Amino Acid Sequence Studies on Rabbit Skeletal Muscle Actin. Cyanogen Bromide Cleavage of the Protein and Determination of the Sequences of Seven of the Resulting Peptides*

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ABSTRACT: Actin prepared from rabbit skeletal muscle has been cleaved with cyanogen bromide and 13 of the resulting peptides, ranging in size from 3 to 44 amino acid residues, have been purified. Actin contains about 420 amino acid residues, and the 13 peptides isolated account for 261 of these amino acids, including the single 3-methyl histidine, 4 of the 5 cysteines, 3 of the 5 tryptophans, and 6 of the 9 histidines. The complete amino acid sequences of 7 peptides containing a total of 66 residues are described. Three of the peptides are recovered either pure or as a defined mixture after a single quantitative gel filtration of the digest on Sephadex G-50. The yields of these peptides are 1 μmole per 49–51 mg of cyanogen bromide treated actin, indicating a maximum molecular weight per polypeptide chain of 49,000–51,000 daltons. This is consistent with molecular weights previously obtained by others using physical methods. One peptide containing 20 amino acid residues lacks homoserine and is thus identified as the peptide which arises from the carboxyl terminus of actin.

Actin participates in a variety of protein–protein and protein–small molecule interactions which may be functionally significant in muscle contraction. These include the binding of a divalent cation (Ca^{2+} or Mg^{2+}) and a nucleotide (ATP or ADP), actin–actin interaction to form fibrous actin, actin–myosin complex formation, interaction with tropomyosin and troponin, and possibly interactions with other myofibrillar proteins. An understanding of the nature of these interactions may facilitate a more complete understanding of contractile systems.

One approach to the study of these interactions is to determine the effect of chemical alterations of specific amino acid side chains in actin upon its functional properties. The literature on the effects of chemical modification of actin has been reviewed (Kielley, 1964; Gergely, 1966; Young, 1969). In general, numerous studies have shown that polymerization, actin–myosin interaction, and nucleotide binding can be affected by a variety of modifications of the SH groups (see also Lusty and Fasold, 1969), and by oxidation of histidine, tryptophan, and possibly methionine and cysteine residues. Information about the amino acid sequence of a protein is essential in order to identify the residue(s) oxidized or otherwise altered during modification of the protein. The results contained in this report have been obtained in a study directed toward elucidation of the amino acid sequence of rabbit skeletal muscle actin.

Previously, the amino terminus of actin has been shown to be acetyl-Asp-Glu-Thr (Alving and Laki, 1966; Gaetjens and Barany, 1966). Johnson and Perry (1968) have determined the sequences of two sulfhydryl containing tryptic peptides; one is a dipeptide, Cys-Phe, which arises from the carboxyl terminus of actin, and another is a hexapeptide, Cys-Asp-Ile-Asp-Ile-Arg.

During the course of this study the author became aware of an investigation on complementary aspects of this problem (Adelstein and Kuehl, 1970). By mutual agreement among the authors, the results of these two studies are being published concurrently.

Experimental Section

Actin Preparation. Actin was prepared from rabbit back and leg muscles essentially as described by Drabikowski and Gergely (1962). Acetone dried muscle powder was extracted at 2°, and the actin was purified by two polymerization cycles.

Alkylation. Reaction of the actin with iodoacetamide (IA) was carried out following the general procedure described by Crestfield *et al.* (1963). In a typical experiment, to a solution of 190 mg (4 μmoles) of ATP-G-actin in 30 ml of 1 mM Tris, 0.2 mM ATP, pH 8, 20 g of urea (Mann Ultra Pure) was added with magnetic stirring, and the mixture stirred under a stream of nitrogen for 1 hr at 25°. The pH was maintained at 8.0 by addition of 1 M Tris to a concentration of 10 mM. Complete reduction of SH groups was ensured by addition of 500 μl of β-mercaptoethanol. This solution was left under a nitrogen stream for an additional hour. IA (450 mg, approximately 120-fold excess over SH groups) dissolved in water was then added, and the solution was stirred in the dark for 30 min. Glacial acetic acid (5 ml) was added to stop the reaction, and the solution was passed over a 4 × 40 cm column of Sephadex G-25 (Pharmacia, fine beads) equilibrated with 25% acetic acid. The column was maintained in darkness by covering it with aluminum foil. The optical density of the

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effluent was monitored at 280 nm, and the protein peak was pooled and dried by rotary evaporation at 40°.

