The Side-Chain Specificity of Pepsin*

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ABSTRACT: The synthesis is described of a series of peptide derivatives of the type Z-His-X-Y-OMe, where X or Y is L-phenylalanyl, and the other amino acid residue is L-alanyl, L-norvalyl, L-valyl, L-norleucyl, L-isoleucyl, *allo*-L-isoleucyl, L-methionyl, β-cyclohexyl-L-alanyl, or *p*-methoxy-L-phenylalanyl.

The kinetics of the peptic cleavage of the X–Y bond of such substrates at pH 4 was determined by means of an automatic ninhydrin method. Under the conditions of these experiments, the compounds in which X = Val, Ile, or *a*Ile were resistant to pepsin action, whereas the compounds in which one of these residues occupied the Y position were cleaved at a measurable rate. Among the members of the series Z-His-X-Phe-OMe tested thus far, the most sensitive substrate is the one in which X = Phe (or *p*-nitro-L-phenylalanyl); the relative resistance of the compound in which X = Tyr is largely overcome if the *p*-hydroxy group is methylated. The replacement of Phe in either the X or Y position by β-cyclohexyl-L-alanyl reduces the rate of peptic cleavage to the low level observed with substrates in which the X or Y position is occupied by an aliphatic amino acid residue (Nva, Nle, or Leu). The results indicate that, for pepsin substrates of the type Z-His-X-Y-OMe, the enzyme exhibits preference for a planar aromatic substituent at the β-carbon of the L-amino acid residue in both the X and Y positions of the substrate.

The objective of the work reported in this communication was to define the specificity of pepsin in relation to the nature of the side chains of the amino acid residues X and Y when pepsin attacks the X–Y bond of a peptide derivative AX–YB. The series of substrates prepared for this study were cationic compounds in which A = Z-His[1] and B = OMe, because previous work in this laboratory had shown Z-His-Phe-Phe-OMe to be an excellent substrate, with a pH optimum for k_{cat}/K_M near pH 4 (Inouye and Fruton, 1967; Hollands and Fruton, 1968; Hollands *et al.*, 1969).

In earlier work, the effect of the replacement of a L-phenylalanyl residue of Ac-Phe-Phe by Tyr, Trp, 3,5-dibromo-L-tyrosyl, or 3,5-diiodo-L-tyrosyl had been examined (Baker, 1951; Jackson *et al.*, 1966; Denburg *et al.*, 1968; Zeffren and Kaiser, 1967), and where comparisons were possible, similar effects were found in substrates related to Z-His-Phe-Phe-OMe (Inouye and Fruton, 1967). Although the nature of the A and B groups of AX–YB has a decisive effect on the catalytic efficiency of pepsin (Hollands *et al.*, 1969), the available data indicate that for any given A and B, a replacement in either the X or Y position may be expected to produce an effect that is relatively independent of the nature of A and B. Consequently, the systematic examination of the effect of the changes in the nature of X or Y in a single series of substrates Z-His-X-Y-OMe should give data relevant to the specificity of pepsin action on other substrates, including long-chain oligopeptides. Furthermore, such data may guide the construction of three-dimensional models of the binding area near the catalytic groups of the enzyme.

The tripeptide derivatives used in this study were all prepared by coupling benzyloxycarbonyl-L-histidinazide with the appropriate dipeptide esters. The initial rate of cleavage at pH 4.0 and 37° was determined by a modification of the automatic ninhydrin method of Lenard *et al.* (1965), and the site of enzymatic action was shown to be restricted to the X–Y bond.

Experimental Section

Chromatography. Examination of the homogeneity of the peptides prepared in this study, 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 employed: (A) methanol–ethyl acetate (1:3, v/v), (B) methanol–benzene (15:85, v/v), (C) 1-butanol–acetic acid–water (50:12:50, v/v, top layer), and (D) 1-butanol–acetic acid–water (3:1:1, v/v). The following reagents were used to detect the chromatographic components: 0.2% ninhydrin in 1-butanol, Pauly's reagent, iodine vapor, and chlorine–tolidine. For the chromatographic examination (solvent D, ninhydrin) of the cleavage products, the peptide (0.5 mM) was incubated with pepsin (1 mg/ml) at pH 4 and 37° for 24 hr. No satisfactory solvent system was found for the separation of Phe-OMe and Ile-OMe, and advantage was taken of the difference in color they give with ninhydrin.

Synthesis of Peptides. The substances used in this study were all of the type Z-His-X-Y-OMe, where X and Y are amino acid residues. The general synthetic procedure involved the coupling of Z-His-N_3 (prepared from 1 mmole of Z-His-$NHNH_2$, mp 172–173°) with an equivalent amount of the appropriate dipeptide ester, in the manner described

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[1] Abbreviations used that are not listed in *Biochemistry* 5, 2485 (1966), are: Osu, *N*-oxysuccinimido; Cha, β-cyclohexyl-L-alanyl; Mpa, *p*-methoxy-L-phenylalanyl; Phe(NO_2), *p*-nitro-L-phenylalanyl. The abbreviated designation of amino acid residues denotes the L form, except where otherwise indicated.

previously (Inouye and Fruton, 1967); the reaction mixture (solvent, ethyl acetate) was kept at 0° for 24–36 hr before it was worked up. Except where otherwise noted, the dipeptide esters were obtained from the corresponding Z-dipeptide esters by removal of the benzyloxycarbonyl group with saturated HBr in glacial acetic acid (1.5 ml, 30 min, room temperature), precipitation of the dipeptide ester hydrobromide with ether (80 ml), extraction of the free base with CH_2Cl_2 from an aqueous solution of the salt made alkaline with 50% K_2CO_3, and concentration of the CH_2Cl_2 extract *in vacuo*.

