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The C-Terminal Determination of Polypeptide by the Selective Tritium-labeling. V.[1] C-Terminal Cleavage of Peptide by the Action of Acetic Anhydride

Hisayuki Matsuo,[2a,c] Yasuo Fujimoto, Hiroko Kobayashi, Takashi Tatsuno,[2a] and Hiroshi Matsubara[2b]

The Institute of Physical and Chemical Research[2a] and Space Sciences Laboratory, University of California[2b]

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During the course of further investigation of Method B, which was previously proposed for the tritiation of C-terminal proline in the peptide chain, it was revealed that the C-terminal amino acid was cleaved to some extent under the heat-treatment of peptide in AcOH–Ac_2O solution.

This type of C-terminal cleavage was confirmed gas- and paper-chromatographically and further evidences were also obtained by the C-terminal assay of the reaction products.

In the present study, it was also revealed that non-C-terminal histidine residue in peptide chain underwent the hydrogen-tritium exchange reaction in its imidazole ring, when the peptide containing histidine residue was heated in a mixture of acetic anhydride-tritiated water or acetic anhydride-[carboxy-^3H]acetic acid in the absence of pyridine.

Selective tritiation of the C-terminal amino acids in polypeptide chains has recently been proposed by the present authors as a new method for identifying the C-terminal amino acids in proteins.[1,3] Proteins are selectively tritiated at their C-terminal amino acids through racemization *via* oxazolone formation by the action of acetic anhydride in the presence of pyridine and tritium oxide. This base-catalyzed tritiation method (Method A) was found to be generally applicable for the C-terminal determination of proteins.[3–5] with only one exception of C-terminal proline, which resists to the oxazolone formation.

On the other hand, our previous paper[3b] described the alternative method for the tritiation of C-terminal proline of peptide. When the peptide, Phe-Arg-Try-Gly-Ser-Pro-Pro, was heated in a mixture of acetic anhydride-tritium oxide or acetic anhydride-[carboxy-^3H]-acetic acid without pyridine, its C-terminal proline underwent tritiation (Method B).

However, in several cases, tritium-incorporation into non-C-terminal residues in the peptide chains has been observed in Method B,[4,5] unlike Method A, which proceeds selectively at C-termini. Even though both of Methods A and B might be based on the racemization reaction of C-terminal amino acids, there was a distinct difference that Method B involved heat treatment with acetic anhydride in acidic media, while Method A was a base-catalyzed reaction at room temperature.

Accordingly, it could be anticipated that anomalies observed in Method B might arise from heat treatment of peptides with acetic anhydride in acetic acid.

The present paper dealt with further investigation on Method B with several model peptides and it was revealed that the C-terminal amino acids of peptides were cleaved to some

1) Part IV: H. Matsuo and H. Matsubara, *Proc. Soc. Exptl. Biol. Med.*, **129**, 564 (1968).
2) Location: *a*) *Yamato-Machi, Saitama* *b*) *Berkeley, California*, 94720, *U.S.A.*; *c*) To whom inquiries may be addressed. Present address: *The Institute for Protein Research. Osaka University, Kita-Ku, Osaka.*
3) *a*) H. Matsuo, Y. Fujimoto, and T. Tatsuno, *Tetrahedron Letters*, **1965**, 3465; *b*) *Idem*, *Biochem. Biophys. Res. Commun.*, **22**, 69 (1966); *c*) *Idem*, *Chem. Pharm. Bull.* (Tokyo), **15**, 716 (1967); *d*) H. Matsuo, *Protein, Nucleic Acid, Enzyme*, **14**, 523, 589 (1969).
4) G.N. Holcomb, S.A. James, and D.N. Ward, *Biochemistry*, **7**, 1291 (1968).
5) D.M.P. Phillips and P. Simon, *Biochim. Biophys. Acta*, **181**, 154 (1969).

extent under the reaction conditions of Method B. This type of C-terminal cleavage reaction was also confirmed by cold experiment involving heat-treatment of peptide in acetic anhydride-acetic acid system in place of acetic anhydride-tritium oxide or acetic anhydride-[carboxy-^3H]-acetic acid in Method B. In addition, non-C-terminal histidine was found to be tritiated through acid-catalyzed path way, when Method B was applied to the peptides having non-C-terminal histidine residue in peptide chains.

