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Restrictions on the Binding of Proline-Containing Peptides to Elastase†

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ABSTRACT: In general, peptide substrates of pancreatic elastase have multiple binding modes to the enzyme and can give rise to heterogeneous reaction products. The presence of proline residues in the substrate is shown to restrict the activity of the enzyme and lead to fewer reaction products. Evidence is presented that the restriction of activity results from the inability of one subsite of the enzyme to bind proline residues of the substrate and the consequent reduction in the number of possible enzyme–substrate binding modes. These observations have led to the design of peptide substrates and inhibitors with a single enzyme binding mode.

The use of X-ray diffraction techniques makes it possible to study the detailed interactions of enzymes with substrate-like molecules. Frequently, however, the functional importance of the interactions observed cannot be deduced from static models of enzyme–substrate complexes. Because of their essentially dynamic nature, the function of enzyme–substrate interactions can often best be determined by studying their effect on the rate and equilibrium constants of the catalytic process. Unfortunately, the derivation of these constants from kinetic data is frequently complicated by the presence of secondary enzyme–substrate complexes.

The long substrate binding site of elastase (EC 3.4.4.7) (Atlas *et al.*, 1970; Thompson and Blout, 1970) and the polymeric nature of its substrates make secondary complex formation particularly likely for this enzyme. For hydrolyses occurring by an acyl-enzyme mechanism with the acylation reaction the rate-determining step, the observed value of the Michaelis constant, K_m, will be related to the dissociation constants for all possible enzyme–substrate complexes by eq 1, where K_p and K_n are the dissociation constants of "productive" and "nonproductive" enzyme–substrate complexes, respectively. Similarly, the observed value of an inhibition constant, K_i, will be related to the dissociation constants for all possible enzyme–inhibitor complexes by

$$\frac{1}{K_m} = \sum_p \frac{1}{K_p} + \sum_n \frac{1}{K_n} \quad (1)$$

$$\frac{1}{K_i} = \sum_n \frac{1}{K_n} \quad (2)$$

The measured value of K_m or K_i is simply related to the dissociation constant of a single enzyme–peptide complex

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only in the event that no other enzyme–peptide complexes are formed. In general, an evaluation of the individual dissociation constants in eq 1 and 2 is not possible if the primary and secondary enzyme–peptide complexes have the same stoichiometry. Consequently, true dissociation constants can be determined only for peptides that form unique complexes with the enzyme. Thus, before the K_m or K_i observed can be accepted as a unique dissociation constant, it is necessary to show that secondary complexes are not formed.

The formation of secondary productive complexes by substrates would lead to heterogeneous reaction products; hence, examination of the reaction products makes it possible to rule out the existence of secondary productive complexes. The possible formation of secondary nonproductive complexes by substrates and inhibitors is much more serious because often it is difficult to detect or exclude their presence. Although strong secondary binding of peptides to an enzyme may be detected by X-ray crystallography, this method is not sufficiently rapid to lend itself generally to demonstrating that inhibitors or substrates bind uniquely. For example, at this time it is quite impractical to compute difference Fourier maps for analogs of each substrate used to investigate the catalytic mechanism of elastase.

The impracticality of dealing individually with the many possible substrates for elastase makes it desirable to have ground rules for the design of peptides which are unable to form secondary complexes with the enzyme. This paper describes how proline residues incorporated into the substrate restrict the number of bonds susceptible to elastase-catalyzed hydrolysis. The restriction is shown to arise from the inability of the enzyme to bind proline residues in certain parts of its active center. This property can be used to reduce the possibility of secondary complex formation. Finally, we discuss the binding to the enzyme of a number of substrates which have been important in forming ideas of the molecular details of elastase-catalyzed amide hydrolysis.

Materials and Methods

Elastase-catalyzed hydrolysis of peptide amides and esters was carried out in a pH-Stat. Reactions were conducted in 1 ml of 10^{-2} M aqueous $CaCl_2$ at pH 9.00 and 37°. The pH of the reaction mixture was maintained by the addition of 2×10^{-3} M aqueous NaOH. Strict Michaelis–Menten kinetics were observed, and k_{cat} and K_m were evaluated from Lineweaver–Burk plots. K_i was evaluated from Dixon plots. In all cases peptide inhibitors were found to be fully competitive within experimental error. On the basis of duplicate measurements of several of these kinetic constants, the values reported are estimated to be reproducible to better than 20% and in most cases better than 10%.

The bond split by the enzyme was established by thin-layer chromatography (tlc) of reaction mixtures. Except for peptides III and IV, a single reaction product was observed which was ninhydrin negative. These findings are consistent with the exclusive hydrolysis of the amide bond. For compounds III and IV, where alaninamide was identified among the products, the relative rates of the amidase and peptidase reactions were calculated from an analysis of the products, conducted with an amino acid analyzer.

Tlc of peptides was carried out with silica gel plates (Q1) purchased from Quantum Industries, N. J. Plates were developed in chloroform–methanol (97:3, system I), 1-butanol–acetic acid–water (4:1:1, system II), *sec*-butyl alcohol–3% aqueous ammonia (100:44, system III), or chloroform–methanol (9:1, system VIII). Spots were visualized by spraying with a 0.3% solution of ninhydrin in acetone and heating at 110° for 10 min. *tert*-Butyloxycarbonyl peptides could generally be visualized by prolonged heating (60 min). After ninhydrin visualization, the plates were exposed to iodine vapor for several hours and sprayed with an aqueous solution of potassium iodide and tolidine (Reagent 32; Waldi, 1965).

tert-Butyloxycarbonylamino acids, amino acid esters, and amides were purchased from the Fox Chemical Co. Los Angeles; Cyclo Chemicals, Los Angeles; or Fluka AG, Switzerland.

