Inhibition of Cathepsin D by Synthetic Oligopeptides*

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A number of synthetic oligopeptides with the COOH terminus α-amino acid situated third from the potentially cleavable phenylalanyl-phenylalanyl bond, typified by < Glu-D-Phe-Pro-Phe-Val-D-Trp (Peptide VI) were shown to be potent competitive inhibitors of cathepsin D and pepsin. Peptide VI, which forms an equimolar nonproductive enzyme-inhibitor complex, inhibited the hydrolysis of methyl[14C]glycinated hemoglobin by porcine pepsin with a $K_i$ of 0.01 M, and the cleavage of the COOH terminus L-tryptophanyl analog at the Phe-Phe bond by cathepsin D with a $K_i$ of 0.06 M. The pH profile of the inhibition of cathepsin D by the peptide followed that of the enzyme reaction. Derivatization of the carboxyl group at the COOH terminus into a nondissociative form diminished the inhibitory activity. The minimum unit to display significant inhibition is probably the segment of Pro-Phe-Phe-Val-D-leucine methyl ester and Cu²⁺ was retarded by about 20%, and the slow inactivation by 1,2-epoxy-3-(p-nitrophenoxy)propane was reduced by about 65%. It is proposed that the potent inhibitory activity of the peptide may be attributable, in part, to the perturbation of the function of a catalytically critical carboxylate group in the active site of the enzyme by the COOH terminus α-amino acid carboxylate group of the peptide, which binds along the primary and secondary binding sites.

The pH profile of the inhibition of cathepsin D by the peptide followed that of the enzyme reaction. Derivatization of the carboxyl group at the COOH terminus into a nondissociative form diminished the inhibitory activity. The minimum unit to display significant inhibition is probably the segment of Pro-Phe-Phe-Val-D-Trp. The interaction contributed by the additional 2 NH₂ terminus residues facilitated binding. Enantioselective substitution at the Phe-Phe unit or the valyl residue reduced the inhibitory capacity. The COOH terminus residue is preferably a large hydrophobic α-amino acid.

This class of inhibitors was effective only on carboxyl proteinases such as cathepsin D and pepsin. Bovine a-chymotrypsin and subtilisin inactivated the inhibitors by cleaving the Phe-Phe bond. In the presence of Peptide VI at a molar ratio of peptide to enzyme of 100:1, the rapid inactivation of cathepsin D by acetyl-DL-norleucine methyl ester and Cu²⁺ was retarded by about 20%, and the slow inactivation by 1,2-epoxy-3-(p-nitrophenoxy)propane was reduced by about 65%. It is proposed that the potent inhibitory activity of the peptide may be attributable, in part, to the perturbation of the function of a catalytically critical carboxylate group in the active site of the enzyme by the COOH terminus α-amino acid carboxylate group of the peptide, which binds along the primary and secondary binding sites.

Cathepsin D is a major endopeptidase of mammalian tissues, which has been implicated as playing a role in intracellular tissue degradation (1, 2). On the basis of its low pH optimum for activity and the property of inactivation by diazoacetyl-DL-norleucine methyl ester, this enzyme is considered to be a member of the family of carboxyl proteinases represented by pepsin, cathepsin D preferentially attacks, among others, the Phe-Phe, Phe-Tyr, and Leu·Yrbonds in polypeptide substrates (1, 5). The rates of the bond cleavage are affected by the nature of secondary enzyme-substrate interactions distant from the cleavage point (4, 6). It is conceivable that by modulation of the secondary interaction sites, analogs of substrates with a productive binding mode may become competitive inhibitors, as was demonstrated by the work of Berger and his collaborators (7-9) with enantiomeric substitution at the critical position in the oligopeptidyl substrates for various proteinases. In fact, Keilová et al. (10) previously reported that the exclusive cleavage of a hexapeptide, Gly-Phe-Leu-Gly-Phe-Leu at the first Phe-Leu bond by bovine spleen cathepsin D was inhibited by d-phenylalanine and d-leucine isomers of this peptide.

