Action of Pepsin on Cationic Synthetic Substrates*

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ABSTRACT: The kinetic parameters for the peptic hydrolysis of a series of cationic peptide derivatives have been determined at several pH values. The substrates used were of the type AX–YB, where A = Z-His, Z-Gly-His, Z-His-Gly, Bz-Lys, Gly-Gly, or Gly-Gly-Gly; X = Phe, Gly, Tyr, or Trp; Y = Phe, Gly, Tyr, or Trp; and B = OMe, OEt, or NH_2. Significant differences were found in the values of K_M (assumed to approximate the dissociation constant of the productive enzyme–substrate complex) for comparable substrates in which A = Gly-Gly of Z-His. Other structural modifications caused relatively small changes in K_M; in numerous cases, significant changes in k_{cat} were found. Of special interest was the large difference in the k_{cat} values for the hydrolysis of Z-Gly-His-Phe-Phe-OEt and of Z-His-Gly-Phe-Phe, Gly, Tyr, or Rrp; Y = Phe, Gly, Tyr, or Rrp; and of the first substrate, but not of the second one, resembled that for Z-His-Phe-Phe-OEt. For a series of comparable substrates AX–YB, when Y = Phe, the presence in the X position of Phe (or *p*-nitro-L-phenylalanyl) led to much higher values of k_{cat} than with Tyr or Trp; replacement of these residues by Gly caused significantly slower hydrolysis. When X = Phe, the presence of Phe, Tyr, or Trp in the Y position gave substrates of approximately equal susceptibility; replacement of these residues by Gly or Leu caused equally slow cleavage. The data in this communication are discussed in relation to the hypothesis that both the substrate and the catalytic region of the enzyme undergo conformational changes as a consequence of multiple cooperative interactions.

In previous communications from this laboratory, it was reported that pepsin hydrolyzes the X–Y bond in synthetic substrates of the general structure AX–YB, where A is Z-His, Z-Gly-His, or Gly-Gly; X is L-phenylalanyl, *p*-nitro-L-phenylalanyl, L-tyrosyl, L-tryptophyl, or L-leucyl; Y is L-phenylalanyl, β-phenyl-L-lactyl, L-tyrosyl, L-tryptophyl, or L-leucyl; and B is OMe, OEt, OBu^t, or NH_2 (Inouye *et al.*, 1966; Inouye and Fruton, 1967). A detailed study of the pH dependence of the kinetic parameters (K_M and k_{cat})[1] in the peptic hydrolysis of Z-His-Phe-Phe-OEt has shown that the Phe–Phe bond of this substrate is cleaved optimally near pH 4.5, and that the dissociated form of a catalytically important prototropic group of $pK_a \simeq 3.8$ (possibly a carboxylate group) in the enzyme may form an ion pair with the imidazolium group of the substrate (Hollands and Fruton, 1968). This finding raised the question whether the pH dependence of the peptic hydrolysis of other cationic substrates resembles that of Z-His-Phe-Phe-OEt, and the present communication reports data for a series of such substrates. The results of this work have also suggested a working hypothesis on the role of the side-chain groups of a substrate in influencing the catalytic efficiency of pepsin at the X–Y bond.

Experimental Section

Chromatography. Examination of the homogeneity of the peptides prepared in this work and of the cleavage products released by pepsin was performed by thin-layer chromatography with silica gel G as the supporting phase (Eastman Chromagram sheets 6061). The following solvent systems were used: (A) 1-butanol–acetic acid–water (4:1:1, v/v), (B) methanol–benzene (15:85, v/v), (C) chloroform–methanol–acetic acid (95:5:1, v/v), and (D) methanol–ethyl acetate (15:85, v/v).

Z-Gly-Gly-Phe(NO_2)-Phe-OMe. To Phe(NO_2)-Phe-OMe[2] prepared from 1.13 g (2.5 mmoles) of the hydrobromide (Inouye and Fruton, 1967) was added a chilled ethyl acetate solution of Z-Gly-Gly-N_3, derived from 0.7 g (2.5 mmoles) of the hydrazide. The reaction mixture was kept at 0° overnight, and worked up in the usual manner to yield 1.04 g (67%) of the product, mp 162–163° (resolidifies and remelts at 190–192°). Chromatography (solvent A) gave a single spot of R_F 0.83 (iodine). *Anal.* Calcd for $C_{31}H_{33}N_5O_9$ (619.6): N, 11.3. Found: N, 11.1.

Gly-Gly-Phe(NO_2)-Phe-OMe Hydrobromide. The above product (0.93 g, 1.5 mmoles) was dissolved in warm glacial acetic acid (5 ml), a saturated solution of HBr in acetic acid (8 ml) was added, and the reaction mixture was kept at room temperature for 1 hr. Ether (100 ml) was added to yield 0.59 g (69%) of the product: $[\alpha]_D^{22}$ +5.4° (*c* 2, methanol), ultraviolet max (H_2O) 278.5

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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, S = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$. For competitive inhibition, K_I denotes the dissociation constant of EI in the process $E + I \rightleftharpoons EI$.

[2] Abbreviation used that is not listed in *Biochemistry 5*, 2485 (1966), is: Phe(NO_2), *p*-nitro-L-phenylalanyl. The abbreviated designation of amino acid residues denotes the L form, except where otherwise indicated.

mμ (ε 9600). Chromatography (solvent A) gave a single spot of R_F 0.67 (iodine, ninhydrin). *Anal.* Calcd for $C_{28}H_{22}BrN_5O_7$ (566.4): C, 48.8; H, 5.0; N, 12.4. Found: C, 48.8; H, 5.1; N, 12.4.

Z-Gly-Gly-Gly-Phe(NO_2)-Phe-OMe. To Phe(NO_2)-Phe-OMe prepared from 1.3 g (2.5 mmoles) of the hydrobromide was added a dimethylformamide solution of Z-$(Gly)_3$-N_3, prepared from 0.84 g (2.5 mmoles) of the hydrazide (Lautsch and Kraege, 1956). The reaction mixture was worked up in the usual manner. After recrystallization from methanol, the product melted at 179–180°; yield 0.9 g (53%). Chromatography (solvent A) gave a single spot of R_F 0.80 (iodine). *Anal.* Calcd for $C_{33}H_{36}N_6O_{10}$ (676.8): N, 12.4. Found: N, 12.6.

Gly-Gly-Gly-Phe(NO_2)-Phe-OMe Hydrobromide. The above compound (0.64 g, 0.95 mmole) was treated with HBr–acetic acid in the manner described for the diglycyl derivative. After recrystallization from methanol–ether, the product (0.59 g, 97%) melted at 187–188° dec: $[\alpha]_D^{23}$ +6.2° (*c* 2, methanol); ultraviolet max (H_2O) 278.5 mμ (ε 9550). Chromatography (solvent A) gave a single spot of R_F 0.54 (iodine). The compound was isolated as the monohydrate. *Anal.* Calcd for $C_{25}H_{31}BrN_6O_8 \cdot H_2O$ (641.5): C, 46.8; H, 5.2; N, 13.1. Found: C, 46.7; H, 5.2; N, 13.0.

Z-Gly-Gly-Phe-Tyr-OEt. A chilled ethyl acetate solution of Z-Gly-Gly-N_3, derived from 1.7 g (6 mmoles) of the hydrazide, was added to Phe-Tyr-OEt prepared from 2.4 g (6 mmoles) of the hydrochloride (obtained by hydrogenolysis of Z-Phe-Tyr-OEt (Inouye *et al.*, 1966)). After being kept for 3 hr at 0°, the reaction mixture was left at room temperature overnight, and worked-up in the usual manner to yield 2.7 g (75%) of the product, mp 187–189°. *Anal.* Calcd for $C_{32}H_{36}N_4O_8$ (604.7): N, 9.3. Found: N, 9.4

Gly-Gly-Phe-Tyr-OEt Acetate. Catalytic hydrogenolysis (palladium black) of 1.5 g (2.5 mmoles) of the above compound in methanol (20 ml) and glacial acetic acid (0.17 ml) gave 1.2 g (93%) of the product: $[\alpha]_D^{23}$ −4.5° (*c* 2, methanol), mp 143.5–145.5°. Chromatography (solvent A) gave a single spot of R_F 0.66 (iodine). *Anal.* Calcd for $C_{26}H_{34}N_4O_8$ (530.6): C, 58.85; H, 6.5; N, 10.6. Found: C, 58.8; H, 6.4; N, 10.4.