Porcine pancreatic elastase (>99.8% pure) was purchased from Whatman Biochemicals, England, and was assayed both by the NBA assay (Visser and Blout, 1972) and with acetylprolylalanylprolylalaninamide.

Alanylalanine Methyl Ester Hydrochloride. Alanine methyl ester hydrochloride (2 g; 14.3 mmol) and *tert*-butyloxycarbonylalanine succinimido ester (4.1 g; 14.3 mmol) were dissolved in chloroform (20 ml) and *N*-methylmorpholine (1.57 ml; 14.3 mmol) was added. After 24 hr at 25°, 5 drops of *uns*-*N*,*N*-dimethylethylenediamine were added, and the solvent was evaporated. The residue was dissolved in ethyl acetate and extracted twice with 0.2 M hydrochloric acid, twice with 5% aqueous sodium bicarbonate, and once with water. The organic phase was dried and evaporated, and the residue was crystallized from ether–hexane to give 2.4 g (61%) of *tert*-butyloxycarbonylalanylalanine methyl ester: mp 110–111°, lit. (Doyle, *et al.*, 1970) mp 112.5–113.5°.

The crystals were dissolved in ethyl acetate saturated with hydrogen chloride (20 ml). After 1 hr at 25°, the solvent was removed and the residue was left *in vacuo* over NaOH pellets for 24 hr; yield, 1.73 g (94%).

Alanylalanylalanine methyl ester hydrochloride was prepared from *tert*-butyloxycarbonylalanine succinimido ester and alanylalanine methyl ester hydrochloride using the coupling and deprotection procedures used to prepare HCl·H-Ala-Ala-OMe.

Acetylalanylalanylalanine Methyl Ester (I). Alanylalanylalanine methyl ester hydrochloride (380 mg; 1.32 mmol) was dissolved in a mixture of pyridine (10 ml) and excess acetic anhydride. After stirring 1 hr at room temperature, the solvent was evaporated; the residue was dissolved in water and cooled to 0°. Excess Rexyn I-300 mixed-bed ion-exchange resin (Fisher) was added and stirred for 15 min at 0°. After filtration the solvent was evaporated, and the residue was crystallized from ethyl acetate–ether to give 310 mg of product (81%), mp 254–255°.

Acetylprolylalanylalanine Methyl Ester (II). Prolylalanylalanine methyl ester hydrochloride was prepared from *tert*-butyloxycarbonylproline succinimido ester and alanylalanine methyl ester hydrochloride by the coupling and deprotection techniques used in the preparation of alanylalanine methyl ester hydrochloride. It was acetylated by the procedure used to prepare acetylalanylalanylalanine methyl ester. Crystallization from ethyl acetate–ether gave 198 mg (46% yield from alanylalanine methyl ester hydrochloride), mp 166–169°.

Acetylalanylalanylalanylalanine Methyl Ester. Alanylalanylalanylalanine methyl ester hydrochloride was prepared from *tert*-butyloxycarbonylalanine succinimido ester and alanylalanylalanine methyl ester hydrochloride using the coupling and deprotection procedures used to prepare HCl·H-Ala-Ala-OMe. The peptide was acetylated by the method

used to prepare Ac-Ala-Ala-Ala-OMe: yield based on Boc-Ala-OSu, 40%; single spot by tlc $R_{F_{II}}$ 0.65; mp 308–311°. *Anal.* Calcd for $C_{15}H_{26}N_4O_6$: C, 50.3; H, 7.3, N, 15.6. Found: C, 50.0; H, 7.3; N, 15.2.

Acetylalanylalanylalanylalaninamide (*III*). Acetylalanylalanylalanylalanine methyl ester was dissolved in a saturated solution of ammonia in hexafluoroisopropyl alcohol. After 48 hr the solvent was removed *in vacuo* and the product was crystallized from aqueous methanol: yield, 49 mg (40%); single spot by tlc $R_{F_{II}}$ 0.5. *Anal.* Calcd for $C_{14}H_{25}N_5O_5 \cdot 0.5H_2O$: C, 47.71; H, 7.44. Found: C, 47.6; H, 7.4.

Acetylprolylalanylalanylalanine Methyl Ester. Acetylproline was coupled to alanylalanylalanine methyl ester hydrochloride by the mixed-anhydride procedure used in the preparation of acetyl-D-alanylprolylalanylprolylalaninamide: yield, 419 mg (70%); mp 248–250°; single spot by tlc $R_{F_{II}}$ 0.6.