Cyanogen Bromide Cleavage. The dried protein was dissolved in 70% formic acid to a concentration of 10–50 mg/ml, and treated with a 5-fold excess (by weight, relative to actin) of cyanogen bromide (CNBr) (Eastman Organic Chemicals) for 16 hr at 25°. Amino acid analyses indicated a 96–99% loss of methionine. The reaction mixture was diluted with 10 volumes of water and dried by rotary evaporation at 40°.

Gel Filtration. The CNBr digest of carbamidomethyl-actin (CAM-actin) was chromatographed on a column system composed of two 1.9 × 200 cm columns of Sephadex G-50 fine beads connected in series (King and Norman, 1962). The columns were equilibrated and developed with 25% acetic acid. The sample was applied in a volume of up to 30 ml in 25% acetic acid at a protein concentration of 10–20 mg/ml. Elution was carried out at room temperature at a flow rate of 11 ml/hr (3.9 ml/cm² per hr) and the effluent was monitored at 280 nm and by ninhydrin reaction after alkaline hydrolysis (Hirs, 1967).

Chromatography on Dowex 50-X2. This procedure was carried out essentially as described by Schroeder (1967) for a 60 × 0.9 cm column. The resin, Bio-Rad AG-50W-X2, 200–400 mesh (Bio-Rad, Richmond, Calif.), was equilibrated with pyridine–acetic acid buffer, 0.2 M in pyridine, pH 3.1. The columns were prepared and developed at 37°. Elution was accomplished with a linear or convex gradient between pH 3.1 and pH 5.0 using a total of 200–400 ml of pyridine–acetic acid buffers which were prepared as described by Schroeder (1967). A flow rate of 15–20 ml/hr was maintained using a Milton Roy pump. The effluent was monitored by ninhydrin reaction after alkaline hydrolysis.

Chromatography on Phosphocellulose. Phosphocellulose, Cellex P (Bio-Rad, Richmond, Calif.), was dry sieved through standard U. S. grade screens, and the 100–200 mesh fraction was used. This was washed with NaOH and HCl as described by Bornstein and Piez (1966), and was equilibrated either with 1 mM sodium acetate buffer, pH 3.8, or with 30% acetic acid.

When the sodium acetate buffer system was used a 0.9 × 15 cm column (maintained at 25°) was poured under 20 psi air pressure. The sample was dissolved in 1 mM sodium acetate. The sample volume was not critical, and ranged from 2 to 25 ml. The conductivity of the sample was kept below 500 μmho. Sodium acetate (30 ml of 1 mM), pH 3.8, was pumped through the column. Elution was accomplished by applying a linear salt gradient over a total volume of 200–400 ml, using 100–200 ml of 1 mM sodium acetate, pH 3.8, and an equal volume of the same buffer containing 0.3 M NaCl. The effluent was monitored at 280 and 230 nm. Fractions representing the peptide peaks were pooled, dried by rotary evaporation, and desalted by passage over a 1 × 30 cm column of Sephadex G-25, fine beads, equilibrated with 25% acetic acid.

When the pyridine–acetic acid buffer system was used, the phosphocellulose was preequilibrated with 30% acetic acid. The sample was dissolved in 5–15 ml of 30% acetic acid, and was applied to the column under air pressure. The column was maintained at 37°.

The peptides were eluted using a linear gradient composed of 100–200 ml of 30% acetic acid and an equal volume of pyridine–acetic acid buffer, pH 4. The pH 4.0 buffer was prepared by mixing appropriate amounts of the pH 3.1 and pH 5.0 pyridine–acetic acid buffers.

