Effect of Secondary Enzyme–Substrate Interactions on the Cleavage of Synthetic Peptides by Pepsin*

Kalman Medzihradszky,† Irene M. Voynick, Hedvig Medzihradszky-Schweiger,‡ and Joseph S. Fruton§

ABSTRACT: A series of synthetic oligopeptide substrates for pepsin has been prepared in which the *p*-nitro-L-phenylalanyl–L-phenylalanyl bond is the only one attacked. Estimates of the kinetic parameters (pH 4, 37°) for substrates of the type A-Phe(NO_2)-Phe-B have shown that when the A group is Phe-Gly-His or Z-His, a change in the B group from OMe to Ala-Ala-OMe or Ala-Phe-OMe leads to a large increase in the value of k_{cat}/K_M. This rate enhancement is attributed to secondary enzyme–substrate interactions, involving hydrophobic groups in the B portion of the substrate, and is largely reflected in an increase in k_{cat}. It is suggested that these secondary interactions affect the catalytic efficiency of pepsin by promoting the better positioning of the catalytic groups of the enzyme in relation to the sensitive peptide bond. Although, with some pairs of substrates (*e.g.*, A = Phe-Gly or Gly-Gly) evidence of nonproductive interaction has been obtained (parallel changes in k_{cat} and K_M), such interactions do not appear to be the determinant factor in most of the rate enhancements observed. Advantage has been taken of the effect of changing the B group from OMe to Ala-Ala-OMe to show that a Phe(NO_2)–Gly bond becomes much more susceptible to pepsin action when secondary interactions are operative, thus providing a partial explanation of the apparently broad side-chain specificity of pepsin in its action on protein substrates.

In previous communications from this laboratory, a series of new pepsin substrates of the type Z-His-X-Y-OMe (or OEt)[1] was described; in these compounds, the enzymic cleavage is restricted to the bond linking the amino acid residues X and Y (Inouye *et al.*, 1966; Inouye and Fruton, 1967; Hollands *et al.*, 1969; Trout and Fruton, 1969). A study of the pH dependence of the kinetic parameters (k_{cat} and K_M)[2] for the substrate Z-His-Phe-Phe-OEt gave a maximum for k_{cat}/K_M near pH 4 (Hollands and Fruton, 1968). An examination of the effect of changes in the nature of X and Y on the rate of pepsin action at pH 4.0 showed that the X–Y bond of Z-His-X-Y-OMe is cleaved most rapidly when X = L-phenylalanyl and Y = L-tryptophyl, L-phenylalanyl, or L-tyrosyl, the other member of the X–Y pair always being L-phenylalanyl (Trout and Fruton, 1969). It was also noted that the replacement of Phe in the X position of Z-His-Phe-Phe-OMe by Phe(NO_2) did not alter the kinetic parameters greatly, and that the hydrolysis of the Phe(NO_2)–Phe bond at pH 4 could be followed spectrophotometrically at 310 mμ (Inouye and Fruton, 1967).

It has been suggested previously (Humphreys and Fruton, 1968; Hollands *et al.*, 1969) that, in the cleavage of peptide substrates by pepsin, secondary interactions at some distance from the locus of catalytic action may play a significant role in determining the sensitivity of a CO–NH bond positioned at the catalytic site. Evidence in support of this view has come from studies on the rate of hydrolysis of substrates of the type A-Phe-Phe-B, where A = benzyloxycarbonyl (Z), Z-Gly, or Z-Gly-Gly, and B is a pyridinium alkyloxy group (Sachdev and Fruton, 1969). Thus, the Phe–Phe bond of Z-Gly-Gly-Phe-Phe-OP4P is cleaved at pH 2 about 300 times more rapidly than the Phe–Phe bond of Z-Phe-Phe-OM4P.

The present communication reports our initial studies on the effect of structural changes in the A and B groups of A-Phe(NO_2)-Phe-B on the kinetic parameters in the cleavage of the Phe(NO_2)–Phe bond by pepsin. As an approach to the understanding of the action of pepsin on proteins and on long-chain natural peptides, the size of the A and B groups has been increased by the addition of amino acid residues to produce oligopeptides (up to heptapeptides) of known structure, in which A = Gly, Gly-Gly, Phe-Gly, Phe-His, Z-His, Phe-Gly-Gly, Phe-Gly-His, or Phe-Gly-Gly-His, and B = OMe, Gly-OMe, His-OMe, Ala, Ala-OMe, Ala-Ala, Ala-Ala-OMe, or Ala-Phe-OMe; in all cases, the only peptide bond hydrolyzed at a measurable rate under the condition of our studies was the Phe(NO_2)–Phe linkage. As will be seen from what follows, such oligopeptides include some of the most sensitive synthetic peptide substrates hitherto found for pepsin.

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† Visiting Research Scholar, Ford Foundation Hungarian Exchange Program. Present address: Institute of Organic Chemistry, University of Budapest, Hungary.

‡ Present address: Institute of Organic Chemistry, University of Budapest, Hungary.

§ To whom requests for reprints should be addressed.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445, 2485 (1966), are: Phe(NO_2), *p*-nitro-L-phenylalanyl; OSu, oxysuccinimido: OM4P, 4-pyridylmethoxy; OP4P, 3-(4-pyridyl)propyl-1-oxy. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

[2] 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 $K_S = k_{-1}/k_1$.

The synthesis of these oligopeptides was effected by stepwise addition of the amino acid units, with isolation, purification, and characterization of the intermediates. In view of the strong binding of the Phe–Phe unit to the catalytic site of pepsin (Inouye and Fruton, 1968), and the inhibitory action thus exerted by substrate analogs that might have arisen as by-products during the course of peptide synthesis, it was essential to assure the chemical identity and purity of the oligopeptides used as substrates.

Experimental Section

Enzyme Studies. Twice-crystallized pepsin (Worthington Biochemical Corp., lot PM693-7) was used in all experiments, and fresh enzyme solutions were prepared before each set of kinetic runs. This enzyme preparation was found to give the same kinetic parameters (within the precision of the measurements) for the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe as those obtained previously with Worthington lot PM708 (Hollands *et al.*, 1969). The initial rate of cleavage (5–15%) of each substrate was determined at pH 4.0 (0.04 M sodium formate buffer) and 37.0 ± 0.1° by means of the spectrophotometric method described previously (Inouye and Fruton, 1967), using a Cary Model 15 recording spectrophotometer, equipped with an automatic sample changer; this method depends on the increase in absorbance at 310 mμ when the Phe(NO_2) residue is converted into its carboxylate form. Usually, 10–20 points were recorded for each run during the first 10–15 min of incubation, and the initial rate was calculated by using a value of $\Delta\epsilon_{310} = 800$. Separate determinations of the absorption spectra of the substrates used in this study showed that they resemble closely that of Z-His-Phe(NO_2)-Phe-OMe, with a maximum at 278.5 mμ (ε 9600 ± 100), and the value of $\Delta\epsilon_{310} = 800 \pm 15$ was found to apply to their cleavage at pH 4.0. It may be noted that the high absorbance of the *p*-nitrophenyl group limits the substrate concentration that can be used for reliable measurements at 310 mμ to about 0.5 mM. Kinetic runs may be made at higher substrate concentrations (up to about 1.5 mM) by using a higher wavelength (*e.g.*, 326 mμ) in the manner described by Hollands and Fruton (1969). In a previous publication from this laboratory (Hollands *et al.*, 1969), kinetic data were reported for the peptic hydrolysis of Gly-Gly-Phe(NO_2)-Phe-OMe and of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe, with substrate concentrations up to 1.5 mM; it was not stated in that paper that the change in absorbance had been measured at 326 mμ.