Chromatographic examination (solvent A) of a peptic hydrolysate of this compound (5 mM substrate, 0.2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of two ninhydrin-positive components, one of R_F 0.62 (identical with that for an authentic sample of Tyr-OEt), and the other of R_F 0.48 (identical with that for an authentic sample of Gly-Gly-Phe). There was no ninhydrin-positive component of R_F 0.74 (the value for an authentic sample of Phe-Tyr-OEt).

Z_2-Tyr-Phe-OEt. Z_2-Tyr (4.5 g, 10 mmoles) and Phe-OEt, derived from 2.5 g (11 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (2.1 g, 10 mmoles) with CH_2Cl_2 as the solvent. The yield was 3.8 g (61%); mp 184–185°. *Anal.* Calcd for $C_{36}H_{36}N_2O_8$ (624.7): N, 4.5. Found: N, 4.5.

Catalytic hydrogenolysis (palladium black) of 3.4 g (5.5 mmoles) of the above product in methanol (20 ml) and HCl (6 mmoles) gave Tyr-Phe-OEt hydrochloride. The yield was 2.0 g (93%); mp 200–201°. *Anal.* Calcd for $C_{20}H_{25}ClN_2O_4$ (392.9): N, 7.1. Found: N, 7.0.

Z-Gly-Gly-Tyr-Phe-OEt. To a chilled tetrahydrofuran solution of Z-Gly-Gly-N_3, derived from 1.4 g (5 mmoles) of the hydrazide, was added a tetrahydrofuran solution of Tyr-Phe-OEt prepared from 2.0 g (5 mmoles) of the hydrochloride. The reaction mixture was kept at 0° overnight, and worked-up in the usual manner to yield 2.2 g (73%) of the product, mp 210–211°. *Anal.* Calcd for $C_{32}H_{36}N_4O_8$ (604.7): N, 9.3. Found: N, 9.4.

Gly-Gly-Tyr-Phe-OEt Acetate. Catalytic hydrogenolysis (palladium black) of 1.5 g (2.5 mmoles) of the above product in ethanol (20 ml) and glacial acetic acid (0.17 ml) gave 1.2 g (93%) of the tetrapeptide acetate: mp 122–124° dec, $[\alpha]_D^{23}$ −4.4° (*c* 2, methanol). Chromatography (solvent A) gave a single spot of R_F 0.66 (iodine). *Anal.* Calcd for $C_{26}H_{34}N_4O_8$ (530.6): C, 58.85; H, 6.5; N, 10.6. Found: C, 58.7; H, 6.7; N, 10.5.

Chromatographic examination (solvent A) of a peptic hydrolysate of this compound (5 mM substrate, 2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of two ninhydrin-positive components, one of R_F 0.64 (identical with that for an authentic sample of Phe-OEt), and the other of R_F 0.39. There was no ninhydrin-positive component of R_F 0.74 (the value for an authentic sample of Tyr-Phe-OEt).

Z-Gly-Gly-Tyr-Tyr-OEt. To a chilled tetrahydrofuran solution of Z-Gly-Gly-N_3, derived from 0.56 g (2 mmoles) of the hydrazide, was added a chilled tetrahydrofuran solution of 0.75 g (2 mmoles) of Tyr-Tyr-OEt (Neumann *et al.*, 1959). After being kept for 24 hr at 0°, the solvent was removed *in vacuo* and the residue was recrystallized from methyl ethyl ketone. The yield was 0.76 g (61%); mp 166–167.5°. *Anal.* Calcd for $C_{32}H_{36}N_4O_9$ (620.7): N, 9.0. Found: N, 8.9.

Gly-Gly-Tyr-Tyr-OEt Acetate. Catalytic hydrogenolysis (palladium black) of 0.62 g (1 mmole) of the above compound in ethanol (20 ml) and glacial acetic acid (0.07 ml) gave 0.51 g (94%) of the tetrapeptide ester acetate: mp 152–154°, $[\alpha]_D^{23}$ −1.3° (*c* 2, methanol). Chromatography (solvent A) gave a single spot of R_F 0.67 (iodine). *Anal.* Calcd for $C_{26}H_{34}N_4O_9$ (546.6): C, 57.1; H, 6.3; N, 10.25. Found: C, 57.0; H, 6.5; N, 10.2.

Chromatographic examination (solvent A) of a peptic hydrolysate of this compound (5 mM substrate, 2 mg of pepsin/ml, pH 4.0, 37°. 24 hr) showed the presence of two ninhydrin-positive components, one of R_F 0.62 (identical with that for an authentic sample of Tyr-OEt), and the other of R_F 0.38. There was no ninhydrin-positive component of R_F 0.72 (the value for an authentic sample of Tyr-Tyr-OEt).

Z-D-His-Phe(NO_2)-Phe-OMe. This compound was prepared in the manner described previously for the L-L-L compound (Inouye and Fruton, 1967). From 0.91 g (3 mmoles) of Z-D-His-$NHNH_2$ (mp 170–171°, $[\alpha]_D^{22}$ +33.8° (*c* 2, 0.1 N HCl)) and 1.13 g (2.5 mmoles) of Phe(NO_2)-Phe-OMe hydrobromide, the yield was 1.0 g (62%): mp 158–160°, $[\alpha]_D^{23}$ −5.2° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.46 (iodine). *Anal.* Calcd for $C_{33}H_{34}N_6O_8$ (642.7): C, 61.7; H, 5.3; N, 13.1. Found: C, 61.5; H, 5.3; N, 13.1.

Z-His-Gly-Phe-OEt. To Gly-Phe-OEt prepared from 0.57 g (2 mmoles) of the hydrochloride was added a chilled ethyl acetate solution of Z-His-N_3 obtained from 0.73 g (2.4 mmoles) of the hydrazide. After being kept at 0° for 40 hr, the reaction mixture was worked up in the usual manner to yield 0.49 g (47%) of the product: mp 128–129°, $[\alpha]_D^{24}$ −3.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.33 (iodine). *Anal.* Calcd for $C_{27}H_{31}N_5O_6$ (521.6): C, 62.2; H, 6.0; N, 13.4. Found: C, 62.3; H, 6.1; N, 13.3.

Chromatographic examination (solvent D) of a peptic hydrolysate of this compound (4 mM substrate, 2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.80 (identical with that for an authentic sample of Phe-OEt). There was no ninhydrin-positive component of R_F 0.31 (the value for an authentic sample of Gly-Phe-OEt).

Z-His-Phe-Gly-OMe. A chilled ethyl acetate solution of Z-His-N_3, derived from 1.97 g (6.5 mmoles) of the hydrazide, was added to Phe-Gly-OMe, prepared from 1.72 g (5.4 mmoles) of the hydrobromide. After being kept at 0° for 4 days, the reaction mixture was worked up in the usual manner, and the product was recrystallized from methanol–water. The yield was 2.2 g (80%): mp 164–165°, $[\alpha]_D^{23}$ −23.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent A) gave a single spot of R_F 0.61 (iodine). *Anal.* Calcd for $C_{26}H_{29}N_5O_6$ (507.6): C, 61.5; H, 5.8; N, 13.8. Found: C, 61.4; H, 5.6; N, 13.8.

Chromatographic examination (solvent A) of a peptic hydrolysate of this compound (4 mM, 2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single major ninhydrin-positive component of R_F 0.30 (identical with that for an authentic sample of Gly-OMe). A faint spot of R_F 0.52 (identical with that for an authentic sample of Phe-Gly-OMe) was also noted, indicating that slight cleavage of the His-Phe had occurred, in addition to the major cleavage at the Phe–Gly bond.

Bz-Lys(Z)-Phe-Phe-OEt. N^{α}-Benzoyl-N^{ϵ}-benzyloxycarbonyl-L-lysine (1.35 g, 3.5 mmoles) and Phe-Phe-OEt hydrobromide (1.5 g, 3.5 mmoles) were coupled by the mixed-anhydride method (0.47 ml isobutylchloroformate), with *N*-methylmorpholine as the base and tetrahydrofuran (65 ml) as the solvent. The reaction mixture was worked up in the usual manner to yield 1.6 g (65%) of the product; after recrystallization from ethyl acetate, it melted at 183–184.5°. Chromatography (solvent C) gave a single spot of R_F 0.72 (iodine). *Anal.* Calcd for $C_{41}H_{46}N_4O_7$ (706.9): N, 7.9. Found: N, 7.9.