Experimental

Materials——Peptides, Ac-Gly-Ala-Val (I), Ac-Gly-Ala-Val-Leu (II), Pro-Phe-His-Leu (III) and Phe-His-Leu-Leu-Val-Tyr (IV) were kindly donated by Dr. T. Mizoguchi, Tanabe Seiyaku Co., to whom our thanks are due. Leu-Gly-Gly (V), Leu-Gly-Val (VI) and Leu-Gly were obtained from Cal. Biochem Co. 99% Deuterium oxide was a product of Merck A.G. and tritiated water was purchased from Japan Radioisotope Association and diluted to the final specific activity of 1 mCi/ml. Reagent grade pyridine was refluxed over NaOH-pellets and distilled. Ac_2O was redistilled and stored in refrigerator. Paper chromatography was carried out with solvent system of BuOH–AcOH–water (200:30:75).

C-Terminal Tritiation of Peptides (Method A)——Peptides (I—IV: 1 mg) was dissolved in a mixture of ^3H_2O (0.2 ml) and pyridine (0.2 ml). To the resulting clear solution was added Ac_2O (0.05 ml) in one portion under ice-cooling and the whole was kept standing in an ice-bath for 0.5 hr and then at room temperature for additional 3 hr. After evaporation of solvent *in vacuo*, addition of 0.5-ml portion of water, followed by evaporation was repeated 5 times to assure complete removal of the washable isotope. The residue thus obtained was hydrolyzed with constant-boiling HCl in an evacuated sealed tube at 110° for 24 hr. The hydrolysate was paper-chromatographed and radioactivity of each amino acids on the papergram was measured by liquid scintillation spectrophotometer by the described manner.[1,3d] Results were listed in Tables I—IV (A; top row).

Treatment of Peptides with Ac_2O-^3H_2O (Method B)——Peptides (I—IV: 1 mg) was heated in a mixture of Ac_2O (0.35 ml) and ^3H_2O (0.05 ml) at 60° for 3 hr. After evaporation, the residue was treated with water (0.5 ml × 5) and worked up further in the same manner as in the case of Method A. Results were listed in Tables I—IV (B: middle row).

C-Terminal Cleavage Reaction with Ac_2O-AcOH (Cleavage)——Peptide (I—IV: 2 mg) was heated in 1 ml of a mixture of Ac_2O–AcOH (1:3 v/v) at 60° for 3 hr. The residue after evaporation was treated with 1 ml of water at 50° for 1 hr and dried up to afford slightly yellow oil, which was directly submitted to the C-terminal assay by Method A.

The results thus obtained were shown in Tables I—IV (Cleavage: bottom row). In the cases of peptides (I and II), radio-gas chromatographic method was also used for radioactivity measurement.

Radio–Gas Chromatography[6]: The hydrolysate of tritiated peptide that obtained by Ac_2O–AcOH treatment, followed by Method A–tritiation was converted to the corresponding N-trifluoroacetyl butyl esters[7] and analyzed by Shimadzu Gaschromatograph Model GC-4APF connected with radioactivity detector, Shimadzu Model RID-2A. Separation was carried out on the glass column (2 m × 4 mm *i.d.*) packed with 2% Apiezon L and 1% butanediol succinate on Celite 545 (HMDS) (60—80 mesh). Column temperature was programmed from 100° to 140° (4°/min). One part of effluent sample gas was converted to tritium-containing hydrogen gas, whose radioactivity was measured on crystalline anthracene scintillator. These results were given in parentheses in Tables I and II (Cleavage).

Gas Chromatography of Ac_2O-Treated Peptide-I——The residue that obtained from peptide (I) by the Ac_2O–AcOH–treatment, followed by mild hydrolysis as described in cleavage procedure, was converted to methyl ester derivatives by the action of diazomethane. The esters were submitted to gas chromatography, using a glass column (3 m × 3 mm *i.d.*) packed with 1.5% OV-17 on Gaschrom P (80—100 mesh). Column temperature was programmed from 100° to 260° (2°/min). Chromatogram shown in Fig. 1 was obtained.

