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Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering

(site-specific mutagenesis/subtilisin/molecular evolution)

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ABSTRACT **The *Bacillus licheniformis* and *Bacillus amyloliquefaciens* subtilisins differ by 31% in protein sequence and by factors of >60 in catalytic efficiency, k_{cat}/K_m, toward various substrates. Despite large differences in sequence and substrate specificity for these serine proteases, only two amino acid substitutions (residues 156 and 217) occur within 4 Å (contact distance) of modeled substrates, and a third substitution (residue 169) is within 7 Å. The three *B. licheniformis* substitutions (Ser-156/Ala-169/Leu-217) were introduced into the wild-type *B. amyloliquefaciens* subtilisin (Glu-156/Gly-169/Tyr-217) by site-directed mutagenesis. The substrate specificity of the triple mutant approaches that of *B. licheniformis* enzyme when assayed with seven different substrates that vary in charge, size, and hydrophobicity. Thus, specificity properties of distantly related and functionally divergent enzymes can be exchanged by limited amino acid replacements, in this case representing <4% of the sequence differences.**

The subtilisins are a homologous family of serine proteases ($M_r \simeq 27{,}000$) that are found in species ranging from bacilli to fungi (1, 2). Although these enzymes have generally broad substrate specificity, they can differ dramatically from each other in catalytic efficiency (k_{cat}/K_m) against a given substrate (1). For example, we show that the *Bacillus amyloliquefaciens* and the *Bacillus licheniformis* subtilisins differ by more than sixty times in catalytic efficiency toward substrates containing a glutamate residue in the P_1 position‡ and by factors >10 with several other neutral and hydrophobic substrates. There are 86 amino acid differences of 275 amino acids (including a deletion) between the two enzymes (4, 5); yet only two substitutions occur for residues that can directly contact a bound substrate.

Previous protein engineering studies of subtilisin (6, 7), tyrosyl-tRNA synthetase (8), trypsin (9), carboxypeptidase Y (10), and alcohol dehydrogenase (11) have shown that substrate specificity can be altered by the modification of residues in direct contact with a bound substrate. Here we evaluate the extent to which two residues in direct contact with a model substrate and a third residue within 7 Å from a model substrate can account for the specificity differences between the two wild-type subtilisins.

Site-directed mutagenesis of the cloned *B. amyloliquefaciens* subtilisin gene (4) was used to produce the *B. licheniformis* binding site sequence in the *B. amyloliquefaciens* enzyme. These studies demonstrate the feasibility for "recruiting" diverse ligand-binding properties from one member of a homologous gene family into another member by limited amino acid substitutions in the immediate vicinity of a bound substrate.

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MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by the Organic Chemistry Department at Genentech using phosphoramidite chemistry and purification by PAGE. Synthetic peptide substrates containing *p*-nitroanilide reporter groups were synthesized by J. Burnier (Genentech), except for succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, which was from Sigma. All enzymes for DNA manipulations were from New England Biolabs, except for *Escherichia coli* DNA polymerase I large fragment (Klenow), which was from Boehringer Mannheim. Subtilisin Carlsberg from *B. licheniformis* was from Sigma. *E. coli* strains JM101 and MM294 were used for phage template and plasmid preparations, respectively.

Cassette Mutagenesis, Plasmid Constructions, and Mutant Characterization. The preparation of Glu-156→Ser-156 in the cloned *B. amyloliquefaciens* gene (4) has been described (7). The mutation of Tyr-217→Leu-217 used a cassette mutagenesis strategy (12). An oligonucleotide starting at codon 211 in the subtilisin gene and having the sequence 5′-GA-AAC-AAA-TAC-GGC-GCC-TAC-GG-ATA-TCA-ATG-GCA-T was used for site-directed mutagenesis of an M13mp11SUBT template (13). This mutation, Δ217, created unique *Nar* I and *Eco*RV restriction sites (underlined) as well as silent mutations (bold type) at codons 215, 216, and 220. Isolation of the Δ217 mutant was simplified by digestion of M13RF pool with *Eco*RV, purification of the linearized DNA by PAGE, and self-ligation (14). The 1.5-kb *Eco*RI–*Bam*HI subtilisin gene fragment from Δ217 was ligated into a similarly cut pBS42 vector (15) fragment to give pΔ217. The pΔ217 was digested with *Nar* I and *Eco*RV, and the cut vector was purified (16). A synthetic DNA cassette having the sequence

