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Alteration of the Specificity of Subtilisin BPN′ by Site-Directed Mutagenesis in Its S_1 and S_1' Binding Sites

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Abstract: The potential of site-directed mutagenesis as a means of controlled alteration of the substrate specificity of subtilisin BPN′, and for differentiating between amide and ester substrates, has been investigated at both the S_1 and S_1' sites. The hydrophobic environment of the S_1 binding site, for which Gly166 is at the bottom in the wild-type enzyme, is inhospitable to polar amino acid side chains such as that of *N*-tosyl-L-arginine methyl ester (TAME). This can be partially compensated for by replacing Gly166 by amino acid residues, such as Asn and Ser, capable of hydrogen bonding with the guanidinium residue of TAME. In wild-type subtilisin BPN′, the Tyr217 located near the end of the S_1' leaving group site restricts somewhat the binding of the *p*-nitroanilide (pna) function of the tetrapeptide substrate succinyl-AAPFpna, for acylation is the rate-determining step in its subtilisin BPN′-catalyzed hydrolysis. Replacement of Tyr217 by the smaller amino acid residue Leu permits the pna group to be better accommodated at S_1'. In contrast, the Tyr217→Leu mutation is without significant effect on k_{cat} for ester substrates, such as TAME or the thiobenzyl (tbe) ester analogue of succinyl-AAPFpna, presumably because the methoxy or tbe leaving groups have departed prior to the deacylation rate-determining step for such ester hydrolyses. Met222→Phe mutation reduces the volume of the S_1' pocket. In this case, the consequence is a reduction in amide hydrolysis rate without affecting catalysis of esters. This mutant is thus an excellent candidate for preparative-scale peptide synthesis applications. Triple replacement of the 156, 169, and 217 amino acid residues of subtilisin BPN′ by those of subtilisin Carlsberg was shown previously to confer Carlsberg-like properties on the BPN′ Glu 156→Ser, Gly169→Ala, Tyr217→Leu mutant for amide hydrolyses. However, the Carlsberg-like properties of this triple mutant are not manifest for ester substrates.

Enzymes are now widely accepted as useful catalysts for asymmetric synthesis.[1] However, despite the large number of enzymes[1] and microorganisms[1,2] of synthetic value now known, it will not always be possible to obtain a natural enzyme capable of catalyzing a desired stereospecific transformation on every substrate structure of synthetic organic or chiral synthon interest. Furthermore, even the synthetically useful enzymes with the broadest specificities, such as chymotrypsin, pig liver esterase, porcine pancreatic and other lipases, and horse liver alcohol dehydrogenase do not accept all chiral synthon precursor structures of current asymmetric synthetic utility.[1] In view of this, and our interest in developing enzyme catalysts with the widest synthetic applicabilities, we have begun to explore the potential of the site-directed mutagenesis techniques of molecular biology for tailoring, in a controlled manner, the specificities of synthetically useful enzymes toward any desired chiron[3] structure. The feasibility of this approach is supported by the recent protein engineering studies on subtilisin[4] and other serine proteases such as trypsin[5a] and α-lytic protease,[5b] tyrosyl-tRNA,[6] carboxypeptidase Y,[7] yeast alcohol dehydrogenase,[8] aspartate transaminase[9a] and aspartate transcarbamoylase,[9b] lactate dehydrogenase,[10] and cytochrome P450,[11] which have shown that substrate specificity can be modified by changing amino acid residue neighbors of a bound substrate in an enzyme–substrate (ES) complex.

The class of enzymes whose synthetic utility is receiving the most current attention is that of the hydrolases, with the serine protease subgroup having been particularly widely applied as catalysts for selective or stereospecific cleavage and formation of ester and peptide bonds.[1,12] The subtilisins are representative serine proteases of proven and potential value for chiron generation for asymmetric synthesis,[13] with subtilisin BPN′ (from *Bacillus amyloliquefaciens*)[14] and subtilisin Carlsberg (from *Bacillus lichenformis*) being among the best documented in this regard. Subtilisin BPN′ is an attractive enzyme for evaluating the parameters that are important in achieving the controlled modification of enzyme specificity toward any preselected substrate structure, since it has been cloned[15] and overexpressed,[15] and its amino acid sequence can be changed at will by site-directed mutagenesis.[4,16] Furthermore, a considerable body of X-ray structural[4,17,18] and kinetic[13a,b,j,k,19] data is available on the wild-type