We describe in this report a study of inhibition of cathepsin D by a class of potent synthetic peptide inhibitors which contain a critical COOH terminus α-amino acid residue. A few salient features of these inhibitors are also presented.

**EXPERIMENTAL PROCEDURES**

**Proteinases**—Cathepsin D was prepared from rabbit liver and bovine spleen according to the method of Barrett (11), with minor modifications. For characterization of inhibitors, cathepsin D, which was extensively purified by isoelectric focusing and polyacrylamide gel electrophoresis and free of known thiol-, and serine-, and metal-dependent proteinases, was used. Crystalline porcine pepsin, pancreatic elastase, and pepsin were obtained from Worthington, N. J. Subtilisin was purchased from Nagase, Japan. Proteolytic activities were assayed with a methyl[14C]glycinated bovine hemoglobin substrate prepared by treating hemoglobin with [14C]glycine methyl ester and EDC (12). The reaction pH values for cathepsin D, pepsin, and serine proteinases were 4.0 (in 0.2 M sodium citrate buffer), 3.0 (in 0.1 M sodium citrate or sodium formate buffer), and 8.0 (0.05 M Tris·HCl buffer), respectively.

In cases where the cleavage of a single site per substrate was determined, < Glu-D-Phe-Pro-Phe-Val-D-Trp (Peptide VII), which was shown to be cleaved at the Phe-Phe bond by cathepsin D and pepsin, was used as the substrate, and the newly generated NH₂ termini were measured by the fluorescamine reaction (13).

Kinetic data of enzyme reactions with or without inhibitors were analyzed by Dixon plots (14) in order to derive inhibition constants, $K_i$. On the basis of duplicate or triplicate measurements, $K_i$ values are estimated to be reproducible within ±20%.

**Peptides**—Pro-Pro-Phe-Val-Leu, Pro-Gly-Phe-Val-D-Leu, Gly-Phe-Val-D-Leu, Pro-Pro-Phe-Val-Ala-D-Leu, and Pro-Gly-Phe(NO₂)·Phe-Val-D-Leu, on a small scale (25 mg), were prepared in this laboratory by Merrifield solid phase synthesis using chloromethylated polystyrene resin and Boc-amino acids (Bachem, Calif.) by the stepwise manual technique (15).

Peptide material was cleaved from the resin by action of HBr in anhydrous trifluoroacetic acid. After purification by thin layer chromatography on silica gel plates (Analtech, Dela.) with chloroform/methanol/ammonia (70:30:3) or chloroform/methanol/ammonia/H₂O (120:66:12.8) and by paper electrophoresis in 0.25 M pyridinium acetate buffer, pH 6.4, or 0.1 M pyridinium acetate buffer, pH 3.6, the peptides were characterized by their amino acid compositions. Pro-Pro-Phe-Val-D-Leu NH(CH₃)₂SO₃H was prepared from Pro-Pro-Phe-Val-d-Leu and 

1 The abbreviations used are: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; < Glu, 5-pyrrolidone-2-carboxylic acid; Boc, t-butylxycarbonyl; OMe, methoxy; Phe(NO₂), p-nitrophenylalanine; DANN, diazoacetyl-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; Me₂SO, dimethyl sulfide.
The active site of many endopeptidases has been suggested to accommodate a peptide segment of 5 to 7 amino acid residues containing the bond cleavage site (7, 24, 25). One of the small peptide fragments previously shown to be susceptible to cathepsin D is a pentapeptide, Gly-Phe-Leu-Gly-Phe, with exclusive cleavage at the Phe-Leu bond (10). There is no evidence indicating that this sequence is representative of an oligopeptide of the highest affinity and susceptibility, since cleavage patterns of various polypeptides by cathepsin D suggest that bonds at the Phe-Phe, Phe-Val, Phe-Tyr, Leu-Glu, and Leu-Val linkages may be cleaved equally well (5, 23, 26, 27). When more than 100 randomly selected synthetic pentapeptides were examined at 0.2 mg/ml (approximately 0.4 and 0.3 mM, respectively) for inhibition of hydrolysis of hemoglobin by horse spleen cathepsin D (about 2 nM), two peptides, Ala-Phe-Val-Phe-Ala-Phe-OMe (I) and Pro-Ala-Pro-Phe-Val-Leu (II), were shown to inhibit about 40% of the enzyme reaction measured for 30 min at 31°C. This was noteworthy since under the same conditions, a synthetic modification of pepstatin A, a potent depsipeptidyl carboxyl proteinase inhibitor isolated from Streptomyces culture media (29) did not change in the inhibitory activity.