Acetylprolylalanylalanylalaninamide (*V*). Acetylprolylalanylalanylalanine methyl ester (256 mg; 0.66 mmol) was dissolved in methanol saturated with ammonia. After 48 hr at room temperature, the solvent was evaporated, and the residue was triturated under ether to give 192 mg (78%) of a hygroscopic solid, mp 288–291°. *Anal.* Calcd for $C_{16}H_{27}N_5O_5 \cdot 1.5H_2O$: C, 48.5; H, 7.6; N, 17.7. Found: C, 48.9; H, 7.6; N, 17.4.

tert-Butyloxycarbonyl-D-alanylproline Benzyl Ester. tert-Butyloxycarbonyl-D-alanine (1.17 g; 6.2 mmol) was dissolved in 50 ml of acetonitrile and cooled to −20° in a Dry Ice–CCl_4 bath. *N*-Methylmorpholine (0.68 ml; 6.2 mmol) was added, followed by isobutyl chloroformate (0.81 ml; 6.2 mmol). After stirring 2 min at −20°, a solution of 1.45 g (6.2 mmol) of proline benzyl ester hydrochloride in 50 ml of chloroform and 0.68 ml (6.2 mmol) of *N*-methylmorpholine were added. The reaction was allowed to warm to room temperature, stirred overnight, and the solvent was evaporated. The oily residue was dissolved in ethyl acetate and washed twice with 0.2 M hydrochloric acid and twice with saturated aqueous sodium bicarbonate. The organic phase was dried and solvent was evaporated to leave 1.9 g (82%) of *tert*-butyloxycarbonyl-D-alanylproline benzyl ester: single spot by tlc R_{F_I} 0.8.

D-Alanylproline Benzyl Ester Hydrochloride. tert-Butyloxycarbonyl-D-alanylproline benzyl ester (0.75 g; 20 mmol) was dissolved in 100 ml of a saturated solution of hydrogen chloride in ethyl acetate. After 1 hr the solvent was evaporated, and the foamy product was left *in vacuo* with NaOH pellets overnight; yield of D-alanylproline benzyl ester hydrochloride; 0.5 g (80%).

Acetyl-D-alanylproline. D-Alanylproline benzyl ester hydrochloride (2.0 g; 6.4 mmol) was dissolved in a mixture of pyridine (50 ml) and acetic anhydride (1 ml). After 1 hr at 25° the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and extracted twice with 0.2 M hydrochloric acid, twice with 5% aqueous sodium bicarbonate, and once with water. The organic phase was dried and evaporated to give acetyl-D-alanylproline benzyl ester as an oil R_{F_I} 0.4.

The oil was dissolved in *tert*-butyl alcohol and hydrogenated over 10% palladium–charcoal at 20 psi for 12 hr. The solution was filtered through Celite and evaporated *in vacuo*. The residue crystallized from 5% aqueous acetone–ether to give 655 mg of crystals (45%), mp 158–161°. *Anal.* Calcd for $C_{10}H_{16}N_2O_4$: C, 52.62; H, 7.07; N, 12.27. Found: C, 52.6; H, 7.0; N, 12.4.

Acetyl-D-alanylprolylalanylprolylalaninamide (VII). Acetyl-D-alanylproline (200 mg; 0.9 mmol) was dissolved in acetonitrile (20 ml), cooled to −20°, and 0.10 ml of *N*-methylmorpholine (0.9 mmol) and 0.115 ml of isobutyl chloroformate (0.9 mmol) were added. After stirring 10 min at −20°, *N,N*-dimethylformamide (10 ml) was added followed by a solution of 257 mg of alanylprolylalaninamide hydrochloride (Thompson and Blout, 1973a) (0.9 mmol) and 0.10 ml of *N*-methylmorpholine (0.9 mmol) in *N,N*-dimethylformamide. The mixture was allowed to warm slowly to room temperature and evaporated after a further 4 hr. The residue was dissolved in water and treated with Rexyn I-300 resin for 10 min. After filtration and evaporation of the water, the product was crystallized from 5% aqueous acetone–ethyl acetate to give 184 mg (44%) of a white solid: single spot by tlc $R_{F_{II}}$ 0.5, $R_{F_{III}}$ 0.5, $R_{F_{VIII}}$ 0.3; $[\alpha]_D^{25}$ −176° (*c* 0.6, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{21}H_{34}N_6O_6 \cdot 0.5H_2O$: C, 53.03; H, 7.41; N, 17.68. Found: C, 53.1; H, 7.6; N, 17.8.

D-Alanylalanylprolylalaninamide Hydrochloride. tert-Butyloxycarbonyl-D-alanylalanylprolylalaninamide was prepared from *tert*-butyloxycarbonyl-D-alanine and alanylprolylalaninamide hydrochloride by the mixed-anhydride coupling procedure used in the preparation of acetyl-D-alanylprolylalanylprolylalaninamide: yield from 5% aqueous acetone–ether, 251 mg (61%); single spot tlc $R_{F_{II}}$ 0.65; mp 177–179°; $[\alpha]_D^{25}$ −125° (*c* 0.2, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{19}H_{33}N_5O_6$; C, 53.38; H, 7.78; N, 16.38. Found: C, 53.9; H, 7.6; N, 16.5.

A total of 140 mg (0.33 mmol) was dissolved in methanol (0.5 ml) and a saturated solution of hydrogen chloride in ethyl acetate (20 ml) was added. After 1 hr at room temperature, the solvent was removed *in vacuo*, and the residue was left *in vacuo* with sodium hydroxide pellets for 24 hr. Trituration under ether gave 101 mg (95%) of D-alanylalanylprolylalaninamide hydrochloride as a hygroscopic white solid: single spot by tlc $R_{F_{II}}$ 0.2 ,$R_{F_{III}}$ 0.4. Amino acid analysis gave Ala:Pro = 2.85.

