A REACTIVE ASPARTYL RESIDUE OF PEPSIN

Keelin T. Fry*

Charles F. Kettering Research Laboratory
Yellow Springs, Ohio 45387

Oh-Kil Kim, Jürgen Spona, and Gordon A. Hamilton**

Department of Chemistry, The Pennsylvania State University
University Park, Pennsylvania 16802

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Recently it was reported (Hamilton, Spona, and Crowell, 1967) that pepsin was inactivated by an equimolar amount of 1-diazo-4-phenylbutanone-2 (DPB). The results indicated that the reaction occurred at or near the active site of pepsin. In the present communication we report evidence indicating that DPB reacts with pepsin to form an ester of 1-hydroxy-4-phenylbutanone-2 (HPB) with the β-carboxyl group of an aspartyl residue, and that the amino acid sequence containing this aspartyl residue is: Ile-Val-Asp-Thr. This aspartyl residue is different from that attacked by another class of pepsin inhibitors, the α-haloketones (Erlanger, Vratsanos, Wassermann, and Cooper, 1966). Therefore, the present results define a different section of the pepsin molecule which may be involved in the enzymic catalysis.

Materials and Methods

Unless specifically noted, the materials and methods were the same as those given previously (Hamilton, Spona, and Crowell, 1967). HPB was prepared by an extension of the method of McPhee and Klingsberg (1944). A crystalline material was obtained from the crude product of the reaction of 1-chloro-4-phenylbutanone-2 with NaOH in methanol by: acidification to pH 5, addition of water, refluxing for 2 hours, evaporation of most of the solvent, extraction with

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ether, distillation at 112-114°C/2 mm, and recrystallization from ether. The product (analysis: calc. for $C_{10}H_{12}O_2$; C, 73.15; H, 7.37: found; C, 73.41; H, 7.43) melts at 44-45°C, and its IR, nmr, and mass spectra are those expected for HPB. The phenylhydrazone ($\lambda_{max}^{methanol}$, 274 mμ, ε, 19,300) of HPB has been described (McPhee and Klingsberg, 1944). The ester, 1-acetoxy-4-phenylbutanone-2 (AcHPB) was prepared by refluxing HPB with excess acetic anhydride. After evaporation of the solvent, the solid was recrystallized from ether. AcHPB (m.p. 43.5-44.5°C) was identified by its IR, nmr, and mass spectra and analysis (calc. for $C_{12}H_{14}O_3$; C, 69.88; H, 6.84; found; C, 70.02; H, 6.85).

Large quantities of C^{14}-labeled pepsin were prepared by reacting 5×10^{-4} M swine pepsin (2x crystallized, Mann Research Laboratories, Lot no. R3456) in an acetate buffer (0.05 M, pH 5.5) with DPB-2-C^{14} (5 or 10×10^{-4}M; specific activity, 1.62×10^8 dpm/mmole) in the presence of Cu(II) (1×10^{-4}M) at 38°C for 2 to 2.5 hr. The solutions were then dialyzed exhaustively (until no further radioactivity appeared in the dialysate) versus the acetate buffer, and the C^{14}-labeled pepsin precipitated by adding 10 volumes of alcohol. After sitting overnight at 4°C the precipitated material was separated by centrifugation, washed several times with 85% alcohol, and dried in vacuo. The overall recovery was ca. 75%; when 5×10^{-4}M DPB-2-C^{14} was used, 60% of the pepsin was labeled with C^{14}, and when 10×10^{-4}M DPB-2-C^{14} was used, 88% of the pepsin was labeled. The labeled pepsin readily dissolves in solutions of pH 6 or above and dissolves with difficulty in pH 5 solution.

Dansyl-Ile was prepared by the method of Morse and Horecker (1966). Other dansyl amino acids were purchased from Pierce Chemical Co. The dansyl amino acids obtained after degradation of the peptides were identified as described by Woods and Wang (1967) by thin-layer chromatography on polyamide sheets. Amino acid analyses were performed by the method of Spackman, Stein, and Moore (1958) on a Phoenix automatic amino acid analyzer.

