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N-Terminal Sequence of Actin*

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ABSTRACT: The N-terminal portion of actin was isolated from a pronase digest by passage through Dowex 50 resin and subsequent fractionation on Dowex 1. This peptide was shown to be acetylated by direct determination of acetic acid by gas chromatography. The smallest residue obtained was acetyl-Asp-Glu, and larger peptides containing threonine and alanine as well were also found. Acetic acid (1 mole) was found in these peptides for each 60,000 g of actin digested, and this accounted for most of the acetic acid found in actin after hydrolysis.

It has been known for about a decade that actin does not contain an N-terminal amino acid that would react with dinitrofluorobenzene (Locker, 1954; Krans *et al.*, 1965; Carsten, 1966). On the other hand, the presence of acetic acid has been reported in the hydrolysate of actin (Krans *et al.*, 1965). These observations suggest that the N-terminal residue of actin is acetylated. We have adapted the technique of gas chromatography for the determination of acetyl groups in the muscle proteins actin, myosin and tropomyosin. Our findings show that actin and tropomyosin contain one acetyl group/mole and confirm the presence of acetyl groups in myosin (Offer, 1965). The details of these investigations will be presented elsewhere. Here we present our experiments which show that the acetyl residue in actin is bound to the N-terminal aspartic acid.

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Experimental Section

New Zealand white rabbits (closed colony since 1930) were used as a source of muscle. Ground muscle

(400 g) was taken to prepare a batch of acetone-dried powder by the method of Feuer *et al.* (1948). This powder was extracted for 4 hr with two changes of 400 ml of ether, and after air drying, it was stored in the freezer until used (not longer than 6 months).

The ether-treated powder was extracted with CO_2-free water at room temperature for 15 min with occasional stirring. The slurry was filtered and the filtrate was made 0.1 N in potassium chloride and 0.006 N in magnesium chloride and was left standing at room temperature for 1 hr and in the refrigerator for another hour before centrifugation of the F-actin gel at 78,000*g*. The F-actin pellets were taken up in 0.6 mM ATP adjusted to pH 7.0, transferred to a dialysis bag, and dialyzed in a cold room (5°) against 2000 ml of 0.3 mM ATP[1] with six changes of the solution every 6–12 hr. The G-actin solution was lypholized and stored in polyethylene boxes in the freezer. G-actin prepared in this manner contains 8–10% tropomyosin and a correction was made for this contaminant in our calculations.

F-actin was prepared by dissolving G-actin in CO_2-free water (usually ^{18}O water used)[2] to which the required amounts of dried potassium and magnesium chloride were added. The pellets obtained after centrifugation were allowed to drain overnight in the refrigerator and then used in the experiments.

Enzymatic Digestion. Actin was digested by Pronase (Calbiochem) at room temperature or at 40° in a water bath. The substrate concentration was 1 mg/ml and the ratio of enzyme:substrate was 1:60 by weight. The pH was adjusted to 8.0 with 15 N NH_4OH, and was found stable during digestion. The suspension of actin and pronase was stirred occasionally at first until a solution was formed.

Isolation of Acetyl Peptides. In a typical experiment, 600 ml of actin digest derived from 10 μmoles of actin was placed directly on a Dowex 50 column (X8, hydrogen cycle, 50–100 mesh, 1 × 10 cm, containing 10 mequiv of resin) without acidification or evaporation. The flow rate was 5 ml/min. The 1200 ml of combined eluate was taken to dryness on a rotary evaporator. The dried material was glassy in appearance and easily dissolved in a small amount of water.

Further purification was achieved using Dowex 1 (X8, formate form, 200–400 mesh, 0.5 × 45 cm). The column was prepared by washing the chloride form of the resin with formic acid until eluate was negative for chloride, and then with water until neutral. The column was then equilibrated in some cases with either formate buffer, or formic acid as specified in the legends. The acidic peptides were applied to the column in about 0.5 ml of water and were eluted using a gradient of formic acid. For this purpose 1.5 N formic acid was run into a mixing chamber of 120-ml capacity which initially contained water (or formic acid in some cases). Fractions (2 ml) were collected, and from these 50- or 100-μl samples were hydrolyzed with 200 μl of 10% sodium hydroxide in tubes with Teflon-lined screw caps at 110° for 2.5 hr. Quantitative determination of the ninhydrin color was carried out on the samples before and after hydrolysis by the method of Moore and Stein (1948), using stannous chloride.