Bz-Lys-Phe-Phe-OEt Hydrobromide. The above product (1.06 g, 1.5 mmoles) was dissolved in glacial acetic acid (5 ml), a saturated solution of HBr in acetic acid (8 ml) was added, and the reaction mixture was kept at room temperature for 1 hr. Ether (150 ml) was added to yield 0.87 g (90%) of the product. Chromatography (solvent A) gave a single spot of R_F 0.61 (iodine, ninhydrin). *Anal.* Calcd for $C_{33}H_{41}BrN_4O_5$ (653.4): C, 60.6; H, 6.3; N, 8.6. Found: C, 60.4; H, 6.1; N, 8.3.

Other Synthetic Substrates. The synthesis of the other substrates used in this work, and the site of their cleavage by pepsin, have been described previously: Gly-Gly-Phe-Phe-OEt acetate, Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, Z-His-Phe-Trp-OEt, and Z-His-Tyr-Tyr-OEt (Inouye *et al.*, 1966); Z-His-Phe-Phe-OMe, Z-His-Phe-Phe-NH_2, Z-His-Phe(NO_2)-Phe-OMe, Z-Gly-His-Phe-Phe-OEt, Z-His-Gly-Phe-Phe-OEt, Z-His-Tyr-Phe-OMe, Z-His-Trp-Phe-OMe, Z-His-Leu-Phe-OMe, and Z-His-Phe-Leu-OMe (Inouye and Fruton, 1967).

Enzyme Studies. Except where otherwise noted, the pepsin preparation (Worthington Biochemical Corp., lot PM 708) used in previous kinetic studies (Inouye and Fruton, 1967; Hollands and Fruton, 1968) was employed; its proteinase activity was 2620 hemoglobin units/mg of pepsin. Several of the kinetic runs were repeated with pepsin-P prepared from crystalline pepsinogen (Worthington Biochemical Corp., lot PG 9163) in the manner described previously (Rajagopalan *et al.*, 1966; Delpierre and Fruton, 1966); its proteinase activity was 2910 hemoglobin units/mg of pepsin. The cleavage of the synthetic substrates was measured at 37.0 ± 0.1° in the presence of sodium formate buffer (0.04 M) to control the pH. The initial rate of cleavage of substrates containing the Phe(NO_2)-Phe group was followed spectrophotometrically, as described by Inouye and Fruton (1967). The kinetics of the hydrolyis of the other substrates was determined by means of the automatic ninhydrin method described previously (Hollands and Fruton, 1968). At each pH value, 6–20 determinations of v were made for each value of S, and satisfactory linear plots of v *vs.* v/S were obtained, from which values of V_m and K_M were estimated. The data were subjected to computer analysis, using the program of Hanson *et al.* (1967), as described previously (Hollands and Fruton, 1968). For the calculation of k_{cat}, it was assumed that 1 mg of pepsin = 0.0286 μmole. The enzyme concentration was determined spectrophotometrically at 278 mμ, with the assumption that pepsin has a molar absorptivity of 50,900 and a molecular weight of 34,163.

In the determination of the initial rates of hydrolysis of the tetrapeptide esters Gly-Gly-X-Y-OEt, it was assumed that the ninhydrin color value of Gly-Gly-X is the same as that of the substrate, since such identity was found for the pair of compounds Gly-Gly-Phe-Phe-OEt and Gly-Gly-Phe.

In the studies on the inhibition of pepsin action by structural analogs, Z-His-Phe(NO_2)-Phe-OMe was used as the substrate (at three concentrations, 0.1–0.35 mM), and at each substrate concentration the inhibitor was tested at four levels of concentration (0–3.6 mM). The four values of v obtained for a given substrate concentration (including the value in the absence of inhibitor, always determined in parallel) were used for $1/v$ *vs.* I (inhibitor concentration) plots according to the method of Dixon (1953). In all cases, satisfactory linear plots were obtained, with intercepts to the left of the ordinate and above the abscissa, indicating competitive inhibition.

Results

In Table I are given data for the kinetic parameters in the action of pepsin on substrates of the type AX–YB, where X and Y are L-phenylalanyl; A is Z-His, Z-Gly-His, Z-His-Gly, Bz-Lys, or Gly-Gly; and B is OEt,

TABLE I: Kinetics of Pepsin Action at Phe–Phe Bond of Synthetic Substrates.[a]

Substrate[b]	pH[c]	k_{cat} (10^2 sec^{-1})	K_M (mM)	k_{cat}/K_M (10^2 sec^{-1} mM^{-1})
Z-His-Phe-Phe-OEt[d]	2.5 (22)	14 ± 1	0.37 ± 0.05	37
(0.05–0.7 mM)	3.5 (19)	21 ± 1	0.21 ± 0.03	99
	4.0 (10)	33 ± 4	0.17 ± 0.05	190
	4.5 (18)	47 ± 2	0.18 ± 0.02	265
Z-His-Phe-Phe-OEt[e]	2.5 (12)	10 ± 1	0.36 ± 0.06	28
(0.05–0.7 mM)	4.0 (12)	31 ± 1	0.17 ± 0.01	186
Z-His-Phe-Phe-OMe[d]	2.5 (21)	8 ± 1	0.47 ± 0.06	17
(0.05–1.0 mM)	4.0 (19)	17 ± 1	0.33 ± 0.04	53
Z-His-Phe-Phe-NH_2[f]	2.5 (10)	7 ± 1	0.52 ± 0.12	14
(0.1–1.0 mM)	3.5 (10)	16 ± 2	0.48 ± 0.05	34
	4.0 (16)	21 ± 3	0.47 ± 0.05	44
	4.5 (10)	28 ± 3	0.29 ± 0.08	96
Z-Gly-His-Phe-Phe-	2.5 (10)	133 ± 31	1.5 ± 0.2	86
OEt[g] (0.1–1.1 mM)	3.5 (12)	237 ± 39	0.6 ± 0.1	381
	4.5 (8)	262 ± 38	0.34 ± 0.06	776
Z-His-Gly-Phe-Phe-	2.5 (9)	3.7 ± 0.3	0.54 ± 0.17	7
OEt[h] (0.1–1.0 mM)	3.5 (11)	5.1 ± 0.3	0.56 ± 0.16	9
	4.5 (10)	3.1 ± 0.3	0.17 ± 0.03	17
Bz-Lys-Phe-Phe-OEt[f]	2.5 (16)	14 ± 2	0.36 ± 0.11	38
(0.08–0.6 mM)	3.5 (12)	15 ± 2	0.32 ± 0.07	47
	4.5 (12)	26 ± 5	0.29 ± 0.11	89
Gly-Gly-Phe-Phe-OEt[f]	2.5 (9)	36 ± 9	2.2 ± 0.8	16
(0.2–1.5 mM)	3.5 (7)	37 ± 9	1.4 ± 0.6	26
	4.0 (11)	49 ± 12	1.6 ± 0.7	31
	4.5 (10)	40 ± 7	1.2 ± 0.4	35
Z-His-Phe(NO_2)-Phe-	2.5 (8)	9 ± 1	0.72 ± 0.17	12
OMe[f] (0.05–0.5 mM)	4.0 (10)	29 ± 5	0.46 ± 0.06	63
Gly-Gly-Phe(NO_2)-	4.0 (10)	12 ± 1	1.5 ± 0.2	8
Phe-OMe[f] (0.2–1.5 mM)				
Gly-Gly-Gly-Phe(NO_2)-	4.0 (10)	9 ± 1	1.5 ± 0.1	6
Phe-OMe[f] (0.1–1.1 mM)				

[a] Formate buffer (0.04 M); 37°. Unless otherwise stated, Worthington pepsin employed. [b] The numbers in parentheses denote the range of substrate concentration. [c] The numbers in parentheses denote the number of runs at this pH value. [d] Enzyme concentration, 0.27 μM. [e] Pepsin-P (see Experimental Section) employed; concentration, 0.27 μM. [f] Enzyme concentration 0.54 μM. [g] Enzyme concentration, 0.07 μM. [h] Enzyme concentration, 2.7 μM.