5′-C-GCG-TTG-AAC-GGT-AC
GC-AAC-TTG-CCA-TG-5′

was ligated into the gap to recreate the wild-type sequence except at codon 217 (underlined), where Tyr-217 (TAC) was converted to Leu-217 (TTG). The DNA sequence for Tyr-217→Leu-217 was confirmed by dideoxy chain-termination sequencing (17) directly on the plasmid (18). The double mutant Ser-156/Leu-217 was prepared by ligation of DNA fragments from the single mutants Glu-156→Ser-156 and Tyr-217→Leu-217 at a common *Ava* II restriction site located at codon 193. The Gly-169→Ala-169 mutation was prepared by cassette mutagenesis (12) and introduced into the Ser-156/Leu-217 mutant to produce Ser-156/Ala-169/Leu-217.

Mutant and wild-type *B. amyloliquefaciens* subtilisin genes were expressed in a protease-deficient strain of *Bacillus subtilis* BG2036 (19), and the secreted mutant and wild-type enzymes were purified (20). Kinetic determinations were

‡Peptide substrate nomenclature can be represented as

$$NH_2—P_n\ldots P_2—P_1—\overset{\overset{O}{\|}}{C}—\overset{\overset{H}{|}}{N}—P_{1'}—P_{2'}\ldots P_n—COOH$$

where the scissile bond is between the P_1 and $P_{1'}$ substrate residues (3).

Table 1. Identification of residues within 4 Å of a model substrate bound to subtilisin

Residue number*	*B. amyloliquefaciens*	*B. licheniformis*	Substrate residue contacted†
64	His	His	P_1, P_2
96	Leu	Leu	P_2, P_4
100	Gly	Gly	P_2, P_3, P_4
101	Ser	Ser	P_4
102	Gly	Gly	P_4
125	Ser	Ser	P_1, P_2
126	Leu	Leu	P_3
127	Gly	Gly	P_1, P_3, P_4
152	Ala	Ala	P_1
154	Gly	Gly	P_1
155	Asn	Asn	P_1, pNA
156	**Glu**	**Ser**	**P_1**
166	Gly	Gly	P_1
217	**Tyr**	**Leu**	**pNA**
218	Asn	Asn	pNA
219	Gly	Gly	P_1
220	Thr	Thr	P_1
221	Ser	Ser	P_1
222	Met	Met	pNA

The main-chain coordinates for the substrate were taken from a preliminary 2.0 Å x-ray crystal structure of a subtilisin–product complex (R. Bott and M. Ultsch, unpublished results). The substrate sequence was Ala-Ala-Pro-Xaa-*p*-nitroanilide, where Xaa was either tyrosine or lysine. The lysine and tyrosine P_1 side chains were modeled to occupy slightly different positions in the P_1 cleft consistent with previous x-ray crystallographic studies (21, 24–26). The *p*-nitroanilide moiety (pNA) was built using the FRODO software package (27) and modeled to fit as closely as possible the contacts made by a similarly bound $P_{1'}$ residue. The side chains for Ala-Ala-Pro were fixed by main-chain atom coordinates. Model building was done on an Evans and Sutherland PS300 computer using the FRODO software package. Residues that are contacted by these substrates are a narrower set than those contacted by longer, more extensively substituted substrates or by protease inhibitors (21–26).

*Numbers refer to the mature *B. amyloliquefaciens* subtilisin sequence (4). The *B. licheniformis* enzyme is aligned for maximal homology.

