The Active Centers of *Streptomyces griseus* Protease 3, α-Chymotrypsin, and Elastase: Enzyme–Substrate Interactions Close to the Scissile Bond†

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ABSTRACT: Kinetic constants are reported for α-chymotrypsin-, *Streptomyces griseus* protease 3 (SGP3)-, and elastase-catalyzed hydrolysis of a number of peptides. SGP3, like α-chymotrypsin, hydrolyzes most readily amide bonds whose immediate acyl group (P_1) is a large, hydrophobic, amino acid residue. SGP3, however, has a broader specificity for P_1 residues than does α-chymotrypsin, primarily because the most important interactions between SGP3 and residue P_1 of the substrate involve the C_β and C_γ groups of the P_1 side chain. For substrates of all three proteases, the amino acid residue contributing the amino group of the scissile bond (P_1') is less important than P_1 in determining k_{cat}/K_m for the hydrolysis reaction. Each enzyme interacts favorably with a hydrophobic P_1' side chain. A substrate with a large P_1' side chain is bound more strongly, but is hydrolyzed less rapidly, than that with P_1' Ala. The observation that strong binding of P_1' is not necessarily conducive to rapid hydrolysis is consistent with the idea that parts of P_1' must undergo a considerable displacement during substrate hydrolysis.

Much of our current knowledge of proteolytic enzymes can be understood in terms of their interactions with the two amino acid residues which form the scissile bond of the substrate. These interactions are the basis of the enzymes' primary specificity. Recently, it has become apparent that these enzymes often interact with other amino acid residues of the substrate, leading to a "secondary specificity" (Fruton, 1975), with long peptides being hydrolyzed more rapidly than short ones. The preceding paper (Bauer et al., 1976) described the secondary specificity of both α-chymotrypsin and *Streptomyces griseus* Protease 3 (SGP3)[1]. To determine the relationship between secondary and primary specificity in these enzymes, we have also studied some of the enzyme–substrate interactions manifested in the primary specificity of α-chymotrypsin, SGP3, and a related serine protease, pancreatic elastase.

α-Chymotrypsin is known to hydrolyze most rapidly peptide bonds whose acyl group (P_1)[2] is an aromatic amino acid, and this specificity is well-understood in terms of the structure of the enzyme–substrate complex (Blow, 1971). The specificity of elastase for hydrolyzing bonds whose acyl group is a small hydrophobic amino acid is also well-established (Geneste and Bender, 1969; Kaplan et al., 1970; Thompson and Blout, 1973b), and a structural basis for this specificity has been presented (Hartley and Shotton, 1971; Shotton et al., 1972). In contrast, the primary specificity of SGP3 is neither well-established nor is it understood in structural terms. This communication will report a comparison of the primary specificity of α-chymotrypsin and SGP3 and suggest ways in which the structure of the S_1–P_1 contact might be expected to differ in these two enzymes.

We also report a comparison of the S_1'–P_1' interaction in α-chymotrypsin, SGP3, and elastase. Fersht et al. (1973) have proposed that the S_1'–P_1' contact in α-chymotrypsin facilitates bond cleavage by a "strain" mechanism (Jencks, 1969). Our results indicate that a similar mechanism may operate in SGP3 and elastase. There are indications, however, that for all three enzymes a strong binding interaction between S_1' and P_1' might retard the hydrolysis of the P_1–P_1' peptide bond.

Materials and Methods

The enzymes, hydrolytic procedures, and analytical methods have been described in Bauer et al. (1976). Pure crystallized elastase was a kind gift from Dr. Eric T. Fossel. K_i values were determined from Dixon plots (Dixon, 1953) using Ac-Pro-Ala-Pro-Phe-NH_2 as a substrate. Inhibition was purely competitive within experimental error.

The values, pK_a(alaninamide) = 7.78, pK_a(glycinamide) = 7.80, and pK_a(phenylalaninamide) = 7.12, were determined titrimetrically at the conditions used in the substrate assays.

