Novel Inhibitors of Human Leukocyte Elastase and Cathepsin G. Sequence Variants of Squash Seed Protease Inhibitor with Altered Protease Selectivity

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ABSTRACT: Novel peptide inhibitors of human leukocyte elastase (HLE) and cathepsin G (CG) were prepared by solid-phase peptide synthesis of P1 amino acid sequence variants of *Curcurbita maxima* trypsin inhibitor III (CMTI-III), a 29-residue peptide found in squash seed. A systematic study of P1 variants indicated that P1 Arg, Lys, Leu, Ala, Phe, and Met inhibit trypsin; P1 Val, Ile, Gly, Leu, Ala, Phe, and Met inhibit HLE; P1 Leu, Ala, Phe, and Met inhibit CG and chymotrypsin. Variants with P1 Val, Ile, or Gly were selective inhibitors of HLE, while inhibition of trypsin required P1 amino acids with an unbranched β carbon. Studies of Val-5-CMTI-III (P1 Val) inhibition of HLE demonstrated a 1:1 binding stoichiometry with a $(K_i)_{app}$ of 8.7 nM. Inhibition of HLE by Gly-5-CMTI-III indicated a significant role for reactive-site structural moieties other than the P1 side chain. Val-5-CMTI-III inhibited both HLE and human polymorphonuclear leukocyte (PMN) proteolysis of surface-bound ^{125}I-labeled fibronectin. Val-5-CMTI-III was more effective at preventing turnover of a peptide *p*-nitroanilide substrate than halting dissolution of ^{125}I-labeled fibronectin. It was about as effective as human serum α_1-proteinase inhibitor in preventing PMN degradation of the connective tissue substrate. In addition to providing interesting candidates for controlling inflammatory cell proteolytic injury, the CMTI-based inhibitors are ideal for studying molecular recognition because of their small size, their ease of preparation, and the availability of sensitive and quantitative assays for intermolecular interactions.

Human leukocyte elastase (HLE)[1] and CG are the predominant neutral proteases contained within azurophil granules of PMN. HLE confers powerful proinflammatory and matrix degradative activity on PMN because of the wide range of substrates that are susceptible to its catalytic activity (Bieth, 1986). The potential physiological roles of CG are less clear, although it has been shown to degrade a number of serum and connective tissue proteins [reviewed by Senior and Campbell (1983)] and to increase transendothelial albumin flux (Peterson, 1989); thus, CG may also play a role in extracellular events mediated by PMN during inflammation. Particular attention has been focused on the role(s) of HLE in the pathogenesis of acute and chronic lung injury in humans; especially interesting was the immunolocalization of HLE at the sites of tissue injury in emphysematous lungs (Damiano et al., 1986). Because of the importance of HLE and CG to human biology and disease, they have been subjected to intense scrutiny, and there has been considerable interest in the development of effective inhibitors of their catalytic activity.

The purpose of the present paper is to report the development of a series of new peptide-based inhibitors of HLE and CG that have potential utility for limiting inflammatory cell proteolysis. These inhibitors were a direct result of altering the protease selectivity of squash seed protease inhibitor (Hojima et al., 1982) through mutation of its reactive-site sequence. Characterization of a representative HLE inhibitor revealed a potent stoichiometric inhibitor capable of inhibiting not only purified HLE but also proteolytic activity of PMN.

Squash Family of Serine Protease Inhibitors. The squash family of serine protease inhibitors is a series of homologous peptides found in the seeds of Curcurbitaceae plants (Hojima et al., 1982; Wieczorek et al., 1985; Joubert, 1984; Otlewski et al., 1987). Members of the family are potent inhibitors of trypsin-like proteases (K_i with trypsin ca. 10^{-12} M; Wieczorek et al., 1985) with the reactive-site peptide bond located between residues 5 (P1,[2] Arg or Lys) and 6 (P1′, Ile). The nature of the interaction of a squash seed protease inhibitor with trypsin has been reported in a recent X-ray crystallographic study (Bode et al., 1989). All of the available evidence indicates that the inhibitors bind in a nonproductive, substrate-like mode (Laskowski & Kato, 1980).

Reactive-Site Mutation of CMTI-III. The basic approach is to manipulate the sequence of a squash family inhibitor in

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[1] Abbreviations: HLE, human leukocyte elastase (EC 3.4.21.11); CG, human leukocyte cathepsin G (EC 3.4.21.20); PMN, polymorphonuclear leukocyte(s); CMTI-III, *Curcurbita maxima* trypsin inhibitor III or squash seed protease inhibitor; pNA, *p*-nitroanilide; FN, purified human plasma fibronectin; ^{125}I-FN, FN labeled with ^{125}I; Aaa-5-CMTI-III, a P1 substitution of Aaa for Arg in the CMTI-III sequence, where Aaa represents the three-letter code for amino acids; $(K_i)_{app}$, apparent equilibrium inhibition (dissociation) constant; $[I]_0$, total concentration of peptide inhibitor; $[E]_0$, total concentration of protease; $[S]_0$, initial substrate concentration; K_m, Michaelis constant; CMTI-I, isoinhibitor related to CMTI-III by substitution of Glu-9 for Lys; α_1-PI, purified human serum α_1-proteinase inhibitor (antitrypsin); $(dA/dt)_{test}$, initial rate of change in absorbance at 405 nm in the *presence* of putative inhibitor; $(dA/dt)_{control}$, initial rate of change in absorbance at 405 nm in the *absence* of putative inhibitor.