†Substrate residues Ala-Ala-Pro-Xaa-pNA correspond to P_4-P_3-P_2-P_1-pNA, respectively (3).

made in 0.1 M Tris, pH 8.6, at 25°C, and reaction progress curves were analyzed as described (20). Briefly, over thirty data points were collected from the initial phase of each progress curve for the hydrolysis reaction to determine k_{cat} and K_m. Kinetic values were averaged from at least four separate progress curves for reactions at two different substrate concentrations.

RESULTS

Structural Considerations. X-ray crystallographic studies of *B. amyloliquefaciens* subtilisin containing bound products (ref. 21; R. Bott and M. Ultsch, personal communication), protease inhibitors (22, 23), transition-state analogues (24), and covalently bound peptide affinity labels (25, 26) delineate an extended substrate binding cleft. The x-ray coordinates for bound product (Fig. 1) were used to identify enzyme residues capable of contacting substrates having the form Ala-Ala-Pro-Xaa-*p*-nitroanilide, where Xaa was either tyrosine or lysine (Table 1). Substrates having this structure are available and have been extensively used for probing changes in specificity for the P_1 amino acid (3) that result from mutagenesis of the P_1 substrate binding cleft in subtilisin (6, 7).

Although the subtilisins from *B. amyloliquefaciens* and *B. licheniformis* are only 69% identical, the positions of the α-carbon atoms from the x-ray structures of these molecules can be superimposed to within about 0.5 Å rms (28). Furthermore, the modes of substrate binding are essentially the same for these two enzymes based on a comparison between x-ray structures of the *B. amyloliquefaciens* (22, 23) and *B. licheniformis* (29) enzymes containing bound protease inhibitors. For 19 residues that are within van der Waals contact distance of the model substrates (Table 1), the only differences between the *B. amyloliquefaciens* and *B. licheniformis* enzyme are at residues 156 and 217. The Glu-156 side chain in the *B. amyloliquefaciens* enzyme forms a salt bridge with lysine P_1 substrate side chain (26); the Tyr-217 side chain can contact the $P_{1'}$ residue (25) and therefore the *p*-nitroanilide leaving group (Fig. 1). Position 169 is a glycine in *B. amyloliquefaciens* subtilisin, and we expect an alanine substitution (as found in the *B. licheniformis* enzyme) to be within 7 Å of a model tyrosine P_1 substrate.

Incorporation of *B. licheniformis* Substrate Specificity into *B. amyloliquefaciens* Subtilisin. To assess the functional consequences of these structural differences in the substrate-binding site mutations of Glu-156→Ser-156, Tyr-217→Leu-217, Gly-169→Ala-169, a double mutant Ser-156/Leu-217, and a triple mutant Ser-156/Ala-169/Leu-217 were prepared by cassette mutagenesis (12) in the *B. amyloliquefaciens* subtilisin gene. Purified mutant subtilisins were analyzed for substrate specificity and compared with the two wild-type