The preparations of Ac-Pro-Ala-Pro-OH, Ac-Pro-Ala-Pro-Gly-NH_2 (I), Ac-Pro-Ala-Pro-Ala-NH_2 (II), Ac-Pro-Ala-Pro-Val-NH_2 (III), and Ac-Pro-Ala-Pro-Leu-NH_2 (IV) have been described by Thompson and Blout (1973b). Bauer et al. (1976) have described the synthesis of Ac-Pro-Ala-Pro-Phe-NH_2 (V) and HCl·H-Ala-Phe-NH_2.

General Method for Preparation of Compounds VI–XII. Acetylprolylalanylproline (1 equiv) was dissolved in 50 ml of *N,N*-dimethylformamide and cooled to −20 °C in a dry ice–CCl_4 bath. *N*-Methylmorpholine (1 equiv) was added, followed after 5 min by isobutyl chloroformate (1 equiv). After 3 min, a precooled solution of amino acid amide or dipeptide amide (1 equiv) 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 an excess of Rexyn I-300 resin (Fisher), filtered, and the water was evaporated. The compounds were crystallized as stated for each compound.

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[1] See Footnote 2 of Bauer et al., 1976.

[2] See Footnote 1 of Bauer et al., 1976.

Table I: Kinetic Parameters for α-Chymotrypsin-Catalyzed Hydrolysis of Tetrapeptide Amides 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-Pro-Ala-Pro-Gly↓-NH_2 (I)	(<1)	(<0.05)	K_i = 120	25–50
Ac-Pro-Ala-Pro-Ala-NH_2 (II)	(<1)	(<0.05)	K_i = 75	20–40
Ac-Pro-Ala-Pro-Val-NH_2 (III)	(<1)	(<0.05)	K_i = 55	20–35
Ac-Pro-Ala-Pro-Leu-NH_2 (IV)	31	0.56 ± 0.05	18 ± 3	1.7–18
Ac-Pro-Ala-Pro-Phe-NH_2 (V)	820	2.8 ± 0.2	3.4 ± 0.4	0.9–7.5
Ac-Pro-Ala-Pro-Tyr-NH_2 (VI)	2470	7.4 ± 0.2	3.0 ± 0.1	1.2–5.2

[a] Range of substrate or inhibitor concentrations.

Table II: Kinetic Parameters for SGP3-Catalyzed Hydrolysis of Tetrapeptide Amides at pH 9.00.

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-Pro-Ala-Pro-Gly↓-NH_2 (I)	0.6	0.012 ± 0.002	20 ± 5	5.3–21
Ac-Pro-Ala-Pro-Ala-NH_2 (II)	38	0.30 ± 0.01	8.0 ± 0.5	0.9–7.3
Ac-Pro-Ala-Pro-Val-NH_2 (III)	33	0.130 ± 0.005	4.0 ± 0.3	0.9–7.3
Ac-Pro-Ala-Pro-Leu-NH_2(IV)	2280	1.80 ± 0.02	0.79 ± 0.04	0.9–7.3
Ac-Pro-Ala-Pro-Phe-NH_2 (V)	10700	5.8 ± 0.1	0.54 ± 0.03	0.4–7.1
Ac-Pro-Ala-Pro-Tyr-NH_2 (VI)	7200	10.1 ± 0.2	1.4 ± 0.1	0.9–7.3
Ac-Pro-Ala-Pro-Tyr-NH_2 (VI)[b]	8000	9.6 ± 0.3	1.2 ± 0.1	0.4–3.6

[a] Range of substrate concentrations. [b] pH 8.00.