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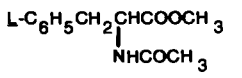
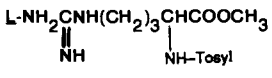
BPN'[17d] and Carlsberg[17c,g] enzymes and also on the BPN' mutant enzymes.[4,17d,f]

In its natural form, subtilisin BPN' exhibits a strong predilection for hydrolysis of hydrophobic amino acid amides or esters such as L-*N*-acetylphenylalanine methyl ester (NAPME, **1**).[13j,k] This



1



2

Succinyl-L-Ala-L-Ala-L-Pro-L-AA-X

3, AA = Arg; X = p-nitroanilide

4, AA = Phe; X = thiobenzyl

5, AA = Phe; X = p-nitroanilide

specificity preference is determined by the binding requirements of the S_1 region[20] of the active site (Figure 1), which is a hydrophobic cleft whose dimensions are appropriate for binding the benzyl group of **1**. In contrast, the hydrophobic character of the S_1 trough discourages the binding of polar groups. This poses a potential restriction on the synthetic utility of subtilisin BPN' since the substrate structure of interest may sometimes require accommodation of a charged moiety at S_1, as illustrated schematically in Figure 1 for L-*N*-tosylarginine methyl ester (TAME, **2**). In this paper, we have examined further the specificity effects of substituting amino acids with side chains capable of hydrogen bonding, such as Asn and Ser,[4a] for the Gly166 residue situated at the bottom of the S_1 pocket of the wild-type enzyme. The specificity of the S_1 region, where the amine or alcohol leaving group moieties bind, is also of interest since structural and stereospecificity discriminations by this active-site region are also possible.[21] Accordingly, the potential of site-directed mutagenesis

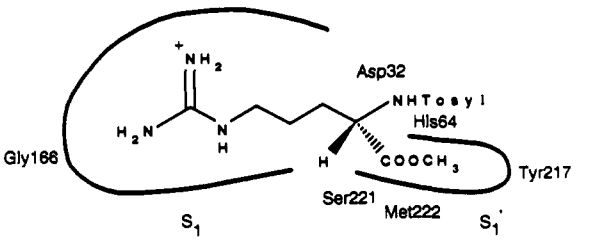


Figure 1. Schematic representation of the active site of subtilisin BPN'. In the wild-type enzyme, the hydrophobic S_1 pocket has a Gly166 located at its end, while the S_1' region that accommodates the leaving group is blocked off by Tyr217. S_1' also contains the serine residue of the catalytically vital Ser221-His64-Asp32 serine protease triad. TAME (**2**) is depicted in an orientation that site-directed mutagenesis of Gly166 to Asn or Ser should promote by creating a favorable hydrogen-bonding environment for the guanidinium function, thereby ameliorating the inhospitality of the hydrophobic milieu of S_1 toward polar groups.

