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Secondary Enzyme–Substrate Interactions and the Specificity of Pepsin*

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ABSTRACT: Kinetic parameters have been determined for the peptic hydrolysis of the L-phenylalanyl–L-phenylalanyl (Phe–Phe) bond in a series of synthetic oligopeptide substrates of the type A-Phe-Phe-B, in which B is 3-(4-pyridyl)propyloxy and A is a benzyloxycarbonyldipeptidyl (or -tripeptidyl) unit; several peptides from which the benzyloxycarbonyl (Z) group had been removed were also tested. The important contribution of the phenyl group of the amino-terminal Z substituent is shown by the slower maximal rate of cleavage and higher K_M upon replacement of the Z group by the methyloxycarbonyl group. The kinetic data indicate that with substrates in which A = Z-Phe-Gly (or Phe-Gly) or Z-Phe-Gly-Gly (or Phe-Gly-Gly), the extent of nonproductive interaction is greater than with comparable substrates in which the Phe residue of the A group has been replaced by a glycyl or L-alanyl residue. For substrates having an amino-terminal Z group, replacement of the Phe or Ala residue of an A group by its D enantiomer markedly decreases the rate of enzymic action; this stereochemical discrimination is not evident, however, when A = Phe-Gly (or Phe-Gly-Gly), as the effect of the introduction of a D-phenylalanyl residue appears to be largely to reduce the extent of nonproductive binding. Further evidence to suggest that substrates of the type examined here interact with pepsin in a manner that reflects the structure of the A group as a whole is provided by kinetic data for substrates in which A = Z-Gly-Gly was replaced by other Z-dipeptidyl units. Of special interest is the finding that when A = Z-Gly-Pro, the Phe–Phe bond is extremely resistant to peptic hydrolysis, whereas when A = Z-Ala-Ala, the rate (k_{cat}/K_M) is the highest hitherto found for a synthetic peptide substrate of pepsin; for these two substrates, the ratio of the rates is about 1:17,500. Such large differences in the catalytic efficiency of pepsin at the Phe–Phe bond of closely related oligopeptide substrates give strong evidence for the decisive importance of interactions involving substrate groups relatively distant from the site of catalytic action, and support the view that such secondary interactions may alter the efficiency of catalysis through an effect on the conformation of the catalytic site of pepsin.

Previous reports from this laboratory have drawn attention to the large effects on the catalytic efficiency of pepsin (as measured by k_{cat}[1]) arising from changes in substrate structure at a distance from the sensitive peptide bond. Thus, for substrates of the type A-Phe-Phe-OP4P,[2] where the Phe–Phe bond is the only pepsin-sensitive linkage, a change in the A group from benzyloxycarbonyl (Z) to Z-Gly-Gly resulted in a 100-fold increase in k_{cat} and only a slight decrease in K_M (Sachdev and Fruton, 1969). Similarly, for substrates of the type Z-His-Phe(NO_2)-Phe-B, where the Phe(NO_2)-Phe bond is the only pepsin-sensitive linkage, a change in the B group from OMe to Ala-Ala-OMe resulted in a 100-fold increase in k_{cat}, with only a 3-fold drop in K_M (Medzihradszky

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[1] The kinetic parameters mentioned in this paper are defined by the equation $v = V_m(S)/(K_M + (S))$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, the maximal velocity $V_m = k_{cat} \times$ total enzyme concentration (E), (S) = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$.

[2] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: Phe(NO_2), *p*-nitro-L-phenylalanyl; OP4P, 3-(4-pyridyl)propyl-1-oxy, Moc, methyloxycarbonyl; Z, benzyloxycarbonyl. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

TABLE I: Synthesis and Properties of Synthetic Substrates A-Phe-Phe-OP4P.