Acetylprolylalanylprolyltyrosinamide. The compound was crystallized from ethyl acetate to give 150 mg (51%) of white crystals: mp 239–240 °C, single spot by TLC, $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{24}H_{33}N_5O_6$: C, 59.1; H, 6.8; N, 14.4. Found: C, 58.5; H, 6.6; N, 14.4. Amino acid analysis (theoretical Ala, 1.00; Tyr, 1.00; Pro, 2.00): Ala, 1.10; Tyr, 1.08; Pro. 2.00.

Acetylprolylalanylprolylphenylalanylglycinamide (*VII*). Crystallization from ethyl acetate yielded 152 mg (74%): mp 226–229 °C, single spot by TLC, $R_{F_{II}}$ 0.5. Amino acid analysis (theoretical Ala, 1.00; Gly, 1.00; Phe, 1.00; Pro, 2.00): Ala, 1.05; Gly, 1.02; Phe, 1.05; Pro, 2.00.

Acetylprolylalanylprolylphenylalanylalaninamide (*VIII*). Crystallization from ethyl acetate gave 157 mg (72%): mp 214–216 °C, single spot by TLC $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{27}H_{38}N_6O_6$: C, 59.8; H, 7.1; N, 15.5. Found: C, 59.7; H, 6.8; N, 15.1. Amino acid analysis (theoretical Ala, 2.00; Phe, 1.00; Pro, 2.00): Ala, 1.92; Phe, 1.00; Pro, 2.00.

Acetylprolylalanylprolylphenylalanylphenylalaninamide (*IX*). The compound was crystallized from ethyl acetate to give 145 mg (81%) of white crystals: mp 203–205 °C, single spot by TLC $R_{F_{II}}$ 0.6. Anal. Calcd for $C_{33}H_{42}N_6O_6$: C, 64.1; H, 6.8; N, 13.6. Found: C, 63.9; H, 6.9; N, 13.7. Amino acid analysis (theoretical Ala, 1.00; Phe, 2.00; Pro, 2.00): Ala, 0.98; Phe, 2.05; Pro, 2.00.

Acetylprolylalanylprolylalanylglycinamide (*X*). The compound was crystallized under ethyl acetate to give 202 mg (58%): mp 190–191 °C, single spot by TLC, $R_{F_{II}}$ 0.35. Amino acid analysis (theoretical Ala, 2.00; Gly, 1.00; Pro, 2.00): Ala, 2.19; Gly, 1.10; Pro, 2.00.

Acetylprolylalanylprolylalanylalaninamide (*XI*). The compound was crystallized from *N,N*-dimethylformamide–ethyl acetate, giving 380 mg (65%): $[\alpha]_D$ −197° (*c* 0.5, 10^{-2} M aqueous $CaCl_2$), mp 233–236 °C, single spot by TLC, $R_{F_{II}}$ 0.65. Anal. Calcd for $C_{21}H_{36}N_6O_7$: C, 52.6; H, 7.5; N, 17.4. Found: C, 52.9; H, 7.2; N, 17.7.

Acetylprolylalanylprolylalanylphenylalaninamide (*XII*). The compound was crystallized from ethanol–hexane to give 140 mg (62%) of white crystals: mp 122–125 °C, single spot by TLC, $R_{F_{II}}$ 0.5. Anal. Calcd for $C_{27}H_{38}N_6O_6 \cdot H_2O$: C, 57.8; H, 7.2; N, 15.0. Found: C, 58.0; H, 6.8; N, 14.6. Amino acid analysis (theoretical Ala, 2.00; Phe, 1.00; Pro, 2.00): Ala, 1.86; Phe, 0.94; Pro, 2.00.

Results

Enzyme-catalyzed hydrolysis of the substrates investigated here is restricted to a single bond in each substrate. This is shown by the observation of a single set of products on thin-layer chromatography of reaction mixtures (peptides I–XII) and by the uptake of 1 equiv (±5%) of base per mole of substrate hydrolyzed (peptides VII–XII).

