Random Mutagenesis of the Substrate-Binding Site of a Serine Protease Can Generate Enzymes with Increased Activities and Altered Primary Specificities†

Lloyd D. Graham,* Kevin D. Haggett, Philip A. Jennings,‡ Darren S. Le Brocque, and Robert G. Whittaker

C.S.I.R.O. Division of Biomolecular Engineering, P.O. Box 184, North Ryde, New South Wales 2113, Australia

Paul A. Schober

Peptide Technology Ltd., P.O. Box 444, Dee Why, New South Wales 2099, Australia

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ABSTRACT: In the past, several point mutations have been introduced individually into the substrate-binding site of α-lytic protease (EC 3.4.21.12) and shown to affect its specificity in a predictable manner [Bone, R., Silen, J. L., & Agard, D. A. (1989) *Nature 339*, 191–195]. One of the resulting mutant enzymes (Met190Ala in the numbering system of Fujinaga et al.) [Fujinaga, M., Delbaere, L. T. J., Brayer, G. D., & James, M. N. G. (1985) *J. Mol. Biol. 183*, 479–502] cleaves at large hydrophobic residues. We chose this enzyme as the parent for a library of mutant proteases. The library was constructed by effecting combinatorial random substitution of up to four other residues (Gly191, Arg192, Met213, and Val218) thought likely to influence the primary specificity of the protease. Active enzymes in the library were screened with a range of synthetic substrates (encompassing 19 different amino acids in the P_1 position) in order to evaluate their primary cleavage preferences. The amino acid sequences of active mutants revealed a strong preference for the replacement of Met213 with a His residue. This substitution also had the greatest observed effect on specificity, conferring a greatly increased and, in some cases, dominant ability to cleave at His residues in synthetic amide substrates. Mutant enzymes with greatly increased proteolytic activity were also found in the library.

The aim of enzyme engineering is to generate proteins with new and useful functional properties. The ability to alter the substrate specificity of an enzyme would be particularly advantageous, and many attempts have been made to change this property using site-directed mutagenesis. While knowledge-based engineering has been rewarding, its success has been limited by large gaps in the current understanding of protein folding and protein–ligand interactions (e.g., Craik et al. (1985), Wilks et al. (1988, 1990), Rutter et al. (1987), Henderson et al. (1991), and Alexander et al. (1991)). In a complementary approach, combinatorial random substitution has been used to generate libraries of variant proteins that contain a proportion of functional mutants (Reidhaar-Olson & Sauer, 1988; Lim & Sauer, 1989, 1991), these often having enzymatic properties different from those of the parent (Oliphant & Struhl, 1989; Dunn et al., 1988; Dunn & Jennings, 1992). Proteases constitute an industrially useful group of enzymes for which substrate specificity is of the utmost importance. While purely rational approaches have been used to change the substrate specificities of several proteases (e.g., Craik et al. (1985), Wells and Estell (1988), Beaumont et al. (1992), Wilson et al. (1991), Carter and Wells (1987), Khouri et al. (1991), and Hedstrom et al. (1992)), there are few examples of combinatorial random substitution being applied to this task (Evnin et al., 1990; Teplyakov et al., 1992). To determine whether proteases of novel specificity could be generated in this way, we simultaneously randomized several of the amino acid residues thought to influence the primary specificity of a serine protease.

The protease chosen for manipulation was α-lytic protease, a serine protease secreted by the soil bacterium *Lysobacter enzymogenes* (Whitaker, 1970). The structure of the protease, which has been determined to high resolution (Fujinaga et al., 1985), shows the enzyme to be a chymotrypsin homologue. Like elastase, α-lytic protease preferentially cleaves on the C-terminal side of small uncharged residues such as Ala (Kaplan et al., 1970; Bauer et al., 1981). The residues responsible for this primary cleavage specificity may be deduced from several crystallographic structures of enzyme–inhibitor complexes (Bone et al., 1989a, 1991a,b). Small amino acid residues such as Ala are preferred at the scissile bond because the pocket that accommodates the substrate P_1 residue is shallow,[1] largely due to the presence of two bulky Met residues at this subsite (Met190 and Met213, in the revised numbering system of Fujinaga et al. (1985)). Replacement of either of these with Ala residues results in a mutant enzyme that prefers large hydrophobic residues in the P_1 position (Bone et al., 1989b). Since we felt that an enlarged S_1 pocket allowed a greater scope for substitution at other positions contributing to this subsite, we selected one of these mutants (Met190Ala; see Figure 1) as the parent for a protease library.

Crystallographic structures reveal that the S_1 pocket of the Met190Ala mutant (Bone et al., 1991a) is largely defined by

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* Author to whom correspondence should be addressed.

‡ Present address: Chief, C.S.I.R.O. Division of Tropical Animal Production, Private Bag No. 3, P.O. Indooroopilly, Queensland 4068, Australia.

[1] In the nomenclature of Schecter and Berger (1967), the substrate residue immediately N-terminal to the scissile bond is termed the P_1 residue, the one before that is the P_2 residue, and so on. Residues C-terminal to the scissile bond are termed P_1', P_2', etc. Cognate residue-binding subsites in the protease are identified by use of the letter S in place of P, e.g., S_1', S_2', etc.

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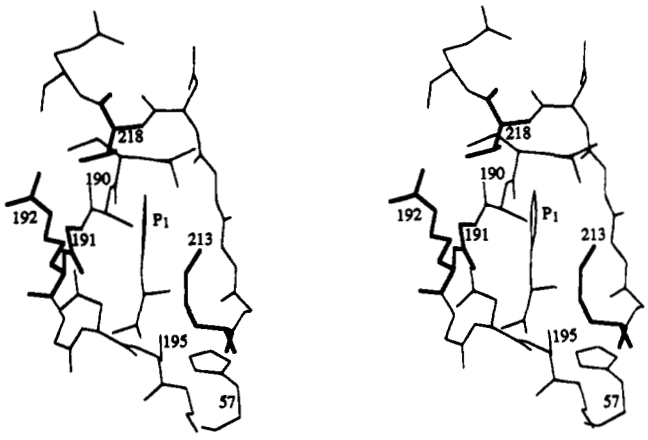

FIGURE 1: Stereoview of the active site of the Met190Ala mutant of α-lytic protease. This enzyme was used as the parent for the library described in this article. The viewer looks directly into the S_1 binding pocket, in which a substrate P_1 side chain (Phe) is shown; the four residues in the protein targeted for random substitution are shown by thick lines. This diagram is derived from file 1P08 in the Brookhaven Protein Data Bank (Abola et al., 1987).

two segments of polypeptide, which we labeled A and B:

segment A segment B

190 192 194 213 215 218 220 226

—AlaGlyArg***GlyAsp*** — //—Met***SerGlyGly***Asn*Val* —*Asn* — *Ser* —

* * * *

Structural information and sequence homology with related enzymes indicate that the residues shown in boldface italics are essential to the overall structure of the protein and suggest that those shown in lightface italics may also be important. Position 190 and our reason for choosing Ala at this position have been discussed above. We did not consider any of the four remaining residues at the S_1 subsite (marked with asterisks) to be essential to the overall architecture of the protein. Our library was therefore constructed using targeted random mutagenesis to effect simultaneous random substitutions at some or all of the positions 191, 192, 213, and 218 (Figure 1).

