(nBu)$_4$NF in THF. The products of **9–11** were analyzed by ^{1}H and ^{13}C NMR and then hydrolyzed without separation under acidic conditions to form a mixture of the same two diols (in the same ratio as the silyloxy precursors).

Equilibrium Studies of Chelates. In a typical experiment, ca. 0.07 mmol of ketone was dissolved in 0.60 mL of CD_2Cl_2, and excess anhydrous magnesium bromide etherate was added. The resulting suspension was stirred for 30 min and then centrifuged until the supernatant liquid was clear. The clear solution was transferred to an NMR tube, and the proton NMR spectrum was recorded. Substantial changes in the chemical shift of many protons in the ketones were observed (see Scheme I). The concentration of $MgBr_2{\cdot}Et_2O$ was equal to the concentration of the ketone according to the integration of the Et_2O peaks relative to those of the ketone.

RINMR Methods. RINMR spectra were recorded in a Bruker WM-250 spectrometer at −70 °C. The rapid injection insert was functionally equilvalent to the one described by McGarrity,[15] which is capable of injecting 10–50 μL of solution into a spinning sample in the probe of a high-resolution NMR apparatus.

Solutions for injection were prepared by removing ca. 0.3 mmol of standard $MgMe_2$ solution, evaporating THF under vacuum, redissolving in 100 μL of THF-d_8, evaporating the solvent again, and redissolving in 600 μL of THF-d_8, which was then drawn into the dried injector syringe under argon. In a typical experiment, a 5-mm NMR tube truncated to a length of 11 cm was dried on a vacuum line and filled with argon. The proper amount of pentamethylbenzene (PMB) as internal standard, 320 μL of THF-d_8, and ca. 0.15 mmol of ketone were added. The tube was placed in the NMR probe where it was under a bath of N_2 from the spinner air and the liquid N_2 used for cooling. The injector containing a solution of $MgMe_2$ was then lowered into the previously shimmed NMR and the injection carried out. The FEDs of single-pulse proton NMR were recorded rapidly following the injection at preset intervals, and the signals due to $MgMe_2$ (−1.78 ppm) were monitored. For fast reactions, fast spectra were obtained in less than 10 s. The rate of disappearance of $MgMe_2$ was determined by integration relative to PMB.

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An Investigation into the Minimum Requirements for Peptide Hydrolysis by Mutation of the Catalytic Triad of Trypsin

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Abstract: The catalytic triad of rat anionic trypsin has been systematically altered by site-directed mutagenesis to determine the activity of alternate combinations of amino acids toward the hydrolysis of peptide bonds. Genetically modified rat trypsins H57A, H57D, H57E, H57K, H57R, H57A/D102N, H57D/D102N, H57L/D102N, H57K/D102N, D102N, S195A, S195T, and H57A/D102N/S195A have been generated. Rigorous steps were taken to show that the resultant catalysis was due to the mutant enzymes and not contaminants. Each of the variants exhibit measurable activity toward the activated amide substrate Z-GPR-AMC. At pH 8.0 k_{cat} ranges from 0.011 to 1.3 min^{-1} (0.0004–0.04% of wild-type). At pH 10.5 k_{cat} ranges from 0.012 to 140 m^{-1} (0.0004–5% of wild-type). The mutant trypsins were subsequently assayed for their ability to hydrolyze the unactivated amide linkages of protein substrates. Trypsins D102N, H57K, and H57K/D102N exhibited the highest level of activity. The k_{cat} for the D102N enzyme was 4 h^{-1} (0.003% of wild-type). The H57A/D102N double mutant was not as active but was chosen for further study since it was the simplest trypsin to exhibit peptidase activity. Its k_{cat} was ~0.1–0.2 h^{-1} at pH 8.0 and 0.7 h^{-1} at pH 10.1. These experiments demonstrate that an intact catalytic triad is not a requirement for peptide bond cleavage and that designed serine peptidases need not include a catalytic histidine or aspartic acid.

The development of peptidases with designed specificities would facilitate the manipulation of peptides and proteins. The challenge in designing such catalysts is the inclusion of interdependent binding and catalytic motifs within a common structural framework to achieve the energetically demanding hydrolysis of peptide bonds.[1] Initial studies have involved the derivatization of small molecules with reactive moieties to partially or fully mimic the chemistry of the serine protease catalytic triad. These catalysts have helped elucidate some aspects of the interactions between members of the triad[2,3] but have not yet been shown to catalyze the cleavage of amide linkages. Recently, this approach has been extended with a de novo designed four helix bundle polypeptide bearing catalytic serine, histidine, and aspartic acid residues. This protein exhibited significant chymotrypsin-like esterase activity.[4] Another strategy has been to elicit monoclonal antibodies to molecules which mimic the transition state of amide hydrolysis.

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Table I. Kinetic Parameters of Wild-Type and Mutant Trypsins at pH 8.0 and 10.1[a]

variant	pH 8.0			pH 10.1		
	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
wild-type	3200	15	210	2700	19	140
H57A	0.054	17	0.0032	0.11	20	0.0055
H57L	0.075	20	0.0037	0.16	21	0.0076
H57D	0.78	13	0.06	0.71	17	0.0042
H57E	0.69	21	0.033	0.63	25	0.0025
H57K	0.83	41	0.020	5.2	48	0.108
H57R	0.017	67	0.00025	0.65	160	0.0041
D102N	1.3	4.2	0.30	140	13	11
H57A, D102N	0.17	87	0.0019	7.5	130	0.058
H57D, D102N	0.18	62	0.0029	0.48	130	0.0037
H57K, D102N	0.41	18	0.023	6.2	130	0.048
H57L, D102N	0.13	41	0.0031	4.9	230	0.021
S195A	0.079	41	0.0019	0.057	45	0.0013
S195T	0.011	21	0.00052	0.012	15	0.0008
H57A, D102N, S195A	0.038	89	0.00043	0.041	170	0.0024
k_{buffer} (min^{-1}) no enzyme		6.0×10^{-7}			1.7×10^{-6}	

[a] The error in these determinations was 10–20%. The buffers were 100 mM NaCl/20 mM $CaCl_2$ and either 50 mM Tris-Cl, pH 8.0, or 50 mM glycine, pH 10.1. Similar results were observed at pH 8.0 with 50 mM MOPS. The experiments were allowed to proceed until more than one turnover had occurred.

