The Active Centers of *Streptomyces griseus* Protease 3 and α-Chymotrypsin: Enzyme–Substrate Interactions Remote from the Scissile Bond†

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ABSTRACT: Kinetic constants are reported for α-chymotrypsin- and *Streptomyces griseus* protease 3 (SGP3)-catalyzed amide hydrolysis of a number of peptide amides of varying substrate chain length. α-Chymotrypsin, but not SGP3, will hydrolyze rapidly specific acetyl amino acid amides. SGP3-catalyzed, but not α-chymotrypsin-catalyzed, hydrolysis is greatly stimulated by the presence of up to four amino acid residues N-terminal to the scissile bond of the substrate. The enzyme–substrate interactions utilized to promote hydrolysis, therefore, differ in these two enzymes, which, in other respects, show marked similarities. α-Chymotrypsin depends mainly on primary enzyme–substrate contacts, those with the amino acid residue (P_1) whose carbonyl group forms part of the scissile bond, whereas SGP3 depends mainly on "secondary" enzyme–substrate contacts with amino acid residues (P_2–P_4) more remote from the scissile bond. A comparison with porcine elastase, a related serine protease, indicates that there is an inverse relation between the importance of primary and secondary enzyme–substrate interactions in this family of enzymes. A rationale is proposed for this effect based on the observation that both types of enzyme–substrate interaction predominantly affect the rate constant for the acylation step of substrate hydrolysis.

The specificities of proteolytic enzymes have been studied extensively both because of their intrinsic value in protein sequencing and because the relationship between specificity and efficiency of catalysis in these enzymes has become a paradigm for understanding enzyme catalysis. Initially, such studies centered on determining which amino acids form the scissile bonds. These investigations defined the specificity of many proteases, including the pancreatic serine proteases, α-chymotrypsin and trypsin. Over the last few years, however, it has become clear that, for certain proteases, definition of the amino acids flanking the scissile bond provides an incomplete picture of those features of the substrate leading to rapid hydrolysis. For such enzymes, interactions with parts of the substrate quite remote from the scissile bond appear to be important in facilitating hydrolysis (see, for example, Berger et al. (1969); Fruton (1973)).

We have previously studied the enzyme–substrate interactions in one of these enzymes, porcine pancreatic elastase, with a view to elucidating the manner in which interactions remote from the scissile bond can facilitate bond cleavage (Thompson and Blout, 1973a–c). One of these interactions has been shown to strain or distort the scissile bond of the substrate toward its transition state configuration (Thompson, 1974). Elastase, however, might be an atypical protease in that the useful enzyme–substrate interactions in the vicinity of the scissile bond are limited by the occlusion of part of the S_1 binding site[1] (Hartley and Shotton, 1971).

We have now extended our studies of remote enzyme–substrate interactions to a bacterial serine protease, *Streptomyces griseus* Protease 3 (SGP3)[2], since initial studies had shown that SGP3 might have interesting enzyme–substrate interactions remote from the scissile bond (Bauer and Löfqvist, 1973). A number of peptide substrates of SGP3 have been synthesized for this investigation. We have also examined the specificity of α-chymotrypsin toward the same set of peptide substrates in order to have a direct comparison with an enzyme whose specificity has already been widely studied.

SGP3 and α-chymotrypsin are known to hydrolyze preferentially amide, peptide, and ester bonds C-terminal to aromatic amino acids. For the most part, we chose to study the hydrolysis of phenylalanyl bonds because phenylalanyl peptides are, in general, easier to synthesize than are their tyrosyl or tryptophanyl analogues.

This paper reports the effect of substrate chain length on the efficiency of amide hydrolysis for both SGP3 and α-chymotrypsin. The following paper will deal with the effect of varying the amino acid residues adjacent to the scissile bond on the ease of peptide and amide bond hydrolysis.

Materials and Methods

α-Chymotrypsin was a three-times crystallized preparation (batch 6JF) from Worthington Biochemical Corpora-

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[1] The nomenclature introduced by Schechter and Berger (1967) is used to facilitate discussion of the interactions between a protease 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}.

