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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}.

and Blout, 1973a). 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).

Elastase was purchased from Whatman Biochemicals, England. *tert*-Butyloxycarbonylamino acids, amino acid esters, and amides were purchased from Fox Chemical Co., Los Angeles; Cyclo Chemicals, Los Angeles; or Fluka AG, Switzerland. Ac-Ala-NH_2 (I) was also purchased from Fox Chemical Co.

The preparations of HCl·H-Ala-Ala-Pro-Ala-NH_2 (VII), Ac-Ala-Ala-Pro-Ala-NH_2 (XVII), Ac-D-Ala-Ala-Ala-Pro-Ala-NH_2 (XIX), Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (XV), Ac-Pro-Ala-Pro-D-Ala-NH_2 (XXIV), Ac-Pro-Ala-Ala-Ala-NH_2 (XXIV), and Ac-Ala-Ala-Ala-NH_2 (XXVIII) have been described previously (Thompson and Blout, 1973a).

tert-Butyloxycarbonylprolylalaninamide. *tert*-Butyloxycarbonylproline (5.2 g., 24 mmol) and *N*-methylmorpholine (2.65 ml; 24 mmol) were dissolved in ethyl acetate (30 ml) and cooled to −20° in a Dry Ice–CCl_4 bath. Isobutyl chloroformate (3.1 ml; 24 mmol) was added with stirring and the mixture was stirred for 10 min. A solution of 3.0 g (24 mmol) of alaninamide hydrochloride and 2.65 ml (24 mmol) of *N*-methylmorpholine in 150 ml of *N,N*-dimethylformamide precooled to −20° was added with stirring and the mixture was allowed to warm to room temperature over a period of 3 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in water and treated with excess Rexyn I-300 resin (Fisher). The resin was removed by filtration, the water was evaporated, and the residue was crystallized from acetone–ether or ethyl acetate to give 3.9 g (57%). The product, mp 178–179°, gave a single spot on tlc $R_{F_{II}}$ 0.8.

Prolylalaninamide Hydrochloride. *tert*-Butyloxycarbonylprolylalaninamide (3.2 g; 11.2 mmol) was dissolved in chloroform (20 ml) and 100 ml of hydrogen chloride saturated ethyl acetate was added. A precipitate was observed after 2 min. After 1 hr, the solvent was removed *in vacuo* and the residue was left *in vacuo* with sodium hydroxide pellets for 2 days. The residue was crystallized from methanol–acetone to give 950 mg (39%) of product: mp 214–216°, single spot by tlc $R_{F_{II}}$ 0.1, $R_{F_{III}}$ 0.6.

Acetylprolylalaninamide (II). Prolylalaninamide hydrochloride (100 mg; 0.45 mmol) was dissolved in a mixture of 0.5 ml of acetic anhydride and 10 ml of pyridine. After 1 hr, the solvent was removed *in vacuo*. The residue was dissolved in water, treated with Rexyn I-300 resin, filtered, and evaporated. The residue was crystallized from 5% aqueous acetone to give 63 mg (62%) of product: mp 170–172°; single spot by tlc $R_{F_{II}}$ 0.4. *Anal.* Calcd for $C_{10}H_{17}N_3O_3$: C, 52.85; H, 7.54; N, 18.49. Found: C, 52.7; H, 7.5; N, 18.5.

Propionylprolylalaninamide (III) was prepared similarly to Ac-Pro-Ala-NH_2 in 51% yield using propionic anhydride: mp 203–205°; single spot by tlc $R_{F_{II}}$ 0.5. *Anal.* Calcd for $C_{11}H_{19}N_3O_3$: C, 54.75; H, 7.94; N, 17.42. Found: C, 54.6; H, 7.7; N, 17.1.

Isobutyrylprolylalaninamide (IV). Prolylalaninamide hydrochloride (100 mg; 0.45 mmol) was dissolved in *N,N*-dimethylformamide (10 ml) and 100 μl (0.91 mmol) of *N*-methylmorpholine was added, followed by 45 μl (47 μg, 0.44 mmol) of isobutyryl chloride in 5-μl lots. After 1 hr, the solvent was removed *in vacuo*. The residue was dissolved in water, treated with I-300 resin, filtered, and evaporated. The residue was crystallized from ethyl acetate–ether to give 75 mg (65%) of a hygroscopic product: mp 145–151°, single spot by tlc R_{F_I} 0.6, $R_{F_{VIII}}$ 0.2. *Anal.* Calcd for $C_{12}H_{21}N_3O_3 \cdot 0.5H_2O$: C, 54.52; H, 8.39; N, 15.90. Found: C, 54.7; H, 8.1; N, 16.0.

tert-Butyloxycarbonylalanylproline. A suspension of 4.5 g (39 mmol) of proline and 4.3 ml (3.9 g, 39 mmol) of *N*-methylmorpholine in 50 ml of *N,N*-dimethylformamide was stirred 30 min at room temperature and 11.4 g of *tert*-butyloxycarbonylalanine succinimido ester (39 mmol) was added. Stirring was continued for a further 12 hr, and the small amount of solid was removed by filtration. The filtrate was evaporated to give an oily residue which was dissolved in saturated aqueous sodium bicarbonate. The aqueous solution was adjusted to pH 8.0 and extracted twice with ethyl acetate. Concentrated hydrochloric acid was then added to the aqueous solution until crystals appeared at approximately pH 4. After standing 6 hr at 4°, the product was removed by filtration: 7.4 g (66%) of Boc-Ala-Pro-OH, mp 155–157°, lit. (Lorenzi *et al.*, 1971) mp 156–158°.

Alanylprolylalaninamide Hydrochloride (V). *tert*-Butyloxycarbonylalanylproline was coupled to alaninamide hydrochloride by the procedure used to prepare Boc-Pro-Ala-NH_2. Boc-Ala-Pro-Ala-NH_2 was obtained as an oil, homogeneous by tlc; $R_{F_{II}}$ 0.65.

The oil was dissolved in ethyl acetate (20 ml), and 50 ml of a solution of hydrogen chloride in ethyl acetate was added. After 1 hr, the solvent was evaporated, and the residue was crystallized from 5% aqueous acetone: 2.0 g (77%), mp 155–158°. *Anal.* Calcd for $C_{11}H_{21}ClN_4O_3$: C, 45.11; H, 7.24; N, 19.14. Found: C, 45.2; H, 7.2; N, 19.0.

