Enzymatic Digestion of C-Terminal ^{3}H-Labeled Peptides: Possible Usefulness for the Structural Study of Proteins* (33370)

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Selective tritiation of the C-terminal amino acids in polypeptide chains has recently been proposed by one of the present authors (1–5) as a new method for identifying the C-terminal amino acids in proteins.

Proteins are selectively tritiated at their C-terminal amino acids through racemization via oxazolone formation by the action of acetic anhydride and pyridine in a medium containing ^3H_2O. The present paper describes the enzymatic digestion of C-terminal tritiated peptides which were readily obtained by the above method. This facilitates the detection of C-terminal fragments in enzymatic digests by radioactivity.

Our investigations were carried out with *Scenedesmus* ferredoxin and peptides derived from it during the course of structural studies of *Scenedesmus* ferredoxin, whose primary structure has recently been established [(6); also Sugeno and Matsubara, in preparation]. The results indicate the usefulness of tryptic and chrymotryptic digestion of the tritiated peptides in identifying C-termini.

Tryptic digestion of C-terminal tritiated peptide. As shown in Fig. 1, the peptide (C-V), one of the C-terminal fragments obtained by chymotryptic digestion of carboxymethylated *Scenedesmus* ferredoxin, had one lysine residue whose peptide linkage is susceptible to trypsin.

To protect the free amino group of lysine from acetylation which might occur during subsequent tritiation reaction, 0.337 μmole of peptide C-V was treated with $F_3CCOSEt$ and 1 N NaOH at pH 9.8 as described by Goldberger and Anfinsen (7). This yielded the corresponding N-trifluoroacetyl peptide

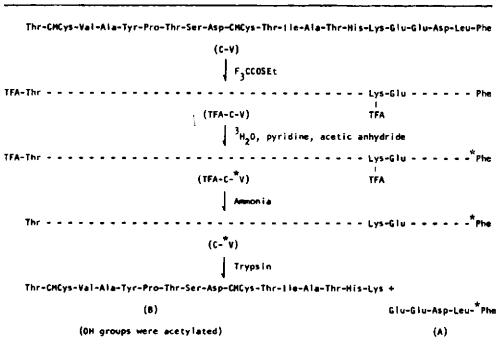

FIG. 1. Schematic representation of trifluoroacetylation, tritiation, and trypsin digestion of C-terminal fragment of *Scenedesmus* ferredoxin.

(TFA-C-V). Purification was carried out on a small Dowex 1×2 column equilibrated with a buffer containing 0.124 M pyridine and 0.003 M acetic acid by successive elutions with the same buffer and 30% acetic acid. Lyophilization of the fraction eluted by acetic acid gave a pure peptide, TFA-C-V (ninhydrin reaction: —, Pauly reaction: +), which was subjected to tritiation reaction by the method described by Matsuo *et al.* (2) as follows: Peptide TFA-C-V was dissolved in 0.1 ml (10 mCi) of ^3H_2O and pyridine (0.2 ml), and acetic anhydride (0.05 ml) was added. The solution was allowed to stand at room temperature overnight to yield the corresponding radioactive peptide (TFA-C-*V), which gave only one spot (+15 cm, ninhydrin: —, Pauly: +) on electrophoresis (pH 6.5, 2000 V, 1 hr). The removal of the TFA group was carried out by exposing the peptide to ammonia in an evacuated desiccator by the method of Perham and Jones (8) to produce a ninhydrin-positive peptide (C-*V), with free NH_2-groups and acetylated hydroxyl groups. The structure of peptide C-*V was confirmed by amino acid analysis and radioactivity measurement of the hydro-

* This work was supported by Grant NsG 479 from the National Aeronautics and Space Administration and Grant NIH HE-11553-01 from the National Institutes of Health.

TABLE I. Amino Acid Composition of Peptides Obtained by Enzymatic Digestion.[a]

Amino acid	A	B	C
Lysine		1.02 (1)	0.69 (1)
Histidine		1.04 (1)	0.63 (1)
Cysteic acid			1.31 (2)
Carboxymethylcysteine		1.49 (2)	
Aspartic acid	1.07 (1)	1.03 (1)	2.07 (2)
Threonine		3.69 (4)	2.63 (4)
Serine		1.07 (1)	0.82 (1)
Glutamic acid	1.97 (2)		1.98 (2)
Proline		1.15 (1)	0.96 (1)
Alanine		2.07 (2)	1.79 (2)
Valine		0.94 (1)	1.14 (2)
Isoleucine		0.92 (1)	0.84 (1)
Leucine	1.02 (1)		1.68 (2)
Tyrosine		0.60 (1)	0.71 (1)
Phenylalanine	0.97 (1)		0.81 (1)

[a] Hydrolysis of peptides was carried out at 110° with 6 *N* HCl in an evacuated tube for 24 hr.

lysate, which indicated that only the C-terminal phenylalanine residue was tritiated. The radioactive peptide (C-*V : 0.168 μmole) was dissolved in 0.2 ml of 0.1 *M* Tris–HCl buffer at pH 8.0 and digested overnight with 0.005 mg of trypsin at 40°. The digest was lyophilized and subjected to preparative electrophoresis (pH 6.5, 2000 V, 1 hr). A guide strip gave two spots, A (+19.5 cm, ninhydrin: +, Pauly: —) and B (+3 cm, ninhydrin: +, Pauly: +). Radioactivity measurement of spots A and B by liquid scintillation spectrometer showed that only A was radioactive. Peptides corresponding to spots A and B were extracted from the ionogram with 30% acetic acid. The amino acid compositions of peptides A and B (Table I) showed that radioactive peptide A was precisely derived from C-terminal portion of peptide C-V by tryptic cleavage at the Lys–Glu linkage as expected, while peptide B was from the N-terminal portion. The results showed that the tritiated peptide underwent tryptic digestion smoothly and the C-terminal fragment was easy to detect by its radioactivity.