These have been shown to hydrolyze both esters and activated amide bonds with efficiencies which approach natural serine protease function for some substrates.[5] In the future, this catalysis may be further enhanced by genetically introducing strategically placed catalytic residues.[6,7] The selective and efficient hydrolysis of unactivated peptide bonds by designed catalysts has not been reported.[8]

This report seeks to determine that the development of agents which hydrolyze peptides by a serine protease-like mechanism is a realistic goal and to discover how this catalysis might be maximized. To accomplish this, the catalytic triad of the serine protease trypsin (His 57, Asp 102, Ser 195) has been mutated to discover a minimal basis for peptidase function. This strategy is similar in its goals to the minimialist approach to protein engineering[9] in that it seeks to simplify the initial basis for catalyst design.

Trypsin was chosen as a target for this analysis because it has already been optimized for highly specific peptidase activity. It contains an oxyanion hole and a highly selective substrate binding site[10] (Figure 1), so that the stage is set for catalysis, *even when the catalytic triad is absent*. Alternate combinations of triad residues can then be auditioned within a framework that maximizes their potential for catalysis. Thus, the trypsin scaffold is a good starting point for defining elements which may be utilized to endow catalytic antibodies or de novo engineered proteins with peptidase activity.[11]

Analysis of point mutations within the catalytic triad of trypsin and subtilisin has provided information on the interplay of the three amino acids in catalysis by serine proteases. The replacement of aspartic acid 102 with asparagine in trypsin yields a mutant

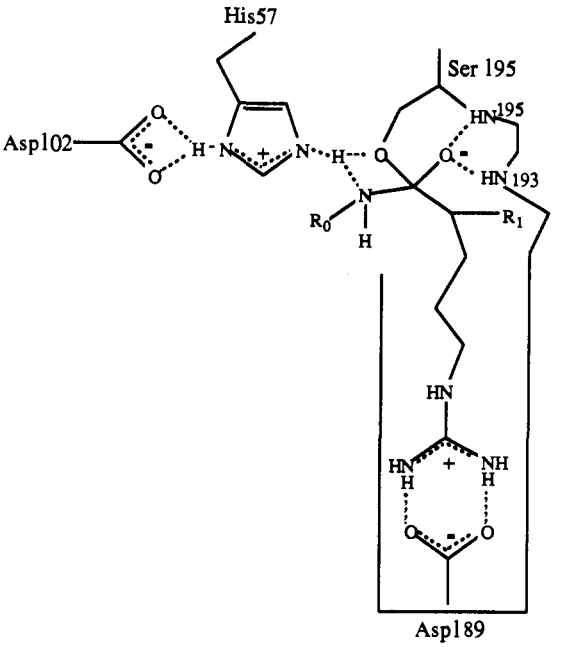

Figure 1. Schematic of the transition state for trypsin catalysis of an arginyl substrate. The catalytic triad consists of Asp 102, His 57, and Ser 195, the oxyanion hole consists of the backbone amides of residues Gly 193 and Ser 195, and the primary determinant of substrate specificity and binding is Asp 189.

which retains 0.1% of the wild-type esterase activity at pH 8.0 and up to 10% of the activity when assayed at pH 10.5.[12] Similarly, studies of the serine protease subtilisin have shown that mutant enzymes in which one or more of the catalytic residues have been replaced with alanine retain activity toward activated amide substrates.[15] Moreover, a detailed understanding of the active site histidine in serine protease amidolysis has lead to the development of a mutant of subtilisin capable of substrate assisted catalysis[13] and to a trypsin variant whose activity could be controlled by the presence of transition metals.[14]

We report here the mutation of trypsin to produce the H57A, H57D, H57E, H57K, H57L, H57R, H57A/D102N, H57D/D102N, H57K/D102N, H57L/D102N, D102N, S195A, S195T,

