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Active Centers of *Streptomyces griseus* Protease 1, *Streptomyces griseus* Protease 3, and α-Chymotrypsin: Enzyme–Substrate Interactions[†]

Carl-Axel Bauer

ABSTRACT: Kinetic constants for *Streptomyces griseus* protease 1 (SGP1) catalyzed hydrolysis of a number of peptides of increasing chain length have been determined and indicate that the active center of the enzyme extends over 6-7 subsites (21-25 Å). The rate of substrate hydrolysis is highly dependent on the peptide chain length, the rate increase being about 10^7-fold, or more, on going from a specific acetyl amino acid amide to an acetyl heptapeptide amide. This rate increase is largely due to an increase in the acylation rate. The specificity of the S_1 and S_1' subsites of SGP1 has been investigated with peptide substrates. New data on the specificity of these subsites in *Streptomyces griseus* protease 3 and α-chymotrypsin are also presented. Generally, the specificities of the S_1 subsites in the three enzymes are similar. However, there are important differences between the microbial and the pancreatic enzymes' ability to hydrolyze peptides with certain P_1 residues, notably Trp. The implications of the kinetic data for the structures of the S_1 subsites are discussed. Exchanging the P_1' NH_2 group of a tetrapeptide for a P_1' amino acid amide increases the hydrolysis rate in all three enzymes, the increase being as large as 100-fold in the most favorable case. The abilities of the enzymes to use strain in facilitating hydrolysis of the P_1' residue differ markedly. Available kinetic and crystallographic data are used to throw light on the role of the active center structure for the rate of peptide substrate hydrolysis in the above three enzymes and trypsin, elastase, and subtilisin BPN'.

Studies of homologous proteins have provided valuable information about several different aspects of protein chemistry such as functionally and structurally essential amino acid residues, the mechanism of action of certain enzyme families and the relationship between the evolution of species and protein structure. The "chymotrypsin-trypsin" family of serine proteases constitutes such a group of homologous proteins, which includes enzymes from microorganisms to mammals (de Haen et al., 1975).

Pronase, the extracellular enzyme mixture from *Streptomyces griseus,* contains at least three proteases homologous to chymotrypsin (Wählby and Engström, 1968). The finding

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of homologous proteases from species of such widely differing phylogenetic origin prompted a closer comparison between the microbial and the mammalian enzymes. The complete primary structures of these three microbial proteases have been determined and show extensive sequence homology with the pancreatic proteases around the catalytic residues and the disulphide bridges (Jurasek et al., 1974; Olafson et al., 1975). The three-dimensional structure, at 2.8 Å, of one of the enzymes, *Streptomyces griseus* protease 1 (SGP1),[1] shows even closer similarity with chymotrypsin, although there are three major regions of difference (Delbaere et al., 1975). Kinetic studies of one of the other microbial enzymes, SGP3,[2] and α-chymotrypsin indicated that the enzymes have similar primary specificities (Bauer et al., 1976a). However, the ability of the enzymes to hydrolyze peptides of varying chain length was fundamentally different (Bauer, 1976; Bauer et al., 1976b), and we suggested that this might partly be due to the differing abilities of the enzymes to form precise contacts with the amino acids which form the scissile bond, notably the P_1 group.

To shed more light on the relationship between active center structure and substrate chain length dependence we here report studies of enzyme-substrate interactions in SGP1. The dynamics of the enzyme, as indicated by the kinetic data, might also be interesting in relation to the structure of the enzyme, which is now being determined at 1.8-Å resolution (M. James, personal communication). In addition, this paper presents further kinetic investigations of S_1-P_1 and S_1'-P_1' interactions in SGP3 and α-chymotrypsin. The results are discussed in relation to earlier investigations on these enzymes, with special reference to the implications for subsite structures and destabilization mechanisms.

Materials and Methods

α-Chymotrypsin was a triply cystallized preparation from Worthington. SGP1 and SGP3 were homogeneous, lyophilized preparations, which have been described earlier (Bauer, 1977; Bauer and Löfqvist, 1973). Determination of the amount of active α-chymotrypsin and SGP3 was performed according to Bauer et al. (1976b). The concentration of active SGP1 was determined as for SGP3.