Results and Discussion

At slightly basic pH's, a radioactive, ether-extractable compound is rapidly released from the C^{14}-labeled enzyme. In 0.05 M tris buffer, pH 8.05, at 30°C, the reaction follows first order kinetics to over 80% completion and $t_{1/2}$ is ca. 5 hours. This result suggests that essentially all the radioactivity is attached at one specific site. Preliminary chromatographic experiments sug-

gested that the ether-extractable radioactive compound is HPB. The results shown in Table 1 verify this conclusion. The phenylhydrazone prepared from the

Table 1

The Equivalence of HPB and the Radioactive Compound Released at pH 8

	Description[a]	Total Radioactivity (dpm x 10^{-5})	Specific Activity (dpm/mmole x 10^{-5})
(1)	1.46×10^{-2} mmole labeled (88%) pepsin	20.8	1620[b]
(2)	ether-extractable radioactivity after hydrolysis of labeled pepsin	17.7	-
(3)	phenylhydrazone prepared from (2) plus 1.86 mmoles HPB	11.8	8.5
(4)	recrystallized phenylhydrazone from (3)	2.25	8.6
(5)	recrystallized phenylhydrazone from (4)	0.29	8.2
(6)	expected[c] specific activity of the phenylhydrazone	-	9.4

[a] The labeled pepsin (1) was incubated in 45 ml 0.05 M tris buffer, pH 8.05, for 48 hours at room temperature. The solution was extracted several times with ether, the organic layer was dried and evaporated, and the residue dissolved in ethanol. The ethanol solution after filtration was counted (2). To the ethanol solution was added 1.86 mmoles unlabeled HPB, and the phenylhydrazone of HPB was prepared by adding phenylhydrazine and a few drops of 1 N HCl. The solid phenylhydrazone (3) was recrystallized twice from ethanol to give (4) and (5).
[b] Specific activity of the DPB-2-C^{14} used to prepared the labeled pepsin.
[c] Calculated from the specific activity of the DPB-2-C^{14}, the known amount of unlabeled HPB added, and the total radioactivity in (2) assuming it is all due to labeled HPB.

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radioactive compound plus unlabeled HPB does not change its specific activity on repeated recrystallizations. Since the expected specific activity of the phenylhydrazone is only slightly higher than that observed, very nearly all of the ether-extractable radioactive compound must be HPB.

The rate of hydrolysis of the model compound, 1-acetoxy-4-phenylbutanone-2 (AcHPB), was investigated to see if simple esters of HPB would hydrolyze at a rate similar to that observed for release of HPB from the labeled pepsin. The hydrolysis of AcHPB was followed using a Radiometer pH stat. Over the pH range 8.5 to 9.6, at 30°C, in 5% dioxane-water, the second order rate constant

for the base catalyzed hydrolysis of AcHPB is 56±7 l-mole^{-1}sec^{-1}. Thus, at pH 8, $t_{1/2}$ for the hydrolysis of AcHPB is 2 to 3 hours. This is comparable to the $t_{1/2}$ observed for the release of HPB from the labeled enzyme and indicates that a reasonable structure for the labeled pepsin is an ester of HPB with a carboxyl group of pepsin.