[2] The notation of Schecter and Berger (1967) is used to denote the relative positions of residues with respect to the reactive-site (scissile) peptide bond of substrate or inhibitor. In this scheme P1, P2, P3, etc. refer to the series of residues proceeding away from the reactive-site peptide bond in the direction of the amino terminus, one residue at a time. A similar definition for P1′, P2′, P3′, etc. obtains, except that the direction is toward the carboxyl terminus. S1 refers to the complementary binding site on the protease for the P1 amino acid of the substrate or inhibitor.

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order to alter its binding selectivity among a series of target (homologous) serine proteases. CMTI-III (Wieczorek et al., 1985; also see footnote *b* of Table I) is a representative member of the squash family consisting of a single chain of 29 amino acids with three disulfide cross-links. The short, highly cross-linked chain suggested a well-defined conformation with features that allow for the strong interaction with the (homologous serine protease) active site(s). The short sequence also meant that CMTI-III, and sequence variations thereof, could be prepared by solid-phase peptide synthesis and folded to give active material (Kupryszewski et al., 1986).

Since the major determinant of inhibitor protease selectivity is the P1 amino acid side chain (Laskowski & Kato, 1980), the initial variants were chosen on the basis of the P1 substrate preference of HLE and CG (Nakajima et al., 1979). A limited number of substitutions at other positions within the reactive site have also been made (not shown). After completion of the experiments for the present study, it was independently suggested that substitution of Val at the P1 residue of CMTI-III could be used to obtain an HLE inhibitor (Bode et al., 1989), although no data were reported.

Materials and Methods

Reagents for peptide synthesis were purchased from Peptides International (Louisville, KY) and from Peninsula Laboratories, Inc. (Belmont, CA). Bovine trypsin (Boehringer Mannheim, Indianapolis, IN), bovine chymotrypsin (Sigma, St. Louis, MO), and HLE and CG (EPC, Inc., Pacific, MO) were obtained from the sources indicated and used without further purification. All other reagents and supplies were of analytical grade or better.

Peptide Synthesis. Peptides were prepared by using the Merrifield method, essentially as described by Stewart and Young (1984), starting with Boc-Gly Merrifield resin (0.5 mequiv/g). A 30-g sample of CMTI-III-(10–29)-resin was prepared as starting material for use in the synthesis of reactive-site variants. Variants were prepared by sequential coupling of the appropriate nine N-terminal Boc-amino acids to between 0.5 and 1.0 g of CMTI-III-(10–29)-resin.

The peptides were cleaved with HF at 0 °C for 60 min in the presence of 5% anisole and 1% mercaptopyridine. The HF-treated resin was washed with ether and then 50% acetic acid. The acid washings were subsequently diluted and lyophilized.

Oxidation of CMTI-III Variants. The CMTI-III variants were oxidized without purification of the reduced peptide. Crude peptides were oxidized at a concentration between 2 and 4 mg/mL in 0.1 M Tris-HCl, pH 8.75, containing 1 mM EDTA, 0.5 mM oxidized glutathione, and 5.0 mM reduced glutathione. After 2 h at room temperature, the presence of active inhibitor in the reaction mixture was verified by an appropriate inhibition assay (see below). A portion of this material was chromatographed by reverse-phase HPLC and the major peak(s) collected for inhibition assays.

Purification of CMTI-III Variants. The oxidized peptides were purified by preparative reverse-phase chromatography (40 × 350 mm, Vydac C_{18}, 10 μm). Peptides were loaded in 5% acetonitrile and eluted at 6 mL/min with a linear gradient of acetonitrile/water with each phase containing 0.1% TFA. Fractions were collected and tested in the appropriate inhibitory assay (see below). Positive fractions with >95% purity (as determined by reverse-phase HPLC) were pooled and lyophilized. The peptides eluted in a range between 22% and 28% acetonitrile; the percentage of acetonitrile at which a variant eluted correlated with the changes in net hydrophobicity introduced by the different amino acid substitutions. Peptide recovery was between 5 and 50 mg. The peptides were analyzed by amino acid composition and sequence analysis. FAB mass spectroscopy was used to confirm the identity of Val-5-CMTI-III.