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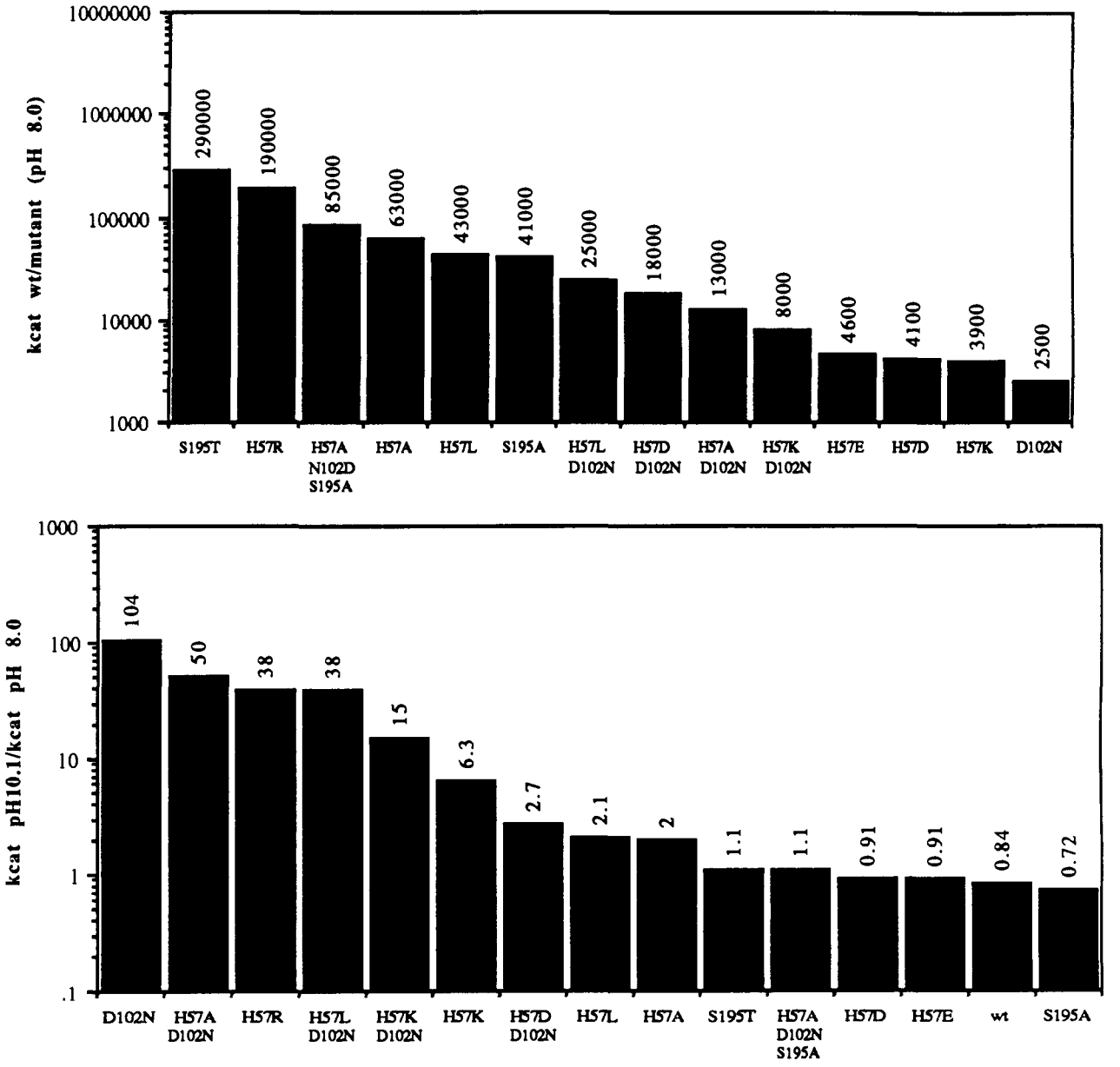

Figure 2. (a, top) Ratio of the k_{cat} values for wild-type and mutant trypsins. (b, bottom) Ratio of the k_{cat} values for wild-type and mutant trypsins at pH 10.1 and 8.0. Bars represent data from determinations on Z-GPR-AMC and were performed as described (Experimental Section). The numbers above each bar represent the numerical value for the relevent ratio.

and H57A/D102N/S195A variants. Initial kinetic analysis of these proteins has been performed using the sensitive fluorogenic substrate Z-GPR-AMC. The variants were then screened against insulin β-chain and galanin peptide to define their activity toward unactivated peptide bonds.

Results and Discussion

Rat anionic trypsin has been modified via site-directed mutagenesis of its catalytic triad, His 57, Asp 102, and Ser 195. The goals of this strategy are (1) to determine the capacity of amino acid substitutions to restore catalytic activity to active site variants and (2) to determine if any of the mutant trypsins thus generated retain the ability to hydrolyze unactivated peptide bonds.

Substitutions for Histidine 57. His 57 has been replaced with either acidic (Asp, Glu), basic (Lys, Arg), or neutral (Ala, Leu) amino acids to assess the effect of these substitutions on catalysis. Kinetic analysis was performed by following the hydrolysis of the fluorogenic substrate Z-Gly-Pro-Arg-7-amino-4-methylcoumarin (Z-GPR-AMC). At pH 8.0, the H57D, H57E, H57K, and H57R mutants retained a limited amount of activity, ranging from 4000- to 190000-fold below wild-type (Table I; Figure 2a). At best, this catalysis was only ~10-fold greater than that exhibited by the nonpolar H57L or H57A variants, indicating that the polarity of the substitution did not have a substantial effect. At pH 10.1 k_{cat} for the H57E and H57D variants was unchanged, reflecting the constant ionization of the acidic side chains over the pH range, while k_{cat} for the H57K and H57R trypsins increased 6.3- and 38-fold, respectively (Figure 2b). These increases may reflect the assumption of some general-base function by these residues, or they may be due to a solvent-mediated hydroxide-dependent mechanism.

Substitutions for Histidine 57 and Asparagine 102. Asp 102 effects catalysis by influencing the polarity of His 57, so that the removal of the histidine negates the catalytic rationale for the presence of the aspartic acid. Therefore, we believed that the mutation D102N should not drastically lower k_{cat} further for mutants already lacking His 57. Moreover, the negative electrostatic potential of Asp 102 may act to destabilize the developing negative charge in the transition state for hydrolysis by the trypsin variants lacking His 57.[15,16] Alternatively, the removal of Asp 102 may increase the rate of hydroxide-catalyzed deacylation by

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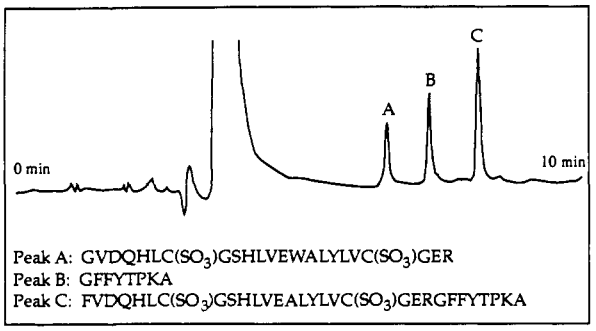

Figure 3. C18 reversed-phase HPLC analysis of the cleavage of insulin β-chain by D102N trypsin. The cleavage reaction was incubated in 20 mM $CaCl_2$/100 mM NaCl/50 mM Tris-Cl, pH 8.0, buffer containing 4 μM enzyme and 240 μM peptide substrate for 16 h at 37 °C. Peak 1 was identified by mass spectral analysis as FVDQHLCGSHLVDALYLVCGDR. Peak 2 was GFFYPKA. Peak 3 was the parent peptide. The gradient was 20–80% buffer B over 10 min (buffer A = 0.1% trifluoroacetic acid/99.9% doubly distilled H_2O; buffer B = 95% CH_3CN/5% H_2O/0.08% TFA).

removing negative potential, thereby making the active site more accessible to hydroxide. In either case, substitution for Asp 102 may actually increase substrate hydrolysis.