**Nature of the D-Amino Acid**—The D-amino acid at the COOH terminus offering the most inhibition is preferably a large hydrophobic amino acid such as D-tryptophan, D-phenylalanine, or D-leucine. D-Proline or even D-alanine derivatives were less effective inhibitors. A critical effect of the fifth amino acid (P') in the inhibitor was shown by the significant reduction in activity after replacement of L-valine by D-valine, β-DL-phenylalanine, or L-proline. Hence, the distance, freedom, and the configuration of the linkage between the Phe-Phe unit and the COOH terminus D-amino acid also appear to affect the inhibitory activity.

**P_0 P_1 Positions**—Whereas proline at the P_1 position may confine the Phe-Phe unit to a favorable binding in the active site, and was found to be most effective, replacement by glycine still yielded an active inhibitor. Proline at P_0 could be replaced by other amino acids without reduction in the inhibitory activity.

* The nomenclature for the subsites of a protease active site and for the corresponding individual amino acid residues in the substrate, follows that of Schechter and Berger (7).
**Table I**

Inhibition of bovine spleen cathepsin D action by synthetic peptides of X-Pro-Phe-Val-D-Leu series

Aliquots of each peptide solution in MeSO, 10 mg/ml, were incubated with bovine spleen cathepsin D (about 0.3 µM) in 0.2 M sodium citrate buffer, pH 4.0 for 15 min at 4°C, before addition of methyl[14C]glycinated hemoglobin substrate for assay of the enzyme activity (15-min reaction at 37°C). At least five concentrations of a peptide at two levels of the substrate were used for obtaining Dixon plots in estimation of the apparent inhibition constant.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_i$ (M)</th>
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<tbody>
<tr>
<td>Pro-Pro-Phe-Phe-Val-Val-Leu (Peptide IV)</td>
<td>$3.4 \times 10^{-6}$ (Ki pepsiin 3.0 $\times 10^{-6}$)</td>
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<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu</td>
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<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu-OH</td>
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</tr>
<tr>
<td>Pro-Pro-Phe-Phe-Val-LeuNH$_2$</td>
<td>$4.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu(NH$_2$)$_2$SO$_3$H</td>
<td>$1.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu, cyclic</td>
<td>$5.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>(Pro-Pro-Phe-Phe-Val-Leu)$_2$ linear</td>
<td>$1.7 \times 10^{-7}$</td>
</tr>
<tr>
<td>Pro-Pro-Phe(H$_2$O)$_2$-Phe-Val-Val-Leu</td>
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<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu</td>
<td>&gt; $5 \times 10^{-2}$ *2</td>
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<tr>
<td>Pro-Gly-Phe-Phe-Val-Leu</td>
<td>$1.5 \times 10^{-5}$</td>
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<tr>
<td>Pro-Pro-Phe-Phe-Leu Val Ala D-Leu</td>
<td>$6.4 \times 10^{-5}$</td>
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<tr>
<td>Pro-Pro-Phe-Phe-Val-Leu</td>
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<td>Pro-Pro-Phe-Phe-Val-Leu</td>
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<td>Phe-Pro-Phe-Phe-Val-Leu</td>
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<td>Tyr-Phe-Pro-Phe-Val-Leu</td>
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<tr>
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<td>Val-D-Phe-Phe-Phe-Val-Leu</td>
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<td>Pro-D-Phe-Phe-Phe-Val-Leu</td>
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<td>Glu-Glu-D-Phe-Phe-Phe-Val-Leu (Peptide V)</td>
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<tr>
<td>Glu-D-Phe-Phe-Phe-Val-Leu-Val-Trp (Peptide VII)</td>
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<td>Glu-D-Glu-D-Phe-Phe-Phe-Val-Leu</td>
<td>$3.9 \times 10^{-8}$ (Kp pepsiin 2.6 $\times 10^{-7}$)</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>$2.5 \times 10^{-9}$ (Kp pepsiin 4.5 $\times 10^{-9}$)</td>
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</tbody>
</table>