Spectrophotometric Assays for Enzyme Inhibition. Spectrophotometric enzyme assays monitored the increase in absorbance at 405 nm resulting from the hydrolysis of peptide *p*-nitroanilide (pNA) substrates. Thus, percent inhibition is determined from the diminution of the observed rate of change in absorbance at 405 nm for a test (putative inhibitor) sample relative to a control that does not contain test sample by using the formula

$$\% \text{ inhibition} = 100\{1 - [(dA/dt)_{test}/(dA/dt)_{control}]\} \quad (1)$$

The analyses were performed as described in the references listed below with the substrates and final concentrations as follows. For trypsin-catalyzed hydrolysis of benzoyl-L-Arg-pNA (BAPNA; Fritz et al., 1974), final concentrations were 0.8 mM BAPNA and 3.3 μg of trypsin in 3 mL of 110 mM triethanolamine and 11 mM $CaCl_2$ at pH 7.8. For HLE-catalyzed hydrolysis of *N*-methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-pNA (Nakajima et al., 1979), final concentrations were 0.9 mM substrate and 1 μg of HLE in 3 mL of 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.03% sodium azide. For HLE-catalyzed hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Ala-pNA (Bieth et al., 1974), final concentrations were 1.0 mM substrate and 50 (K_i determinations) or 500 nM (stoichiometry titration) HLE in the above pH 7.5 Tris-HCl buffer. For CG-catalyzed hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-pNA (DelMar et al., 1979; Nakajima et al., 1979), final concentrations were 0.5 mM substrate and 10 μg of CG in 3 mL of 100 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and 0.03% sodium azide. For chymotrypsin-catalyzed hydrolysis of *N*-methoxysuccinyl-L-Arg-L-Pro-L-Tyr-pNA (Kabi Diagnostik, Stockholm, Sweden), final concentrations were 0.5 mM substrate and 2.5 μg of chymotrypsin in 3 mL of the trypsin assay buffer.

^{125}I-FN Assay. This is an assay designed to model the degradation of insoluble extracellular matrix components by viable inflammatory cells. The assay, carried out essentially as described by Campbell et al. (1982), used ^{125}I-FN coated as a solid substrate in the wells of a microtiter plate. Assays were performed in triplicate and employed defined amounts of proteolytic activity in the form of either purified HLE or freshly separated PMN stimulated with phorbol myristate acetate (10 ng/mL); the amounts of proteolytic activity were predetermined in spectrophotometric assays. A standard curve showing the linearity of released ^{125}I counts to the amount of purified HLE added was obtained concurrently with the assay. Inhibition was determined from the decrease in released counts for a test sample relative to a control without inhibitor added.

Stoichiometry of Inhibitor/Enzyme Complexes. The binding stoichiometry of CMTI-III to trypsin and Val-5-CMTI-III to HLE was determined by first equilibrating the inhibitor/protease pair at various ratios and then measuring the remaining amount of free enzyme with the spectrophotometric assay. At the concentrations employed, the substrates do not effectively compete for enzyme with these inhibitors. The conditions used in the assay are given in the legend to Figure 1.

Determination of $(K_i)_{app}$ for Val-5-CMTI-III with HLE. The determination of $(K_i)_{app}$ employed the same procedure as the stoichiometry experiment except that the final concentration of HLE was decreased to 50 nM in order to observe some free HLE under the conditions of the experiment. The

Table I: Inhibition of Serine Proteases by CMTI-III Sequence Variants[a]

variants	trypsin	HLE	CG	chymotrypsin
Arg-5[b]	+	–	–	–
Val-5	–	+	–	–
Ile-5	–	+	–	–
Gly-5	–	+	–	–
Leu-5	+	+	+	+
Ala-5	+	+	+	+
Phe-5	+	+	+	+
Met-5	+	+	+	+

[a] Symbols denote the following: +, greater than 10% inhibition; –, less than 10% inhibition. See text for experimental details. [b] Arg-5 is the wild-type sequence of CMTI-III, shown here with the one-letter code for amino acids. The reactive-site peptide bond is shown by the vertical line.

$$\overset{P1}{R}\ V\ C\ P\ \overset{P1'}{R}\ |\ I\ L\ M\ K\ C\ K\ K\ D\ S\ D\ C\ L\ A\ E\ C\ V\ C\ L\ E\ H\ G\ Y\ C\ G$$

procedures of Empie and Laskowski (1982) for working at high dilution were employed. The inhibition (dissociation) constant was determined from the data according to the method of Bieth (1974) for tight-binding inhibitors.

Results and Discussion

We have prepared and studied a variety of sequence variants of a protease inhibitor (CMTI-III) derived from *Curcurbita maxima*; these variants display distinct spectra of inhibitory activity toward various serine proteases. For the purpose of altering protease selectivity, the sequence of CMTI-III was divided into two portions: a reactive-site portion (residues 1–9) and a framework portion (residues 10–29). The reactive site dictates protease selectivity, and the framework portion provides the structure needed to maintain the proper reactive-site geometry. Important features of the present work are the following: (1) CMTI-III's small size for a polypeptide inhibitor (29 residues) facilitates preparation and screening of CMTI-III variants; and (2) high-resolution structures of polypeptide inhibitor/serine protease complexes have been described that have both guided (Ruhlmann et al., 1973; Sweet et al., 1974; Huber et al., 1974, 1975; Bode et al., 1978, 1986; Mitsui et al., 1979; Fujinaga et al., 1982, 1987) and supported (Bode et al., 1989) our decisions regarding apportionment of framework versus reactive-site portions of CMTI-III. This work provides insights into the constraints of high-affinity binding of polypeptide inhibitors to serine proteases and has resulted in synthesis of novel peptide inhibitors of HLE and CG.