Previous workers have demonstrated the strong specificity of the S_1 subsite of α-chymotrypsin using acetyl amino acid esters and amides. Our results in Table I are in good agreement with earlier work and show that α-chymotrypsin has a preference for the acyl bonds of tyrosine, phenylalanine, and leucine (peptides IV–VI) in that order. The high primary specificity of α-chymotrypsin is well-illustrated by our observation that the cleavage of bonds of smaller amino acids (peptides I–III) is so slow as to be unmeasurable by the methods employed here.

Our results indicate that the S_1 subsite of SGP3, like that of α-chymotrypsin, shows a preference for aromatic amino acids. However, SGP3 has a lower primary specificity for P_1. Whereas α-chymotrypsin hydrolyzes the P_1 Phe peptide more than 800 times better than the P_1 Ala and Val peptides and 30 times better than the P_1 Leu peptide (Table I), for SGP3 these ratios are only 300 and 5, respectively (Table II). Due to this broader specificity, SGP3 hydrolyzed all the peptides described here at measurable rates.

The S_1' subsites of α-chymotrypsin (Table III), SGP3 (Table IV), and elastase (Table V) show very similar specificities, with k_{cat}/K_m increasing on going from Gly to Ala to Phe. k_{cat}/K_m for the P_1' Gly peptide is about 2–3-fold

Table III: Kinetic Parameters for α-Chymotrypsin-Catalyzed Hydrolysis of Pentapeptide Amides at pH 8.00.

P_5 P_4 P_3 P_2 P_1 P_1'	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	[S][a] (mM)
Ac-Pro-Ala-Pro-Phe↓Gly-NH_2 (VII)	2390	11.0 ± 0.6	4.6 ± 0.5	1.0–5.4
Ac-Pro-Ala-Pro-Phe-Ala-NH_2 (VIII)	11700	18.7 ± 0.9	1.6 ± 0.2	0.7–3.6
Ac-Pro-Ala-Pro-Phe-Phe-NH_2 (IX)	38100	8.0 ± 0.1	0.21 ± 0.01	0.3–3.6

[a] Range of substrate concentrations.

Table IV: Kinetic Parameters for SGP3-Catalyzed Hydrolysis of Pentapeptide Amides at pH 9.00.

P_5 P_4 P_3 P_2 P_1 P_1'	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	[S][a] (mM)
Ac-Pro-Ala-Pro-Phe↓Gly-NH_2 (VII)	20700	15.1 ± 0.2	0.73 ± 0.04	0.9–3.7
Ac-Pro-Ala-Pro-Phe-Ala-NH_2 (VIII)	310000	40.0 ± 0.7	0.13 ± 0.01	0.2–3.7
Ac-Pro-Ala-Pro-Phe-Phe-NH_2 (IX)	510000	16.3 ± 0.2	0.032 ± 0.002	0.1–3.6
Ac-Pro-Ala-Pro-Ala-Gly-NH_2 (X)	125	1.00 ± 0.04	8.0 ± 0.5	1.6–6.3
Ac-Pro-Ala-Pro-Ala-Ala-NH_2 (XI)	600	2.10 ± 0.05	3.5 ± 0.2	0.9–7.3
Ac-Pro-Ala-Pro-Ala-Phe-NH_2 (XII)	1340	0.94 ± 0.02	0.70 ± 0.07	0.4–7.4

[a] Range of substrate concentrations.

Table V: Kinetic Parameters for Elastase-Catalyzed Hydrolysis of Pentapeptide Amides at pH 9.00.