EXPERIMENTAL PROCEDURES

Materials. The limited range of commercially available amino acid–chromophore conjugates meant that our suite of synthetic substrates could not all be made with the same leaving group. In consequence, screening was done using some chromogenic substrates of the form *Suc*AlaProXaa-pNa[2] and some fluorogenic substrates of the form *Suc*AlaProXaa-βNap (where Xaa denotes any natural amino acid residue or D-Ala). With the exception of *Suc*AlaProAla-pNa (Peptide Institute, Japan), these substrates were synthesized from (*tert*-butyloxycarbonyl)-AlaPro and the appropriate amino acid-pNa or -βNap, succinylated using succinic anhydride, and purified by reversed-phase HPLC. In each case, the composition was confirmed by amino acid analysis. Peptides with P_1 residues as follows were verified as enzyme substrates by digestion with the enzyme named: Ala, D-Ala, Gly, Leu, Met, and Val, wild-type α-lytic protease; Arg, bovine trypsin (Sigma); Asp and Glu, endoprotease Glu-C (Boehringer Mannheim); Phe, α-chymotrypsin (Sigma); and Pro, proline-specific endoprotease (Seikagaku Kogyo, Japan). Substrate purity was estimated in each case from the total amount of *p*-nitroaniline released. Any additional peptide substrates were purchased from Bachem. Molecular biology and related procedures were according to Sambrook et al. (1989), unless otherwise specified.

Cloning and Sequencing. We used PCR (95 °C, 30 s; 50 °C, 30 s; 72 °C, 1.5 min; 30 cycles) with primers having sequences 5′-ATTTATGCATGCCGATCAGGTCGATCCTCAG-3′ and 5′-TCTCATCGATCTATTAACCCGTGACCAGGCTCAGGCC-3′ in the presence of 7-deaza-2′-dGTP (Innis, 1990) to amplify the segment encoding the pro and enzymatic regions of the α-lytic protease gene (Silen et al., 1988) from *L. enzymogenes* genomic DNA. After it was end-filled with Klenow, the blunt-ended fragment was cloned into Bluescript (Stratagene Inc.). Dideoxy sequencing of DNA was done with Taq polymerase using end-labeled primers in the presence of 7-deaza-2′-dGTP; the best results were obtained when undenatured DNA (250 ng) was used as the template for the Promega "fmol" linear amplification method (95 °C, 30 s; 70 °C, 30 s; 30 cycles). After verification of the cloned sequence, a fragment encoding the *pelB* secretion leader (Lei et al., 1987) was inserted upstream of the protease gene, and the resulting cassette was ligated into pBS(+) (Stratagene Inc.) so that it would be expressed by the indigenous *lac* promoter (Silen et al., 1989; K. D. Haggett, unpublished results).

Mutagenesis. A *Bal*1–*Eco*R1 fragment (0.36 kb) that encoded the active-site region of the protease was subcloned into M13mp19, and the oligonucleotide 5′-CCGAATCGCCGCGGCCCGCGCAGGCGTTGC-3′ was used (Nakamaye & Eckstein, 1986; Sayers et al., 1988) to introduce the mutation Met190Ala. Further mutagenesis of the Met190Ala mutant was effected using the oligonucleotide 5′-TTGCCGTTGGACTGTGAGTTGCCGCTGCTCATCACGCCCTGC-3′ to introduce an *EcoB* restriction site ($TGAN_8TGCT$) into the region encoding the S_1 pocket, which resulted in two amino acid substitutions (Gly215Ser and Val218Ser). Replacement of the corresponding portion in the pBS(+) expression construct with the mutated *Bal*1–*Eco*R1 fragment gave us the construct to be used as the template for the library. The *EcoB* site served as a genetic marker and later allowed us to select against the unmutated template sequence (Carter, 1991).

Oligo-A, which had the sequence 5′-CACGAACCGCCCGAATCGCC$^{G}/_{C}$NN$^{G}/_{C}$NNCGCGCAGGCGTTGCCCTGG-3′, was designed to bind to the sense strand encoding segment A in the library template and to effect random substitutions at positions 191 and 192. Oligo-B, which had the sequence 5′-CAGTTGTTGCCGTTGGACTG$^{G}/_{C}$NNGTTGCCGCCGCT$^{G}/_{C}$NNCACGCCCTGCGCCTGGCCGG-3′, was designed to bind to the sense strand encoding segment B in the library template and to effect random substitutions at positions 213 and 218, as well as to restore Gly at position 215. The codon format $NN^{G}/_{C}$ ensured that each amino acid could be represented and eliminated two of the three stop codons (Dunn et al., 1988).

Mutagenic priming by oligo-B destroyed the *EcoB* selection sequence, allowing direct selection for incorporation of this oligonucleotide. To effect targeted random mutagenesis, existing methods (Foss & McClain, 1987; Kramer & Fritz, 1987; Inouye & Inouye, 1991) were adapted to maximize coupled priming. A sample of double-stranded library template construct was digested with *Eco*N1 and *Eco*R1 to remove a small region (0.2 kb) around the target sequence (i.e., the DNA encoding segments A and B), while another sample was cleaved at one position only using *Hin*dIII. About 460 ng of the *Eco*N1–*Eco*R1 fragment (4.3 kb) and 50 ng of the *Hin*dIII fragment were denatured together (100 °C, 3

[2] Abbreviations: *Suc*, succinyl; pNa, *p*-nitroanilide; βNap, β-naphthylamide.