Acetic Acid Determination. Acetic acid was determined by the method of Jackson (1964), using a 20% sebacic acid column ($^3/_{32}$ in. diameter × 6 ft long) at 145° and flame detector. Peptides and proteins were hydrolyzed in constant boiling hydrochloric acid and the hydrolysate was injected directly into the column. The peaks were broad when aqueous solutions were injected, but there were no interfering peaks in our samples, and the retention time for acetic acid was sufficiently long to allow the detector to stabilize after the water vapor passed through. Peptides dissolved in acid without heating were used as controls. Reproducibility was ±10% and recovery of added acetic acid was within this error.

Amino Acid Sequence of the Peptides. C-Terminal amino acids were identified by a modification of the method of Gladner (as quoted by Harris, 1955). Carboxypeptidase A was carefully washed with water several times before dissolving in 10% LiCl. Dipeptides generally are not hydrolyzed by carboxypeptidase, and *N*-acetylated dipeptides are hydrolyzed only slowly. In addition, the attack of peptide bonds involving acidic amino acids is unusually slow. Indeed, we found that the attack of carboxypeptidase on our di- and tripeptides was negligible with the usual amounts of enzyme used, and therefore very large amounts of the enzyme were used for these peptides.

About 0.2 mg of peptide was digested with 1.5 ml of a solution of carboxypeptidase containing 12 mg/ml. The peptide digest and control containing enzyme alone were passed through twin columns of Dowex 1 (X8, hydroxyl form, 50–100 mesh, 0.6 × 20.0 cm) and the columns were washed with water. Hydrochloric acid (6 N) was used to eluate the amino acids from the columns, and the eluates were taken to dryness. The amino acids were separated by paper electrophoresis carried out at pH 2.25 for 3 hr (16 v/cm) (Visakorpi and Puranen, 1958).

Total amino acid content of the peptides was determined by hydrolysis for 18 hr at 110° in sealed vials with constant boiling hydrochloric acid. The evaporated hydrolysates were either analyzed on a Stein–Moore amino acid analyzer or determined semiquantitatively after separating them by electrophoresis at pH 2.25.

Hydrazinolysis was carried out using 0.06 mg of peptide and 1 ml of 95% anhydrous hydrazine (Eastman Kodak). After heating in sealed vials for 10 hr in boiling water, hydrazine was evaporated down in a desiccator over H_2SO_4. The residue was taken up in water, chromatographed with a mixture of pyridine–aniline–water (9:1:4), and sprayed with ammonia–silver nitrate (Akabori *et al.*, 1952), or the material was subjected to

[1] Abbreviations used: ATP, adenosine triphosphate; FDNB, 1-fluoro-2,4-dinitrobenzene.

[2] The use of ^{18}O water has no bearing on the experiments presented here.

paper electrophoresis and the free amino acid detected with ninhydrin.

Electrophoresis. Electrophoresis was carried out using Whatman 3MM paper pressed between glass plates. Voltage was 10–16 v/cm depending on buffer used. The plates were cooled by placing a polyethylene bag of crushed ice on them. The buffers used were mixtures of pyridine–acetic acid–water (pH 6.5, 200, 8, 1800; pH 4.0, 10, 30, 1960; pH 3.2, 15, 250, 1750). A mixture of 0.375 N formic acid–0.05 N acetic acid served for pH 2.25.

Staining Reagents. Ninhydrin stain was prepared using a 0.25% solution in acetone with 2% pyridine added. Electrophoresis strips were dipped into the solution, dried, then steamed and heated in an oven at 110° for 1 min.