The hydrolytic procedure was the same as described by Bauer et al. (1976b). The values pK_a(leucinamide) = 7.58 and pK_a(tyrosinamide) = 7.18 were determined titrimetrically under the conditions used in the substrate assays. Other pK_a values have been given by Bauer (1976) and Bauer et al. (1976a,b). Steady-state kinetic parameters and K_i values were evaluated according to Bauer et al. (1976a,b).

Thin-layer chromatography (TLC) of peptides and reaction mixtures (to establish the bond split by the enzymes) was carried out as described previously (Bauer, 1976), with the addition that the reaction mixtures were developed in an ethanol-25% aqueous ammonia (92:8) system.

Amino acid derivatives and peptide amides were purchased from Cyclo Chemicals and Sigma Chemical Co. All amino acids were of the L configuration. The preparations of Ac-Pro-Ala-Pro-OH and substrates VI and XI-XIII (see Tables I and II for identification) have been described by Thompson and Blout (1973a). The synthesis of peptides II-V, VIII, XIV, XVI, and XVIII have been described by Bauer et al. (1976a,b). Bauer (1976) has described the preparations of peptides IX and X. Peptide VII was a kind gift from Drs. E. R. Blout and R. C. Thompson.

Synthesis of Acetylprolylalanylprolyltryptophanamide (XV). Acetylprolylalanylproline (130 mg, 0.4 mmol) was dissolved in 10 mL of *N*,*N*-dimethylformamide and cooled to −20 °C in a dry ice-CCl_4 bath. *N*-Methylmorpholine (44 μL, 0.4 mmol) was added, followed after 5 min by isobutyl chloroformate (52 μL, 0.4 mmol). After 3 min a precooled solution of tryptophanamide hydrochloride (96 mg, 0.4 mmol) and *N*-methylmorpholine (44 μL, 0.4 mmol) in 10 mL of *N*,*N*-dimethylformamide was added and the solution stirred overnight. The solvent was evaporated in vacuo, the residue dissolved in water, treated with excess Rexyn I-300 resin (Fisher Scientific Co.), filtered, and the water evaporated. The residue was crystallized from ethanol-ether to give 136 mg (67%) of crystals of mp 148-151 °C, and a single tolidine positive spot by TLC, R_f 0.3. Anal. Calcd for $C_{26}H_{34}N_6O_5 \cdot H_2O$: C, 59.1; H, 6.87; N, 15.9. Found: C, 58.9; H, 7.02; N, 15.6.

Synthesis of Acetylprolylalanylprolylphenylalanylleucinamide (XVII). Acetylprolylalanylproline and phenylalanylleucinamide hydrochloride were coupled by the procedure used to prepare compound XV. The solvent was evaporated and the residue was dissolved in 150 mL of chloroform and extracted twice with 0.2 M HCl, twice with 5% aqueous $NaHCO_3$, and once with water. The chloroform phase was dried and evaporated and the residue crystallized from ethyl acetate-ether to give 84 mg (48%) of crystals of mp 119-122 °C, and a single tolidine positive spot by TLC, R_f 0.4. Anal. Calcd for $C_{30}H_{44}N_6O_6 \cdot H_2O$: C, 59.8; H, 7.69; N, 13.9. Found: C, 60.1; H, 7.77; N, 13.9.

Synthesis of Acetylprolylalanylprolylphenylalanyltyrosinamide (XIX). Acetylprolylalanylproline and phenylalanyltyrosinamide were coupled and purified by the procedures used for compound XV. The residue was crystallized from ethanol-ether, giving 66 mg (30%) of crystals of mp 145-148 °C, and a single tolidine positive spot by TLC, R_f 0.4. (The comparatively poor yield is probably due to a partial removal of the coupling product by the resin.) Anal. Calcd for $C_{33}H_{42}N_6O_7 \cdot 2H_2O$: C, 59.1; H, 6.91; N, 12.5. Found: C, 59.3; H, 7.02; N, 12.7.