Formylalanylprolylalaninamide (VI). Formic acid, 97% (0.05 ml; 1.3 mmol), was dissolved in 5 ml of acetonitrile and cooled to −20° in Dry Ice–CCl_4. *N*-Methylmorpholine (0.14 ml; 1.3 mmol) was added, followed by 0.17 ml (1.3 mmol) of isobutyl chloroformate, and the mixture was stirred 5 min at −20°. *N,N*-Dimethylformamide (2 ml) was added, followed by a solution of 38 mg of alanylprolylalaninamide hydrochloride (0.13 mmol) in 1 ml of *N,N*-dimethylformamide and 0.14 ml of *N*-methylmorpholine. The solution was allowed to warm to room temperature over a period of 1 hr and stirred for a further 6 hr. The solvent was evaporated, and the residue was dissolved in water and treated with sufficient Rexyn I-300 resin to maintain a blue color in some of the beads after 15 min. The solution was filtered, the water was evaporated, and the residue was crystallized from ethyl acetate–hexane to yield 27 mg (73%) of product: single spot $R_{F_{II}}$ 0.55, $[\alpha]_D$ −217° (*c* 0.3, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{12}H_{20}N_4O_4$: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.5; H, 7.0; N, 19.4.

Acetylalanylprolylalaninamide (VII) was prepared from HCl·H-Ala-Pro-Ala-NH_2 by the procedure used to prepare Ac-Pro-Ala-NH_2. Trituration under ether gave 83 mg of product (82%): homogeneous by tlc, single spot $R_{F_{II}}$ 0.53, mp 207–208°, $[\alpha]_D^{25}$ −173° (*c* 0.5, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{13}H_{22}N_4O_4$: C, 52.3; H, 7.4; N, 18.8. Found: C, 52.2; H, 7.3; N, 18.7.

Formylalanylalanylprolylalaninamide (XVI) was prepared from alanylalanylprolylalaninamide hydrochloride by the formylation procedure used to prepare formyl-Ala-Pro-Ala-NH_2. The product was crystallized in 67% yield from acetone–ethyl acetate: single spot on tlc $R_{F_{II}}$ 0.4, $R_{F_{III}}$ 0.4, $R_{F_{VIII}}$ 0.2; $[\alpha]_D^{25}$ −235° (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{15}H_{25}N_5O_5$: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.6; H, 7.1; N, 19.6.

Alanylalanylalanylprolylalaninamide Hydrochloride. Carbobenzoxyalanylalanylalanylprolylalaninamide was prepared

from carbobenzoxyalanine and alanylalanylprolylalaninamide hydrochloride by the mixed-anhydride coupling procedure used to prepare Boc-Pro-Ala-NH_2: yield from acetone, 250 mg (68%); single spot by tlc $R_{F_{II}}$ 0.6, $R_{F_{III}}$ 0.6, $R_{F_{VIII}}$ 0.4. *Anal.* Calcd for $C_{25}H_{36}N_6O_7$: C, 56.38; H, 6.81; N, 15.78. Found: C, 56.6; H, 6.6; N, 15.9.

Carbobenzoxyalanylalanylalanylprolylalaninamide (220 mg; 0.414 mmol) was dissolved in methanol (5 ml), 1 M aqueous hydrochloric acid (1 ml), and *tert*-butyl alcohol (20 ml). The mixture was hydrogenated for 12 hr at 20 psi over a 10% palladium–charcoal catalyst. The catalyst was removed by filtration through Celite and the solvents were evaporated: yield of alanylalanylalanylprolylalaninamide hydrochloride from trituration under 2% aqueous acetone, 136 mg (71%); single spot by tlc $R_{F_{II}}$ 0.1, $R_{F_{III}}$ 0.2. *Anal.* Calcd for $C_{17}H_{31}ClN_6O_5 \cdot 1H_2O$: C, 45.07; H, 7.34. Found: C, 44.9; H, 7.4.

Acetylalanylalanylalanylprolylalaninamide (*XVIII*) was prepared from HCl·H-Ala-Ala-Ala-Pro-Ala-NH_2 by the procedure used to prepare acetylprolylalaninamide: yield from 5% aqueous acetone, 54 mg (82%); $[\alpha]_D^{25}$ −226° (*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, 51.3; H, 7.3; N, 18.7.

Alanylprolylalanylprolylalaninamide Hydrochloride (*XIII*). Carbobenzoxyalanylproline (Cyclo Chemical Co.) was coupled to alanylprolylalaninamide hydrochloride by the procedure used to prepare Boc-Pro-Ala-NH_2. The product, homogeneous by tlc ($R_{F_{II}}$ 0.6, $R_{F_{III}}$ 0.6), failed to crystallize. It was hydrogenated under the conditions used to prepare HCl·H-Ala-Ala-Ala-Pro-Ala-NH_2 to give a product which crystallized from 5% aqueous isopropyl alcohol–acetone: 75 mg (yield based on Z-Ala-Pro-OH, 20%); single spot $R_{F_{II}}$ 0.2, $R_{F_{III}}$ 0.3; $[\alpha]_D^{25}$ −215° (*c* 0.5, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{19}H_{33}ClN_6O_5 \cdot H_2O$: C, 47.65; H, 7.37; N, 17.55. Found: C, 47.6; H, 7.4; N, 17.0.

Glycylprolylalanylprolylalaninamide Hydrochloride (*XII*). *tert*-Butyloxycarbonylglycylproline was coupled to alanylprolylalaninamide hydrochloride by the procedure used to prepare Boc-Pro-Ala-NH_2: yield 233 mg (67%) from aqueous acetone–ether, single spot by tlc $R_{F_{II}}$ 0.5, mp 124–126°. *Anal.* Calcd for $C_{23}H_{38}N_6O_7 \cdot H_2O$: C, 52.25; H, 7.63; N, 15.90. Found: C, 52.7; H, 7.9; N, 16.2.

The *tert*-butyloxycarbonyl group was removed in 91% yield under the conditions used to prepare HCl·H-Pro-Ala-NH_2. The product was hygroscopic but homogeneous by tlc $R_{F_{II}}$ 0.1, $R_{F_{III}}$ 0.3; $[\alpha]_D^{25}$ −205° (*c* 1.15, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{18}H_{31}ClN_6O_5 \cdot 1.5H_2O$: C, 45.61; H, 7.23; N, 17.73. Found: C, 45.6; H, 7.0; N, 17.4.

Alanylproline Benzyl Ester Hydrochloride. *tert*-Butyloxycarbonylalanine (11.8 g; 62 mmol) was dissolved in 50 ml of tetrahydrofuran and cooled to −20° in a Dry Ice–CCl_4 bath. *N*-Methylmorpholine (6.9 ml; 62 mmol) was added, followed by isobutyl chloroformate (8.1 ml; 62 mmol). After stirring 2 min at −20°, a solution of 15 g (62 mmol) of proline benzyl ester hydrochloride in 50 ml of chloroform and 6.9 ml (62 mmol) of *N*-methylmorpholine were added. The reaction was allowed to warm to room temperature 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 the solvent was evaporated to leave 21.3 g (92%) of *tert*-butyloxycarbonylalanylproline benzyl ester: single spot by tlc R_{F_I} 0.8.

tert-Butyloxycarbonylalanylproline benzyl ester (7.5 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* over NaOH pellets overnight: yield of alanylproline benzyl ester hydrochloride, 5.2 g (83%).

Acetylalanylproline. Alanylproline benzyl ester hydrochloride (2.1 g; 6.7 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 acetylalanylproline 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 was crystallized from 5% aqueous acetone–ether to give 1.0 g (65%) of white crystals: mp 171–175°, single spot by tlc $R_{F_{II}}$ 0.6. *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, 12.1.