A series of variants was constructed containing D102N and either alanine, aspartic acid, lysine, leucine, or histidine at position 57. The k_{cat} values for the H57A/D102N and H57L/D102N variants at pH 8.0 were slightly higher and at pH 10.1 were much higher than those for the analogous H57A and H57L enzymes (Figure 2b; Table I). This increase in k_{cat} may support an inhibitory effect of Asp 102 on catalysis by the latter variants. However, the k_{cat}/K_m values do not reflect this increase, leaving the effect of Asp 102 on catalysis by these trypsin variants uncertain.

At pH 8.0 the D102N mutant was approximately 10-fold more active than the other variants (Table I) (3000-fold lower than wild-type) (Figure 2a), perhaps reflecting a residual role for the retained histidine. At pH 10.1 the variants, with the exception of H57D/D102N, exhibited substantial increases in activity (Figure 2a). The observation that the H57A/D102N and H57L/D102N variants exhibited rate enhancements at pH 10.1 that are similar to that of the H57K/D102N enzyme suggested that the Lys 57 did not play a pivotal role in catalysis and that the increase in k_{cat} was primarily due to a hydroxide-mediated reaction. The D102N variant possessed a much greater k_{cat} than the other mutants (140 m^{-1}, 20-fold higher), suggesting that His 57 continued to contribute to catalysis in the absence of Asp 102.

Substitutions for Serine 195. Serine 195 is the residue most directly involved in catalysis. We assessed the effects on catalysis of its replacement with either threonine or alanine. Both mutants resulted in large reductions in k_{cat} (Table I). Previously the replacement S195C had been shown to reduce k_{cat} to 0.0036 m^{-1},[17] possibly due to obstruction of the oxyanion hole.[18] Similarly, the mutation of serine to alanine in subtilisin reduced catalysis by a factor of 10^{-5}.[15] The H57A, D102N, and S195A mutant, the simplest variant included in these studies, was also constructed. It also exhibited a greatly decreased k_{cat} (85 000-fold) relative to wild-type.

Hydrolysis of Peptide Substrates. The hydrolysis of unactivated amide bonds was assayed using oxidized insulin β-chain. This peptide was chosen as a substrate because it contains an internal arginine which is flanked by residues which can fill the S2, S3, S1′, S2′, and S3′ subsites. This should maximize the binding energy available for catalysis. The progress of the digestions was monitored by C18 reversed-phase HPLC, and the resultant products were identified by mass spectral analysis. No hydrolysis was observed in the absence of added protease. Kinetic analysis was performed using C18 reversed-phase HPLC (Figure 3) by analyzing the initial rates of a series of hydrolysis reactions containing differing amounts of substrate. The enzymatic digests were incubated at 37 °C in 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0, buffer.

Table II. Effect of Leaving Group on Catalysis by Wild-Type and D102N Trypsins

	wild-type		D102N	
substrate	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
Z-GPR-S-benzyl	9.2	2900	2.8	3.0
Z-GPR-*p*-nitroanilide	7.1	3800	0.27	1.0
Z-GPR-AMC	15	3300	2.7	1.2
insulin peptide, β-chain	140	2500	90	0.066

[a] The error in these determinations was 10% for the ester and the activated amide substrate and 20% for insulin β-chain. The buffer was 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0.

We found that wild-type protease cleaved the Arg–Gly bond with a k_{cat} of 2500 m^{-1} and a K_m of 140 μM. All of the mutant trypsins which contained Ser 195 exhibited a much lower but still detectable level of activity toward the Arg–Gly linkage. No hydrolysis of substrate was observed upon incubation of substrate with the mutants which lacked Ser 195. The D102N, H57A/D102N, H57K, and D102N/H57K variants showed substantially more catalysis than the other mutants. The D102N trypsin had a k_{cat} of 0.066 m^{-1} and a K_m of 90 μM. The activity did not increase over a pH range of 8–10.1. The kinetic constants k_{cat} and K_m for the H57K and H57K/D102N variants toward insulin β-chain at pH 8.0 were determined to be 1.26 m^{-1} and 72 μM and 0.84 m^{-1} and 125 μM, respectively.

We chose to examine the D102N/H57A protein in depth because it represented the simplest triad configuration to be active toward the peptide. The ensure that the observed hydrolysis was not due to contaminating serine peptidase activity, the mutant enzyme was treated with TLCK, which inactivates wild-type trypsin by labeling the active site histidine (see below). The TLCK-treated enzyme had the same specific activity toward both Z-GPR-AMC and insulin β-chain as did the untreated enzyme.