min) and reannealed (65 °C, 10 min and then cooled to 0 °C over 30 min) in 10 μL of 100 mM Tris containing 500 mM KCl, pH 8, to generate approximately 50 ng of gapped duplex. At this stage, 8.5 pmol of phosphorylated oligo-A and 8.5 pmol of phosphorylated oligo-B were added, and the mixture was incubated at 65 °C for 3 min and then cooled to 0 °C over 30 min. T4 DNA polymerase (0.17 unit) and *E. coli* DNA ligase (0.85 unit) were added, and extension/ligation was achieved by incubation in 22.4 μL of 50 mM Tris containing 18 μg/mL T4 Gene 32 protein, 0.19 mM NAD^+, 0.23 mM each dNTP, 4.7 mM dithiothreitol, 35 mM KCl, 56 mM ammonium acetate, and 4.7 mM $MgCl_2$, pH 8, at 28 °C for 3.3 h. The DNA was ethanol precipitated and electroporated into *E. coli* HB2155 (Carter et al., 1985) using a BioRad gene pulser (five 40-μL aliquots at 2.5 kV, 25 μF, 600 Ω, with an electrode gap of 0.2 cm). Constructs retaining the *EcoB* site (i.e., those that had failed to incorporate oligo-B) were destroyed in this *E. coli B* strain. Following discharge, cells were allowed to recover in 1 mL of SOC broth (Sambrook et al., 1989) for 50 min at 28 °C before the titer of primary transformants was measured. The SOC outgrowths were then pooled and used as the inoculum for 50 mL of L-broth containing 0.2 mg/mL ampicillin and 2% (w/v) glucose, which was agitated at 25 °C for 21 h (A_{660} = 3.9). Plasmid isolation using a Quiagen-100 column (Diagen GmbH) yielded 67.5 μg of library DNA. Electroporation of *E. coli* JM109 with a small portion (100 ng) of this provided a sample of transformants in an amber suppressor strain, where the only nonsense codon possible at randomized triplets (TAG) would be suppressed.

Screening Methods. Preliminary screening of transformants was done by culturing colonies at 25 °C on L-agar plates (pH 7.2) containing 2% skim milk powder, where secretion of active protease resulted in localized clearing of the opaque growth medium due to degradation of the casein. After 11 days, the ratio of the diameter of the cleared zone to that of the colony was converted to an index value on a scale of 0–20. Obviously, this screening procedure (and those described below) can only identify enzymes that retain activity long enough after secretion to exert a detectable effect. In consequence, there is a bias toward active enzymes that also possess reasonable stability.

The substrate preferences of active proteases were first characterized in a qualitative fashion by a substrate overlay method, as follows. Colonies were grown in separate compartments of multiwell plates (such as Nunclon Δ Multidishes) on LAmp-broth (pH 7.5, 50 μg/mL ampicillin) that had been solidified with 1.5% (w/v) Seaplaque (FMC Marine Colloids). Growth for 10 days at 25 °C produced sufficient enzyme for sensitive detection. At this time, each well was filled with 1 mL of a molten solution (40 °C) containing 2 mM pNa or 10 mM βNap substrate, 1% (w/v) Seaplaque, and 11% *N,N*-dimethylformamide in 100 mM Hepes buffer, pH 8.0. Plates were incubated at 37 °C and inspected repeatedly over a period of 48 h to estimate visually the release of yellow *p*-nitroaniline or fluorescent β-naphthylamine (using illumination at 366 nm for the latter). Finally, to enhance detection of low levels of *p*-nitroaniline or β-naphthylamine, plates were developed by diazotization (Ohlsson et al., 1986) or by reaction with Fast Blue Salt BN (Barrett, 1972), respectively. Transformants were scored on a nonlinear scale of 0–20 to reflect the observed rate of substrate hydrolysis. The partly logarithmic nature of the scale emphasizes weak activities.

Initial rates of substrate hydrolysis in solution were measured spectrophotometrically at 410 nm for pNa (Erlanger et al., 1961) and at 340 nm for βNap substrates (Lee et al., 1971). Because reaction rates only afford a true reflection of k_{cat}/K_m when the substrate concentration is small relative to K_m, we used the lowest substrate concentrations consistent with workable assay durations. Reactions were conducted at 25 °C in 100 mM Hepes, pH 8, containing 9% *N,N*-dimethylformamide and pNa substrate (0.5 mM) or βNap substrate (5 mM). Under these conditions, the amount of enzyme activity releasing 1 μmol of chromophore/fluorophore per minute was defined as 1 unit.

RESULTS

Construction of the Library. The gene in the expression construct used as the template for the library encoded the Met190Ala variant of α-lytic protease and also contained a selectable genetic marker that directed the additional substitutions Gly215Ser and Val218Ser. When the template construct was cultured in *E. coli* JM109 on skim milk plates, the triple-mutant enzyme it produced (Met190Ala/Gly215Ser/Val218Ser) was shown to be inactive. Molecular modeling (not shown) had indicated that this would be the case, since the side chain of Ser215 protrudes into the space normally occupied by the main-chain atoms of the substrate P_2 residue. It was advantageous that unmutated template constructs escaping the genetic selection did not give rise to active enzymes in the library. The targeted random mutagenesis procedure was designed to remove the inactivating mutation at position 215 and to randomize up to four other positions (namely, 191, 192, 213, and 218) at the S_1 subsite. Combinatorial replacement by all 20 amino acids at each of the target positions permits a total of 1.6×10^5 permutations. The mutagenesis procedure generated a library containing 9.7×10^4 primary transformants.

Active Enzymes in the Library. Preliminary tests with endoprotease Glu-C, endoprotease Lys-C, and trypsin confirmed that even proteases with narrow substrate specificities could be detected using the skim milk screen. When 8.4×10^3 clones from the library were cultured in *E. coli* JM109 on skim milk plates, 0.57% of the colonies expressed active enzymes. All 47 of the active enzymes in this sample of the library hydrolyzed casein with activities equal to or greater than that of the parent (Table I), and a number (such as mutants 1 and 2) were exceptionally active in this assay.

Using wild-type and Met190Ala protease (WT and MA, Table I), the relative magnitudes of scores from the plate–overlay screening procedure were shown to be in general agreement with those of reaction rates determined spectrophotometrically.[3] Rate values for βNap substrates had to be adjusted before inclusion to compensate for the different (and usually slower) rate of hydrolysis of the leaving group, whereas this difference was accommodated in substrate overlays by using different scoring systems for βNap and pNa substrates. Reaction rates at the chosen substrate concentrations were shown to provide a reliable reflection of the relative magnitudes of specificity constants (k_{cat}/K_m) published for the test enzymes (Wilson et al., 1991; Bone et al., 1989b).

[3] An apparent discrepancy arises at the upper limit to the scale for plate screen scores, where very strong activities are all awarded the maximum value of 20 points. While discrimination between strong reactions is not possible in the plate screen due to rapid color or fluorescent saturation of the culture wells, such restrictions do not apply to the spectrophotometric rates. Thus with mutant 55 it is possible for spectrophotometric rates as varied as 1437 units/L (P_1 = Leu), 4207 units/L (P_1 = Ala), 10 741 units/L (P_1 = Phe), and 13 674 units/L (P_1 = Met) all to be awarded the same (maximum) score in the screen data.