Results

The Bond Split by the Enzymes. All the substrates described here, except for peptide IV, are hydrolyzed at a single bond, as indicated by arrows in Tables I-IV. Evidence in support of this conclusion is the observation of a single set of products on TLC of reaction mixtures as well as the uptake of only 1 equiv (±5%) of base per mol of substrate hydrolyzed.[3] Peptide IV is cleaved both at the Ala-Phe and the Phe-NH_2 bonds, as indicated by TLC.

Kinetic Effects in SGP1 of Peptide Substrates of Increasing Chain Length. SGP1 hydrolyzes Ac-Phe-NH_2 (cf. Table I) so slowly as to be unmeasurable with the assay method used, although the P_1 residue is optimal (Narahashi, 1972; this work). However, substrate elongation to the acetyl dipeptide amide, II, results in a more than 160-fold increase in k_{cat}/K_m. Further elongations to the tripeptide, III, and the tetrapeptide, V, cause additional increase in k_{cat}/K_m of about 20- and 10-

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[1] SGP1 is identical with "PNPA-hydrolase II" (Wählby, 1969), "alkaline-proteinase c" (Narahashi, 1970), "*Streptomyces griseus* enzyme III" (Gertler and Trop, 1971), "*Streptomyces griseus* protease B (=SGPB)" (Johnson and Smillie, 1971), and "guanidine-stable chymoelastase" (Siegel and Awad, 1973).

[2] SGP3 is identical with "PNPA-hydrolase I", described by Wählby (1969), "alkaline proteinase a" (Narahashi, 1970), "*Streptomyces griseus* enzyme II" (Gertler and Trop, 1971), "*Streptomyces griseus* protease A (=SGPA)" (Johnson and Smillie, 1971), and "lysine-free chymoelastase" (Siegel and Award, 1973).

[3] The uptake of base was tested only for substrates with $k_{cat} \geq 6.7$ s^{-1}.

TABLE I: Kinetic Parameters for SGP1-Catalyzed Hydrolysis of Peptides of Increasing Chain Length (pH 9.00 and 37 °C).

P_6 P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3'		k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	$[S]^a$ (mM)
↓					
Ac-Phe-NH_2	(I)	<0.1	<0.003	K_i = 30	30–60
Ac-Pro-Phe-NH_2	(II)	16	0.079 ± 0.003	4.9 ± 0.6	3–46
Ac-Ala-Pro-Phe-NH_2	(III)	300	1.4 ± 0.2	4.6 ± 1.1	0.9–7.5
Ac-Pro-Ala-Phe-NH_2	$(IV)^b$	<17	<0.16 ± 0.01	>9.5 ± 1.4	2.3–19
Ac-Pro-Ala-Pro-Phe-NH_2	(V)	2 800	6.7 ± 0.3	2.4 ± 0.2	0.5–7.4
Ac-Pro-Ala-Pro-Ala-NH_2	(VI)	6	0.16 ± 0.03	25 ± 5	3.4–14
Ac-Ala-Pro-Ala-Pro-Ala-NH_2	(VII)	13	0.16 ± 0.01	12 ± 1	2.3–18
Ac-Pro-Ala-Pro-Phe-Ala-NH_2	(VIII)	25 200	15.1 ± 0.3	0.60 ± 0.03	0.3–1.4
Ac-Pro-Ala-Pro-Phe-Ala-Ala-NH_2	(IX)	420 000	143 ± 3	0.34 ± 0.02	0.2–1.3
Ac-Pro-Ala-Pro-Phe-Ala-Ala-Ala-NH_2	(X)	780 000	125 ± 2	0.16 ± 0.01	0.2–1.3

[a] Range of peptide concentrations used. [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: Specificity of Subsite S_1 and S_1' of SGP1 (pH 9.00 and 37 °C).