Other studies have obtained sequence variants from naturally occurring homologous sequences [avian ovomucoid third domain (Empie & Laskowski, 1982)], enzymatic semisynthesis [soybean trypsin inhibitor (Kowalski et al., 1974; Jering & Tschesche, 1976), bovine pancreatic trypsin inhibitor (Beckmann et al., 1986)], or site-directed mutagenesis [α_1-PI (Courtney et al., 1985)]. The homologous sequence approach is limited to those substitutions found in nature, while the semisynthetic and recombinant DNA techniques are relatively complicated. In contrast, the CMTI-III variants are easily prepared and are not limited to either single-site substitutions or the usual 20 L-amino acids.

Detection of Protease Binding by CMTI-III Variants. This paper focuses on the effects of sequence variation in CMTI-III upon selectivity toward trypsin, HLE, CG, and chymotrypsin. The first experiments were designed to screen variants in a plus/minus fashion (Table I) for moderate to strong binding to these proteases. In order to interpret the results of these experiments, it is necessary to consider the limit of detection for interacting species and how these limits can be used to estimate protease selectivity. The limit of detection for interacting species, that is, the threshold $(K_i)_{app}$ above which inhibition will not be detected in the screen, can be estimated from the following considerations. It can be shown (Bieth, 1974) that at equilibrium

$$\frac{[I]_0}{[E]_0} = \frac{(K_i)_{app}(1 - \text{activity})}{[E]_0(\text{activity})} + (1 - \text{activity}) \qquad (2)$$

where $[I]_0$ and $[E]_0$ are the total concentrations of inhibitor and protease, $(K_i)_{app}$ is the apparent equilibrium inhibition (dissociation) constant, and the activity, determined from the turnover of substrate, is $[E]/[E]_0$. Substituting an upper limit estimate[3] of 50 for the $[I]_0/[E]_0$ ratio and the limit of detection for inhibition (activity ≤ 0.9) into eq 2 gives, after rearrangement, a threshold $(K_i)_{app} < 500[E]_0$. If the actual value of $(K_i)_{app}$ exceeds this threshold, or if the association kinetics are slow, then binding will not be detected in an assay. In this way, the threshold $(K_i)_{app}$s *for this plus/minus screen* are estimated to be about 10 μM. Positive results in Table I cannot be ranked by potency because many of the inhibitors simply titrate the proteases under the assay conditions (see below). The level of inhibition for several of the inhibitors was found to be independent of the order of addition of substrate and inhibitor and was detectable within the dead time of manual mixing (ca. 15 s, not shown), thus indicating that the associations were relatively rapid.

A detailed understanding of the results of Table I would require a quantitative determination of inhibitor binding; it might also require structural data in order to account for conformational differences in inhibitors and proteases. Inhibitor selectivity between two proteases can be evaluated by taking the ratio of the $(K_i)_{app}$s. The data of Table I only categorize the $(K_i)_{app}$s relative to the thresholds as described above. Thus, a peptide with a plus entry for one protease and a minus for another, corresponds to a minimum selectivity [ratio of $(K_i)_{app}$s] on the order of 10. In many cases, data not shown indicate that this ratio is actually larger, but the data of Table I alone do not provide such information. Nevertheless, the data are quite interesting with regard to trypsin selectivity, HLE inhibition, and inhibition of chymotrypsin-like enzymes. All of the following discussion of protease selectivity is made with reference to the threshold $(K_i)_{app}$s described above.

Trypsin Selectivity. Beginning with trypsin selectivity, it appears that trypsin is quite tolerant of P1 amino acid substitution (Table I), this despite the well-known preference for basic residues at the P1 position (Bergmann & Fruton, 1941). Arg, Leu, Ala, Phe, and Met are permissible, while Val, Ile, and Gly are not. P1 Lys is also known to be functional (Wieczorek et al., 1985). Comparing the literature K_i value of CMTI-III/trypsin with the threshold $(K_i)_{app}$ of 10 μM

[3] The limit of detection for percent inhibition (see eq 1 of text) is determined by the uncertainty in the measurement of the velocity for both the control and test samples. The precision of each of these velocity measurements is conservatively estimated to be ±5%, and thus, inhibition can be safely concluded at or above 10% inhibition; this corresponds to activity ≤ 0.9. When a peptide resulted in activity >0.9, its concentration was increased and the activity measured again until either the activity dropped below 0.9 or the upper limit in peptide concentration possible with the peptide stock solution had been reached. From the estimated concentration of peptide stock solutions, the upper limit on the $[I]_0/[E]_0$ ratios for all of the negative entries of Table I was estimated to be 50; note that most of the $[I]_0/[E]_0$ ratios in Table I were lower, with many being less than 1.