Table I: Activity Data for Reference Enzymes and Enzymes from the Library

			pNa substrates, P_1 as shown[a]										βNap substrates, P_1 as shown[a]									positions[a]					
no.[b]	data[c]	SM[d]	G	dA	A	V	L	D	E	M	F	R	S	T	I	N	Q	H	Y	W	Adj[e]	191	192	213	218	*n*[f]	%[g]
Reference Enzymes[h]																											
WT	Sc	11	2	+	20	6	0	0	0	2	0	0	+	0	0	0	0	0	0	0		G	R	M	V		
	Ra		+	0	140	3	0	0	0	4	0	0	2	0	0	0	0	0	0	0	5.8						nd
	Pu		33		2100	79	+			180	+		200	20	+	3	68										
MA	Sc	3	+	0	6	2	12	0	0	20	20	0	0	1	0	+	0	0	2	0		G	R	M	V	0	
	Ra		0	0	10	1	67	0	0	308	321	0	+	+	0	+	0	0	13	0	5.0						60
	Pu				1000	300	11000			35000	31000																
Active Library Enzymes[h,i]																											
1	Sc	18	3	0	3	0	+	0	0	20	10	0	+	0	0	8	1	16	0	0		G	V	H	M	1	
	Ra		2	0	4	0	0	+	0	87	8	0	0	2	0	7	2	92	+	+	3.7						nd
2	Sc	20	3	0	3	0	0	1	0	16	10	0	+	0	0	8	1	16	0	0		G	R	H	M	1	
7	Sc	18	2	0	2	0	1	+	0	12	2	0	0	0	0	2	0	12	0	0		G	R	H	I	1	
	Ra		+	0	2	0	0	0	0	55	3	0	+	0	0	6	+	117	0	+	2.6						60
9	Sc	13	2	0	6	0	+	+	0	12	6	0	0	0	0	1	0	12	0	0		G	R	H	L	4	
	Ra		1	0	5	0	0	0	0	37	6	0	+	0	0	3	+	196	0	0	4.3						95
10	Sc	9	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	+	0	0		G	R	H	E	1	
11	Sc	10	+	0	+	0	0	+	0	1	+	0	0	0	0	0	0	2	0	0		G	T	H	S[j]	2	
	Ra		0	0	+	0	0	0	0	1	0	0	+	0	0	+	0	+	+	+	0.5						nd
12	Sc	4	0	0	0	0	0	1	0	3	1	0	0	0	0	0	0	4	0	0		G	R	H	S	2	
14	Sc	8	1	0	1	0	0	1	+	6	2	0	0	0	0	+	0	2	0	0		G	R	H	A	2	
	Ra		+	0	+	0	0	0	0	7	+	0	0	0	0	+	+	4	0	0	nk						nd
16	Sc	10	0	0	+	0	+	0	0	3	2	0	0	0	0	0	0	4	0	0		S	M	H	L	1	
19	Sc	10	+	0	+	0	0	+	0	1	+	0	0	0	0	+	0	1	0	0		G	H	H	T	1	
	Ra		0	0	0	0	0	0	0	+	0	0	0	0	0	+	0	+	0	0	nk						nd
22	Sc	14	2	0	1	0	+	+	0	12	2	0	0	0	0	1	0	4	0	0		G	R	H	V	3	
	Ra		+	0	+	0	0	0	0	20	1	0	0	0	0	1	+	22	0	+	1.5						st
36	Sc	7	+	0	1	0	0	+	0	1	+	0	0	0	0	0	0	2	0	0		G	R	H	N	4	
	Ra		0	0	+	0	0	0	0	+	+	0	+	0	0	0	0	5	0	0	1.1						nd
17	Sc	4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	+	0	0		Q	A	I	T[k]	1	
31	Sc	9	3	0	16	10	16	0	0	20	20	0	4	1	0	1	0	1	12	0		G	R	M	L	7	
35	Sc	15	3	0	16	2	16	0	0	20	20	0	2	1	0	+	0	+	8	0		G	L	L	M	1	
37	Sc	15	2	0	16	3	16	0	0	20	20	+	2	1	0	+	0	+	12	4		A	Q	T	M	1	
38	Sc	11	0	0	3	2	16	0	0	20	20	0	0	+	0	+	0	+	2	1		G	S	T	V	1	
39	Sc	8	2	0	20	+	12	0	0	16	20	0	+	+	0	+	0	0	4	+		G	R	F	M	1	
	Ra		2	0	198	0	26	0	0	1443	1238	0	2	+	0	2	0	2	94	2	8.2						st
41	Sc	14	6	+	20	12	20	+	+	20	20	+	2	1	+	2	1	1	16	4		G	R	T	M	1	
	Ra		5	0	1071	11	630	0	+	6646	6514	+	2	1	+	7	1	10	1013	52	4.8						50
43	Sc	7	1	0	10	10	16	0	0	20	20	0	2	1	0	1	0	+	8	0		G	R	M	I	2	
44	Sc	8	1	0	10	10	20	0	0	20	20	0	0	1	0	0	0	+	2	1		A	T	T	I	1	
45	Sc	7	2	0	20	1	12	0	0	20	20	0	1	1	0	0	0	0	1	+		A	W	T	L	1	
	Ra		+	0	305	+	24	0	0	228	133	0	2	0	0	0	0	0	6	1	5.0						15
47	Sc	9	2	0	20	12	12	0	+	20	20	0	2	1	1	1	2	2	4	1		G	R	S	L	3	
	Ra		+	+	148	25	50	+	+	583	546	0	7	1	3	3	5	28	70	4	6.0						20
50	Sc	15	1	0	16	10	16	0	0	20	20	0	2	1	0	+	0	1	12	0		G	R	M	M	1	
51	Sc	9	3	0	16	1	16	0	0	20	20	0	2	1	0	+	0	1	8	+		G	R	L	L	2	
55	Sc	11	+	+	20	12	20	+	+	20	20	0	2	1	0	2	1	2	16	4		G	R	T	L	1	
	Ra		37	0	4207	23	1437	+	2	13674	10741	0	8	+	0	8	+	6	480	16	2.4						15

[a] Substrate P_1 residues and amino acid residues at target positions in the enzymes are indicated using one-letter codes; dA is D-Ala. Since no enzyme in this table had detectable activity with substrate *Suc*AlaProPro-pNa, there is no column for P_1 = Pro. [b] Enzyme identifier: WT, wild-type α-lytic protease; MA, Met190Ala mutant (library parent); numbers, library enzymes. [c] Sc: Plate screen scores (substrate overlay) on pseudo-log scale. Ra: Spectrophotometric rates (units/L). Pu: One-tenth of published values for k_{cat}/K_m (s^{-1} M^{-1}) with *Suc*AlaAlaProXaa-pNa (where Xaa denotes an amino acid residue) taken from Wilson et al. (1991) and Bone et al. (1989b). [d] SM: Index of skim milk clearing (see text). [e] Adj: The adjustment factor applied to rate data to normalize for different leaving groups (see text). nk: Factor not known (no normalization applied). [f] *n*: Number of instances of each sequence in the 47 active library enzymes sequenced. [g] %: The approximate percentage of activity remaining in cell-free supernatant after 260 days at 4 °C, pH 9. nd: not determined. st: stable over at least 80 days. [h] +, value below 1 (number omitted to improve clarity). [i] Active library enzymes are segregated into those with His213 (top set) and those without (bottom set). [j] Spurious mutation Ser219aAla also present. [k] Spurious mutations Gln219His and ΔSer219a also present.