Acetylalanylprolylalanylprolylalaninamide (*XIV*). Acetylalanylproline and alanylprolylalaninamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. The product was crystallized from 5% aqueous acetone–ethyl acetate in 44% yield: single spot by tlc $R_{F_{II}}$ 0.4, $R_{F_{III}}$ 0.4, $R_{F_{VIII}}$ 0.3; $[\alpha]_D^{25}$ −240° (*c* 0.9, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{21}H_{34}N_6O_6$: C, 54.06; H, 7.35; N, 18.02. Found: C, 53.7; H, 7.3; N, 17.9.

Acetylglycylalanylprolylalaninamide (*XXXIX*). *tert*-Butyloxycarbonylglycine and alanylprolylalaninamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. Boc-Gly-Ala-Pro-Ala-NH_2 was crystallized from tetrahydrofuran–ether to give two fractions, the first 34 mg (15%), mp 99–104°, and the second 126 mg (56%), mp 117–118°. The second fraction had $[\alpha]_D^{25}$ −148° (*c* 0.2, 10^{-2} M aqueous $CaCl_2$), single spot $R_{F_{II}}$ 0.65. *Anal.* Calcd for $C_{18}H_{31}N_5O_6$: C, 52.28; H, 7.56. Found: C, 52.1; H, 7.5.

Glycylalanylprolylalaninamide hydrochloride was prepared from Boc-Gly-Ala-Pro-Ala-NH_2 by the procedure used to prepare HCl·H-Pro-Ala-NH_2. Crystallization from 5% aqueous acetone gave a 92% yield of a hygroscopic solid which was homogeneous by tlc $R_{F_{II}}$ 0.1, $[\alpha]_D^{25}$ −122° (*c* 0.5, MeOH). *Anal.* Calcd for $C_{13}H_{24}ClN_5O_4 \cdot H_2O$: C, 42.44; H, 7.12; N, 19.04. Found: C, 42.7; H, 6.8; N, 18.6.

HCl·H-Gly-Ala-Pro-Ala-NH_2 was acetylated by the procedure used to prepare Ac-Pro-Ala-NH_2: yield from acetone, 17 mg (36%); single spot by tlc $R_{F_{II}}$ 0.2; mp 178–182°; $[\alpha]_D^{25}$ −179° (*c* 0.3, 10^{-2} aqueous $CaCl_2$). *Anal.* Calcd for $C_{15}H_{25}N_5O_5$: C, 50.69; H, 7.09. Found: C, 50.5; H, 7.1.

Acetyl-α-aminobutyrylalanylprolylalaninamide (*XXX*) was prepared from *tert*-butyloxycarbonylaminobutyric acid and alanylprolylalaninamide·HCl by coupling, deprotection, and acetylation reactions analogous to those used in the preparation of Ac-Gly-Ala-Pro-Ala-NH_2; yield from acetone–ethyl acetate, 22%; single spot on tlc $R_{F_{II}}$ 0.4; $[\alpha]_D^{25}$ −203° (*c* 0.35, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{17}H_{29}N_5O_5$: C, 53.25; H, 7.62; N, 18.27. Found: C, 52.8; H, 7.6; N, 18.1.

Acetylnorvalylalanylprolylalaninamide (*XXXI*) was prepared from carbobenzoxynorvaline and alanylprolylalaninamide hydrochloride using coupling, deprotection, and acetylation reactions analogous to those used in the preparation of Ac-Ala-Ala-Ala-Pro-Ala-NH_2: yield from acetone–ethyl acetate, 16%; single spot on tlc $R_{F_{II}}$ 0.5; $[\alpha]_D^{25}$ −193° (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{18}H_{31}N_5O_5$:

C, 54.39; H, 7.86; N, 17.62. Found: C, 54.1; H, 7.9; N, 17.5.

tert-Butyloxycarbonylprolylalanylproline Benzyl Ester. *tert*-Butyloxycarbonylproline (3.6 g; 16.7 mmol) was coupled to alanylproline benzyl ester hydrochloride (5.2 g; 16.6 mmol) by the procedure used to prepare Boc-Ala-Pro-OBzl. Boc-Pro-Ala-Pro-OBzl (6.8 g; 87%) was obtained as an oil.

tert-Butyloxycarbonylprolylalanylproline. *tert*-Butyloxycarbonylprolylalanylproline benzyl ester (4.6 g; 9.8 mmol) was dissolved in *tert*-butyl alcohol and hydrogenated over a 10% palladium–charcoal catalyst at 20 psi for 12 hr. The reaction mixture was filtered through Celite and evaporated to give an oil. The oil was dissolved in 5% aqueous sodium bicarbonate (100 ml) and extracted with an equal volume of ethyl acetate. The aqueous phase was brought to pH 3 by addition of 1 M hydrochloric acid and extracted twice with equal volumes of ethyl acetate. The organic phases from the acid extractions were dried and evaporated to give a glassy solid: 1.2 g (32%); single spot on tlc $R_{F_{II}}$ 0.7.

tert-Butyloxycarbonylprolylalanylprolylalaninamide. *tert*-Butyloxycarbonylprolylalanylproline (400 mg; 1.04 mmol) and alaninamide hydrochloride (155 mg; 1.04 mmol) were coupled by the method used to prepare Boc-Pro-Ala-NH_2. Boc-Pro-Ala-Pro-Ala-NH_2 (332 mg; 71%) was obtained as an amorphous solid: single spot on tlc $R_{F_{II}}$ 0.7, $R_{F_{VIII}}$ 0.5.

Prolylalanylprolylalaninamide Hydrochloride (IX). *tert*-Butyloxycarbonylprolylalanylprolylalaninamide (300 mg; 0.66 mmol) was dissolved in ethyl acetate (5 ml) and hydrogen chloride saturated ethyl acetate (10 ml) was added. After 1 hr, the solvent was evaporated *in vacuo* and the residue was left *in vacuo* over sodium hydroxide pellets for 2 days. On trituration under ethyl acetate, the residue gave 239 mg (93%) of a hygroscopic solid, single spot by tlc $R_{F_{III}}$ 0.5. *Anal.* Calcd for $C_{16}H_{28}ClN_5O_4 \cdot 2H_2O$: C, 45.12; H, 7.57; N, 16.44. Found: C, 44.7; H, 7.8; N, 15.9. Amino acid analysis gives Pro:Ala:NH_3:2.1:2.0:0.95.

Formylprolylalanylprolylalaninamide (X) was prepared from prolylalanylprolylalaninamide hydrochloride by the procedure used to prepare formyl-Ala-Pro-Ala-NH_2. The product was obtained in 40% yield as a hygroscopic solid: single spot on tlc $R_{F_{II}}$ 0.5, $R_{F_{III}}$ 0.6, $R_{F_{VIII}}$ 0.3; $[\alpha]_D^{25}$ −246° (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{17}H_{27}N_5O_5 \cdot 0.5H_2O$: C, 52.29; H, 7.23; N, 17.94. Found: C, 51.9; H, 7.2; N, 17.5.

