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A Kinetic Investigation of Subsites S_1' and S_2' in α-Chymotrypsin and Subtilisin BPN'

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A kinetic study was conducted to investigate the properties of subsites S_1' and S_2' of α-chymotrypsin and subtilisin BPN', which were deduced from model complexes with a pancreatic trypsin inhibitor and a hexapeptide substrate, respectively. For this purpose, Cbz-Gly-Pro-Phe-AA and Cbz-Gly-Pro-Phe-AA-AA (AA, various amino acid residues) were synthesized. Since they were susceptible to cleavage at the positions shown by the arrows, we could examine the effect of P_1' or P_2' amino acid residue on hydrolysis [amino acid residues in peptide substrates and the corresponding subsites in enzymes are numbered according to the system of Schechter and Berger (1967) *Biochem. Biophys. Res. Commun.* 27, 157-162]. The results agreed well with interactions of the leaving group with the corresponding subsites in both enzymes, which were deduced from the model complexes.

Both α-chymotrypsin (2) and subtilisin BPN' (2, 3) have a large active site corresponding to at least five or six amino acid residues, three or four (P_1–P_3 or P_1–P_4)[1] to the N-terminal and two (P_1' and P_2') to the C-terminal from the splitting point in peptide substrates. The subsites S_1–S_3 or S_1–S_4 and S_1'–S_2', respectively, are numbered by the nomenclature of Schechter and Berger (1). X-ray study of both γ-chymotrypsin (4) and subtilisin BPN' (5) inactivated with peptide chloromethyl ketone derivatives indicated that their polypeptide portions (P_1–P_3) are bound in antiparallel β-sheet fashion to Ser(214)-Trp(215)-Gly(216) and Ser(125)-Leu(126)-Gly(127), respectively. This can be applied to the binding of true polypeptide substrates and their acyl enzyme intermediates for several reasons (4–6). The positions of both enzymes corresponding to the polypeptide portions (P_1–P_3) of the inhibitors mentioned above can thus be regarded as subsites S_1–S_3. On the other hand, it was difficult to deduce the positions of subsites S_1' and S_2' directly from X-ray study. The corresponding positions of α-chymotrypsin were therefore assumed from a model complex with a pancreatic trypsin inhibitor (7, 8) and those of subtilisin BPN' were assumed from a model enzyme–substrate complex with a hexapeptide substrate (9).

In chymotrypsin, the P_1' amino acid residue (Ala 16) of the pancreatic trypsin inhibitor is closely in contact with His 57, Cys 42, Met 192, Gly 193, and Ser 195, which constitute the active site; a hydrogen bond contact can be made between the main chain NH of the P_1' amino acid residue and $N^{\epsilon 2}$ of His 57 in the enzyme (acyl enzyme). As for the P_2' amino acid residue (Arg 17), the main chain NH forms a hydrogen bond with CO of Phe 41 of the enzyme, and the residue forms numerous van der Waals contacts with His 40, Phe 41, Trp 141, Gly 142, Leu 143, Met 192, Gly 193, Asp 194, and Ser 195 of the enzyme. With subtilisin BPN', the polypeptide portion (P_1' and P_2') of the hexapeptide substrate interacts with Asn(218)–Thr(220); the main chain CO and NH groups of the P_2' amino acid residue form hydrogen bonds with the corresponding backbone NH and CO of Asn 218.

[1] Abbreviations used: Amino acid residues in peptide substrates and the corresponding subsites in enzymes are numbered according to the system of Schechter and Berger (1). AA, various amino acid residues; OEt, ethyl ester; ONph, nitrophenyl ester; OBu^t, t-butyl ester; OMe, methyl ester.