Chromatography on DEAE-cellulose. Whatman DEAE-cellulose (DE-32) was washed with 0.5 N HCl and 0.5 N NaOH (Peterson and Sober, 1962) and equilibrated with 1 mM Tris, pH 8.0. A 0.9 × 15 cm column was used at 25°. The sample was dissolved in 1 mM Tris, pH 8.0, and applied to the column under 20 psi air pressure. After 30 ml of the same buffer was pumped through, a linear gradient (200 ml of 1 mM Tris, pH 8.0, and 200 ml of 1 mM Tris, pH 8.0, 0.3 M NaCl) was applied. The effluent peaks were detected at 280 nm and 230 nm.

Amino Acid Analysis. Analyses were performed on Phoenix amino acid analyzers using a two-column system. Samples to be analyzed were routinely hydrolyzed in 6 N HCl for 22 hr at 110° in sealed, evacuated test tubes. Analyses for the basic amino acids were performed using a 0.9 × 15 cm column of Phoenix XX8-20-0 resin, developed with a pH 5.35 buffer (0.35 M in sodium) at a buffer flow rate of 60 ml/hr and a temperature of 51.5°. Under these conditions, 3-methylhistidine emerged between histidine and ammonia. Cysteine was measured as cysteic acid following performic acid oxidation carried out as described by Moore (1963), or as carboxymethylcysteine after alkylation of the sulfhydryl groups. Asparagine and glutamine were determined by comparison of amino acid analyses of acid and enzymatic digest of peptides (Elzinga *et al.*, 1968).

Tryptic Digestion. Peptides were dissolved in 0.2 M *N*-ethylmorpholine–acetic acid buffer, pH 8.0, to a concentration of 1 μmole/ml. Trypsin (Worthington, TRTPCK) dissolved (1 mg/ml) in 1 mM HCl was added to give an enzyme:substrate weight ratio of 1:20–50, and digestion was allowed to proceed for 16 hr at 25°. The reaction mixture was then acidified to pH 3 by addition of glacial acetic acid, and dried by rotary evaporation.

Chymotryptic Digestion. The digestion conditions for chymotrypsin (Worthington,CDI) were essentially the same as those for trypsin; the amount of enzyme used and the time of digestion varied with the peptide being digested; the conditions used for specific peptides are given in the text.

Aminopeptidase M Digestion. This enzyme (Rohm & Haas, Gmbh, obtained through Henley & Co., New York) was used for total digestion of peptides as described by Elzinga *et al.* (1968).

Carboxypeptidase A and B Digestions. Carboxypeptidase A (Worthington, COADFP) was dissolved in 0.067 M sodium phosphate, pH 7.5, containing 10% LiCl, to a concentration of 2 mg/ml, while carboxypeptidase B (Worthington, COB-DFP) was dissolved in 0.067 M sodium phosphate, pH 7.5, to a concentration of 1 mg/ml. Digestions were carried out in 0.067 M sodium phosphate buffer using 100 μg of enzyme/μmole of peptide; the times of incubation are given in the text.

Thermolysin Digestion. Peptides CB-5 and CB-7-C-1 were digested with thermolysin using the following reaction conditions. The peptide, 2.5 μmoles, was dissolved in 1 ml of 0.05 M Tris, pH 7.5; 350 μl of a 1 mg/ml aqueous solution of thermolysin (Calbiochem, A grade, containing 65% enzyme protein) was added. This represented an enzyme:substrate weight ratio of approximately 1:15. The solution was incubated for 6 hr at 37°, and the digestion was stopped by lowering the pH to 3 by addition of glacial acetic acid.

Edman Degradation. This procedure was carried out

TABLE I: Amino Acid Composition of Actin.