Z-His-Ala-Ala-OMe. Z-Ala-Osu (5 mmoles, mp 119–120°, lit. (Anderson *et al.*, (1964)) mp 123–123.5°) was coupled with Ala-OMe hydrochloride (5 mmoles) in the presence of triethylamine (5 mmoles), with 1,2-dimethoxyethane (20 ml) as the solvent. After 20 min, water (100 ml) was added to precipitate Z-Ala-Ala-OMe, which was recrystallized from ethyl acetate–petroleum ether (bp 30–60°): yield 56%, mp 107.5–108°, lit. (Hofmann *et al.* (1965) mp 104–105° for the product prepared by the mixed-anhydride method). Coupling of Z-His-N_3 with Ala-Ala-OMe derived from the above product gave the tripeptide derivative; after recrystallization from ethyl acetate–petroleum ether, it melted at 153.3–155.5°: yield 46%, $[\alpha]_D^{24}$ −47.0° (*c* 0.5, methanol). Chromatography (solvent B) gave a single spot of R_F 0.37 (Pauly). *Anal.* Calcd for $C_{21}H_{27}N_5O_6$ (445.5): C, 56.6; H, 6.1; N, 15.7. Found: C, 56.7; H, 6.1; N, 15.8.

Z-His-Ala-Phe-OMe. Z-Ala-Phe-OMe (mp 100.5–101.5°) was obtained in 90% yield by the coupling of Z-Ala-Osu and Phe-OMe in the manner described above. *Anal.* Calcd for $C_{21}H_{24}N_2O_5$ (400.4): N, 7.0. Found: N, 7.0. Coupling of Z-His-N_3 with Ala-Phe-OMe gave the tripeptide derivative; after recrystallization from ethanol–water, it melted at 180–180.5°: yield 63%, $[\alpha]_D^{24}$ −19.3° (*c* 1.2, methanol). Chromatography (solvent B) gave a single spot of R_F 0.30 (Pauly); peptic cleavage gave a single ninhydrin-positive component whose R_F (0.52) was the same as that for Phe-OMe. *Anal.* Calcd for $C_{27}H_{31}N_5O_6$ (521.6): C, 62.2; H, 6.0; N, 13.4. Found: C, 62.0; H, 5.8; N, 13.0.

Z-His-Phe-Ala-OMe. Z-Phe-Osu (mp 137.5–138.5°, lit. (Anderson *et al.* (1964)) mp 140–140.5°) was coupled with Ala-OMe in the manner described above to yield Z-Phe-Ala-OMe: yield 88%, mp 130–131°, lit. (Grassmann *et al.* (1958)) mp 130–131°). Coupling of Z-His-N_3 with Phe-Ala-OMe gave the tripeptide derivative; after recrystallization from ethanol–water, it melted at 203° dec: yield 43%, $[\alpha]_D^{24}$ −43.7° (*c* 1, methanol). Chromatography with solvent A gave a single spot of R_F 0.61 (Pauly, chlorine–tolidine) and with solvent B a single spot of R_F 0.42 (Pauly). Peptic cleavage gave a single major ninhydrin-positive component with the R_F of Ala-OMe (0.46); a faint spot of R_F 0.52 indicated that slight cleavage of the His–Phe bond had also occurred. *Anal.* Calcd for $C_{27}H_{31}N_5O_6$ (521.6): C, 62.6; H, 6.0; N, 13.4. Found: C, 62.4; H, 6.0; N, 13.0.

Z-His-Nva-Phe-OMe. Z-Nva-Phe-OMe (mp 108–110°; Fox Chemical Co.) was used to prepare Nva-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 182–183°: yield 73%, $[\alpha]_D^{24}$ −25.0° (*c* 1, methanol). Chromatography with solvent A gave a single spot of R_F 0.68 (Pauly) and with solvent B a single spot of R_F 0.46 (chlorine–tolidine); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52). *Anal.* Calcd for $C_{29}H_{35}N_5O_6$ (549.6): C, 63.4; H, 6.4; N, 12.7. Found: C, 63.3; H, 6.3; N, 12.5.

Z-His-Phe-Nva-OMe. Z-Phe-Nva-OMe (mp 147°; Fox Chemical Co.) was used to prepare Phe-Nva-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 186–186.5°: yield 60%, $[\alpha]_D^{24}$ −39.6° (*c* 1, methanol). Chromatography with solvent A gave a single spot of R_F 0.66 (Pauly) and with solvent B a single spot of R_F 0.46 (chlorine–tolidine); peptic cleavage gave a single ninhydrin-positive component with the R_F of Nva-OMe (0.46). *Anal.* Calcd for $C_{29}H_{35}N_5O_6$ (549.6): C, 63.4; H, 6.4; N, 12.7. Found: C, 63.3; H, 6.3; N, 13.0.

Z-His-Val-Phe-OMe. Z-Val-Phe-OMe (mp 138–140°; Fox Chemical Co.) was used to prepare Val-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 201–202.5°: yield 48%, $[\alpha]_D^{24}$ −33.2° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.46 (Pauly). *Anal.* Calcd for $C_{29}H_{35}N_5O_6$ (549.6): C, 63.4; H, 6.4; N, 12.7. Found: C, 63.0; H, 6.4; N, 12.8.

Z-His-Phe-Val-OMe. Z-Phe-Val-OMe (mp 113–114.5°; Fox Chemical Co.) was used to prepare Phe-Val-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 198.5–199.5°: yield 74%, $[\alpha]_D^{24}$ −37.7° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.60 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Val-OMe (0.46). *Anal.* Calcd for $C_{29}H_{35}N_5O_6$ (549.6): C, 63.4; H, 6.4; N, 12.7. Found: C, 63.0; H, 6.0; N, 12.7.

Z-His-Nle-Phe-OMe. Z-Nle-Phe-OMe (mp 94–96°; Fox Chemical Co.) was used to prepare Nle-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 176–177°: yield 52%, $[\alpha]_D^{24}$ −23.7° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.59 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52, brown). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 63.6; H, 6.8; N, 12.6.

Z-His-Phe-Nle-OMe. Z-Phe-Nle-OMe (mp 123–124.5°; Fox Chemical Co.) was used to prepare Phe-Nle-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 193–194°: yield 58%, $[\alpha]_D^{24}$ −36.7° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.57 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Nle-OMe (0.54, purple). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 63.8; H, 6.6; N, 12.6.