indicates that P1 Arg provides a factor of about 10^6–10^7 in discriminating between trypsin and the other three proteases of Table I. With regard to inhibitor binding to trypsin, the acceptability of nonsubstrate side chains at the P1 position of trypsin inhibitors has been noted for Met (α_1-PI; Johnson & Travis, 1978, 1979) and Phe (soybean trypsin inhibitor variant; Kowalski et al., 1974). By use of molecular graphics, an inspection of the trypsin–BPTI crystal structure (Marquart et al., 1983) suggests that steric interactions will prevent trypsin from binding β-branched P1 residues in the S1 subsite, in agreement with the data of Table I. By comparison of the threshold $(K_i)_{app}$ for the trypsin assay with the literature CMTI-III/trypsin K_i, an estimated loss of at least 6 orders of magnitude in affinity for trypsin results from substitution of P1 Val, Ile, and Gly. The example of P1 Leu and Ala as trypsin inhibitors appears to be new. Moreover, an unexpected finding was that a variant with P1 Ala, but not P1 Gly, was effective. With regard to the latter finding, it is not known if the simple loss of a methyl group interaction would be sufficient to explain the loss of binding or if substitution of Gly in the P1 position merely increases reactive-site flexibility and thereby raises the $(K_i)_{app}$ above the threshold of 10 μM. Alternative explanations, such as differences in solvation or binding modes, may also be important.

HLE Inhibition. The P1 substrate preferences of HLE are for moderately bulky hydrophobic groups (Nakajima et al., 1979). A much wider range of P1 substitutions (Val, Ile, Leu, Ala, Phe, Met, and Gly; Table I) were found to be functional. Of these, Val, Ile, and Gly showed the sharpest selectivity for HLE, giving no detectable inhibition of trypsin, CG, and chymotrypsin. In the case of Val-5-CMTI-III, the difference in selectivity $[(K_i)_{app}]$ between HLE and the other three proteases is at least 3 orders of magnitude. In contrast to the situation with trypsin (see above), P1 Gly functioned, thus indicating the importance of the reactive-site backbone conformation and its interaction with HLE. Indeed, the CMTI-I/trypsin complex does show a number of hydrogen bonds from main-chain atoms of the inhibitor reactive site to main-chain atoms of the protease active site (Bode et al., 1989); it may be that these types of interactions have more importance in the interaction of CMTI-III variants with HLE than with trypsin. Other factors that could be important for P1 Gly functionality include flexibility, solvation, and the possibility of a different bound-state conformation. It may also be relevant to note that P1′ substitutions (McWherter, unpublished results) suggest a possible role for this side chain, either directly or indirectly, in the interaction with HLE.

Inhibition of Chymotrypsin-like Proteases. CG and chymotrypsin have P1 substrate preferences that strongly favor bulky hydrophobic residues, especially aromatic residues (Nakajima et al., 1979). P1 substitutions of Phe, Leu, Met, and Ala were found to be functional (Table I) with both CG and chymotrypsin. There was no strict selectivity for either of the chymotrypsin-like enzymes, although the very bulky tryptophan residue has not been tested.

Binding Stoichiometry and $(K_i)_{app}$ for Val-5-CMTI-III with HLE. The ability to change protease selectivity by reactive-site variation having been established, the stoichiometry and affinity were investigated for a representative HLE inhibitor, Val-5-CMTI-III. The linear titration curve of Val-5-CMTI-III/HLE (Figure 1B) with an $[I]_0/[E]_0$ intercept of about 1 establishes that the inhibitor/protease pair forms a 1:1 stoichiometric complex. For comparison purposes, the titration of synthetic CMTI-III with trypsin was shown to give similar results (Figure 1A and Kupryszewski et al., 1986).