	This Report[a]	Assumed Number of Residues	CNBr Treated Actin	Asatoor and Armstrong (1967)[b]	Carsten (1963)[b]
Lysine	23.6	24	24.0	22.0	24.3
Histidine	8.76	9	8.77	8.62	8.40
3-Methylhistidine	0.95	1	0.95	1.10	
Arginine	21.4	21	21.3	20.4	20.8
Aspartic acid	39.5	40	38.2	38.4	38.7
Threonine[c]	29.5	30	28.0	28.2	31.2
Serine[c]	24.6	25	25.3	25.9	26.6
Glutamic acid	46.1	46	46.0	44.7	45.2
Proline	20.7	21	20.5	20.4	20.8
Glycine	31.9	32	30.5	29.8	30.8
Alanine	33.6	34	33.1	32.9	33.4
Valine	20.2	20	19.7	21.9	20.6
Methionine	16.4	16–17	0.30	17.2	18.0
Methionine sulfone[d]	15.9				
Isoleucine	28.7	29	28.5	31.4	30.3
Leucine	30.5	31	30.0	29.0	28.5
Tyrosine	17.2	17	16.5	17.2	17.7
Phenylalanine	12.9	13	12.8	13.3	13.0
Carboxymethylcysteine	4.75	5	4.37		
Cysteic acid[c,d]	5.23				
Tryptophan[e]	5	5			4.2
Homoserine[f]			14.7		
Total residues		419–420			

[a] Average of seven preparations. There was no significant difference between analyses of native actin and CAM-actin. [b] For purposes of comparison, the published numbers were normalized to a total of about 420 amino acid residues. [c] Corrections for destruction during hydrolysis: threonine, 0.95; serine, 0.90; cysteic acid, 0.93. [d] Based upon analyses of three performic acid oxidized preparations. [e] Estimated spectrophotometrically. [f] Homoserine plus homoserine lactone

essentially as described by Elzinga *et al.* (1968). Aliquots of peptides containing 0.05–0.1 μmole were pipetted into heavy wall Pyrex test tubes and dried under a stream of nitrogen. The number of tubes used for a particular peptide was equal to the number of steps of sequential degradation to be performed. A pyridine solution (200 μl, 3 parts of twice distilled pyridine and 1 part of water) and 10 μl of phenyl isothiocyanate (Pierce Sequanal Grade, Pierce Chemicals, Rockford, Ill.) were added to each tube. The tubes were flushed with nitrogen and incubated at 40° for 1 hr. The reagents were then removed by evaporation at 40° under a steam of nitrogen. Cyclization was effected by adding 200 μl of anhydrous trifluoroacetic acid (Matheson Coleman and Bell) and incubating the solution at 40° for 20 min. The trifluoroacetic acid was removed by evaporation under a stream of nitrogen. At this point one tube was removed. The residue in this tube was dissolved in 1 ml of 1 N HCl and was extracted three times at 0° with 1 ml of butyl acetate to remove derivatized amino acids and excess reagents. The HCl was removed by evaporation at 40° under a stream of nitrogen; the residue was hydrolyzed with 6 N HCl, and subjected to amino acid analysis. The above cycle was repeated for the remaining tubes until the desired number of steps had been completed.

Results

Amino Acid Composition of Actin. The amino acid composition of the actin used in this study is shown in Table I and is compared with two sets of literature values. There is good agreement within experimental error for all amino acids. The determination of cysteine as the carboxymethyl derivative is difficult because of the ease with which it may be oxidized or otherwise destroyed during acid hydrolysis. The value given for carboxymethylcysteine is the average of analyses of nine different preparations; the range was from 4.25 to 5.19 residues per mole. Performic acid oxidation of native actin followed by amino acid analysis yields a value of 5.23 residues of cysteic acid per mole of protein. If actin has an integral number of half-cysteine residues, these data would suggest that this number is probably five. About 3% of the methionine remained after cyanogen bromide treatment, while the other amino acids were unchanged.

The value of about 1 mole of 3-methylhistidine per 47,000 g is consistent with previous reports (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967).

Purification of Peptides CB-1 through CB-13. Sephadex G-50 gel filtration of a cyanogen bromide digest of 203 mg of

TABLE II: Amino Acid Analyses of Selected Peaks Obtained after Sephadex G-50 Chromatography of 203 mg (4.32 μmoles, Assuming a Mol Wt of 47,000) of CNBr Treated CAM-Actin.