Group A	Yield (%)	Mp (°C)	R_F[a]	$[\alpha]_D^{24}$[b] (deg)	Calcd (%) C	Calcd (%) H	Calcd (%) N	Found (%) C	Found (%) H	Found (%) N
Z-Gly-Ala-	82	164–165	0.57 (A)	−19.1	67.5	6.2	10.1	67.4	6.4	10.2
Z-Gly-D-Ala-	83	156–157	0.30 (B)	−10.5	67.5	6.2	10.1	67.3	6.3	10.1
Z-Ala-Gly-	85	154–155	0.57 (A)	−11.5	67.5	6.2	10.1	67.5	6.3	10.1
Z-D-Ala-Gly-	87	143–144	0.56 (A)	−6.2	67.5	6.2	10.1	67.6	6.3	10.0
Z-Ala-Ala-	85	152–153	0.55 (A)	−19.6	67.9	6.4	9.9	67.8	6.5	9.9
Z-Gly-Sar-	71	68–70	0.59 (A)	−11.1	67.5	6.2	10.1	67.4	6.4	10.2
Z-Sar-Gly-	91	142–143	0.44 (A)	−9.2	67.5	6.2	10.1	67.3	6.4	10.0
Z-Gly-Pro-	73	142–144	0.54 (A)	−48.3	68.4	6.3	9.7	68.7	6.1	9.5
Z-Gly-Leu-	80	124–125	0.64 (A)	−22.5	68.55	6.7	9.5	68.6	6.6	9.4
Z-Gly-Ile-	74	104–105	0.71 (A)	−15.3	68.55	6.7	9.5	68.5	6.8	9.6
Z-Gly-His-	44	187–188	0.57 (A)	−26.5	66.4	6.0	12.9	66.3	6.0	13.0
Z-Phe-Gly-	67	110–112	0.65 (A)	−23.2	70.2	6.15	9.1	70.3	6.1	9.0
Z-D-Phe-Gly-	88	143–144	0.51 (A)	−2.7	70.2	6.15	9.1	70.1	6.2	9.2
Z-Phe-Gly-Gly-	75	155–156	0.47 (A)	−21.4	68.3	6.1	10.2	68.2	6.2	10.3
Z-D-Phe-Gly-Gly-	89	163–164	0.45 (A)	+4.2	68.3	6.1	10.2	68.4	6.1	10.2
Moc-Gly-Gly-	84	161–162	0.70 (A)	−10.5	63.7	6.2	11.6	63.8	6.0	11.7
Phe-Gly-	83	120–122	0.47 (C)	−34.1	69.9	6.5	11.0	69.8	6.7	10.8
D-Phe-Gly-	78	115–117	0.45 (C)	+33.3	69.9	6.5	11.0	69.9	6.7	11.1
Phe-Gly-Gly-	89	79–81	0.44 (C)	−35.2	67.6	6.4	12.1	67.6	6.3	12.1
D-Phe-Gly-Gly-	86	80–82	0.41 (C)	+5.2	67.6	6.4	12.1	67.6	6.4	12.2

[a] The letter in parentheses denotes the solvent system used (see Experimental Section). [b] A Perkin-Elmer Model 141 automatic polarimeter was employed; concentration, 1% in dimethylformamide.

et al., 1970). In these cases, therefore, chain elongation in either direction from the sensitive peptide bond, with alterations in the position and number of hydrophobic groups and amide bonds flanking the reactive Phe–Phe unit, markedly enhanced the catalytic efficiency, without a comparable increase in total binding energy (as measured by K_M, assumed to approximate K_S; Inouye and Fruton, 1967). It has been suggested (Hollands *et al.*, 1969) that this rate enhancement is a consequence of enzyme–substrate interactions at loci relatively distant from the catalytic site, and that such "secondary" interactions may promote mutual conformational adjustment of the catalytic groups of the enzyme and of the sensitive peptide bond of the substrate to increase catalytic efficiency. Such secondary interactions are likely to be generally important in the action of enzymes that act on oligomeric substrates (Fruton, 1970); recent studies on subtilisin (Ottesen *et al.*, 1970) and lysozyme (Chipman and Sharon, 1969) may be cited in this connection. In a sense, the effect of relatively distant parts of an oligomeric substrate on the efficiency of the catalytic groups of the enzyme may be considered to represent a special case of the more general phenomenon of the alteration of the kinetic behavior of an enzyme by "allosteric" interactions (Koshland and Neet, 1968).