[2] SGP3 has been shown (Löfqvist and Sjöberg, 1971; Bauer and Löfqvist, 1973) to be identical with "PNPA-hydrolase I", described by Wählby (1969), "alkaline protease a" (Narahashi, 1970), "*Streptomyces griseus* Enzyme II" (Gertler and Trop, 1971), "*Streptomyces griseus* protease A" (Johnson and Smillie, 1971), and "lysine-free chymoelastase" (Siegel and Awad, 1973).

tion, N.J. The concentration of active α-chymotrypsin was determined with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (Eastman), as described by Kezdy and Kaiser (1970).

Homogeneous, lyophilized SGP3 was prepared from Pronase-P (Lot No. 592045 from Kaken Chemical Co.) by the method of Bauer and Löfqvist (1973). The concentration of active SGP3 in a lyophilized preparation is 70% of that in the fresh, gel-chromatographed enzyme, as determined by its activity toward Ac-Pro-Ala-Pro-Phe-NH_2. The loss of activity affected only the value of k_{cat}. All k_{cat}'s reported here have been corrected for the loss of activity and refer to the gel-chromatographed enzyme.

Initial rates of amide hydrolyses were measured in a pH-stat. Reactions were conducted under N_2 atmosphere in 1 ml of 10 mM $CaCl_2$, 3 mM NaN_3 at pH 8.00 (α-chymotrypsin), or pH 9.00 (SGP3) and 37 °C. The concentrations of active enzymes were 0.56–5.3 μM (α-chymotrypsin) and 0.25–7.5 μM (SGP3). The pH of the reaction mixture was maintained by the addition of 1 mM aqueous NaOH (α-chymotrypsin) or 2 mM aqueous NaOH (SGP3). The molarity of the NaOH solutions were determined with potassium hydrogen phthalate. The pK_a of ammonia, determined titrimetrically under the conditions used in the substrate assays, is 8.90. Product ionization was corrected according to Kurtz and Niemann (1962).

The parameters, k_{cat} and K_m of the Michaelis–Menten equation, and their standard deviations were determined by iterative regression analysis according to Pettersson and Pettersson (1970). Strict Michaelis–Menten kinetics were observed. Preliminary parameter estimates, necessary for the iterative regression analysis, were obtained graphically from Lineweaver–Burke plots.

The bond split by the enzymes was established by thin-layer chromatography (TLC) of reaction mixtures. Except for Ac-Pro-Ala-Phe-NH_2 (IV), a single reaction product was observed which was ninhydrin negative. These findings are consistent with the exclusive hydrolysis of the amide bond. For compound IV, Phe-NH_2 was identified among the products.

TLC of peptides was carried out with silica gel plates, as described previously (Thompson and Blout, 1973a). Amino acid composition of the peptides was determined with a Beckman 121C amino acid analyzer. *tert*-Butyloxycarbonyl amino acids, amino acid esters, and amides were purchased from Fox Chemical Co., Los Angeles, and Cyclo Chemicals, Los Angeles.

Unless otherwise specified, all amino acids are of the L configuration. The preparations of Ac-Ala-Pro-OH, Ac-Pro-Ala-Pro-OH, Ac-Ala-Ala-Pro-Ala-NH_2 (VI), Ac-Pro-Ala-Pro-Ala-NH_2 (VII), Ac-Ala-Pro-Ala-Pro-Ala-NH_2 (VIII), and Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (IX) have been described previously (Thompson and Blout, 1973a,b).

Synthesis of Acetylprolylphenylalaninamide (II). Acetylproline monohydrate (525 mg, 3 mmol) was dissolved in 50 ml of *N,N*-dimethylformamide and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (0.33 ml, 3 mmol) was added, followed after 5 min by 0.39 ml (3 mmol) of isobutyl chloroformate. After 1 min, a precooled solution of 492 mg (3 mmol) of phenylalaninamide in 50 ml of *N,N*-dimethylformamide was added, and the solution was stirred overnight. The solvent was evaporated in vacuo; the oily residue was dissolved in water and shaken with an excess of Rexyn I-300 resin (Fisher). The resin was removed by filtration, the water was evaporated, and the residue was crystallized from ethyl acetate to give 585 mg (64%) of product: mp 178–179 °C, single spot by TLC $R_{F_{II}}$ 0.9. Anal. Calcd for $C_{16}H_{21}N_3O_3$: C, 63.4; H, 7.0; N, 13.9. Found: C, 63.3, H, 6.9; N, 13.9. Amino acid analysis (theoretical Phe, 1.00; Pro, 1.00): Phe, 1.07; Pro, 1.00.