	Peak CB-1[a]	Residues in CB-1	Peak CB-2[a]	Residues in CB-2	Peak CB-3,4[a]	Residues in CB-3 and CB-4
Lysine					0.34 (0.08)	
Histidine						
Arginine					3.04 (0.76)	1
Aspartic acid			0.47 (0.24)		7.90 (1.97)	2
Threonine			4.14 (2.20)	2	4.31 (1.08)	1
Serine			1.81 (0.93)	1	0.61 (0.15)	
Glutamic acid			1.37 (0.70)		4.22 (1.05)	1
Proline					7.56 (1.89)	2
Glycine	4.02 (0.98)	1	3.84 (1.94)	2	4.88 (1.22)	1
Alanine			0.37 (0.19)		7.76 (1.94)	2
Valine	4.25 (1.04)	1			3.59 (0.90)	1
Isoleucine			1.06 (0.54)		4.10 (1.02)	1
Leucine					0.39 (0.09)	
Tyrosine					3.31 (0.83)	1
Phenylalanine					7.83 (1.95)	2
Homoserine[b]	4.09 (1.00)	1	1.89 (0.97)	1	7.99 (1.99)	2
Estimated μmoles/peptide[c]	4.10		1.95		4.01	
μmoles of peptide recovered/47 mg of protein	0.95		0.45		0.93	

[a] Values on the left represent total μmoles in the designated peak; values in parentheses represent the calculated number of residues per mole of peptide(s). [b] Homoserine plus homoserine lactone. [c] Obtained by summing the columns headed "Peak CB-1 ...CB-4" (excluding the values for amino acids not present in the constituent peptides), and dividing this sum by the total number of residues in the peptides that make up the peak.

actin (4.32 μmoles, assuming a molecular weight of 47,000 daltons) is shown in Figure 1. Fractions representing the peaks were pooled as indicated, and amino acid analyses of the peaks from which peptides CB-1 through CB-4 were subsequently isolated are shown in Table II.

The compositions of peptides CB-1 through CB-13 are shown in Table III. (The number assigned to a peptide is not intended to reflect its position in the intact protein. In general, the peptides were numbered in order by size, CB-1 being the smallest.) CB-1 and CB-2 were obtained essentially pure after the single G-50 gel filtration step, although CB-2 was further purified on Dowex 50-X2. CB-3 and CB-4 cochromatographed, as would be expected from their similarity in size, and were resolved on Dowex 50-X2 (see below).

CB-5, CB-6, and CB-7, which contain 12, 14, and 14 residues, respectively, appeared as a double, unresolved peak. This region was pooled and the three peptides were purified on phosphocellulose (see Figure 2). Sequence determinations of peptides CB-1 through CB-7 are described below.

CB-8 and CB-9 also cochromatographed and were resolved using phosphocellulose (see Figure 3). CB-8 contains 16 residues, including one homoserine and one carboxymethylcysteine; CB-9 also contains one carboxymethylcysteine but is devoid of homoserine, indicating that CB-9 must have arisen from the C-terminus of actin.

The peak designated CB-10,12 was chromatographed on DEAE-cellulose, and the two peptides were resolved as shown in Figure 4. CB-12 contains one carboxymethylcysteine among its 42 residues, while CB-10 contains the single 3-methylhistidine residue. The sequences of CB-10, CB-9, and CB-8 have been determined and will be reported in a subsequent publication.

Peptides CB-11 and CB-13 were purified using phosphocellulose, as shown in Figure 5. CB-13 contains one residue of carboxymethylcysteine while CB-11 is characterized by a lack of glycine and serine.

Sequence Determinations of Peptides CB-1 through CB-7. In presenting the results of subtractive Edman degradations, the amino acid assumed to be lost at a given degradation step is indicated by boldface type. Homoserine was assumed to be the carboxyl-terminal amino acid in each peptide in which it was found. The value given for homoserine is actually homoserine plus homoserine lactone. A dash (–) indicates that an amino acid was not determined quantitatively. The sequence data are summarized in Figure 8.