In the present experiments, 9–13 determinations of the initial velocity (v) were made for each value of the substrate concentration (S), and satisfactory linear plots of $1/v$ *vs.* $1/S$ were obtained, from which the values of V_m and K_M were estimated (method of least squares). For the calculation of k_{cat}, the enzyme concentration (E) was determined spectrophotometrically at 278 mμ, with the assumption that pepsin has a molar absorptivity of 50,900 and a molecular weight of 34,200.

For a few of the oligopeptide substrates, the maximum concentration that could be used for kinetic measurements at 310 mμ was too low for reliable estimates of k_{cat} and K_M to be made by linear plotting methods. In these instances, the first-order rate constants for a series of substrate concentrations were determined, and extrapolated to $S = 0$, to give an estimate of k_{cat}/K_M by means of the integrated Michaelis–Menten equation (Neurath and Schwert, 1950).

In the determination of the cleavage products formed upon prolonged incubation (14–16 hr) at pH 4.0 and 37°, the substrate concentration ranged from 0.10 to 0.25 mM, and the enzyme concentration was approximately that used in the individual kinetic runs.

Since some of the peptide substrates were obtained as trifluoroacetate salts, separate experiments were performed to determine the effect of trifluoroacetic acid (1 mM) on the kinetics of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe at pH 4.0 and 37°; within the precision of the measurements, the values of k_{cat} and K_M were unchanged by the addition of trifluoroacetic acid.

Chromatography. Examination of the homogeneity of the synthetic products prepared in this work, and of the cleavage products released by pepsin, was performed by thin-layer chromatography[3] with silica gel G as the supporting phase (Eastman Chromatogram sheets 6061). Except where otherwise indicated, the chromatograms were developed by means of iodine vapor. Other reagents used were: (a) 0.2% ninhydrin in 1-butanol; (b) chlorine–tolidine. The chromatographic behavior of the synthetic compounds described below is given as: thin-layer chromatography (solvent) R_F value (reagent, except where iodine was used). In all cases, single spots were found for the reported final recrystallized products.

Synthesis of Peptides

Phe-His-Phe(NO_2)-Phe-OMe·2Trifluoroacetate. His-Phe(NO_2)-Phe-OMe·2HBr was prepared in quantitative yield by the treatment of 643 mg (1 mmole) of the Z-protected peptide ester (Inouye and Fruton, 1967) with HBr–acetic acid in the usual manner; thin-layer chromatography (B) 0.52; (C) 0.71. *Anal.* Calcd for $C_{25}H_{30}Br_2N_6O_6$ (670.4): N, 12.5. Found: N, 12.3. To a chilled solution of the product in dimethylformamide (3 ml) were added triethylamine (0.28 ml, 2 mmoles) and Boc-Phe-OSu (362 mg, 1 mmole) (Anderson *et al.*, 1964). After 24 hr, the mixture was concentrated *in vacuo*, the residue was extracted with ethyl acetate, and the ethyl acetate solution was washed successively with ice-cold 0.5 N HCl (saturated with NaCl), 5% $NaHCO_3$, and water. After being dried over $MgSO_4$, the solution was evaporated *in vacuo* to yield 620 mg (82%) of the Boc-tetrapeptide ester, mp 134–136°; thin-layer chromatography (A) 0.60; (B) 0.75. *Anal.* Calcd for $C_{39}H_{45}N_7O_9$ (755.85): N, 13.0. Found: N, 13.1. Saponification of this product in the usual manner yielded the Boc-tetrapeptide, mp 212–213° dec. *Anal.* Calcd for $C_{38}H_{43}N_7O_9$ (741.8): N, 13.2. Found: N, 13.4. The Boc-tetrapeptide ester (1.51 g, 2 mmoles) was treated with trifluoroacetic acid (20 ml) for 45 min at room temperature, the solution was evaporated *in vacuo*, and the residue was taken up in methanol (5 ml). The addition of ether (20 ml) yielded 1.2 g (67%) of the product, which was recrystallized from methanol–ether; thin-layer chromatography (B) 0.65; (C) 0.78 (ninhydrin, iodine). *Anal.* Calcd for $C_{38}H_{39}F_6N_7O_{11}$ (883.8): C, 51.7; H, 4.5; N, 11.1; CH_3O, 3.5. Found: C, 51.7; H, 4.9; N, 11.4; CH_3O, 3.6.

[3] The following solvent systems were used: (A) benzene–methanol (85:15, v/v); (B) 1-butanol–acetic acid–water (4:1:1, v/v); (C) 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v).

Phe-Gly-His-Phe(NO_2)-Phe-OMe·2Trifluoroacetate. Boc-Phe-Gly-OEt (Schröder, 1964) was saponified in the usual manner to yield Boc-Phe-Gly (mp 163–164°). This product (3.2 g, 10 mmoles) was coupled in the usual manner with *N*-hydroxysuccinimide (1.3 g, 11 mmoles) in the presence of dicyclohexylcarbodiimide (2.3 g, 11 mmoles), with dioxane (50 ml) as the solvent, to yield Boc-Phe-Gly-OSu (4.1 g, 97%), mp 116–118°; thin-layer chromatography (A) 0.74. *Anal.* Calcd for $C_{20}H_{25}N_3O_7$ (419.4): N, 10.0. Found: N, 10.1. This product (2.1 g, 5 mmoles) was coupled with His-Phe(NO_2)-Phe-OMe in the manner described above for Boc-Phe-His-Phe(NO_2)-Phe-OMe to yield 3.4 g (84%) of the Boc-pentapeptide ester. After recrystallization from dimethylformamide–water (150 ml), the product (3.1 g) melted at 222–223° dec; thin-layer chromatography (A) 0.43. *Anal.* Calcd for $C_{41}H_{48}N_8O_{10}$ (812.9): N, 13.8. Found: N, 13.9. Saponification of this product in the usual manner yielded the Boc-pentapeptide, mp 162° dec. *Anal.* Calcd for $C_{40}H_{46}N_8O_{10}$ (798.9): N, 14.0. Found: N, 13.8. The Boc-pentapeptide ester (1.2 g, 1.5 mmoles) was treated with trifluoroacetic acid in the manner described above for the Boc-tetrapeptide ester to yield 1.0 g (74%) of the product; mp 128–132°; thin-layer chromatography (B) 0.65; (C) 0.76 (ninhydrin, iodine). *Anal.* Calcd for $C_{40}H_{42}F_6N_8O_{12}\cdot H_2O$ (958.8): C, 50.1; H, 4.6; N, 11.7; CH_3O, 3.2. Found: C, 50.0; H, 4.5; N, 12.1; CH_3O, 3.0.

Z-His-Phe(NO_2)-Phe-Ala-OMe. Z-His-Phe(NO_2)-Phe-OMe (6.43 g, 10 mmoles) was dissolved in dimethylformamide (32 ml), methanol (80 ml) was added, followed by 85% hydrazine hydrate (2.4 ml, *ca.* 40 mmoles). After the reaction mixture had been kept for 48 hr at room temperature, the hydrazide (6.0 g, 93%) was collected. After recrystallization from dioxane–water, it melted at 218–219°; thin-layer chromatography (A) 0.48 (R_F (A) for Z-His-Phe(NO_2)-Phe-OMe, 0.71). *Anal.* Calcd for $C_{32}H_{34}N_8O_7$ (642.7): C, 59.8; H, 5.3; N, 17.4. Found: C, 59.5; H, 5.2; N, 16.9. The hydrazide (1 mmole) was dissolved in dimethylformamide (4 ml), and to the chilled solution (−10°) 6 N HCl (0.5 ml) and $NaNO_2$ (1.1 mmoles) were successively added with vigorous stirring. After 5 min, an ice-cold mixture of 50% K_2CO_3 (1 ml) and water (16 ml) was added, the resulting precipitate was collected, washed with a few milliliters of ice-cold water, and the azide was promptly allowed to react with Ala-OMe (1.5 mmoles each of the hydrochloride and of triethylamine) in dimethylformamide (3 ml). After 20 hr at 0°, the reaction mixture was evaporated *in vacuo*, and the residue was treated with water. After crystallization from acetic acid, the product melted at 165–167°; $[\alpha]_D^{20}$ −31.0° (*c* 1, dimethylformamide); thin-layer chromatography (A) 0.58; (C) 0.90. *Anal.* Calcd for $C_{36}H_{39}H_7O_8$ (713.8): N, 13.75. Found: N, 13.7.