Synthesis of Acetylalanylprolylphenylalaninamide (III). Acetylalanylproline (79 mg, 0.34 mmol) was dissolved in 50 ml of *N,N*-dimethylformamide and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (37 μl, 0.34 mmol) was added, followed after 5 min by 44 μl (0.34 mmol) of isobutyl chloroformate. After 3 min, a precooled solution of 62 mg (0.38 mmol) of phenylalaninamide in 50 ml of *N,N*-dimethylformamide was added, and the solution was stirred overnight. The solvent was evaporated in vacuo, the residue was dissolved in water and treated with Rexyn I-300 resin, filtered, and the water was evaporated. The solid was dissolved in a small amount of ethanol and crystallized from ethyl acetate–hexane to give 80 mg (63%) of white, slightly hygroscopic crystals: mp 94–97 °C, single spot by TLC $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{19}H_{26}N_4O_4 \cdot 0.5H_2O$: C, 59.5; H, 7.1; N, 14.6. Found: C, 59.9; H, 7.1; N, 14.7. Amino acid analysis (theoretical Ala, 1.00; Phe, 1.00; Pro, 1.00): Ala, 0.97; Phe, 1.03; Pro, 1.00.

Synthesis of tert-Butyloxycarbonylalanylphenylalaninamide. tert-Butyloxycarbonylalanine (0.76 g, 4 mmol) was dissolved in 25 ml of tetrahydrofuran in a 200-ml round-bottomed flask and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (0.44 ml, 4 mmol) was added and, after 5 min, isobutyl chloroformate (0.53 ml, 4 mmol) was added with stirring. After 3 min, a solution of 0.8 g (4 mmol) of phenylalaninamide hydrochloride and 0.44 ml (4 mmol) of *N*-methylmorpholine in 25 ml of tetrahydrofuran, precooled to −20 °C, was added with stirring, and the mixture was allowed to warm to room temperature overnight. The solvent was evaporated in vacuo, and the solid residue was dissolved in chloroform. The chloroform solution was extracted twice with 100 ml of 0.2 M hydrochloric acid, once with distilled water, and twice with 100 ml of a 5% aqueous sodium bicarbonate solution. The organic phase was dried with $MgSO_4$, the $MgSO_4$ was filtered off, and the chloroform was evaporated. The residue was crystallized from ethyl acetate to give 0.56 g (40%) of *tert*-butyloxycarbonylalanylphenylalaninamide: mp 174–178 °C, single spot by TLC $R_{F_{II}}$ 0.9.

Synthesis of Alanylphenylalaninamide Hydrochloride. tert-Butyloxycarbonylalanylphenylalaninamide (0.56 g, 1.6 mmol) was suspended in 100 ml of ethyl acetate and cooled in an ice bath. Hydrogen chloride was bubbled through for 15 min, during which time the solid rapidly dissolved. During the first few minutes the reaction mixture warmed up, but heat soon ceased to be produced. The ice bath was then removed, and the reaction was allowed to warm to room temperature. After 1 h, the solvent was removed in vacuo, and the residue was left in vacuo with sodium hydroxide pellets overnight. Alanylphenylalaninamide hydrochloride (0.45 g, ~100%; highly hygroscopic) was obtained: single spot by TLC, $R_{F_{II}}$ 0.3.

Synthesis of Acetylprolylalanylphenylalaninamide (IV). Acetylproline monohydrate (222 mg, 1.2 mmol) was dissolved in 50 ml of *N,N*-dimethylformamide and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (0.13 ml, 1.2 mmol) was added and, after 5 min, followed by isobutyl chloroformate (0.156 ml, 1.2 mmol). After 1 min, a precooled solution of alanylphenylalaninamide hydrochloride (320 mg, 1.1 mmol) and *N*-methylmorpholine (0.12

Table I: Kinetic Parameters for the SGP3-Catalyzed Hydrolysis of Peptide Amides at pH 9.00.