P_5 P_4 P_3 P_2 P_1 P_1'	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	[S][a] (mM)
Ac-Pro-Ala-Pro-Ala↓Gly-NH_2 (X)	6500	26.0 ± 1.0	4.0 ± 0.3	0.5–5.0
Ac-Pro-Ala-Pro-Ala-Ala-NH_2 (XI)	24700	37.0 ± 0.8	1.5 ± 0.1	0.5–7.6
Ac-Pro-Ala-Pro-Ala-Phe-NH_2 (XII)	28800	18.4 ± 0.2	0.64 ± 0.03	0.5–3.7

[a] Range of substrate concentrations.

greater than that of the corresponding peptide with P_1' NH_2. The most prominent increase in k_{cat}/K_m is almost always associated with the replacement of P_1' Gly by Ala, and in SGP3 this increase can be as great as 15-fold (cf. peptides VII and VIII). A smaller increase in k_{cat}/K_m generally results on going from P_1' Ala to P_1' Phe, although in elastase this increase is hardly perceptible (cf. peptides XI and XII).

Discussion

We have demonstrated above that all our peptides are hydrolyzed at a single bond. k_{cat}/K_m for the hydrolysis reaction may, therefore, be used in discussion of the enzymes' specificity. It is our belief that, for most of the peptides, the unique productive mode of enzyme–peptide binding (S_{54321} and $S_{543211'}$) is the only kinetically significant substrate binding mode. The observed parameters, K_m and k_{cat}, may, therefore, be equated, respectively, with K_s, the dissociation constant of the enzyme–substrate complex, and k_2, the rate constant for acylation of the enzyme (Bender and Kezdy, 1965; Thompson and Blout, 1973a). The absence of significant, nonproductive enzyme–substrate binding is indicated by the fact that nonproductive binding modes either require a proline residue of the substrate to fill the S_3 subsite of these enzymes (S_{65432}, S_{654321} and $S_{43211'}$, $S_{43211'2'}$), an interaction thought to be unfavorable, or will not form the S_1–P_1 interaction (S_{76543} and S_{765432}), which is a strong binding interaction in α-chymotrypsin and SGP3–substrate complexes (Bauer et al., 1976). An exception is the case of acetyl pentapeptide amides X–XII binding to elastase and SGP3. With these enxyme–peptide complexes, the S_1–P_1 interaction is less critical, and both the productive and S_{765432} enzyme–peptide binding modes may be of similar strength (Thompson and Blout, 1973a).

Table VI: Free Energy of the S_1–P_1 Side-Chain Interaction in SGP3 and α-Chymotrypsin.

Peptide	P_1 Side Chain	ΔF^a (SGP3)	ΔF^a (α-Chymotrypsin)
Ac-Pro-Ala-Pro-Ala-NH_2 (II)	Me	−0.6	−0.3
Ac-Pro-Ala-Pro-Val-NH_2 (III)	*i*-Pr	−1.0	−0.5
Ac-Pro-Ala-Pro-Leu-NH_2 (IV)	*i*-Bu	−2.0	−1.2
Ac-Pro-Ala-Pro-Phe-NH_2 (V)	Bzl	−2.2	−2.2
Ac-Pro-Ala-Pro-Tyr-NH_2 (VI)	*p*-OHBzl	−1.6	−2.3

[a] In kcal/mol.

The data in Tables I and II show that α-chymotrypsin and SGP3 have a greater affinity for and a higher rate constant for hydrolysis of substrates with large hydrophobic amino acids at P_1. The improved enzyme–substrate affinity is due entirely to the S_1–P_1 interaction. By comparing the binding energy of each peptide with that of Ac-Pro-Ala-Pro-Gly-NH_2, we can calculate a minimum free energy of binding for each P_1 side chain. These minimum binding energies are presented in Table VI. Of course, the free energy of the S_1–P_1 side chain interaction may be more negative than the binding energy listed if the improved S_1–P_1 interaction causes a rearrangement of other enzyme–substrate contacts. It can be seen from Table VI that the net change in the free energy of binding due to a P_1 benzyl group is the

same for SGP3 and α-chymotrypsin. There are important differences, however, for with α-chymotrypsin a large part of the binding energy is due to hydrophobic parts of the side chain distal to the peptide backbone (cf. the P_1 Phe and P_1 Leu peptides), while for SGP3 these distal interactions are much less important. Instead, for the latter enzyme, interaction with parts of the side chain proximal to the peptide backbone assumes increased importance (cf. the binding energies of P_1 methyl and isopropyl side chains).