C-Terminal Cleavage of N-Trifluoroacetyl-peptide——One mg of peptide, Leu-Gly-Gly (V) or Leu-Gly-Val (VI) was suspended in 1 ml of anhydrous dioxane, trifluoroacetic anhydride (0.1 ml) was added under ice-cooling and the whole was allowed to stand at 0° for 1 hr to give a clear solution. After evaporation *in vacuo*, the resulting N-TFA peptide showed single spot (Ninhydrin positive), corresponding to the starting peptide (*Rf*: 0.25 for V; 0.55 for VI), by the paper-chromatography after removal of TFA group by exposing to ammonia vapor.[1,8]

The TFA–peptide thus obtained (TFA–(V) or TFA–(VI)) was submitted to C-terminal cleavage reaction with Ac_2O–AcOH under the same conditions as described in cleavage-procedure. After mild treat-

6) H. Matsuo, Y. Fujimoto, T. Tatsuno, and N. Ikekawa, to be published in *Anal. Biochem.* See ref. 3*d*).
7) C.W. Gehrke and D.L. Stalling, *Separation Science*, **2**, 101 (1967).
8) R.N. Perham and G.M.T. Jones, *European J. Biochem.*, **2**, 84 (1967).

ment with water, followed by evaporation, the residue was dissolved in 10-μl of water, 10-μl portion of which was spotted on a line (3 cm wide) on the paper (Toyo Roshi No. 50). After removal of TFA-protective group by exposing to ammonia vapor in an evacuated chamber,[8] paper-chromatography was carried out. Ninhydrin spray on reference paper-strip showed two spots, corresponding to Leu-Gly (*Rf*: 0.35) and the starting peptide (V or VI). In each cases, two peptides thus obtained were extracted from the paper-chromatogram with 5% AcOH, and hydrolyzed with 6N HCl in an evacuated sealed tube at 110° for 24 hr, respectively. Recovery yields of each peptides were determined by the aid of amino acid analysis of the hydrolysate obtained above. Calculated from analysis data, molar ratios of Leu-Gly *vs.* starting peptides were determined as 0.5 for peptide (V) and 0.3 for peptide (VI), respectively.

Deuteration of N-Acetyl-histidine——N-Acetyl-DL-histidine[9] (50 mg) was heated in a mixture of Ac_2O (1.4 ml) and ^2H_2O (0.2 ml) at 60° for 3 hr. After evaporation *in vacuo*, the residue was dissolved in 0.5 ml of water and then allowed to stand at room temperature overnight. The residue after evaporation was recrystallized from water–acetone to afford colorless prisms, which showed one spot (Pauli+; Ninhydri−) corresponding to N-acetyl histidine on the electrophoretogram at pH 3.5.

NMR spectrum of this sample at 60 M Hz was taken in a solution of ^2H_2O with TMS as an external reference, as shown in Fig. 2.

Results and Discussion

C-Terminal Cleavage of Peptide

In the base-catalyzed C-terminal tritiation by Method A, peptides (I—IV) were selectively tritiated at their C-terminal amino acids and no radioactivity was observed at other constituent

Table I. Tritium–incorporation in Ac–Gly-Ala-Val

Method	cpm		
	Gly	Ala	Val
A	30	32	1535
B	225	452	1005
Cleavage	85	225	1268
	(55)	(148)	(602)[a]

a) The value of cpm in parentheses was obtained by radio-gas chromatographic measurements.

Table II. Tritium-incorporation in Ac-Gly-Ala-Val-Leu

Method	cpm			
	Gly	Ala	Val	Leu
A	27	25	34	1328
B	115	485	786	1487
Cleavage	95	275	540	1360
	(60)	(175)	(262)	(655)[a]

a) The value of cpm in parentheses was obtained by radio-gas chromatographic measurements.

Table III. Tritium-incorporation in Pro-Phe-His-Leu

Method	cpm			
	Pro	Phe	His	Leu
A	22	20	38	1910
B	320	960	2380	1590
Cleavage	98	181	417	2215

9) J.P. Greenstein and M. Winitz, "Chemistry of Amino Acids," Vol. 3, John Wiley & Sons, Inc., New York, 1961, p. 1990.

Table IV. Tritium-incorporation in Phe-His-Leu-Leu-Val-Tyr

Method	cpm				
	Phe	His	Leu (×1/2)	Val	Tyr
A	30	35	60	45	1860
B	160	2200	240	590	1750
Cleavage	110	180	300	507	2150

amino acid residues, as seen from Tables I—IV (A). On the contrary, when peptides (I—IV) were treated with Ac_2O–^3H_2O in the absence of pyridine under acidic conditions of Method B, tritiation patterns listed in Tables I—IV (B), were quite different from those obtained by Method A.