In the present communication, data are presented on the action of pepsin at the Phe–Phe bond of a series of synthetic substrates of the type A-Phe-Phe-OP4P, in which the A group has been varied systematically. The results give more definitive evidence than obtained heretofore of the importance of secondary enzyme–substrate interactions in the catalytic action of pepsin.

Experimental Section

The chromatographic examination of the peptides prepared in this work and of the cleavage products released by pepsin was performed with Eastman Chromagram sheets 6061, with the following solvent systems: (A) ethyl acetate–methanol (9:1, v/v), (B) ethyl acetate–methanol (95:5, v/v), and (C) 1-butanol–acetic acid–water (4:1:1, v/v). Chlorine–tolidine, iodine, and (where applicable) ninhydrin were used as reagents.

In the synthesis of the substrates of the type Z-X-Y-Phe-Phe-OP4P, where X and Y are amino acid residues other than histidyl, the general procedure described earlier (Sachdev and Fruton, 1969) for the preparation of Z-Gly-Gly-Phe-Phe-OP4P was used. The dipeptide derivative Z-X-Y (*ca.* 1.8 mmoles of crystalline material, checked for purity by melting point, chromatography, elementary analysis, and optical rotation) was coupled in the usual manner with Phe-Phe-OP4P (derived from 1.8 mmoles of Z-Phe-Phe-OP4P by treatment with HBr–acetic acid and preparation of the free base) in the presence of dicyclohexylcarbodiimide (1.8 mmoles), with tetrahydrofuran as the solvent. In the case of Z-Gly-His-Phe-Phe-OP4P, Z-Gly-His-N_3 (derived from 1.8 mmoles of the hydrazide) was used. The substrates in which A = Z-Phe-Gly-Gly or Z-D-Phe-Gly-Gly were prepared by coupling Z-Phe (or Z-D-Phe) with Gly-Gly-Phe-Phe-OP4P, described previously (Sachdev and Fruton, 1969); the same products were also obtained by coupling Z-Phe-Gly (or Z-D-Phe-Gly) with Gly-Phe-Phe-OP4P (prepared by treatment of the Z-protected peptide with HBr–acetic acid). The peptide

TABLE II: Kinetics of Pepsin Action at Phe–Phe Bond of A-Phe-Phe-OP4P.[a]

A	Substrate (mM)[b]	Enzyme (μM)	k_{cat}[c] (sec^{-1})	K_M[c] (mM)	k_{cat}/K_M (sec^{-1} mM^{-1})
Z-Gly-Gly-[d]	0.03–0.4 (11)	0.0023	71.8 ± 3.6	0.42 ± 0.08	171
Moc-Gly-Gly-	0.03–0.63 (11)	0.0095	19.1 ± 2.2	0.75 ± 0.11	25.5
Z-Phe-Gly-	0.015–0.16 (8)	0.0027	24.7 ± 3.1	0.11 ± 0.08	225
Z-D-Phe-Gly-	0.015–0.12 (9)	0.054	0.92 ± 0.13	0.11 ± 0.02	8.4
Z-Phe-Gly-Gly-	0.03–0.28 (10)	0.00068	127 ± 22	0.13 ± 0.03	978
Z-D-Phe-Gly-Gly-	0.02–0.13 (8)	0.0027	40 ± 6	0.10 ± 0.02	400
Gly-Gly-[d]	0.08–0.8 (10)	0.087	3.8 ± 0.6	1.3 ± 0.3	2.9
Phe-Gly-	0.13–1.3 (12)	0.17	0.49 ± 0.06	0.3 ± 0.1	1.7
D-Phe-Gly-	0.04–0.4 (10)	0.27	1.1 ± 0.1	0.6 ± 0.2	1.9
Phe-Gly-Gly-	0.10–0.8 (9)	0.034	6.3 ± 1.1	0.6 ± 0.2	10
D-Phe-Gly-Gly-	0.09–0.9 (10)	0.034	10.2 ± 1.5	1.0 ± 0.2	10
Z-Gly-Ala-	0.04–0.4 (10)	0.00027	409 ± 52	0.11 ± 0.02	3720
Z-Gly-Leu-	0.02–0.12 (9)	0.00027	134 ± 10	0.032 ± 0.006	4195
Z-Gly-Ile-	0.04–0.2 (9)	0.0014	12.6 ± 1.0	0.07 ± 0.01	180
Z-Gly-His-	0.04–0.4 (11)	0.0054	15.8 ± 2.1	0.44 ± 0.12	35.6
Z-Gly-D-Ala-	0.08–0.4 (10)	0.27	0.19 ± 0.02	0.35 ± 0.05	0.56
Z-Gly-Sar-	0.045–0.45 (11)	0.092	1.04 ± 0.11	0.40 ± 0.07	2.6
Z-Gly-Pro-	0.08–0.4 (9)	0.50	0.056 ± 0.003	0.14 ± 0.03	0.4
Z-Ala-Gly-	0.04–0.4 (10)	0.00068	145 ± 30	0.25 ± 0.04	579
Z-D-Ala-Gly-	0.04–0.35 (10)	0.054	2.1 ± 0.5	0.51 ± 0.12	4
Z-Sar-Gly-	0.09–0.4 (10)	0.011	3.5 ± 0.3	0.17 ± 0.03	21
Z-Ala-Ala-	0.02–0.19 (9)	0.00011	282 ± 38	0.04 ± 0.01	7050