P_6 P_5 P_4 P_3 P_2 P_1 ↓	k_{cat}/K_m ($s^{-1}M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	[S][a] (mM)
Ac-Phe-NH_2 (I)	0.28	0.007 ± 0.001	25 ± 4	16–65
Ac-Pro-Phe-NH_2 (II)	27	0.130 ± 0.002	4.9 ± 0.2	2.3–18
Ac-Ala-Pro-Phe-NH_2 (III)	700	3.0 ± 0.1	4.3 ± 0.3	0.8–3.7
Ac-Pro-Ala-Phe-NH_2 (IV)[b]	<11	<0.16 ± 0.01	14 ± 2	2.3–19
Ac-Pro-Ala-Pro-Phe-NH_2 (V)	10700	5.8 ± 0.1	0.54 ± 0.03	0.5–7.1
Ac-Ala-Ala-Pro-Ala-NH_2 (VI)	24	0.33 ± 0.03	14 ± 2	1.3–7.2
Ac-Pro-Ala-Pro-Ala-NH_2 (VII)	38	0.30 ± 0.01	8.0 ± 0.5	0.9–7.3
Ac-Ala-Pro-Ala-Pro-Ala-NH_2 (VIII)	43	0.30 ± 0.02	7.0 ± 0.9	0.9–7.3
Ac-D-Ala-Pro-Ala-Pro-Ala-NH_2 (IX)	23	0.28 ± 0.02	12 ± 1	0.9–7.3

[a] Range of peptide concentrations used to determine K_m. [b] The kinetic constants denote maximal values for amidase activity. However, evidence was also obtained for a considerable peptidase activity. The relative rates of these two activities have not been assessed.

Table II: Kinetic Parameters for α-Chymotrypsin-Catalyzed Amide Hydrolysis at pH 8.00.

P_5 P_4 P_3 P_2 P_1 ↓	k_{cat}/K_m ($s^{-1}M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	[S][a] (mM)
Ac-Phe-NH_2 (I)	10	0.22 ± 0.01	21 ± 2	9.0–71
Ac-Pro-Phe-NH_2 (II)	29	0.70 ± 0.02	24 ± 1	4.6–37
Ac-Ala-Pro-Phe-NH_2 (III)	1040	2.3 ± 0.1	2.2 ± 0.2	0.9–7.4
Ac-Pro-Ala-Phe-NH_2[b] (IV)	24	0.44 ± 0.02	18 ± 2	4.6–37
Ac-Pro-Ala-Pro-Phe-NH_2 (V)	820	2.8 ± 0.2	3.4 ± 0.4	0.9–7.5

[a] Range of peptide concentrations used to determine K_m. [b] The kinetic constants denote maximal values for amidase activity. However, evidence was also obtained for a considerable peptidase activity. The relative rates of these two activities have not been assessed.

ml, 1.1 mmol) in 50 ml of *N,N*-dimethylformamide was added, and the solution was stirred overnight.

The solvent was evaporated in vacuo, the residue was dissolved in water, treated with excess Rexyn I-300 resin, and filtered, and the water was evaporated. The residue was crystallized from ethyl acetate–hexane to give 320 mg of product (78%): mp 214–217 °C, single spot on TLC, $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{19}H_{26}N_4O_4$: C, 60.9; H, 7.0; N, 15.0. Found: C, 60.7; H, 7.5; N, 14.9. Amino acid analysis (theoretical Ala, 1.00; Phe, 1.00; Pro, 1.00): Ala, 1.01; Phe, 1.09; Pro, 1.00.