Table I. Kinetic Parameters for Subtilisin-Catalyzed Hydrolyses of **2–5**

enzyme	substrate **2**[b]	**3**[c]	**4**[c]	**5**[c]
Subtilisin BPN'				
wild-type[e]				
k_{ca}, s^{-1}	5.4	3.9	2000	50[d]
K_m, M × 10^{-3}	34	0.74	0.11	0.14[d]
k_{cat}/K_m,[a] M^{-1} s^{-1}	160	5.3 × 10^3	1.8 × 10^7	3.6 × 10^5[d]
Gly166→Asn				
k_{cat}, s^{-1}	0.39	1.7	2700	28
K_m, M × 10^{-3}	21	1.2	0.17	0.15
K_{ca}/K_m,[a] M^{-1} s^{-1}	19	1.4 × 10^3	1.6 × 10^7	1.8 × 10^5
Gly166→Ser				
k_{ca}, s^{-1}	2.7	3.9	4600	14
K_m, M × 10^{-3}	14.4	0.72	0.15	0.06
k_{cat}/K_m,[a] M^{-1} s^{-1}	190	5.4 × 10^3	3.1 × 10^7	2.3 × 10^5
Met222→Phe				
k_{cat}, s^{-1}	10		4300	3.6
K_m, M × 10^{-3}	88		0.28	0.56
k_{cat}/K_m,[a] M^{-1} s^{-1}	110		1.5 × 10^7	6.4 × 10^3
Tyr217→Leu				
k_{ca}, s^{-1}	4.4		3200	280[d]
K_m, M × 10^{-3}	42		0.3	0.47[d]
k_{ca}/K_m,[a] M^{-1} s^{-1}	104		1.1 × 10^7	5.9 × 10^5[d]
Glu156→Ser/ Gly169→Ala/ Tyr217→Leu				
k_{ca}, s^{-1}	2.4[f]		1200	250[d]
K_m, M × 10^{-3}	80[f]		0.1	0.09[d]
k_{ca}/K_m,[a] M^{-1} s^{-1}	30[f]		1.2 × 10^7	2.6 × 10^6[d]
Subtilisin Carlsberg				
wild-type				
k_{ca}, s^{-1}	73		2700	510[d]
K_m, M × 10^{-3}	33		0.11	0.2[d]
k_{cat}/K_m,[a] M^{-1} s^{-1}	2200		2.4 × 10^7	2.5 × 10^6[d]

[a] The k_{ca} and K_m numbers are apparent values, with error limits ≤±10%. [b] Determined by pH-stat kinetics at pH 8.0 and 25 °C. [c] Determined by UV at pH 8.6 and 25 °C and corrected for product inhibition. [d] From ref 18. [e] Subtilisin Carlsberg and BPN' have respectively Ser156, Ala169, Leu217 and Glu156, Gly169, Tyr217. [f] Due to the limited amount of enzyme available, broader error limits (±40%) apply to these values.

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for inducing changes in the specificity of S_1' has also been investigated, using the Met222→Phe[4c] and Tyr217→Leu[18] mutants of subtilisin BPN′ as representative probes. In addition, the effects of simultaneous mutations in both the S_1 and S_1' pockets have been further evaluated with the Glu156→Ser, Gly169→Ala, Tyr217→Leu trimutant[18] of subtilisin BPN′. This was prepared initially[18] with the goal, achieved for acylation rate-determining amide substrates, of creating a BPN′ mutant that was catalytically equivalent to subtilisin Carlsberg. In order to evaluate the generality of this concept, we have extended the study of the triple mutant to ester substrates to see if the Carlsberg-like properties are maintained when deacylation is rate-determining.

The substrates used to investigate these effects were TAME (**2**), succinyl-L-Ala-L-Ala-L-Pro-L-Arg-*p*-nitroanilide (sAAPRpna, **3**), succinyl-L-Ala-L-Ala-L-Pro-L-Phe-thiobenzyl ester (sAAPFtbe, **4**), and succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPFpna, **5**).

Results and Discussion

Table I summarizes the kinetic data obtained for the hydrolyses of TAME (**2**), sAAPRpna (**3**), sAAPFtbe (**4**), and sAAPFpna (**5**) catalyzed by wild-type subtilisin BPN′ and Carlsberg and by the BPN′ mutant enzymes. The selection of the mutants surveyed was based on graphics analyses of their potential enzyme–substrate complexes with **2**–**5**.

Mutations in the S_1 Pocket. Our goal was to make the S_1 trough more receptive to polar groups of substrates by changing the Gly166 residue at the bottom of S_1 to amino acids such as Asn and Ser whose side chains are capable of hydrogen bonding to polar functions, of which the guanidinium residue of TAME was selected as being representative.

For the Gly166→Asn mutant-catalyzed hydrolysis of TAME, the k_{cat} and K_m values are reduced by factors of 14 and 1.6, respectively, relative to the wild-type enzyme constants. The X-ray structure of the Gly166→Asn mutant[17f] shows that a major effect of the replacement of Gly166 by Asn is hydrogen bonding of the new CH_2CONH_2 side chain with the side-chain carboxyl group of Glu156. This induces a conformation change that is catalytically beneficial for hydrolysis of protein substrates, which Bott et al.[17f] suggested could be due to the reaction of a more pocket-like S_1 shape, and to the tethering down of the Glu156 side chain at the enzyme's surface. This S_1 shape change may also contribute to the improved binding of TAME to the Gly166→Asn enzyme, with binding augmented further by hydrogen bonding of the Asn CH_2CONH_2 with the guanidinium function of TAME. The lowered k_{cat} for Gly166→Asn-catalyzed hydrolysis of TAME reflects a less favored substrate orientation. This may be due to the S_1 shape change, and also to the steric hindrance to productive accommodation of the side chain of TAME resulting from the introduction of the relatively bulky CH_2CONH_2 side chain in place of glycine's hydrogen atom at the bottom of S_1.