The present kinetic study was undertaken to investigate the properties of subsites S_1' and S_2' of α-chymotrypsin and subtilisin BPN' in relation to their tertiary structures deduced from a model complex, as given above. For this purpose, peptide substrates such as Cbz-Gly-Pro-Phe-AA and Cbz-Gly-Pro-Phe-AA-AA (AA = various amino acid residues) were synthesized, in which susceptible peptide bonds were expected at the positions shown by the arrows. We considered that with hydrolysis (or acylation), the acyl portion of the peptides (Cbz-Gly-Pro-Phe) binds tightly and correctly to the active site of the respective enzyme for the following reasons. (1) The Phe (P_1) side chain is held in the hydrophobic pocket, (2) the Pro (P_2) residue cannot occupy subsite S_1 or S_3, but can occupy S_2 because its amino group cannot form a hydrogen bond (the main chain NH groups of P_1 and P_3 amino acid residues are joined by hydrogen bonds to the corresponding CO groups of the enzymes), and (3) the main chain NH and CO groups of Gly (P_3) are connected by hydrogen bonds to the corresponding NH and CO groups of subsite S_3. These have been observed by X-ray study (4, 5).

MATERIALS AND METHODS

(a) Enzymes

α-Chymotrypsin (3× recrystallized) and subtilisin BPN' (crystals, "Nagarse") were supplied by Worthington Biochemicals Corporation, New Jersey and by Nagase Co., Ltd., Osaka, Japan, respectively.

(b) Substrates

Ac-Tyr-OEt was obtained from the Protein Research Foundation, Osaka. The other peptides were synthesized as follows.

Cbz-Gly-Pro-Phe-AA. Cbz-Gly-Pro-ONph was synthesized from Cbz-Gly-Pro (supplied from the Protein Research Foundation, Osaka) by coupling with *p*-nitrophenol (carbodiimide method). Phe-AA-OBu^t (AA: glycine, L-alanine, L-valine, L-leucine, D-leucine, and L-proline) was prepared by hydrogenolysis (Pd-black) of the corresponding Cbz derivatives, which had been synthesized by coupling Cbz-Phe-ONph with the tertiary butyl esters of various amino acids. Phe-NH_2 and Phe-OEt were obtained commercially. Cbz-Gly-Pro-Phe-AA was thus prepared by coupling Cbz-Gly-Pro-ONph with Phe-AA-OBu^t, Phe-NH_2, and Phe-OEt and by degradation of the tertiary butyl esters (if necessary) with an excess of dry HCl in acetic acid (N/2, 5-7 mole equivalent).

Cbz-Gly-Pro-Phe-AA-NH_2. Cbz-Phe-AA-NH_2 was synthesized by coupling AA-NH_2 (AA, glycine and L-leucine; commercially available) with Cbz-Phe-ONph. Coupling of Cbz-Gly-Pro-ONph with Phe-AA-NH_2, prepared by hydrogenolysis of the corresponding Cbz derivatives, gave the final products.

Cbz-Gly-Pro-Phe-AA-Ala. Cbz-Phe-AA-Ala-OBu^t was synthesized by stepwise elongation using Ala-OBu^t, Cbz-AA-ONph (AA, D- and L-leucine), and Cbz-Phe-ONph as the starting materials. The final products were obtained by coupling Cbz-Gly-Pro-ONph with Phe-AA-Ala-OBu^t, and then degrading the tertiary butyl ester.

Cbz-Gly-Pro-Phe-Pro-Leu. Cbz-Phe-Pro-Leu-OBu^t was synthesized by stepwise elongation using Leu-OBu^t, Cbz-Pro-ONph, and Cbz-Phe-ONph as the starting materials. Coupling Cbz-Gly-Pro-ONph with Phe-Pro-Leu-OBu^t and degradation of the tertiary butyl ester gave the final product.

Cbz-Gly-Pro-Phe-Gly-AA. Cbz-Phe-Gly-AA-OBu^t was synthesized by stepwise elongation using AA-OBu^t (AA: glycine, D- and L-leucine, and L-proline), Cbz-Gly-ONph, and Cbz-Phe-ONph as the starting materials. Coupling of Cbz-Gly-Pro-ONph with Phe-Gly-AA-OBu^t, prepared by hydrogenolysis of the corresponding Cbz derivatives, gave Cbz-Gly-Pro-Phe-Gly-AA-OBu^t, from which the final products were obtained by degradation of the tertiary butyl ester.

The analytical data and melting points of these compounds are listed in Table 1.