Z-His-Ile-Phe-OMe. Z-Ile-Phe-OMe (mp 149–150°; Fox Chemical Co.) was used to prepare Ile-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 207°: yield 59%, $[\alpha]_D^{24}$ −33.8° (*c* 1, methanol). Chromatography with solvent A gave a single spot of R_F 0.72 (Pauly) and with solvent B a single spot of R_F 0.56 (Pauly). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 63.9; H, 6.6; N, 12.3.

Z-His-Phe-Ile-OMe. Z-Phe-Ile-OMe (mp 103.5–104°; Fox

Chemical Co.) was used to prepare Phe-Ile-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 170–171°: yield 57%, $[\alpha]_D^{24}$ −30.8° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.61 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Ile-OMe (0.52, purple). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 64.0; H, 6.8; N, 12.4.

Z-His-aIle-Phe-OMe. Z-*a*Ile (Winitz *et al.*, 1956) and Phe-OMe were coupled in the presence of dicyclohexylcarbodiimide in the usual manner, to give Z-*a*Ile-Phe-OMe (75%), mp 134–136°. *Anal.* Calcd for $C_{24}H_{30}N_2O_5$ (426.5): N, 6.6. Found: N, 6.7. This product was converted into *a*Ile-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 198–198.5°, yield 64%. Chromatography (solvent B) gave a single spot of R_F 0.42 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52, brown), *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 63.9; H, 6.5; N, 12.4.

Z-His-Phe-aIle-OMe. Z-Phe and *a*Ile-OMe (derived from the hydrochloride of mp 118–120°) were coupled in the presence of dicyclohexylcarbodiimide, in the usual manner, to give Z-Phe-*a*Ile-OMe (64%), mp 94.5–95°. *Anal.* Calcd for $C_{24}H_{30}N_2O_5$ (426.5): N, 6.6 Found: N, 6.4. This product was converted into Phe-*a*Ile-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; after recrystallization from ethanol–water, it melted at 193–193.5°: yield 73%, $[\alpha]_D^{24}$ −30.3° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.42 (Pauly). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 64.2; H, 6.6; N, 12.5.

Z-His-Met-Phe-OMe. Boc-Met-Osu (1 mmole; Anderson *et al.*, 1964) and Phe-OMe were coupled in the manner described above for Z-Ala-Ala-OMe to yield Boc-Met-Phe-OMe (84%), mp 84–86°. *Anal.* Calcd for $C_{20}H_{30}N_2O_5S$ (410.5): N, 6.8. Found: N, 6.7. This product (0.5 mmole) was treated with trifluoroacetic acid (4 ml) for 1 hr at room temperature, the solution was concentrated *in vacuo* to dryness, and the residue was converted in the usual manner into Met-Phe-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative. After recrystallization from ethanol–water, it melted at 167.5–168.5°, yield 38%. Chromatography with solvent A gave a single spot of R_F 0.64 (Pauly, chlorine–tolidine) and with solvent B a single spot of R_F 0.49 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52). *Anal.* Calcd for $C_{29}H_{35}N_5O_6S$ (581.7): C, 59.9; H, 6.1; N, 12.0. Found: C, 59.9; H, 6.0; N, 12.1.

Z-His-Phe-Met-OMe. Boc-Phe-Osu (Anderson *et al.*, 1964) and Met-OMe were coupled to yield Boc-Phe-Met-OMe (66%), mp 78–80°. *Anal.* Calcd for $C_{20}H_{30}N_2O_5S$ (410.5): N, 6.8. Found: N, 6.5. The tripeptide derivative was prepared in the manner described above. After recrystallization from ethanol–water, it melted at 186°: yield 48%, $[\alpha]_D^{24}$ −39.5° (*c* 0.6, methanol). Chromatography with solvent A gave a single spot of R_F 0.62 (Pauly) and with solvent B a single spot of R_F 0.49 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Met-OMe (0.44). *Anal.* Calcd for $C_{29}H_{35}N_5O_6S$ (579.7): C, 59.9; H, 6.1; N, 12.0. Found: C, 60.2; H, 6.2; N, 11.8.

Z-His-Cha-Phe-OMe. β-Cyclohexyl-L-alanine hydrochloride was prepared from L-phenylalanine by catalytic hydrogenation with platinum oxide (Sela and Arnon, 1960). Chromatography (solvent C) gave a single spot of R_F 0.62 (ninhydrin). Treatment of the product (20 mmoles) with benzyloxycarbonyl chloride in the usual manner gave Z-Cha as an oil that resisted crystallization. It was converted into Z-Cha-Osu in the manner described by Anderson *et al.* (1964) for Z-Phe-Osu; yield 92%, mp 121–122° after recrystallization from chloroform–petroleum ether. *Anal.* Calcd for $C_{21}H_{26}N_2O_6$ (402.4): N, 7.0. Found: N, 6.8. The coupling of Z-Cha-Osu and Phe-OMe in the manner described above for Z-Ala-Ala-OMe gave Z-Cha-Phe-OMe in 86% yield, mp 121.5–122° after recrystallization from ethanol–water. Chromatography (solvent B) gave a single spot of R_F 0.69 (chlorine–tolidine). *Anal.* Calcd for $C_{27}H_{34}N_2O_5$ (466.6): N, 6.0. Found: N, 5.8. This product was treated with HBr in glacial acetic acid to yield the dipeptide ester hydrobromide, which was converted into the free base and coupled with Z-His-N_3 to give the tripeptide derivative; yield 77%, mp 174–175° after recrystallization from ethanol–water; $[\alpha]_D^{24}$ −30.4° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.51 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52). *Anal.* Calcd for $C_{33}H_{41}N_5O_6$ (603.7): C, 65.65; H, 6.8; N, 11.6. Found: C, 65.5; H, 6.9; N, 11.5.