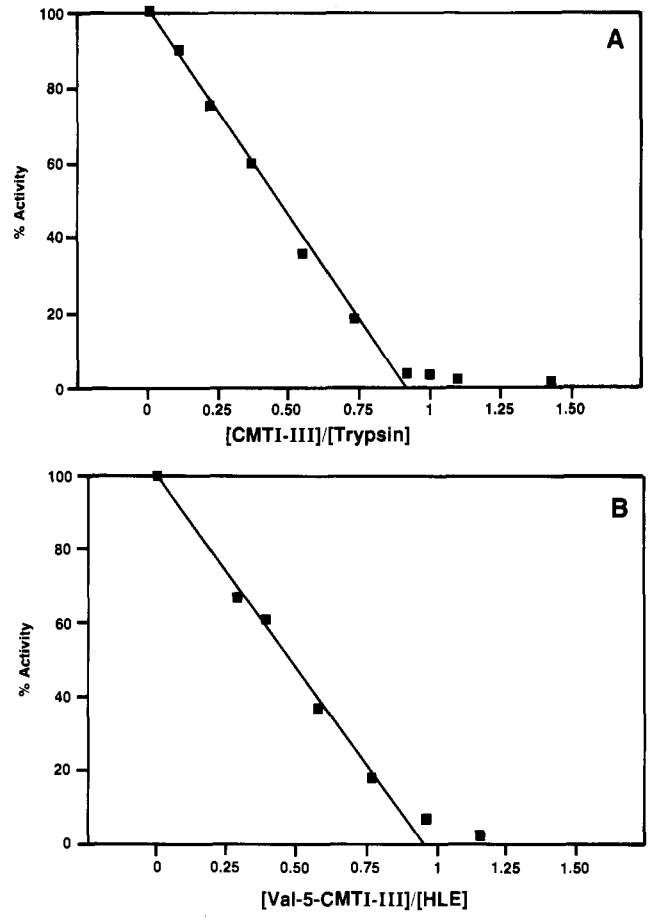

FIGURE 1: Stoichiometry of binding. Aliquots of stock inhibitor (concentration from amino acid analysis) were equilibrated with protease (trypsin concentration from active-site titration, HLE from UV absorbance), and free enzyme was determined from activity assays. (A) Titration of trypsin (1.3 nM) with CMTI-III detected by turnover of 89 μM tosyl-Gly-L-Pro-L-Arg-pNA ($10 \times K_m$) in 0.2 M Tris-HCl, pH 8.3, 20 mM $CaCl_2$, and 0.005% Triton X-100. The stoichiometry is 1:1 on the basis of a linear least-squares fit of the data with an abscissa intercept of 0.92. (B) Titration of HLE (500 nM) with Val-5-CMTI-III detected by 3.7 mM *N*-acetyl-L-Ala-L-Ala-L-Ala-pNA in HLE assay buffer (see Materials and Methods). The abscissa intercept of 0.95 from the least-squares fit indicates 1:1 binding.

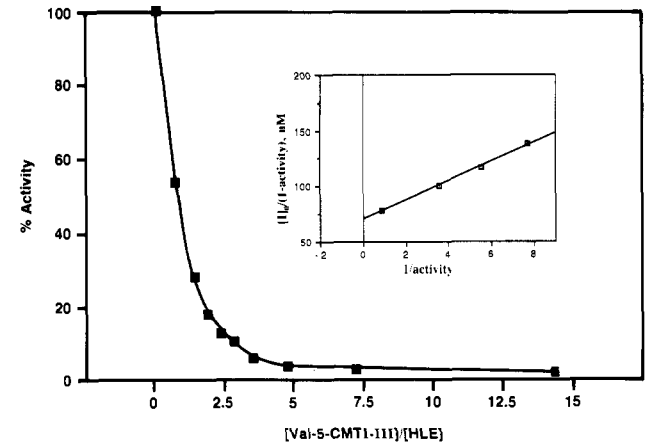

FIGURE 2: Titration of HLE with Val-5-CMTI-III for determination of $(K_i)_{app}$. After equilibration, free enzyme is determined by using the HLE assay (see Materials and Methods). The curve is drawn to aid the eye of the reader. Inset: A replot of the data. The curve is a linear least-squares fit, the slope of which gives a $(K_i)_{app}$ of 8.7 nM.

The titration curve of Val-5-CMTI-III with HLE was repeated (Figure 2) with a more dilute concentration of HLE (50 nM), a condition required for detection of free HLE and

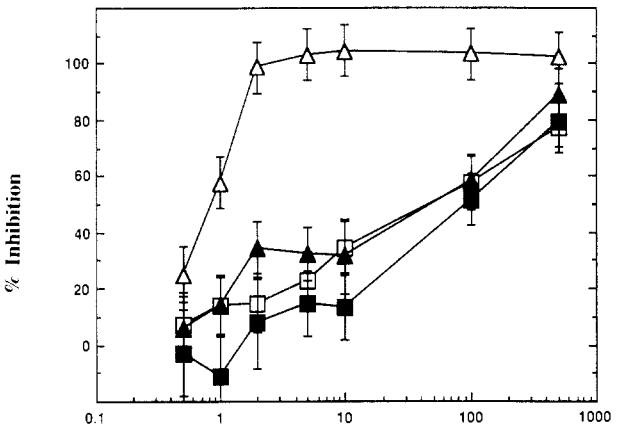

FIGURE 3: Comparison of Val-5-CMTI-III and α_1-PI using the ^{125}I-FN assays (see Materials and Methods). The inhibitors and source of proteolysis are as follows: (△) α_1-PI/HLE; (▲) α_1-PI/PMN; (■) Val-5-CMTI-III/HLE; (□) Val-5-CMTI-III/PMN. The curves are drawn to aid the eye of the reader.

subsequent determination of $(K_i)_{app}$ for tight-binding inhibitors (Green & Work, 1953; Bieth, 1974; Empie & Laskowski, 1982). The data from the resulting titration curve is replotted (Figure 2, inset) according to Bieth (1974), and a $(K_i)_{app}$ of 8.7 nM was determined from the slope of a linear least-squares analysis. The lower limit on K_i can then be estimated to be 4.4 nM from the expression

$$(K_i)_{app} = K_i[1 + [S]_0/K_m] \quad (3)$$

where $[S]_0$ is the initial substrate concentration and K_m is the Michaelis constant of substrate with HLE. The $(K_i)_{app}$ for CMTI-III/trypsin can be calculated from the data of Wieczorek et al. (1985) to be 1.5×10^{-12} M. The corresponding value of CMTI-III/HLE is unknown, but is too weak to be determined by the approach for tight-binding inhibitors. The $(K_i)_{app}$ for the Val-5-CMTI-III/HLE pair is 3 orders of magnitude less than the corresponding value for CMTI-III/trypsin. The large differences between HLE and trypsin make it difficult to draw meaningful conclusions from a comparison of these $(K_i)_{app}$ values.