*1 $P_1, P'_1$ etc. denote the individual amino acid residues assumed to bind at the corresponding subsites of the active site, $S_1, S'_1$ etc. (*7).

*2 Due to inaccuracy associated with the determination of low inhibitory activities within the reasonable concentration ranges tested, the inhibition constants could be larger than those indicated.

*3 No detectable inhibition under the conditions used.

*4 The hydrolysis of Pro-Pro-Phe-Phe-Val-Leu and Peptide VII at the Phe-Phe bonds catalyzed by cathepsin D gave $k_m$ values $3.2 \times 10^{-4}$ and $4.6 \times 10^{-4}$ M$^{-1}$ s$^{-1}$ respectively.
itory activity. It is not known if any of these sites has to be occupied by at least 1 proline residue. Extension beyond the S1 site was favorable. Elimination of P3 reduced the potency (e.g., Pro-Phe-Phe-Val-d-Leu). Since blocking of the terminal NH2 group by a large and hydrophobic residue increased the inhibitory efficiency, the S1 region may contain a hydrophobic environment. The stereoisomeric configuration of the amino acid at P3 did not affect activity of the peptide, because proline at P3 could be substituted by D-amino acids such as D-phenylalanine.

The most potent form of inhibitor found in this series of studies has the general structure represented by <Glu-n-Phe-Pro-Phe-Phe-Val-d-Trp (VI) From Peptide II (Pro-Ala-Pro-Val-Leu; K, 4.1 × 10^{-4} M) to Peptide IV (Pro-Pro-Phe-Val-Phe-Val-Leu; K, 3.4 × 10^{-5} M) an increase in inhibiting potency of about 130-fold and from Peptide IV to Peptide V (K, 3.1 × 10^{-5} M) a change of 100-fold was observed (Fig. 1, Table I).

**Mode of Inhibition of Cathepsin D by Peptide Inhibitors**

Fig. 2 shows the Dixon plots for the effect of <Glu-n-Phe-Pro-Phe-Phe-Val-d-Trp (VI) (M, 937.1), the D-tryptophan analog of Peptide V, and pepstatin A (M, 685.9) on hemoglobin-cathepsin D and hemoglobin-pepsin reaction systems. The effect of these inhibitors on the cathepsin D-catalyzed cleavage of <Glu-n-Phe-Pro-Phe-Phe-Val-Trp (VII) at the Phe-Phe bond was also examined. These peptides appeared to be competitive inhibitors of the enzymes in all systems. The inhibition constants obtained by the kinetic data using hemoglobin as substrate (K, for pepstatin, 2 nM, and for Peptide VI, 12 nM with cathepsin D; K, for pepstatin, 5 nM and for Peptide VI, 400 nM with pepsin) were comparable with those derived from the cathepsin D reaction on Peptide VII with a single cleavage site at the Phe-Phe linkage (K, for pepstatin, 4 nM, and for Peptide VI, 60 nM).