The same product was obtained by stepwise synthesis. Boc-Phe-Ala-OMe (mp 101–102°) was prepared in 86% yield by the reaction of Boc-Phe-OSu (4 mmoles) with Ala-OMe (derived from 5 mmoles of the hydrochloride) in the presence of triethylamine (5 mmoles), with dioxane (20 ml) as the solvent. *Anal.* Calcd for $C_{18}H_{26}N_2O_5$ (350.4): N, 8.0. Found: N, 7.9. Z-Phe(NO_2)-OSu (mp 124–125°) was prepared in 83% yield by the reaction of Z-Phe(NO_2) (10 mmoles) and *N*-hydroxysuccinimide (11 mmoles), in the presence of dicyclohexylcarbodiimide (11 mmoles), with dioxane (25 ml) as the solvent. *Anal.* Calcd for $C_{21}H_{19}N_3O_8$ (441.4): N, 9.5. Found: N, 9.6. Z-Phe(NO_2)-OSu (2 mmoles) and Phe-Ala-OMe·trifluoroacetate (derived from 2 mmoles of Boc-Phe-Ala-OMe by treatment with trifluoroacetic acid) were coupled in the usual manner, with dimethylformamide (4 ml) as the solvent, to yield 0.9 g (78%) of the Z-tripeptide ester, mp 214–215°. *Anal.* Calcd for $C_{30}H_{32}N_4O_8$ (576.6): N, 9.7. Found: 9.8. This product (0.5 mmole) was treated with HBr–acetic acid to yield the tripeptide ester HBr, which was coupled with Z-His-N_3 (derived from 0.5 mmole of the hydrazide) in the presence of triethylamine (0.5 mmole), with dimethylformamide (1.5 ml) as the solvent, to yield the Z-tetrapeptide ester. After recrystallization from acetic acid, the product (0.225 g, 63%) had the same melting point, optical rotation, and R_F as that given above.

Z-His-Phe(NO_2)-Phe-Ala. Saponification of the above product (1 mmole) in the usual manner yielded the Z-tetrapeptide in 91% yield. After recrystallization from methanol–water, it melted at 226–227°; thin-layer chromatography (C) 0.81. *Anal.* Calcd for $C_{35}H_{37}N_7O_9$ (699.8). N, 14.0. Found: N, 14.2.

Phe-Gly-His-Phe(NO_2)-Phe-Ala. Boc-Phe-Gly-OSu (1 mmole) and His-Phe(NO_2)-Phe-Ala-OMe·2HBr (1 mmole, derived from the above Z-tetrapeptide ester by treatment with HBr–acetic acid; thin-layer chromatography (C) 0.68) were coupled in the presence of triethylamine (2 mmoles), with dimethylformamide (5 ml) as the solvent, to yield 200 mg (84%) of the Boc-hexapeptide ester (thin-layer chromatography (A) 0.38). This product was saponified in the usual manner to yield the Boc-hexapeptide (71%), mp 213–214°. *Anal.* Calcd for $C_{43}H_{51}N_9O_{11}$ (869.95): N, 14.5. Found: N, 14.3. Treatment of this product (300 mg) with trifluoroacetic acid (4 ml) gave the hexapeptide in 87% yield, mp 199–201°; thin-layer chromatography (C) 0.68. *Anal.* Calcd for $C_{38}H_{43}N_9O_9$ (769.8): C 59.3; H, 5.6; N, 16.4. Found: C, 59.5; H, 6.2; N, 16.7.

Z-His-Phe(NO_2)-Phe-Ala-Ala-OMe. Z-His-Phe(NO_2)-Phe-N_3 (derived in the manner described above from 1 mmole of the hydrazide) was coupled with Ala-Ala-OMe·HCl (derived from 1.5 mmoles of Z-Ala-Ala-OMe (Hofmann *et al.*, 1965) by catalytic hydrogenolysis in the presence of HCl) in the presence of triethylamine (1.5 mmoles). After crystallization from dimethylformamide–water (1:1, 50 ml), the product melted at 244–245°; yield 0.63 g (81%); thin-layer chromatography (B) 0.63; (C) 0.85. *Anal.* Calcd for $C_{39}H_{44}N_8O_{10}$ (784.85): C, 59.7; H, 5.65; N, 14.3. Found: C, 60.0; H, 6.05; N, 14.3.

Phe-Gly-His-Phe(NO_2)-Phe-Ala-Ala-OMe·2Trifluoroacetate. The above Z-pentapeptide ester (1 mmole) was treated with HBr–acetic acid in the usual manner to yield His-Phe(NO_2)-Phe-Ala-Ala-OMe·2HBr; thin-layer chromatography (C) 0.70. *Anal.* Calcd for $C_{31}H_{40}Br_2N_8O_8$ (812.6): N, 13.8. Found: N, 13.7. This product was coupled with Boc-Phe-Gly-OSu (1 mmole) in the presence of triethylamine (2 mmoles), with dimethylformamide (6 ml) as the solvent. The reaction mixture was worked up in the manner described above for Boc-His-Phe(NO_2)-Phe-OMe, to yield 0.72 g (75%) of the Boc-heptapeptide ester, mp 226–228° dec; thin-layer chromatography (A) 0.38 (ninhydrin, chlorine). *Anal.* Calcd for $C_{47}H_{58}N_{10}O_{12}$ (955.1): N, 14.7. Found: N, 14.7. Saponification of this product (0.5 mmole) in the usual manner gave 0.44 g (93%) of the Boc-heptapeptide. *Anal.* Calcd for $C_{46}H_{56}N_{10}O_{12}$ (941.0): N, 14.9. Found: N, 15.2. Treatment of this product (0.25 mmole) with trifluoroacetic acid gave the peptide in 86% yield; thin-layer chromatography (C) 0.68 (ninhydrin, iodine). *Anal.* Calcd for $C_{41}H_{48}N_{10}O_{10}$

(840.9): C, 58.55; H, 5.75; N, 16.7. Found: C, 58.7; H, 6.0; N, 16.2.

Treatment of the Boc-heptapeptide ester (0.2 mmole) with trifluoroacetic acid (6 ml) gave the heptapeptide·2 trifluoroacetate in 80% yield; thin-layer chromatography (C) 0.72 (ninhydrin, iodine). *Anal.* Calcd for $C_{46}H_{52}F_6N_{10}O_{14}$ (1083.0): C, 51.0; H, 4.8; N, 12.9. Found: C, 51.1; H, 5.1; N, 12.95.

Z-His-Phe(NO_2)-Phe-Ala-Phe-OMe. Z-His-Phe(NO_2)-Phe-N_3 (derived from 0.64 g (1.0 mmole) of the hydrazide) was coupled in the usual manner with Ala-Phe-OMe (derived from 0.43 g (1.5 mmoles) of the hydrochloride), with dimethylformamide as the solvent. After recrystallization from methanol–water, the product (0.61 g, 71%) melted at 230–232° dec; thin-layer chromatography (C) 0.88. *Anal.* Calcd for $C_{45}H_{48}N_8O_{10}$ (861.0): N, 13.0. Found: N, 12.8.

Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe·2Trifluoroacetate. The above compound (0.6 g, 0.7 mmole) was treated with HBr–acetic acid in the usual manner to yield the pentapeptide ester hydrobromide; after recrystallization from methanol–ether, the product (0.58 g, 94%) melted at 169–172°; thin-layer chromatography (C) 0.80 (ninhydrin, iodine). *Anal.* Calcd for $C_{37}H_{44}Br_2N_8O_8$ (888.7): N, 12.6. Found: N, 12.3. This product (0.44 g, 0.5 mmole) was coupled with Boc-Phe-Gly-OSu (0.21 g, 0.5 mmole) in the manner described above for the Ala-Ala compound to yield the Boc-heptapeptide ester; after recrystallization from dimethylformamide–water, the product (0.24 g, 46%) melted at 210–212° dec; thin-layer chromatography (B) 0.56. *Anal.* Calcd for $C_{53}H_{62}N_{10}O_{12}$ (1031.2): N, 13.6. Found: N, 13.4. Treatment of this product (0.2 mmole) with trifluoroacetic acid in the usual manner gave the heptapeptide ester·2trifluoroacetate (0.23 g, 100%); thin-layer chromatography (C) 0.79 (ninhydrin, iodine). *Anal.* Calcd for $C_{52}H_{56}F_6N_{10}O_{14}$ (1159.1): C, 53.9; H, 4.9; N, 12.1. Found: C, 53.8; H, 4.7; N, 11.7.

Z-His-Phe(NO_2)-Phe-His-OMe. Z-His-Phe(NO_2)-Phe-N_3 (derived from 1 mmole of the hydrazide) was coupled with His-OMe (derived from 1.5 mmoles of the dihydrochloride), in the manner described above for Z-His-Phe(NO_2)-Phe-Ala-OMe, to yield 0.63 (81%) of the product. After recrystallization from dioxane–water, it melted at 196–198°; thin-layer chromatography (A) 0.22; (C) 0.80. *Anal.* Calcd for $C_{39}H_{41}N_9O_9$ (779.8): C, 60.0; H, 5.3; N, 16.2. Found: C, 60.1; H, 5.5; N, 16.0.

Z-His-Phe(NO_2)-Phe-Gly-Ala-OMe. Z-His-Phe(NO_2)-Phe-N_3 (derived from 1 mmole of the hydrazide) was coupled with Gly-Ala-OMe (derived from 1.2 mmoles of the hydrochloride), in the manner described above for Z-His-Phe(NO_2)-Phe-Ala-OMe, to yield 0.6 g (78%) of the product. After recrystallization from dimethylformamide–water, it melted at 224–226°; thin-layer chromatography (A) 0.38. *Anal.* Calcd for $C_{38}H_{42}N_8O_{10}$ (770.8): C, 59.2; H, 5.5; N, 14.5. Found: C, 59.0; H, 5.6; N, 14.8.

Z-His-Phe(NO_2)-Gly-OMe. Z-Phe(NO_2) (1.7 g, 5 mmoles) and Gly-OMe·HCl (0.63 g, 5 mmoles) were coupled, in the presence of isobutyl chloroformate (0.67 ml, 5 mmoles) and *N*-methylmorpholine (1.1 ml, 10 mmoles), with tetrahydrofuran (25 ml) as the solvent, to yield the Z-dipeptide ester; after recrystallization from ethyl acetate–petroleum ether (30–60°), the product (1.35 g, 65%) melted at 175–176°; thin-layer chromatography (B) 0.79. *Anal.* Calcd for $C_{20}H_{21}N_3O_7$ (415.4): N, 10.1. Found: N, 9.8. This product (1 g, 2.5 mmoles) was treated with HBr–acetic acid in the usual manner to yield the dipeptide ester hydrobromide (0.9 g, 100%); thin-layer chromatography (C) 0.75 (ninhydrin). Z-His-N_3 (prepared from 0.55 g (1.8 mmoles) of the hydrazide) and Phe(NO_2)-Gly-OMe (prepared from 0.54 g (1.5 mmoles) of the hydrobromide) were coupled in the usual manner, with ethyl acetate as the solvent. After recrystallization from methanol–water, the resulting product (0.46 g, 57%) melted at 185–187°; thin-layer chromatography (B) 0.60. *Anal.* Calcd for $C_{26}H_{28}N_6O_8$ (552.5): N, 15.2. Found: N, 15.1.

Z-His-Phe(NO_2)-Gly-Ala-Ala-OMe. Z-Gly-Ala-Ala-OMe (mp 123–124°) was prepared in 71% yield by the coupling of Z-Gly-OSu (1.2 g, 4 mmoles) with Ala-Ala-OMe (derived from 0.84 g, 4 mmoles, of the hydrochloride), with dimethylformamide (5 ml) as the solvent. *Anal.* Calcd for $C_{17}H_{23}N_3O_6$ (365.4): N, 11.5. Found: N, 11.5. Hydrogenolysis of this product (1.0 g, 2.7 mmoles) in the presence of HCl (2.7 mmoles) gave 0.61 g (83%) of the tripeptide ester hydrochloride, mp 266–268°; thin-layer chromatography (C) 0.53 (ninhydrin). *Anal.* Calcd for $C_9H_{18}ClN_3O_4$ (267.7): N, 15.7. Found: N, 15.6. This product (2 mmoles) was coupled with Z-Phe(NO_2)-OSu (2 mmoles) in the presence of triethylamine (2 mmoles), with dimethylformamide (10 ml) as the solvent, to yield 1.0 g (90%) of the Z-tetrapeptide ester. After recrystallization from methanol (50 ml), it melted at 215–216°. *Anal.* Calcd for $C_{26}H_{31}N_5O_9$ (557.6): N, 12.6. Found: N, 12.7. Treatment of this product with HBr–acetic acid gave the tetrapeptide ester hydrobromide, thin-layer chromatography (C) 0.73 (ninhydrin). *Anal.* Calcd for $C_{18}H_{26}BrN_5O_7$ (504.4): N, 13.9. Found: N, 13.5. This product (1.3 mmoles) was coupled with Z-His-N_3 (prepared from 1.3 mmoles of the hydrazide) in the presence of triethylamine (1.3 mmoles), with dimethylformamide (5 ml) as the solvent, to yield 0.76 g (84%) of the product. After recrystallization from dioxane–water, it melted at 184–186°; thin-layer chromatography (A) 0.35; (C) 0.82. *Anal.* Calcd for $C_{32}H_{38}N_8O_{10}$ (694.7): C, 55.3; H, 5.5; N, 16.1. Found: C, 55.1; H, 5.7; N, 16.3.

Z-Gly-His-Phe(NO_2)-Phe-OMe. Z-Gly-OSu (0.31 g, 1 mmole) and His-Phe(NO_2)-Phe-OMe·2HBr (0.67 g, 1 mmole) were coupled in the presence of triethylamine (0.28 ml, 2 mmoles), with dimethylformamide (4 ml) as the solvent, to yield 0.52 g (74%) of the product, mp 195–197°; thin-layer chromatography (A) 0.40; (C) 0.80. *Anal.* Calcd for $C_{35}H_{37}N_7O_9$ (699.75): C, 60.1; H, 5.3; N, 14.0. Found: C, 60.2; H, 5.3; N, 13.9.