OMe, or NH_2. These data were obtained by means of the automatic ninhydrin procedure described previously (Hollands and Fruton, 1968). As noted in the Experimental Section, a commercial preparation of crystalline pepsin was used in this work, and it was desirable to compare its catalytic activity with that of a sample of the enzyme prepared from crystalline pepsinogen. It will be seen from Table I that the values of K_M and k_{cat} at pH 2.5 and 4.0 for the peptic hydrolysis of Z-His-Phe-Phe-OEt were nearly the same for the two enzyme preparations. It may be concluded, therefore, that at the levels of enzyme concentration used, the low molecular weight peptides present in commercial pepsin preparations (Rajagopalan *et al.*, 1966) do not affect the kinetics of the peptic hydrolysis of Z-His-Phe-Phe-OEt to a significant extent. The possibility exists, however, that when proteins or long-chain peptides are used as substrates, the peptide impurities may inhibit peptic action by interactions at secondary binding sites of the enzyme (Humphreys and Fruton, 1968).

In considering the data in Table I, attention may first be given to the effect of variation in the nature of B in substrates of the type Z-His-Phe-Phe-B. It will be seen that, for the three compounds having B = OEt, OMe, or NH_2, the effect of an increase in pH from 2.5 to 4.0 (or 4.5) is similar. The higher value of k_{cat}/K_M near pH 4 for the ethyl ester than for the methyl ester (or the amide) is a consequence of a slightly higher value of k_{cat} and a slightly lower value of K_M. For comparison with the data on other substrates, in which A, X, or Y are varied, it may be concluded that for the peptic hydrolysis at pH 4–4.5 of substrates such as Z-His-Phe-Phe-B, where B = OEt or OMe, k_{cat} = 0.2–0.5 sec^{-1}, K_M = 0.2–0.4 mM, and k_{cat}/K_M = 0.5–2.7. The data for

B = NH_2 (Table I) and for B = OBu^t (Inouye and Fruton, 1967) fall in this range of values.

Whereas the above changes in the nature of the B component of AX–YB cause relatively small changes in the kinetic parameters, changes in the A component affect decisively the values of k_{cat} and K_M, and their variation over the pH range 2.5–4.5. It will be seen that Z-Gly-His-Phe-Phe-OEt is hydrolyzed about three times more rapidly than Z-His-Phe-Phe-OEt, with a similar pH dependence of k_{cat}/K_M. The effect of changing A = Z-His to A = Z-Gly-His is largely to increase k_{cat}. In sharp contrast to the rapid hydrolysis of Z-Gly-His-Phe-Phe-OEt, the isomeric compound (in which A = Z-His-Gly) is not only much more resistant to peptic cleavage of the Phe–Phe bond, but the pH dependence of k_{cat} has disappeared, the slight increase in k_{cat}/K_M in the pH range 2.5–4.5 being entirely attributable to a decrease in K_M. It is clear, therefore, that the favorable effect on catalysis caused by displacement of the benzyloxycarbonyl group, away from the sensitive Phe–Phe bond, has been more than counteracted by the displacement of the histidyl residue.

In Table I are given data for a substrate in which A = Bz-Lys, and it will be noted that k_{cat} and K_M for its hydrolysis at pH 2.5 are the same as for the corresponding Z-His-Phe-Phe-OEt, but that the effect of increased pH on the kinetic parameters is not very marked. The values of k_{cat}/K_M for Bz-Lys-Phe-Phe-OEt thus place the peptic hydrolysis of this substrate in the range given above for Z-His-Phe-Phe-OR, the change in the cationic side chain having an effect on the pH dependence of hydrolysis.

The data in Table I also include the kinetic parameters for the peptic hydrolysis of Gly-Gly-Phe-Phe-OEt. The rapid cleavage of this peptide ester at the Phe–Phe linkage has been described in a previous communication (Inouye *et al.*, 1966); the values (at pH 4.0) of k_{cat} = 0.18 sec^{-1} and K_M = 0.28 mM reported there require correction, however. It will be noted from Table I that in the present work, conducted over the pH range 2.5–4.5, k_{cat} = *ca.* 0.4 sec^{-1} and K_M = *ca.* 1.5 mM. Because of the large number of replicate determinations in the present work, we believe that the earlier values are incorrect. No explanation can be offered for this discrepancy, except to note that the precision of the kinetic data obtained by the ninhydrin method for substrates of the type Gly-Gly-X-Y-OEt is less satisfactory than in the hydrolysis of substrates such as Z-His-Phe-Phe-OEt, because of the large base-line ninhydrin color at relatively high concentrations of the tetrapeptide esters.

Because of the limited precision of the data for Gly-Gly-Phe-Phe-OEt, only tentative conclusions can be drawn about the effect of a change in the A group from Z-His to Gly-Gly. It would appear, however, that although k_{cat} is approximately the same K_M is significantly greater. Furthermore, within the limits of error, there does not seem to be a marked change in either k_{cat} or K_M over the pH range 2.5–4.5, in distinct contrast to the behavior of Z-His-Phe-Phe-OEt. It would seem, therefore, that the striking change in k_{cat}/K_M with pH, seen with cationic substrates of the type A-Phe-Phe-OEt when A = Z-His or Z-Gly-His, is not seen when A = Gly-Gly, and is evident to a lesser degree when A = Bz-Lys.

The limitations of the ninhydrin method for the study of the kinetics of the hydrolysis of substrates related to Gly-Gly-Phe-Phe-OEt led us to synthesize the analogous Gly-Gly-Phe(NO_2)-Phe-OMe for use in the spectrophotometric assay developed in this laboratory (Inouye and Fruton, 1967). Separate experiments showed that at pH 4.0, the change in molar absorptivity at 310 mμ in the cleavage of the Phe(NO_2)–Phe bond of the tetrapeptide amide was 800, as in the case of the cleavage of Z-His-Phe(NO_2)-Phe-OMe. The data in Table I indicate that the change in A = Z-His to Gly-Gly causes a similar decrease in the value of k_{cat}/K_M for the pair of substrates having the Phe(NO_2)–Phe bond as for the comparable pair having the Phe–Phe bond. It was of interest to find that elongation of the peptide chain at the amino terminus to yield Gly-Gly-Gly-Phe(NO_2)-Phe-OMe caused a significant diminution in the value of k_{cat} at pH 4. Studies are in progress in this laboratory on the synthesis of long-chain unblocked peptides having the Phe(NO_2)–Phe bond as the site of peptic cleavage, and in which one or more Gly has been replaced by amino acid residues having hydrophobic side chains.

The kinetic data in Table II refer to substrates of the general type AX–YB in which X or Y are varied. Since the work of Baker (1951, 1954), it has been recognized that pepsin preferentially attacks peptide bonds linking two L-amino acid residues with apolar side chains. Quantitative data on the changes in the kinetic parameters upon the replacement of one of these residues by a glycyl residue have been lacking, however. It will be noted from Table II that if either L-phenylalanyl residue of the substrate Z-His-Phe-Phe-OR is replaced by a glycyl residue, the value of k_{cat} at pH 4–4.5 drops from about 0.35 to about 0.002 sec^{-1}, whereas the value of K_M is only about twice as high. Because of the extremely slow hydrolysis of the substrates Z-His-Gly-Phe-OR or Z-His-Phe-Gly-OR, the precision of the kinetic data is not great.

