THE COMPLEX ACTIVE SITES OF BACTERIAL NEUTRAL PROTEASES IN RELATION TO THEIR SPECIFICITIES

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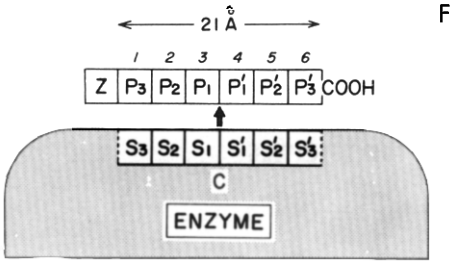
Received February 14, 1968

Schechter and Berger (1967) have shown that papain has a large active site which extends over about 25 Å and can be divided into 7 "subsites", each accommodating one amino acid residue of peptide substrate. The conclusion was obtained from an elaborated study using 40 diastereoisomeric peptides of alanine as substrate. Carboxypeptidase A has also been shown as having an active site which extends over about 18 Å and accommodating 5 amino acids of the substrate, by a similar method (Abramowitz, Schechter and Berger, 1967). The sizes of these enzymes are nearly the same as the binding site in lysozyme which accommodates 6 sugar units (Phillips, 1966). These findings may indicate that any protease has active site of comparable complexity and length.

This paper presents the relationships between the specificities of three bacterial neutral proteases and their complex active sites. The size of the active site of the respective enzyme was measured by a modification of the method of Schechter and Berger (1967) which was applied to papain. The result indicates that these bacterial enzymes have a large active site which covers at least 21 Å and can be divided into 6 "subsites" each accommodating one amino acid residue of the substrate. A further study demonstrates that these subsites show a cooperative effect for the appearance of the specificity in these enzymes. (See Fig. 1)



C = catalytic site; S = subsite; Z = carbobenzoxy-; P = amino acid

Fig. 1. Schematic representation of the enzyme-substrate complexes of bacterial neutral proteases. The subsite S'_1 possesses an ability to discern the specific amino acid residue such as L-leucine or L-phenylalanine. The other subsites also show specific interactions with different amino acid side-chains, in addition to their stereo-specificity. All the subsites show a cooperative effect for appearance of the specificity which is decided primarily by the subsite S'_1.

MATERIALS AND METHODS

Some peptides were obtained commercially, and the others were prepared in our laboratory, the procedures of which will be reported in another paper. Their purity was checked by elementary analysis and by thin layer chromatography. Except when specified, the constituent amino acids were all of the L-configuration. A crystalline neutral protease of Bacillus subtilis (abbr. BS-) was supplied from Seikagaku Kogyo Co., Tokyo. A thermostable protease (crystals) of Bacillus thermoproteolyticus (abbr. BT-) was kindly donated by Dr. Endo of Daiwa Kasei Co., Osaka. An elastase of Pseudomonas aeruginosa (abbr. PA-) was crystallized by a method described previously (Morihara et al., 1965). The three enzyme preparations were ascertained to be of homogeneous nature by sedimentation and electrophoresis studies. These enzymes are regarded as neutral proteases from their pH-optimum and their behaviour against inhibitors (Morihara, 1967). The proteolytic reactions were determined either by the ninhydrin method as described in a previous paper (Morihara and Ebata, 1967) or by the aid of a Radiometer type TTTI pH-stat equipped with a type SBUIa syringe burette, a type SBR2c recorder, and a thermostat controlled reaction vessel. In the former case, the rate of hydrolysis was calculated using authentic compounds as a standard. In some cases, dimethyl formamide (abbr. DMF) was added in the reaction mixture owing to the low solubility of peptides. The bonds hydrolyzed were identified by paper chromatography of the hydrolysates compared to authentically known compounds and by the usual dinitrophenylation technique.