Z-His-Phe-Cha-OMe. β-Cyclohexyl-L-alanine was esterified with methanol and SO_2Cl_2 to give the methyl ester hydrochloride; yield 90%, mp 155.5–156°. Chromatography (solvent D) gave a single spot of R_F 0.58 (ninhydrin). *Anal.* Calcd for $C_{10}H_{20}ClNO_2$ (221.7): N, 6.3. Found: N, 6.2. This product was used for coupling with Z-Phe-Osu to give Z-Phe-Cha-OMe in 89% yield; mp 76–78° after recrystallization from ethyl acetate–petroleum ether. *Anal.* Calcd for $C_{27}H_{34}N_2O_5$ (466.6): N, 6.0. Found: N, 5.8. This product was converted into Phe-Cha-OMe, which was coupled with Z-His-N_3 to give the tripeptide derivative; yield, 66%, mp 175–176° after recrystallization from ethanol–water; $[\alpha]_D^{24}$ −38.1° (*c* 1, methanol). Chromatography in solvent A gave a single spot of R_F 0.63 (Pauly) and in solvent B a single spot of R_F 0.40 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Cha-OMe (0.64). *Anal.* Calcd for $C_{33}H_{41}N_5O_6$ (603.7): C, 65.65; H, 6.8; N, 11.6. Found: C, 65.4; H, 7.0; N, 11.7.

Z-His-Mpa-Phe-OMe. *p*-Methoxy-L-phenylalanine hydrochloride was prepared in 81% yield by treatment of *N*-formyl-*O*-methyl-L-tyrosine (Izumiya and Nagamatsu, 1952) with 6 N HCl for 30 min on the steam bath: mp 231° dec, lit. (Baker *et al.* (1955)) mp 230–232° dec). Chromatography (solvent D) gave a single spot of R_F 0.51 (ninhydrin). Z-Mpa was prepared in the manner described by Baker *et al.* (1955) and used for coupling with Phe-OMe (1 mmole) by the mixed-anhydride method (isobutyl chlorocarbonate), with *N*-methylmorpholine as the base, and tetrahydrofuran as the solvent: yield of Z-Mpa-Phe-OMe 87%, mp 166–166.5° after recrystallization from ethanol–water. Chromatography (solvent B) showed a single spot of R_F 0.91 (chlorine–tolidine). *Anal.* Calcd for $C_{28}H_{30}N_2O_6$ (490.6): N, 5.65. Found: N, 5.5. The removal of the benzyloxycarbonyl group was effected by catalytic hydrogenolysis (palladium black) in the presence of HCl, to yield the dipeptide ester hydrochloride, Treatment with HBr in glacial acetic acid was not satisfactory.

presumably because of cleavage of the methyl ether (Law and duVigneaud, 1960). Coupling of the free dipeptide ester with Z-His-N_3 gave the tripeptide derivative in 53% yield: mp 195.5–196° after recrystallization from ethanol–water: $[\alpha]_D^{24}$ −27.0° (*c* 0.5, methanol). Chromatography (solvent B) gave a single spot of R_F 0.41 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Phe-OMe (0.52). *Anal.* Calcd for $C_{34}H_{37}N_5O_7$ (627.7): C, 65.1; H, 5.9; N, 11.2. Found: C, 65.4; H, 6.1; N, 11.0.

Z-His-Phe-Mpa-OMe. *p*-Methoxy-L-phenylalanine was converted into the methyl ester hydrochloride (Law and duVigneaud, 1960), mp 191°. Chromatography in solvent D gave a single spot of R_F 0.49 (ninhydrin). *Anal.* Calcd for $C_{11}H_{16}ClNO_3$ (245.7): N, 5.7. Found: N, 5.7. This product was used for coupling with Z-Phe by the mixed-anhydride method, as described above for the isomeric compound: yield of Z-Phe-Mpa-OMe 89%, mp 134.5–135° after recrystallization from ethanol–water. Chromatography (solvent B) gave one spot of R_F 0.89 (chlorine–tolidine). *Anal.* Calcd for $C_{28}H_{30}N_2O_6$ (490.6): N, 5.65. Found: N, 5.5. This compound was also prepared in 89% yield by the coupling of Z-Phe-Osu and Mpa-OMe. After removal of the benzyloxycarbonyl group by catalytic hydrogenolysis, the resulting dipeptide ester was coupled with Z-His-N_3 to give the tripeptide derivative in 54% yield: mp 198–198.5° after recrystallization from ethanol–water, $[\alpha]_D^{24}$ −25.6° (*c* 0.5, methanol). Chromatography (solvent B) gave a single spot of R_F 0.40 (Pauly); peptic cleavage gave a single ninhydrin-positive component with the R_F of Mpa-OMe (0.45). *Anal.* Calcd for $C_{34}H_{37}N_5O_7$ (627.7): C, 65.1; H, 5.9; N, 11.2. Found: C, 65.1; H, 6.1; N, 11.2.

Z-His-D-Phe-D-Phe-OMe. Z-D-Phe-D-Phe-OMe (mp 145–146°) was prepared in 89% yield from Z-D-Phe and D-Phe-OMe by the mixed-anhydride method (isobutyl chlorocarbonate) with *N*-methylmorpholine as the base and tetrahydrofuran as the solvent. *Anal.* Calcd for $C_{27}H_{28}N_2O_5$ (460.5): N, 6.1. Found: N, 6.0. The tripeptide derivative was prepared in the usual manner: mp 141–143° after recrystallization from ethanol–water, yield 52%, $[\alpha]_D^{24}$ +18.0° (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.51 (Pauly). *Anal.* Calcd for $C_{33}H_{35}N_5O_6$ (597.7): C, 66.4; N, 5.9; N, 11.7. Found: C, 66.5; H, 5.9; N, 11.5.

Z-His-Gly-Gly-OMe. This compound was prepared in the usual manner by coupling Z-His-N_3 with Gly-Gly-OMe. After recrystallization from methanol–water, it melted at 158–159°: yield 43%, $[\alpha]_D^{24}$ −6.2 (*c* 1, methanol). Chromatography (solvent B) gave a single spot of R_F 0.52 (Pauly). *Anal.* Calcd for $C_{19}H_{23}N_5O_6$ (417.4): C, 54.7; H, 5.6; N, 16.8. Found: C, 54.8; H, 5.5; N, 16.6.