Phe-Gly-Gly-His-Phe(NO_2)-Phe-OMe. The above product (0.42 g, 0.6 mmole) was treated with HBr–acetic acid to give 0.42 g (100%) of the tetrapeptide ester dihydrobromide; thin-layer chromatography (C) 0.64 (ninhydrin). *Anal.* Calcd for $C_{27}H_{33}Br_2N_7O_7$ (727.45): N, 13.5. Found: 13.3. This product (0.5 mmole) was coupled with Boc-Phe-Gly-OSu (0.5 mmole) in the presence of triethylamine (1 mmole), with dimethylformamide (3 ml) as the solvent, to yield 0.31 g (71%) of the Boc-hexapeptide ester; after recrystallization from dimethylformamide–water, it melted at 217–218°; thin-layer chromatography (A) 0.38. *Anal.* Calcd for $C_{43}H_{51}N_9O_{11}$ (869.95): N, 14.5. Found: N, 14.7. This product (0.2 mmole) was treated with trifluoroacetic acid to give a quantitative yield of the hexapeptide ester salt; thin-layer chromatography (C) 0.74 (ninhydrin, iodine). *Anal.* Calcd for $C_{42}H_{45}F_6N_9O_{13}$ (997.9): C, 50.5; H, 4.55; N, 12.6. Found: C, 50.3; H, 4.65; N, 12.7.

Gly-Phe(NO_2)-Phe-OMe·HBr. Z-Gly (0.63 g, 3 mmoles) and Phe(NO_2)-Phe-OMe·HBr (1.36 g, 3 mmoles) (Inouye and Fruton, 1967) were coupled in the presence of isobutyl chloroformate (0.4 ml, 3 mmoles) and *N*-methylmorpholine (0.65 ml, 6 mmoles), with tetrahydrofuran (20 ml) as the solvent, to yield the Z-tripeptide ester; after recrystallization from methanol–water, the product (1.53 g, 91%) melted at 167–169°; thin-layer chromatography (B) 0.84. *Anal.* Calcd for $C_{29}H_{30}N_4O_8$ (562.6): N, 10.0. Found: N, 9.9. This product (1.13 g, 2 mmoles) was treated with HBr–acetic acid in the usual manner to yield the tripeptide ester hydrobromide; after recrystallization from methanol–ether, the product (0.98 g, 96%) melted at 228–230° dec; thin-layer chromatography (B) 0.36. *Anal.* Calcd for $C_{21}H_{25}BrN_4O_6$ (509.4): N, 11.0. Found: N, 10.8.

Phe-Gly-Phe(NO_2)-Phe-OMe·HBr. Boc-Phe-Gly-OSu (0.63 g, 1.5 mmoles) and Phe(NO_2)-Phe-OMe·HBr (0.68 g, 1.5 mmoles) were coupled in the presence of triethylamine (0.21 ml, 1.5 mmoles), with dimethylformamide (9 ml) as the solvent, to yield the Boc-tetrapeptide ester; after recrystallization from methanol–water, the product (0.77 g, 76%) melted at 184–186°; thin-layer chromatography (B) 0.82. *Anal.* Calcd for $C_{35}H_{41}N_5O_9$ (675.8): N, 10.4. Found: N, 10.2. This product (0.47 g, 0.7 mmole) was treated with HBr–acetic acid (5 ml) to yield the tetrapeptide ester hydrobromide; after recrystallization from methanol–ether, the product (0.4 g, 87%) melted at 233–235° dec; thin-layer chromatography (C) 0.85. *Anal.* Calcd for $C_{30}H_{34}BrN_5O_7$ (656.6): C, 54.9; H, 5.3; N, 10.7. Found: C, 55.3; H, 5.2; N, 10.8.

Phe-Gly-Gly-Phe(NO_2)-Phe-OMe·Trifluoroacetate. Boc-Phe-Gly (0.26 g, 0.8 mmole) and Gly-Phe(NO_2)-Phe-OMe·HBr (0.41 g, 0.8 mmole) were coupled by the mixed-anhydride method (isobutyl chloroformate) in the usual manner, with *N*-methylmorpholine as the base, and tetrahydrofuran as the solvent, to yield the Boc-pentapeptide ester; after recrystallization from methanol–water, the product (0.37 g, 63%) melted at 178–180°; thin-layer chromatography (B) 0.76. *Anal.* Calcd for $C_{37}H_{44}N_6O_{10}$ (732.8): N, 11.5. Found: N, 11.3. This product (0.29 g, 0.4 mmole) was treated with trifluoroacetic acid in the usual manner to yield 0.25 g (83%) of the pentapeptide ester trifluoroacetate, mp 101–103°; thin-layer chromatography (C) 0.90 (ninhydrin, iodine). *Anal.* Calcd for $C_{34}H_{37}F_3N_6O_{10}$ (746.7): C, 54.7; H, 5.0; N, 11.3. Found: C, 54.6; H, 5.1; N, 11.3.

Gly-Gly-Phe(NO_2)-Phe-Gly-OMe·HBr. Boc-Phe(NO_2) was prepared by the treatment of *p*-nitro-L-phenylalanine·H_2O (11.4 g, 0.05 mole) with *t*-butyloxycarbonyl azide (14.3 g, 0.1 mole) at 45° for 20 hr, in the presence of MgO (4 g), water (87 ml), and dioxane (150 ml). After extraction with ethyl acetate, the aqueous layer was carefully acidified (pH 1) with 2 N HCl and extracted with ethyl acetate (4 × 50 ml). The ethyl acetate solution was washed with water, dried over $MgSO_4$, and concentrated *in vacuo* to yield the product, which was recrystallized from CCl_4 (220 ml); yield 11.4 g (74%), mp 68–69°; thin-layer chromatography (B) 0.70. *Anal.* Calcd for $C_{14}H_{13}N_2O_6$ (310.3): N, 9.0. Found: N, 9.0. Phe-Gly-OMe·HBr (mp 128–129°) was prepared in 93% yield by the treatment of Z-Phe-Gly-OMe (mp 115–117°) with HBr–acetic acid in the usual manner; thin-layer chromatography (B) 0.37 (ninhydrin). Boc-Phe(NO_2) (1.55 g, 5 mmoles) and Phe-Gly-OMe (prepared from 1.6 g (5 mmoles) of the hydrobromide by neutralization with cold 50% K_2CO_3) were coupled in the presence of dicyclohexylcarbodiimide (1.0 g, 5 mmoles), with acetonitrile (30 ml) as the solvent. A portion of the product separated with the dicyclohexylurea and was extracted with dioxane. After recrystallization from ethyl acetate, the product melted at 167–168°; yield 1.6 g (61%); thin-layer chromatography (A) 0.71. *Anal.* Calcd for $C_{26}H_{32}N_4O_8$ (528.6): N, 10.6. Found: N, 10.5.