RESULTS AND DISCUSSION

We interpret the observed variations in the proteolytic activities of these enzymes on the basis of the following assumption which has previously been made by Schechter and Berger (1967): the substrates are lined up on the enzyme in such a way that the

CO-NH group being hydrolyzed always occupies the same place (the catalytic site); the amino acid residues occupy adjacent subsites, those towards the NH_2-end occupying subsites S_1, S_2 etc., and those towards the COOH-end occupying subsites S'_1, S'_2 etc. The positions of the amino acid residues (P) in the peptide are numbered according to the subsites they occupy, i.e., the residues which are adjacent to subsites S_1, S_2 etc. are referred to as P_1, P_2 etc., respectively, and those adjacent to subsites S'_1, S'_2 etc. are referred to as P'_1, P'_2 etc., respectively (see Fig. 1).

Table I shows the proteolytic activities of the three neutral proteases against the various diastereoisomeric peptides, in which the peptide bond containing the amino

TABLE I.
Effects of Diastereoisomeric Peptides for Catalysis of Bacterial Neutral Proteases

	Peptide*a (P_4—P_3—P_2—P_1↓P'_1—P'_2—P'_3—P'_4)	BS-Enz	BT-Enz	PA-Enz
		(μM/min·mg enzyme)		
(I)*b	Z—Gly—Leu—NH_2	2.2	18.0	1.2
	Z—Gly—Leu—L-Ala	60	76.1	86
	Z—Gly—Leu—D-Ala	0.0	0.3	0.0
	Z—Gly—Leu—Gly—L-Ala	16	25	11.6
	Z—Gly—Leu—Gly—D-Ala	0.2	2.4	1.4
	Z—Gly—Leu—Gly—Gly—L-Ala	8.8	29	7.1
	Z—Gly—Leu—Gly—Gly—D-Ala	4.8	25	8.6
(II)*c	Z—Gly—Leu—NH_2	0.9	6.6	0.5
	Z—L-Ala—Leu—NH_2	2.6	13.6	3.9
	Z—D-Ala—Leu—NH_2	0.0	0.0	0.0
	Z—L-Ala—Gly—Leu—NH_2	9.0	15.2	13.6
	Z—D-Ala—Gly—Leu—NH_2	0.1	3.0	0.1
	Z—L-Ala—Gly—Gly—Leu—NH_2	0.12	0.8	0.3
	Z—D-Ala—Gly—Gly—Leu—NH_2	0.04	5.0	0.1

*a Prepared by our laboratory. The arrows show the bond split.
*b pH-stat: 4 mM peptide, pH 8, 0.1 M KCl, 30°C.
*c Ninhydrin: 4 mM peptide, 10% DMF, 0.05 M Tris (pH 7), 0.002 M $CaCl_2$, 40°C, 8 min.

group of L-leucine is hydrolyzed as has been expected from the results of many workers (Matsubara et al., 1965 and 1966; Matsubara, 1966; Morihara and Tsuzuki, 1966[a,b], Morihara and Ebata, 1967; Morihara, 1967; Feder, 1967; Feder and Lewis, 1967; Morihara et al., 1968). It indicates that the inhibiting effect of the D-residue is

strongest in S_1 and S'_2 similarly as have been seen in S'_1 by D-leucine (Morihara, 1967; Morihara et al., 1968), decreasing with the increasing distance from the catalytic point. It further indicates the fact that the active site of each of the three enzymes corresponds to at least 6 amino acid residues (subsite S_1-S_3 and S'_1-S'_3). Each of them has a size of about 21 Å as a whole and a length of 3.5 Å per residue.

