ON THE ACTIVE SITE OF PROTEASES. III. MAPPING THE ACTIVE SITE OF PAPAIN; SPECIFIC PEPTIDE INHIBITORS OF PAPAIN

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It was found that in papain one of the subsites of the active site - namely "S_2" - specifically interacts with phenylalanine residues. The evidence is that: (a) the presence of Phe in positions three or higher from the C-terminal of the substrate enhances the susceptibility of a peptide to hydrolysis; (b) Phe directs the enzymic attack to the bond next-but-one to it towards the C-terminal; (c) peptides containing Phe as the second residue from the C-terminal are strong competitive inhibitors of papain ($\bar{K}_i \sim 10^3 M^{-1}$). It is assumed that these inhibitors occupy part of the active site (S_1, S_2, etc.) in the same manner as substrates. Thus they may help, by means of X-ray analysis of their complexes with papain, to show how peptide chains are bound to the active site of the enzyme.

In previous work (Schechter and Berger, 1967; Abramowitz et al., 1967), we showed that the size of the active site of proteolytic enzymes could be measured by comparing the rates of hydrolysis of a series of pairs of peptides, in which composition was systematically varied. In the case of papain, diastereoisomeric pairs of alanine peptides (up to the size of hexapeptides) were used, and it was concluded that the size of the active site corresponds to that of seven amino acid residues. The whole binding site was visualized as subdivided into subsites, and it was assumed that the substrate is lined up in the site so that each of its amino acid residues occupies one subsite. The residues were designated P_1, P_2, etc. and P_1', P_2', etc. (counting from the bond cleaved towards the amino- and carboxyl terminal respectively), and the corresponding subsites were designated S_1, S_2, S_3, S_4 and S_1', S_2', S_3' (see Fig. 1). The three-dimensional structure of papain worked out by Drenth and his group (1968) may make it possible to test this picture and identify the subsites on either side of the reactive sulfhydryl group.

In continuation of these studies we now attempted to map the active site by identifying specific interactions in the various subsites.

RESULTS. A number of peptides containing L-alanine plus a single residue of L-phenylalanine and/or L-lysine were prepared and checked for hydrolysis by papain.

All reaction products were identified by comparison with authentic samples, using chromatography in butanol-acetic acid-water and high voltage paper electrophoresis at pH 1.5 and 6.5. The results are given in Table I.

It is seen that peptides in which Phe is the third or further residue from the C-terminal end are good substrates for papain. Peptides containing Phe as the second residue from the C-terminal are hardly attacked. The bond split is always the next-but-one to the Phe residue in the direction of the C-terminal. In our terminology: whenever a split occurs the Phe residue is "P_2" of the substrate, i.e., it always occupies subsite S_2 on the enzyme (see Fig. 1). This means that S_2 has a definite preference for the Phe-side chain. This is also seen from the fact that the introduction of Phe into an appropriate position (third or further from the C-terminal) markedly increases the susceptibility of a given substrate to hydrolysis. It is interesting to note that the "specific" splits are always purely hydrolytic, whereas in the peptides Ala_3, Ala_4, Ala_2Phe and Ala_3Phe transpeptidation occurs (Schechter and Berger, 1967).

The evidence for strong interaction of Phe in subsite S_2 raised the possibility that peptides with Phe as the penultimate residue, while not undergoing cleavage, might bind strongly to subsites S_1, S_2 and S_3 on the one side of the active sulfhydryl group of the enzyme (see Fig. 1). By blocking part of the active site they would act as competitive inhibitors. Indeed it was observed (by semiquantitative paper electrophoresis) that the hydrolysis of Ala_5 (DL_4) ($k_{cat}/K_m \sim 6$ sec^{-1} M^{-1}, Schechter and Berger, 1967) was inhibited to an extent of about 50% by 10^{-3} M AlaPheLys, Ala_2PheAla or Ala_2PheLys (Table I).

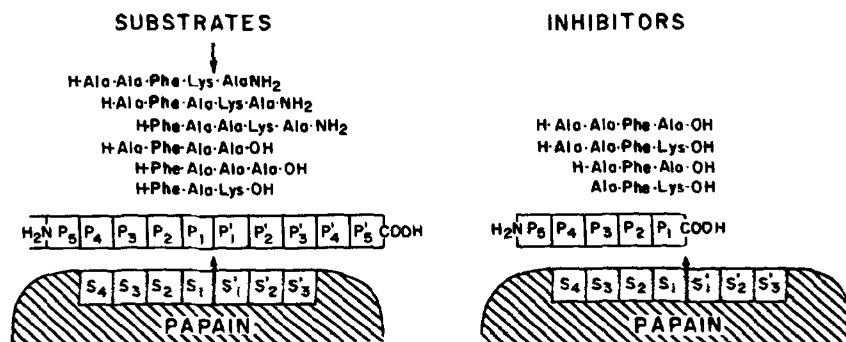

Fig. 1. - Mode of binding of substrates and inhibitors in the active site of papain. The Phe-residue always occupies S_2. The arrow indicates the point of cleavage.

TABLE 1. Specificity of papain in the hydrolysis of peptides containing phenylalanine and their inhibitory effect.

Hydrolysis: Reaction mixtures (36°) contained peptide (0.02 M), citrate-phosphate buffer pH 3.7 (citric acid 0.025 M; Na_2HPO_4 0.016 M), EDTA (0.002 M), 2-mercaptoethanol (0.01 M). Arrows indicate the bond split. "tp": transpeptidation. Inhibition of hydrolysis of $Ala_5(DL_4)$: medium as above. Ala_5 (0.01 M), test peptide (0.001 M). Inhibition of hydrolysis of BAEE: see Text.