Inhibition of Inflammatory Cell Proteolysis of Connective Tissue. The spectrophotometric assays were very revealing in terms of detecting strong interactions between peptides and proteases. However, the substrates employed do not mimic extracellular matrix substrates that are relevant in vivo. Thus, such assays may not accurately reflect the ability to inhibit in the presence of more natural and effective substrates (Laurent et al., 1987; Bruch & Bieth, 1986; Morrison et al., 1987). Furthermore, the relevant goal in vivo is to control proteolysis not only by purified enzymes but also by inflammatory cells (Campbell & Campbell, 1988; Ossanna et al., 1986; Sibille et al., 1986; Weis et al., 1984, 1986; Weis, 1989; Weitz et al., 1988). For the above reasons, a purified sample of Val-5-CMTI-III was tested for its ability to inhibit proteolysis of a radiolabeled extracellular matrix component (^{125}I-FN) by HLE or by stimulated PMN. For comparison, the natural HLE inhibitor α_1-PI was also tested.

Figure 3 summarizes the inhibition of solid ^{125}I-FN degradation at 4 nM HLE. The data points represent the mean of several replicates (n = 3–9) compared to controls that contained no inhibitor. Val-5-CMTI-III had nearly the same inhibitory profile against purified HLE and PMN-derived activity. α_1-PI was superior against purified HLE, behaving essentially as a titrating inhibitor. When stimulated PMN were used, however, α_1-PI effectiveness dropped to a level similar to that of Val-5-CMTI-III. Possible reasons for this difference have been discussed previously (Campbell et al., 1982; Campbell & Campbell, 1988).

Calculation of theoretical inhibition curves (percent inhibition vs $[I]_0/[E]_0$, not shown) indicated a $(K_i)_{app}$ of about 500 nM, which is about 50 times higher than that with the soluble peptide-pNA substrate. Differences in soluble and solid connective tissue substrates have been noted in the HLE/elastin (Morrison et al., 1987) and the collagenase/collagen systems (Welgus et al., 1980, 1985). The data reported here suggest the same is also true for the HLE/^{125}I-FN system.

Summary and Conclusion

Apart from implications for control of PMN proteolysis, the approach of the present work provides a powerful new tool for exploring molecular recognition. Our results demonstrate that reactive-site variants of CMTI-III can be prepared with altered selectivity. The conclusions regarding changes in selectivity are limited by the threshold of detection, which is a threshold $(K_i)_{app}$ on the order of 10 μM for the assays as employed here. While some of the selectivities were changed in a predictable manner, others were not. For example, a broader range of P1 substitutions were tolerated by trypsin than would be predicted from its substrate specificity. An intolerance toward β-branched P1 residues was observed, while the need for a β carbon was also indicated. Several new inhibitors of CG were found with P1 Leu, Ala, Phe, and Met functioning in the screening assay. None of the inhibitors were uniquely selective for chymotrypsin-like enzymes.

A number of new inhibitors of HLE were also established with Val, Ile, and, surprisingly, Gly giving the sharpest selectivity toward HLE. The nature of the Val-5-CMTI-III/HLE interaction was investigated in detail and was shown to involve a 1:1 stoichiometric complex with a $(K_i)_{app}$ of 8.7 nM. Since Gly-5-CMTI-III does not have a P1 side chain, a prominent role for other reactive site to HLE interactions may be indicated by the data. Val-5-CMTI-III was found to inhibit connective tissue proteolysis by purified HLE and stimulated PMN. In particular, Val-5-CMTI-III showed an ability to inhibit stimulated-PMN proteolysis of ^{125}I-FN that was comparable to α_1-PI. The data suggested an increase (ca. 50-fold) in $(K_i)_{app}$ for the Val-5-CMTI-III/HLE pair when assayed with the surface-bound substrate.

The ability to manipulate the protease selectivity of CMTI variants has distinct advantages over other natural inhibitor variants. With solid-phase peptide synthesis it is straightforward to prepare and screen variants in a short period of time, and the sequence substitutions are not limited to a single site. The CMTI inhibitors are amenable to X-ray (Bode et al., 1989) and 2D NMR (Likos, 1989) structural studies. The structure of HLE in complex with turkey ovomucoid third domain has been determined (Bode et al., 1986), and a comparison of structures for Val-5- and Gly-5-CMTI-III/HLE complexes, along with $(K_i)_{app}$ values, could lead to a detailed understanding of the factors affecting protease selectivity. This, in turn, could lead to synthesis of more selective and potent inhibitors.

It has been the goal of many researchers to develop new inhibitors of HLE as a method of studying and controlling inflammatory cell proteolysis [reviewed by Stein et al. (1985)]. To achieve this goal, an inhibitor should be selective for the target proteases and be effective against both purified enzymes and inflammatory cell proteolysis. The results obtained here emphasize the importance of characterizing the interaction of inhibitors with connective tissue substrates (Campbell et

al., 1982, 1987) since the insoluble substrates do not behave in a classical fashion (Morrison et al., 1987; Welgus et al., 1980, 1985). Indeed, the connective tissue studies should also include PMN proteolysis since recent results (Campbell & Campbell, 1988) indicate that limited PMN proteolysis of certain connective tissue surfaces proceeds even in the presence of large excesses of an inhibitor. Potential therapeutic candidates for controlling proteolysis should preferably be nonreactive, nontoxic and nonimmunogenic. Also, delivery and stability of these agents in the circulation and in the target tissues are important considerations. In this regard, the highly cross-linked nature of CMTI-III may explain its resistance to proteolysis (McWherter, unpublished results). Indeed, when fully active ^{125}I-labeled Val-5-CMTI-III was injected into rats, no proteolytic fragments could be detected in blood samples drawn from 3 to 45 min after injection, with only intact peptide being recovered. Although many hurdles remain, such stability may be an important asset in an in vivo setting. While there is considerable interest in limiting inflammatory cell proteolysis, its ultimate utility awaits verification. The peptide inhibitors of the present work, and their derivatives, may be useful for this purpose.