Acetyl-D-alanylalanylprolylalaninamide (*X*) was prepared from D-alanylalanylprolylalaninamide hydrochloride by the acetylation procedure used to prepare acetylalanylalanylalanine methyl ester: Yield, 102 mg (89%); single spot by tlc $R_{F_{II}}$ 0.5; $[\alpha]_D^{25}$ −118° (*c* 0.7, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{16}H_{27}N_5O_5$: C, 52.0; H, 7.4. Found: C, 51.7; H, 7.3. Amino acid analysis gave Ala:Pro = 3.15.

Alanylalanylprolylalaninamide Hydrochloride. Carbobenzoxyalanylalanylprolylalaninamide was prepared from carbobenzoxyalanine and alanylprolylalaninamide hydrochloride (Thompson and Blout, 1973a) by the mixed-anhydride coupling procedure used in the preparation of acetyl-D-alanylprolylalanylprolylalaninamide above: yield from ethyl acetate, 800 mg (73%); single spot by tlc $R_{F_{II}}$ 0.7, $R_{F_{VIII}}$ 0.6; mp 141–143°. *Anal.* Calcd for $C_{22}H_{31}N_5O_6$: C, 57.25; H, 6.77; N, 15.18. Found: C, 57.5; H, 6.8; N, 15.3.

A total of 780 mg (1.7 mmol) was dissolved in *tert*-butyl alcohol (20 ml), 2 M hydrochloric acid (1 ml) was added, and the mixture was hydrogenated over 10% palladium–charcoal at 20 psi for 3 hr. After filtration through Celite and evaporation of solvent, the residue was triturated under acetone to give 556 mg (90%) of a hygroscopic solid: single spot by tlc $R_{F_{II}}$ 0.2; $R_{F_{III}}$ 0.4; $[\alpha]_D^{25}$ −152° (*c* 0.6, 10^{-2} M aqueous $CaCl_2$). Amino acid analysis gave Ala:Pro = 3.0.

Acetylalanylalanylprolylalaninamide (*VI*) was prepared in 66% yield from alanylalanylprolylalaninamide hydrochloride by the acetylation procedure used to prepare acetylalanylalanylalanine methyl ester: single spot on tlc $R_{F_{II}}$ 0.5; mp 240–243°; $[\alpha]_D^{25}$ −218° (*c* 0.5, 10^{-2} M aqueous $CaCl_2$).

Anal. Calcd for $C_{16}H_{27}N_5O_5$: C, 52.0; H. 7.4; N, 19.0. Found: C, 52.2; H, 7.4, N, 18.7.

Acetyl-D-alanylalanylalanylprolylalaninamide (VIII). Carbobenzoxy-D-alanylalanylalanylprolylalaninamide was prepared from carbobenzoxy-D-alanine andal anylalanylprolylalaninamide hydrochloride by the mixed-anhydride coupling procedure used to prepare acetyl-D-alanylprolylalanylprolylalaninamide. The product (217 mg), obtained in 59% yield from acetone, is a single spot by tlc: $R_{F_{II}}$ 0.6, $R_{F_{III}}$ 0.6, $R_{F_{VIII}}$ 0.4. *Anal.* Calcd for $C_{25}H_{36}N_6O_7 \cdot 1H_2O$: C, 54.53; H, 6.96; N, 15.26. Found: C, 55.1; H, 7.0; N, 15.4.

The carbobenzoxy group was removed by the procedure used to prepare alanylalanylprolylalaninamide hydrochloride: yield of D-alanylalanylalanylprolylalaninamide hydrochloride from trituration under 2% aqueous acetone, 72%; single spot by tlc $R_{F_{II}}$ 0.1. *Anal.* Calcd for $C_{17}H_{31}ClN_6O_5 \cdot H_2O$: C, 45.07; H, 7.34; N, 18.56. Found: C, 45.0; H, 7.4; N, 18.1.

Acetylation was by the procedure used to prepare acetylalanylalanylalanine methyl ester above; yield from 5% aqueous acetone, 64%; $[\alpha]_D^{25}$ −162° (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{19}H_{32}N_6O_6$: C, 51.80; H, 7.32; N, 19.08. Found: C, 51.6; H, 7.3; N, 18.8.

Acetylprolylalanylprolyl-D-alaninamide (XI). Acetylprolylalanylproline (Thompson and Blout, 1973a) and D-alanine benzyl ester tosylate were coupled by the procedure used to prepare *tert*-butyloxycarbonyl-D-alanylproline benzyl ester. The oily peptide benzyl ester was dissolved in methanol saturated in ammonia for 48 hr, evaporated, dissolved in water, and treated with Rexyn I-300 resin. After evaporation of the water, the product was triturated under ether giving a 62% yield: single spot by tlc $R_{F_{II}}$ 0.5. *Anal.* Calcd for $C_{18}H_{29}N_5O_5$: C, 54.67; H, 7.39; N, 17.71. Found: C, 54.7; H, 7.7; N, 17.6.