Examination of the data in Table II for substrates of the type A-X-Phe-OR, where X = Leu, Tyr, or Trp shows that the values of K_M (near pH 4) are similar to those found previously for A-Phe-Phe-OR, and that a change from A = Z-His to A = Gly-Gly causes a significant increase in K_M. The values of k_{cat}, however, are all much lower (*ca.* 0.02 sec^{-1}) than for the substrates in which X = Phe or Phe(NO_2) (*ca.* 0.35 sec^{-1}). Furthermore, for the substrates Z-His-X-Phe-OMe, where X = Tyr or Trp, the change in k_{cat} over the pH range 2.5–4.0 is relatively small as compared with the behavior of Z-His-Phe-Phe-OMe. It may be added that Dr. G. E. Trout of this laboratory has found (data to be published) that the kinetic parameters for the peptic hydrolysis of Z-His-Nle-Phe-OMe are similar to those for the cleavage of Z-His-Leu-Phe-OMe, and that Z-His-Val-Phe-OMe and Z-His-Ile-Phe-OMe are much more resistant than the substrates with X = Leu or Nle. It would appear, therefore, that although the apolar side chains of several amino acids (Leu, Trp) enhance the rate of cleavage of the X–Phe bond to a significant extent (about tenfold), as compared with the cleavage of the Gly–Phe

TABLE II: Kinetics of Pepsin Action on Synthetic Substrates.[a]

Substrate[b]	pH[c]	k_{cat} (10^2 sec^{-1})	K_M (mM)	k_{cat}/K_M (10^2 sec^{-1} mM^{-1})
Z-His-Gly-Phe-OEt[d] (0.6–2.7 mM)	4.0 (8)	0.08 ± 0.01	0.5 ± 0.2	0.16
Z-His-Leu-Phe-OMe[e] (0.2–1.0 mM)	4.0 (10)	1.7 ± 0.2	0.52 ± 0.15	3.1
Z-His-Tyr-Phe-OMe[d] (0.1–1.0 mM)	2.5 (11)	0.4 ± 0.1	0.42 ± 0.09	1.0
	4.0 (11)	0.9 ± 0.1	0.30 ± 0.06	3.0
	4.5 (9)	1.1 ± 0.1	0.36 ± 0.06	3.1
Gly-Gly-Tyr-Phe-OEt[d] (0.1–1.5 mM)	2.5 (12)	1.1 ± 0.3	0.7 ± 0.3	1.5
	3.5 (14)	3.0 ± 0.2	1.6 ± 0.6	1.9
	4.0 (16)	2.7 ± 0.2	1.2 ± 0.4	2.3
	4.5 (15)	5.2 ± 0.6	1.3 ± 0.6	3.9
Z-His-Trp-Phe-OMe[e] (0.1–1.0 mM)	2.5 (12)	0.7 ± 0.1	0.39 ± 0.08	1.8
	3.5 (14)	1.2 ± 0.1	0.24 ± 0.03	5.0
	4.0 (6)	1.4 ± 0.2	0.26 ± 0.03	5.4
Z-His-Phe-Gly-OMe[d] (0.5–5.0 mM)	2.5 (10)	0.10 ± 0.01	1.2 ± 0.4	0.09
	4.5 (6)	0.28 ± 0.02	0.8 ± 0.2	0.35
Z-His-Phe-Leu-OMe[d] (0.2–1.0 mM)	4.0 (8)	0.25 ± 0.02	0.54 ± 0.1	0.5
Z-His-Phe-Tyr-OMe[f] (0.5–1.0 mM)	2.5 (19)	11 ± 1	0.55 ± 0.04	20
	3.5 (20)	14 ± 1	0.29 ± 0.03	48
	4.0 (14)	17 ± 2	0.29 ± 0.06	58
	4.5 (14)	24 ± 3	0.29 ± 0.06	63
Gly-Gly-Phe-Tyr-OMt[f] (0.1–1.5 mM)	2.5 (11)	35 ± 10	1.2 ± 0.3	29
	3.5 (14)	59 ± 15	1.4 ± 0.6	42
	4.0 (15)	57 ± 10	0.9 ± 0.3	67
	4.5 (12)	56 ± 9	0.8 ± 0.3	71
Z-His-Phe-Trp-OEt[g] (0.1–1.0 mM)	2.5 (12)	29 ± 2	0.24 ± 0.04	121
	3.5 (12)	58 ± 3	0.16 ± 0.02	365
	4.0 (6)	51 ± 4	0.23 ± 0.03	222
Z-His-Tyr-Tyr-OEt[d] (0.1–1.0 mM)	4.0 (8)	1.0 ± 0.1	0.29 ± 0.05	3.5
Gly-Gly-Tyr-Tyr-OEt[d] (0.1–1.5 mM)	2.5 (10)	1.8 ± 0.4	1.6 ± 0.6	1.1
	3.5 (11)	2.7 ± 0.8	1.5 ± 0.4	1.9
	4.0 (16)	3.9 ± 0.6	1.8 ± 0.4	2.3
	4.5 (14)	4.0 ± 0.6	1.8 ± 0.3	2.2

[a] Formate buffer (0.04 M); 37°. [b] The numbers in parentheses denote the range of substrate concentration. [c] The numbers in parentheses denote the numbers of runs at this pH value. [d] Enzyme concentration, 13.6 μM. [e] Enzyme concentration, 2.7 μM. [f] Enzyme concentration, 0.54 μM. [g] Enzyme concentration, 0.27 μM.

bond, there is clear evidence of a preference for Phe or Phe(NO_2) residue in the X position when the Y position is occupied by a Phe residue. The significant role of the amino acid residue in the X position is consistent with the view that pepsin catalysis involves the nucleophilic attack by a carboxylate group of the enzyme at the carbonyl group of the sensitive peptide bond (Delpierre and Fruton, 1965).

As regards the effect of changes in the nature of Y with substrates of the type A-Phe-Y-OR, it will be seen from Table II that the replacement of Y = Gly by Y = Leu causes no significant change in k_{cat} or K_M, and similar values have been found for the hydrolysis of Z-His-Phe-Val-OMe and of Z-His-Phe-Ile-OMe (Dr. G. E. Trout, unpublished experiments). On the other hand, the introduction of a L-tyrosyl residue in the Y position renders the Phe–Y bond as sensitive to pepsin action as the Phe–Phe bond of comparable substrates. It will be noted that the values of k_{cat} and K_M near pH 4 for A-Phe-Tyr-OR are nearly the same as for A-Phe-Phe-OR, with similar differences in the kinetic parameters for the substrates in which A = Z-His or Gly-Gly. Because of the lack of precision in the kinetic data for Gly-Gly-Phe-Tyr-OEt, it is not possible to compare with confidence the pH dependence of k_{cat}/K_M for this substrate with the data for Z-His-Phe-Tyr-OMe, which exhibits a marked increase in the value of k_{cat} in the pH range 2.5–4.5.

As reported previously (Inouye *et al.*, 1966) the replacement of the L-phenylalanyl residue in the Y position of Z-His-Phe-Phe-OEt by a L-tryptophyl residue increases the rate of pepsin action at pH 4. This finding has been confirmed in the present studies, and a change

in pH from 2.5 to 4.0 has been found to cause a large increase in k_{cat}, the value of K_M remaining essentially unchanged. It should be added that during the course of the kinetic studies with Z-His-Phe-Trp-OEt and Z-His-Trp-Phe-OMe it was found that although Michaelis–Menten kinetics were obeyed in the pH range 2.5–4.0 over the range of substrate concentration tested (0.1–1.0 mM), marked inhibition was observed at pH 4.5 for substrate concentrations above 0.5 mM. This observation merits further study.

The data on the effect of changes in Y of substrates of the type A-Phe-Y-B lead to the conclusion that there is a decided preference for the residue of an aromatic amino acid in the Y position when X is Phe. The greater importance of the nature of the residue in X position is brought out, however, by the data in Table II for pepsin substrates of the type AX–YB in which X–Y is Tyr-Tyr. It will be noted that the values for K_M are comparable with the cases in which X–Y is Tyr-Phe or Phe-Tyr, with higher values of K_M when A = Gly-Gly than when A = Z-His. The values of k_{cat}, however, resemble only those for substrates in which X–Y is Tyr-Phe, and are markedly lower than for X–Y as Phe-Tyr. This result gives further support to the view that, for substrates of the type studied in this work, the nature of the amino acid in the X position is decisive in determining the catalytic efficiency in the peptic hydrolysis of the X–Y bond.

It is clear from the foregoing, as well as from the studies of Inouye and Fruton (1968) on the inhibition of pepsin action by substrate analogs, that the active site of the enzyme interacts readily with the benzyl side chains of adjacent phenylalanyl residues in synthetic substrates. Such interaction may be expected to limit the degrees of freedom of the remainder of the substrate molecule. In view of the indication that, at pH values near 4, the imidazolium group of Z-His-Phe-Phe-OEt forms an ion pair with a catalytically important carboxylate group in the enzyme (Hollands and Fruton, 1968), it seemed of interest to examine the effect of the replacement of the L-histidyl residue of such a substrate by its D enantiomer. Accordingly, Z-D-His-Phe(NO_2)-Phe-OMe was synthesized for study of the pH dependence of its kinetic parameters, as determined by the spectrophotometric method. If the above considerations are correct, this D-L-L compound should be hydrolyzed at the same rate as the L-L-L substrate at pH values near 2, and at a different rate at pH values near 4. The objective could not be fully realized, however, as the D-L-L compound proved to be much more insoluble in aqueous buffered solutions than its L-L-L diastereoisomer. The rate of its hydrolysis was determined at a substrate concentration of 0.1 mM and at an enzyme concentration of 0.02 mg/ml. Replicate (8–10) runs gave the following initial rates of hydrolysis (expressed as millimicromoles per minute per milligram of pepsin): pH 2.0, 7 ± 1; pH 4.0, 35 ± 2. Parallel determinations of the rate of cleavage of the L-L-L diastereoisomer under the same experimental conditions gave a value of 9.5 ± 1 at pH 2.0 and a value of 60 ± 4 at pH 4.0. Earlier work had shown that replacement of either of the L-phenylalanyl residues of Z-His-Phe-Phe-OEt by its D enantiomer blocked pepsin action at the Phe–Phe bond. Such stereospecificity is clearly not evident with respect to the L-histidyl residue.