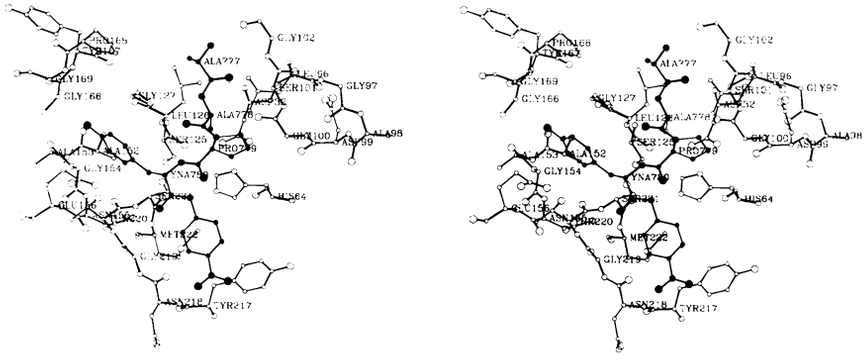

FIG. 1. Stereoview of a model substrate, Ala-Ala-Pro-Tyr-*p*-nitroanilide, bound to *B. amyloliquefaciens* subtilisin. The model is based on a preliminary 2.0 Å x-ray structure of a product bound to subtilisin (R. Bott and M. Ultsch, personal communication). The main-chain coordinates and general features of this model are in close agreement with previous substrate models (21, 24–26). The α-carbon atoms are labeled for all potential contact residues (Table 1) including the nonconserved residues (Glu-156, Gly-169, and Tyr-217), the catalytic triad (Asp-32, His-64, and Ser-221), and the substrate residues (filled atoms) from P_4 to P_1 [Ala-777, Ala-778, Pro-779, and Tyr-780-*p*-nitroanilide (YNA)].

Table 2. Kinetics of wild-type and mutant *B. amyloliquefaciens* subtilisins and wild-type *B. licheniformis* subtilisin against substrates differing in P_1 residue

	P_1 residue, $k_{cat}/K_m \times 10^{-3}$ (k_{cat}; $K_m \times 10^3$)*						
	Glu	Gln	Ala	Lys	Met	Phe	Tyr
B. amyloliquefaciens							
Wild-type†	0.035	8.7	14	40	140	360	1400
	(0.18; 5.2)	(3.3; 0.38)	(1.9; 0.15)	(30; 0.75)	(13; 0.090)	(50; 0.14)	(25; 0.018)
Ser-156‡	0.39	24	18	2.3	590	1000	2100
	(0.47; 1.2)	(3.9; 0.16)	(2.0; 0.11)	(4.7; 2.0)	(11; 0.019)	(37; 0.035)	(18; 0.0086)
Leu-217	0.15	16	28	46	240	590	1700
	(0.68; 4.4)	(14; 0.85)	(8.6; 0.31)	(88; 1.9)	(73; 0.30)	(280; 0.47)	(230; 0.14)
Ala-169	0.10	15	22	66	270	1100	2800
	(0.22; 2.1)	(3.9; 0.26)	(2.2; 0.099)	(26; 0.40)	(12; 0.043)	(52; 0.050)	(23; 0.0084)
Ser-156/Leu-217	0.58	44	35	4.2	920	1500	2400
	(1.2; 2.0)	(21; 0.47)	(8.9; 0.26)	(14; 3.3)	(89; 0.097)	(300; 0.20)	(180; 0.079)
Ser-156/Ala-169/	1.1	59	40	9.2	1500	2600	3800
Leu-217	(1.3; 1.3)	(18; 0.31)	(6.1; 0.15)	(15; 1.6)	(76; 0.050)	(250; 0.094)	(140; 0.036)
B. licheniformis							
Wild-type	2.2	160	86	16	2000	2500	2900
	(3.7; 1.7)	(46; 0.29)	(14; 0.16)	(68; 4.3)	(87; 0.044)	(510; 0.20)	(230; 0.079)

Substrates have the form succinyl-Ala-Ala-Pro-Xaa-*p*-nitroanilide, where Xaa is the indicated P_1 amino acid (3).
*Variation in the values of k_{cat} (sec^{-1}), K_m (M), and k_{cat}/K_m (sec^{-1}, M^{-1}) were below 5%.
†Kinetic values were taken from Estell *et al.* (6) or Wells *et al.* (7).
‡Kinetic values for glucine, glutamine, lysine, and methionine substrates were taken from Wells *et al.* (7).

enzymes (Table 2)§. To facilitate comparisons between variant enzymes and to scale the data in proportion to free energy, the logarithms of k_{cat} and logarithms of $1/K_m$ are plotted in bar graph form for each substrate (Fig. 2).