The above Boc-tripeptide ester (1.2 g, 2.3 mmoles) was treated in the usual manner with HBr–acetic acid (10 ml). After recrystallization from methanol–ether, the tripeptide ester hydrobromide (1 g, 89%) melted at 218–219° dec; thin-layer chromatography (B) 0.56. *Anal.* Calcd for $C_{21}H_{25}BrN_4O_6$ (509.3): N, 11.0. Found: N, 10.8. This product (0.92 g, 1.8 mmoles) was coupled with Z-Gly (0.38 g, 1.8 mmoles) in the presence of isobutyl chloroformate (0.25 ml, 1.8 mmoles) and *N*-methylmorpholine (0.4 ml, 3.6 mmoles), with tetrahydrofuran (15 ml) as the solvent to yield the Z-tetrapeptide ester; after recrystallization from methanol the product (0.95 g, 85%) melted at 192–194°; thin-layer chromatography (A) 0.60. *Anal.* Calcd for $C_{31}H_{33}N_5O_9$ (619.6): N, 11.3. Found: N, 11.0. This product (0.8 g, 1.3 mmoles) was treated with HBr–acetic acid (5 ml) in the usual manner to yield the tetrapeptide ester hydrobromide; after recrystallization from methanol–ether, the product (0.7 g, 95%) melted at 214–215° dec; thin-layer chromatography (B) 0.42 (ninhydrin, iodine); $[\alpha]_D^{30}$ +7.6° (*c* 1, MeOH). This product (0.57 g, 1.0 mmole) was coupled with Z-Gly (0.21 g, 1.0 mmole) in the presence of isobutyl chloroformate (0.14 ml, 1 mmole) and *N*-methylmorpholine (0.22 ml, 2 mmoles), with tetrahydrofuran (11 ml) as the solvent, to yield the Z-tetrapeptide ester; after recrystallization from methanol, the product (0.56 g, 82%) melted at 171–173° dec; thin-layer chromatography (B) 0.71. Treatment of this product (0.45 g, 0.7 mmole) with HBr–acetic acid (3 ml) in the usual manner gave the pentapeptide ester hydrobromide; after recrystallization from methanol–ether, the product (0.36 g, 82%) melted at 212–214° dec; thin-layer chromatography (B) 0.34; $[\alpha]_D^{28}$ −3.6° (*c* 1, MeOH). *Anal.* Calcd for $C_{25}H_{31}BrN_6O_8$ (623.5): C, 48.2; H, 5.0; N, 13.5. Found: C, 48.4; H, 5.0; N, 13.2.

Phe-Gly-Phe(NO_2)-Phe-Gly-OMe·HBr. Z-Phe (0.23 g, 1 mmole) and Gly-Phe(NO_2)-Phe-Gly-OMe·HBr (0.57 g, 1 mmole) were coupled by means of the mixed-anhydride method as described above for Z-Gly-Gly-Phe(NO_2)-Phe-Gly-OMe. After recrystallization from methanol, the product (0.57 g, 74%) melted at 191–192°; thin-layer chromatography (A) 0.74. *Anal.* Calcd for $C_{40}H_{41}N_6O_{10}$ (765.8): N, 11.0. Found: N, 10.8. Treatment of the Z-pentapeptide ester (0.46 g, 0.6 mmole) with HBr–acetic acid (5 ml) gave the desired product; after recrystallization from methanol–ether, the product (0.34, 79%) melted at 195–197° dec; thin-layer chromatography (B) 0.56; $[\alpha]_D^{28}$ +16.7° (*c* 1, MeOH). *Anal.* Calcd for $C_{32}H_{37}BrN_6O_8$ (713.6): C, 53.85; H, 5.2; N, 11.8. Found: C, 53.6; H, 5.3; N, 11.8.

Results

In Table I are collected the chromatographic data for the peptic cleavage of the oligopeptides used as substrates in this study; upon prolonged incubation (14–16 hr at pH 4.0 and 37°), with enzyme concentrations (0.003–5.8 μM) that gave 5–10% hydrolysis in 10 min, the only products found were

TABLE I: Products of the Peptic Cleavage of Oligopeptide Substrates.[a]

Substrate	Products	R_F[b]
Phe-His-Phe(NO_2)-Phe-OMe		0.78 (N, C)
	Phe-His-Phe(NO_2)	0.61 (N, C)
	Phe-OMe	0.74 (N)
Phe-Gly-His-Phe(NO_2)-Phe-OMe		0.76 (N, C)
	Phe-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-OMe	0.74 (N)
Phe-Gly-Gly-His-Phe(NO_2)-Phe-OMe		0.74 (N, C)
	Phe-Gly-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-OMe	0.74 (N)
Phe-Gly-His-Phe(NO_2)-Phe-Ala		0.68 (N, C)
	Phe-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-Ala	0.58 (N)
Phe-Gly-His-Phe(NO_2)-Phe-Ala-Ala		0.68 (N, C)
	Phe-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-Ala-Ala	0.56 (N)
Phe-Gly-His-Phe(NO_2)-Phe-Ala-Ala-OMe		0.72 (N, C)
	Phe-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-Ala-Ala-OMe	0.73 (N)
Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe		0.81 (N, C)
	Phe-Gly-His-Phe(NO_2)	0.62 (N, C)
	Phe-Ala-Phe-OMe	0.83 (N, C)
Phe-Gly-Phe(NO_2)-Phe-OMe		0.85 (N, C)
	Phe-Gly-Phe(NO_2)	0.69 (N, C)
	Phe-OMe	0.74 (N)
Phe-Gly-Gly-Phe(NO_2)-Phe-OMe		0.80 N, C)
	Phe-Gly-Gly-Phe(NO_2)	0.70 (N, C)
	Phe-OMe	0.74 (N)
Phe-Gly-Phe(NO_2)-Phe-Gly-OMe		0.86 (N, C)
	Phe-Gly-Phe(NO_2)	0.72 (N, C)
	Phe-Gly-OMe	0.79 (N)
Gly-Phe(NO_2)-Phe-Gly-OMe		0.78 (N, C)
	Gly-Phe(NO_2)	0.56 (N, C)
	Phe-Gly-OMe	0.79 (N)
Gly-Gly-Phe(NO_2)-Phe-Gly-OMe		0.76 (N, C)
	Gly-Gly-Phe(NO_2)	0.52 (N, C)
	Phe-Gly-OMe	0.79 (N)
Z-His-Phe(NO_2)-Phe-Ala		0.81 (C)
	Z-His-Phe(NO_2)	0.70 (C)
	Phe-Ala	0.58 (N)
Z-His-Phe(NO_2)-Phe-Ala-OMe		0.90 (C)
	Z-His-Phe(NO_2)	0.70 (C)
	Phe-Ala-OMe	0.81 (N)
Z.His-Phe(NO_2)-Phe-Ala-Ala-OMe		0.85 (C)
	Z-His-Phe(NO_2)	0.70 (C)
	Phe-Ala-Ala-OMe	0.73 (N)
Z-His-Phe(NO_2)-Phe-His-OMe		0.80 (C)
	Z-His-Phe(NO_2)	0.70 (C)
	Phe-His-OMe	0.57 (N, C)
Z-His-Phe(NO_2)-Gly-Ala-Ala-OMe		0.82 (C)
	Z-His-Phe(NO_2)	0.70 (C)
	Gly-Ala-Ala-OMe	0.55 (N)
Z-Gly-His-Phe(NO_2)-Phe-OMe		0.80 (C)
	Z-Gly-His-Phe(NO_2)	0.68 (C)
	Phe-OMe	0.74 (N)

[a] Thin-layer chromatography performed with solvent C. For details, see Experimental Section. [b] N, ninhydrin reagent; C, chlorine–tolidine reagent.