At pH 8.0 the cleavage was too slow to allow accurate determination of the kinetic constants, but the turnover rate could be estimated at 0.0017–0.0034 m^{-1}. At pH 10.1 the rate was enhanced, allowing the determination of K_m (130 μM) and k_{cat} (0.012 m^{-1}). While these rates were far below the turnover number for wild-type trypsin, it is necessary to recall that restriction enzymes are often not much faster (k_{cat} for *Eco*RI is 4 m^{-1}).[19] *If a catalyst is selective and available in substantial amounts, it need not process substrate rapidly in order to achieve practical uses.*

Dependence of k_{cat} on the Leaving Group. The leaving group of the GPR substrate was varied to assay the effect of that variable on catalysis (Table II). For wild-type trypsin, k_{cat} remained the same, approximately 3000–4000 m^{-1}, regardless of whether the leaving group was a benzyl thioester, *p*-nitroanalide, or aminomethylcoumarin. Moreover, the k_{cat} for the insulin β-chain substrate was almost as high (2500 m^{-1}), indicating that catalysis by the wild-type protease was surprisingly insensitive to the lability of the substrate amide linkage. The D102N mutant protein also had similar turnover numbers for the ester and activated amide linkages (1–3 m^{-1}), while k_{cat} for the unactivated insulin β-chain was much lower (0.066 m^{-1}). In contrast to the data for wild-type trypsin, these results indicate that acylation becomes the rate-determining step as the lability of the sissile bond decreases. This underscores the importance of determining the activity of amidase catalysts toward unactivated peptide linkages to gain an understanding of their catalytic limits.

Contamination due to Codon Usage. Whenever relatively inactive mutant enzymes are examined, care must be taken to ensure that the observed function is not due to contaminating protein.

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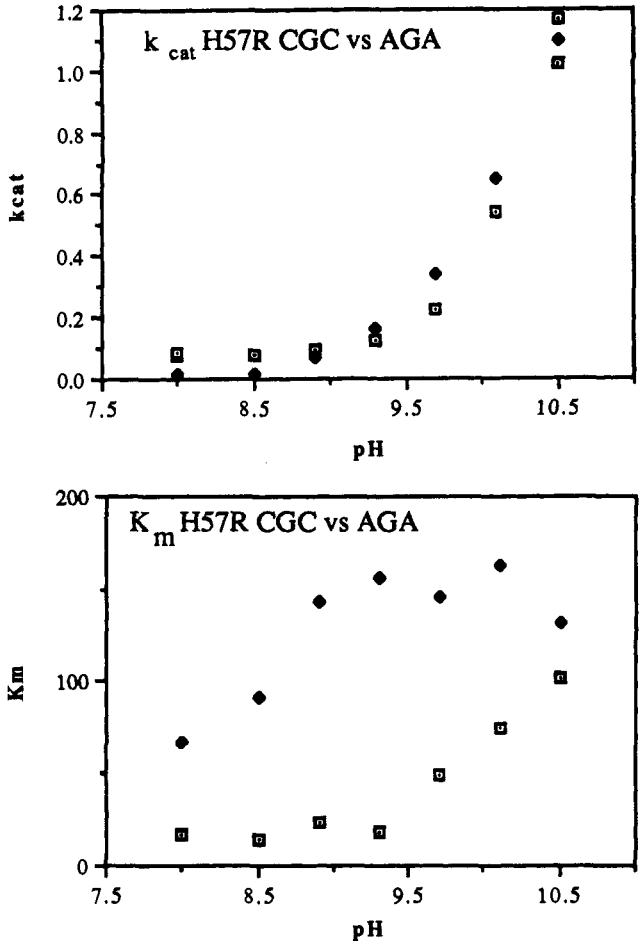

Figure 4. (a, top) k_{cat} versus pH–rate profile for H57R trypsin variants with position 57 encoded by either CGC or AGA. (b, bottom) K_m versus pH–rate profile for H57R trypsin variants with position 57 encoded by either CGC or AGA. The reaction buffer was 20 mM $CaCl_2$/100 mM NaCl/50 mM glycine-Cl (pH 8.5, 8.9, 9.3, 9.7, 10.1, 10.5) or 20 mM $CaCl_2$/100 mM NaCl/50 mM Tris-Cl, pH 8.0. Substrate concentrations were 400, 200, 100, 50, 25, and 12.5 μM. The concentrations of the CGC and AGA encoded enzymes were 125 and 40 nM, respectively. The kinetic parameters were determined using Z-GPR-AMC as a substrate as described in the Experimental Section. Key: ◻, CGC-H57R; ◆ AGA-H57R.

One way in which this contamination might arise is through the misreading of the mutant codon at the tRNA level and the subsequent insertion of a wild-type amino acid into the nascent polypeptide.[20]

In the course of our investigations we initially made the H57R mutation with the codon CGC. This codon is very similar to the histidine codon CAC. The resulting protein had the same K_m as wild-type at pH 8.0, but unlike wild-type, it showed a dependence of both K_m and k_{cat} on pH (Figure 4) (k_{cat} and K_m for wild-type rat trypsin are invariant over pH 8.0–10.1[12]). A second preparation of the enzyme gave similar results. Since contamination by wild-type protein would cause the K_m to be that of the wild-type at pH 8.0, we chose to redo the mutagenesis using a primer containing a different arginine codon, AGA. The K_m for the AGA-H57R protein at pH 8.0 was now significantly higher (67 μM).

Our explanation for the difference between the AGA and the CGC protein was that the putative CGC-encoded protein preparation contained some wild-type contamination. The contamination was estimated at approximately one part in 50000 as judged by the difference in turnover number at pH 8.0 between the two protein preparations. This is a minute quantity, but given the low activity of H57R trypsin at pH 8.0, it was sufficient to yield misleading kinetic data. The presence of contamination was further indicated by the observation that the CGC-encoded protein readily cleaved oxidized insulin β-chain within 12 h, while the AGA-encoded enzyme required a much longer incubation (70 h) to achieve a perceptible amount of hydrolysis. These observations emphasize that, for a mutant enzyme preparation, a pH-rate profile that is different from that of the wild-type enzyme does not necessarily indicate that there is no contribution *at all pHs* from wild-type contamination.