The k_{cat}/K_m ratios of the *B. licheniformis* subtilisin exceed those of the *B. amyloliquefaciens* enzyme by factors ranging from 2 to 60 on all P_1 substrates except for lysine. The k_{cat} term is greater for the *B. licheniformis* on all P_1 substrates, and smaller differences between these enzymes appear in K_m. The specificity profile of Ser-156/Ala-169/Leu-217 is much closer to the *B. licheniformis* enzyme than it is to its parent *B. amyloliquefaciens* subtilisin. The k_{cat}/K_m ratios for the triple mutant are slightly smaller than for the *B. licheniformis* enzyme on small and charged P_1 substrates (to a factor of three times k_{cat}/K_m on the glutamine substrate) but exceed the *B. licheniformis* enzyme on large hydrophobic substrates. The values of k_{cat} and K_m are similarly close between these two enzymes. The Ser-156/Leu-217 double mutant has k_{cat}/K_m ratios that are close to the triple mutant but intermediate between the triple mutant and the wild-type *B. amyloliquefaciens* except for the lysine substrate.

The kinetic effects of the multiple mutants can be dissected by analysis of the single mutants. The Glu-156→Ser-156 mutation causes the P_1 substrate preference to resemble that of the *B. licheniformis* subtilisin. There is a prominent increase in the catalytic efficiency toward the glutamic acid P_1 substrate and a decrease in efficiency toward the lysine P_1 substrate (Table 2 and Fig. 2). Smaller improvements in k_{cat}/K_m appear with the glutamine and hydrophobic substrates for Ser-156. The changes in k_{cat}/K_m are predominantly caused by alterations in the K_m term.

In contrast to the position 156 mutation, the Tyr-217→Leu-217 change causes no alteration in the order of substrate preference from *B. amyloliquefaciens* wild-type. However, all substrates show an increase in k_{cat}/K_m that is dominated by increases in the k_{cat} values that are similar to the k_{cat} values for the *B. licheniformis* enzyme. Like Leu-217, the Gly-169→Ala-169 mutation causes a general increase in k_{cat}/K_m ranging from one and one-half to three times for all substrates. However, unlike Leu-217, the changes in k_{cat}/K_m are dominated by a lowering of the K_m value.

In general, the change in substrate preference ($\Delta\log k_{cat}/K_m$) for the multiple mutants relative to the wild-type *B. amyloliquefaciens* enzyme is nearly the sum of the component single mutants (Fig. 3). The sum of the change in substrate preference for Ser-156 and Leu-217 is within 0.05 log k_{cat}/K_m units of the double mutant, Ser-156/Leu-217, except on the glutamic acid and lysine substrates where the sum of the single mutants is larger than the double mutant by 0.48 and 0.19, respectively. The change in substrate preference for Ser-156/Ala-169/Leu-217 nearly equals the sum of values for Ala-169 plus the double mutant, Ser-156/Leu-217.

DISCUSSION

These data suggest that much of the difference in kinetics between the *B. amyloliquefaciens* and *B. licheniformis* subtilisins results from two substitutions at positions 156 and 217 that are within van der Waals contact of the substrate. The Glu-156→Ser-156 mutation in the *B. amyloliquefaciens* enzyme produces the same order of P_1 substrate preference as the *B. licheniformis* enzyme (Fig. 2) and is consistent with position 156 being in the P_1 binding site (Table 1). The Glu-156→Ser-156 mutation improves binding of a negatively charged substrate and decreases binding of positively charged substrate in a manner that is similar to other electrostatic substitutions made in the P_1 binding site (7). The Tyr-217→Leu-217 mutation has no effect on the P_1 substrate preference (Fig. 2), which is consistent with position 217 not being in the P_1 binding site. The increase in k_{cat} for Leu-217, that is comparable to the k_{cat} value for the *B. licheniformis* enzyme, may result from reduced steric hindrance with the *p*-nitroanilide leaving group of the substrate in the transition-state complex. These results may be expected because previous studies (5–11) have shown that large effects occur on substrate specificity by modification of substrate contact residues.

§The k_{cat} represents the turnover number, and the K_m approximates the dissociation constant for the enzyme–substrate complex K_s (14, 30). The k_{cat}/K_m ratio, a measure of the catalytic efficiency, is the second-order rate constant for catalysis of substrate to product. The logarithm of k_{cat}/K_m is proportional to the free-energy difference ($\Delta G_T^{\neq}$) between the free enzyme and substrate and the transition-state complex (31).