Gly-Gly-Phe-OMe Hydrobromide. Z-Gly-Osu and Gly-Phe-OMe were coupled in the usual manner to give the protected tripeptide in 75% yield, mp 74–75° after recrystallization from ethanol–water. Chromatography (solvent B) gave a single spot of R_F 0.59 (chlorine–tolidine). *Anal.* Calcd for $C_{22}H_{25}N_3O_6$ (427.5): N, 9.8. Found: N, 9.8. Removal of the Z group with HBr–acetic acid gave the tripeptide ester salt, mp 158–159° dec. Chromatography (solvent D) gave a single spot of R_F 0.47 (ninhydrin). *Anal.* Calcd for $C_{14}H_{20}BrN_3O_2$ (374.2): N, 11.2. Found: N, 11.1.

Gly-Phe-Gly-OMe Hydrobromide. Z-Gly-Phe-Gly-OMe was prepared in the manner analogous to that described above. It melted at 107–108° after recrystallization from ethyl acetate–petroleum ether, yield 62%. Chromatography (solvent B) gave a single spot of R_F 0.63 (chlorine–tolidine). *Anal.* Calcd for $C_{22}H_{25}N_3O_6$ (427.5): N, 9.8. Found: N, 9.8. Removal of the Z group with HBr–acetic acid gave a hygroscopic product; chromatography (solvent D) gave a single spot of R_F 0.44 (ninhydrin).

Enzyme Studies. The pepsin preparations (Worthington Biochemical Corp., lots PM 708 and 693-7) used in this work had the same specific activity toward Z-His-Phe-Phe-OEt, and the kinetic parameters for this substrate agreed with those reported previously (Hollands and Fruton, 1968). The initial rate of cleavage of the synthetic substrates was measured at 37.0 ± 0.1° at pH 4.0 (0.04 M sodium formate buffer) by means of the automatic ninhydrin method, and the kinetic parameters k_{cat} and K_M were estimated in the manner described previously (Hollands and Fruton, 1968; Hollands *et al.*, 1969). In all cases, satisfactory Michaelis–Menten kinetics were found over the ranges of substrate concentration, *S*, used. The data were subjected to computer analysis (Hanson *et al.*, 1967) and the precision is given in terms of 95% confidence limits. For the calculation of k_{cat}, it was assumed that 1 mg of pepsin = 0.0286 μmole. 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 the studies on the inhibition of pepsin by structural analogs or resistant substrates, the spectrophotometric method was used, with Z-His-Phe(NO_2)-Phe-OMe (0.05–0.25 mM) as the substrate, in the manner described previously (Inouye and Fruton, 1968; Hollands *et al.*, 1969).

Results

The kinetic data in Table I show that in the peptic hydrolysis of substrates of the type Z-His-X-Y-OMe, where X or Y is Phe, K_M is relatively invariant; except for the case of X or Y = Ala, all the K_M values fall in the range 0.3–1.0 mM. On the other hand, relatively large variations are found for k_{cat}. When X = Nva, Nle, or Leu, the values of k_{cat} are very similar (*ca.* 0.015 sec^{-1}), and about six times greater than that for X = Ala. It is of special interest that the substrates Z-His-Val-Phe-OMe, Z-His-Ile-Phe-OMe, and Z-His-*a*Ile-Phe-OMe were found to be completely resistant to pepsin under the conditions used (S = 1 mM, E = 13.6 μM, 30 min). The replacement of X = Nle by X = Met, whose side chain is isosteric with that of Nle, causes relatively little change.

Among the substrates of the type Z-His-X-Phe-OMe examined thus far, the two that are cleaved most rapidly are those in which X = Phe or Phe(NO_2). The favorable effect of the aromatic and planar substituent at the β-carbon of the X residue is emphasized by the finding that where X = β-cyclohexyl-L-alanyl (Cha), the value of k_{cat} is approximately one-tenth that for X = Phe, and resembles that for Z-His-Leu-OMe; it is of interest that the apparent binding affinity of Z-His-Cha-Phe-OMe is greater than that for Z-His-Phe-Phe-OMe.

In contrast to the sensitivity of substrates in which X = Phe(NO_2), the introduction of Tyr at the X position leads to a marked diminution in k_{cat}, as compared with the standard substrate Z-His-Phe-Phe-OMe. That this apparent inhibitory effect is related to the presence of the phenolic hydroxyl group is suggested by the effect of introducing a *p*-methoxy-

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

Substrate	S (mM)[b]	E (μM)[c]	k_{cat} (10^2 sec^{-1})	K_M (mM)	k_{cat}/K_M (10^2 sec^{-1} mM^{-1})
Z-His-Ala-Phe-OMe	0.3–3.4 (9)	8.1	0.23 ± 0.02	1.7 ± 0.3	0.14
Z-His-Nva-Phe-OMe	0.1–3.2 (12)	13.6	1.1 ± 0.1	1.0 ± 0.1	1.1
Z-His-Nle-Phe-OMe	0.1–1.0 (11)	2.7	1.6 ± 0.2	0.3 ± 0.1	5.3
Z-His-Leu-Phe-OMe[d]	0.2–1.0 (10)	2.7	1.7 ± 0.2	0.5 ± 0.1	3.4
Z-His-Met-Phe-OMe	0.05–0.7 (10)	8.1	0.96 ± 0.06	0.51 ± 0.06	1.9
Z-His-Phe-Phe-OMe	0.05–1.0 (19)	0.27	17 ± 1	0.33 ± 0.04	52
Z-His-Phe(NO_2)-Phe-OMe	0.05–0.5 (10)	0.54	29 ± 1	0.46 ± 0.06	63
Z-His-Cha-Phe-OMe	0.03–0.8 (11)	5.4	1.5 ± 0.1	0.17 ± 0.02	8.8
Z-His-Tyr-Phe-OMe[d]	0.1–1.0 (11)	13.6	0.9 ± 0.1	0.30 ± 0.06	3.0
Z-His-Mpa-Phe-OMe	0.05–0.6 (10)	0.7	8.8 ± 0.9	0.34 ± 0.07	26
Z-His-Phe-Ala-OMe	0.3–3.6 (8)	8.1	0.37 ± 0.04	1.8 ± 0.4	0.21
Z-His-Phe-Nva-OMe	0.1–3.2 (18)	8.1	0.59 ± 0.05	0.6 ± 0.1	1.0
Z-His-Phe-Val-OMe	0.1–3.3 (8)	8.1	0.38 ± 0.04	0.5 ± 0.1	0.8
Z-His-Phe-Nle-OMe	0.1–1.0 (8)	5.4	0.7 ± 0.1	0.4 ± 0.1	1.8
Z-His-Phe-Ile-OMe	0.1–3.3 (19)	8.1	0.7 ± 0.1	0.4 ± 0.1	1.8
Z-His-Phe-*a*Ile-OMe	0.4–4.6 (7)	8.1	0.31 ± 0.03	0.93 ± 0.06	0.33
Z-His-Phe-Leu-OMe[d]	0.2–1.0 (8)	13.6	0.52 ± 0.02	0.5 ± 0.1	1.0
Z-His-Phe-Met-OMe	0.06–1.5 (10)	8.1	1.4 ± 0.1	0.9 ± 0.1	1.6
Z-His-Phe-Cha-OMe	0.2–0.7 (8)	8.1	0.26 ± 0.06	0.3 ± 0.1	0.9
Z-His-Phe-Tyr-OMe[d]	0.05–1.0 (14)	0.54	17 ± 2	0.29 ± 0.06	59
Z-His-Phe-Mpa-OMe	0.07–0.52 (12)	1.4	7.7 ± 0.3	0.4 ± 0.1	19