Acetyl-D-alanylalanylalanylalaninamide (IX). *tert*-Butyloxycarbonylalanylalaninamide was prepared by coupling *tert*-butyloxycarbonylalanine and alaninamide hydrochloride by the mixed-anhydride procedure used to prepare acetyl-D-alanylprolylalanylprolylalaninamide: yield from ethyl acetate, 85%; mp 168–169°.

tert-Butyloxycarbonylalanylalaninamide was deprotected by treatment with hydrogen chloride in ethyl acetate as described in the preparation of alanylalanine methyl ester hydrochloride. The product was then coupled to *tert*-butyloxycarbonylalanine by the mixed-anhydride procedure: yield of *tert*-butyloxycarbonylalanylalanylalaninamide, 85%; mp 207–209°.

tert-Butyloxycarbonylalanylalanylalaninamide was deprotected with hydrogen chloride in ethyl acetate, and the product was coupled to *tert*-butyloxycarbonyl-D-alanine by the mixed-anhydride procedure: yield of *tert*-butyloxycarbonyl-D-alanylalanylalanylalaninamide from trituration under ether, 59%; single spot by tlc $R_{F_{II}}$ 0.7.

tert-Butyloxycarbonyl-D-alanylalanylalanylalaninamide was deprotected with hydrogen chloride in ethyl acetate, and the product was acetylated by the procedure used to prepare acetylalanylalanylalanine methyl ester; yield from trituration under ethyl acetate, 67%; single spot by tlc $R_{F_{II}}$ 0.5; $[\alpha]_D^{25}$ −56° (*c* 0.5, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{14}H_{25}N_5O_5$: C, 48.97; H, 7.34. Found: C, 48.6; H, 7.3.

Acetylalanylprolylalanylalaninamide (IV). Alanylalaninamide hydrochloride, prepared as described above, was coupled to *tert*-butyloxycarbonylproline by the mixed-anhydride procedure used to prepare acetyl-D-alanylprolylalanylprolylalaninamide: yield of *tert*-butyloxycarbonylprolylalanylalaninamide, 75%; mp 236–237°.

TABLE I: Effect of P_3 Proline Residues on the Esterase, Amidase and Peptidase Activity of Elastase.

P_5 P_4 P_3 P_2 P_1 P_1' ↓[a]	k_{cat}/K_m (M^{-1} sec^{-1})	[S][b] (mM)
Ac-Ala-Ala-Ala-OMe (I)	370,000	0.2–10
Ac-Pro-Ala-Ala-OMe (II)	830	0.9–9
Ac-Ala-Ala-Ala-Ala-NH_2 (III)	2,070	0.5–5
Ac-Ala-Pro-Ala-Ala-NH_2 (IV)	<50	0.5–9
Ac-Ala-Ala-Ala-Ala-NH_2 (III)	90	0.5–5
Ac-Pro-Ala-Ala-Ala-NH_2 (V)	<2	0.5–8

[a] Vertical arrow indicates bond subject to elastase-catalyzed hydrolysis. [b] Range of substrate concentrations.

tert-Butyloxycarbonylprolylalanylalaninamide was deprotected by hydrogen chloride in ethyl acetate as described in the preparationof alanylalanine methyl ester hydrochloride. The product was coupled to *tert*-butyloxycarbonyl alanine by the mixed-anhydride procedure: yield of *tert*-butyloxycarbonylalanylprolylalanylalaninamide, 68%; mp 144–146°.

tert-Butyloxycarbonylalanylprolylalanylalaninamide was deprotected by hydrogen chloride in ethyl acetate. The product was acetylated by the procedure used to prepare acetyl alanylalanylalanine methyl ester. Trituration under ethyl acetate gave a 77% yield of a white hygroscopic solid; single spot by tlc $R_{F_{II}}$ 0.5. *Anal.* Calcd for $C_{16}H_{27}N_5O_5 \cdot H_2O$: C, 49.60; H, 7.55; N, 18.1. Found: C, 49.5; H, 7.7; N, 17.7.

Results

Restriction of Elastase Activity by Proline Residues of the Substrate. Investigations of peptide substrates of elastase have shown that certain substrates can form more than one complex with the enzyme, making the k_{cat} and K_m for hydrolysis of such substrates impossible to interpret. This situation is most obvious where two productive complexes are formed. For example, when Ac-Ala-Ala-Ala-Ala-NH_2 is digested by elastase, both ammonia and alaninamide are observed as products (Table I), indicating the existence of two binding modes, S_{54321} and $S_{43211'}$,[1] respectively, for this substrate.

We have found that it is possible to restrict the action of elastase to just one bond of a substrate by using substrates containing proline residues. With Ac-Pro-Ala-Ala-Ala-NH_2, for example, only the amide bond is cleaved at a significant rate (see Tables I and II). To explore the basis for this restriction of the enzyme's activity, we compared the kinetic parameters for elastase-catalyzed hydrolysis of several substrates containing proline and analogous substrates where this residue is replaced by alanine. It is clear from the data

[1] In this and subsequent papers the nomenclature introduced by Schechter and Berger (1967) is used to facilitate discussion of the interactions between elastase and bound peptides. Amino acid residues and partial amino acid residues (*e.g.*, acetyl groups) of substrates are numbered P_1, P_2, P_3, etc., in the N-terminal direction, and P_1', P_2', etc., in the C-terminal direction from the scissile bond. The complementary subsites of the enzyme's active center are numbered S_1, S_2 and S_1', S_2', etc., in an analogous fashion. The binding mode of a peptide which occupies, for example, the S_1, S_3, S_2, and S_1 subsites of the enzyme will be denoted by the abbreviation S_{1321}.

TABLE II: Influence of P_2 and P_4 Proline and P_5 D-Alanine Residues on the Amidase Activity of Elastase.