[a] pH 3.5 (0.04 M formate buffer), 37°. [b] The numbers in parentheses denote the number of runs. [c] The precision of the data is given as 95% confidence limits, estimated by computer analysis (Hanson *et al.*, 1967). [d] Data taken from Sachdev and Fruton (1969).

esters lacking an amino-terminal benzyloxycarbonyl group were prepared in the usual manner by treatment with HBr–acetic acid and were converted to the free base. For the preparation of the methyloxycarbonyl (Moc) analog of Z-Gly-Gly-Phe-Phe-OP4P, Moc-Gly-Gly (mp 126–127°) was coupled with Phe-Phe-OP4P in the manner described above. The products were recrystallized to constant melting point and chromatographic homogeneity; the yields, properties, and elementary analysis of the recrystallized products are given in Table I.

The pepsin preparation (Worthington Biochemical Corp. lot 693-7) employed in this work is the same one used in earlier studies in this laboratory, and the kinetic parameters k_{cat} and K_M were estimated by means of an automatic ninhydrin method in the manner described previously (Sachdev and Fruton, 1969). The site of cleavage was determined by chromatographic examination of a peptic hydrolysate of the compound after 24-hr incubation at the enzyme concentration used in the kinetic runs.

Results

In Table II are summarized the kinetic data for the action of pepsin at pH 3.5 and 37° on the series of substrates of the structure A-Phe-Phe-OP4P. In all cases, the Phe–Phe bond was the only one cleaved to a detectable extent under the conditions of these studies; it may be noted that this also applies to the substrate in which A = Z-Gly-Leu, as no cleavage of the Leu–Phe bond was observed.

In considering the data in Table II, attention may first be drawn to the fact that the presence of the benzyloxycarbonyl group (Z) at the amino terminus of the substrates makes a significant contribution in lowering the value of K_M and in increasing the value of k_{cat}. This is evident from the effect of the replacement of the benzyloxycarbonyl group of Z-Gly-Gly-Phe-Phe-OP4P by a methyloxycarbonyl group (Moc). Furthermore, for the pairs of substrates in which A = Z-Gly-Gly or Gly-Gly, Z-Phe-Gly or Phe-Gly, and Z-Phe-Gly-Gly or Phe-Gly-Gly, the ratios of the k_{cat}/K_M values are 59, 132, and 98, respectively. The variation in these numbers is much less than the range of k_{cat}/K_M values (1.7 to 978) involved; if the confidence limits for the individual values for k_{cat} and K_M are taken into account, the introduction of the benzyloxycarbonyl group may be considered to exert a roughly similar effect in the three cases. Clearly, this effect may be a consequence of the blocking of the amino-terminal cationic group of the peptide substrate as well as the addition of the hydrophobic benzyl group in the substituent.