As shown in the above, these enzymes show their specificities against the peptide bond containing an amino group of L-leucine, which indicates that their subsite S'_1 possesses an ability to discern the specific amino acid residues. Table II demonstrates that the other four subsites S_1, S_2, S'_2 and S'_3 also show specific interactions with different amino acid side-chains. The subsite S_3 may also show a similar interaction with different amino acids, although the experiment was not made. The rate of hydrolysis of a peptide is shown to be remarkably promoted when glycine at the position of P_1, P_2, P'_2 or P'_3 in the peptide is replaced with L-tyrosine or L-alanine. Such a promoting effect influenced the hydrolysis of the peptide bond containing an amino group of even an insignificant amino acid residue; Z-Gly$\underset{\uparrow}{-}$Ala-NH_2 is a poor substrate for these enzymes (Morihara, 1967; Morihara et al., 1968), the rate of hydrolysis being about from 1 to 2% in comparison with that of Z-Gly$\underset{\uparrow}{-}$Leu-NH_2 (the arrow shows the bond split). Z-Tyr$\underset{\uparrow}{-}$Ala-NH_2, however, was found to be hydrolyzed by enzymes of both BS- and BT- to a considerably large extent; the rate of hydrolysis was about one-half as compared with that of Z-Gly-Leu-NH_2. A striking feature was the cooperative effect of a double replacement. The ratios of catalytic activity (k_3/K_m) of Z-Gly-Leu-NH_2, Z-Gly$\underset{\uparrow}{-}$Leu-Ala and Z-Phe$\underset{\uparrow}{-}$Leu-Ala were found to be 1 : 37 : 349 in BS-enzyme and 1 : 10 : 180 in BT-enzyme.

The above results would mean that the six "subsites" in the active sites of these enzymes show cooperative effects for appearance of the specificity which must be decided primarily by the subsite S'_1. In other words, the reactivity of a certain bond in a protein depends not only on the one residue such as P'_1 (although being most important) but also on the nature of at least five residues in its neighbourhood, indicating multiple

TABLE II.
Effects of Various Amino Acid Residues for Each Subsite of Active Site in Bacterial Neutral Proteases

	Peptide*a $P_3 — P_2 — P_1 \downarrow P'_1 — P'_2 — P'_3$	BS-Enz	BT-Enz	PA-Enz
		(μM/min.mg enzyme)		
(I)*b	Z— Gly — Leu — NH_2	2.2	18.0	1.2
	Z— Gly — Leu — Gly	1.7	7.6	5.2
	Z— Gly — Leu — Ala	60.0	76.1	86.0
	Z— Gly — Leu — Leu	39.2	51.4	98.4
	Z— Gly — Leu — Phe	19.6	18.2	64.2
	Z— Gly — Leu — Gly — Gly	3.9	11.2	6.8
	Z— Gly — Leu — Gly — Ala	16.0	25.0	11.6
	Z— Gly — Leu — Gly — Phe	7.6	18.6	6.1
(II)*c	Z— Gly — Leu — NH_2	0.05	0.13	0.10
	Z— Ala — Leu — NH_2	0.15	0.30	0.80
	Z— Leu — Leu — NH_2	0.25	0.40	0.10>
	Z— His — Leu — NH_2	0.11	0.30	0.10>
	Z— Pro — Leu — NH_2	0.00	0.00	0.00
	Z— Phe — Leu — NH_2	1.57	2.63	0.10>
	Z— Tyr — Leu — NH_2	2.05	3.76	0.15
	Z— Try — Leu — NH_2	1.76	2.40	0.08
(III)*d	Z— Gly — Leu — NH_2	0.9	6.6	0.5
	Z— Gly— Gly — Leu — NH_2	0.1	1.5	0.15
	Z— Ala — Gly — Leu — NH_2	9.0	15.2	13.6
	Z— Phe — Gly — Leu — NH_2	21.9	13.0	11.4

*a (I) and (III) were prepared by our laboratory. (II) were supplied by the "Peptide Center" of Osaka University.

*b pH-stat: 4 mM peptide, pH 8, 0.1 M KCl, 30°C.

*c Ninhydrin: 1 mM peptide, 25% DMF, 0.05 M Tris (pH 7), 0.002 M $CaCl_2$, 40°, 3 min.

*d Ninhydrin: 4 mM peptide, 10% DMF, 0.05 M Tris (pH 7), 0.002 M $CaCl_2$, 40°, 8 min.

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attachment of the substrate to the active site. Our study concerning bacterial neutral proteases supports the previous assumption of Schechter and Berger (1967), which describes that the active sites in proteases in general are large, it might be possible to explain "unexpected" cleavages observed in protein.

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