[a] Formate buffer (0.04 M, pH 4.0), 37°. [b] Range of substrate concentration; the numbers in parentheses denote the number of runs. [c] Pepsin concentration. [d] Data taken from Hollands *et al.* (1969).

L-phenylalanyl (Mpa) residue at the X position; as will be seen from Table I, the K_M values for the substrates in which X = Phe, Tyr, or Mpa are the same, but the value of k_{cat} for Z-His-Mpa-Phe-OMe is about ten times that for the substrate in which X = Tyr, and only about one-half for the substrate in which X = Phe.

Turning now to the series of substrates of the type Z-His-Phe-Y-OMe, it may first be noted that all the substrates having an aliphatic side chain in the Y residue (including those with branching at the β-carbon) are cleaved slowly at rates that are quite similar (k_{cat} = 0.004–0.008 sec^{-1}). The introduction of Met into the Y position appears to promote hydrolysis slightly.

Previous work (Inouye and Fruton, 1967) had shown that whereas substrates of the type Z-His-X-Y-OR in which X = Tyr (or Trp) and Y = Phe are cleaved slowly, those with X = Phe and Y = Tyr (or Trp) are hydrolyzed as rapidly (or more rapidly) as is Z-His-Phe-Phe-OR. That this preference for Phe, Tyr, and Trp in the Y position may be related to their aromatic character is suggested by the relative resistance of the compound in which Y = β-cyclohexyl-L-alanyl. Methylation of the *p*-hydroxy group of Tyr in the Y position appears to cause a significant decrease in k_{cat}, but the value for Z-His-Phe-Mpa-OMe is still considerably greater than those for the substrates in which Y carries an aliphatic side chain.

Examination of the inhibition of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe (Inouye and Fruton, 1968) at pH 4 by the relatively resistant substrates Z-His-Nva-Phe-OMe (0.2–1.0 mM) and Z-His-Phe-Ile-OMe (0.2–1.0 mM) has shown them to be competitive inhibitors with K_I values of 1.1 ± 0.2 and 0.5 ± 0.2 mM, respectively; these values approximate closely the K_M values given in Table I for these two substrates. The K_I value found for the resistant compound Z-His-Ile-Phe-OMe was 3.0 ± 0.3 mM. Other completely resistant compounds examined as competitive inhibitors of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe at pH 4 were Z-His-Gly-Gly-OMe (2–10 mM; K_I = *ca.* 100 mM), Gly-Phe-Gly-OMe (4–20 mM; K_I = 6.4 ± 0.8 mM), and Gly-Gly-Phe-OMe (5–50 mM; K_I = *ca.* 25 mM). Because of their sparing solubility, the benzyloxycarbonyl derivatives of the latter two compounds could not be tested at sufficiently high concentrations to show significant inhibition.

Other compounds prepared during the course of this work, and tested as substrates of pepsin, were Z-His-Ala-Ala-OMe and Z-His-D-Phe-D-Phe-OMe; they were found to be completely resistant under the conditions used (S = 1 mM, E = 13.6 μM, 30 min).

Discussion

The specificity of several enzymes that act at amide or ester linkages is characterized by a preference for substrates in which the apolar side chains of L-amino acids such as phenylalanine, tyrosine, or tryptophan (and to lesser extent of leucine and methionine) are in the immediate vicinity of the sensitive bond. Until recently, the most extensive data on this question have been obtained with chymotrypsin;

the studies of Niemann (1964) have notably extended the initial work (for a review, see Neurath and Schwert, 1950) on the side-chain specificity of this enzyme. These studies have led to the conclusion that the action of chymotrypsin is favored by the presence of an apolar side-chain B in substrates of the type ACO-NHCH(B)CO-X (where X is the leaving group), and that there is a correlation between the hydrophobic character of the B group (as measured by the partition ratio of the free amino acid between water and an organic solvent) and its effect in promoting chymotrypsin catalysis (Hymes *et al.*, 1965; Ingles and Knowles, 1967). Other enzymes that exhibit marked preference for the presence of apolar side chains on one side of the sensitive bond, or at locations one amino acid residue away from that bond are carboxypeptidase A (Stahmann *et al.*, 1946; Neurath and Schwert, 1950), papain (Mycek and Fruton, 1957; Schechter and Berger, 1968), streptococcal proteinase (Gerwin *et al.*, 1966), and bacterial neutral proteinases (Morihara *et al.*, 1968).

The data presented in this communication suggest that although the apolar nature of the amino acid side chain in the X and Y positions of a pepsin substrate Z-His-X-Y-OMe is a significant factor in the rate of hydrolysis of the X–Y bond, additional factors must also be involved. The marked decrease in the susceptibility of the substrates in which X or Y is β-cyclohexyl-L-alanyl, as compared with Z-His-Phe-Phe-OMe, speaks for a preference for a planar aromatic side chain over the similarly hydrophobic (Nemethy and Scheraga, 1962) but alicyclic nonplanar ring. In this respect, the specificity of pepsin appears to be more restricted than that of chymotrypsin, which cleaves acetyl-(β-cyclohexyl)-L-alaninamide at approximately the same rate as acetyl-L-phenylalaninamide (Foster and Niemann, 1955).