Of special interest is the finding that the replacement of A = Z-Gly-Gly or Gly-Gly by A = Z-Phe-Gly or Phe-Gly respectively leads to a parallel decrease (within the precision of the values) in k_{cat} and K_M, suggesting that the benzyl side chain of the Phe residue of the A group may contribute to nonproductive enzyme–substrate interaction, in the manner

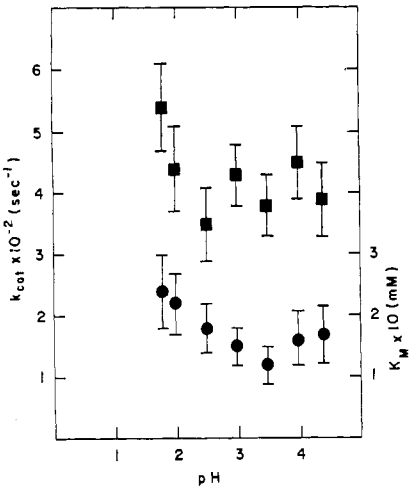

FIGURE 1: pH dependence of kinetic parameters for the peptic hydrolysis of Z-Gly-Ala-Phe-Phe-OP4P. Range of substrate concentration, 0.018–0.25 mM (10–12 runs at each pH value); enzyme concentration, 2×10^{-4} μM; 37°. The pH was maintained by means of 0.04 M sodium maleate–HCl buffers. The mean values for k_{cat} are denoted by squares (left ordinate), and K_M by circles (right ordinate); the vertical bars denote the 95% confidence limits, as estimated by computer analysis.

noted with other pepsin substrates (Medzihradszky *et al.*, 1970). It is noteworthy, however, that the replacement of Phe-Gly by D-Phe-Gly causes a doubling of both k_{cat} and K_M, suggesting that the stereochemical discrimination with respect to the Phe residue is expressed in terms of decreased nonproductive interaction in the case of A = D-Phe-Gly, as compared with A = Phe-Gly. This result is not seen, however, with the pair of substrates in which A = Z-Phe-Gly or Z-D-Phe-Gly; here K_M is the same, and a large stereochemical discrimination is evident in a 25-fold difference in the value of k_{cat}. The presence of the benzyloxycarbonyl group thus alters decisively the nature of the interaction, with the enzyme, of the entire A group of the substrate, and this result may be taken as further evidence of the participation of the benzyloxycarbonyl group in the formation of a productive enzyme–substrate complex.

The fact that a similar behavior is observed with the pair of substrates in which A = Phe-Gly-Gly or D-Phe-Gly-Gly as compared to the corresponding pair bearing a benzyloxycarbonyl group indicates that the interaction of the enzyme with this group may occur even when it is separated from the sensitive peptide bond by four amino acid residues. It is clear, however, that the substrate in which A = Z-Phe-Gly-Gly, though having the same K_M value as that with A = Z-Phe-Gly, is cleaved at a significantly greater rate. Furthermore, the replacement of A = Z-Phe-Gly-Gly by Z-D-Phe-Gly-Gly results in a smaller decrease in k_{cat} than in the case of the enantiomeric Z-Phe-Gly and Z-D-Phe-Gly substituents (a 3-fold decrease instead of a 25-fold decrease); the K_M values for the four substrates are the same, within the precision of the measurements.

These results indicate that a substrate of the type Z-X-Y-Phe-Phe-OP4P (where X and Y are amino acid residues) appears to interact with pepsin in a manner that reflects the structure of the Z-X-Y group as a whole. To examine this possibility further, a number of variations were made in the nature of Y in substrates of the type Z-Gly-Y-Phe-Phe-OP4P. It will be noted in Table II that when Y = Ala, k_{cat} is increased to about 400 sec^{-1} (the highest value hitherto observed for a synthetic substrate of pepsin) and K_M is lowered. With the more hydrophobic Leu residue in the Y position, both k_{cat} and K_M are lowered to approximately the same degree, as compared to Y = Ala, suggesting the possibility that the presence of the Leu residue may contribute to nonproductive enzyme–substrate interaction. It is noteworthy that when Y = Ile, K_M is similar to the case in which Y = Ala, but the value of k_{cat} is lowered more than 30-fold. The k_{cat} value for the substrate in which Y = Ile is similar to that having the cationic His residue in the Y position, but the latter substrate is evidently bound less tightly, as indicated by its higher K_M value, which is comparable to the case where Y = Gly. Indeed, as judged by the k_{cat}/K_M values for the pair of substrates in which Y = Gly or Ile, the presence of the Ile residue appears to contribute significantly to nonproductive bonding, rather than to the enhancement of catalytic efficiency as seen for the substrate with Y = Ala.