Hydrolysis			Inhibition (v_i/ v)	
			Substrates	
Papain	Peptide	% hydrol. (approx)	$Ala_5(DL_4)$	BAEE
330 µg/ml	AlaAlaAla	40 tp		1.02
(7 hrs)	PheAla↓Ala	60		0.87
	AlaPheAla	trace		[0.57]
	AlaAlaPhe	30 tp		
330 µg/ml	AlaAlaAlaAla	50 tp		1.00
(45 min)	PheAla↓AlaAla	90		0.94
	AlaPheAla↓Ala	90		0.79
	AlaAlaPheAla	trace tp	0.5	[0.60]
	AlaAlaAlaPhe[1]	tp		
	AlaAlaPheLys	0	0.5	[0.37]
100 µg/ml	AlaAlaLys	0	1	1.00
(3 hrs)	PheAla↓Lys	5	1	0.90
	AlaPheLys	0	0.5	[0.37[2]]
30 µg/ml	$AlaAlaLysAlaNH_2$	0		
(3 hrs)	PheAla↓$LysAlaNH_2$	5		
	AlaPheLys↓$AlaNH_2$	30		
30 µg/ml	$AlaAlaAlaLysAlaNH_2$	0		
(1 hr)	PheAla↓$AlaLysAlaNH_2$	60		
	AlaPheAla↓$LysAlaNH_2$	60		
	AlaAlaPheLys↓$AlaNH_2$	60		

[1] Substrate sparingly soluble [2] 0.80 at pH 5.6; 0.93 at pH 7.0.

The inhibitory effect of AlaPheLys on the hydrolysis of benzoylarginine ethyl ester (BAEE) at pH 4.35 was studied in more detail in the pH-stat (36^0, KCl 0.33 M, 2-mercaptoethanol 0.01 M, EDTA 0.002 M, BAEE 0.01 to 0.05 M). In a Lineweaver-Burk plot the substrate gave a K_m of 3×10^{-2} M (Sluyterman, 1964; Whitaker and Bender, 1965). Inhibition by AlaPheLys was purely competitive and gave an inhibition constant $\overline{K}_i = 2500\ M^{-1}$ (determined at $[I] = 10^{-3}$ M). Determinations of relative inhibition at $[S] = 0.02$ M and $[I] = 10^{-3}$ M for other peptides are given in Table I. Strong inhibition is shown by Ala_2PheLys ($\overline{K}_i \sim 2500$) AlaPheAla ($\overline{K}_i \sim 1000$) and Ala_2PheAla ($\overline{K}_i \sim 1000$). The inhibition by AlaPheLys was also checked at higher pH: at pH 5.6 $v_i/v = 0.80$ and at pH 7.0 $v_i/v = 0.93$. This indicates that like in the case of benzoylarginine, ($\overline{K}_i = 33$ at pH 4.35 ; Sluyterman, 1964; Whitaker and Bender, 1965) inhibition is mainly due to the protonated acid form. At pH 4.35 (about one pH unit above the pK) the protonated form of AlaPheLys would then have an association constant of about 3×10^4.

DISCUSSION. Papain is considered to have a very wide specificity (Smith and Kimmel, 1960; Hill, 1965) which is one way of stating that it shows little specificity. Peptide bonds formed by the carboxyl groups of α-amino substituted lysine and arginine are said to be most susceptible to papain (Hill, 1965). The experiments reported here define a new kind of specificity, namely -Phe-X$\overset{\downarrow}{-}$Y- where the bond between X and Y is split specifically if X is preceded by phenylalanine. In our peptides containing both phenylalanine and lysine the Phe-specificity as defined above dominates the pattern of hydrolysis. The same conclusion can be reached from the inhibition experiments: whereas AlaPheAla is a strong inhibitor, AlaAlaLys does not inhibit at 10^{-3} M (although it does seem to inhibit at much higher concentrations). However, it can be seen that Lys is bound (in S_1) stronger than Ala since in both cases (AlaPheLys vs. AlaPheAla and Ala_2PheLys vs. Ala_2PheAla) it more than doubles the association constant. This corresponds to about 0.5 Kcal/mole of free energy of binding in favor of the lysine side chain over the methyl group of alanine. As to the nature of the interaction of the Phe-residue with the active site, there may be aromatic interaction, possibly with the tryptophane residue 176 (Drenth et al., 1968) in the active site in addition to "hydrophobic binding". There are indications in the literature (Hill, 1965) that bonds of the type Val-X$\overset{\downarrow}{-}$Y and Leu-X$\overset{\downarrow}{-}$Y are frequently attacked by papain. This type of specificity is similar to the one described for streptococcal proteinase (Gerwin et al., 1966), but seems to have been overlooked in the case of papain.

An essential step towards the understanding of enzyme action (binding and

catalysis) is the elucidation, by X-ray crystallography, of the precise geometrical relationship between substrate and enzyme in the active complex. Since the latter is not stable, enzyme-inhibitor complexes are the second best choice. However, in order to yield relevant information, the inhibitor should interact with the active site, or with part of it, in the same manner as the substrate, i.e. utilize the same points of attachment. The inhibitor should also be large enough to interact with as many subsites as possible. It seems that inhibitors like AlaAlaPheLys meet these requirements and it is hoped that the mode of substrate binding in one part of the active site can be elucidated. From there on one might be able to fit the substrate in the whole of the active site using the three-dimensional structure of the enzyme and information such as stereochemical requirements of the various subsites. We intend to do this in collaboration with the Groningen group.

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