The overall effect of the Gly166→Asn replacement with respect to the polar substrate TAME is quite negative, with the specificity constant of 19 M^{-1} s^{-1} being ≈8-fold lower than for the native subtilisin BPN′-catalyzed reaction. This result, and the indications that the steric bulk of the Asn side chain was partly responsible for the low k_{cat}, prompted a study of the Gly166→Ser enzyme. This was considered a beneficial change at position 166 since the CH_2OH side chain has hydrogen-bonding capabilities similar to those of asparagine's CH_2CONH_2, but is sterically smaller. The improved catalytic properties of the Ser166 enzyme supported this expectation. For the Gly166→Ser-catalyzed hydrolysis of TAME, the K_m value was reduced by a factor of 2.4 relative to that with the wild-type BPN′ enzyme. Furthermore, although for TAME the k_{cat} value of the Ser166 enzyme was 2-fold lower than for the wild-type enzyme, it was 7-fold higher than for its Asn166 counterpart. In fact, the Gly166→Ser mutant, with its specificity constant of 190 M^{-1} s^{-1}, is the most effective catalyst of the above three enzymes for the hydrolysis of TAME. An ES complex compatible with the improved hydrogen bonding envisaged for Gly166→Ser with TAME is depicted graphically in Figure 2.

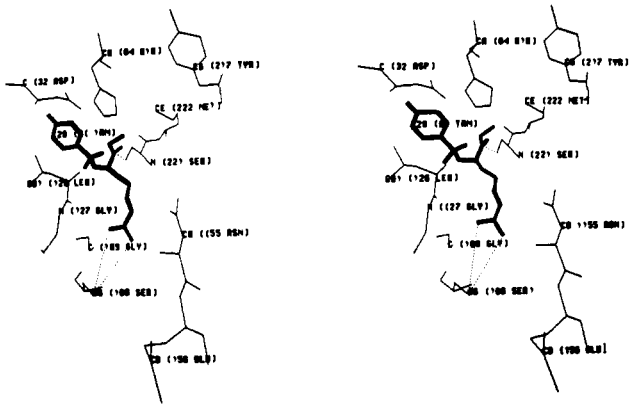

Figure 2. Graphics representation, in "cross-eyed" stereo, of TAME (**2**; shown in heavy lines) in the type of ES complex envisaged for the Gly166→Ser mutant, in which hydrogen bonding between the guanidinium residue of **2** and Ser166 (shown in dotted lines) can take place. The boundaries of the S_1 pocket, which in BPN′ is a groove on the surface of the enzyme, are traced by residues 126, 127, 169, 166, 156, and 155. The S_1' pocket is bounded on one side by Ser221 and Met222, and is blocked off at the end by the phenolic side chain of Tyr217. The catalytically active Asp32-His64-Ser221 triad is also in the S_1' region, with the Ser221 OH correctly oriented for facile attack of the ester carbonyl of TAME (dotted line) in the acylation step. In the Met222→Phe mutant, the volume of S_1' is sharply reduced, while the Tyr217→Leu change increases the capacity of S_1' for large leaving groups.

The kinetic results are in accord with the relatively poor binding of TAME (**2**) to native BPN′ being due to the charged guanidinium function having to locate in the hydrophobic environment of S_1. The data for the tetrapeptide substrate **3** are also consistent with location of the arginine side chain in the S_1 cleft being disfavored for the wild-type enzyme, but improving when hydrogen bonding with Asn- or Ser166 is possible. However, because of the extra binding contributions at the other subtilisin S sites[20] of the additional peptide functions of **3**, the kinetic consequences of its S_1–arginine interactions are not as marked as for TAME.