CB-1. The amino acid composition of this peptide is given in Table III. After one step of Edman degradation the compo-

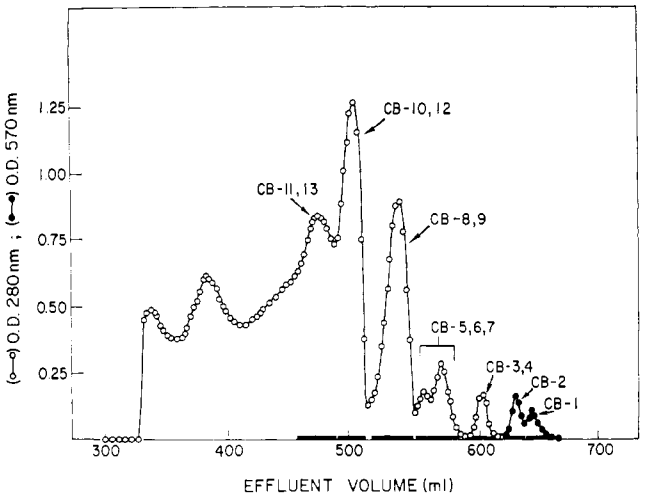

FIGURE 1: Sephadex G-50 chromatography of a CNBr digest of 203 mg of CAM-actin. Peaks were pooled as indicated by the heavy bars.

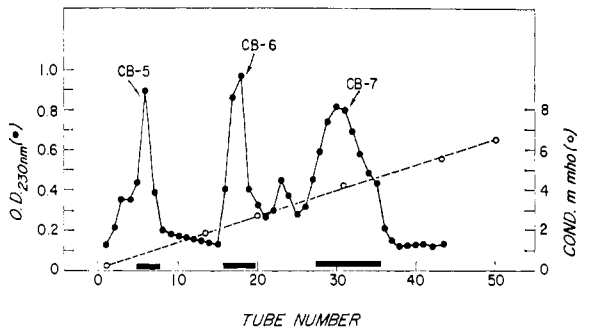

FIGURE 2: Phosphocellulose chromatography of peak CB-5, 6, 7 from the Sephadex G-50 column (Figure 1). The sodium acetate buffer system was used, and 4-ml fractions were collected. The tubes which were pooled are indicated by the heavy bars.

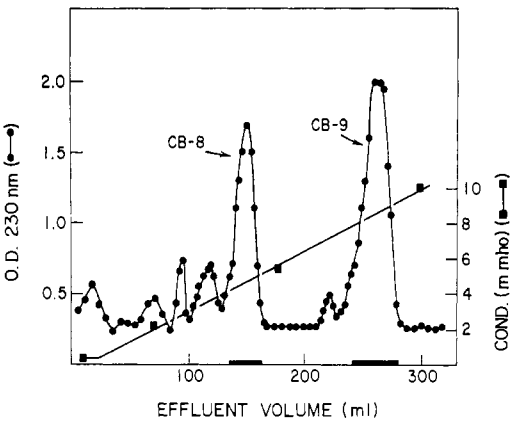

FIGURE 3: Phosphocellulose chromatography of peak CB-8, 9 from the Sephadex G-50 column (Figure 1). The sodium acetate buffer system was used. The fractions pooled are indicated by the heavy bars.

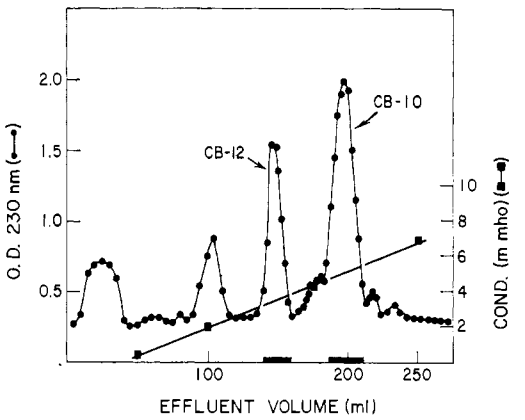

FIGURE 4: DEAE-cellulose chromatography of peak CB-10, 12 from the Sephadex G-50 column (Figure 1). The fractions pooled are indicated by the heavy bars.

sition was: **Val.** 0.14; Gly, 1.00; Hse,[1] 0.77. Thus the sequence is: Val-Gly-Hse.