Precautions against Contamination. As noted above, the low activities of the mutant trypsins required that contaminating enzymes be eliminated as a possible explanation of the observed catalysis. *Escherichia coli* cultures containing a truncated trypsin gene exhibited no proteolytic activity, indicating that endogenous bacterial enzyme activity was not significant.[21] Nondenaturing SDS gel electrophoresis of the mutants followed by application of a substrate-impregnated overlay membrane[22] showed that enzyme activity migrated at the same location as with wild-type trypsin, further suggesting that the activity was not due to any endogenous contamination. Finally, the cleavage of either insulin β-chain or porcine galanin peptide occurred at arginine, confirming that the mutant preparations possessed a trypsin-like specificity.

Another source of contamination could be small amounts of wild-type trypsin, introduced either through codon level misincorporation or by plasmid contamination. Several lines of evidence indicate that the observed activity is due to the mutant protein: (1) All mutants titrate with 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) except for the H57E, H57A, and H57L proteins and those mutants lacking Ser 195. (2) Several mutants have K_m's which significantly differ from wild type (H57K, H57R, all mutants with the D102N mutation). (3) All mutants have pH–rate profiles different from those of the wild-type except for H57E, H57D, and those mutants lacking serine 195.

Taken together, these results indicate that the activity of each mutant enzyme preparation was predominantly due to protein with the mutation. However, they do not rule out the presence of an amount of wild-type contamination too small to be observed in the kinetic assays. Such a minute amount of activity might account for the activity against insulin β-chain. One line of evidence against this is that those mutants containing Ser 195 uniformly retained at least a minimal level of activity against the peptide, while those lacking Ser 195 showed no activity. In addition, variants containing mutations at both His 57 and Asp 102 retained activity, eliminating any source of contamination involving amino acid misincorporation since two misincorporation events would be needed to generate wild-type protein.

The possibility of contamination was further tested by the treatment of the H57A/D102N variant with TLCK, a compound which inactivates wild-type trypsin by labeling the active site histidine. Both H57A/D102N and wild-type bovine trypsin were treated with 1 mM TLCK and then subsequently dialyzed against 1 mM HCl to remove unreacted label. Titration with MUGB demonstrated that the wild-type protein had lost 94% of its original reactivity, while the H57A/D102N protein was fully active. The treated mutant also showed no alteration in its activity toward Z-GPR-AMC or insulin β-chain (see above).

Conclusion

Proteolytic function depends on the complex interaction of amino acid chemistry and transition-state stabilization to hydrolyze amide bonds, which otherwise would have a half-life of 7 years.[1] For trypsin and other similar serine proteases this involves three finely balanced catalytic residues, His 57, Asp 102, and Ser 195, an oxyanion hole formed by backbone amides, and transition-state stabilization due to substrate binding. The incorporation of all of these characteristics into catalytic antibodies, designed peptides,

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or preexisting unrelated proteins would be a very demanding project. Each addition would need to be fit in a precise spatial orientation while simultaneously avoiding the disruption of substrate binding or preexisting catalytic features.

Our results indicate that this problem can be simplified because, of the three members of the triad, only serine is essential for the cleavage of unactivated amide bonds by trypsin. The presence of an adjacent basic residue, lysine or histidine, can increase this catalysis further. Moreover, designed systems need not be limited to serine since it is the intrinsic structural framework of trypsin which prevents other nucleophilic side chains from functioning. For other protein scaffolds the presence of cysteine, threonine, or tyrosine may also be sufficient. Thus, a small, but measurable level of activity can be obtained by a simplified protease, indicating that initial attempts at introducing peptidase function into proteins should focus on the introduction of an oxyanion hole,[23,24] transition-state stabilization via substrate binding, and a strategically positioned nucleophile instead of an entire triad. Subsequent studies could then be undertaken to maximize this catalysis.

Experimental Section

Materials. Rat anionic trypsin was obtained by expression of plasmid pT3 containing the tac promoter and the *Salmonella typhimurium* his J coding sequence[25] in *E. coli* strain X90 (F', *lac*IQ *lac*ZY, *pro*A/Δ-(*lac–pro*) *ara*, *nal*A, *arg*Eam, *thi*, *rif*R) or HB101 (*supE*44*hsd*S20-($r_b^-m_b^-$)*rec*A13*ara*-14*pro*A2*lac*Y1*gal*K2*rsp*L20*xyl*-5*mtl*-1). Z-GPR-AMC, Z-LGR-pNA, porcine galanin peptide, and Z-GPR-pNA were obtained from Bachem Bioscience. Z-GPR-AFC, Z-GPR benzyl thioester, and cellulose diacetate membranes were purchased from Enzyme Systems Products. S-Sepharose Fast Flow resin was obtained from Pharmacia, and benzamidine-derivatized agarose was from Pierce. Oxidized insulin β-chain and 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) were from Sigma. DNA oligonucleotides were synthesized using an Applied Biosystems 391 PCR Mate DNA synthesizer. Fluorescent kinetic assays were done using a Perkin-Elmer LS-5B spectrometer. Microplate assays were accomplished with a Molecular Devices UV MAX kinetic microplate reader. UV spectrophotometric kinetic assays were performed using a Kontron 860 spectrophotometer. Bovine cationic trypsin and goat–anti-rabbit antibody/horseradish peroxidase conjugate were obtained from Boerhinger Mannheim.

Site-Directed Mutagenesis and Protein Isolation. Oligonucleotide-directed mutagenesis was performed using single-stranded template by the method of Kunkel.[26] The primers for mutagenesis (with the mutated codon underlined) were 5'-GTG-TCT-GCA-GCT-GAT-TGC-TAT-AAG-TCC-3' (H57D), 5'-GTG-TCT-GCA-GCT-AGA-TGC-TAT-AAG-TCC-3' (H57R), 5'-GTG-TCT-GCA-GCT-AAG-TGC-TAT-AAG-TCC-3' (H57K), 5'-GTG-TCT-GCA-GCT-GCG-TGC-TAT-AAG-TCC-3' (H57E), 5'-GTG-TCT-GCA-GCT-GCC-TGC-TAT-AAG-TCC-3' (H57A), 5'-GTG-TCT-GCA-GCT-CTG-TGC-TAT-AAG-TCC-3' (H57L), 5'-GC-CAG-GGT-GAC-GCA-GGT-CCT-GT-3' (S195A), 5'-GC-CAG-GGT-GAC-ACC-GGT-CCT-GT-3' (S195T), and 5'-CTG-AAC-AAC-AAC-ATC-ATG-CTG-3' (D102N).