Acetylprolylalanylproline Benzyl Ester. Acetylproline (2.6 g; 16.6 mmol) was dissolved in tetrahydrofuran, cooled to −20° in a Dry Ice–CCl_4 bath, and 1.8 ml of *N*-methylmorpholine (16.5 mmol) was added, followed by 2.1 ml (16.5 mmol) of isobutyl chloroformate. After 2 min, 5.2 g (16.5 mmol) of alanylproline benzyl ester hydrochloride in chloroform solution was added, followed by 1.8 ml (16.5 mmol) of *N*-methylmorpholine. The reaction mixture was allowed to warm to room temperature over a period of 3 hr. The solvent was evaporated, and the residue was dissolved in chloroform. The organic phase was washed twice with 0.2 M hydrochloric acid and twice with 5% aqueous sodium bicarbonate. After drying, the solvent was evaporated to give 6.2 g (90%) of a hygroscopic white solid: single spot by tlc $R_{F_{VIII}}$ 0.6, mp 137–140°.

Acetylprolylalanylproline. Acetylprolylalanylproline benzyl ester (4.2 g; 10 mmol) was dissolved in *tert*-butyl alcohol and hydrogenated at 20 psi for 24 hr with a 10% palladium–charcoal catalyst. After filtration through Celite, the solvent was evaporated. The foamy residue crystallized from tetrahydrofuran to give 2.8 g (86%) of product: single spot by tlc $R_{F_{II}}$ 0.4, mp 177–179°. *Anal.* Calcd for $C_{15}H_{23}N_3O_5$: C, 55.4; H, 7.1; N, 12.9. Found: C, 55.7; H, 7.2; N, 12.7.

Acetylprolylalanylprolylalaninamide (XI). Acetylprolylalanylproline and alaninamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. The product was crystallized from 5% aqueous acetone in 85% yield: single spot by tlc $R_{F_{II}}$ 0.5, mp 117–119°, $[\alpha]_D^{25}$ −203° (*c* 0.7, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{18}H_{29}N_5O_5$: C, 54.67; H, 7.39; N, 17.71. Found: C, 54.7; H, 7.5; N, 17.8.

Acetylprolylalanylprolylvalinamide (XX). Acetylprolylalanylproline and valinamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. The product crystallized from ethyl acetate in 93% yield: single spot on tlc $R_{F_{II}}$ 0.6, mp 208–210°. *Anal.* Calcd for $C_{20}H_{33}N_5O_5$: C, 56.72; H, 7.85; N, 16.54. Found: C, 56.6; H, 8.0; N, 16.4.

Acetylprolylalanylprolylleucinamide (XXI). Acetylprolylalanylproline and leucinamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. The product crystallized from ethyl acetate–ether in 70% yield: single spot on tlc $R_{F_{II}}$ 0.6, mp 114–116°. *Anal.* Calcd for $C_{21}H_{35}N_5O_5$: C, 57.64; H, 8.06; N, 16.01. Found: C, 57.7; H, 8.3; N, 15.9.

Acetylprolylalanylprolylglycinamide (XXII). Acetylprolylalanylproline and glycinamide hydrochloride were coupled by the procedure used to prepare Boc-Pro-Ala-NH_2. The product crystallized from 5% aqueous acetone in 82% yield: single spot by tlc $R_{F_{II}}$ 0.3, mp 115–117°. *Anal.* Calcd for $C_{17}H_{27}N_5O_5 \cdot H_2O$: C, 51.11; H, 7.32; N, 17.53. Found: C, 50.9; H, 7.3; N, 17.2.

Acetylprolylalanylprolylglycolamide (XXIII). Acetylprolylalanylproline (500 mg; 1.5 mmol) was dissolved in pyridine (50 ml) and cooled to 0°. Benzenesulfonyl chloride (0.38 ml; 3.0 mmol) was added over a period of 5 min with stirring. After a further 10 min, 225 mg (3.0 mmol) of glycolamide in pyridine (10 ml) was added and stirring was continued for a further 30 min at 0° and 48 hr at room temperature. The solvent was removed *in vacuo* and the residue was dissolved in water and treated with Rexyn I-300 resin for 15 min at 0°. After filtration, the water was partially evaporated *in vacuo* and the residue was crystallized from ethyl acetate–ether to give 200 mg (32%) of a hygroscopic solid homogeneous by tlc, $R_{F_{II}}$ 0.6. *Anal.* Calcd for $C_{17}H_{26}N_4O_6 \cdot 2H_2O$: C, 48.79; H, 7.23. Found: C, 48.9; H, 7.3.

tert-Butyloxycarbonylglycylproline Benzyl Ester. *tert*-Butyloxycarbonylglycine was coupled to proline benzyl ester hydrochloride by the procedure used to prepare Boc-Ala-Pro-OBzl: yield, 2.3 g (61%); mp 77–79°, lit. (Deber *et al.*, 1970) mp 76–77°.

tert-Butyloxycarbonylglycylproline was prepared from *tert*-butyloxycarbonylglycylproline benzyl ester by a procedure similar to that used to prepare Ac-Ala-Pro-OH from its benzyl ester: yield from ethyl acetate, 1.26 g (75%); mp 142–143°, lit. (Deber *et al.*, 1970) mp 126–135°.

tert-Butyloxycarbonylglycylprolylalaninamide was prepared by coupling *tert*-butyloxycarbonylglycylproline and alaninamide hydrochloride using the coupling procedure employed to prepare Ac-Pro-Ala-NH_2: yield from ethyl acetate–ether, 921 mg (73%); mp 127–129°; single spot by tlc $R_{F_{II}}$ 0.6. *Anal.* Calcd for $C_{15}H_{26}N_4O_5$: C, 52.62; H, 7.65; N, 16.36. Found: C, 52.6; H, 7.7; N, 16.4.

Acetylglycylprolylalaninamide (XXVII) was prepared from *tert*-butyloxycarbonylglycylprolylalaninamide by removal of the *tert*-butyloxycarbonyl group and subsequent acetylation of the product according to the procedure used to prepare Ac-Pro-Ala-NH_2: yield, 92% mp 236–238°; $[\alpha]_D^{25}$ −142° (*c* 2.5, 10^{-2} M aqueous $CaCl_2$); single spot on tlc $R_{F_{II}}$ 0.3. *Anal.* Calcd

TABLE I: Kinetic Parameters for the Elastase-Catalyzed Hydrolysis of Di- and Tripeptide Amides.