The variations in K_m values are also compatible with the effects of position 166 side-chain changes on the volume of the S_1 pocket. This has been calculated to be 160 ± 30 Å^3 in the native enzyme.[4a] The side chain of TAME represents a volume[22] of 136 Å^3, so the dimensions of the S_1 pocket of wild-type BPN′, with glycine at 166, are clearly more than adequate. Substitution of Gly166 by any other amino acid is liable to decrease the volume of S_1. For an Asn166–TAME complex, the minimum combined volume requirement of the Asn CH_2CONH_2 plus TAME guanidinium side chains is 205 Å^3. This would exceed somewhat the space available and preclude optimum catalytic orientation of TAME. In contrast, the combined volume of Ser166's CH_2OH and the TAME side chain is 169 Å^3, a sterically optimal S_1 value. In this regard, it is interesting to note that for the excellent subtilisin BPN′ substrate NAPME (**1**) (k_{cat} = 318 s^{-1}, K_m = 44.5 mM),[13a] the benzyl side-chain volume of 137 Å^3 does not quite fill S_1, indicating that an optimal S_1 fit is not essential for high catalytic activity.

The influence of the Gly166→Asn/Ser mutations on substrates with hydrophobic S_1 groups was further explored with the tetrapeptides sAAPFtbe (**4**) and sAAPFpna (**5**), each of which locates the benzyl group of the P_1 Phe residue in S_1, but whose rate-determining steps of subtilisin BPN′-catalyzed hydrolysis are different. For the amide **5**, acylation is rate-determining, while for the ester **4**, the rate-determining step occurs after acylation and presumably corresponds to deacylation, as for other serine protease-catalyzed hydrolyses of esters.[23]

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(22) The values for volumes of side chains are taken as the average packing volumes determined from X-ray crystal structures of proteins: Chothia, C. *Annu. Rev. Biochem.* **1984**, *53*, 537.

For the tetrapeptide ester **4**, the k_{cat} values were increased over the wild-type constants by factors of 1.4 and 2.3 with the Gly166→Asn and Gly166→Ser mutants, respectively. In contrast, for the tetrapeptide amide **5**, the k_{cat} values were decreased by factors of 1.8 and 3.6, respectively, with the same mutants. These differences in k_{cat} behavior reflect the change in shape, the decrease in volume, or both, of the S_1 cleft caused by the mutations at position 166. The influence is negative for the acylation rate-determining hydrolysis of the amide **5** but is a positive factor in the deacylation rate-limiting processes of ester **4** hydrolysis. With the exception of the **5** Ser166 case, the K_m's for **4** and **5** are similar for both wild-type and mutant enzyme catalyses.

Mutations in the S_1' Site. The S_1' site is the region in which the leaving groups of amide and ester substrates locate prior to, and during, the acylation step. In S_1', methionine 222 is directly adjacent to the catalytic Ser221 of subtilisin BPN'. Met222 is not involved in the catalytic process but, because of its proximity to the catalytic site, it does affect hydrolysis adversely when it is oxidized to its bulkier sulfoxide derivative.[17d,24] Such steric hindrance effects would be expected to be much greater on replacement of Met222 by Phe. On the other hand, replacement of the relatively bulky tyrosine 217 at the end of the S_1' region of BPN' by the smaller Leu residue (as present in subtilisin Carlsberg) should increase the space available for accommodating a large leaving group. The catalytic and specificity effects of the previously made Met222→Phe[4c] and Tyr217→Leu[18] mutants were therefore evaluated with substrates **2**–**5** (Table I).

For the Met222→Phe enzyme, the increased bulk of the Phe222 side chain decreases the volume of the S_1' significantly, thereby reducing its capacity to accept large leaving groups. This is manifest in its catalysis of **5**, for which the large pna group must be accommodated by S_1' during the rate-determining acylation step, with k_{cat} being reduced to 3.6 s^{-1} from the 50-s^{-1} value for the wild-type, Met222, enzyme. In contrast, for TAME (**2**) and **4**, with their small (methoxy) and large (tbe) leaving groups, respectively, no longer present at S_1' during the rate-determining deacylation steps, the k_{cat} values are not lowered by the Phe222 mutation. In fact, the k_{cat} values for the Phe222-catalyzed hydrolyses of **2** and **4** are both ≈2-fold higher than for the wild-type situation. This reflects improved orientations of the respective Met222→Phe acyl enzymes in the hydrolytic deacylation steps.