After expression in *E. coli* strain X-90 the resultant mutants were differentiated from wild-type by their greatly decreased ability to hydrolyze Z-LGRpNA (100 μM in 100 mM Tris-Cl, pH 8.0, buffer) on a microplate assay as measured at 405 nm. Putative mutants were then retransformed, and the presence of the mutation was confirmed by Sanger dideoxy sequencing of the double-stranded plasmid. An overnight culture of *E. coli* X-90 or HB101 containing the mutant plasmids was used to inoculate 3 L of LB media containing 60 mg/L ampicillin and 0.2 mM IPTG. The cultures were grown with vigorous shaking for 6–12 h. The cells were then pelleted, and the periplasmic fraction was obtained as described.[17] This fraction was exhaustively dialyzed against 50 mM glycine, pH 2.5, after which solid precipitate was removed by centrifugation. The supernatant was applied to a column consisting of 10 mL of S-Sepharose Fast Flow cation-exchange resin to separate the trypsin from ecotin,[27] an endogenous *E. coli* protease inhibitor. Trypsin was eluted using a gradient of 0–1.5 M NaCl/50 mM glycine, pH 2.5. Fractions containing ecotin-free trypsin were identified by western blot with anti-trypsin and anti-ecotin antibodies or, in the case of the more active variants, by a microplate assay using Z-LGRpNA in either 100 mM Tris-Cl, pH 8.0, or 50 mM glycine, pH 10.5, buffer. The trypsin-containing fractions were pooled and dialyzed against 10 mM MES, pH 6.0, buffer. The solution was then applied to a 1.0-mL *p*-aminobenzamidine-agarose affinity column and eluted as described.[17] The fractions containing trypsin were identified by their UV absorbance at 280 nm and concentrated using a Centricon 10 microconcentrater (Amicon). Trypsin stocks were stored in 1 mM HCl at 4 °C and showed no decrease in activity over the course of these experiments (6 months).

Determination of Active Site Concentration. The concentration of trypsin active sites for some of the mutants was determined spectrofluorometrically using 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), which reacts with serine 195. The titrations were performed at 24 °C in 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0, buffer. The titrations required from 5 to 90 min depending on the mutant. For those mutants which did not titrate (H57A, H57L, H57E, S195A, S195T, and the triple mutant H57A/D102N/S195A) the concentration of trypsin was determined spectrophotometrically at 280 nm ($\epsilon = 1.4$). The concentration of active sites was then estimated at 80% of the spectrophotometric value, a specific activity which was typical of all of the mutants which could be titrated.

TLCK Treatment of Bovine Wild-Type and H57A/D102N Trypsin. H57A/D102N or wild-type bovine enzyme (1 mg) was treated with 1 mM TLCK in 2 mL of 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0. The reactions were incubated for 1 h at 24 °C after which they were exhaustively exchanged using a Centricon 10 microconcentrator against 1 mM HCl to remove unreacted TLCK. The activity of the treated enzymes was determined by MUGB titration and Z-GPR-AMC hydrolysis.

Activity Assays. Fluorescence assays were performed by following the release of aminomethylcoumarin from Z-GPR-AMC as described[16] (ex = 380, em = 460). Base-line hydrolysis was monitored over 30 min and subtracted from the hydrolysis by the enzyme. Hydrolysis was allowed to proceed until greater than one turnover event had occurred to demonstrate catalysis. The concentrations of trypsin variants used were 22 nM (H57R), 60 nM (H57A, D102N), 110 nM (H57A, D102N, S195A), 57 nM (S195A), 22 nM (S195T), 2.5 nM (wild-type), 18 nM (H57E), 21 nM (H57D), 29 nM (H57K), 42 nM (H57L), 20 nM (H57A), 30 nM (D102N, H57K), 11 nM (D102N), and 12 nM (D102N, H57L and D102N, H57D). Ester hydrolysis was monitored spectrophotometrically at 324 nm using a coupled assay of Z-GPR-thiobenzyl ester and 4,4'-dithiobis(pyridine) as described.[12] The hydrolysis of Z-GPR-pNA was followed at 410 nm.

The hydrolysis of unactivated amide bonds was assayed by following the cleavage of oxidized insulin β-chain peptide [FVNQHLC(SO_3)-GSHLVEALYLVC(SO_3)GERGFFYLPKA]. The peptide substrate was dissolved in 1:1 DMF/H_2O. Cleavage reactions were performed at 37 °C in 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0, buffer. Assays with wild-type trypsin (4 nM) were quenched after 8–12 min with an equal volume of 25% acetic acid and were stored on dry ice prior to analysis. Assays with D102N or H57A/D102N were terminated after either 16–20 or 40–48 h, respectively by storage on dry ice. The cleavage fragments were resolved by reversed-phase HPLC using a C18 Vydex column and an acetonitrile gradient (0–80%, 0.1 trifluoroacetic acid). The identity of the fragments was determined by mass spectral analysis. The cleavage of porcine galanin peptide (GWTLNSAGYLLGPHAID-NHRSFHDKYGLA-amide) was analyzed similarly.