Synthesis of Acetylprolylalanylprolylphenylalaninamide (V). Acetylprolylalanylproline (200 mg, 0.6 mmol) was dissolved in 50 ml of *N,N*-dimethylformamide and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (66 μl, 0.6 mmol) was added, followed after 5 min by 78 μl (0.6 mmol) of isobutyl chloroformate. After 3 min, a precooled solution of phenylalaninamide hydrochloride (133 mg, 0.66 mmol) and *N*-methylmorpholine (73 μl, 0.66 mmol) in 50 ml of *N,N*-dimethylformamide was added, and the solution was stirred overnight. The solvent was evaporated in vacuo, and the residue was dissolved in water and treated with an excess of Rexyn I-300 resin. The resin was removed by filtration, the water was evaporated, and the residue was triturated under ether to give 185 mg (65%) of a slightly hygroscopic solid, homogeneous by TLC $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{24}H_{33}N_5O_5 \cdot H_2O$: C, 58.9; H, 7.2; N, 14.3. Found: C, 59.1; H, 7.2; N, 14.0. Amino acid analysis (theoretical Ala, 1.00; Phe, 1.00; Pro, 2.00): Ala, 0.95; Phe, 1.05; Pro, 2.00.

Results

Despite the fact that α-chymotrypsin and SGP3 show similar specificities in the hydrolysis of amino acid *p*-nitrophenyl esters (Bauer and Löfqvist, 1973), a comparison of their ability to hydrolyze the peptide amides I–V (Tables I and II) shows several striking differences between these enzymes. α-Chymotrypsin can be seen to be the more efficient amidase for the acetyl amino acid amide, Ac-Phe-NH_2 (I), whereas, for short peptide amides (e.g., II and III), α-chymotrypsin and SGP3 catalyze amide hydrolysis about equally well. With the longer tetrapeptide amide, V, SGP3 is a considerably better amidase than α-chymotrypsin.

The differences between the enzymes result from SGP3 showing a large (15–100-fold) increase in k_{cat}/K_m for each addition of an amino acid residue of the substrate between 1 and 4, whereas α-chymotrypsin shows an increase of similar magnitude only on going from a di- to a tripeptide amide (cf. peptides II, III, and V).

With SGP3, which has a broader specificity for P_1 residues than α-chymotrypsin (Bauer et al., 1976), we were also able to study the effect of adding a fifth amino acid residue N-terminal to the scissile bond of the substrate with a series of P_1 alanyl peptides (VI–IX) used previously to study the active center of pancreatic elastase (Thompson and Blout, 1973b). Studies on the hydrolysis of these peptides show that no significant increase in k_{cat}/K_m results from elongating the acetyl tetrapeptide amide to an acetyl pentapeptide amide with either a D- or L-amino acid residue at P_5 (Table I).

Our results are also of interest in that they show that SGP3 shares with some other serine proteases an inability to hydrolyze efficiently substrates with a P_3 Pro residue (cf. peptides III and IV). The discrimination against such substrates is so marked that it can overcome the primary specificity of SGP3 and α-chymotrypsin for P_1 Phe. In peptide IV, for example, a significant amount of hydrolysis of the Ala–Phe bond is observed. Discrimination against P_3 Pro residues has been noted previously in α-chymotrypsin by

Segal (1972) and independently in elastase by Thompson and Blout (1973a).

Discussion

Both α-chymotrypsin and SGP3 have been shown to hydrolyze long peptide amides more efficiently than short ones. Before any conclusions can be drawn as to whether the increased catalytic efficiency results from better substrate binding or an increased turnover number, it is necessary to discuss the meaning of the observed parameters, k_{cat} and K_m, which are commonly assumed to reflect changes in rate of substrate turnover and enzyme–substrate affinity, respectively.

Substrate hydrolyses by α-chymotrypsin and SGP3 can be described by the kinetic scheme (eq 1) (Bender and Kezdy, 1965; Bauer et al., 1974):

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES^* \xrightarrow[(\ k_3]{H_2O} E + Pr_2 \qquad (1)$$

$$ES \xrightarrow{k_2} Pr_1$$

For both enzymes there is evidence that, where the first product, Pr_1, is an amine, the rate-determining step of the hydrolysis is that corresponding to transfer of the acyl group from the amine to the enzyme to form the intermediate acyl–enzyme ES* (Fastrez and Fersht, 1973; Bauer and Pettersson, 1974). For such substrates, therefore, K_m will be a true binding constant (Bender and Kezdy, 1965). However, it is not clear that K_m will be equal to K_s, the dissociation constant of the productive enzyme–substrate complex, since other, nonproductive, enzyme–substrate complexes may be formed. Several factors lead us to believe that these complexes, if they are formed, will be weak and of little kinetic importance.