In contrast to the reduction in volume of the S_1' site induced by the Met222→Phe mutation, replacement of Tyr217 by Leu relieves somewhat the steric congestion toward the end of the S_1' region. Larger leaving groups should therefore be better accommodated. This is borne out by the Table I data, with k_{cat} for hydrolysis of the *p*-nitroanilide **5** by the Tyr217→Leu enzyme being 5.6-fold higher than for the wild-type enzyme. Again, as expected, for hydrolyses of the esters **2** and **4**, the k_{cat} values are basically unaffected by the improved S_1' accessibility because the leaving groups are no longer present when the rate-determining deacylations take place.

Evaluation of Simultaneous Mutations in S_1 and S_1' as a Basis for BPN'-to-Carlsberg Conversion. The active site of subtilisin BPN', shown graphically in Figure 2, is known to be closely related to that of subtilisin Carlsberg.[13k,17,26] Indeed, for the residues that are usually within van der Waals contact with substrates interacting at the active site, the most significant differences between subtilisin BPN' and Carlsberg are at positions 156, 169, and 217, being Glu, Gly, Tyr and Ser, Ala, Leu in BPN' and Carlsberg, respectively. Building on this observation, it was shown recently[18] that the Glu156→Ser, Gly169→Ala, Tyr217→Leu trimutant of subtilisin BPN' became virtually kinetically equivalent to Carlsberg for amide substrates such as **5** (cf. Table I).

In view of the pattern of kinetic differences observed with the various S_1 and S_1' mutants of BPN' in their catalyses of the hydrolyses of the ester and amide substrates **2**–**5**, it was of interest to evaluate whether the triple mutation of BPN' at positions 156, 169, and 217 was also effective in creating a Carlsberg-like enzyme with respect to ester substrates. The results for TAME (**2**) and sAAPFtbe (**4**) are recorded in Table I. They show that, in contrast to the behavior observed[18] with the amide sAAPFpna (**5**), the 156, 169, 217-BPN' trimutant remains BPN'-like in its catalytic properties toward **2** and **4**, with none of the k_{cat} enhancement of the Carlsberg catalyst being conferred by the triple site directed mutagenesis. The pattern for both ester substrates is similar, with the k_{cat}'s and k_{cat}/K_m's being somewhat lower in each case for the trimutant than for wild-type BPN'. Because the thiobenzyl ester **4** is such an excellent substrate for both the BPN' and Carlsberg enzymes, the differences between the kinetic parameters are more compressed for **4** than for TAME, and the specificity constants for hydrolysis of **4** by BPN', its trimutant, and Carlsberg end up being very close to one another. This is in contrast to the situation with the *p*-nitroanilide **5** as the substrate, for which the k_{cat}/K_m's for the trimutant and Carlsberg enzymes are 7 times higher than for BPN' itself. Thus, triple mutation does not generally convert BPN' into Carlsberg-like enzyme, but appears to be a valid approach in this regard only for substrates for which acylation is the rate-determining step.

Synthetic Potential of S_1 and S_1' Mutants. These initial results of applying site-directed mutagenesis of the S_1 and S_1' pockets of subtilisin BPN' to improve binding of polar substrates at S_1 by increasing hydrogen-bonding possibilities, and for exploiting steric factor changes in S_1', provide further indications of the eventual feasibility of tailoring the specificity of an enzyme toward any preselected structure. The Met222→Phe mutant, in which the S_1' volume is reduced, could be of particular synthetic interest in that its efficiency of amide hydrolysis is sharply reduced, while its esterase activity is largely unaffected. This mutant should therefore be an excellent catalyst for the formation of peptides by coupling of amino acid ester components, in that the ester activation steps will proceed normally, but little subsequent hydrolysis of the newly formed peptide bond can occur. The yields of the desired peptide products should thus be significantly enhanced. Enzyme-catalyzed methods of peptide synthesis are receiving considerable attention.[12,27,28] The Met222→Phe mutant thus represents an attractive potential addition to chemical[27] or multiple-mutation[4i] approaches to modification of the esterase–amidase properties of serine proteases.