That the productive interaction of Z-Gly-Ala-Phe-Phe-OP4P with pepsin depends importantly on the position of the side-chain methyl group of the Ala residue is evident from the results for the substrate with A = Z-Gly-D-Ala; here K_M is increased 3-fold, but k_{cat} is decreased by a factor of about 2000. The replacement of Y = Ala by a sarcosyl residue has a similar effect on K_M and decreases k_{cat} by a factor of about 450; the introduction of a sarcosyl residue in place of an L-alanyl residue may be expected to cause a marked conformational change, because of the presence of the *N*-alkyl group. In this connection, it is noteworthy that the most resistant of the substrates tested in the present experiments was the compound in which A = Z-Gly-Pro; despite the presence of a L-amino acid residue in the Y position, the conformational restriction caused by the fact that the peptide nitrogen is part of a pyrrolidine ring renders the sensitive Phe–Phe bond relatively resistant to pepsin action.

It was noted above that replacement of A = Z-Gly-Gly by Z-Phe-Gly was characterized by little change in k_{cat}/K_M, and the parallel changes in k_{cat} and K_M were consistent with greater nonproductive interaction for the case with A = Z-Phe-Gly. This does not appear to be the situation when A = Z-Ala-Gly, since there is a 2-fold enhancement of k_{cat} and a decrease in K_M, leading to a 4-fold increase in k_{cat}/K_M. It will be noted that the replacement of the Ala residue of A = Z-Ala-Gly by D-Ala or by Sar causes a marked decrease in k_{cat}/K_M, although not as large as in the case of the substrates analogous to Z-Gly-Ala-Phe-Phe-OP4P.

The most sensitive pepsin substrate found in the present study, as judged by its k_{cat}/K_M value, is Z-Ala-Ala-Phe-Phe-OP4P. Although its k_{cat} value (280 sec^{-1}) is somewhat lower than that for A = Z-Gly-Ala, its K_M value (0.04 mM) is among the lowest thus far observed for a synthetic substrate of pepsin.

In connection with these studies, it was important to examine the pH dependence of the kinetic parameters for one of the more sensitive substrates of the type Z-A-B-Phe-Phe-OP4P, and the data for Z-Gly-Ala-Phe-Phe-OP4P are given in Figure 1. It will be noted that in the pH range near 3.5,

where the data for the substrates listed in Table II were obtained, the variation of k_{cat} and K_M was within the precision of the measurements. We do not consider the data in Figure 1 to allow any conclusion to be drawn about the nature of catalytically important prototropic groups either in the free enzyme (on the basis of k_{cat}/K_M values) or the enzyme–substrate complex (on the basis of k_{cat} values).