For the preparation of labeled peptide fragments, denatured C^{14}-labeled pepsin was digested with native pepsin as follows. A 133 mg sample of inhibited pepsin (60% labeled) was dissolved in 2.5 ml tris buffer (0.05 M, pH 7.2) to yield a slightly turbid solution of pH 6.0. After standing at room temperature for 30 min, the solution was brought to pH 7.8 by adding 0.3 ml tris buffer (0.1 M, pH 8.5). After ca. 10 min, the pH was adjusted to 2.02 by adding in succession 16.5 ml of 0.02 M HCl and 0.1 ml of 1.0 M HCl. Digestion of the C^{14}-labeled pepsin at 37°C was initiated by adding 2.35 ml of a pepsin solution (5 mg of 2x crystallized Worthington pepsin per ml in 0.01 M HCl). In a few minutes essentially all the precipitate, which had formed when the solution was brought to pH 2.02, went into solution. After 4.3 hours, an additional 2.35 ml of pepsin solution was added and digestion was allowed to continue for a total of 21 hr. The digest was then treated with 0.2 ml of 1.0 M acetate buffer, pH 5.0 to neutralize the excess HCl. The resulting solution, (pH 2.95) was lyophilized to dryness and the residue suspended in 0.2 M acetic acid. After centrifugation to remove a small amount of white precipitate, the digest was chromatographed on a column of sephadex G-25 (Figure 1). The recovery of C^{14} in fractions 222-355 was 90% of that present in the original digest.

Fractions 283-292 (peak I) were pooled and lyophilized to dryness. Gel filtration of the residue in 0.2 M acetic acid on a 0.9 x 157 cm column of Sephadex G-15 gave a single peak of radioactivity with 78% recovery. From this material a C^{14}-labeled tetrapeptide was isolated by performing in succession (a) descending paper chromatography in 1-butanol-acetic acid-water, 200:30:75, v/v (Nolan and Margoliash, 1966); (b) paper electrophoresis on a Savant flat plate electrophoresis apparatus in 8% formic acid for 5 hours at 40 watts/cm; and (c) paper electrophoresis at pH 3.6 in pyridine-acetic acid-water, 1:10:289 (Katz, Dreyer, and Anfinsen, 1959). At each stage of purification good recovery (69, 96, and 91% in steps (a)-(c) respectively) of radioactivity was obtained in narrow regions of the chromatograms. The amino acid composition of the labeled tetrapeptide, determined after 69 hr. hydrolysis in constant-boiling

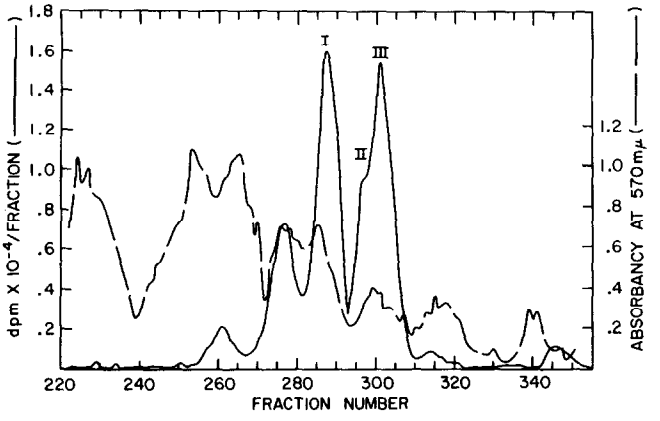

Figure 1. Gel filtration at room temperature of the pepsin digest of denatured, C^{14}-labeled pepsin. The 1.9 x 243 cm column, which was packed with Sephadex G-25 (fine bead form) in 0.2 M acetic acid (Eaker, 1962), had a void volume of 275 ml. The digest sample volume was 3.7 ml and the eluant was 0.2 M acetic acid. Two-ml fractions were collected at a flow rate of 28.5 ml/hr. The ninhydrin color (absorbancy at 570 mμ) of base-hydrolyzed 0.05 ml aliquots of each fraction was determined by the method of Rosen (1957).

HCl at 110°C, is: Asp, (1.00); Ile, 0.95; Thr, 0.89; Val, 0.95. Other amino acids were present to less than 5% of the amount of Asp. In the labeled tetrapeptide the molar ratio of radioactive compound to Asp is 1.0. Also, the β-carboxyl group of the Asp is not free because the labeled tetrapeptide does not migrate on electrophoresis at pH 6.5. If the labeled tetrapeptide is incubated in tris buffer, pH 8.5, for 4 hr. at 37°C, the radioactivity can be extracted from the aqueous solution with ether. This result is similar to that obtained under comparable conditions with labeled pepsin, and suggests that the C^{14}-labeled compound is attached to the tetrapeptide in the same way as to pepsin.