Chymotryptic digestion of Scenedesmus *ferredoxin. Scenedesmus* ferredoxin (0.87 μmole) was tritiated by treatment with ^3H_2O (0.2 ml, 100 mCi), pyridine (0.3 ml) and acetic anhydride (0.05 ml) to yield radioactive *N,O*-acetylferredoxin (0.80 μmole). The tritiated protein (0.1 μmole), was hydrolyzed with HCl and subjected to paper chromatography (butanol:acetic acid:water, 200:30:75). The radioactivity of the ninhydrin-positive spots was measured as described above and only C-terminal phenylalanine was found to be radioactive, coinciding with the result obtained by carboxypeptidase A (9).

Radioactive *N,O*-acetylferredoxin (0.9 μmole) was digested with α-chymotrypsin (0.5 mg) in 0.05 *M* Tris–HCl buffer (pH 8.0) at 40° overnight. After lyophilization, the digest was subjected to two-dimensional paper chromatography (butanol:pyridine:acetic acid:water, 15:10:3:12) and electrophoresis (pH 6.5, 2000 V, 1 hr). The radioactive spot from the map was easily detected by scintillation spectrometric measurement of all 15 spots which were cut out of the peptide map. The spot corresponding to the radioactive peptide (R_f 0.53; +11.0 cm) was cut out of another map and extracted with 30% acetic acid to obtain the radioactive peptide fragment (C). The amino acid composition of peptide C (Table I) agreed well with the theoretical value for the C-terminal peptide which is presumed to be derived by cleavage at the Phe–Val linkage (Sugeno and Matsubara, in preparation).

The C-terminal tritiated peptides were easily obtained by the same method as that used routinely for C-terminal amino acid determination. These peptides smoothly underwent enzymatic digestion and the resulted radioactive peptide fragments could be unambiguously characterized as C-terminal peptides.

Tritiation procedures using acetic anhydride for oxazolone formation result in simultaneous acetylation of hydroxyl groups. This hinders the direct comparison of peptide maps after enzymatic digestion with those of nontritiated peptides, because acetylation changes the behavior of peptide fragments in chromatography or electrophoresis.

Attempts at reversible masking of hydroxyl groups, and use of other oxazolone formation reagents in place of acetic anhydride have given encouraging results that are being investigated.

The authors wish to express their thanks to Dr. T. H. Jukes and Dr. J. E. Casida for their support and interest in this work, to Dr. K. Sugeno for helpful suggestions and discussions, and to Mrs. Dorinne Tsuchiya for skillful technical assistance.

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Received July 1, 1968. P.S.E.B.M., 1968, Vol. 129.

Respiration Rate of Muscle Mitochondria from Genetically Dystrophic Chickens* (33371)

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Muscle mitochondria of genetically dystrophic animals have been observed to be abnormal morphologically (1, 2). Isolated mitochondria from chicken dystrophic muscle show greater efflux of protein during incubation in the absence of respiratory substrate than do similarly treated mitochondria from normal birds (3). There seem to have been few studies of the respiration of mitochondria from dystrophic muscle. Muscle mitochondria of dystrophic mice reportedly show normal oxygen uptake and normal coupling of oxidation and phosphorylation (4). There are descriptions of some myopathies in human subjects in which there is abnormal respiration involving loosely coupled oxidative phosphorylation in the muscle mitochondria (5, 6). In view of the lack of information on the subject the present investigation was conducted to determine if there is any abnormality in the respiration rate or in respiratory decline in mitochondria from dystrophic chicken muscle.

Materials and Methods. Mitochondria were isolated from the pectoral muscle of 11 normal and 11 genetically dystrophic New Hampshire cockerels. Birds of the two strains had been subjected to similar management and dietary practice since hatching and were one year old at the time of the experiment.

Approximately 15 g of muscle from each bird was immersed in ice-chilled 0.15 *M* KCl solution. The muscle was cut into small pieces and rinsed with the KCl solution. The tissue was then homogenized with 10 vol of ice-chilled Tris-KCl medium (7) using a Virtis homogenizer at a setting of 70 for 45 sec. Myofibrils, nuclei, and unbroken cells were removed by two successive centrifugations at 600*g* for 10 min at 0–4°. The final supernatant fluid was then centrifuged at 8500*g* for 10 min at 0–4°. The mitochondria were washed by resuspension in the isolation medium and recentrifuged at 8500*g*. The mitochondrial pellet was rinsed with 0.15 *M* KCl at a concentration of approximately 3 mg of mitochondrial protein per ml. Protein determination on the mitochondrial suspension was by the biuret reaction (8) after clearing with 0.5% sodium deoxycholate.

Oxygen consumption was measured manometrically. Each Warburg vessel contained 0.9 ml of mitochondrial suspension in a total volume of 3 ml. The medium contained, in addition to the suspension, 50 m*M* KCl, 25 m*M* Tris buffer, 0.02 m*M* cytochrome *c*, 10

* Supported by a grant from The Muscular Dystrophy Association of Canada.