The number of the interaction sites calculated from the data of equilibrium dialysis of Peptide VI and cathepsin D (Fig. 3) is about 1.2 according to the method of Scatchard (29), with an association constant of about 1.2 × 10^{7} M^{-1}, suggesting formation of a stoichiometric inhibition complex of the enzyme and the peptide. When kinetics of inhibition of cathepsin D activity by Peptide VI was analyzed in the presence of three concentrations of the inhibitor at various amounts of the enzyme (Fig. 4), the results suggest that the enzyme-peptide interaction involves a pseudo-reversible equimolar complex formation (30) similar to that observed by

![Fig. 1. Comparison of the inhibitory activities of representative synthetic peptides and pepstatin on the cathepsin D action.](http://www.jbc.org/)
Barrett and Dingle (31) for pepstatin inhibition of human liver cathepsin D. The inhibition constants of some of the other peptide inhibitors derived from kinetic data of proteolysis are also indicated in Table I.

As shown in Fig. 5, the difference ultraviolet spectra of the pepsin-Peptide VI complex against pepsin indicate distinct increases in absorption at 293, 285, 282, and 280 nm. These increases depend on the concentration of the peptide, and were altered by the presence of pepstatin. The spectra of pepsin with pepstatin is similar to those for pepsin-Peptide VI complex but shows more dominant increases in absorption at the 287 and 280 nm regions than the absorption at 294 nm. Thus these peptide inhibitors and pepstatin may share at least some common domains in the active site of the enzyme, and cause similar but not identical conformational changes affecting the environment around tryptophanyl and tyrosyl residues.

In preliminary analyses, difference spectra of the rabbit cathepsin D-Peptide VI complex against cathepsin D, which were similar to those for pepsin were obtained, but further studies on cathepsin D were not carried out due to limited amounts of pure enzyme.

Effect of pH and Ionic Strength on Peptide Inhibition of Cathepsin D Activity

The pH effect on the inhibition as illustrated in Fig. 6 indicates that the binding of peptide inhibitor to the enzyme follows closely the profile of the enzymatic activity. The affinity of the peptides to the proteinase could thus be related to the catalytic processes or to the dissociation properties of

Fig. 3. A plot of binding data from an equilibrium dialysis study of <Glu-o-Phe-Pro-Phe-Pro-Val-Trp (Peptide VI) and rabbit liver cathepsin D. Concentrations of the peptide (25 to 200 ng/ml) in the compartments were determined by the inhibition of a known enzyme activity. r, moles of bound peptide per mol of cathepsin D; c, the concentration of free peptide at equilibrium. The line was drawn according to linear regression analysis.

Fig. 4. Dependence of the proteolytic activity of rabbit liver cathepsin D on the concentration of the enzyme at various levels of <Glu-o-Phe-Pro-Phe-Pro-Val-Trp (Peptide VI). The proteolytic activity was measured on methyl[14C]glycinated hemoglobin (0.44 mg/ml) for 20-min reaction at 37°C in 0.4 M sodium citrate buffer, pH 4.0, at various levels of the enzyme concentration in the presence of 0 (○), 20 (■), 50 (▲), and 100 (■) nM of Peptide VI, respectively. Inset, the proteolytic activities at pH 4.0 of the mixture of cathepsin D and Peptide VI were plotted against the molar ratio for the inhibitor concentration [I] to the enzyme concentration [E]. At the molar ratio of 1, the enzyme activity approaches to zero.

Fig. 5. Effects of peptide inhibitors on absorption spectra of pepsin. The concentration of pepsin was 29.5 μM in 0.02 M sodium formate buffer, pH 4.3. Aliquots of peptide (10 mg/ml) in MeSO were mixed with pepsin solution. All measurements were done at 25°C. A, difference spectra of pepsin-Peptide VI complex against pepsin. Concentrations of the peptide: (a) 24.3 μM, (b) 16.2 μM, and (c) 8.1 μM. B, difference spectra of pepsin with pepstatin. Concentrations of pepsta-
the catalytically critical functional groups in the enzyme. The inhibitory effect of the peptide increased as the ionic strength increased to about 0.5, then leveled off at the higher salt concentrations.