Discussion

As was indicated previously (Sachdev and Fruton, 1969) the study of the pyridyl esters of peptides had led to the finding of exceptionally sensitive substrates for pepsin. It still remains for future work to establish the reasons for the large rate enhancement caused by the introduction of the pyridylpropyloxy group on the carboxyl side of the sensitive Phe–Phe unit of pepsin substrates. Earlier work (Medzihradszky *et al.*, 1970) had shown similar large rate enhancement by the replacement of OMe by Ala-Ala-OMe on the carboxyl side of sensitive dipeptidyl unit, suggesting a contribution of the Ala–Ala unit to increased productive enzyme–substrate interaction. Whether the pyridinium ion of the pyridyl ester substrates participates in enzyme–substrate interaction is not definitely known; however, with more resistant substrates, such as Z-Phe-Phe-OP4P or Z-Gly-Phe-Phe-OP4P, a shift in pH from 2.0 to 3.0–3.5 is accompanied by a marked decrease (*ca.* 3-fold) in the value of K_M without a large change in k_{cat} (Sachdev and Fruton, 1969), suggesting the possibility that the pyridinium ion of such substrates may form ion pairs with carboxylate groups of the enzyme. It should be noted, however, that the kinetic parameters for comparable 4-(3-pyridyl)propyl and 3-(3-pyridyl)propyl esters were approximately the same (Sachdev and Fruton, 1969), so that the precise location of the cationic group does not appear to be crucial for optimum enzyme–substrate interaction.

In the present study, the Phe-Phe-OP4P portion of the pepsin substrates was invariant, and large changes were observed in the kinetic parameters upon modifying the nature of the Z-dipeptidyl group attached to the amino terminus of the Phe-Phe-OP4P unit. The most striking difference was that found for the pair of substrates in which the Z-dipeptidyl group was Z-Gly-Ala or Z-Gly-Pro; the K_M values were the same (within the precision of the data) but the k_{cat} values were in the ratio 7300:1. Furthermore, the replacement of Z-Gly-Gly by Z-Ala-Ala has yielded the most sensitive synthetic substrate for pepsin found thus far (k_{cat}/K_M = 7000 sec^{-1} mM^{-1}); this may be compared to the action of pepsin on Ac-Phe-TyrI_2 (pH 2, 37°) for which a value of k_{cat}/K_M = 2.5 has been reported (Jackson *et al.*, 1965); a similar k_{cat}/K_M value has been found for Z-His-Phe-Phe-OEt at pH 4.5 and 37° (Hollands and Fruton, 1968). Indeed, the rate of cleavage of Z-Ala-Ala-Phe-Phe-OP4P by pepsin at the Phe–Phe bond approaches the k_{cat}/K_M values for the chymotryptic cleavage of the *p*-nitrophenyl esters of Ac-Trp, Ac-Phe, and Ac-Leu (Ingles and Knowles, 1967). To our knowledge, no synthetic amide or peptide substrate of chymotrypsin or trypsin has been found to be cleaved at a rate comparable to that reported for the best of the pepsin substrates listed in Table II.

As indicated above, the available data are consistent with the conclusion that the Z-dipeptidyl group of a substrate Z-X-Y-Phe-Phe-OP4P interacts with the enzyme as a unit, and the benzyl portion of the benzyloxycarbonyl group participates importantly in this interaction. Furthermore, the presence or absence of the terminal benzyloxycarbonyl group may be reflected in differences in the apparent stereospecificity with respect to the configuration of an amino acid residue two or three residues away from the sensitive peptide bond; this suggests the need for caution in the use of such stereochemical discrimination as a means of "mapping" the active sites of enzymes that act on oligomeric substrates (Berger and Schechter, 1970).

At the present state of our knowledge regarding the three-dimensional structure of pepsin, it is clearly premature to attempt a detailed interpretation of the data offered in Table II in terms of subsites in a cleft, as has been done for lysozyme (Blake *et al.*, 1967). It may be hoped, however, that these data will be useful in future studies on the three-dimensional structure of enzyme–substrate complexes involving pepsin. Although a detailed analysis of the effect of changes in substrate structure on pepsin action is not yet possible, the present results offer more striking evidence than obtained before to show the importance of secondary interactions in enzymic catalysis, and to suggest that these interactions may affect the catalytic efficiency by causing conformational changes at the catalytic site of the enzyme (Fruton, 1970).