P_6 P_5 P_4 P_3 P_2 P_1 ↓	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	[S][a] (mM)
Ac-Ala-Ala-Ala-Ala-NH_2 (III)	2070	6.0	2.9	0.5–5
Ac-Ala-Ala-Pro-Ala-NH_2 (VI)	2900	6.1	2.1	0.5–9
Ac-Pro-Ala-Ala-Ala-NH_2 (V)	1200	4.7	3.9	0.5–8
Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (VII)	1050	8.3	7.9	0.5–10
Ac-D-Ala-Ala-Ala-Pro-Ala-NH_2 (VIII)	2200	3.8	1.7	0.5–5

[a] Range of substrate concentrations.

in Table I that an ester, amide, or peptide bond C-terminal to Y in the sequence –Pro-X-Y– is relatively resistant to elastase-catalyzed hydrolysis. In the nomenclature of Schechter and Berger,[1] proline is residue P_3 of these substrates, and the subsite of the enzyme interacting with this residue is termed S_3. The restriction of the enzyme's action, therefore, lies in its decreased ability to form productive complexes with a substrate when proline is required to fill the S_3 subsite of the enzyme's active center. This could arise either from the prevention of enzyme–substrate binding in this mode, or from the formation of a nonproductive complex.

Effects on Enzyme–Peptide Binding of Proline and D-*Alanine Residues in the Peptide.* To determine whether the inability of elastase to cleave the Y–Z bond in the sequence Pro-X-Y-Z arose in the binding or acylation step of reaction, we set out to study the effects on enzyme–peptide binding of proline residues in the peptide. As it turned out, the most interesting enzyme–peptide interaction, that of proline with the S_3 subsite, could not be studied in a direct fashion. It could, however, be approached using the results presented below by the indirect method described in the Discussion section.

Positive evidence for a favorable interaction between a particular subsite and an amino acid residue of a bound peptide can usually be drawn from comparisons of the kinetic parameters for substrate hydrolysis. Identification of the subsite is possible only because the hydrolysis reaction allows us to assign the productive binding mode to the substrates. Collected in Table II are the kinetic data for amide hydrolysis from some substrates containing prolyl and D-alanyl residues. The kinetic parameters found for Ac-Ala-Ala-Pro-Ala-NH_2 (VI) and Ac-Pro-Ala-Ala-Ala-NH_2 (V) are typical of those of other tetrapeptide amides (Thompson and Blout, 1973a), indicating unambiguously that both the S_4 and S_2 subsites of elastase bind proline residues satisfactorily. The results for Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (VII) and Ac-D-Ala-Ala-Ala-Pro-Ala-NH_2 (VIII), which are typical of other pentapeptides (Thompson and Blout, 1973a), indicate that the S_5 subsite is capable of binding D-alanyl residues.

Evidence for an unfavorable interaction between a particular subsite and an amino acid residue of a peptide can sometimes be obtained by comparing the enzyme–peptide dissociation constants of an inhibitor and an analogous substrate. Again, the hydrolysis reaction of the substrate is the key to identifying the particular subsite involved. A comparison of the K_i (350 mM) of Ac-D-Ala-Ala-Pro-Ala-NH_2 (X) and the K_m (2.1 mM) of Ac-Ala-Ala-Pro-Ala-NH_2 (VI) shows that the S_4 subsite is unable to bind internal D-alanine residues of a peptide. A similar, but less powerful, ability to discriminate against D-alanine residues may be ascribed to the S_1 subsite by comparing the K_i (48 mM) of Ac-Pro-Ala-Pro-D-Ala-NH_2 (XI) with the K_m (3.9 mM) of Ac-Pro-Ala-Pro-Ala-NH_2 (Thompson and Blout, 1973a).

A comparison of the enzyme–peptide dissociation constants of two inhibitors can often give information about the properties of a subsite, but in the absence of further information localizing the binding mode of one of these inhibitors, that subsite cannot be identified. A comparison of the K_i's of Ac-D-Ala-Ala-Pro-Ala-NH_2 (X), 350 mM, and Ac-D-Ala-Ala-Ala-Ala-NH_2 (IX), 5.5 mM, shows quite unambiguously the existence of a subsite capable of binding alanine, but not proline, residues of a peptide. Identification of this subsite is the subject of the discussion below.

Discussion

Identification of S_3 as the Subsite Unable to Bind Proline Residues. The existence of a subsite discriminating against the proline residues of a bound peptide has been shown above through a comparison of the K_i's of Ac-D-Ala-Ala-Ala-Ala-NH_2 and Ac-D-Ala-Ala-Pro-Ala-NH_2. To identify the subsite responsible for the high K_i of the latter peptide, it is necessary to identify the binding mode(s) giving rise to the low K_i of the former compound.

Of the subsites of the active center of elastase, only S_5 has been shown capable of binding internal D-alanyl residues of peptides; whereas, S_4 and S_1 have been shown to be incapable of doing this (*vide supra*). The properties of the S_2 and S_3 subsites, although not studied in any detail, might be thought to resemble the other internal subsites, S_4 and S_1, rather than the terminal S_5 subsite (Thompson and Blout, 1973a). In the most favorable situation a D-alanyl residue binding to the S_2 or S_3 subsites should mimic a glycine residue, since no contact with the side-chain binding site for L-amino acid residues will be possible, At least in the case of subsite S_3, contact with the side-chain binding site has been shown to be important for good peptide binding (*cf.* Ac-Pro-Gly-Pro-Ala-NH_2 and Ac-Pro-Ala-Pro-Ala-NH_2; Thompson and Blout, 1973a). It is likely, therefore, that the S_4, S_3, and S_1 subsites, and probably the S_2 subsite also, will prove incapable of interacting favorably with D-alanyl residues of a bound peptide.