With this information in hand, all 47 of the active enzymes in the library sample were screened by substrate overlay (Table I). In addition, 13 transformants that expressed active enzymes were cultured in rich liquid medium. Since all of the liquid cultures contained comparable levels of mutant enzyme protein (as judged by HPLC), and zymograms (not shown) confirmed the absence of other proteases, samples of cell-free supernatants were used directly in spectrophotometric assays to measure reaction rates. The scaling factors used to adjust the rates of βNap substrates (calculated by comparing the activities of each mutant on the *Suc*AlaProAla-pNa and *Suc*AlaProAla-βNap substrates) are shown in Table I. As before, spectrophotometric rates and screening scores were in broad agreement. Assays that monitored the hydrolysis of ester substrates (benzyloxycarbonyl)-AlaLys-OMe and (benzyloxycarbonyl)-AlaCys-OMe by measurement of proton release (R. G. Whittaker, unpublished results) failed to find any library enzymes (among the 13) with a major capacity for cleavage at the two natural P_1 residues not already tested (data not shown). Moreover, spectrophotometric measurement of the rate of hydrolysis of tetrapeptide *Suc*AlaAlaProLys-pNa by the Met190Ala enzyme and library enzymes 7, 9, and 55 confirmed that none had a major ability to cleave this substrate (data not shown).

From Table I it is evident that about one-half of the enzymes in the library sample exhibited substrate preferences that

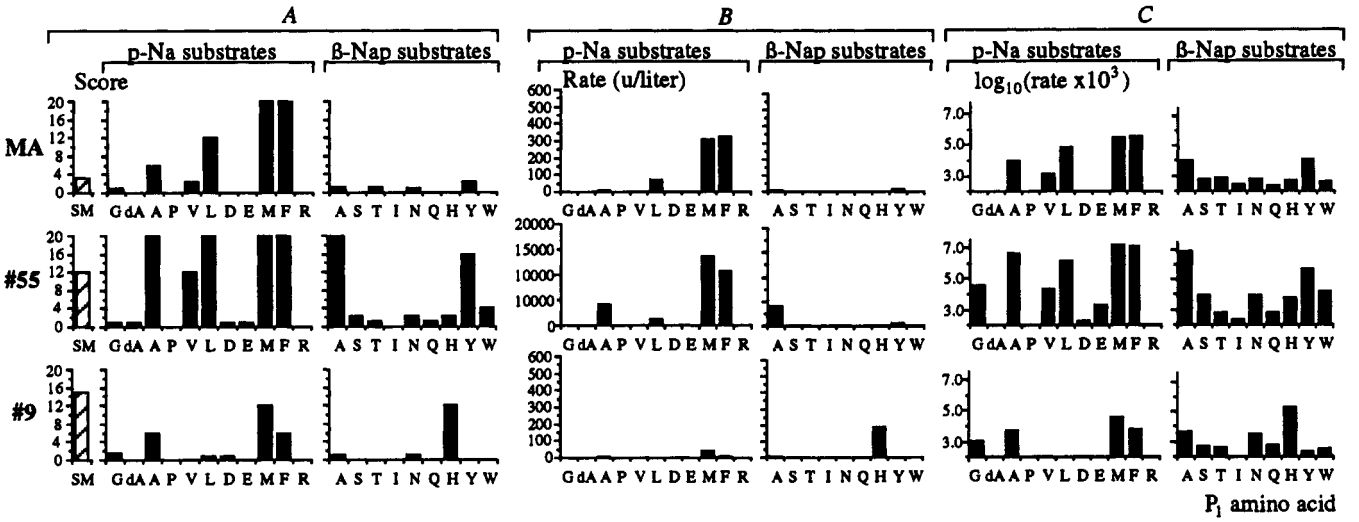

FIGURE 2: Activity data for the library parent (MA) and two enzymes found in the library (mutants 55 and 9). Three histograms are shown for each enzyme: (A) the plate–screen scores for skim milk clearing (hatched bars) and substrate overlays (solid bars); (B) linear plot of spectrophotometric rate data (expressed as units of activity per liter of supernatant); (C) $\log_{10}$ plot of the spectrophotometric rate data. In addition to skim milk clearing (SM), the horizontal axes show substrate P_1 residues in one-letter codes; dA is D-Ala. Vertical axes without markings relate to the first marked scale to the left. The partly logarithmic nature of the plate–screen scores (explained in Experimental Procedures) is evident. Logarithmic scales serve to emphasize weak activities.

reflected the specificity of the parent protease, giving the best scores with the Met and Phe substrates and good scores with Ala and Leu. The remaining enzymes constituted a distinct group that showed greater selectivity and preferred to cleave the His and Met substrates. In the enzymes of both groups, additional (but lesser) cleavage capabilities were featured to different extents. There were also large differences between the overall levels of activity exhibited by individual enzymes, with some (e.g., mutants 41 and 55) showing rates 20–45 times faster than that of the library parent or wild-type protease. Some activity data for selected enzymes have been plotted to illustrate the functional diversity contained in the library (Figure 2). In general, however, we found that enzymes in the library had little or no ability to act on charged P_1 residues (His being predominantly uncharged at the pH of the assay) or to accommodate those which were sterically unusual (such as D-Ala and Pro).

Substitutions in the Library. DNA sequencing of 21 clones selected at random from the library (not shown) revealed that 29% of the transformants were invalid, mostly because of partial incorporation of oligo-B (resulting in destruction of the *EcoB* selectable marker but retention of the inactivating mutation Gly215Ser). Some 52% of the transformants were valid clones that resulted from coupled priming by oligo-A and oligo-B (changes possible to positions 191, 192, 213, and 218), while the remaining 19% of the transformants were valid clones that resulted from mutagenic priming by oligo-B alone (changes possible to positions 213 and 218 only). Sequence data (not shown) revealed that the incorporation of nucleotides at randomized positions was somewhat biased, with different nucleotides being favored in different positions in an unpredictable fashion (cf. Dunn et al. (1988) and Oliphant and Struhl (1989)). Nevertheless, the extent of nucleotide substitution was sufficient for adequate diversity to occur at the amino acid level (see below), and the biases cannot have been seriously limiting because many clones encoding active enzymes featured substitutions poorly represented in the library. We presume that the biases inherent to the library served mostly to reduce its yield of active mutants. As statistically expected from the frequency of active enzymes in the library (0.57%), all 21 clones picked at random encoded inactive enzymes. Amino acid combinations for the valid members of this sample are shown in Table II.