TABLE II: Kinetics of Pepsin Action on Oligopeptide Substrates of the Type A-Phe(NO_2)-Phe-B.[a]

Substrate A	Substrate B	S[b] (mM)	E (μM)	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1} mM^{-1})
Z-His	OMe	0.05–0.5 (10)	0.54	0.29 ± 0.05	0.46 ± 0.06	0.63
Phe-His	OMe	0.08–0.3 (9)	5.8	0.009 ± 0.001	0.22 ± 0.02	0.04
Phe-Gly	OMe	0.06–0.4 (11)	5.4	0.007 ± 0.001	0.07 ± 0.01	0.1
Gly-Gly[c]	OMe	0.2–1.5 (10)	0.54	0.12 ± 0.01	1.5 ± 0.2	0.08
Gly-Gly-Gly[c]	OMe	0.1–1.0 (10)	0.54	0.09 ± 0.01	1.5 ± 0.1	0.06
Phe-Gly-Gly	OMe	0.05–0.4 (10)	0.54	0.09 ± 0.01	0.52 ± 0.04	0.17
Phe-Gly-His	OMe	0.08–0.24 (12)	1.2	0.10 ± 0.01	0.40 ± 0.06	0.25
Z-Gly-His	OMe	0.08–0.24 (9)	0.58	0.37 ± 0.02	0.95 ± 0.08	0.38
Phe-Gly-Gly-His	OMe	0.08–0.24 (12)	0.58	0.27 ± 0.01	0.56 ± 0.03	0.48
Gly	Gly-OMe	0.1–0.4 (6)	0.54			0.035[d]
Gly-Gly	Gly-OMe	0.1–0.4 (6)	0.54			0.4[d]
Phe-Gly	Gly-OMe	0.1–0.4 (6)	0.54	0.16 ± 0.02	0.8 ± 0.1	0.2 (0.2[d])
Z-His	His-OMe	0.08–0.24 (13)	0.58	0.33 ± 0.08	0.7 ± 0.2	0.5
Z-His	Ala	0.08–0.28 (11)	0.12	2.9 ± 0.4	0.8 ± 0.1	3.6
Phe-Gly-His	Ala	0.06–0.3 (11)	0.12	1.65 ± 0.02	1.2 ± 0.1	1.4
Z-His	Ala-OMe	0.06–0.3 (9)	0.06	3.3 ± 0.3	0.4 ± 0.1	8.3
Phe-Gly-His	Ala-Ala	0.08–0.2 (12)	0.006	19 ± 1	0.27 ± 0.03	71
Phe-Gly-His	Ala-Ala-OMe	0.08–0.28 (12)	0.003	28 ± 1	0.16 ± 0.02	175
Z-His	Ala-Ala-OMe	0.05–0.15 (9)	0.003	28 ± 2	0.13 ± 0.01	215
Phe-Gly-His	Ala-Phe-OMe	0.02–0.28 (14)	0.003	20 ± 2	0.04 ± 0.005	500

[a] pH 4.0, 37°. [b] The number in parentheses denotes the number of runs. [c] Data taken from Hollands *et al.* (1969). [d] Calculated from rate data using integrated Michaelis–Menten equation.

those to be expected from the scission of the Phe(NO_2)–Phe bond. The hydrolysis appeared to be complete, indicating the absence of significant racemization of either the Phe(NO_2) or Phe residues of the Phe(NO_2)–Phe unit in the recrystallized peptides used for kinetic studies. Earlier work (Inouye and Fruton, 1967) had shown that Z-His-D-Phe-Phe-OEt and Z-His-Phe-D-Phe-OEt are both resistant to the action of pepsin.

The kinetc data obtained during the course of this study are presented in Table II. It will be noted that, for substrates of the type A-Phe(NO_2)-Phe-OMe, a change in the A group from Z-His to Phe-His, Phe-Gly, or Gly-Gly leads to a marked decrease in k_{cat}/K_M. This effect may be caused in part by an unfavorable influence of the α-ammonium group, a view consistent with the results for the pair of substrates in which A = Gly or Gly-Gly and B = Gly-OMe. However, a comparison of the kinetics of the hydrolysis of substrates in which A = Phe-Gly with those in which A = Gly-Gly indicates a significant contribution of the amino-terminal Phe residue in lowering both K_M and k_{cat}. Since the values of k_{cat}/K_M for Phe-Gly-Phe(NO_2)-Phe-OMe and Gly-Gly-Phe-(NO_2)-Phe-OMe are nearly the same, the possibility of greater nonproductive binding of the compound with A = Phe-Gly is strongly suggested. A similar situation appears to apply to the pair of substrates in which A = Phe-Gly or Gly-Gly and B = Gly-OMe; in this case, a change in the B group from OMe to Gly-OMe increased K_M to such an extent that it could not be estimated reliably, and only a k_{cat}/K_M value was determined for the compound with A = Gly-Gly. It may be inferred, therefore, that substrates with A = Phe-Gly or Phe-His are relatively resistant to pepsin action not only because of the possible inhibitory effect of the α-ammonium group, but also because of a significant contribution of the amino-terminal Phe residue to nonproductive interaction.

It is of interest that the effect of the Phe-Gly unit in lowering both k_{cat} and K_M is not seen with A = Phe-Gly-Gly; here, only K_M is markedly decreased, as compared with the case in which A = Gly-Gly-Gly. Furthermore, a comparison of corresponding pairs of substrates that differ only in having A = Z-His or Phe-Gly-His (see the pairs in which B = OMe, Ala, or Ala-Ala-OMe) suggests that Phe-Gly-His unit in the A position of a substrate has an effect roughly equivalent to that of a Z-His unit. These findings raise the possibility that the apparent nonproductive binding seen with compounds having A = Phe-Gly may be a consequence of an interaction of the Phe residue of this dipeptidyl unit with an enzymic locus that binds the benzyl group of Phe-Gly-Gly, Phe-Gly-His, or Z-His in the enzyme–substrate complex.

The data in Table II indicate that the replacement of the His residue of Phe-Gly-His by Gly does not alter the kinetic parameters significantly. In earlier work (Inouye *et al.*, 1966), the His residue was introduced to confer solubility in water on peptides blocked at both the α-amino and α-carboxyl groups. It was also found, however, that the location of a His residue in substrates of the type A-Phe-Phe-OEt could markedly affect the value of k_{cat}; in contrast to the rapid cleavage of the substrate with A = Z-Gly-His, the compound with A = Z-His-Gly was relatively resistant to cleavage at the Phe–Phe bond (Inouye and Fruton, 1967; Hollands *et al.*, 1969). It is of interest that the replacement of the benzyloxycarbonyl

group of Z-His-Phe(NO_2)-Phe-OMe by a Z-Gly group causes no change in k_{cat} and an increase in K_M. This finding differs from that for the pair of substrates A-Phe-Phe-OEt, where k_{cat}/K_M for the substrate with A = Z-Gly-His is about 40 times greater than for the one with A = Z-His (Hollands *et al.*, 1969). A lower ratio of k_{cat}/K_M values has also been found for the pair of substrates A-Phe(NO_2)-Phe-OP4P, where A = Z-Gly-Gly or Z (Sachdev and Fruton, 1969).

It will be seen in Table II that when A = Z-His or Phe-Gly-His, the replacement of B = OMe by Ala leads to a large increase in both k_{cat} and K_M; it may be surmized that the effect on K_M is in part a consequence of the proximity of the terminal carboxylate group to the sensitive bond. Earlier work (Inouye *et al.*, 1966) had shown that Z-His-Phe-Phe is markedly more resistant to pepsin action at pH 4 than is Z-His-Phe-Phe-OEt, and this difference was ascribed to the unfavorable effect of the terminal α-carboxylate group adjacent to the sensitive Phe–Phe bond; this effect also explains the difference in the apparent pH optima for the peptic cleavage of these two substrates. That a similar effect may be exerted by a carboxylate group one amino acid residue away from a sensitive Phe(NO_2)-Phe unit is indicated by the data in Table II for the two substrates with A = Z-His and B = Ala or Ala-OMe. The k_{cat} values for the two compounds appear to be the same, but the K_M value for the Z-tetrapeptide is approximately twice that for the corresponding methyl ester.

A comparison of the kinetic parameters for the two substrates Z-His-Phe(NO_2)-Phe-B, with B = OMe or Ala-OMe, indicates that the introduction of the Ala residue had only a slight effect on K_M, but increased k_{cat} about tenfold. On the other hand, the replacement of B = OMe by His-OMe caused little change in either kinetic parameter. It may be noted from Table II that the introduction of a Gly residue (B = Gly-OMe) appears to increase both k_{cat} and K_M. Further work is needed to determine the effect of the introduction of other amino acid residues, but it may be tentatively surmised that the favorable effect of the Ala residue in B = Ala-OMe is a consequence of hydrophobic interaction involving its side chain.