**Proteolytic Susceptibility of Peptide Inhibitors**

The activity of this class of peptide inhibitors appeared to be confined to carboxyl proteinase inhibitors such as cathepsin D and pepsin; the actions of trypsin, papain, and thermolysin were not inhibited by these peptides. Chymotrypsin, subtilisin, and elastase inactivated these carboxyl proteinase inhibitors (Fig. 7) by cleaving mainly at the Phe-Phe bond. Such inactivation by serine proteinases of the chymotrypsin family was not observed on pepstatin. When an equimolar mixture of Peptide VI and chymotrypsin was incubated at 37°C, pH 8.0 for 1 h, and analyzed by thin layer chromatography on silica gel plates in chloroform/methanol/ammonia (70:30:3), two peptides migrating slower than Peptide VI (Rf 0.61) could be detected. The amino acid composition of the acid hydrolysate of these materials corresponded to the fragments, <Glu-n-Phe-Pro-Phe (molar ratio of Glu/Pro/Phe, 1.00:0.87:1.95) (Rf 0.45) and Phe-Val-b-Trp (molar ratio of Phe/Val/Trp, 1.00:0.88:0.89) (Rf 0.29). Similar results were also obtained with Peptide V.

Selectivity among these peptides for the rate of proteolytic cleavage was noted as Peptide VI and <Glu-n-Phe-Pro-Phe-Val-n-Phe (data not shown) were resistant to the elastase action in comparison with other analogs such as Peptide V (Fig. 7). Formation of a nonproductive complex between Peptide VI and elastase through the Phe-Phe or Val-D-Trp regions is possible, but the peptide did not show strong inhibition of the elastase activity, nor were reactions of chymotrypsin and subtilisin (20 nM) on hemoglobin affected by the peptides at 1 mM.

Peptide VI was also slowly hydrolyzed by pepsin through the Phe-Phe bond during incubation at 37°C, pH 3 for 1 h at a molar ratio of enzyme to peptide of 1:1, with retention of more than 80% of the native inhibitory activity. Little degradation of Peptide IV by pepsin was detected under the same conditions even though it is less potent as an inhibitor than Peptide VI. Degradation of these peptide inhibitors by cathepsin D under the same conditions was not observed.

**Effect of Peptide Inhibitors on Inactivation of Cathepsin D**

by Diazoacetyl-m-norleucine Methyl Ester and Cu²⁺ (DANM-Cu²⁺) or by 1,2-Epoxy-3-(p-nitrophenoxy)propene (EPNP)

The proposed mechanism for carboxyl proteinase catalysis involves the participation of β-carboxyl groups of aspartyl residues (6,32). Pepsin and cathepsin D can be inactivated by EPNP (22,23), and in pepsin the specific modification of the carboxylate of Asp-32 (33) or Asp-32 and Asp-215 (34) by the reagent has been described. Rapid inactivation of pepsin and cathepsin D resulted with treatment by DANM-Cu²⁺ (2,3,20-23). This inactivation is attributed to the modification at a single β-carboxylic acid group of the aspartyl residue 215 in the sequence of porcine and bovine pepsin (35,36).

The presence of Peptide VI at the molar ratio of cathepsin D to peptide of 1:100, retarded both the inactivation reactions with EPNP and DANM-Cu²⁺, although definite characterization of the nature of the modified residues in cathepsin D by these reagents has not been made (Fig. 8). The protection effect of the peptide was distinctly more significant on the slow inactivation by EPNP as the time required for 50% inactivation increased from an average of 17 h to about 36 h. After 24 h reaction at 25°C about 65% of the activity remained in the presence of the peptide with an average 1.1 EPNP residues bound/mol of enzyme while about 35% of the native activity was detected in the absence of peptide with an average 1.9 EPNP residues bound/mol of enzyme.

The rapid inactivation of cathepsin D by DANM-Cu²⁺ was affected only moderately in the presence of Peptide VI. The pseudo-first order rate constant for the initial reaction with or without peptide binding was about 3.2 × 10⁻² min⁻¹ and 4.6 × 10⁻² min⁻¹, respectively. Although within 15 min of reaction the presence of the peptide caused about 20% less inactivation with incorporation of 0.8 norleucine residue/mol enzyme versus 0.9 residue in the absence of the peptide, no protection could be shown when the reaction proceeded beyond 60 min with an average 1.3 norleucine residues incorporated/mol of enzyme with or without the peptide.