As noted earlier (Medzihradszky *et al.*, 1970), the availability of highly sensitive peptide substrates for pepsin makes possible a reinvestigation of various aspects of the problem of the mechanism of pepsin action. The data in Figure 1, showing a pH dependence of k_{cat} and of k_{cat}/K_M quite different from that reported for relatively resistant substrates such as Ac-Phe-Phe-NH_2 (Cornish-Bowden and Knowles, 1969), is noteworthy in this regard. Examination of the data reported earlier for the effect of pH on the kinetics of the peptic cleavage of the Phe–Phe bond in various pyridyl ester substrates (Sachdev and Fruton, 1969), together with the data presented in Figure 1, suggests that substrates for which K_M is relatively high (greater than 0.5 mM) at pH 2 exhibit a larger decrease in K_M at higher pH values than do substrates (such as Z-Gly-Ala-Phe-Phe-OP4P) with a low K_M at pH 2. If it is assumed that K_M approximates K_S, it may be surmised that the contribution to the binding energy of any ion pairing between the cationic group of the substrate and carboxylate groups in the enzyme would be proportionately greater when the binding of the rest of the substrate molecule to the enzyme is relatively weak. Further studies are needed, however, to establish the validity of the assumption that $K_M \simeq K_S$, and of the inferences drawn from it, since the question of the rate-limiting step in the overall process of the cleavage of substrates such as Z-Gly-Ala-Phe-Phe-OP4P is open, and requires experimental study.

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Fragmentation of Bovine Chymotrypsinogen A and Chymotrypsin Aα. Specific Cleavage at Arginine and Methionine Residues and Separation of Peptides, Including B and C Chains of Chymotrypsin*

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ABSTRACT: Chymotrypsinogen A and chymotrypsin Aα have been quantitatively degraded and separated into homogeneous peptide fragments utilizing simple aqueous reaction and solvent systems. Succinylation both solubilized the peptide derivatives and blocked the lysine residues to the action of trypsin. Disulfide bonds were cleaved by reduction with dithiothreitol and the newly formed cysteine residues alkylated with iodoacetamide. Specific trypsin cleavage at three arginine residues of the chymotrypsinogen derivative produced four pure peptides containing amino acid residues 1–15, 16–145, 146–230, and 231–245, respectively. Under the chosen conditions, no significant cleavage occurred at the fourth arginine residue, Arg 154. Size and solubility differences among the peptides allowed their complete separation and isolation in high yield by gel filtration on Sephadex columns in 0.01 M ammonium acetate. The succinylated, reduced, and carboxamidomethylated B and C chains (131 and 97 amino acids, respectively) of chymotrypsin Aα were isolated similarly except that no trypsin step was employed. Succinylated and carboxamidomethylated chymotrypsinogen was treated with cyanogen bromide to produce additional, and different, peptide fragments. By these methods many amino acid residues which have a role in enzyme function or which exhibit chemical reactivity can be readily segregated in unique peptide fragments.

Amino acid analyses of the individual peptides were in complete agreement with present knowledge of the sequences of chymotrypsinogen A and chymotrypsin Aα.

The characterization of chemically modified bovine chymotrypsinogen A and chymotrypsins A is hampered by the insolubility of the denatured protein and the tendency of large fragments to aggregate and precipitate. For example, upon digestion of the zymogen with trypsin over two-thirds of the protein is found in an insoluble aggregate, sometimes called the "core" (Hartley, 1964). For purposes of sequence analysis, various investigators have used methods of separation based on ion-exchange chromatography in 8 M urea after conversion of the protein into the oxidized, *S*-sulfo, *S*-carboxymethyl, or *S*-aminoethyl derivative (Hartley, 1963; van Hoang *et al.*, 1963; Meloun *et al.*, 1966, 1967). Separation of the B and C chains of *S*-sulfochymotrypsin Aα may also be achieved by fractional precipitation from solutions containing 7 M urea and 0.035 M sodium dodecyl sulfate (Richmond, 1966). Chymotryptic digestion of *S*-carboxymethylchymotrypsinogen leaves only 10% of the protein in the form of insoluble peptides (Kluh *et al.*, 1966). Most of the numerous soluble peptides are small, however, and quantitative recovery and identification would be exceedingly difficult. In all of these applications, difficulties resulting from aggregation have been only partially overcome and recovery of all portions of the protein has been far from quantitative. Burkhardt and Wilcox (1967) obtained a quantitative separation of fragments of the *S*-aminoethyl derivative of chymotrypsinogen

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