The unfavorable effect of the *p*-hydroxybenzyl side chain in substrates in which a tyrosyl residue occupies the X position is largely overcome by methylation of the phenolic hydroxyl group. A possible explanation of this finding is that the hydroxyl group may be so located in the enzyme–substrate complex as to be able to form a hydrogen bond with a catalytically important carboxylate group. The fact that the *p*-nitro group does not appear to block enzymic action may be interpreted in terms of the coplanarity of this group and the benzene ring.

The data in Table I indicate that all the substrates in which the X position is occupied by an aliphatic amino acid residue larger than L-alanyl are cleaved more rapidly than is Z-His-Ala-Phe-OMe, suggesting that the side chains of norvaline, norleucine, leucine, and methionine can interact with a portion of the enzymic region that binds planar aromatic groups. The notable resistance of the substrates in which the X position is occupied by an amino acid residue that is branched at the β-carbon (valyl, isoleucyl) suggests that steric hindrance is operative, and invites the hypothesis that one of the catalytic groups of pepsin (presumably a carboxyl group) is prevented from attacking the carbonyl group of the sensitive peptide bond. If the side-chain ethyl group at the β-carbon of isoleucine is considered to be attracted more strongly than the β-methyl group to the enzymic region that binds the phenyl group, the position of the β-methyl group relative to the peptide CO group would be different when X = *allo*-L-isoleucyl. However, since Z-His-*a*Ile-Phe-OMe was also found to be resistant to pepsin action under the condition of these experiments, no conclusion can be drawn as to the validity of the above hypothesis. A more demanding test of the hypothesis would be the examination of substrates in which the X position is occupied by each of the diastereoisomeric β-methyl-L-phenylalanyl residues; here the strong interaction with the benzene nucleus may be expected to fix the β-methyl group so that a measurable difference might be observed in the action of pepsin on the two substrates. It may be added that the resistance of the Ile–Y bond in synthetic substrates of pepsin appears to be reflected in the peptic cleavage of proteins (Tang, 1963) and in the inability of the enzyme to synthesize this bond in condensation reactions (Determann *et al.*, 1965).

In contrast to the resistance of substrates of the type Z-His-X-Y-OMe, where X = Val or Ile, the comparable substrates with these amino acid residues in the Y position are cleaved at approximately the same rate as the corresponding compounds with Nva, Nle, or Leu residues. Although the substrate with Y = *allo*-L-isoleucyl appears to be somewhat more resistant than its counterpart with Y = Ile, the difference is not great. In this case also, the examination of the susceptibility of substrates having each of the diastereoisomeric β-methyl-L-phenylalanyl residues in the Y position will be of interest. It is clear, however, that the steric hindrance postulated above to explain the resistance of Z-His-Ile-Phe-OMe does not apply to Z-His-Phe-Ile-OMe, and suggests that the stereochemical relations in the enzyme–substrate complex are such as to place the catalytic groups of the enzyme in a manner that is not hindered by the β-methyl group of Val or Ile, when they occupy the Y position.

It will be noted from Table I that there is little correlation between the values of K_M and k_{cat} for substrates of different susceptibility to peptic hydrolysis. Thus, the large difference in k_{cat}/K_M between Z-His-Phe-Phe-OMe and substrates in which X or Y is leucyl or β-cyclohexylalanyl is largely reflected in a difference in k_{cat}. In view of the likelihood that in the cleavage of amide substrates by pepsin K_M approximates K_S (the dissociation constant of the enzyme–substrate complex), such data imply that binding specificity is not correlated with kinetic specificity, and provide further evidence for the possibility that complementary conformational changes in the enzyme and the substrate may be associated with a lowering of the energy of activation, rather than a decrease in K_M (Hollands *et al.*, 1969).

Although the available knowledge about the structure of pepsin does not permit extensive speculation based on the above data, the following comments may be offered as a working hypothesis. The primary contribution of the Phe–Phe unit to the binding of Z-His-Phe-Phe-OMe at the catalytic site of pepsin has been shown previously (Inouye and Fruton, 1968), and is further documented here by the relatively large K_I value for Z-His-Gly-Gly-OMe; because of the weak inhibition observed, the reported value (*ca.* 100 mM) can be taken only as a rough estimate. The replacement of one of the Gly residues of Z-His-Gly-Gly-OMe by L-phenylalanyl markedly increases the extent of binding (K_M = *ca.* 1 mM) and leads to a measurable, though very slow, hydrolysis of the Phe–Gly or Gly–Phe bond (Hollands *et al.*, 1969). The data presented in this communication indicate that the replacement of the other Gly residue by L-alanyl causes only a slight enhancement in hydrolysis, and that Z-His-Ala-Ala-OMe is resistant to peptic attack under the conditions of