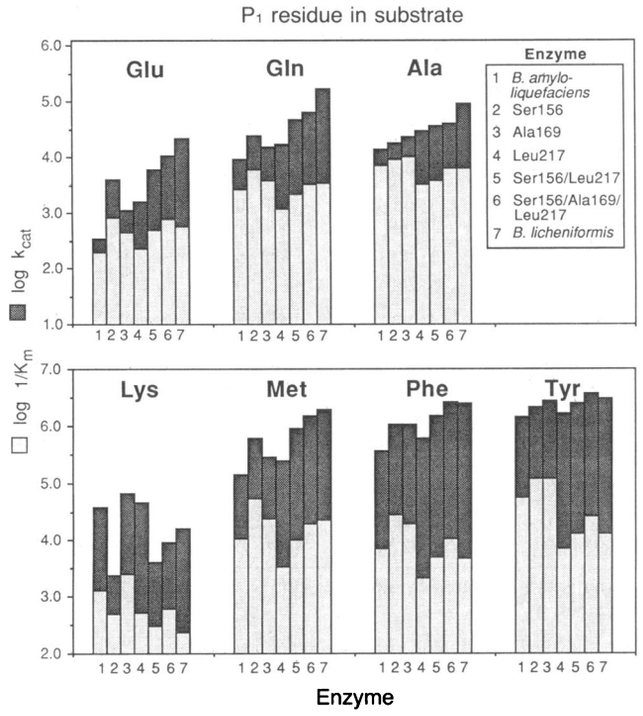

FIG. 2. Substrate-specificity profiles of mutant and wild-type *B. amyloliquefaciens* and wild-type *B. licheniformis* subtilisin. Each full bar represents the logarithm of k_{cat}/K_m for the P_1 substrate indicated below it. The upper bar represents logarithm k_{cat} values, and the lower bar represents logarithm $1/K_m$ values. Because k_{cat} values for the Glu P_1 substrate are <1, the data for this substrate has been scaled such that the full bar represents (log k_{cat}/K_m) + 1 and the upper bar represents (log k_{cat}) + 1. All substrates have the form succinyl-Ala-Ala-Pro-Xaa-*p*-nitroanilide, where Xaa is the P_1 amino acid (3).‡ Enzymes are as follows: *B. amyloliquefaciens* wild-type (1); Ser-156-substituted, 2; Leu-217-substituted, 3; Ala-169-substituted, 4; Ser-156/Leu-217-substituted, 5; Ser-156/Ala-169/Leu-217 mutant, 6; and *B. licheniformis* wild-type subtilisin, 7. Data is taken from Table 2.

The Gly-169→Ala-169 mutation shows that substantial effects on substrate binding can be produced by substitution of a residue just outside of direct substrate contact distance. The position 169 mutation has no effect on the order of substrate preference but causes a general lowering of K_m for all substrates tested. A β-methyl group from a model of an alanine substitution at position 169 (data not shown) is outside direct contact distance of a tyrosine P_1 substrate (Fig. 1) but is within 4 Å of the carbonyl oxygen at position 152. In the *B. licheniformis* structure (28) this contact is accommodated by a slight shift (<0.3 Å) in the position of the α-carbon atom at position 169. It is possible that the effect of the Ala-169 mutation on P_1 substrate binding is mediated indirectly by contact with residue 152 and/or by a change in the hydrophobicity of the P_1 binding site that has been shown to be important in P_1 substrate binding (6). Although the triple mutant is within a factor of three of the *B. licheniformis* wild-type in k_{cat}/K_m on all substrates, the residual differences must be accounted for by other residues also outside direct contact distance of the substrate. The basis for these small, but significant, indirect effects on substrate specificity awaits further structural and kinetic analysis. Modeling of these mutations does not predict substantial structural alterations and, indeed, x-ray structural determinations of more than twenty point mutants at other functionally important residues in subtilisin reveal only minor structural perturbations except for the direct amino acid side-chain substitutions (refs. 32 and 33; R. Bott, B. Katz, M. Ultsch, and T. Kossiakoff, personal communication).