P_3 P_2 P_1 ↓[a]	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_i (mM)	[P][b] (mM)
Ac-Ala-NH_2 (I)	<0.005	<0.0008	160	50–100
Ac-Pro-Ala-NH_2 (II)	0.07	0.007	100	25–100
Propionyl-Pro-Ala-NH_2 (III)	0.12	0.006	52	25–60
Isobutyryl-Pro-Ala-NH_2 (IV)	0.06	0.009	160	10–70
H-Ala-Pro-Ala-NH_2[c] (V)	0.11	0.017	K_m = 160	30–300

[a] The vertical arrow denotes the bond subject to elastase-catalyzed hydrolysis. [b] Range of peptide concentrations used to determine K_i or K_m. [c] Kinetic constants are from rates determined by the amino acid analyzer technique (see Methods) between the peptide concentrations shown.

for $C_{12}H_{20}N_4O_4$: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.6; H, 7.1; N, 19.7.

Acetylprolylglycylprolylalaninamide (*XXV*) was prepared by removal of the *tert*-butyloxycarbonyl group from *tert*-butyloxycarbonylglycylprolylalaninamide according to the procedure used to prepare HCl·H-Pro-Ala-NH_2 and subsequent coupling to acetylproline by the coupling procedure used to prepare Boc-Pro-Ala-NH_2. The product was obtained in 85% yield: mp 190–191°, $[\alpha]_D^{25}$ −195° (*c* 0.39, 10^{-2} M aqueous $CaCl_2$), single spot on tlc $R_{F_{II}}$ 0.3. *Anal.* Calcd for $C_{17}H_{27}N_5O_5$: C, 53.53; H, 7.14; 18.36. Found: C, 53.6; H, 7.1; N, 18.5.

Results

Dependence of the Kinetic Parameters for Hydrolysis on Substrate Length. P_3 AND P_2. Listed in Table I are the smallest substrates for which we have been able to measure the rate of elastase-catalyzed amide hydrolysis. Although the separate k_{cat} and K_m values of these substrates have been listed, the exact significance of these parameters is unclear since certain of these short substrates may have strong nonproductive binding modes. For example, peptides II, III, and IV may bind in the S_{543} subsites of the active center. The quotient, k_{cat}/K_m, is the only kinetic parameter unaffected by nonproductive binding (Bender and Kezdy, 1965) and is, therefore, the only basis for a meaningful comparison of the ease of hydrolysis of these substrates.

The increase in k_{cat}/K_m on going from P_2 Ac to P_{32} Ac-Pro (*cf.* I and II) clearly shows that the latter residues increase the enzyme's ability to cleave the scissile amide bond. We cannot calculate the exact magnitude of the increase, but a similar replacement of P_2 Ac by P_{32} Ac-Ala was found to stimulate the esterase activity of the enzyme more than 40-fold (Thompson and Blout, 1970). Groups directly attached to the P_3 α-carbon atom, by comparison, have little effect on the ease of amide hydrolysis (*cf.* II with III, IV, and V). The change from P_3 Ac to P_3 Ala has previously been shown to have very little effect on the susceptibility to hydrolysis of P_1–P_1' ester bonds (Thompson and Blout, 1970).

P_4. The substrates listed in Table II are bound sufficiently strongly in the productive mode (S_{4321}) to make nonproductive modes kinetically insignificant. The kinetic parameters, k_{cat} and K_m, may therefore be equated with k_2, the rate constant for the acylation reaction, and K_s, the dissociation constant of the productive enzyme–substrate complex, respectively (for a discussion and further references, see Thompson and Blout, 1973a).

TABLE II: Kinetic Parameters for the Elastase-Catalyzed Hydrolysis of Tri- and Tetrapeptide Amides.

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)
Formyl-Ala-Pro-Ala-NH_2 (VI)	5	0.10	20	1–12
Ac-Ala-Pro-Ala-NH_2 (VII)	21	0.09	4.2	0.5–5
H-Ala-Ala-Pro-Ala-NH_2 (VIII)	74	1.4	19	3–30

[a] Range of substrate concentrations.

The K_m of peptide VI is much lower than that of peptide V, showing that the formyl group of the former binds very well to the S_4 subsite of the enzyme. If V were bound entirely in the S_{321} mode, the binding energy of the P_4 formyl group to S_4 could be calculated from the K_m's to be about −1.3 kcal/mol. If peptide V is bound in other modes to any significant extent, the free energy of binding of the P_4 formyl group to S_4 will be in excess of this value. Whether the formyl group also leads to changes in the rate of amide hydrolysis is uncertain. The fraction of the reference peptide, V, binding S_{321}, and hence its true rate of reaction, cannot be determined at present; a direct comparison with VI is therefore impossible. However, the P_4 carbonyl group has been shown to facilitate the deacylation step of hydrolysis (*cf.* the k_{cat}'s of H-Ala-Ala-Ala-OMe and Ac-Ala-Ala-Ala-OMe, Thompson and Blout, 1970). It is therefore logical to assume that the same group will similarly enhance the rate of the acylation reaction.

The fivefold decrease in K_m on going from formyl- (VI) to Ac-Ala-Pro-Ala-NH_2 (VII) is indicative of a strong interaction between the additional methyl group and the S_4 subsite of the enzyme, which contributes about −1.0 kcal/mol to the enzyme–substrate binding energy. This interaction leads to no significant change in the rate of hydrolysis.

The changes in the kinetic parameters for hydrolysis on going from substrates with P_4 acetyl groups to those with P_4 amino acid residues are complex. These changes are discussed fully in the following paper (Thompson and Blout, 1973b), where it is shown that the α-amino group of residue P_4 requires about 2 kcal/mol to bind it to the enzyme and

TABLE III: Kinetic Parameters for the Elastase-Catalyzed Hydrolysis of Tetra- and Pentapeptide Amides.

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)
H-Pro-Ala-Pro-Ala-NH_2 (IX)	900	3.5	4.0	1–5
For-Pro-Ala-Pro-Ala-NH_2 (X)	1780	8.5	4.8	1–9
Ac-Pro-Ala-Pro-Ala-NH_2 (XI)	2200	8.5	3.9	1–10
H-Gly-Pro-Ala-Pro-Ala-NH_2 (XII)	1160	5.8	5.0	1–12
H-Ala-Pro-Ala-Pro-Ala-NH_2 (XIII)	880	4.6	5.2	0.5–7.5
Ac-Ala-Pro-Ala-Pro-Ala-NH_2 (XIV)	1360	5.3	3.9	1–10
Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (XV)	1050	8.3	7.9	0.5–10
H-Ala-Ala-Pro-Ala-NH_2 (VIII)	74	1.4	19	3–30
For-Ala-Ala-Pro-Ala-NH_2 (XVI)	1230	4.4	3.6	1–10
Ac-Ala-Ala-Pro-Ala-NH_2 (XVII)	2900	6.1	2.1	0.5–9
Ac-Ala-Ala-Ala-Pro-Ala-NH_2 (XVIII)	850	3.0	3.6	1–10
Ac-D-Ala-Ala-Ala-Pro-Ala-NH_2 (XIX)	2200	3.8	1.7	0.5–5

[a] Range of substrate concentrations.

leads to an approximate 10-fold increase in the rate of the acylation reaction.