these studies. On the other hand, when some aliphatic amino acid residues (*e.g.*, Nle, Leu, or Met) are introduced into the X or Y position of Z-His-X-Y-OMe (the other position being occupied by Phe), a significant increase in the catalytic rate is observed, suggesting that the hydrophobic side chains of these amino acids can interact, to some degree, with the enzymic region near the catalytic site that binds strongly the phenyl rings of the Phe–Phe unit. If it is assumed that, in the initial enzyme–substrate complex, the backbone of the substrate Z-His-Phe-Phe-OMe is extended as fully as possible (the CONH groups are assumed to be *trans*, and the N–C^{α} and C^{α}–CO bond angles are subject to the restrictions imposed by the presence of the side-chain groups), the phenyl groups of the Phe–Phe unit extend out from the axis of the peptide backbone, with the X side chain above the plane of the sensitive CONH group and the Y side chain below it. In terms of this model, the resistance of Z-His-X-Phe-OMe when X = Val or Ile invites the speculation that one of the catalytic groups of pepsin attacks the CO group of the sensitive peptide bond in a manner that is hindered by branching at the β-carbon of the X residue. If it is further assumed that the interaction of the Phe–Phe unit with the enzyme fixes the sensitive CONH groups for attack by the catalytic groups of the enzyme (Hollands and Fruton, 1969), a significant displacement of the side-chain groups of the Phe–Phe unit may be expected to cause a concomitant displacement of the sensitive peptide group. Such an effect would be caused by the replacement of one of the L-phenylalanyl residues of the Phe-Phe unit by its D enantiomer. As shown previously (Inouye and Fruton, 1967), both Z-His-D-Phe-Phe-OEt and Z-His-Phe-D-Phe-OEt are resistant to peptic attack, but their K_I values as competitive inhibitors are nearly the same as the K_M values for Z-His-Phe-Phe-OEt. In terms of the above model, the side-chain phenyl group of D-Phe in the X position is below the plane of the sensitive CONH group, and the side chain of a D-Phe in the Y position is above it. It may be surmized that these two side chain groups of the three diastereoisomeric peptides bind to the enzyme equally strongly, but that the position of the sensitive peptide bond in the initial enzyme–substrate complex is unfavorable for catalysis in the case of the LDL and LLD compounds. As noted above, the LDD compound also is resistant to pepsin action under the conditions of these studies. From the above considerations it would seem therefore that the stereochemical specificity of pepsin with respect to the Phe–Phe unit of Z-His-Phe-Phe-OMe is primarily an expression of the catalytic specificity of the enzyme, rather than of its binding specificity. Furthermore, the significant, but relatively slower cleavage of substrates in which X or Y bears an aliphatic side chain (as in Leu) or an alicyclic group (as in Cha) may be taken to indicate partial interaction with the enzymic region that interacts strongly with the planar aromatic side chains of Phe, Tyr, and Trp. This partial interaction would appear to be largely reflected in the magnitude of k_{cat}, rather than K_M, again suggesting that the interaction of the X–Y unit of Z-His-X-Y-OMe with the catalytic site is accompanied by conformational changes in the enzyme–substrate complex.

In connection with the above model, it may be noted that Baker (1951) reported that acetylaminocinnamoyl-L-tyrosine is resistant to peptic cleavage, indicating that the orientation of the phenyl group in the X position is critical for enzymic attack at the CONH group. It will be of interest to examine the action of pepsin on substrate analogs in which the X or Y position is occupied by a L-phenylglycyl residue; from the above considerations, it would be predicted that such substrate analogs would be resistant to enzymic cleavage. It should be added that, in contrast to chymotrypsin, pepsin does not cause measurable hydrolysis of acetyl-L-tyrosinanilides.

Pepsin is frequently termed a proteinase of broad side-chain specificity because of the variety of peptide bonds found to be cleaved in protein substrates after prolonged incubation with the enzyme. Although further work is needed to examine the kinetics of pepsin action on small substrates in which the sensitive peptide bond is provided by X–Y units that include amino acid residues (*e.g.*, Glu, Lys, Ser, Pro, etc.) not present in the substrates listed in Table I, it would appear that the seeming discrepancy between the specificity of pepsin toward small synthetic substrates and long-chain oligopeptides may be attributed in large part to the effect of secondary interactions at some distance from the catalytic site (Humphreys and Fruton, 1968; Hollands and Fruton, 1968). Additional evidence for this view has recently been obtained in this laboratory; thus, Z-His-Phe(NO_2)-Gly-Ala-Ala-OMe is cleaved by pepsin (pH 4, 37°) at the Phe(NO_2)–Gly bond with a k_{cat}/K_M value of 0.03 sec^{-1} mM^{-1}, a rate much greater than that for the hydrolysis of the relatively resistant Z-His-Phe(NO_2)-Gly-OMe. (K. Medzihradszky, I. M. Voynick, H. Medzihradszky-Schweiger, and J. S. Fruton, paper in preparation).

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Dissociation of Chicken Egg-White Macroglobulin into Subunits in Acid. Hydrodynamic, Spectrophotometric, and Optical Rotatory Measurements*

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ABSTRACT: The high molecular weight globulin from egg white, variously referred to as "line 18," "component 18," and "ovomacroglobulin," has been characterized by physical techniques including sedimentation velocity and equilibrium, diffusion, viscosity, optical rotatory dispersion, and ultraviolet absorption. The native macroglobulin is a glycoprotein of mol wt 6.5×10^5. It appears to have little α-helix content. Spectrophotometric titrations show that most of its phenolic chromophores are abnormal. The transition temperature, T_m, of the native globulin at neutral pH is approximately 60°. In acid solution, the macroglobulin dissociates into two subunits of equal weight which have essentially the same frictional ratio (1.6) as the native molecule. The pH dependence of subunit formation was determined by hydrodynamic and optical methods. The change in ultraviolet absorption parallels the appearance of subunit, but changes in optical rotation do not. The change in the ultraviolet absorption spectrum observed upon separation of subunits and solvent perturbation difference spectra of both the native protein and its subunits indicates that, upon dissociation, 24 phenolic and 2 indole chromophores per subunit become newly exposed to solvent.

The high molecular weight globulin of avian egg white has been called "component 18" or "line 18" because of its position on starch gel electrophoresis (Lush, 1961). Miller and Feeney (1966), who propose the name "ovomacroglobulin," have shown that this protein prepared from the egg white of different avian species exhibits extensive cross-reactivity when tested against antibodies to the chicken protein. The amino acid composition of this globulin is similar to that of the α_2-macroglobulin from human plasma (Dunn and Spiro, 1967), which binds trypsin. The bound trypsin loses most of its proteolytic activity but little of its esterolytic activity (Haverback *et al.*, 1962). We find that the macroglobulin from chicken egg white dissociates into subunits like those formed by the human macroglobulin (Razafimahaleo and Bourrillon, 1968). This paper presents a study of the pH dependence of this dissociation and of the molecular weights and conformations of the subunits.

Experimental Methods[1]

Preparation of Ovomacroglobulin. Fresh egg white (4 l.) was blended and twice dialyzed overnight at 4° against fresh portions of 40 l. of deionized water. The precipitate was centrifuged and discarded. The supernatant was adjusted to

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