(c) Enzymatic Assay

Esterase activity was determined using a Radiometer Type TTT1 pH stat equipped with a syringe buret, a Type SBR2c recorder, and a thermostatically controlled reaction vessel (30°C). Reactions were carried out in 0.1 M KCl at pH 7.5 with 0.05 N NaOH as titrant. The hydrolyses of various synthetic peptides were measured as follows. A reaction mixture (1 or 5 ml) containing 0.05 M Tris buffer (pH 7.5), an appropriate concentration of peptide, and a suitable amount of enzyme was incubated at 40°C. At various intervals, 0.1 ml (or 1 ml) of the reaction mixture was withdrawn and put into a test tube containing 1 or 2 ml of 0.05 M citrate buffer (pH 5.0), which then was cooled in an ice bath to prevent further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking (10).

The ninhydrin color yield of NH_3, Gly-NH_2, Leu-NH_2, Gly-Gly, Gly-Leu, Gly-Pro, or Leu-Ala, based on L-leucine as 100%, was described in the preceding papers (3, 11, 12). The color yield of Gly-D-Leu or D-Leu-Ala was regarded as being identical to that of the corresponding L peptide. With the other

TABLE I

MELTING POINTS AND ELEMENTARY ANALYSES OF VARIOUS SYNTHETIC PEPTIDES

Compounds	Melting point (°C)		Elementary analysis (%)[a]		
			C	H	N
Cbz-Gly-Pro-Phe-OEt	Oil	Cd	64.85	6.49	8.73
		Fd	64.81	6.39	8.84
Cbz-Gly-Pro-Phe-NH_2	170.5–171.5	Cd	63.70	6.24	12.38
		Fd	63.79	6.23	12.21
Cbz-Gly-Pro-Phe-Gly-OH	206–208	Cd	61.17	5.92	10.97
		Fd	60.99	6.04	10.96
Cbz-Gly-Pro-Phe-Ala-OH	162–164	Cd	61.82	6.15	10.58
		Fd	61.81	6.19	10.80
Cbz-Gly-Pro-Phe-Val-OH	93–95	Cd	63.03	6.57	10.14
		Fd	63.09	6.68	9.97
Cbz-Gly-Pro-Phe-Leu-OH	90–92	Cd	63.59	6.76	9.89
		Fd	63.58	6.77	9.69
Cbz-Gly-Pro-Phe-D-Leu-OH	86–88	Cd	63.59	6.76	9.89
		Fd	63.52	6.78	9.73
Cbz-Gly-Pro-Phe-Pro-OH	91–93	Cd	63.26	6.22	10.18
		Fd	63.40	6.32	9.68
Cbz-Gly-Pro-Phe-Gly-NH_2	175–178	Cd	61.29	6.13	13.74
		Fd	61.14	6.31	13.57
Cbz-Gly-Pro-Phe-Leu-NH_2	167–169	Cd	63.70	6.95	12.38
		Fd	63.91	6.77	12.45
Cbz-Gly-Pro-Phe-Leu-Ala-OH	188–190	Cd	62.15	6.80	10.98
		Fd	62.33	6.83	10.79
Cbz-Gly-Pro-Phe-D-Leu-Ala-OH	100–102	Cd	62.15	6.80	10.98
		Fd	62.26	6.85	10.94
Cbz-Gly-Pro-Phe-Pro-Leu-OH	95–97	Cd	63.33	6.84	10.55
		Fd	63.47	6.80	10.40
Cbz-Gly-Pro-Phe-Gly-Gly-OH	92–94	Cd	59.25	5.86	12.34
		Fd	59.30	6.12	12.09
Cbz-Gly-Pro-Phe-Gly-Leu-OH	98–100	Cd	61.62	6.63	11.23
		Fd	61.69	6.63	11.47
Cbz-Gly-Pro-Phe-Gly-D-Leu-OH	96–98	Cd	61.62	6.63	11.23
		Fd	61.32	6.72	11.02
Cbz-Gly-Pro-Phe-Gly-Pro-OH	110–112	Cd	61.27	6.14	11.53
		Fd	61.30	5.83	11.57

[a] Cd, calculated; Fd, found.

products, calculations were made using L-leucine as the standard.

The sites of enzyme action upon the substrates were determined by paper chromatography of the hydrolyzates or by the standard dinitrophenyl method. The release of ammonia from the substrates was examined using Conway's apparatus.