Table II: Inactive Combinations[a]

	position			
	191	192	213	218
103	Pro	Arg	Thr	Arg
104	Val	Lys	Met	His
107	Gly	Arg	Met	Cys
108	Tyr	Lys	His	Ala
109	Glu	Gly	Met	Leu
110	Val	Asn	Gln	Ala
112	Gly	Arg	Arg	Gln
113	Tyr	Lys	Leu	Arg
114	Gly	Arg	Pro	Ser
121	Gly	Arg	Asn	Ser
124	His	Asn	Lys	Asn
128	Gly	Tyr	Arg	Ala
129	Gln	Cys	Leu	Lys
146	Lys	Val	Pro	Thr

[a] Column 1 contains the clone identification numbers. Each combination was observed only once.

The substitutions present in each of the 47 active enzymes from the library sample (see above) are listed in Table I. About 75% of the active enzymes resulted from mutagenic priming by oligo-B alone (changes possible to positions 213 and 218 only). The remaining active mutants resulted from coupled priming by oligo-A and oligo-B (changes possible to positions 191, 192, 213, and 218). Many residue combinations were observed more than once (Table I) to the extent that about one-half of the active enzymes were sequence replicates. Only slight differences (not shown) were observed in the substrate preference profiles of mutants having the same sequence. We thought it useful to have independent clones of particular mutant genes, since the existence of replicates would to some extent offset the effects of any spurious mutations in library clones. However, one of the few unplanned changes detected (Ser219aAla) was found to occur in two clones having the same S_1 sequence (mutants 8 and 11). Interestingly, the only other spurious mutation observed (Gln219His/ΔSer219a) affected the same position in the protein, which was located in a surface loop adjacent to the S_1 site. While changes in this region are thought to be unlikely to influence enzymatic activity, we have been careful to draw

our main conclusions about structure–activity relationships from trends rather than from data for individual mutants.

Structure–Activity Relationships. Figure 3 compares the frequency of substitutions observed in the active enzymes with data for substitutions in the (inactive) clones chosen at random from the library. While there was some bias in the distribution of residues in the randomly chosen clones, representation of the 20 alternatives was broadly comparable. In contrast, selection for biological function of the mutants resulted in substitutions being confined to more limited sets of amino acids. In active enzymes, Pro (which might be disruptive to structure) was avoided at all four of the positions open to substitution. Given that His should be largely unprotonated, charged residues were scarce. The lack of charged substituents was surprising, considering that all four positions have access to solvent and than one of them (position 192) is occupied by Arg in the wild-type and parental sequences. Cys was also avoided in active enzymes, perhaps because disulfide bond formation in the protein is vulnerable to interference from additional Cys residues. In this connection, we note that mutants 31, 43, and 50 (identical but for the substitution of Leu, Ile, and Met, respectively, at position 218) were strongly active, whereas mutant 107 (identical to the previous mutants but for the substitution of Cys at position 218) was completely inactive (Tables I and II).

In addition to the general trends described above, each of the four randomized positions in active enzymes displayed characteristic substitution preferences. Position 191 showed a very strong preference for Gly (as found in the wild-type sequence) and accommodated other small residues with a frequency inversely related to their size, which suggested that this position was subject to tight steric constraints. Position 192 was quite permissive, allowing polar and nonpolar substitutions of very different sizes. Position 213 accommodated a limited set of residues, with a strong preference for His. Remarkably, all of the enzymes in the substrate-selective group that preferred to cleave His and Met substrates (Table I) were found to contain His at position 213. The remainder of the active mutants, which had broad substrate specificity, all contained residues other than His at this position, with Met (the wild-type residue) occurring most frequently. Position 218 accepted a range of residues, but had a strong preference for Leu. A more detailed examination (Figure 3b) revealed that the strong preference for Leu at position 218 was a feature of enzymes that did not contain a His at position 213. For His213 mutants, the preference for Leu at position 218 was slight, and there was a notable increase in the occurrence of small polar residues such as Ser and Asn.

Spectrophotometric rate data for the activity of mutant enzymes on substrates with different P_2 (and, in some cases, P_3 and P_4) residues are presented in Figure 4. The correlations presented in this figure indicate that different mutants responded similarly to the same change in the P_2 residue (and, where tested, in the P_3 and P_4 residues). This strongly suggests that subsites S_2–S_4 have not been greatly affected by the amino acid substitutions we introduced in the S_1 subsite. In contrast, a comparison of reaction rates obtained for the action of each enzyme on *Suc*AlaProAla-pNa and *Suc*AlaProAla-βNap substrates (Adj, Table I) revealed differences in how mutants (other than those of identical sequence) responded to the switch in chromophore. This might be an indication of changes to the S_1' subsite caused unintentionally by our substitutions in the S_1 pocket. However, enzyme–substrate interactions C-terminal to the scissile bond are considered to be of limited importance in α-lytic protease (Bauer et al., 1981).