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-Gly-NH_2	(XI)	<0.06	<0.005	K_i = 80	35–67
Ac-Pro-Ala-Pro-Ala-NH_2	(VI)	6.4	0.16 ± 0.03	25 ± 5	3.4–14
Ac-Pro-Ala-Pro-Val-NH_2	(XII)	3.4	0.044 ± 0.003	13 ± 3	6–46
Ac-Pro-Ala-Pro-Leu-NH_2	(XIII)	240	1.2 ± 0.1	5.0 ± 0.8	0.5–8.6
Ac-Pro-Ala-Pro-Phe-NH_2	(V)	2 800	6.7 ± 0.3	2.4 ±0.2	0.5–7.4
Ac-Pro-Ala-Pro-Tyr-NH_2	(XIV)	2 100	7.8 ± 0.2	3.7 ± 0.2	0.5–7.4
Ac-Pro-Ala-Pro-Trp-NH_2	(XV)	120	0.53 ± 0.04	4.5 ± 0.7	1.2–9.4
Ac-Pro-Ala-Pro-Phe-Gly-NH_2	(XVI)	9 500	9.5 ± 0.2	1.0 ± 0.1	0.7–5.7
Ac-Pro-Ala-Pro-Phe-Ala-NH_2	(VIII)	25 200	15.1 ± 0.3	0.60 ± 0.03	0.3–1.4
Ac-Pro-Ala-Pro-Phe-Leu-NH_2	(XVII)	49 000	10.3 ± 0.2	0.21 ± 0.01	0.2–1.9
Ac-Pro-Ala-Pro-Phe-Phe-NH_2	(XVIII)	135 000	11.6 ± 0.2	0.086 ± 0.007	0.2–1.4
Ac-Pro-Ala-Pro-Phe-Tyr-NH_2	(XIX)	48 000	9.1 ± 0.2	0.19 ± 0.01	0.1–1.0

[a] Range of peptide concentrations used.

TABLE III: Specificity of Subsite S_1 and S_1' of SGP3 (pH 9.00 and 37 °C).[a]

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)	$[S]^b$ (mM)
↓					
Ac-Pro-Ala-Pro-Phe-NH_2	$(V)^c$	10 700	5.8 ±0.1	0.54 ± 0.03	0.4–7.1
Ac-Pro-Ala-Pro-Tyr-NH_2	$(XIV)^c$	7 200	10.1 ± 0.2	1.4 ± 0.1	0.9–7.3
Ac-Pro-Ala-Pro-Trp-NH_2	(XV)	115	0.10 ± 0.01	0.87 ± 0.08	0.3–4.6
Ac-Pro-Ala-Pro-Phe-Leu-NH_2	(XVII)	1 000 000	20.0 ± 0.2	0.020 ± 0.002	0.06–1.9
Ac-Pro-Ala-Pro-Phe-Tyr-NH_2	(XIX)	380 000	56.8 ±1.7	0.15 ±0.02	0.1–0.9

[a] For additional data, see Bauer et al. (1976a). [b] Range of peptide concentrations. [c] Data from Bauer et al. (1976a).

TABLE IV: Specificity of Subsite S_1 and S_1' of α-Chymotrypsin (pH 8.00 and 37 °C).[a]

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)	$[S]^b$ (mM)
↓					
Ac-Pro-Ala-Pro-Phe-NH_2	$(V)^c$	820	2.8 ± 0.2	3.4 ± 0.4	0.9–7.5
Ac-Pro-Ala-Pro-Trp-NH_2	(XV)	1 300	2.1 ± 0.1	1.6 ± 0.1	0.7–9.4
Ac-Pro-Ala-Pro-Phe-Leu-NH_2	(XVII)	8 500	10.2 ± 1.1	1.2 ± 0.3	0.4–2.9
Ac-Pro-Ala-Pro-Phe-Tyr-NH_2	(XIX)	38 000	9.9 ± 0.4	0.26 ± 0.03	0.1–1.0

[a] For additional data, see Bauer et al. (1976a). [b] Range of peptide concentrations. [c] Data from Bauer et al. (1976a).

fold, respectively. The tripeptide with a P_3Pro (IV) constitutes an exception in this series of substrates (I–V) in that, unlike peptide III, there is no increase in k_{cat}/K_m on going from the dipeptide, II, to this tripeptide. The similarity in k_{cat}/K_m for peptides VI and VII provides evidence that there are probably no important enzyme–substrate interactions beyond the α carbon of residue P_5.