A Rydon–Smith test was performed by carefully drying the paper in an oven at 110° for 1 hr, then exposing it to chlorine gas for 10 min in a large polyethylene bag. The paper was then hung in the fume hood for 30 min and sprayed with a solution of 1% potassium iodide and 1% starch (Rydon and Smith, 1952). "Neutral bromocresol green (0.2%) in butanol" was obtained from Mann Research Laboratories in an aerosol can.

Results

The eluates from Dowex 50 after hydrolysis contained chiefly aspartic and glutamic acids and variable amounts of threonine and alanine. An attempt was made to separate these peptides by paper electrophoresis but this was not practical because of difficulties in detection. The smaller peptides were negative to both ninhydrin and chlorine–starch–iodine staining, and could only be detected with bromocresol green spray. The latter reagent is difficult to use on paper after electrophoresis because of high background, and it is rather insensitive. Therefore, the separation of the peptides was carried out on Dowex 1 as described in legends to the figures.

Actin (10 μmoles) digested with pronase yielded mostly dipeptide. The unretained peak (I, Figure 1) was found to contain only traces of aspartic and glutamic acids, alanine, and glycine. The nature of this peak has not been determined. Amino acid analysis carried out on peak II (Figure 1) showed the presence of 1.08 μmoles of aspartic acid, 1.08 μmoles of glutamic acid, and 0.14 μmole of threonine/μmole of actin digested. The peak was found to have a major and minor component on electrophoresis at pH 4.0 and staining with bromocresol green. The mobilities were 11.5 (minor) and 12.3 (major), compared to 6.6 and 11.5 cm for glutamic and aspartic standards. This peak appears to be predominantly a dipeptide with tripeptide contamination.

Larger peptides were obtained by different conditions of pronase digestion. For example, after 3 hr at 40°, tetra- and tripeptides were obtained (Figure 2). Peak II (Figure 2) is a larger peptide containing aspartic acid, glutamic acid, threonine, valine, serine, glycine, and alanine. Peak III (Figure 2) contained no amino acids.

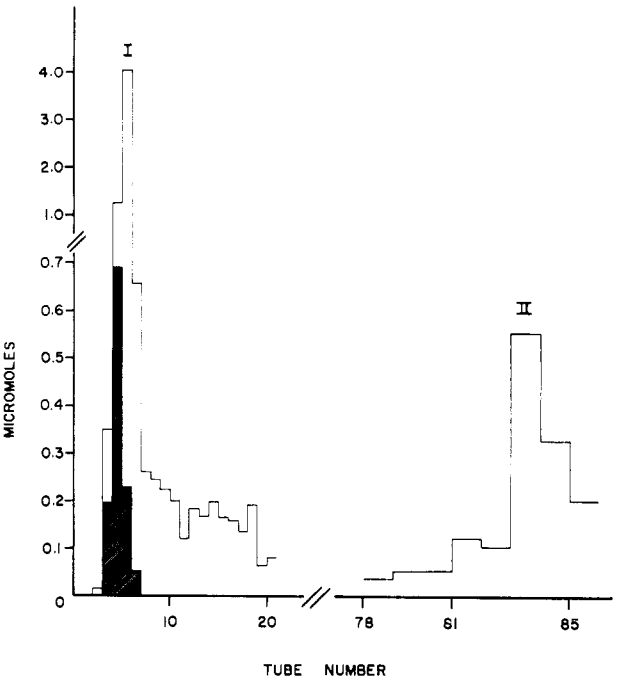

FIGURE 1: Chromatogram of acidic peptides eluted with water from Dowex 50. F-actin was digested 60 hr with pronase at 25°. Pronase (30% more) was added after 36 hr of digestion. Column was equilibrated with and eluted with 0.1 N sodium formate–0.1 N formic acid. Elution with 0.2 N hydrochloric acid started at tube 78. Initially 2-ml samples were collected and changed to 6 ml at tube 60. Ordinate units are micromoles of ninhydrin color (based on glycine standard) per micromole of actin. Shaded areas are ninhydrin color before hydrolysis.

Paper electrophoresis showed that peak IV (Figure 2) contained roughly equal amounts of aspartic acid, glutamic acid, threonine, and alanine, while peak V is similar but without the alanine.