Consistent with all the data is the hypothesis that $S_{(6)5432}$ is a major, if not the sole, binding mode of Ac-D-Ala-Ala-Ala-Ala-NH_2. This mode should ensure maximum enzyme–peptide contact while avoiding interactions which are seriously detrimental to binding. The obvious inability of Ac-D-Ala-Ala-Pro-Ala-NH_2 to occupy this particular binding mode must,

therefore, result from the inability of the Pro residue to occupy the S_3 subsite. From a comparison of the K_i's of Ac-D-Ala-Ala-Pro-Ala-NH_2 and Ac-D-Ala-Ala-Ala-Ala-NH_2, the S_3–Pro interaction occurring in the former peptide can be calculated to be at least 2.6 kcal/mol less favorable than the S_3–Ala interaction occurring in the latter. The poor binding of proline to S_3, then, is clearly sufficient to account for the poor reactivity of the P_3 proline peptides listed in Table I.

The kinetic data argue strongly for an S_3 subsite unable to bind proline residues of the substrate. It would, however, be desirable to compare these results with difference Fourier maps of elastase–substrate analog complexes, both to test this hypothesis and further to establish a physical basis for the exclusion of proline from the S_3 subsite.

Difference electron density maps of elastase–peptide complexes are currently available at 3.5 Å. At this resolution interpretation of structure is beset with difficulties. However, the structures currently favored by Shotton *et al.* (1972) are consistent with the idea that the S_3 subsite will not bind proline residues. The elastase–Ac-Pro-Ala-Pro-Ala-OH complex appears to have a unique structure which would be unlikely if the proline residues were not constraining the binding in some way. There is, for example, no evidence of the multiple binding modes implicated for the analogous peptide Ac-Ala-Ala-Ala-Ala-OH by the multiple hydrolysis products of Ac-Ala-Ala-Ala-Ala-NH_2 (*vide supra*).

Design of Peptides with Unique Binding Modes to Elastase. The difficulties involved in interpreting the kinetic parameters for elastase-catalyzed hydrolysis of substrates with multiple binding modes were described earlier. The results presented above show that the number of binding modes available to peptide substrates containing proline residues is limited by the inability of proline to occupy the S_3 subsite of elastase. In searching for peptides which would have unique binding modes to this enzyme, we have, therefore, examined the type of enzyme–peptide binding expected for peptides with various defined sequences of alanine and proline residues.

We now consider the number of modes expected for peptides binding to elastase and the degree to which these are circumscribed by the unfavorable S_3–Pro interaction. The enzyme has five known binding subsites for amino acid residues of the substrate N terminal to the scissile bond (the contribution of subsite S_6 to peptide binding is so small as to be negligible (Thompson and Blout, 1973a)). Since, in general, acetyl tripeptides bind tightly to elastase (dissociation constant, $K_d \sim 5$ mM), while tripeptides and acetyl dipeptides bind much less well ($K_d > 50$ mM) (Thompson and Blout, 1973a, and unpublished observations), it may be concluded that at least four of the five subsites must be occupied to ensure good enzyme–peptide binding. For acetyl tetrapeptides, therefore, three principle binding modes might be expected; *viz.*, $S_{(6)5432}$, S_{54321}, and $S_{43211'}$. For acetyl tetrapeptides of the general formula Ac-Pro-Ala-Pro-X, the first and last of these complexes require a proline residue to bind the S_3 subsite. According to the evidence presented above, such complexes will be extremely weak and may be assumed to be kinetically insignificant. Effectively then, the peptides Ac-Pro-Ala-Pro-X should have a unique binding mode, S_{54321}, in which the residue X occupies the S_1 subsite.

The ability of the sequence Pro-Ala-Pro to orient a peptide in this fashion has been used to study both the binding and kinetic specificities of the S_1 subsite. This work is reported in the following papers (Thompson and Blout, 1973a, and to be published).

For peptides shorter than acetyl tetrapeptides, the problems of ensuring a single enzyme–peptide binding mode become more difficult. For tetrapeptides and acyl tripeptides, for example, the binding modes S_{5432}, S_{4321}, and $S_{3211'}$ are possible. With peptides of the general formula X-Ala-Pro-Ala-NH_2, the first mode will be weak on account of the proline residue occupying the S_3 subsite of the enzyme. Exclusion of the $S_{3211'}$ binding mode from consideration is based on the hypothesis that the S_1 subsite will also discriminate against proline residues of the substrate. This hypothesis is consistent with the inability of elastase to hydrolyze the ester bond of carbobenzoxyproline *p*-nitrophenyl ester (Geneste and Bender, 1969) and with the general tendency of the S_1 subsite to reject amino acid residues with large side chains. The binding of a P_1 proline residue would also require a drastic modification of the S_1–P_1 interaction tentatively proposed by Shotton *et al.* (1972). These findings lead us to believe that peptides of general formula X-Ala-Pro-Ala-NH_2 will bind only weakly in the $S_{3211'}$ mode. Their most important binding mode will therefore be S_{4321}, in which the residue X binds to the S_4 subsite. Accordingly, we have used such peptides to elucidate the binding and kinetic specificities of the S_4 subsite (Thompson and Blout, 1973b).