Inhibition Experiments. Several of the compounds tested as pepsin substrates, and found to be relatively resistant to cleavage, were tested as competitive inhibitors in the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe, using the spectrophotometric assay. At the enzyme concentrations employed (0.54 μM), the rate of cleavage of the inhibitory compounds was negligible during the period of the experiment. Satisfactory plots of $1/v$ *vs.* I were obtained at pH 4.0 and 37° for Z-His-Gly-Phe-OEt, Z-His-Phe-Gly-OMe, and Gly-Gly-Tyr-Phe-OEt, and the K_I values were estimated to be (in millimolar) 1.9 ± 0.2, 2.1 ± 0.1, and 1.7 ± 0.4, respectively. The K_I values for Z-His-Gly-Phe-OEt and Z-His-Phe-Gly-OMe at pH 2.5 were found to be 1.5 ± 0.2 and 1.7 ± 0.3, respectively. These values of K_I are near the corresponding values of K_M (Table II), within the precision of the measurements. When taken together with the earlier data on the inhibition of pepsin action by substrate and alogs (Inouye and Fruton, 1967, 1968), these results give further support to the view that K_M approximates K_S in the present studies.

Effect of Buffers on the Peptic Hydrolysis of Cationic Substrates. In the connection with the above studies, it was necessary to examine the effect of changes in the buffer species on the kinetic parameters of the peptic cleavage of substrates such as Z-His-Phe-Phe-OEt. Although the action of pepsin on small synthetic substrates appears to be relatively independent of ionic strength (Zeffren and Kaiser, 1967; Hollands and Fruton, 1968), work in this laboratory pointed to an inhibitory effect of relatively high concentrations of acetate buffer, as compared with formate or citrate buffer. It seemed desirable therefore to examine more closely the rate of pepsin action at pH 4.0 in the presence of various concentrations of acetate (pK_a = 4.7), formate (pK_a = 3.8), and chloroacetate (pK_a = 2.9) buffers. The data in Table III on the kinetic parameters for the hydrolysis of Z-His-Phe-Phe-OEt, as determined by the automatic ninhydrin method, indicate that among the three buffer systems used, formate gives the highest values of k_{cat}, and does not show the inhibitory effect observed with increasing concentrations (up to 0.4 M) of acetate or chloroacetate. This inhibitory effect does not appear to be a reflection of the ratio of COOH to COO^-, as there is no correlation with the pK_a values of the three buffer acids, but may rather be attributed to an interaction of the enzyme with the apolar group of the buffer pair. In kinetic studies with pepsin, therefore, buffers such as formate, citrate, or phosphate appear to be more suitable than acetate, although citrate (and possibly also phosphate) at high concentrations also inhibits pepsin action (Inouye and Fruton, 1968), presumably because of its polyvalent nature. For this reason, 0.04 M formate was selected as the standard buffer in the present studies; it may be added that, except for the case of Gly-Gly-Phe-Phe-OEt (as noted above), the kinetic data obtained with this buffer were the same, within the precision of the methods employed, as those obtained previously in the presence of 0.04 M citrate buffer.

TABLE III: Effect of Buffer on Kinetics of Hydrolysis of Z-His-Phe-Phe-OEt by Pepsin.[a]

	Chloroacetate		Acetate		Formate	
Buffer Concn (M)	k_{cat} (sec^{-1})	K_M (mM)	k_{cat} (sec^{-1})	K_M (mM)	k_{cat} (sec^{-1})	K_M (mM)
0.04	0.23	0.15	0.36	0.24	0.43	0.23
0.10	0.20	0.15	0.33	0.31	0.47	0.27
0.40	0.12	0.10	0.27	0.30	0.45	0.23

[a] The buffers were prepared by mixing equimolar solutions of the acid and its sodium salt to give pH 4.0 (Beckman Expandomatic pH meter). In the kinetic runs, the range of substrate concentration was 0.05–0.60 mM, the enzyme concentration was 0.27 μM; temperature, 37°. At each concentration of a given buffer, 8–15 determinations were performed for v *vs.* v/S plots. The precision of the kinetic values is approximately ±10%.

Discussion

The available data on the kinetics of the peptic cleavage of the X–Y bond of substrates AX–YB (Inouye and Fruton, 1967; Hollands and Fruton, 1968), and on the competitive inhibition of such hydrolysis by substrate analogs (Inouye and Fruton, 1968) are consistent with the assumption that the kinetically determined value of K_M approximates a dissociation constant (or a combination of dissociation constants). If this assumption is accepted as a basis for the discussion of the data presented in Tables I and II, one may first ask whether these data give information about the extent to which unreactive ("nonproductive") enzyme–substrate complexes (Hein and Niemann, 1961, 1962) play a role in determining the observed values of K_M and k_{cat}. Such nonproductive interaction, if characterized by a lower dissociation constant than the K_S of the reactive ("productive") complex, may lead to competitive inhibition by the substrate. In these circumstances, Michaelis–Menten kinetics are obeyed, but the observed value of K_M is equal not to K_S, but to $K_S K_N/(K_S + K_N)$, where K_N is the dissociation constant (or combination of constants) of the inhibitory nonproductive complex. Clearly, if K_N is much larger than K_S, K_M will approximate K_S. A situation where K_N is less than K_S will make K_M much lower than K_S and will also lead to a decrease in the value of k_{cat}, since $k_{cat}(obsd) = K_M k_{cat}(uninhibited)/K_S$. Thus, when a structural change in a substrate leads to a significant decrease in both K_M and k_{cat}, the occurrence of increased nonproductive inhibitory binding must be considered as a possibility. Niemann and his colleagues applied such considerations to the interpretation of the extensive data gathered in Niemann's laboratory on the cleavage of acylamino acid esters and amides by chymotrypsin (Hein and Niemann, 1961, 1962; Wolf and Niemann, 1963; Jones *et al.*, 1965).

Examination of the data in Tables I and II, for the peptic hydrolysis of substrates of the type AX–YB, gives little evidence for significant differences in the extent of nonproductive inhibitory interaction of the kind discussed above. One of the most striking features of these data is that for the two series of substrates Z-His-X-Y-OR and Gly-Gly-X-Y-OR, variations in the nature of X or Y (one of them is always Phe) have only a minor effect on the magnitude of K_M, but exert major effects on k_{cat}. Although the possible occurrence of nonproductive interaction near the catalytic site is not excluded, the data suggest that such interactions are not characterized by K_N values much smaller than the value of K_S in a given pepsin–substrate interaction.

If the measured value of K_M is indeed a measure of the dissociation of the reactive complex (or complexes) leading to products, the data presented above, taken together with those presented previously (Inouye and Fruton, 1968), indicate that the binding area in the region of the catalytic site of pepsin has loci for interaction with the hydrophobic groups of the two L-amino acids forming the sensitive peptide bond. The finding that K_M for the hydrolysis of Z-His-X-Y-OR, where X–Y is either Gly-Phe or Phe-Gly is significantly greater than when X–Y = Phe-Phe supports the view that the binding area is so constructed that it can engage in cooperative interaction with the side chains of both L-phenylalanyl residues.

That a suitably located hydrophobic group in the A portion of a substrate AX–YB may assist binding is suggested by the lower K_M values for substrates in which the A portion includes the benzyl group of a benzyloxycarbonyl group (*e.g.*, A = Z-His, Z-Gly-His, Z-His-Gly), as compared with analogous substrates in which A = Gly-Gly. If this conclusion is correct, it may be expected from the data in Table I that peptides of the type Phe-Gly-His-Phe(NO_2)-Phe-B should be tightly bound and should be rapidly cleaved by pepsin at the Phe(NO_2)–Phe bond; this question is currently under investigation. In addition to the effect on K_M of a hydrophobic group in the A portion of AX–YB, comparison of the data for the compounds in which B is OMe or OEt suggests that a hydrophobic group in B also promotes binding, and this possibility is also being examined through the synthesis of pentapeptide derivatives of the type mentioned above, in which the B portion contains one or more L-alanyl residues.