**DISCUSSION**

The introduction of a D-amino acid into a polypeptide chain inevitably affects both the orientation of the side chain and the conformation of the peptide backbone. The effect of a single COOH terminus D-amino acid substitution on the function of a peptide found in this study is quite significant. Assuming that the NM value derived from the hydrolysis of <Glu-n-Phe-Pro-Phe-Val-Trp (VII) by cathepsin D could approximate K₉, ΔG° for the interaction of the enzyme and the peptide may be in the order of 7.5 kcal/mol, while −ΔG° for the binding of an inhibitor, the D-tryptophanyl isomer (Peptide VI), would be 11.2 kcal/mol. The COOH
Liver cathepsin D (15 μg) was incubated with equimolar quantity of bovine α-chymotrypsin or porcine pancreatic elastase in 0.5 ml of 0.06 M Tris-HCl buffer, pH 8.0 at 37°C. At the indicated intervals, 50-μl aliquots were taken, added to 10 μl of a 10 mg/ml solution of phenylmethanesulfonyl fluoride in dioxane. The effect of this material was determined on the activity of bovine spleen cathepsin D (about 0.1 μm) on methyl[1-14C]glycinated hemoglobin (0.4 mg) in 0.5 ml of 0.4 M sodium citrate buffer, pH 4.0, at 37°C for 30 min and the remaining inhibitory activity estimated. From left to right, (1) pepstatin treated with (○—○) or without (□—□) chymotrypsin; (2) < Glu-n-Phe-Pro-Phe-Val-n-Trp (Peptide VI) treated with (●—●) or without (Δ—Δ) chymotrypsin, < Glu-n-Phe-Pro-Phe-Val-n-Leu (Peptide VI) treated with (●—●) or without (Δ—Δ) chymotrypsin; essentially same results were obtained with subtilisin. (3) Peptide VI treated with elastase (▲—▲), and Peptide V treated with elastase (■—■).

The ineffective protection of the enzyme activity by the inhibitor from the inactivation caused by DANM-Cu2+, would suggest that the modifying agent may still have access to the catalytically critical groups in the enzyme-inhibitor (E–I) complex. More likely, the residue which is susceptible to DANM-Cu2+ is and corresponding to Asp-215 of pepstatin (39) may be different from the one assumed to be interfered with by the carboxyl of the terminal d-amino acid in the peptide inhibitor. The inactivation of cathepsin D by EPNP was found to be much slower than the similar reaction on pepstatin (22), but the protection effect by the peptide inhibitor was distinct. It is tempting to assume that the main catalytic group modified by EPNP, is possibly a carboxyl of an aspartyl residue leads to a large enhancement of the sensitivity of the Phe-Phe bond. Such secondary interactions particularly at S1 site were considered to alter the conformation of catalytically important groups in the enzyme so as to change greatly the efficiency of catalysis. Our findings on the unique inhibitory effect of a large aromatic and aliphatic d-amino acid at P3 may be a related phenomenon. Results of the effect of ionic strengths on the inhibitory activity of the peptides does not suggest the significance of a salt bridge formation between the terminus carboxyl and any positively charged group in the active site.
peptide in \textit{E}. \textit{I}. In this context, it is noteworthy that these peptides could not inhibit the activity of proteinas of the chymotrypsin family which cleaved these peptides at the Phe-Phe bond. Thus, the effect of the COOH terminus \( \alpha \)-amino acid with respect to the Phe-Phe bond discussed above is not operative in the active center of a serine protease.