An intermediate period of digestion (10 μmoles of actin for 40 hr at 25°) gives a bigger ratio of tripeptides to dipeptides (see Figure 3). Peak I (Figure 3) contains roughly equal amounts of aspartic acid, glutamic acid, and threonine by electrophoresis. The identity of peak II (Figure 3) is obscure since most of its ninhydrin color was not accounted for by the amino acids found in it. (Aspartic acid was equal to glutamic acid and threonine was about one-third of the aspartic.) Amino acid analysis of peak III (Figure 3) (tubes 26–34) showed 0.23 μmole of glutamic, 0.22 μmole of aspartic acids, and 0.053 μmole of threonine/μmole of actin digested. The threonine comes from tailing of peak I since the middle tube (tube 30) of peak III was pure dipeptide (Asp-Glu) on electrophoresis.

Acetic acid was determined on peak III and tube 30 (Figure 3). In both cases, the molar amount of acetic acid was equal to the glutamic or aspartic acid content. By the same procedure, acid hydrolysates of

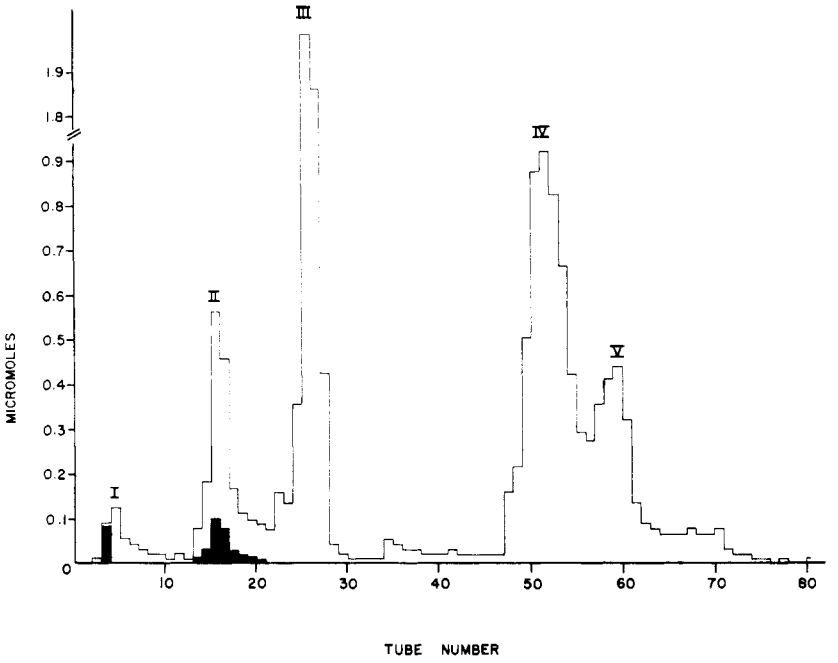

FIGURE 2: Chromatogram of acidic peptides eluted with water from Dowex 50. From a digestion of G-actin with pronase, 3 hr at 40°. Column in formate form was equilibrated with water and developed with a 2 N formic acid gradient (see text). Samples (2 ml) were collected. Ordinate units are micromoles of ninhydrin color per micromole of actin. Shaded areas are ninhydrin color before hydrolysis.

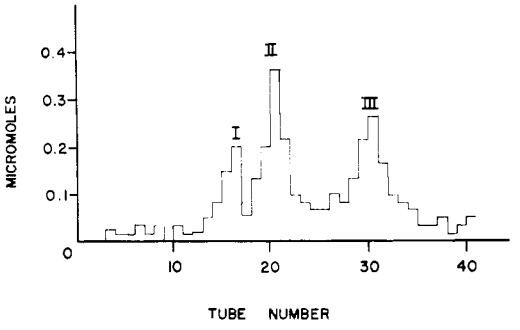

FIGURE 3: Acidic peptides from G-actin digested 48 hr at 25°. The material applied to this column was first eluted from Dowex 50 with water, then applied to Dowex 1 in formate phase and eluted with hydrochloric acid (similar to peak II, Figure 1). Column initially was equilibrated with 1 N formic acid and developed using 2.5 N formic acid gradient (see text). Samples (2 ml) were collected. Ordinate units are micromoles of ninhydrin per micromole of actin.

actin were shown to contain 1.2 μmoles of acetic acid. Thus, about 90% of the acetic acid in actin is accounted for by the acetyl peptides.