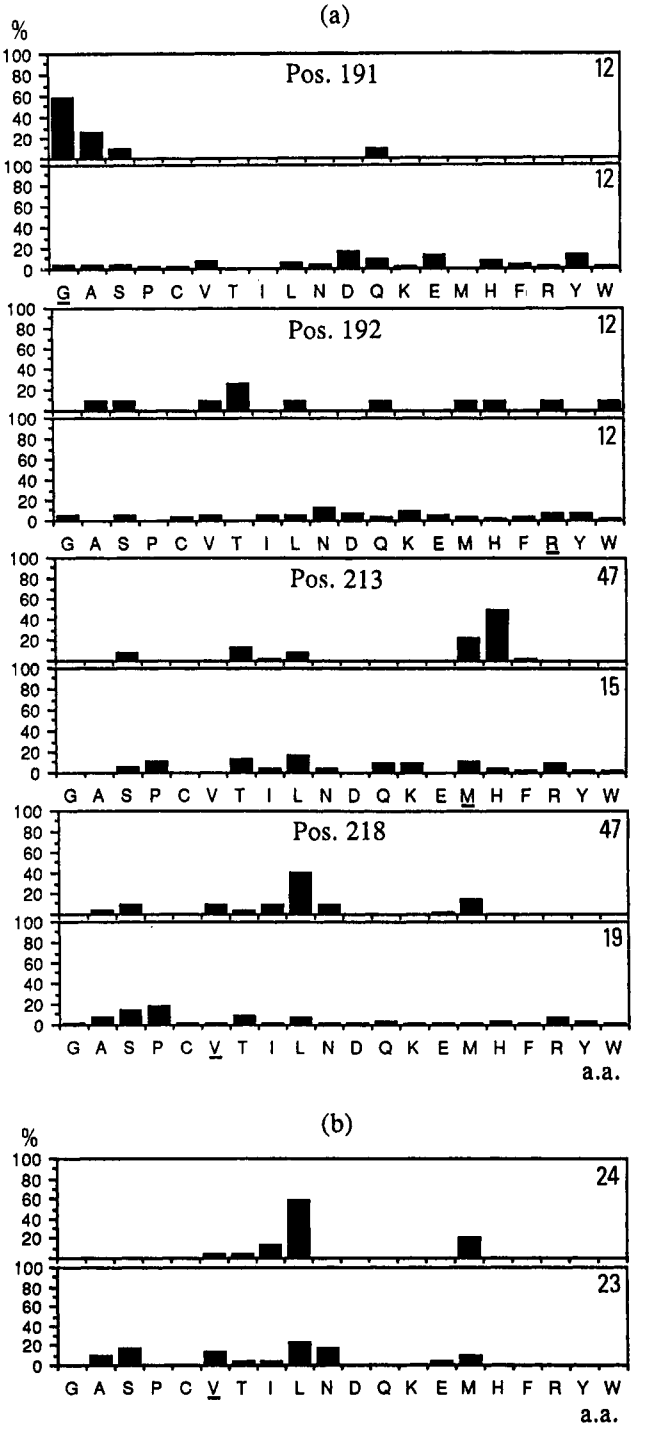

FIGURE 3: Frequency of substitutions in the library. (a) Each double panel refers to one of the target positions (Pos) in the protein and shows the incidence (%) of amino acid substituents (a.a.) in active library enzymes (upper panel), along with data for clones chosen at random from the library (lower panel). Amino acids are indicated using one-letter codes, and the wild-type residue for each position is underlined. The number in the top right-hand corner of each panel indicates the sample size (i.e., number of clones). Each upper panel is a histogram of the actual amino acid frequencies observed in active enzymes, excluding for positions 191 and 192 data from enzymes that were unmutated in segment A. In view of the more limited sample size for clones picked at random, each lower panel shows an amino acid distribution calculated from the observed nucleotide incorporation data for the corresponding codon. The histograms of actual amino acid substitutions (not shown) reflect these plots. (b) This double panel uses a similar format to show the incidence of amino acid substitutions at position 218 in active enzymes with residues other than His at position 213 (upper panel), along with the corresponding incidence in those having His at position 213 (lower panel).

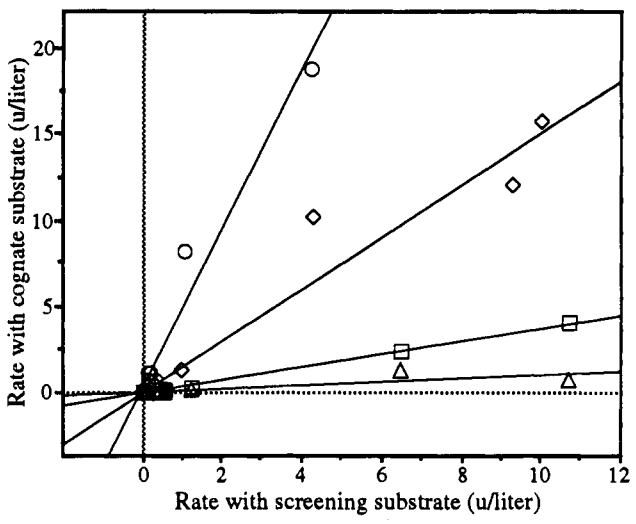

FIGURE 4: Effect of S_1 mutagenesis on S_2–S_4 binding sites. Using 12 library enzymes of different sequences, rates (expressed as units of activity per liter of supernatant) were measured with four pNa substrates, and each result was plotted against the rate obtained for the same enzyme with the same concentration of the corresponding screening substrate (0.5 mM). Cognate substrates were *Suc*AlaAlaValAla-pNa (○), *Suc*AlaAlaVal-pNa (◇), *Suc*AlaAlaPhe-pNa (□), and *Suc*GlyGlyPhe-pNa (△) for screening substrates *Suc*AlaProAla-pNa, *Suc*AlaProVal-pNa, *Suc*AlaProPhe-pNa, and *Suc*AlaProPhe-pNa, respectively. In order to fit all of the data on the same graph, rate values for the following substrates have been multiplied by the factors shown: *Suc*GlyGlyPhe-pNa, 50; *Suc*AlaAlaVal-pNa, 3000; *Suc*AlaProVal-pNa, 400.

A number of cell-free supernatants showed a steady decline in activity during the period of study (Table I). One of the most unstable enzymes (mutant 55) has broad specificity and very high activity, which suggests that autolysis may play a role in this process.

DISCUSSION

We have demonstrated that combinatorial random substitution of a few chosen positions in the substrate-binding site of a protease, followed by screening with multiple substrates, is a useful method for production and identification of mutant enzymes of altered structure and function. This approach is not limited to proteases and can be applied to any enzyme for which a structural model is available and for which appropriate assays can be devised. In fact, the approach we have adopted should be applicable in an iterative manner to enzymes for which structural information is limited.[4]

In our experiment, 0.57% of the library constructs expressed active enzymes, indicating the presence of some 550 active mutants in total. However, DNA sequencing revealed that 29% of the primary transformants were invalid (because of retention of Gly215Ser or for other reasons), indicating that the true frequency of active mutants must be about 0.8%. Moreover, sequencing also disclosed a level of duplication among clones that suggested the number of different active mutants in our library was closer to 300. Although most of the valid mutants in the library resulted from coupled priming by oligo-A and oligo-B, such double mutants (with four positions affected) retained activity much less often than mutants resulting from oligo-B alone (with only two positions affected). In consequence, while all of the active mutants in the library contained substitutions in the positions making the greater contribution to the S_1 pocket (namely, 213 and 218; Figure 1), only a limited number contained additional changes at the other two positions. This was a favorable situation for analyzing structure–activity relationships in the S_1 subsite.

Calculations indicated that 11.9% of the oligo-A sequences and 2.3% of the oligo-B sequences were compatible with enzymatic function.[5] This result strongly suggests that (when considered as pairs) positions 191 and 192 are much more tolerant of substitution than positions 213 and 218, an observation that is not directly apparent from independent consideration of substitutional preferences at each of these sites (Figure 3). The difference could be a reflection of the fact that positions 213 and 218 contribute considerably more to the surface that defines the S_1 pocket (Figure 1). Overall, the positions we investigated in α-lytic protease display a tolerance to substitution slightly greater than that calculated elsewhere for substrate-binding residues in β-lactamase (Palzkill & Botstein, 1992).