Although the available data are limited in scope, it would seem justifiable to accept, as a working hypothesis, the view that the binding area in the region of the catalytic site of pepsin can interact with a number of hydrophobic groups in a substrate. The data in Table I indicate not only multiple cooperative hydrophobic interactions but also show that the presence of a suitably located imidazolium group in the A portion of A-Phe-Phe-OR promotes the rate of peptic action at the Phe–Phe bond. This rate enhancement is reflected both in a decrease in K_M and an increase in k_{cat} when the pH is shifted from 2.5 to near 4, a finding consistent with

the suggestion (Hollands and Fruton, 1968) that the imidazolium group of Z-His-Phe-Phe-OEt interacts with a suitably located carboxylate group of the enzyme. A comparison of the K_M values for the two substrates A-Phe-Phe-OEt in which the A portion is A-Gly-His and Z-His-Gly indicates, however, that the marked difference in the rates of their cleavage at pH 4.5 cannot be attributed to a difference in the extent to which they are bound at the catalytic site. It may be inferred, therefore, that the postulated ion pair formed when A is Z-His or Z-Gly-His does not in itself contribute significantly to binding, and that such electrostatic interaction is a consequence of the cooperative interactions of hydrophobic groups in the substrate molecule with appropriate loci in the region of the catalytic site of the enzyme.

Examination of the data in Table II indicates that, for substrates of the type AX–YB, where A is Z-His or Gly-Gly, and B is OMe or OEt, variations in the nature of the hydrophobic side chains present in X and Y affect the value of K_M only slightly but have large effects on the magnitude of k_{cat}. When Y = Phe, the variations in the nature of X examined thus far suggest a special preference for the apolar side chain of a L-phenylalanyl (or *p*-nitro-L-phenylalanyl) residue, with a much lesser positive contribution by the nonionized side chains of other amino acid residues (Leu, Tyr, and Trp). The question whether the retardation of catalysis caused by the replacement of Phe by Tyr in the X position is caused by an unfavorable interaction of the phenolic hydroxyl group of the substrate may perhaps be answered by the examination of the susceptibility of the comparable substrate in which X is a *p*-methoxy-L-phenylalanyl residue. Also, it will be of interest to examine the effect of the presence of a β-cyclohexyl-L-alanyl residue in the X position of Z-His-X-Phe-OR, to determine whether there is a special preference for an aromatic side chain. If the catalytic center of pepsin has a binding locus with a preference for the benzyl group of a L-phenylalanyl residue in the X position, that locus must also readily interact with the *p*-nitrobenzyl group, and it may be noted in this connection that the most stable conformation of the *p*-nitrophenyl group is one in which the nitro group and the benzene ring are coplanar (Wheland, 1955). Furthermore, in view of the marked enhancement of the rate of cleavage by the replacement of the glycyl residue of Z-His-Gly-Phe-OR by a leucyl or a tryptophyl residue, the presumed locus of preferred interaction with a benzyl group should be capable of more limited hydrophobic interaction with the side chains of these two amino acid residues.

The kinetic data in Table II indicate that when X = Phe, and the nature of Y is varied, the K_M and k_{cat} values for the rate of cleavage of the Phe–Tyr or Phe–Trp bonds are similar to the values for the cleavage of the Phe–Phe bond in comparable substrates. The much lower susceptibility of the Phe–Leu bond in substrates of the type Z-His-Phe-Y-OR indicates a preferential interaction with the aromatic side chain of Phe, Tyr, or Trp in the Y portion of the substrate. It will be of special interest, therefore, to examine the kinetic parameters for the peptic hydrolysis of a substrate of this type in which Y is β-cyclohexyl-L-alanine; this work is in progress.

It has been reported previously that the diastereoisomeric compounds Z-His-D-Phe-Phe-OEt and Z-His-Phe-D-Phe-OEt are resistant to pepsin action, and that they are competitive inhibitors with K_I values near the value of K_M for Z-His-Phe-Phe-OEt (Inouye and Fruton, 1967). The possibility exists, therefore, that the hydrophobic groups of the inhibitors and of the substrate interact with similar loci in the binding area of the catalytic center, but that the changed spatial orientation of the CONH group between the two phenylalanyl residues of the L-D-L and L-L-D compounds renders that peptide bond inaccessible to attack at the catalytic site. The similarity between the values of K_I for these inhibitors and the value of K_M for the L-L-L substrate may be taken as evidence in favor of the assumption that the kinetically determined value of K_M approximates the dissociation constant K_S. Further support is provided by the data given above for the inhibition of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by more resistant substrates (Z-His-Gly-Phe-OEt, Z-His-Phe-Gly-OMe, Gly-Gly-Tyr-Phe-OEt); in these cases, the observed value of K_I was near the value of K_M found when the inhibitor served as a substrate.

If, as seems probable, $K_M = K_S$ for the substrates listed in Table II, it is difficult to explain the large differences in their rates of cleavage solely in terms of differences in binding affinity. It is appropriate, therefore, to consider other factors (in addition to nonproductive inhibitory interaction) that may be involved in causing the wide variation in the susceptibility of the X–Y bond of substrates of the type Z-His-X-Y-OR.

One possiblity that may be envisaged is a form of nonproductive binding, which might either inhibit or activate catalysis, involving an interaction of the substrate at secondary binding sites of pepsin (Humphreys and Fruton, 1968) to produce a change in the conformation of the groups forming the catalytic site, with a resulting decrease or increase in its catalytic efficiency. Such effects have been extensively discussed in the recent literature for ligands that are structurally different from substrates ("allosteric" effects). There is no resason *a priori* why an enzyme such as pepsin, which clearly has the capacity for multiple cooperative interaction with long-chain peptides, should not bind a small peptide substrate at some distance from the catalytic site, and exhibit a conformational change at that site in response to what may be termed an "autosteric" effect (Koshland and Neet, 1968). Such an interaction has been described for the nonproductive interaction of lysozyme with a trisaccharide (Blake *et al.*, 1967), and may well be operative in some of the cases for which data are given in Tables I and II. It is also possible that the effect of acetylation of pepsin in increasing k_{cat} for the cleavage of Z-His-Phe-Phe-OEt (Hollands and Fruton, 1968) may be a consequence of the fact that such nonproductive interaction does occur, and is diminished by acetylation of the enzyme. At the present stage of our knowledge, however, it seems difficult to ascribe so such "autosteric" interaction the very large differences in k_{cat} for such pairs of substrates as Z-Gly-His-Phe-Phe-OEt and Z-His-Gly-Phe-Phe-OEt.

A more attractive general hypothesis to explain the

kinetic parameters of pepsin action on small substrates is to assume that, in the interaction of the catalytic region of the enzyme with a series of substrates of different structure, a number of different productive complexes can be formed. The available data suggest that this binding region is asymmetric, with at least two parts (P and Q), and that for the catalytic cleavage of AX–YB the side chain of X must interact with P, and the side chain of Y must interact with Q. It is reasonable to expect that the cooperative interaction of X and Y with P and Q, as affected by other interactions (both hydrophobic and electrostatic interactions) further removed from the catalytic site, may alter the conformational relationship of the enzymic groups concerned with the bond-breaking step in pepsin action. In particular, such changes in conformation may affect the efficiency of catalysis by influencing the geometry of intermediate states of the enzyme–substrate complex, with an effect on such processes as proton transfer *via* directed hydrogen bonds between groups in the enzyme and the amide group of the substrate (Wang, 1968). The possibility may also be considered that the cooperative interactions postulated above may induce strain at the CO–NH bond (Lumry, 1959; Jencks, 1966) leading to steric inhibition of amide resonance (Pauling and Corey, 1951; Holley, 1953) and thus reducing the activation energy for hydrolysis.