SGP1 hydrolyzes the peptide with a P_1' Ala residue 9-fold more efficiently than the corresponding analogue with a P_1' NH_2 group (V). Further C-terminal elongation with a P_2' Ala residue results in an additional 17-fold increase in k_{cat}/K_m and shows that S_2'–P_2' interactions are of considerable importance.

A comparatively small increase in k_{cat}/K_m is observed on addition of a P_3' Ala residue (IX), which indicates that there are no important enzyme–substrate interactions beyond the nitrogen atom of residue P_3'.

S_1–P_1 and S_1'–P_1' Interactions in SGP1, SGP3, and α-Chymotrypsin. The specificity of subsite S_1 in SGP1 has been investigated with a number of tetrapeptide amides of the general formula Ac-Pro-Ala-Pro-X-NH_2 (cf. Table II). SGP1 is unable to hydrolyze the peptide with a P_1 Gly at a measurable rate. However, addition of the C_β-methyl group of the P_1 Ala analogue increases k_{cat}/K_m more than 100-fold. Although the P_1 Val compound does not have a k_{cat}/K_m any greater than that of the Ala analogue, further elongation of the P_1 side chain with isobutyryl and benzyl side chains (substrates XIII and V) increases k_{cat}/K_m about 40- and 440-fold, respectively. The high k_{cat}/K_m values for the aromatic P_1 Phe and Tyr analogues show that SGP1 has a preference for such P_1 residues, while the P_1 Leu and Trp compounds are only moderately good substrates.

Table III shows that Ac-Pro-Ala-Pro-Trp-NH_2 is a poor substrate for SGP3, in striking contrast to the other two aromatic analogues (V and XIV). For α-chymotrypsin, on the other hand, the Trp compound is a very good substrate (cf. Table IV).

SGP1 hydrolyzes all the pentapeptides (VIII and XVI–XIX) more or much more rapidly than the tetrapeptide analogue Ac-Pro-Ala-Pro-Phe-NH_2, which has a P_1' amino group only, those pentapeptides with aromatic or long aliphatic P_1' residues being hydrolyzed fastest (cf. Table II).

The new P_1' Leu and Tyr analogues are very good substrates for SGP3 (cf. Table III). However, α-chymotrypsin hydrolyzes the pentapeptides more slowly than SGP1 and much more slowly (about 10–100-fold slower) than SGP3.

Discussion

Catalytic Mechanism of SGP1 and Binding Modes of the Peptides. Prior to a more detailed interpretation of the kinetic data, it is necessary to consider briefly the mechanism of SGP1 as well as the binding modes of the peptides. It will be assumed that SGP1 conforms to the same basic mechanism as α-chymotrypsin (Bender and Kezdy, 1965) and SGP3 (Bauer et al., 1974), because of the close structural similarities between SGP1 and SGP3 (Jurasek et al., 1974; M. James, personal communication). The finding that the deacylation rate for the SGP1-catalyzed hydrolysis of Ac-Pro-Ala-Pro-Phe OMe is 750 s^{-1} (Bauer, unpublished) indicates that acylation is rate determining and K_m a true binding constant for all the peptides described here. However, nonproductive enzyme–substrate complexes of significant strength may be formed, making it impossible to equate K_m with K_s, the dissociation constant of the productive enzyme–substrate complex, and k_{cat} with k_2, the true acylation rate (Bender and Kezdy, 1965; Thompson and Blout, 1973b). By using proline-containing peptides, nonproductive binding to α-chymotrypsin, elastase and SGP3 can be minimized, because at least one subsite in these enzymes, S_3, interacts unfavorably with proline (Thompson and Blout, 1973b; Bauer et al., 1976b). The unfavorable kinetic constants for the tripeptide with a P_3 Pro (cf. Table I, peptides II–IV) indicate that SGP1 shows a similar discrimination toward P_3 Pro.

