionization mass spectrometry.[8b,c] The purity of the CMMs was ascertained by native PAGE on 8−25% gels using the Phast system from Pharmacia. The extent of chemical modification of the cysteine mutants was determined by thiol titration with DTNB for the 62, 217, and 166 mutants and with I_2[22] for the more sterically hindered 222 mutants, which do not react with DTNB. Active site titrations were performed on all enzymes by monitoring the burst of fluoride released upon addition of phenylmethanesulfonyl fluoride to the enzyme.[23]

(a) Rapid Screen on Microtiter Plates. Detailed procedures and validation of this assay have been published previously.[24] Briefly, enzyme solutions were prepared in 5 mM MES with 2 mM $CaCl_2$, pH 6.5, at ca. 10^{-7} M for amidase and 10^{-8} M for esterase. Substrate solutions in DMSO were 1.6 mM (amidase) and 1.0 mM (esterase). The assay was performed at pH 8.6 in the same buffer used for kinetics (see below). Enzyme solutions were arranged on a microtiter plate (loading plate) along columns, with the last well in each column as a buffer blank. On a separate plate (assay plate) 10 μL of substrate and 180 μL of buffer were added to each well. Reactions were initiated by transferring 10 μL of enzyme from an appropriate column on the loading plate to the assay plate. Reactions were monitored on a Multiskan MCC 340 96-well reader programmed in the kinetic mode at 414 nm, with no time lag, at 5 s intervals for total times of 1 min (amidase) and 30 s (esterase). Background hydrolysis was subtracted automatically. The k_{cat}/K_M was estimated from the rate of substrate hydrolysis (v) using the low-substrate approximation: $v \approx k_{cat}/K_M[E][S]$, where $[S] \ll K_M$.

(b) Kinetics. Assays were done in 0.1 M Tris, pH 8.6, containing 0.005% Tween. Substrate solutions were prepared in DMSO. In the esterase assay, substrate solutions also contained 0.0375 M DTNB.[6a] Concentrations of substrate stock solutions ranged from 0.013 to 0.3 M for amidase and from 0.0015 to 0.3 M for esterase, and 9−10 different concentrations were tested in duplicate for each enzyme. Enzyme solutions were prepared in 20 mM MES, 1 mM $CaCl_2$, pH 5.8, at ca. 10^{-6} M for amidase and 10^{-7} M for esterase. Reactions were monitored spectrophotometrically on a Perkin-Elmer Lambda 2 instrument equipped with a thermostated cell compartment.

Prior to an assay, 980 μL of Tris buffer in a cuvette was equilibrated to 25 °C. Substrate stock solution (10 μL) was added to the buffer and the reading set to zero. Reactions were initiated by addition of 10 μL of enzyme solution and were monitored at 410 nm (amidase) and 412 nm (esterase). Extinction coefficients for the chromophores were 8800 M^{-1} cm^{-1} for *p*-nitroaniline[6a] and 13 470 M^{-1} cm^{-1} for 3-carboxylate-4-nitrothiophenolate in 0.1 M Tris, pH 8.6, with 0.005% Tween. Initial rates were obtained by linear fitting up to 5% conversion; r values exceeded 0.9996. In the case of esterase, rates in the presence of enzyme were corrected for uncatalyzed background hydrolysis of the thiobenzyl ester. Kinetic constants were obtained by fitting the rate data to the Michaelis−Menten equation using Grafit.

(c) Reaction of the Cysteine Mutants with DTNB. Since [DTNB] ≫ [enzyme] and [DTNB] ≈ constant over 30 s (time for 5% conversion), the pseudo-first-order rate constant for the reaction of N62C, L217C, and S166C mutants with DTNB was determined under the same conditions as used in the assay, using enzyme concentrations from 10^{-6} to 10^{-4} M. The pseudo-first-order rate constants of reaction of N62C, L217C, and S166C with DTNB under the esterase assay conditions were 1.8×10^{-4} s^{-1} (0.5% = maximum amount of cysteine mutant reacted with DTNB over the time of the esterase assay), 1.4×10^{-3} s^{-1} (4.2% reacted), and 1.4×10^{-4} s^{-1} (0.4% reacted). The M222C mutant did not detectably react with DTNB.

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