In the cases of peptides (I and II) (Table I (B) and II (B)), major radioactivity, of course, located at C-terminal Leu and Tyr, respectively, but significant tritium-incorporation was also observed at non-C-terminal residues.

As seen from Table III (B) and IV (B), peptides (III and IV) also afforded similar tritiation patterns except abnormally high ^3H-incorporation in His, which will be discussed in the next section.

From these results, it was anticipated that the cleavage of C-terminal residue might occur under the reaction conditions of Method B to afford another new peptide having original penultimate amino acid of the starting peptide as a new C-terminus. This assumption was confirmed by following cold experiment, using Ac_2O–AcOH system in place of Ac_2O–^3H_2O or Ac_2O–AcO^3H in Method B mentioned above, followed by gas chromatographic analysis.

In order to exclude complexity due to His which showed abnormal behavior in Method B as mentioned above, peptide (I) was chosen as a test-sample for this purpose. Peptide, Ac-Gly-Ala-Val (I) was heated at 60° for 3 hr in a mixture of Ac_2O–AcOH (1:3 v/v). The ratio of Ac_2O/AcOH was chosen as to be the same to that, calculated from $Ac_2O/^3H_2O$-ratio used in Method B. After heating, the residue was mildly treated with water to assure hydrolysis of the probably resulting oxazolone or mixed anhydride derivative. After drying up, the product mixture thus obtained was analyzed by gas chromatography as their methyl ester derivatives. Gas-chromatogram (Fig. 1) clearly showed two new peaks, which corresponded to a newly formed peptide, Ac-Gly-Ala and Ac-Val, respectively, along with doublet peaks due to a diastereomeric pair of the recovered starting peptide, Ac-Gly-Ala-Val (I), whose C-terminal Val must be racemized during this reaction. Therefore, it was unambiguously confirmed that C-terminal Val was cleaved as its N-acetyl derivative from the parent peptide (I) by the action of acetic anhydride under the acidic reaction conditions to afford a new pept ideAc-Gly-Ala,

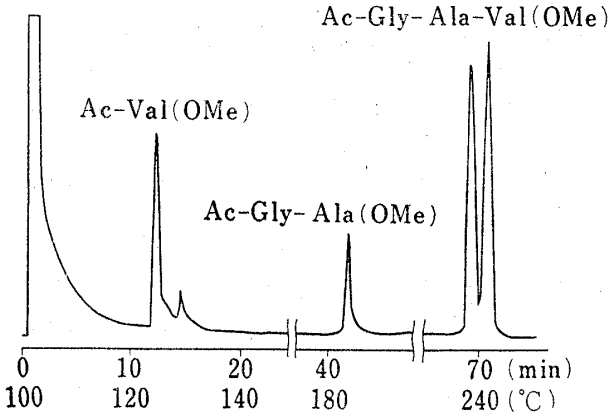

Fig. 1. Gas Chromatogram of Ac_2O-AcOH treated Peptide (I)

1.5% OV-17 on Gaschrom P (80—100 mesh) 3 m×3 mm *i.d.*

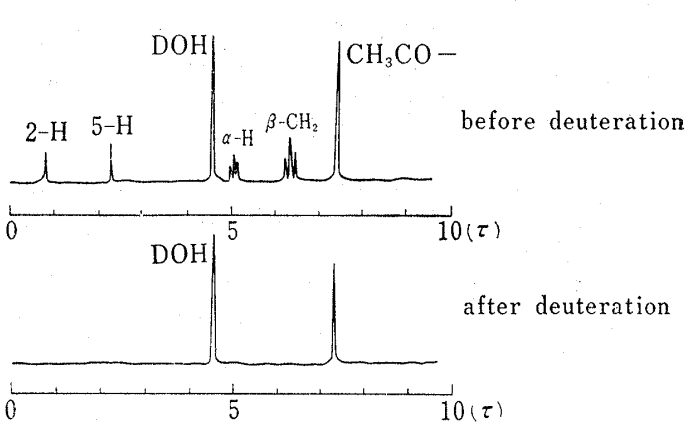

Fig. 2. NMR Spectra of Acetyl-Histidine before and after Deuteration

at 60 Hz in D_2O solution (TMS as external reference)

whose C-terminal Ala was originally penultimate residue in peptide (I).