CB-2. This peptide was purified by chromatography on Dowex 50-X2, and it was eluted at pH 3.5. The amino acid composition of the peptide as purified and after 3 steps of Edman degradation is as follows: peptide: Ser, 0.97; Gly, 2.22; Thr, 1.94; Hse, 0.96; step 1: **Ser, 0.04**; Gly, 2.06; Thr, 1.95; Hse, –; step 2: Ser, 0.12; **Gly, 1.35**; Thr, 2.00; Hse, –; step 3: Ser, 0.17; **Gly, 0.67**; Thr, 2.00 Hse, –. Thus the sequence is Ser-Gly-Gly-Thr-Thr-Hse.

CB-3 and CB-4 were resolved by chromatography on Dowex 50-X2; peptide CB-4 appeared at pH 3.90 (52% yield) and peptide CB-3 at 4.13 (30% yield). The compositions of these peptides are shown in Table III.

CB-3. This peptide was digested with trypsin (enzyme: substrate, 1:20) and the product was chromatographed on Dowex 50-X2. One peptide, designated CB-3-T-1, was eluted at pH 4.30 and recovered in 35% yield. Homoserine was not recovered. Aminopeptidase M digestion revealed the presence of aspartic acid. The analysis of CB-3-T-1 and the composition after each of six steps of Edman degradation are as follows: peptide: Tyr, 0.84; Pro, 0.80; Gly, 1.09; Ile, 0.95; Ala, 1.00; Asp, 0.97; Arg 1.01; step 1: **Tyr, 0.16**; Pro, 0.91; Gly, 1.05; Ile, 0.97; Ala, 1.00; Asp, 1.00; Arg, –; step 2: Tyr, 0.14; **Pro, 0.08**; Gly, 1.08; Ile, 0.94; Ala, 1.03; Asp, 1.00; Arg, –; step 3: Tyr, 0.12; Pro, 0.05; **Gly, 0.52**; Ile, 0.93; Ala, 0.97; Asp, 1.00; Arg, –; step 4: Tyr, –; Pro, 0.09; Gly, 0.48; **Ile, 0.35**; Ala, 0.91; Asp, 1.00; Arg, –; step 5: Tyr, –; Pro, 0.07; Gly, 0.52; Ile, 0.11; **Ala, 0.35**; Asp, 1.00; Arg, –; step 6: Tyr, –; Pro, 0.09; Gly, 0.41; Ile, 0.06; Ala, 0.27; **Asp, 0.54**; Arg, 1.00.

Placing homoserine at the carboxyl terminus, the sequence of CB-3 is Tyr-Pro-Gly-Ile-Ala-Asp-Arg-Hse.

CB-4. CB-4 was digested with chymotrypsin (enzyme: substrate, 1:50; 6 hr) and two peptides (CB-4-C-1 and CB-4-C-2) were obtained after chromatography of the digest on Dowex 50-X2. Peptide CB-4-C-1 was eluted at pH 3.65 and was recovered in 40% yield. The composition of CB-4-C-1 and results after each of three steps of Edman degradation on this peptide were: peptide: Phe, 2.02; Glx, 0.95; Thr, 1.00; step

[1] Unusual abbreviations used are: Hse, homoserine; Asx, aspartic acid *or* asparagine; Glx, glutamic acid *or* glutamine.

TABLE III: Amino Acid Analyses of Cyanogen Bromide Peptides from Actin.[a]