(d) Kinetic Study

In all cases, the data were in accordance with Michaelis-Menten kinetics over the range of substrate concentrations employed (~10 mM). Five to eight runs were performed for each determination of K_m and k_{cat} (V per molar equivalent of enzyme). The enzyme concentration was chosen to give reliable data for the initial rate of hydrolysis. In the calculation of k_{cat}, the molecular weights of α-chymotrypsin and subtilisin BPN' were taken as 25,000 (13) and 27,600 (14), respectively.

The inhibition constant (K_i) was determined from plots of $1/v$ vs I for three substrate concentrations (2, 3, and 5 mM). Ac-Tyr-OEt was used as substrate. The value on the abscissa that corresponds to the point where these three lines intersect is equal to $-K_i$.

RESULTS AND DISCUSSION

Table II shows the esterase activity against Cbz-Gly-Pro-Phe-OEt by α-chymotrypsin and subtilisin BPN', and a comparison is made with the hydrolyses of a peptide ester series of Ac-$(Ala)_n$-Phe-OMe (n = 0-2). In the latter series, increased esterase activity of both enzymes is found on elongating the peptide chain in the ester substrates. The proteolytic coefficient of both enzymes with Cbz-Gly-Pro-Phe-OEt is comparable to that with Ac-$(Ala)_2$-Phe-OMe. Thus, each amino acid residue of the acyl portion (Cbz-Gly-Pro-Phe) of the peptide series used in this study probably binds to the corresponding subsite, as had been expected.

Hydrolyses of Cbz-Gly-Pro-Phe-AA by α-chymotrypsin and subtilisin BPN' are shown in Table III (A). This peptide series is insensitive to α-chymotrypsin. A similar effect is observed with subtilisin BPN', although the hydrolysis depends partly upon the nature of the amino acid residue, such as glycine and L-alanine. The peptide series, however, shows competitive inhibition of hydrolysis of Ac-Tyr-OEt by both enzymes, the K_i values corresponding to the K_m of the relative peptide series Cbz-Gly-Pro-Phe-AA-AA, which is susceptible to the enzymes (Table III, C and D). The results indicate that the carboxylate ion at the P_1' position disturbs catalysis, but not binding. Possibly, the carboxylate ion interferes with the van der Waals' contact with Met 192 in α-chymotrypsin or Met 222 in subtilisin BPN', resulting in an incorrect orientation of the susceptible carbonyl carbon of substrates toward the oxygen of the active serine residue in the enzymes (in acylation process). This may be a reason for both enzymes being endopeptidases.

The previous study (2) using Cbz-Tyr-AA-NH_2 as substrate indicated that the P_1' specificity of α-chymotrypsin is L-tyrosine > L-leucine, L-alanine > glycine > D-alanine. Baumann *et al.* (16) have independently shown that the proteolytic coefficient (k_{cat}/K_m) of α-chymotrypsin for hydrolysis of Ac-Tyr-Ala-NH_2 is much higher (>10 times) than that for Ac-Tyr-Gly-NH_2, mainly related to catalysis (k_{cat}). These results were not inconsistent with the model complex with pancreatic trypsin inhibitor as shown by Fersht, Blow, and Fastrez (8). The P_1' specificity of subtilisin BPN', using either Cbz-Tyr-AA-NH_2 (2) or Cbz-Gly-Leu-AA (3), was not clear because of the considerably small

TABLE II

ESTERASE ACTIVITY AGAINST N-ACYLATED TRIPEPTIDE ESTERS

Substrate					α-Chymotrypsin		Subtilisin BPN'	
P_4	P_3	P_2	P_1	P_1'	K_m (mM)	k_{cat}/K_m (mM^{-1} sec^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} sec^{-1})
		Ac	Phe	OMe[a]	0.57	55	28	3.6
	Ac	Ala	Phe	OMe[a]	0.05	330	1.1	230
Ac	Ala	Ala	Phe	OMe[a]	0.03	2000	0.32	930
Cbz	Gly	Pro	Phe	OEt	0.044	1060	0.2	2300

[a] Data from Morihara and Oka (15).