Because self-processing of the precursor at a Thr–Ala junction is required for release of the active mature protease (Silen et al., 1989), we expected that all of the active enzymes in our library would have a significant ability to cleave at Thr residues. However, we found that the activity of the wild-type protease and the library parent on a synthetic substrate with Thr as the P_1 residue was slight and that some of the enzymes in the library were even less active with this substrate. It seems that processing of the precursor junction by these enzymes is still sufficiently fast for good expression of the mature protease, perhaps due to the unimolecular nature of the reaction or due to unusual strain at this bond. Another surprise was the poor correlation between the activity of library enzymes on skim milk and on synthetic substrates ($r^2 = 0.8$ at best; data not shown). Transformants with the greatest capacity for milk clearing during growth (mutants 1 and 2) were not those with the highest overall activity when overlaid with synthetic substrates (mutants 41 and 55). Since the substrate preferences of the former group were not directed toward amino acids common in casein, and since we have evidence that our mutagenesis has not affected interactions at the other subsites critical to substrate binding (Figure 4), we are unable to offer a satisfying explanation for this phenomenon.

In general, residue changes in the protease that were compatible with enzymatic activity had only modest effects on substrate specificity (Table I). The one exception was the substitution of Met213 with its strongly favored replacement, His. This substitution severely reduced the (otherwise large) capacity of the enzyme for cleavage at Phe and conferred instead a greatly increased ability to cleave the fluorogenic substrate having His in the P_1 position. Neither the wild-type α-lytic protease nor the library parent (Met190Ala) displayed

[4] If an initial library is made by the randomization of a substantial number of different positions that may be involved in substrate binding, then a very low yield of active mutants should result. The sequences of these mutants will reveal which positions are largely restricted to the wild-type residue (such as position 191 in our study). A subsequent library in which such positions are fixed as the wild-type residue, while the remaining positions of the original choice are once again varied, should be much more productive in terms of active mutant enzymes and, for that reason, afford useful functional diversity.

[5] Oligo-B: Since 75% of the active enzymes (i.e., 75% of 0.57% of clones) resulted from priming by oligo-B alone, and 19% of the library constructs were valid clones that resulted from mutagenic priming by oligo-B alone, we concluded that 2.3% of the oligo-B sequences were compatible with enzymatic function. Oligo-A: Since 25% of the active mutants (i.e., 25% of 0.57% of clones) resulted from coupled priming by oligo-A and oligo-B, but only 2.3% of oligo-B sequences would be expected to allow activity, and 52% of the library constructs were valid clones that resulted from coupled priming, we calculated that 11.9% of the oligo-A sequences were compatible with enzymatic function.

any significant capacity for cleavage at His, whereas His was actually the preferred P_1 residue for some of the Met213His mutants (e.g., mutant 9). Thus it appears that the presence of a key residue in a key position can cause an abrupt switch in primary specificity.

In the absence of perturbations of pK_a in the S_1–P_1 complex, neither of the two imidazole rings should have been substantially ionized at the pH used in the study. We therefore chose to view His as an uncharged residue. The poor activity of library enzymes with substrates containing charged P_1 residues is likely to be a reflection of the fact that charged residues were almost absent from the repertoire of acceptable S_1 substituents, which would preclude the opportunity for electrostatic balance in enzyme–substrate complexes. Interestingly, a recently discovered homologue of α-lytic protease from *Streptomyces griseus* contains a His residue at position 213 (Svendsen et al., 1991) and cleaves preferentially at Glu in protein and synthetic substrates (Yoshida et al., 1988), even though molecular modeling predicts no charged amino acids near the S_1 region at the pH used. However, since the ability of the *S. griseus* protease (protease E) to cleave at His in protein or synthetic substrates is unknown, we are unable to compare it further with our Met213His enzymes. We do, however, note that *S. griseus* protease E contains a Ser at position 190. This invites speculation that a preference for His at position 213 is a response to the presence of a small residue at position 190 and would not have been observed if we had used wild-type α-lytic protease (rather than the Met190Ala variant) as the parent for our library.

In addition to their propensity for cleaving at His residues, all Met213His mutants efficiently cleaved the synthetic substrate with Met as the P_1 residue, further supporting the presumption that His213 is largely uncharged. Un-ionized His has polar aromatic character. Attention has recently been paid to the interactions of His with other aromatic residues in proteins (Lowenthal et al., 1992; Jasanoff et al., 1992), but His–His and His–Met interactions have not been studied in any detail. For our Met213His mutants of α-lytic protease, molecular modeling suggests that it is mainly the $N_{\epsilon 2}$–$C_{\epsilon 1}$ edge of the His213 imidazole that contacts the substrate P_1 residue, regardless of the precise orientation of the ring (L. D. Graham, unpublished results). In the more common histidine tautomer, i.e., the $N_{\epsilon 2}$-imino form (Walters & Allerhand, 1980), resonance theory (and Huckel molecular orbital calculations) predicts the $N_{\epsilon 2}$–$C_{\epsilon 1}$ edge to be electron-deficient. Modeling also suggests that His and Met are among the few uncharged P_1 side chains capable of positioning an electron-rich atom (the tertiary nitrogen and thioether sulfur, respectively) in good contact with this potentially electropositive region. The observed substrate preference of our Met213His enzymes may therefore be explained in terms of favorable polar interactions at the S_1 subsite.

We suggest from a comparison of mutants 7, 9, 14, 22, and 36 (identical but for the substitution of Ile, Leu, Ala, Val, and Asn, respectively, at position 218) that the overall activity of the His213 enzymes may increase in response to increased hydrophobicity of residue 218 (Table I). Inspection of the other rate data in Table I suggested that this effect may apply, at least loosely, to non-His213 enzymes as well. However, it was difficult to find further examples of this kind of structure–function correlation. While other residue substitutions in active enzymes resulted in substantial differences being observed in some minor activities (particularly in the ability to cleave at Val, Tyr, and Asn), changes in these activities did not show any systematic dependence upon the sizes or hydrophobicity indices of the S_1 substituents (not shown). We presume in such cases that many individual steric and electrostatic considerations combine to determine changes in specificity and that they do so in a nonadditive way that exceeds our present ability to model and understand them.

We do not consider that we have exhausted the functional diversity likely to be attained by this approach and expect that other mutants of altered primary specificity can be obtained by mutating a different combination of residues in the S_1 subsite, e.g., by randomizing position 190 in place of position 191. Moreover, we suggest that a library constructed to contain His at position 213 with random substitutions at other amenable S_1 positions (e.g., positions 190, 192, and 218) is likely to have a large proportion of His-cleaving proteases and may well include enzymes with tighter P_1 specificity for His. Work is in progress to test these hypotheses. In the meantime, we are engaged in the characterization of the more interesting of the existing library mutants in greater detail. The mutants most likely to find application as research enzymes (e.g., as tools in peptide mapping) are those with high activity and good selectivity, such as mutants 9 and 1. In addition, some library mutants (e.g., mutants 41 and 55) display a large increase in proteolyic activity over wild-type α-lytic protease and the library parent. Enzymes such as these, with very high activity and broad substrate specificity, could have applications as general-purpose reagents for protein degradation.

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