In general, the sum of the kinetic effects for the single mutants, Ser-156 and Leu-217, gives a reasonable approximation of the Ser-156/Leu-217 double mutant, and the sum of Ala-169 plus the double mutant gives a fair approximation of the triple mutant. These results suggest that these sites function generally in an independent fashion. Similar additive and independent kinetic effects for single mutations have also been found for double mutants in tyrosyl-tRNA synthetase (34). The basis for slight deviations from additivity, observed largely for the charged substrates, may reflect differences in substrate binding as has been shown crystallographically for phenylalanine (25) and lysine (26) P_1 substrate analogues.

Exchange of single variant residues near the active site between two functionally similar and highly homologous tyrosyl-tRNA synthetases (99%) produces single mutants that retain essentially wild-type function (35). It is significant that we show that natural substitutions in the substrate binding can produce binding specificity nearly equivalent to a natural variant that is considerably different from the parent.

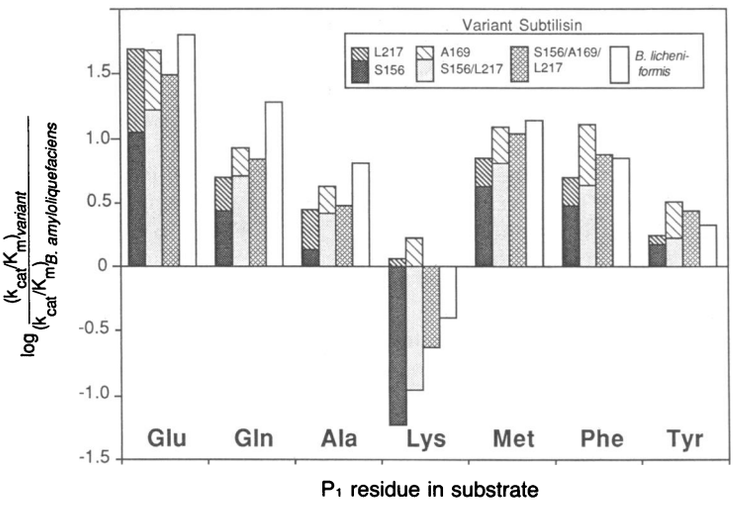

FIG. 3. Effects of variant subtilisins on substrate preference relative to the *B. amyloliquefaciens* wild-type subtilisin. Difference in logarithm k_{cat}/K_m value for each enzyme on each substrate was calculated versus wild-type *B. amyloliquefaciens* subtilisin. The Δlogarithm k_{cat}/K_m values for the single mutants Ser-156, Leu-217, and Ala-169 are stacked to evaluate the additivity to the relevant double and triple mutants.

Previous studies have shown that specificity properties from a heterologous source can be recruited by exchange of antigen binding loops (36), DNA recognition helices (37, 38), or whole domains (39) involved in ligand binding. For example, fusion of hapten binding-site loops from a 4-hydroxy-3-nitrophenyl-acetylcaproic acid-specific mouse monoclonal antibody into a human monoclonal antibody (36) produces a human–mouse hybrid antibody capable of binding the dinitrophenyl hapten. These loop, helix, and domain exchange experiments demonstrate the independent nature of their function. It is notable that subtilisin is a single-domain protein and that the intradomain function can be similarly exchanged by selective amino acid substitution.

Recruitment of natural variant enzyme properties by protein engineering not only provides direct information on the function of particular amino acids, but it may provide a practical solution to problems associated with cloning and expression of a desirable natural variant. Levels of expression among homologous proteins (40) can vary dramatically in the same heterologous host. Thus, in cases where a strong structure–function database exists, it may be simpler to recruit the function of a natural variant by selective amino acid substitution than to clone and express the natural variant. More importantly, within a homologous gene family protein recruitment should permit the production of hybrid enzymes and proteins that exhibit the most desirable properties of the family, such as thermal stability, pH profile, and substrate specificity.

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