For peptides longer than acetyl tetrapeptides the restrictions of enzyme–peptide binding arising from the inability of proline to occupy S_3 and S_1 are probably insufficient to ensure a unique binding mode. Since studies of the peptidase activity of elastase will require the use of penta- and hexapeptide substrates (Atlas *et al.*, 1970; Thompson and Blout, 1970), it is probable that further restrictions on enzyme–substrate binding will have to be found before the peptidase activity of the enzyme can be investigated fully.

In this paper we have discussed the conditions under which the observed values of K_m and K_i can be equated with the dissociation constant of a single enzyme–peptide complex. From the equation (Bender and Kezdy, 1965)

$$k_{cat}/K_m = k_2/K_p$$

it is clear that under conditions where $K_m = K_p$, the observed catalytic constant, k_{cat}, can be equated with the rate constant for the acylation reaction, k_2. Peptides with unique binding modes may, therefore, be exploited to obtain true rate and equilibrium constants for the catalytic process. The use of such data to explore the mechanism of catalysis is the subject of the following papers in this series.

Summary

The inability of the S_3 subsite of elastase to accept proline residues of bound peptides has been demonstrated using peptides containing both proline and D-alanine residues. This observation has made possible the design of a number of peptides with a single strong binding mode to the enzyme.

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Dependence of the Kinetic Parameters for Elastase-Catalyzed Amide Hydrolysis on the Length of Peptide Substrates†

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ABSTRACT: The kinetic parameters for elastase-catalyzed amide hydrolysis have been measured for a number of peptide amides. k_{cat}/K_m for this reaction increases with increasing peptide chain length up to a maximum with pentapeptide amides. Using substrates for which the kinetic constants for the binding and acylation steps of reaction can be separated, the binding and kinetic specificities of several subsites of the enzyme have been explored. The most important response of the kinetic parameters to increasing substrate chain length is shown to be an increased rate constant for the acylation reaction. The magnitude of this increase on going from acyl tri- to acyl tetrapeptide amides is almost independent of the amino acid sequence of the substrate. This observation is consistent with the hypothesis that the increased rate constant results from a conformational change in the enzyme.

Elastase (EC 3.4.4.7) is a pancreatic serine proteinase related by sequence homology, tertiary structure, and mechanism to α-chymotrypsin and trypsin (Hartley and Shotton, 1971). Both the similarities and differences within this family of enzymes promise to illumine the relationship between their structure and function. One property of elastase not shared by α-chymotrypsin and trypsin is the marked dependence of the kinetic parameters for substrate hydrolysis on substrate chain length (Atlas *et al.*, 1970; Thompson and Blout, 1970). Preliminary work on this phenomenon indicated that the efficiency of ester, amide, and peptide bond hydrolysis increased with increasing substrate chain length. A particularly important role was ascribed to the S_4–P_4[1] enzyme–substrate contact which appeared to increase the rate constant for substrate hydrolysis.

This paper reports a more extensive study of the relationship between substrate chain length and the efficiency of amide hydrolysis. Wherever possible, substrates which bind to elastase in a single mode are used (Thompson and Blout, 1973a), and the separate binding and kinetic specificities of the enzyme are thereby determined. The results confirm the previous inference of a rate acceleration due to enzyme–substrate contacts remote from the scissile bond. Additionally, they allow a further definition of the mechanism through which this rate acceleration can occur.

Materials and Methods

Elastase-catalyzed hydrolysis of peptide amides was followed in a pH-Stat as described previously (Thompson and Blout, 1973a), or, in the case of amides having $k_{cat} = <0.1$ sec^{-1}, using an amino acid analyzer. In this latter case the amide, at a concentration in excess of its K_i, was incubated at 37° in 0.05 M bicarbonate–carbonate buffer (pH 9.00) with sufficient enzyme to ensure 5–10% hydrolysis in 1 hr. The concentration of ammonia in samples taken from the reaction mixture at 15, 30, 45, 60, and 90 min was measured by its ninhydrin reaction after chromatography on the short column of a Beckman 120B amino acid analyzer. These measurements allowed a calculation of the initial velocity of hydrolysis, v. The K_i of the amide was measured by its effect on the hydrolysis of Ac-Pro-Ala-Pro-Ala-NH_2 at 37°, pH 9.00, in 10^{-2} M aqueous $CaCl_2$. Inhibition was found to be fully competitive for all the peptides examined. Since K_i and K_m for a peptide amide may be assumed equal for physically plausible models of the peptide binding site, the measured values of v and K_i allow us to calculate V_{max} and k_{cat} from the Michaelis–Menten equation. For several substrates the k_{cat} and K_m measured by this method were identical, within experimental error, to those observed using the pH-Stat.

In all cases, thin-layer chromatography (tlc) of the reaction products showed a single product which was ninhydrin negative for ninhydrin-negative substrates. This result is consistent with exclusive hydrolysis of the amide bond. Tlc of peptides was carried out as described previously (Thompson

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[1] In this, and subsequent papers, the nomenclature introduced by Schechter and Berger (1967) is used to facilitate discussion of the interactions between elastase and bound peptides. Amino acid residues and partial amino acid residues (*e.g.*, acetyl groups) of substrates are numbered P_1, P_2, P_3, etc., in the N-terminal direction, and P_1', P_2', etc., in the C-terminal direction from the scissile bond. The complementary subsites of the enzyme's active center are numbered S_1, S_2 and S_1', S_2', etc., in an analogous fashion. The binding mode of a peptide which occupies, for example, the S_4, S_3, S_2, and S_1 subsites of the enzyme will be denoted by the abbreviation S_{4321}.