For the purpose of further confirmation of C-terminal cleavage, the same reaction was applied to peptides (II—IV). In these cases, gas chromatographic method was unable to be used for product-analysis. The product mixtures obtained by above reaction were directly submitted to C-terminal analysis by Method A.

For the convenience, the C-terminal cleavage reaction, followed by C-terminal analysis as mentioned above will be denoted as cleavage in Table I—IV, which summarized the results obtained above. Even though quantitative conclusion can not be drawn from these Tables, because quantitation of C-terminal analysis by Method A was not completed yet, these cpm-value might be helpful to estimate the approximate yield of C-terminal cleavage. Thus, the C-terminal cleavage at the lst step in above cases seemed to proceed in the yields of 10—20%.

As seen from the comparison of Table I—IV (cleavage) with Tables I—IV (A), the significant radioactivity which located at every constituent amino acid residues in a decreasing order from C-termini to N-termini lead us to anticipate that this cleavage reaction might proceed stepwise or sequentially, in spite of the lack of clear evidences for this assumption at present time.

In order to determine the yield of cleavage reaction, quantitative survey was carried out with N-trifluoroacetyl derivatives of peptides, Leu-Gly-Gly (V) and Leu-Gly-Val (VI).

After C-terminal cleavage reaction of TFA–V and TFA–VI, the resulted products were spotted on the paper, respectively.

In order to make peptide-spots Ninhydrin-positive, removal of protecting TFA-groups was carried out by exposing the spotted paper to ammonia vapor according to the method of Perham, *et al.*[8] and then chromatographed. In each cases, the recovered parent peptide (V or VI) and a newly formed dipeptide, Leu-Gly were characterized on the paper-chromatogram by Ninhydrin staining. Two peptides, corresponding to the starting one and Leu-Gly, were extracted from the papergram, hydrolyzed with HCl and then analyzed by amino acid analyser, respectively. Thus, the molar ratios of Leu-Gly/starting V and VI in the products mixture were determined to be 0.5 and 0.3, respectively. From these values, the yields of C-terminal cleavage were calculated as 33% for V and 25% for VI, respectively. In the above experiments, Leu, a probable product by the 2nd cleavage, was unable to be detected on paper-chromatograms. Study on the reaction mechanism is now going on. If this reaction will be generally applicable, sequential degradation of polypeptide from its C-terminus would be hopefully expected.

^{3}H-Incorporation in Non-C-Terminal Histidine Residue

As discussed in the foregoing section, tritiation patterns obtained by the treatment of Method B of peptides I and II (Tables I (B) and II (B)) were found to be interpreted by the C-terminal cleavage.

In the casesof peptides (III and IV) having His residue in their chain, however, only C-terminal cleavage mentioned above was unable to give a satisfactory explanation for the extraordinary ^{3}H-incorporation in non-C-terminal His observed in tritiation patterns listed in Tables III (B) and IV (B).

In order to elucidate such an anomaly in His, deuteration expeliment was carried out with acetylhistidine as a model compound, by using deuterium oxide in place of tritium oxide in Method B.

Acetyl-histidine was heated with acetic anhydride in a medium of deuterium oxide under the same conditions as in Method B. The NMR spectrum of the deuteration product was taken in a deuterium oxide, as shown in Fig. 2.

It was surprising enough that not only α-proton but also all protons in histidine residue were completely replaced by deuterium under this reaction conditions, as seen from Fig. 1.

It is still difficult to draw a complete interpretation for extraordinary ^{3}H-incorporation in non-C-terminal histidine from the deuteration experiment mentioned above, because acetyl-

histidine is not always a good model for non-C-terminal histidine in peptide chain. However, it is quite likely enough to conclude that imidazole ring in histidine, whether it is C-terminal or not, readily undergoes isotope-exchange reaction under the present conditions.

Furthermore, in the C-terminal determination of aspartate transferase isozyme, the group of Kagamiyama[10] reported the unexpected tritium-incorporation in non-C-terminal histidine when tritiation was carried out by using AcONa as a catalyst in place of pyridine in the standard conditions (Method A). This might be also explainable by the similar reason as discussed above.

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10) H. Kagamiyama, T. Watanabe, H. Wada, Y. Fujimoto, and T. Tatsuno, *Biochem. Biophys. Res. Commun.*, **32**, 678 (1968).