Enzyme Overlay Membranes. The wild-type and mutant variants were loaded onto a nondenaturing SDS–polyacrylamide gel. After electrophoresis, the gel was soaked for 1 h in 1% aqueous Triton X-100 to remove the SDS and then soaked for 5 min in 20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0, buffer. The substrate-impregnated cellulose diacetate membrane (prepared as suggested by the manufacturer by soaking in a 50 μM solution of Z-GPR-AFC in 100 mM NaCl/20 mM $CaCl_2$/50 mM Tris-Cl, pH 8.0) was then applied to the surface of the gel. The resultant bands due to the release of the AFC leaving group were visualized by short-wave ultraviolet light. Wild-type protein (100 ng/lane) produced a visible band within 10 min, while the mutant trypsins (1–2 μg/lane) required from 30 to 60 min.

Abbreviations: TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Z-GPR-AMC, [N^{α}-(benzyloxycarbonyl)-L-glycylprolylargininyl]-7-amino-

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4-methylcoumarin; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane; DMF, *N*,*N*-dimethylformamide; Boc-LGRpNA, Boc-lysylglycylarginyl-*p*-nitroanilide; IPTG, isopropyl β-D-thiogalactopyranoside; AFC, 7-amino-4-(trifluoromethyl)-coumarin; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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Total Synthesis of (±)-Chondrillin, (±)-Plakorin, and Related Peroxy Ketals. Development of a General Route to 3,6-Dihydro-1,2-dioxin-3-ols

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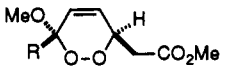
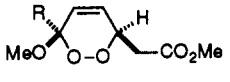
Abstract: Seven-step syntheses of the antitumor cyclic peroxy ketals **1a**, **2a**, chondrillin (**1b**), and plakorin (**2b**) from (methoxymethoxy)benzene (**8**) have been achieved in 26–28% overall yields. The key step is the photooxygenation of enone **4** with a sun lamp using rose bengal lactone or $CuSO_4$ as a sensitizer which gives a mixture of peroxy hemiketals **15** and **16** in 75–85% yields. Acetal formation in acidic methanol completes the syntheses of **1** and **2**. The mechanism of photooxygenation was ascertained using 3-nonen-2-one (**22**) as a model for **4**. Irradiation converts **22** to the *cis*-enone **23** which undergoes photoenolization to give **24**. Dienol **24** undergoes a sensitized reaction with oxygen to give **29** and **30**. The detailed mechanism of this last step is not known, although singlet oxygen is probably not involved. This reaction is general for any enone or enal which can undergo photoenolization to give a dienol. Peroxy hemiketals **33a**, **41**, and **43–46** were prepared in 30–80% yields. Peroxy ketals can be used for the synthesis of furans, diones, and pyridazines.

Introduction

A wide variety of biologically active cyclic peroxides have been isolated from marine organisms. Chondrillin (**1b**) was first isolated in 1976 from a sponge of the genus *Chondrilla* by Wells.[1] More recently, xestins A (**2c**) and B (**1c**) have been isolated from a sponge of the genus *Xestospongia* by Crews,[2] chondrillin (**1b**) and a series of related ketals (**2a,f–h**) have been isolated from *Plakortis lita* by Higa and Christophersen,[3] chondrillin (**1b**), *epi*-chondrillin (**2b**), and a series of unsaturated analogues (**1d,e**, **2d,e**) have been isolated from *P. lita* by De Guzman and Schmitz,[4] and *epi*-chondrillin (called plakorin) (**2b**) has been isolated from a *Plakortis* species by Kobayashi.[5]



1



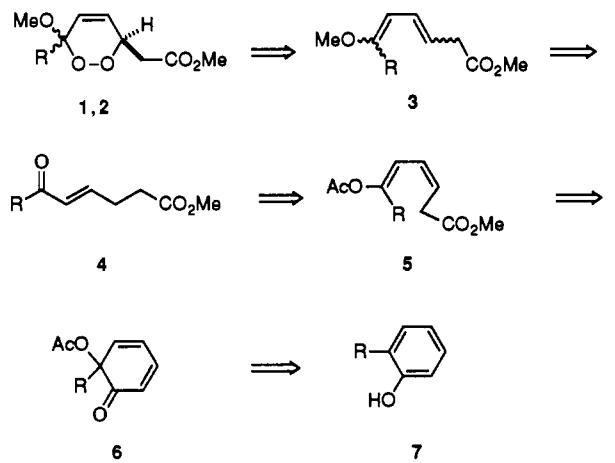
2

a, R = $C_{12}H_{25}$
b, R = $C_{16}H_{33}$
c, R = $C_{13}H_{26}CH{=}CHCH{=}CHCH_3$
d, R = $C_{13}H_{26}CH{=}CHCH_3$
e, R = $C_{11}H_{22}CH{=}CHCH{=}CHCH_3$
f, R = $C_9H_{18}CH{=}CHCH_3$
g, R = $C_7H_{14}CH{=}CHCH{=}CHCH_3$
h, R = $C_9H_{18}CH{=}CHCH{=}CHCH_3$

Peroxy ketals **2a,c,f–h** have been shown to be active against P388 mouse leukemia cells in vitro with IC_{50} values of 0.05–0.3 μg/mL.[2,3] The isomers **1b,c** are approximately 1 order of magnitude less active. Plakorin (**2b**) (10^{-5} M) activated SR Ca^{2+}-ATPase activity by 30% and exhibited antineoplastic activity against L1210 cells and KB cells in vitro with IC_{50} values of 0.85 and 1.8 μg/mL, respectively.[5] A variety of related cyclic peroxides with branched skeletons, including the norsesterterpenes trunculins A and B,[6] plakortin,[7] plakortic acid,[8] and plakinic acid B,[8] have been shown to possess antitumor and antimicrobial activity.

The structural novelty and potent biological activity of peroxy ketals **1** and **2** prompted us to undertake their synthesis. Despite the biological activity and ostensible simplicity of these cyclic

Scheme I



1,2 3 4 5 6 7

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