TABLE III

KINETIC PARAMETERS FOR HYDROLYSIS OF THE Cbz-Gly-Pro-Phe PEPTIDE SERIES BY α-CHYMOTRYPSIN AND SUBTILISIN BPN′

Group	Peptides						α-Chymotrypsin				Subtilisin BPN′			
		P_3	P_2	P_1	P_1'	P_2'	Hydrolysis[a] (%) 0.5 h	Hydrolysis[a] (%) 20 h	K_m or K_i[b] (mM)	k_{cat}/K_m (M^{-1} sec^{-1})	Hydrolysis[a] (%) 0.5 h	Hydrolysis[a] (%) 20 h	K_m or K_i[b] (mM)	k_{cat}/K_m (M^{-1} sec^{-1})
	Cbz	Gly	Pro	Phe ↓	Gly			0	(1.4)		8	68	2.9	50
	Cbz	Gly	Pro	Phe	Ala			0	(2.0)		11	100	1.9	70
A	Cbz	Gly	Pro	Phe	Val			0	(1.9)			0	(0.7)	
	Cbz	Gly	Pro	Phe	Leu			0	(1.3)			0	(1.1)	
	Cbz	Gly	Pro	Phe	D-Leu			0	(1.6)			0	(2.1)	
	Cbz	Gly	Pro	Phe	Pro			0	(1.1)			0	(2.5)	
	Cbz	Gly	Pro	Phe	NH_2		0	37	2.4	40	52	100	8.2	1,100
B[c]	Cbz	Gly	Pro	Phe	Gly	NH_2	4	35	2.0	40	91	100	1.6	1,200
	Cbz	Gly	Pro	Phe	Leu	NH_2	32	100	1.5	150	14	85	13.6	34
	Cbz	Gly	Pro	Phe	Leu	Ala	40	100	0.5	700	87	100	0.3	9,000
C	Cbz	Gly	Pro	Phe	D-Leu	Ala		0	(1.2)			0	(2.3)	
	Cbz	Gly	Pro	Phe	Pro	Leu		0	(0.3)			0	(0.9)	
	Cbz	Gly	Pro	Phe	Gly	NH_2	23	100	1.9	200	85	100	3.7	2,300
	Cbz	Gly	Pro	Phe	Gly	Gly	14	100	1.3	200	92	100	3.7	6,300
D	Cbz	Gly	Pro	Phe	Gly	Leu	73	100	0.4	1,700	100	100	0.6	34,800
	Cbz	Gly	Pro	Phe	Gly	D-Leu	8	65	(0.7)		94	100	0.9	5,300
	Cbz	Gly	Pro	Phe	Gly	Pro	0	20	(1.3)		20	100	2.4	200

[a] The reaction mixture (1 ml) contained 2 mM substrate, 0.05 mg of enzyme, and 0.1 M Tris buffer (pH 7.5) and was kept at 40°C.

[b] The inhibition constants (K_i) are shown in parentheses.

[c] Due to the low solubility of the peptide group, the reaction was performed in the presence of 10% dimethylformamide.

hydrolysis. The result, however, indicated that a small amino acid residue, such as alanine or glycine, is better than a bulky one. A similar case is observed with the hydrolyses of Cbz-Gly-Pro-Phe-AA by subtilisin BPN′, as seen in Table III (A).

The P_1' specificity of both enzymes was further studied using Cbz-Gly-Pro-Phe-AA-NH_2 as substrate (Table III, B). The results supported the above view. The previous study of subtilisin BPN′ (3, 17), using either Cbz-AA-Leu-NH_2 or Cbz-AA-Lys-OMe as substrate, showed that alanine is the most effective residue at the P_2 position, bulkier residues tend to inhibit the hydrolysis. This is supported by X-ray study (5). Nevertheless, it has been shown (18) that Cbz-Phe-AA-NH_2 is susceptible at the amide bond when AA is hydrophobic or bulky. These results may thus indicate that residues bulkier than alanine reject the P_1' position more markedly than the P_2. Subsite S_1' of subtilisin BPN′ is a shallow surface, pocket-lined with the side chains of Tyr 217 and His 64, with Met 222 deeper inside the surface. Probably, the P_1' group comes particularly close to the active site imidazole of His 64, and the bulky residues would be poor choices of P_1'.