Kinetic and crystallographic investigations on chymotrypsin and trypsin have shown that, whereas a P_3 amino acid residue forms 2 H bonds and several van der Waals contacts with the S_3 subsite, a P_3Pro forms, at best, a single weak H bond and few van der Waals contacts (Segal et al., 1971; Blow et al., 1972; Rühlmann et al., 1973). It is likely that the P_3 Pro interactions with the S_3 subsites in SGP1/SGP3 are similarly unfavorable. The only strong binding mode for the peptides used here is therefore, probably, the productive binding mode indicated in the tables, justifying the approximations $K_m \approx K_s$ and $k_{cat} \approx k_2$. A discussion of the binding modes of the peptides to SGP3 and α-chymotrypsin has been presented earlier (Bauer, 1976; Bauer et al., 1976a), and will be equally applicable to SGP1.

Kinetic Effects in SGP1 of Peptide Substrates of Increasing Chain Length. As can be seen from Table I, the extremely low k_{cat}/K_m value for the SGP1-catalyzed hydrolysis of Ac-Phe-NH_2 is due to the enzyme's inability to form an acyl-enzyme ($k_{cat} \approx k_2$), but not an enzyme–substrate complex, with this substrate. N-terminal elongations of the substrate peptide chain up to the tetrapeptide (V), however, result in large increases in the acylation rate. The overall increase in k_2 is greater than 2000-fold on going from I to V, whereas the increase in enzyme–substrate affinity is only about 12-fold. A similar marked dependence on substrate chain length has been noted for SGP3, while α-chymotrypsin shows a weak dependence (Bauer et al., 1976b).

C-terminal elongations of the peptide chain (cf. Table I, peptides V and VIII–X) result in a more efficient hydrolysis by SGP1 due to increases in both acylation rates and enzyme–substrate affinities. The most remarkable increase observed in this series of substrates is the approximately 10-fold increase in acylation rate which occurs on going from the pentapeptide (VIII) to the hexapeptide (IX). The relatively small increase in binding energy on going from VIII to IX indicates that most of the free energy of binding of the P_2' Ala group is used to reduce the free energy of activation for acylation. Similar results have been observed for SGP3 and, although less pronounced, also for α-chymotrypsin (Bauer, 1976).

S_1–P_1 Interactions in SGP1, SGP3, and α-Chymotrypsin. SGP1 is unable to hydrolyze Ac-Pro-Ala-Pro-Gly-NH_2 at a measurable rate, due to poor binding and extremely low acylation rate (cf. Table II). The addition of the C_β-methyl group of the P_1 Ala analogue increases the acylation rate at least 30-fold. This is the single most important rate increase observed for the tetrapeptides and points to important interactions between the enzyme's S_1 subsite and the P_1 C_β-methyl group. A similarly important rate increase in the hydrolysis of P_1 Ala containing peptides has been noted for SGP3, but not for α-chymotrypsin (Bauer et al., 1976a). These findings indicate some significant difference in the S_1 structures of the microbial and the pancreatic enzymes.

The data of Table II clearly demonstrate that Phe and Tyr are the optimal P_1 residues, in good agreement with earlier specificity studies on acetyl amino acid *p*-nitrophenyl esters (Narahashi, 1972), and on the insulin B chain (Narahashi and Yoda, 1973). Both SGP1- and SGP3-catalyzed hydrolysis of the P_1 Tyr analogue is characterized by K_ms and k_{cat}'s higher than those for the P_1 Phe analogue (cf. Tables II and III). Apparently the microbial enzymes can use the phenolic OH of the Tyr peptide to destabilize the Michaelis complex of this substrate with respect to its transition state.

Although SGP1 and SGP3 show a similar specificity to that of α-chymotrypsin in their high activities toward the Phe and Tyr analogues, this is clearly not the case with the Trp analogue (cf. Tables II–IV). While α-chymotrypsin binds the Trp analogue tightest of the various P_1 amino acid residues and is rapidly acylated (cf. Table IV and Bauer et al., 1976a), both SGP1 and SGP3 bind the Trp analogue significantly less well than the Phe analogue, and, more important, are acylated much slower by the former compound.