The most sensitive substrates found during the course of the present study were those in which A = Phe-Gly-His or Z-His, and B = Ala-Ala, Ala-Ala-OMe, or Ala-Phe-OMe. In particular, it is noteworthy that the kinetic parameters for the two substrates A-Phe(NO_2)-Phe-Ala-Ala-OMe, which A = Phe-Gly-His or Z-His, are the same (k_{cat}/K_M = *ca.* 200 sec^{-1} mM^{-1}), within the precision of the data. The highest k_{cat}/K_M value of about 500 sec^{-1} mM^{-1} was found for the substrate with B = Ala-Phe-OMe; the effect of replacing an Ala residue of Ala-Ala-OMe by a Phe residue is to lower K_M considerably, with a slight decrease in k_{cat}, suggesting some contribution of the added Phe residue to nonproductive binding. These relatively high k_{cat}/K_M values are comparable with those found for the most sensitive synthetic peptide substrates previously known for pepsin (*e.g.*, Z-Gly-Gly-Phe-Phe-OP4P, Z-Ala-Ala-Phe-Phe-OP4P) (Sachdev and Fruton, 1969).

It is clear from the foregoing data, and from previous studies in this laboratory, that an increase in the size and hydrophobic character of the group attached to the carboxyl terminus of a sensitive dipeptidyl unit can enhance markedly the catalytic efficiency of pepsin. Further work is needed to test the validity of this conclusion, and compounds of the type A-Phe(NO_2)-Phe-B, with various dipeptidyl units in the B portion, are in preparation. It may be mentioned here that the attempt to determine the effect of replacing the Ala-Ala-OMe portion of the substrate Z-His-Phe(NO_2)-Phe-Ala-Ala-OMe by Gly-Ala-OMe was unsuccessful, because Z-His-Phe(NO_2)-Phe-Gly-Ala-OMe proved to be too sparingly soluble for reliable studies of its cleavage by pepsin. The greater solubility of substrates with A = Phe-Gly-His makes them preferable to those with A = Z-His, despite the additional labor involved in the synthesis of such longer oligopeptides.

The 300-fold increase in the value of k_{cat}/K_M for the cleavage of Z-His-Phe(NO_2)-Phe-B when the B group was changed from OMe to Ala-Ala-OMe suggested the possibility that a compound such as Z-His-Phe(NO_2)-Gly-Ala-Ala-OMe might be hydrolyzed by pepsin at the Phe(NO_2)–Gly bond under conditions where Z-His-Phe(NO_2)-Gly-OMe is resistant to pepsin action. This was found to be the case; when tested at pH 4 and 37° (0.08–0.24 mM substrate), the pentapeptide derivative was cleaved at a slow but measurable rate (k_{cat} = 0.018 ± 0.003 sec^{-1}; K_M = 0.56 ± 0.12 mM), and the products of hydrolysis (Table I) showed the site of enzymic action to be the Phe(NO_2)–Gly bond. Under the same experimental conditions (0.24 mM substrates, 5.8 μM pepsin), no measurable hydrolysis of Z-His-Phe(NO_2)-Gly-OMe was observed during time periods as long as 2 hr.

Discussion

Previous studies in this laboratory (Inouye and Fruton, 1968; Hollands *et al.*, 1969; Trout and Fruton, 1969) and by other investigators (Denburg *et al.*, 1968; Zeffren and Kaiser, 1968; Knowles *et al.*, 1969) have provided evidence for the view that, in the hydrolysis of some peptide substrates by pepsin, the kinetically determined value of K_M approximates the dissociation constant (K_S) of the enzyme–substrate complex, and that the release of the first reaction product occurs after the rate-limiting step in the overall process (Hollands and Fruton, 1969). It may be assumed, therefore, that in these cases K_M provides a measure of binding energy in the enzyme–substrate interaction. The extent to which values of K_M for a series of peptide substrates are a reflection of different degrees of nonproductive binding is difficult to estimate; as noted by previous workers (Hein and Niemann, 1962; Bender and Kézdy, 1965), however, when Michaelis–Menten kinetics are obeyed, nonproductive binding has the same effect on K_M and k_{cat}, thus making the ratio k_{cat}/K_M independent of such nonproductive interaction. As regards the substrates listed in Table II, attention has been drawn to the possibility that pepsin action on substrates with A = Phe-Gly may involve significant nonproductive binding. It is difficult, however, to explain the kinetic data in Table II solely on the basis of differences in nonproductive binding, in view of the marked differences in k_{cat} for pairs of substrates for which K_M is nearly the same. The most striking example is the pair of substrates in which the A group is changed from Phe-His to Phe-Gly-His and the B group is changed from OMe to Ala-Ala-OMe; these structural changes cause little alteration in K_M (*ca.* 0.2 mM) but lead to a 3000-fold increase in k_{cat}.

In considering possible explanations, other than nonproductive binding, for such rate enhancements, it should be noted that the available data support the view that, in the binding of small A-Phe-Phe-B substrates by pepsin, the primary binding energy appears to come from the interaction of

the Phe–Phe unit with the enzyme (Inouye and Fruton, 1968; Knowles *et al.*, 1969). The data reported in Table II, together with earlier findings (Hollands *et al.*, 1969; Sachdev and Fruton, 1969), indicate that if the A and B portions are increased in size by the introduction of hydrophobic groups, these groups (if properly located) may also interact with loci on the enzyme to cause large increases in k_{cat}. Since such secondary interactions are not always reflected in large changes in the total binding energy in the enzyme–substrate interaction, it may be suggested that they lead to increased catalytic efficiency by promoting the better positioning of the catalytic groups of the enzyme in relation to the sensitive peptide bond. Such better positioning might be a consequence either of a change in the conformation of the catalytic site itself (Koshland and Neet, 1968), or by a redistribution of the total binding energy so that the binding of the sensitive Phe–Phe unit is weakened, thus favoring more efficient directed proton transfer and nucleophilic attack (Wang, 1968; Hollands and Fruton, 1969). In addition, the secondary interactions may promote catalysis by increasing the distortion of the sensitive peptide bond, thus making possible the utilization of the potential binding energy in the enzyme–substrate interaction to lower the free energy of activation in the catalytic process (Lumry, 1959; Jencks, 1969).

The data presented for the cleavage of the Phe(NO_2)–Gly bond of Z-His-Phe(NO_2)-Gly-Ala-Ala-OMe give evidence of the importance of such secondary interactions in promoting the hydrolysis of peptide bonds that are relatively resistant when present in smaller substrates (as in Z-His-Phe(NO_2)-Gly-OMe). This finding has obvious relevance to the apparently broad side-chain specificity of pepsin in the cleavage of proteins (Tang, 1963; Hill, 1965), as contrasted to its narrower specificity toward small substrates of the type Z-His-X-Y-OMe (Trout and Fruton, 1969).

Most of the earlier studies on the mechanism of pepsin action have involved the use of relatively resistant substrates (*e.g.*, Z-Glu-Tyr, Ac-Phe-Tyr-OEt, and Z-His-Phe-Phe-OEt). The availability of the much more sensitive substrates, both of the type reported above and of the pyridinium alkoxy derivatives, makes possible the reinvestigation of such aspects of the problem as transpeptidation (Neumann *et al.*, 1959), exchange of labeled cleavage products (Fruton *et al.*, 1961), deuterium isotope effect (Clement *et al.*, 1968; Hollands and Fruton, 1969), and inhibition by resistant substrate analogs (Inouye and Fruton, 1968; Knowles *et al.*, 1969). In particular, it will be important to determine whether the earlier conclusions regarding the nature of the rate-limiting step in the hydrolytic process also apply to the more sensitive substrates.

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