The hypothesis outlined above thus assumes complementary conformational changes in the substrate (to promote its transformation) and of the catalytic region of the enzyme (to enhance or diminish its efficiency), and has been discussed previously by many investigators (for a recent summary, see Koshland and Neet, 1968). Experimental evidence in favor of conformational changes at the catalytic center of a proteolytic enzyme has come from the study of the effect of the binding of Gly-Tyr on the X-ray diffraction pattern of crystalline carboxypeptidase (Reeke *et al.*, 1967; Steitz *et al.*, 1967), and from the examination of the effect of modifiers on the catalytic activity of this enzyme (Davies *et al.*, 1968). In the case of pepsin, whose binding region at the catalytic site interacts with hydrophobic side chains on both sides of the sensitive bond, such conformational changes in the enzyme are extremely probable, and the effect of a suitably located cationic group (as in Z-His-Phe-Phe-OEt) may be to contribute an additional conformational change favorable for catalysis.

The assumption of complementary conformational changes in the enzyme–substrate complex may apply not only to the positioning of the enzymic groups concerned with the bond-breaking step, but to the binding area as well. For proteinases such as pepsin, chymotrypsin, and papain, whose side-chain specificity is relatively broad, the effect on binding of a change in the nature of amino acid residue Y in AX–YB may be different for the series of substrates in which X = Phe from a comparable series in which X is a different type of amino acid residue. Insufficient data are available for the cleavage of synthetic peptides by these proteinases to do more than to raise this question for future study. In the case of chymotrypsin, most of the extensive studies have dealt with variations in the A and X components of AX–B, where the B component of the sensitive X–B bond has been OMe, OEt, NH_2, $NHNH_2$, or $NHCH_2CONH_2$. An interesting case is that studied by Hofstee (1957) whose kinetic data for the cleavage of a series of *n*-fatty acyl *m*-hydroxybenzoic acids by chymotrypsin showed a different relationship between K_M and k_{cat} from that observed with methyl esters of acylamino acids (Jones *et al.*, 1965; Ingles and Knowles, 1966).

The likelihood of flexibility in the substrate binding region of an enzyme such as pepsin, with relatively broad side-chain specificity and the capacity for multiple cooperative interactions, suggests caution in designating the apparent loci of hydrophobic interaction for a substrate such as Z-Gly-His-Phe-Phe-OEt as "subsites." Such a designation involves the assumption that these loci of interaction are associated with a relatively rigid structure at the substrate binding area of pepsin. If this assumption is correct, one may hope to define the relative position of the subsites of the enzyme. An example of such mapping has been reported for papain by Schechter and Berger (1967). On the other hand, if the cooperative effect of multiple interactions may lead to such changes in the conformation of pepsin so as to create one set of apparent subsites for one type of substrate, and a different group of subsites for another type of substrate, it may not be possible to define the binding area in terms of a unique structure.

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References

Baker, L. E. (1951), *J. Biol. Chem. 193*, 809.
Baker, L. E. (1954), *J. Biol. Chem. 211*, 701.
Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc. (London) B167*, 378.
Davies, R. C., Auld, D. S., and Vallee, B. L. (1968), *Biochem. Biophys. Res. Commun. 31*, 628.
Delpierre, G. R., and Fruton, J. S. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1161.
Delpierre, G. R., and Fruton, J. S. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1817.
Dixon, M. (1953), *Biochem. J. 55*, 170.
Hanson, K. R., Ling, R., and Havir, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 194.
Hein, G. E., and Niemann, C. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1341.
Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc. 84*, 4495.
Hofstee, B. H. J. (1957), *Biochim. Biophys. Acta 24*, 211.
Hollands, T. R., and Fruton, J. S. (1968), *Biochemistry 7*, 2045.
Holley, R. W. (1953), *Science 117*, 23.
Humphreys, R. E., and Fruton, J. S. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 519.
Ingles, D. W., and Knowles, J. R. (1966), *Biochem. J. 99*, 275.

Inouye, K., and Fruton, J. S. (1967), *Biochemistry 6*, 1765.
Inouye, K., and Fruton, J. S. (1968), *Biochemistry 7*, 1611.
Inouye, K., Voynick, I. M., Delpierre, G. R., and Fruton, J. S. (1966), *Biochemistry 5*, 2473.
Jencks, W. P. (1966), *in* Current Aspects of Biochemical Energetics, Kaplan, N. O., and Kennedy, E. P., Ed., New York, N. Y., Academic, p 273.
Jones, J. B., Kunitake, T., Niemann, C., and Hein, G. E. (1965), *J. Am. Chem. Soc. 87*, 1777.
Koshland, D. E., Jr., and Neet, K. E. (1968), *Ann. Rev. Biochem. 37*, 359.
Lautsch, W., and Kraege, H. J. (1956), *Chem. Ber. 89*, 737.
Lumry, R. (1959), *Enzymes 1*, 157.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1959), *Biochem. J. 73*, 33.
Pauling, L., and Corey, R. B. (1951), *Proc. Natl. Acad. Sci. U. S. 37*, 251.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *J. Biol. Chem. 241*, 4940.
Reeke, G. N., Hartsuck, J. A., Ludwig, M. L., Quiocho, F. A., and Lipscomb, W. N. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2220.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157.
Steitz, T. A., Ludwig, M. L., Quiocho, F. A., and Lipscomb, W. N. (1967), *J. Biol. Chem. 242*, 4662.
Wang, J. H. (1968), *Science 161*, 328.
Wheland, G. W. (1955), Resonance in Organic Chemistry, New York, N. Y., Wiley, p 159.
Wolf, J. P., and Niemann, C. (1963), *Biochemistry 2*, 82.
Zeffren, E., and Kaiser, E. T. (1967), *J. Am. Chem. Soc. 89*, 4204.

A Functional Role of Metal Ions in a Class II Aldolase*

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ABSTRACT: A crucial role of the metal ion in the catalytic activity of yeast aldolase has been demonstrated. Zinc can be removed from the native enzyme resulting in an inactive apoenzyme. The activity can be reconstituted by addition of Zn^{2+} or by certain ions of the first transition period, namely Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+}. The Zn^{2+} protein has the highest specific activity; the other metal ions restore the enzymatic activity to varying degrees.

A number of other metal ions (Cu^{2+}, Hg^{2+}, Cd^{2+}, Mg^{2+}, and Fe^{3+}) fail to restore activity to any measurable extent. All active metalloaldolases exhibit similar K_m values for fructose diphosphate and are stimulated by K^+ ion.

Studies on fructose diphosphate aldolases from phylogenetically divergent organisms suggest that these enzymes can be grouped into two distinct classes (Rutter, 1964). The class I enzymes are found in animals, plants, protozoa, and algae. Rabbit muscle aldolase, the prototype of the class I enzymes, has a molecular weight of 160,000, a tetrameric structure (Kawahara and Tanford, 1966; Penhoet *et al.*, 1966, 1967), and a mechanism which involves Schiff-base formation between dihydroxyacetone phosphate and a lysyl residue on the protein molecule (Grazi *et al.*, 1962; Horecker *et al.*, 1963; Model *et al.*, 1968).

Class II aldolases are found in most bacteria, fungi, yeast, and blue-green algae (Rutter, 1964). Yeast aldolase, the prototype of the class II enzymes, has a molecular weight of approximately 80,000, a dimeric structure (C. E. Harris, R. D. Kobes, D. C. Teller, and W. J. Rutter, in preparation), and is activated by K^+ ion. Little is known about the catalytic mechanism of the yeast enzyme.

Warburg and Christian (1943) first observed that yeast aldolase was inhibited by chelating agents and proposed that it was activated by a metal ion (presumably Fe^{2+}). Later it was found that significant quantities of zinc, and only traces of iron were present in this enzyme prepared by various methods. Thus, zinc contents of 1800 μg/g of protein (Rutter and Ling, 1958), 1150 μg/g of protein (Richards and Rutter, 1961), and 1200 μg/g of protein (B. L. Vallee, unpublished observations) have been found, although much lower zinc contents (465–643 μg/g of protein) have been reported by other workers (Vanderheiden *et al.*, 1962). These results suggested that yeast aldolase might be a zinc metalloprotein, but evidence for participation of the metal atom in either the structure or the catalytic action of this enzyme has not been reported.

Previous attempts to study the molecular and catalytic properties of this enzyme were hampered by the instability of the purified preparations. We have recently been able to obtain yeast aldolase in a stable and homogeneous form, thus permitting us to undertake studies on the molecular architecture and mechanism of action of this enzyme.

In this context, we now have been able to directly dem-

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