P_5 AND P_6. The effects of residues P_5 and P_6 of the substrate on the kinetic parameters for hydrolysis depend on the nature of the P_4 residue (Table III). In the P_4 Pro series, residues P_5 and P_6 appear to have very little effect apart from a small increase in k_{cat}, apparently due to the P_5 carbonyl group (*cf.* IX and X). Even the change from P_6P_5 Ac-Ala to P_6P_5 Ac-D-Ala hardly affects the ability of the enzyme to hydrolyze the P_1 amide bond.

In the P_4 Ala series the P_5 residue has a much more pronounced effect. A P_5 formyl group, for example, leads to a threefold increase in rate and a fivefold increase in enzyme–substrate binding (*cf.* VIII and XVI). Small further increases in rates and binding result from the enzyme's interaction with the P_5 acetyl methyl group (*cf.* XVI and XVII). In the P_4 Ala series of substrates, as in the P_4 Pro peptides, the enzyme appears to show little stereoselectivity with regard to the P_5 residue (*cf.* XVIII and XIX).

The changes in k_{cat} and K_m corresponding to structural changes in the substrate distal to the P_5 α-carbon atom are small and could conceivably result from differences in the solution conformation of the peptides. At present, therefore, we have no evidence for the existence of any subsites beyond S_5 in the active center of elastase.

In the P_4 Pro series of peptides, even the S_5–P_5 contact appears to have very little effect on the kinetic parameters for substrate hydrolysis (Table III). The major difference between the P_4 Pro and P_4 Ala peptides in this respect may be explicable in terms of differing dihedral angles of the P_4 residue in their respective enzyme–substrate complexes. The ϕ angle in the former complexes will be determined by the geometry of the pyrollidine ring. A P_4 Pro residue may therefore restrict the range of enzyme–substrate contacts beyond P_4, and optimal contact between S_5 and P_5 may not be possible. The wider range of ϕ values accessible to the P_4 alanine peptides will allow a greater chance of forming the optimal enzyme–substrate contact in the S_5 subsite.

Dependence of the Kinetic Parameters for Hydrolysis on Substrate Amino Acid Sequence. P_1. The results of Geneste and Bender (1969) on elastase-catalyzed hydrolysis of *N*-carbobenzoxyamino acid *p*-nitrophenyl esters, those of Kaplan *et al.* (1970) on *N*-benzoylamino acid methyl esters, and those of Narayanan and Anwar (1969) on the oxidized insulin A and B chains, have shown that elastase cleaves most readily bonds C terminal to alanine residues. By studying the kinetics of amide cleavage from a series of peptides of general formula Ac-Pro-Ala-Pro-X-NH_2 (Table IV), we have been able to ascribe the overall specificity observed in these prior investigations to the binding or acylation steps of the hydrolysis reaction. The evidence presented in a previous paper (Thompson and Blout, 1973a) indicates that the Pro-Ala-Pro sequence in these peptides should bind exclusively to the S_{432} subsites of the active center, thereby confining X to the S_1 subsite and allowing us to equate K_m with K_s and k_{cat} with k_2.

The rate of the acylation reaction in the P_1 valine peptide (XX) is very similar to that of the P_1 alanine analog (XI), indicating that the P_1 side chain of the former peptide can bind without severe distortion of the spatial relationship between the scissile bond and the catalytic residues of the enzyme. The K_m of the valine peptide, however, is about 10-fold greater than the alanine peptide. Thus, the discrimination against P_1 valine residues reported by the other authors appears to come almost entirely in the binding step of reaction and probably results from unfavorable contacts between the enzyme and one, or both, of the P_1 β-methyl groups. The P_1 glycine peptide, by way of contrast, reacts at almost one-hundredth the rate of the alaninamide, showing that discrimination against P_1 glycine appears most strongly in the acylation step of reaction (*cf.* XXII and XI). The enzyme's interaction with the α-methyl group of P_1 alanine may therefore be more important for the correct orientation of the scissile amide bond to the catalytic residues than as a source of enzyme–substrate binding energy. With the P_1 leucine peptide (XXI), both the k_{cat} and K_m for hydrolysis are less favorable than in the alanine analog; discrimination against this P_1 amino acid appears about equally in the binding and acylation steps of reaction.

As shown by the respective k_{cat}/K_m values of XXII and XXIII, the sensitivity of the P_1–P_1' bond to hydrolysis is lower when P_2 and P_1 are joined by an ester, rather than an amide bond. The K_m of the substrate with a P_2–P_1 ester bond is lower than that of its amide analog. The lowered sensitivity

TABLE IV: Effect of the P_1 Residue on the Kinetic Parameters for Elastase-Catalyzed Amide Hydrolysis.

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)	[P][a] (mM)
Ac-Pro-Ala-Pro-Ala-NH_2 (XI)	2200	8.5	3.9	1–10
Ac-Pro-Ala-Pro-Val-NH_2 (XX)	208	6.0	35	0.1–10
Ac-Pro-Ala-Pro-Leu-NH_2 (XXI)	270	3.0	11	0.8–17
Ac-Pro-Ala-Pro-Gly-NH_2 (XXII)	5	0.1	22	1–23
Ac-Pro-Ala-Pro-O-CH_2-CO-NH_2 (XXIII)	0.1	0.001	K_i = 9.0	2–40
Ac-Pro-Ala-Pro-D-Ala-NH_2 (XXIV)			K_i = 48	10–25

[a] Range of peptide concentrations used to determine K_m and K_i.

TABLE V: A Comparison of the Effect of the P_3 and P_2 Residues on the Kinetic Parameters for Tetra- and Tripeptide Amide Hydrolysis.

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)	[P][a] (mM)
Ac-Pro-Ala-Pro-Ala-NH_2 (XI)	2200	8.5	3.9	1–10
Ac-Pro-Gly-Pro-Ala-NH_2 (XXV)	64	2.8	43	5–110
Ac-Pro-Ala-Ala-Ala-NH_2 (XXVI)	1200	4.7	3.9	0.5–8
Ac-Ala-Pro-Ala-NH_2 (VII)	21	0.09	4.2	0.5–7.5
Ac-Gly-Pro-Ala-NH_2 (XXVII)	0.5	0.02	K_i = 33	6–40
Ac-Ala-Ala-Ala-NH_2 (XXVIII)	13	0.03	K_i = 2.5	0.5–5

[a] Range of peptide concentrations used to determine K_m or K_i.

to hydrolysis of the depsipeptide (XXIII) is therefore entirely due to it having an acylation rate constant about 100-fold lower than that of the peptide XXII.

The resistance of peptide XXIV to hydrolysis shows that peptide amides with P_1 D-alanine residues are not substrates of elastase. The rate constant for the acylation reaction with this peptide is too small to be measurable by the pH-Stat method. Significant discrimination against P_1 D-amino acid residues also appears in the binding step of reaction (*cf.* the K_i of XXIV and the K_m of XI).