The mode of inhibition of carboxyl proteinases by pepstatin proposed by Tang and his associates (38) emphasizes that the unusual amino acid residue, \( 4\)-amino-3-hydroxy-6-methylheptanoyl ("statyl"), is responsible for the unusually strong inhibitory capability of pepstatin and that statine is an analog of the proposed transition state for catalysis by pepsin and other carboxyl proteinases. Although the basis of the inhibitory properties of pepstatin and the inhibitor exemplified by Peptide VI may be different, the binding to the Phe-Phe segment of the latter to the critical region of the active site of the enzyme would be comparable to the binding of "statine" portion of pepstatin to the protease. The competitive nature of binding is suggested by the results of the effect of the presence of pepstatin on the binding of various amounts of Peptide VI to pepsin analyzed by the difference absorption spectra of the pepstatin inhibitor complex and pepsin. The difference spectra especially due to tryptophan residues is more distinct in binding of pepstatin with Peptide VI than binding of pepstatin. It is intriguing that in an analogous acid protease, penicillopepsin, a profound conformation change occurs when certain peptides bind in the region near Trp-39, Trp-7, and Tyr-75. Tyr-75, which is hydrogen-bonded to Trp-39, is located on a \( \beta \)-bend loop involving residues 70 to 83, and probably undergoes a major conformational change to accommodate the substrate binding (32).

The inhibitory potency of pepstatin is known to differ for various carboxyl proteinases (38, 39). Although inhibition of pepsin and cathepsin D by pepstatin was found to be similar, the inhibitors of the Peptide VI series exert less inhibition toward pepsin (Table I). This is probably due to increasing susceptibility to pepsin-catalyzed hydrolysis of the peptide inhibitor, from peptide IV to the more potent cathepsin D inhibitor, Peptide VI.

Inouye and Fruton (40) previously demonstrated that the Phe-Phe unit is essential to the prime affinity and the cleavage for pepsin catalyzed hydrolysis of the synthetic peptides of \( \alpha \)-Phe-Phe-B type, and that this cleavage is competitively inhibited by peptides of \( \alpha \)-Phe-Phe-D type. The work of Keilová et al. (10) also indicated that Gly-Phe-Leu-Gly-Phe-Val-Leu has a high inhibitory activity toward cathepsin D-catalyzed cleavage of the peptide with all \( L \)-amino acids at Phe-2-Leu-3 bond. Replacement with \( d \)-amino acids at the site of cleavage in the native sequence of the tetradecapeptide renin substrate (41) or of the octapeptide segment (42) was shown to form effective competitive inhibitors of a carboxyl proteinase, renin. However, \( L \) configuration of amino acids at the P1 and P1′ positions in Peptide IV series was strictly required for strong inhibition of pepsin and cathepsin D. Perhaps this is due in part to the dominant contribution of the COOH terminus \( \alpha \)-amino acid carboxyl group to the overall inhibitory activity displayed by this class of inhibitors.

This study also indicated that a minimum peptide unit required for significant inhibitory activity on cathepsin D or pepsin is a pentapeptidyl segment such as Pro-Phe-Phe-Val-Leu (\( K_0 \), 40 \( \mu \)M). Elongation of two \( \alpha \)-amino acid units from the NH\(_2\) terminus end to reach the P2 subsite possibly with a hydrophobic environment (6) markedly enhance the activity, such as shown in Glu-Phe-Pro-Phe-Phe-Val-Leu (\( K_0 \), 30 \( \mu \)M). This is consistent with the proposed size of the extended substrate binding site in pepsin as being composed of seven subsites, \( P_1 \rightarrow P_7 \) (25). Thus this class of inhibitor binds to the active site of carboxyl protease along the primary and the secondary subsites similar to the substrate.

From the statistical analysis of the cleavage sites of known proteins by pepsin, Powers et al. (25) suggested that L-proline is unfavorable to any subsites except \( P_2 \) and \( P_6 \). The preference of placing proline at the \( P_2 \) position for good inhibitors in the peptide IV series implies that proline may affect the orientation of the Phe-Phe unit, and thus its topographic relationship to the catalytic groups in the active site and the maximal functional role exerted by the COOH terminus \( \alpha \)-amino acid carboxylate group.

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