The amino acid sequence of this peptide was shown to be Ile-Val-Asp-Thr by using the subtractive Edman degradation procedure, and identifying the N-terminal amino acid liberated at each stage by the dansylation technique (Gray, 1967). Most of the radioactivity associated with the labeled peptide remained in the aqueous solution during the first 2 steps of the Edman degradation. However, following the third step, the radioactivity was extracted into the organic solvent, and free threonine was found in the aqueous layer.

These results indicate that the C^{14}-labeled compound is attached to the β-carboxyl group of the aspartyl residue.

The radioactivity in peaks II and III (Figure 1) appears to be associated with two C^{14}-labeled peptides which have been separated and purified by procedures similar to those employed for the purification of the labeled tetrapeptide. Amino acid and radioactivity analysis of an impure sample of material from peak II indicate it contains a labeled tripeptide composed of Val, Asp, and Thr. Preliminary experiments on material from peak III indicate that it contains a labeled tripeptide with the sequence Ile-Val-Asp. Further work with larger amounts of materials will be necessary to substantiate these conclusions. However, these results further suggest that only a single aspartyl residue on pepsin reacts with DPB.

All of the results reported here are consistent with the hypothesis that DPB reacts with pepsin to form an ester of HPB with the β-carboxyl group of one specific aspartyl residue which is in the sequence Ile-Val-Asp-Thr. Further work will be necessary to determine whether this aspartyl residue is intimately involved in the enzymic catalysis, and where in the enzyme this sequence occurs. This tetrapeptide is not present in the known sequence of the carboxyl terminal section of pepsin (Dopheide, Moore, and Stein, 1967; Stepanov et al, 1967). It will also be of interest to see if the observed reactions of pepsin with other diazo compounds (Rajagopalan, Stein and Moore, 1966; Delpierre and Fruton, 1966; Ong and Perlmann, 1967; Erlanger, Vratsanos, Wassermann, and Cooper, 1967) occur at the same or different sites.

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References

Delpierre, G. R., and Fruton, J. S. (1966) Proc. Natl. Acad. Sci., U.S. 56, 1817.
Dopheide, T.A.A., Moore, S., and Stein, W.H. (1967) J. Biol. Chem., 242, 1833.
Eaker, D.L. (1962) Ph.D. Dissertation, The Rockefeller Institute, p. 29.
Erlanger, B. F., Vratsanos, S.M., Wassermann, N. and Cooper, A.G. (1966) Biochem. Biophys. Res. Commun. 23, 243.
Erlanger, B.F., Vratsanos, S.M., Wassermann, N. and Cooper, A.G. (1967) Biochem. Biophys. Res. Commun. 28, 203.
Gray, W.R. (1967) Methods in Enzymology, 11, 469.

Hamilton, G.A., Spona, J. and Crowell, L.D. (1967) Biochem. Biophys. Res. Commun. 26, 193.
Katz, A.M., Dreyer, W. J., and Anfinsen, C.B. (1959) J. Biol. Chem., 234, 2897
McPhee, W.D. and Klingsberg, E. (1944) J. Am. Chem. Soc., 66, 1132.
Morse, D. and Horecker, B.L. (1966) Anal. Biochem., 14, 429.
Nolan, C. and Margoliash, E. (1966) J. Biol. Chem. 241, 1049.
Ong, E.B. and Perlmann, G.E. (1967) Nature, 215, 1492.
Rajagopalan, T.G., Stein, W.H. and Moore, S. (1966) J. Biol. Chem., 241, 4295.
Rosen, H. (1957) Arch. Biochem. Biophys. 67, 10.
Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem., 30, 1190.
Stepanov, V.M., Ostoslavskaya, V.I., Krivtzov, V.F., Muratova, G. L. and Levin, E.D. (1967) Biochem. Biophys. Acta., 140, 182.
Woods, K. R. and Wang, K. T. (1967) Biochem. Biophys. Acta, 133, 369.