It appears that the degree to which long peptides show nonproductive binding is reduced by their containing proline residues. Proline residues differ from amino acids in that no rotation can occur about the N–C_α bond. A peptide containing proline will, therefore, be unable to bind to an enzyme's active center unless the ϕ angle required for a close enzyme–peptide fit is that of proline (−60°). There is evidence for some serine proteases that subsites S_2 and S_4 of their active centers can accommodate proline residues, whereas subsites S_1 and S_3 cannot. With chymotrypsin, for example, the kinetics of hydrolysis of several peptide methyl esters indicate that hydrolysis is hindered in peptides with P_3 proline residues, and a model has been proposed to explain the discrimination against proline by S_3 (Segal, 1972). A proline residue is unable to fit in the S_1 subsite of this enzyme, based on the substrate binding mode proposed by Steitz et al. (1969) and on the structure proposed for the soybean trypsin inhibitor·chymotrypsin complex by Blow et al. (1972). The affinity of Ac-Pro-NH_2 for the S_1 subsite of α-chymotrypsin is less than $^1/_{15}$ that of Ac-Phe-NH_2, based on a K_i of 350 mM for the former compound (Bauer, unpublished result) and a K_m of 21 mM for the latter compound (Table II). For a related serine protease, pancreatic elastase, there is kinetic evidence indicating that the S_3 subsite will not bind proline residues (Thompson and Blout, 1973a).

Our initial investigation of the specificity of SGP3 revealed a similar difficulty in forming productive complexes when residue P_3 of the substrate is proline (cf. peptides III and IV). While it is by no means certain that the discrimination against P_3 proline occurs in the binding step rather than the acylation step of the reaction, this would seem to be a reasonable hypothesis in light of the above data on other homologous serine proteases. It is likely, therefore, that the presence of proline residues will severely curtail nonproductive binding of our peptides to both α-chymotrypsin and SGP3. A more detailed discussion of the effect of proline in preventing nonproductive binding has been presented in a previous paper from this laboratory (Thompson and Blout, 1973a).

Because the rate-limiting step of reaction is acylation and because the productive mode is likely to be the only strong enzyme–substrate binding mode, we feel justified in equating the observed K_m with K_s, the dissociation constant of the productive binding mode, and k_{cat} with k_2, the acylation rate constant. When this is done, several interesting conclusions can be drawn from the data in Tables I and II.

The data in Table I indicate that, for SGP3, the increased efficiency of hydrolysis of acetyl dipeptide amides, as compared with acetyl amino acid amides, is due to both a 5-fold increase in enzyme–substrate affinity and a 20-fold increase in the rate of the acylation reaction. Elongating the substrate further to a tripeptide and a tetrapeptide results in selective increases in the acylation rate constant (23-fold) and enzyme–substrate affinity (8-fold), respectively. No further significant changes are observed on going to acetyl pentapeptide amides, showing that the active center of SGP3 does not interact strongly with parts of the substrate beyond the α-carbon of residue P_5. In this respect SGP3 has an active center similar in size to that of elastase (Thompson and Blout, 1973b).

With α-chymotrypsin, small (about 3-fold) increases in k_{cat} are observed on adding a second and third amino acid residue N-terminal to the scissile bond (Table II). These increases should be compared with the 20-fold increases in k_{cat} observed with SGP3. The largest effect of substrate chain length on the kinetic parameters for α-chymotrypsin-catalyzed hydrolyses is the 10-fold increase in enzyme–substrate binding on extending the substrate to an acetyl tripeptide amide. The similarity of the kinetic constants for the acetyl tripeptide amide, III, and the acetyl tetrapeptide amide, V, indicates that there are probably no significant enzyme–substrate interactions beyond the α-carbon atom of residue P_4. An alternative explanation, which at present cannot be excluded, is that the above statement is true only for substrates containing P_4 proline residues. Our results are in essential agreement with those of Baumann et al. (1973) who studied the effect of substrate chain length of α-chymotrypsin-catalyzed peptide hydrolysis.