Experimental Section

Materials. Subtilisin Carlsberg (P5380, lot 40F-0430), L-*N*-tosylarginine methyl ester hydrochloride (**2**), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Co., St. Louis, MO, and succinyl-L-Ala-L-Ala-L-Pro-L-Phe-thiobenzyl ester (**4**) and succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (**5**) from Bachem, Inc., Torrance, CA. Succinyl-L-Ala-L-Ala-L-Pro-L-Arg-*p*-nitroanilide (**3**) was a gift from J. Burnier.[29] Subtilisin BPN' and its mutant enzymes were prepared, purified, and assayed by the literature procedures.[4a,15,16] The Tris buffer used was a 0.1 M solution of pH 8.6, containing 0.005% Tween 80.

Graphics Analyses. The selection of mutants was based on graphics analyses performed for each substrate using X-ray structural data for subtilisin BPN'[17d] and subtilisin Carlsberg,[17e,26a] with the molecular modeling performed with the MIDAS[30] program on a Silicon Graphics Iris 2400 terminal.

Rate Studies. The procedure applied was determined by the substrate used. In each case the initial rates were determined on reactions pro-

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ceeding to ≤5% total hydrolysis. Error limits for k_{cat} and K_m are ≤±10%. Each enzyme was assayed prior to preparation of stock solutions. All quantities of enzymes quoted in stock solutions refer to active enzyme content.

(A) For TAME (2). Enzyme stock solutions were prepared as follows: wild-type subtilisin BPN′ and its mutants Gly166→Ser, Gly166→Asn, Met222→Phe, 8.3, 5.1, 27.2, and 4.2 mg, respectively, per milliliter of 0.1 M Tris buffer (pH 8); BPN′ mutant Tyr217→Leu and the 156, 169, 217 trimutant, 2.7 and 0.35 mg, respectively, per milliliter of 1:1 water–propylene glycol mixture; subtilisin Carlsberg, 1.1 mg/mL of 0.01 M phosphate buffer (pH 8). These enzyme solutions were prepared immediately prior to use, except for the subtilisin mutants Tyr217→Leu and the trimutant, which were used as obtained from their purification from the fermentation procedure. The stock solution of TAME (**2**; 0.185 M) was prepared in water and was stable for several days at 4 °C.

General Procedure. To a pH-stat vessel (45 mL, Radiometer) thermostated at 25 °C were added 1 M aqueous KCl (1 mL) and the desired volume of TAME (0.4, 0.6, 0.8, 1.2, 1.6, 2.0, 4.0, 6.0, and 8.0 mL). In each case, water was added to bring the final volume to 10 mL to give final concentrations of substrate in the range 7.4×10^{-3} to 0.148 M. After equilibration for 5 min, the pH was adjusted to 8.0 with 0.2 M aqueous NaOH and the reaction initiated by addition of 50 μL of subtilisin stock solution (except with the subtilisin BPN′ mutant Tyr217→Leu and the trimutant, where 75 μL and 0.6 mL were used, respectively). Each run was performed in duplicate and the initial rates at each substrate concentration were analyzed in a Lineweaver–Burk manner by linear regression least-squares analysis. Correlation coefficients of ≥0.993 were obtained in each case. The same basic procedure was used with the subtilisin BPN′ mutants Gly166→Ser, Gly166→Asn, Met222→Phe, and Tyr217→Leu and the Glu156→Ser, Gly169→Ala, Tyr217→Leu trimutant, except that with the BPN′ mutants Gly166→Ser and →Asn, the 6.0- and 8.0-mL TAME runs were replaced by 0.2- and 1.0-mL substrate solution volumes.

(B) For sAAPRpna (3), sAAPFtbe (4), and sAAPFpna (5), the kinetic parameters were determined as follows with a Hewlett-Packard 8451A diode-array spectrophotometer.