S_1'-P_1' Interactions in SGP1, SGP3, and α-Chymotrypsin. A comparison of the S_1'-P_1' interactions in the three enzymes points to several interesting differences between the S_1' subsites. For example, there is evidence that α-chymotrypsin is able to employ strain in the hydrolysis of peptides containing P_1' Gly and Ala, due to C_α and C_β of P_1' forming short contacts with the side chain of Ser-195 in the ES complex, but not in the ES transition state complex (Fersht et al., 1973). SGP3 probably uses a similar strain mechanism in the hydrolysis of such peptides (Bauer et al., 1976a). However, these enzymes react differently toward a P_1' Phe containing peptide, the kinetics indicating tight binding and a decreased acylation rate. It was suggested that the tight binding of the benzyl side chain to the S_1' subsite of the enzymes might result in a displacement of the $P_1'C_\alpha$ and C_β relieving the strain in the ES complex (Bauer et al., 1976a).

The kinetic data for α-chymotrypsin (cf. Table IV) indicates tight binding and comparatively modest acylation rates for the P_1' Leu and Tyr peptides and provides evidence that these peptides show the same behaviour as the P_1' Phe analogue. SGP3-catalyzed hydrolysis of the P_1' Leu analogue (cf. Table III) is also similar to the P_1' Phe peptide, in terms of binding and acylation. However, SGP3 binds a P_1' Tyr (XIX) much weaker and cleaves that substrate much more rapidly than the P_1' Leu peptide, indicating that there is strain in the ES complex with the former peptide.

SGP1 reacts differently to SGP3 and α-chymotrypsin toward several of the P_1' pentapeptides. The effects of the P_1' Gly residue of substrate XVI on the activity of SGP1 are to increase the enzyme–substrate affinity about 2.4-fold and the acylation rate about 1.4-fold, compared with the P_1' NH_2 analogue (cf. Table II). Exchanging the P_1' Gly for aliphatic (Ala or Leu) or aromatic (Phe or Tyr) amino acid residues results in increased enzyme–substrate affinities but has little effect on the acylation rate. It appears, therefore, that SGP1 does not use a strain mechanism to facilitate hydrolysis of any of the P_1' residues of these peptides.

Implications of the Kinetic Data for the Active Center Structures. Since there were no important enzyme–substrate interactions in SGP1 beyond the α carbon of a P_5 residue or beyond the nitrogen atom of a P_3' residue, the active center of the enzyme extends over 6–7 subsites corresponding to 21–25 Å. The active center of SGP1 should therefore be of a similar size to those of SGP3 (Bauer, 1976) and elastase (Atlas et al., 1970; Thompson and Blout, 1970, 1973a), but probably slightly larger than that of α-chymotrypsin (Bauer, 1976).

The S_1 subsites of all three enzymes have similar affinities for a P_1 Phe residue, both in Ac-Phe-NH_2 (dissociation constants of 20–30 mM) and in the tetrapeptide Ac-Pro-Ala-Pro-Phe-NH_2 (apparent binding energies of the P_1 Phe being about −2.2 kcal/mol), indicating that also SGP1 and SGP3 have a hydrophobic S_1 subsite (Bauer et al., 1976b; this work). An important difference, however, is the extremely low acylation rates for Ac-Phe-NH_2 observed in the microbial enzymes ($k_{cat}(SGP1) < 0.003\ s^{-1}$ and $k_{cat}(SGP3) = 0.007\ s^{-1}$) compared with α-chymotrypsin ($k_{cat} = 0.22\ s^{-1}$). This points to an inability of SGP1 and SGP3 to orient the scissile bond properly in relation to the catalytic residues when the substrate is an acetyl amino acid amide. The S_1 subsites of SGP1 and SGP3 might therefore be less restrictive with a looser fit than that of α-chymotrypsin. Only when the microbial enzymes are able to form contacts with additional amino acid residues, such as in the tetrapeptide, are they able to compensate for this and achieve full catalytic activity. The kinetic indications that SGP1 and SGP3 have S_1 subsites of looser fit than α-chymotrypsin are consistent with crystallographic data at 2.8 Å resolution (Delbaere et al., 1975; M. James, personal communication).