For SGP3, k_2 (k_{cat}), like K_s (K_m), is affected most markedly by those parts of the P_1 side chain proximal to the peptide chain. Thus, k_2 increases 25-fold on going from the P_1 Gly substrate to the P_1 Ala analogue (Table II). The 3-fold increase in k_2 going from the P_1 Leu to the P_1 Phe substrate is comparatively small.

For α-chymotrypsin we were unable to measure the very slow rate of hydrolysis of the P_1 Gly, Ala, and Val peptides. The limited data available, however, tend to support the results of Jones et al. (1965) who measured k_{cat}'s for a series of acetyl amino acid methyl esters. These results indicate that the contacts made by C_γ and/or C_δ atoms of the P_1 side chain are particularly important in determining the rate of substrate hydrolysis.

We have pointed out (Bauer et al., 1976) that in SGP3 the S_1–P_1 contact is much less effective in promoting substrate hydrolysis than it is in α-chymotrypsin. We now find that the parts of the P_1 side chain important to SGP3 and α-chymotrypsin are fundamentally different. For α-chymotrypsin the structural details of the S_1–P_1 interaction have been elucidated by x-ray crystallography of complexes between the enzyme and a specific substrate (Steitz et al., 1969). Beyond C_β, the P_1 side chain fits into a deep slot in the enzyme. The all-enveloping nature of this enzyme–substrate contact, together with its capacity to severely restrict the motion of C_α in the enzyme–substrate complex, accounts quite well for the great importance of interactions beyond C_β in determining the kinetic parameters for substrate hydrolysis. The lesser importance of these distal interactions in SGP3 implies a quite different structure for the S_1–P_1 interaction. If there is an S_1 specificity pocket in this enzyme, it might be expected to be larger than that of α-chymotrypsin with a correspondingly looser fit with distal parts of the P_1 side chain. At the same time, SGP3 must form a closer contact with the C_β group of a P_1 side chain than does α-chymotrypsin.

Turning now to the S_1'–P_1' interaction, we can see from Tables III, IV, and V that α-chymotrypsin, SGP3, and elastase respond very similarly to changes in residue P_1'. We find that, in all three enzymes, substrates with P_1' glycine residues are bound less tightly, but are hydrolyzed more rapidly, than those with P_1' amide groups, while substrates with P_1' alanine are bound more tightly and hydrolyzed more rapidly than those with P_1' glycine or amide groups. Similar results have been obtained previously for α-chymotrypsin by Baumann et al. (1970) and Fersht et al. (1973). The latter workers have suggested that this is the result of C_α and C_β of P_1' forming short contacts with the side chain of Ser 195 in the Michaelis complex, but not in the transition state complex for the acylation reaction. The similar P_1' specificities of elastase and SGP3 make it likely that a similar strain mechanism facilitates hydrolysis in these enzymes, although the substrate binding subsites of elastase may not be analogous to these of α-chymotrypsin (Shotton et al., 1972).

The most puzzling aspect of the S_1'–P_1' interaction, however, is the parallel drop in k_{cat} and K_m that results when the P_1' residue is changed from Ala to Phe. While the P_1' Phe peptide could show some nonproductive binding, we consider it unlikely that the existence of a grossly different, nonproductive mode gives rise to this behavior. Instead, it seems possible that the strong interaction of the P_1' Phe side chain with the S_1' subsite, evidenced by the low K_m, may have to be disrupted as the transition state complex is formed. The resulting increase in $\Delta F^\ddagger$, the activation free energy for acylation, would account for the lower value of k_{cat}. Given the large displacement of parts of P_1' which accompany the acylation reaction (Fersht et al., 1973), this is not an unlikely sequence of events.

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