(i) sAAPRpna (3) and sAAPFpna (5). Enzyme stock solutions of the following concentrations were prepared in the Tris buffer. For sAAPRpna (**3**), wild-type subtilisin Carlsberg and BPN′: 1×10^{-5} and 6×10^{-4} M, respectively; BPN′ mutants Gly166→Ser and Gly166→Asn, 5×10^{-4} and 6×10^{-5} M, respectively. For sAAPFpna (**5**), wild-type subtilisin Carlsberg and BPN′: 1×10^{-6} and 1×10^{-5} M, respectively; BPN′ mutants Gly166→Ser, Gly166→Asn, Met222→Phe, Tyr217→Leu, and trimutant, 2×10^{-5}, 3×10^{-5}, 4×10^{-4}, 4×10^{-6}, and 1×10^{-6} M, respectively. Stock solutions of substrates **3** (0.01 and 0.1 M) and **5** (0.02 and 0.2 M) were prepared in dimethyl sulfoxide. These are stable at room temperature for several months.

General Procedure. To a 1-cm cuvette containing 0.1 M pH 8.6 Tris buffer containing 0.005% Tween 80 (0.98 mL) and thermostated at 25 °C was added substrate **3** or **5** (10 μL). The reaction was then initiated with the addition of a subtilisin stock solution (10 μL) to give a final volume of 1 mL in the cuvette and a substrate concentration range from 10^{-4} to 10^{-3} M. The reaction progress was followed by monitoring the 410-nm (ϵ = 8800 M^{-1} cm^{-1}) absorption of *p*-nitroaniline. At pH 8.6, hydrolysis of the substrates in the absence of enzyme is negligible for the duration of the reaction. Data for the reaction progress curves were taken at regular intervals and pairs of absorbance–time values were stored in the spectrophotometer's internal memory. Upon completion of the reaction a first derivative of the data set was calculated and absorbance values were converted to product concentration. The reaction velocity vs product concentration for each progress curve was then fitted to the Michaelis–Menten equation by using a nonlinear regression algorithm.[31] For each enzyme, at least two progress curves were measured, using initial substrate concentrations differing by a factor of 10. This allowed the data to be corrected for any inhibition due to the peptide product generated during the reaction.

(ii) sAAPFtbe (4). Enzyme stock solutions of the following concentrations were prepared in the Tris buffer: wild-type subtilisin Carlsberg and BPN′, 4×10^{-8} and 6×10^{-8} M, respectively; BPN′ mutants Gly166→Ser, Gly166→Asn, Met222→Phe, Tyr217→Leu, and trimutant, 8×10^{-7}, 6×10^{-8}, 3×10^{-8}, 4×10^{-8}, and 9×10^{-8} M, respectively. Five stock solutions of substrate **4** ranging from 0.008 to 0.24 M and a 0.075 M solution of 5,5′-dithiobis(2-nitrobenzoic acid) were prepared daily in dimethyl sulfoxide.

General Procedure. To a 1-cm cuvette thermostated at 25 °C were added 0.1 M pH 8.6 Tris buffer containing 0.005% Tween 80 (0.98 mL), a stock solution of substrate **4** (5 μL), and 5,5′-dithiobis(2-nitrobenzoic acid) (5 μL). Reactions were initiated by addition of a subtilisin stock solution (10 μL) to give a final volume of 1 mL in the cuvette and a substrate concentration range from 4×10^{-5} to 1.2×10^{-3} M. Initial reaction rates for hydrolysis of substrate **4** were measured over a 15-s time period by monitoring the 412-nm (ϵ = 13600 M^{-1} cm^{-1})[32] absorption of the 3-carboxy-4-nitrothiophenoxide anion. The final enzyme concentration in the reaction mixture was adjusted such that substrate depletion for the duration of the measurement remained below 5% of the initial substrate concentration. Duplicate runs were performed and the initial rates were corrected for substrate hydrolysis in the absence of enzyme. The kinetic parameters were then calculated in the Michaelis–Menten manner for each run from the average of five initial reaction rates measured at five different substrate concentrations.

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Registry No. **1**, 3618-96-0; **2**, 901-47-3; **3**, 131068-47-8; **4**, 131068-48-9; **5**, 70967-97-4; Gly, 56-40-6; L-Asn, 70-47-3; L-Ser, 56-45-1; L-Met, 63-68-3; L-Phe, 63-91-2; L-Tyr, 60-18-4; L-Leu, 61-90-5; L-Glu, 56-86-0; L-Ala, 56-41-7; subtilisin BPN′, 9014-01-1.

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