A second major difference between the S_1 subsites is indicated by the inability of the microbial enzymes to hydrolyze the P_1 Trp tetrapeptide, which is an excellent substrate for α-chymotrypsin (cf. Tables II–IV). The high activity of α-chymotrypsin for this substrate is consistent with the finding of Steitz et al. (1969) that the indolyl side chain of a Trp residue fits well into the hydrophobic S_1 pocket of the enzyme. The relatively low affinities of SGP1 and SGP3, compared with α-chymotrypsin, for the P_1 Trp residue, might reflect S_1 subsites too small to accommodate completely that residue in the former enzymes. This is further substantiated by the very low acylation rates for SGP1 and SGP3, despite the substrate being a tetrapeptide, where the S_{432}-P_{432} enzyme–substrate interactions should contribute to a favorable orientation of the scissile bond.

The comparatively high affinities of the enzymes for certain aliphatic and aromatic P_1' amino acid residues indicate that the S_1' subsites are able to form a number of favorable contacts with the P_1' residues. This is consistent with the S_1'-P_1' interactions deduced from the model for the association of pancreatic trypsin inhibitor with chymotrypsin (Blow et al., 1972). For SGP1 and SGP3 the kinetic properties of the S_1' subsites of the enzymes (cf. Tables II and III and Bauer et al., 1976a) should help in localizing the subsites in the three-dimensional models of the enzymes.

The kinetic data presented here, and by Bauer et al. (1976a,b), indicate close structural similarity between the active centers of SGP1 and SGP3. However, several results point to small structural differences between the two microbial proteases. One such difference is that k_{cat} of many of the substrates is at least twofold lower for SGP1 than for SGP3 (cf. Bauer et al., 1976a,b). An exception is the P_1 Trp analogue which is cleaved five-fold faster by SGP1 than by SGP3, thus indicating a considerably better orientation of the Trp residue in SGP1 than in SGP3. Another difference is the approximately fourfold better binding of the P_{54} Ac-Pro group to SGP3 compared with SGP1 (cf. the K_m values of peptide V), a difference which is reflected in the K_m of all other peptides containing this group.

The Role of the Active Center Structure for the Rate of Hydrolysis of Peptide Substrates in Serine Proteases. The active centers of several serine proteases have been extensively characterized, both by studies of the kinetics of enzyme–substrate interactions and by determination of the tertiary structures with x-ray crystallography. These investigations point to some major differences between the enzymes with regard to the structure of the S_1 subsite and the importance of enzyme–substrate contacts outside the S_1 subsite. Thus, chymotrypsin and trypsin have S_1 subsites with good to excellent fit for their optimal P_1 residues (Steitz et al., 1969; Rühlmann et al., 1973; Sweet et al., 1974). Substrate contacts outside the S_1 subsite are relatively unimportant, the increase in k_{cat}/K_m on going from an acetyl amino acid amide to a peptide which covers the entire active center being about 4000-fold (Bauer, 1976; Bauer et al., 1976b) and 300-fold (Izumiya and Uchio, 1959; Yamamoto and Izumiya, 1967), respectively. In contrast, elastase, SGP1, SGP3, and subtilisin BPN′ all have S_1 subsites characterized as ill defined, wide, or less restrictive (Shotton et al., 1972; Wright, 1972; Delbaere et al., 1975; M. James, personal communication). These enzymes are all highly dependent on substrate contacts outside the S_1 subsite, the increase in k_{cat}/K_m, except for subtilisin BPN′, being of the order of 10^7-fold or more, under the same conditions as above (Morihara et al., 1970; Thompson and Blout, 1970, 1973a

Bauer, 1976; Bauer et al., 1976b; this work).

It is clear that the above x-ray crystallographic and kinetic data are entirely consistent with the suggestion of Bauer et al. (1976b) that enzymes which are able to form precise S_1–P_1 interactions are little dependent on enzyme–substrate contacts remote from the scissile bond, while the reverse is true for enzymes with less restrictive S_1 subsites. However, the molecular details by which these remote enzyme–substrate interactions in certain proteases so dramatically affect the turnover rate are largely unknown. Efforts should now be directed toward an elucidation of this mechanism, which also might be relevant for our understanding of the "one substrate specificity" of some of the proteases involved in "biological control systems".

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