An interesting comparison may be made between α-chymotrypsin, SGP3, and the closely related serine protease, pancreatic elastase. The chemical nature of the scissile bonds of peptide substrates of these enzymes is very similar, and their catalytic residues are probably identical. However, of these enzymes only α-chymotrypsin achieves anything approaching the optimal rate of substrate turnover ($k_{cat} = k_2$) when the only specificity determinants available are those present in P_1 and the C_α and C′ groups of P_2. For SGP3, these P_1 and P_2 specificity determinants are significantly less effective (Table I), while for elastase they are ineffective in facilitating amide bond hydrolysis (k_{cat}(Ac-Ala-NH_2) < 0.0008 s^{-1}; Thompson and Blout, 1973b). These "primary specificity determinants" (Fruton, 1970, 1975), therefore, become more useful on going from elastase to SGP3 to α-chymotrypsin.

Whatever the inadequacies of the primary contacts in el-

astase and SGP3 enzyme–substrate complexes, they are completely rectified when additional, "secondary", enzyme–substrate contacts (Fruton, 1975) are formed by the P_4, P_3, and P_2 amino acid residues of the substrate. The effectiveness of the secondary contacts in increasing k_{cat} (k_2) becomes greater on going from α-chymotrypsin to SGP3 to elastase (cf. these results and those of Thompson and Blout, 1973b). The rank for important secondary enzyme–substrate interactions is, therefore, the inverse of that for important primary interactions. A similar phenomenon has been noted previously by Morihara and Oka (1973), although the use of ester substrates by those investigators precluded their determining that the acylation rate constant (k_2) was the most important kinetic parameter responding to substrate chain length.

When it is recognized that the major effect of both primary and secondary enzyme–substrate contacts is to increase k_2, it becomes possible to propose a rationale for the inverse importance of primary and secondary specificity in these enzymes. While specificity might be manifested in an enzyme–substrate affinity, it would seem to have no natural limit beyond the ability of the enzyme to provide binding sites. In contrast, where specificity is manifested primarily in a rate constant, it has a natural limit beyond which factors other than the relationship between enzyme and substrate determine the rate of substrate hydrolysis. (The fact that for α-chymotrypsin, elastase, and SGP3 there is a striking similarity between the highest values of k_2 observed for amide hydrolysis ($\sim 10\ s^{-1}$) may indicate that this upper limit is in sight.) Where the overall contribution of enzyme–substrate contacts is limited, and two distinct types of enzyme–substrate contact may be identified, it follows that the increased importance of one of these types must be associated with a decreased role for the other. Evolution seems to have presented proteolytic enzymes with two potential strategies in catalyzing substrate hydrolysis. The first strategy has these enzymes forming precise contacts with those amino acids immediately flanking the scissile bond; i.e., evolving a strong primary specificity. A disadvantage of this choice is that the enzyme is severely restricted in the type of bond it may cleave. The second strategy has the enzyme using elements of the peptide bonds several residues removed from the scissile bond to facilitate catalysis. The disadvantage of this choice is that the enzyme is an inefficient catalyst of the hydrolysis of short peptides. Since the efficiency of the catalytic process is subject to limits other than those stemming from the contacts between enzyme and substrate, one of these strategies can only be exploited fully at the expense of the other.

Of course, the argument presented above is only valid where "specificity" arises from the necessity for forming certain enzyme–substrate contacts if there is to be catalysis. Where specificity is an end in itself, as might be the case with the proteolytic enzymes involved in blood clotting or complement fixation, the inverse relation between primary and secondary specificity might not hold, and it should be possible to find enzymes with both strong primary and secondary specificities. That the inverse relation does hold for the enzymes considered here may be a consequence of these enzymes having a purely digestive function, where the evolution of "excess" specificity might be detrimental to function.

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