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Elementary Steps in the Formation of Horseradish Peroxidase Compound I: Direct Observation of Compound 0, a New Intermediate with a Hyperporphyrin Spectrum†

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ABSTRACT: The reaction of horseradish peroxidase (HRP) with H_2O_2 has been studied in 50% v/v methanol/water over the 25.0 to −36.0 °C temperature range by using the low-temperature stopped-flow technique. All reactions were carried out under pseudo-first-order conditions with $[H_2O_2] \gg [HRP]$. Arrhenius plots for the pseudo-first-order rate constant k_{obs} were linear over the 17.6 to −36.0 °C temperature range studied with an activation energy of 4.8 ± 0.5 kcal/mol. Above 0 °C, k_{obs} varies linearly with peroxide concentration. However, saturation kinetics are observed below −16.0 °C, indicating that there is at least one reversible elementary step in this reaction. Double-reciprocal plots at −26.0 °C at pH* 7.3 for the reaction give k_{obs}^{max} = 163 s^{-1} and K_M = 0.190 mM. Rapid-scan optical studies carried out at −35.0 °C with $[H_2O_2] \gg K_M$ reveal the presence of a transient intermediate referred to as compound 0 whose conversion to compound I is rate limiting. The Soret region of the optical spectrum of compound 0 resembles that of a "hyperporphyrin" with prominent bands near 330 and 410 nm. The temperature dependencies of k_{obs}^{max} and K_M have been measured over the −16.0 to −26.0 °C range and give an activation energy for k_{obs}^{max} of 1.6 ± 0.7 kcal/mol and an enthalpy of formation for compound 0 of 4.0 ± 0.7 kcal/mol.

One of the most intensively studied reactions in enzymology is that between the hydroperoxidases and H_2O_2. Early pioneering studies indicated that horseradish peroxidase (HRP)[1] (Theorell, 1941) and catalase (Chance, 1947) each react with H_2O_2 to give a discrete, catalytically active reaction intermediate that is referred to as compound I. Subsequent studies have shown that this reaction is common to all of the hydroperoxidases and is characterized by the two-electron oxidation of these heme enzymes (Frew & Jones, 1984). An important goal in the elucidation of the mechanism of action of any enzyme is to identify and characterize both thermodynamically and kinetically all possible elementary steps in the catalytic pathway (Hammes & Schimmel, 1970; Gutfreund, 1975; Auld, 1977). In spite of intensive study, the elementary steps that lead to the formation of compound I have remained largely undefined.

Jones and Dunford (1977) have summarized the kinetic evidence which indicates that the reaction of HRP with H_2O_2 to form compound I is not a diffusion-controlled elementary bimolecular reaction

$$HRP + H_2O_2 \xrightarrow{k} I \qquad (1)$$

but rather a chemically controlled reaction in which there is a preequilibrium of reactants to form at least one precursor complex, HRP–H_2O_2 (where this designation implies that no electron transfer has occurred between the reactants, but is unspecific with regard to its structure), followed by a rate-limiting redox step. The simplest preequilibrium mechanism is

$$HRP + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HRP\text{–}H_2O_2 \xrightarrow{k_2} I \qquad (2)$$

The first piece of evidence in favor of mechanism 2 is the observation that the second-order rate constant of $\sim 10^7$ M^{-1} s^{-1} is lower than expected for an elementary diffusion-controlled bimolecular reaction (Dunford & Hewson, 1977). Second, the rate constants for compound I formation from some of the larger peroxybenzoic acids (e.g., *m*-chloroperoxybenzoic acid) are *larger* than for H_2O_2 (Davies et al., 1976). Third, the second-order rate constant for compound I formation from H_2O_2 is independent of viscosity, while that for *m*-chloroperoxybenzoic acid is viscosity dependent and diffusion controlled (Dunford & Hewson, 1977).

In principle, mechanisms 1 and 2 can be distinguished kinetically. If the first step in mechanism 2 equilibrates rapidly ($k_1[H_2O_2] + k_{-1} \gg k_2$) and $[HRP] \ll [H_2O_2]$, the pseudo-first-order rate constants, k_{obs}, for mechanisms 1 and 2 are

$$k_{obs} = k[H_2O_2] \qquad (3)$$

$$k_{obs} = k_{obs}^{max}[H_2O_2]/([H_2O_2] + K_M) \qquad (4)$$

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[1] Abbreviations: HRP, horseradish peroxidase; pH*, apparent protonic activity; OEP, octaethylporphyrin.

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