Both Cbz-Gly-Pro-Phe-D-Leu-Ala and Cbz-Gly-Pro-Phe-Pro-Leu were not hydrolyzed at all by α-chymotrypsin or by subtilisin BPN′, which was determined by lack of an increase of the ninhydrin color value of the reaction mixture or by paper chromatography of the hydrolyzate (Table III, C). In the former case, the P_1' carbonyl group cannot form a van der Waals contact with Met 192 in α-chymotrypsin or with Met 222 in subtilisin BPN′; thus, the acylation process is blocked as discussed above. In the latter case, the P_1' amino acid (L-proline) cannot accept a proton from $N^{\epsilon 2}$ of the active histidine during catalysis; this is an essential process for acylation, which was deduced from X-ray (9, 19). Cbz-Gly-Pro-Phe-Leu-Ala is considerably hydrolyzed by subtilisin BPN′ (Table III, C), overcoming the inhibitory effect of L-leucine at the P_1' position, as mentioned above, which may suggest the significance of the alanine residue at the P_2' position.

The effects of the kind of amino acid residues at the P_2' position for hydrolysis of both enzymes were studied using Cbz-Gly-Pro-Phe-Gly-AA as substrate. The results in Table III (D) indicate that the proteolytic coefficients (k_{cat}/K_m) differ with the kind of amino acid residue at the position, as follows: L-leucine > glycine, amide > D-leucine > L-proline in α-chymotrypsin and L-leucine > glycine, D-leucine > amide >> L-proline in subtilisin BPN′. The marked effect of the L-leucine residue at the position is mainly related to the binding (K_m), which may be due to the hydrophobic contact between the side chain of the residue and the corresponding subsite (S_2') of the respective enzyme. The reason for the L-proline residue being the poorest may not solely be ascribed to an inability to form hydrogen bonds between the imino group and the CO group of Phe 41 in α-chymotrypsin or Asn 218 in subtilisin BPN′, but also to the steric hindrance at the enzyme subsite. Cbz-Gly-Pro-Phe-Gly-D-Leu is hydrolyzed weakly by α-chymotrypsin, but is hydrolyzed to some extent by subtilisin BPN′. This may be related to the looseness of subsite S_2' in the latter enzyme in comparison to that in the former.

The model complex study (7-9) of both enzymes indicates that one or two hydrogen bonds are formed in the P_2'-S_2' interaction. Comparison of the kinetic parameters between Cbz-Gly-Pro-Phe-NH_2 and Cbz-Gly-Pro-Phe-Gly-NH_2 (Table III, B) and between Cbz-Gly-Pro-Phe-Gly-NH_2 and Cbz-Gly-Pro-Phe-Gly-Gly (Table III, D) shows small differences in both. It was previously shown (16) that the k_{cat}/K_m of α-chymotrypsin for hydrolysis of Ac-Tyr-Gly-NH_2 is about fivefold higher than that for Ac-Tyr-NH_2, mainly related to k_{cat}. Probably no hydrogen bond is formed between P_2' and S_2' when Cbz-Gly-Pro-Phe-NH_2 (or Ac-Tyr-NH_2) is used as a substrate, while one or two bonds must be formed when Cbz-Gly-Pro-Phe-Gly-NH_2 (or Ac-Tyr-Gly-NH_2) or Cbz-Gly-Pro-Phe-Gly-Gly is used, respectively. This may suggest that the contribution of hydrogen bonding, assumed to exist between P_2' and S_2', is negligible or is not so large as seen with subsite S_3 in both enzymes,

which has been described in previous papers (15, 17).

We conclude that the interaction of the leaving group of peptide substrates with the active site of α-chymotrypsin or subtilisin BPN′ assumed from the model complex study is essentially consistent with the present kinetic study using the Cbz-Gly-Pro-Phe peptide series. As for subtilisin BPN′, however, a more detailed study is required using an enzyme-protein inhibitor complex. This probably would help us to understand the P_1' specificity against residues smaller than alanine, as well as the broad specificity of the P_2' position in comparison with that of α-chymotrypsin.

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