P_3 AND P_2. We have not attempted to determine the specificity of the S_2 and S_3 subsites completely. Instead, we have concentrated on examining any differences that might exist between the S_2–P_2 and S_3–P_3 contacts in poor (tripeptide) and good (tetrapeptide) substrates. To this end, the kinetic parameters for hydrolysis of the P_2 Ala and P_3 Gly analogs of peptides VII and XI have been measured and are listed in Table V along with those of the reference peptides.

The P_3 Gly peptides (XXVII and XXV) are significantly poorer substrates than their P_3 Ala analogs (VII and XI, respectively). In both the tri- and tetrapeptides, the major change is in the K_m value which corresponds to losses of -1.3 and -1.5 kcal per mol of binding energy, respectively. The most straightforward explanation of this phenomenon is that the S_3 subsite has a good hydrophobic binding region for the P_3 alanine side chain. The P_3 Ala → Gly exchange results in a fivefold and threefold drop in k_{cat} in the tri- and tetrapeptide series, respectively. The changes in both K_m and k_{cat} corresponding to the exchange are therefore similar in the tri- and tetrapeptides and reveal only minor differences in the P_3–S_3 contact between the two classes of peptides.

On the basis of their k_{cat}/K_m values, the P_2 Ala peptides can be seen to be slightly poorer substrates than their P_2 Pro analogs. Unfortunately, comparison of the individual kinetic parameters of these compounds is complicated by the fact that nonproductive binding may occur with the former peptides. Thus, XXVI and XXVIII, by virtue of their $(Ala)_3$ sequence, can bind in the $S_{(6)5432}$ and S_{5432} subsites, respectively, in addition to their productive S_{54321} and S_{4321} modes (Thompson and Blout, 1973a). The occurrence of two, about equally strong,[2] mutually exclusive binding modes in the P_2 Ala peptides leads to the k_2 and K_s values of both being about a factor of 2 greater than the observed k_{cat} and K_m.

When the correction factor is taken into account, it can be seen that neither k_2 nor K_s change greatly due to the P_2 Pro → Ala exchange. k_2 remains virtually unchanged in the tetrapeptide and falls by about one-third in the tripeptide, while the change in K_s corresponds to losses of about -0.4 and -0.1 kcal per mol of binding energy, respectively. As in the case of the P_3 Ala → Gly exchange, the P_2 Pro → Ala exchange produces very similar responses in the kinetic parameters for hydrolysis of tri- and tetrapeptides. There appear to be only minor differences between the S_2–P_2 enzyme–substrate contact in the two classes of peptides.

P_4. The importance of residue P_4 in determining the rate of substrate hydrolysis (Thompson and Blout, 1970, 1973b) encouraged us to explore whether or not that rate depended

[2] Estimated by comparison of the K_m's of Ac-Ala-Ala-Ala-NH_2 (XXVIII) and Ac-Ala-Pro-Ala-NH_2 (VII) and the K_i of Ac-Pro-Ala-Ala-NH_2 (8.5 mM, unpublished data), which should bind solely in the S_{5432} mode.

TABLE VI: Effect of the P_4 Residue on the Kinetic Parameters for Elastase-Catalyzed Amide Hydrolysis.

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- Gly-Ala-Pro-Ala-NH_2 (XXIX)	118	1.8	15	1–7
Ac- Ala-Ala-Pro-Ala-NH_2 (XVII)	2900	6.1	2.1	0.5–9
Ac-Abu-Ala-Pro-Ala-NH_2 (XXX)	1090	4.2	2.3	0.5–9
Ac-Nva-Ala-Pro-Ala-NH_2 (XXXI)	715	3.2	4.5	0.7–9
Ac- Pro-Ala-Pro-Ala-NH_2 (XI)	2200	8.5	3.9	1–10

[a] Range of substrate concentrations.

on the size of the P_4 side chain. In Table VI are presented the kinetic parameters for hydrolysis of a series of peptides, Ac-X-Ala-Pro-Ala-NH_2, where X is one of a homologous series of amino acid residues and a cyclic analog of one of them.

The data indicate that the first methyl group of the P_4 side chain results in an increase in the enzyme–substrate affinity and a somewhat smaller increase in the rate of substrate hydrolysis (*cf.* XXIX and XVII). This result is typical and is found in two other series of peptides (Thompson and Blout, 1973b). Further increases in the length of the P_4 side chain are slightly deleterious to hydrolysis, both to the binding and acylation steps of reaction. Cyclization to give a P_4 Pro residue, however, facilitates hydrolysis and may indicate the presence of another methylene binding site adjacent to the P_4 nitrogen atom. Taken together, the results in Table VI indicate that, apart from the α-methyl group, the P_4 side chain is not of tremendous importance in determining the ease of substrate hydrolysis.

Discussion

Bender and Marshall (1968) have shown that elastase catalyzed hydrolysis of esters proceeds by an acyl-enzyme mechanism. We shall assume that basically the same mechanism is involved in elastase-catalyzed amide and peptide hydrolysis, only, by analogy with α-chymotrypsin, with the acylation reaction (k_2) rate limiting.

$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{Pr_1}{\overset{k_2}{\rightarrow}} ES' \xrightarrow{k_3} E + Pr_2 \qquad (1)$$

Pr_1 = ROH $\quad k_2 > k_3$

Pr_1 = RNH_2 $\quad k_3 > k_2$

The marked facilitation of elastase-catalyzed hydrolysis in long peptide substrates documented above and earlier (Atlas *et al.*, 1970; Thompson and Blout, 1970) could conceivably result from better enzyme–substrate binding or increased acylation or deacylation reaction rates. An increase in the rate of the deacylation reaction has recently been shown to be an unimportant factor in the increased rate of hydrolysis of ester, amide, or peptide bonds. Preliminary evidence has been obtained to support the proposition that an increased acylation reaction rate is the major factor contributing to the increased facility of bond cleavage in all three cases (Thompson and Blout, 1970). However, distinguishing between an increased acylation rate and increased productive enzyme–substrate binding is difficult unless the substrates considered bind exclusively in the productive mode. As indicated above, we have obtained evidence that certain tri- and tetrapeptide substrates, *e.g.*, formyl-Ala-Pro-Ala-NH_2 (VI) and Ac-Ala-Ala-Pro-Ala-NH_2 (XVII), do not bind nonproductively to any significant extent (Thompson and Blout, 1973a). Both the binding and acylation steps of hydrolysis proceed more efficiently with the latter than with the former substrate. The P_{54} $CH_3CONHCH(CH_3)$-group of Ac-Ala-Ala-Pro-Ala-NH_2 appears to contribute about -1.4 kcal/mol to the free energy of enzyme–substrate binding and accelerates the acylation reaction about 60-fold. The improved enzyme–substrate binding in the tetrapeptide can be easily rationalized in terms of an enzyme binding site for the additional $CH_3CONHCH(CH_3)$-unit (Thompson and Blout, 1973b). However, the higher acylation rate, which is the dominant factor in the increased k_{cat}/K_m, is less easily explained.