Pronase digestion of F-actin for 5 hr at 37° produced predominantly tri- and tetrapeptides. Partial purification of these peptides by column chromatography gave a peptide mixture with the following relative molar amino acid content: 1.00 aspartic acid, 0.98 glutamic acid, 0.94 threonine, and 0.28 alanine. The yield of acetic acid from this peak was 1 μmole/μmole of glutamic or aspartic acid. Carboxypeptidase A digestion of this sample released alanine, threonine, and a small amount of aspartic acid, but no glutamic acid. A control run at the same time showed no alanine and aspartic or glutamic acids, and about one-third of the threonine found in the sample.

Since carboxypeptidase A released aspartic acid from this peptide preparation, the implication was that aspartic acid must be penultimate to the amino terminal. However, a small amount of contaminating peptide that would yield aspartic acid could not be excluded; thus, the appearance of small amounts of aspartic acid could not be taken as the final proof.[3] In order to settle this question, the dipeptide fraction (containing aspartic and glutamic acids, peak III, Figure 3) was subjected to hydrazinolysis. In this procedure, only the C-terminal amino acid is released unmodified. When the hydrolysis mixture was subjected to paper electrophoresis at pH 6.5, the free amino acid in the digest could be identified as glutamic acid. Therefore, it may be concluded that in the dipeptide, glutamic acid is the C-terminal residue and the acetylated aspartic acid is N-terminal.

[3] This conclusion became even more questionable when we learned from Dr. M. Barany (Institute for Muscle Disease, Inc., N. Y.) that he and his co-workers have isolated a tripeptide from actin in which acetylated aspartic acid was the N-terminal residue.

Discussion

The results indicate that actin contains one peptide chain of sequence acetyl-Asp-Glu per 60,000 mol wt, and that the next two amino acids are probably threonine and alanine. When actin is digested under conditions which yield the N-terminal dipeptide, the recovery of acetylated aspartic acid is nearly quantitative, but under conditions which give mainly the N-terminal tetrapeptide, the total yield of acetylaspartic acid in all the recovered peptides is low, and no pentapeptide is detected. This strongly suggests that the next amino acid in the chain is basic since any pentapeptide produced in the digestion would be trapped by the Dowex 50 column. Probably a longer sequence could be elucidated by a similar digestion and fractionation of acetylated actin or FDNB-actin. The amount of acetic acid recovered from the actin digests was 1 mole/actin molecule of 60,000 g. This lends added support to the idea that the minimum molecular weight of actin is in the order of 60,000 g.

Actin prepared by our method contains about 10% tropomyosin, but this is not a likely source of the acetylated peptides since the molecular weight of tropomyosin is close to that of actin and there would have to be about 10 moles of acetyl peptide/mole of tropomyosin to account for our yields. Direct determination of acetic acid in hydrolysates of actin and tropomyosin show that tropomyosin does not contain more acetyl groups than actin.

The possibility that the acetyl group is present in the actin molecule originally as *O*-acetylthreonine cannot be completely excluded, but it seems extremely unlikely in view of the high yields of the acetylated dipeptide and in view of the distances such a transfer would involve. Furthermore, if the acetyl peptide is not the N-terminal fragment, then some other ninhydrin-negative peptide should have been found under one of the various conditions of digestion. Such a peptide might not be recovered if the linkage of the blocking group to the N-terminal were extremely sensitive to lysis by pronase, or if the N-terminal peptide were very resistant to pronase digestion and also contained basic groups causing it to be retained by Dowex 50. We believe the experiments presented in this paper show that actin contains an acetylated N-terminal aspartic acid followed in the chain by glutamic acid, threonine, and alanine.

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