It is reasonable to believe that the rate of the acylation reaction of elastase with the substrates in Tables I–VI will depend almost exclusively on the relative orientation of the scissile bond, P_1–P_1', and the protein's S_1 and S_1' subsites. A P_{54}-induced acceleration of the acylation reaction therefore implies that the S_{54}–P_{54} enzyme–substrate contact is capable of correctly orienting the P_1–P_1' bond with respect to S_1 and S_1'. The orientation corresponds to a transfer of information from the S_{54}–P_{54} to the $S_{11'}$–$P_{11'}$ contact, and the mechanism of information transfer may turn out to be one of the most interesting features of this enzyme.

Information transfer between the S_{54}–P_{54} and $S_{11'}$–$P_{11'}$ contacts could occur in one of two ways. The information could be transmitted directly along the substrate peptide chain. In this substrate-mediated information transfer process, the binding mode forced on residues P_5 and P_4 by subsites S_5 and S_4 may be envisioned as causing a bodily displacement of substrate, including residues P_1 and P_1', within the enzyme's active center. Alternatively, the information may be carried through the three-dimensional network of bonds that forms the tertiary structure of the enzyme. This latter model of enzyme-mediated information transfer requires an S_{54}–P_{54} induced rearrangement of the S_1 and S_1' subsites of the active center. It can be considered a particular case of the "induced-fit" model of Koshland (Koshland and Neet, 1968). *A priori*, it would appear that either of the models could describe the information transfer process in elastase. The possibilities can be distinguished in several ways.

A significant difference between the models lies in the role of the S_2–P_2 and S_3–P_3 enzyme–substrate contacts. In the substrate-mediated model a movement of the P_2 and P_3 residues is necessary to transmit information from S_{54}–P_{54} to $S_{11'}$–$P_{11'}$. The S_2–P_2 and S_3–P_3 contacts, and their contribution to the free energy of enzyme–substrate binding, would then depend on the nature of the S_{54}–P_{54} contact and would be expected to differ between slow and fast reacting substrates. A variation in the free energy of the S_2–P_2 and S_3–P_3 contacts is also possible in the induced-fit model, but is not inevitable, since an information channel in the enzyme may not involve any of the residues forming the subsites of the active center.

Variation of the S_2–P_2 and S_3–P_3 enzyme–substrate contacts between the fast- and slowly reacting substrates in Table V has been discussed above, where it was shown that the free energy of these contacts is not greatly dependent on the nature of the S_{54}–P_{54} contact. Variations in the S_2–P_2 and S_3–P_3 con-

tacts between fast- and slowly reacting substrates are, therefore, minor, and a bodily displacement of substrate resulting from the formation of the S_{54}–P_{54} contact is correspondingly unlikely. This evidence then favors an enzyme rather than substrate-mediated information transfer mechanism.

Secondly, the integrity of a substrate-mediated information transfer process might reasonably be expected to depend on the rigidity of the substrate's peptide chain. For an S_{54}–P_{54} induced repositioning of P_1 and P_1' to be completely accurate, the intervening substrate would have to be rigid. Any flexibility would be likely to decrease the integrity of substrate-mediated information transfer. The rigidity of the substrate's peptide chain can be systematically varied by variation of the P_2 and P_3 amino acid residues. The data in Table V allow us to assess the importance of substrate rigidity in the information transfer process. The increased rate constant resulting from the P_4 Ac → P_{54} Ac-Pro substitution may be taken as an index of the accuracy of information transfer. This rate increase is 95-fold in the P_3–P_2 Ala–Pro peptides (*cf.* VII and XI), 140-fold in the P_3–P_2 Gly–Pro peptides (*cf.* XXVII and XXV), and 157-fold in the P_3–P_2 Ala–Ala peptides[3] (*cf.* XXVIII and XXVI). The rigidity of these substrates should vary considerably by virtue of the increased freedom of rotation about the N–C^α and C^α–C′ bonds of the Gly–Pro and Ala–Ala peptides. The integrity of information transfer, however, does not appear to vary greatly. Such variation as does occur is in entirely the opposite direction to that expected if information transfer were substrate mediated. Thus arguments based both on the invariance of enzyme–substrate contacts in the S_3 and S_2 subsites and on the lack of dependence of the information transfer process on substrate rigidity support the enzyme-mediated, as opposed to the substrate-mediated, model of information transfer.

The above evidence, suggesting the existence of an enzyme-mediated mode of information transfer, sets elastase apart from another serine proteinase, α-chymotrypsin. Many features of the substrate specificity of both elastase and α-chymotrypsin can be explained in terms of substrate-mediated information transfer; *e.g.*, the positioning of the scissile bond by the interactions of the P_1 side chain and P_2–P_1 amide bond with the S_1 and S_2 subsites. However, no clear response of the acylation reaction rate constant to enzyme–substrate contacts outside the S_1 and S_2 subsites has been demonstrated for chymotrypsin (Yamashita, 1960a,b).

The increased reaction rates of elastase associated with increased substrate chain length are largely the result of the enzyme recognizing the carbonyl group or amide nitrogens of the substrates' peptide groups. Thus a 30-fold rate increase is associated with enzyme recognition of the P_5–P_4 peptide group (*cf.* the k_{cat}'s of formyl-Ala-Ala-Pro-Ala-NH_2, 4.4 sec^{-1}, and propionyl-Ala-Pro-Ala-NH_2 (0.15 sec^{-1}, Thompson and Blout, 1973b). The P_4–P_3 peptide group similarly results in a rate increase (*cf.* the k_{cat}'s of formyl-Ala-Pro-Ala-NH_2, 0.1 sec^{-1}, and propionyl-Pro-Ala-NH_2, 0.006 sec^{-1}). Additionally, the P_3–P_2 peptide group is probably responsible for first bringing the hydrolysis rate within a measurable range (*cf.* the k_{cat}'s of Ac-Pro-Ala-NH_2, 0.007 sec^{-1}, and Ac-Ala-NH_2, <0.0008 sec^{-1}).

The molecular basis for enzyme recognition of elements of the substrates' peptide groups is unknown. The large rate increase, noted above, due to the P_4 amino group is also observed when this group is replaced by a methyl group and is always associated with a decreased enzyme–substrate interaction energy. In this case the increased rate has been attributed to the rearrangement of enzyme–substrate contacts consequent to an unfavorable steric interaction between P_4 and S_4 (Thompson and Blout, 1973b). With other substrates, however, the rate increase is associated with an increased enzyme–substrate interaction energy, *e.g.*, the threefold rate and fivefold binding increase due to the P_5 carbonyl group in formyl-Ala-Ala-Pro-Ala-NH_2. The manner in which these substrate peptide groups may, on absorption to the enzyme from aqueous solution, reorient other enzyme–substrate contacts in an energetically favorable process is particularly interesting and worthy of further study. The important role of these hydrophilic groups in the protein–peptide recognition process may be relevant to our understanding of the protein–protein interactions in multisubunit enzymes.

[3] The ratio of the k_2's of these peptides will be approximately equal to the ratio of their k_{cat}'s; see Results and footnote 2.

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