	CB-1	CB-2	CB-3	CB-4	CB-5	CB-6	CB-7	CB-8	CB-9	CB-10	CB-11	CB-12	CB-13
Lysine					1.05 (1)			2.04 (2)	2.01 (2)	3.08 (3)	2.99 (3)	1.00 (1)	1.04 (1)
Histidine						1.04 (1)			0.98 (1)		2.68 (3)		1.02 (1)
3-Methylhistidine										0.84 (1)			
Arginine			1.01 (1)				1.76 (2)	0.98 (1)	1.07 (1)	1.04 (1)	1.86 (2)	2.00 (2)	3.03 (3)
Aspartic acid			0.97 (1)	1.08 (1)		1.06 (1)	2.90 (3)	5.10 (5)	1.01 (1)	4.78 (5)	2.99 (3)	2.01 (2)	5.42 (6)
Threonine		1.94 (2)		0.95 (1)	2.07 (2)	1.84 (2)	1.20 (1)		0.90 (1)	1.87 (2)	2.75 (3)	2.73 (3)	1.78 (2)
Serine		0.97 (1)			1.04 (1)	1.86 (2)			0.84 (1)	1.98 (2)	0.20	5.56 (6)	1.81 (2)
Glutamic acid				1.00 (1)	2.01 (2)	2.00 (2)			3.02 (3)	3.91 (4)	6.63 (6)	5.95 (6)	2.97 (3)
Proline			0.80 (1)	0.84 (1)	0.90 (1)				0.80 (1)	0.90 (1)	4.66 (5)	2.71 (3)	3.16 (3)
Glycine	1.10 (1)	2.22 (2)	1.09 (1)			1.02 (1)	1.18 (1)		0.99 (1)	3.77 (4)	0.25	3.06 (3)	5.63 (6)
Alanine			1.00 (1)	1.17 (1)	2.20 (2)	1.12 (1)	1.07 (1)	1.14 (1)	0.99 (1)	0.89 (1)	3.32 (3)	3.09 (3)	4.80 (5)
Valine	0.98 (1)			1.01 (1)				1.07 (1)	0.76 (1)	1.22 (1)	1.39 (1)	0.80 (1)	4.25 (5)
Isoleucine			0.95 (1)		1.00 (1)	1.83 (2)		1.87 (2)	1.64 (2)	3.84 (4)	1.08 (1)	2.48 (3)	0.87 (1)
Leucine					1.02 (1)		3.70 (4)	1.02 (1)		1.98 (2)	3.62 (4)	2.96 (3)	2.06 (2)
Tyrosine			0.84 (1)			0.87 (1)	0.99 (1)	0.98 (1)	1.04 (1)	1.97 (2)	0.88 (1)	1.02 (1)	
Phenylalanine				2.00 (2)					0.95 (1)		0.84 (1)	3.00 (3)	1.85 (2)
Tryptophan									0.93 (1)[b]	0.90 (1)[b]	(1)[c]		
Carboxymethylcysteine								0.85 (1)	0.83 (1)			0.70 (1)	0.76 (1)
Homoserine[d]	0.94 (1)	0.96 (1)	0.90 (1)	0.90 (1)	0.85 (1)	0.84 (1)	0.79 (1)	1.02 (1)		0.80 (1)	0.83 (1)	0.72 (1)	0.85 (1)
Total residues	3	6	8	9	12	14	14	16	20	35	38	42	44
Yield,[e] %	95	46	52	35	90	54	70	85	89	80	50	52	65

[a] Hydrolysis was for 22 hr at 110° in 6 N HCl. Values of less than 0.2 residue per peptide are not reported. The number in parentheses is the assumed number of residues in the peptide. Details regarding the isolation of these peptides are given in the text. [b] Based upon sequence studies on these peptides. [c] Estimated from extinction coefficient of peptide. [d] Homoserine plus homoserine lactone. [e] Uncorrected, based upon starting CAM-actin.

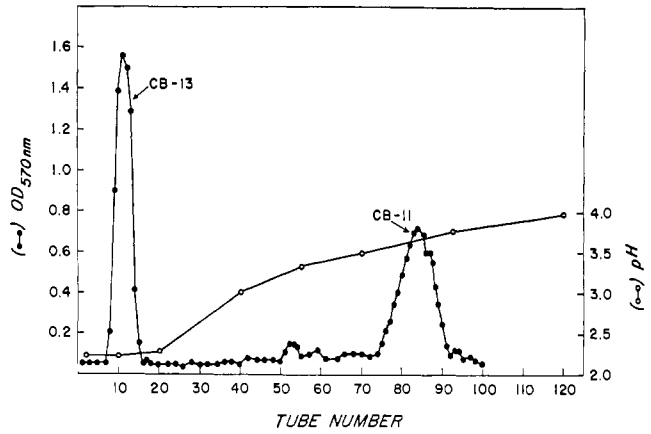

FIGURE 5: Phosphocellulose chromatography of peak CB-11, 13 from the Sephadex G-50 column (Figure 1). The pyridine–acetic acid buffer system was used, and 3-ml fractions were collected. Tubes 9–16 were pooled for CB-13, and tubes 73–90 for CB-11.

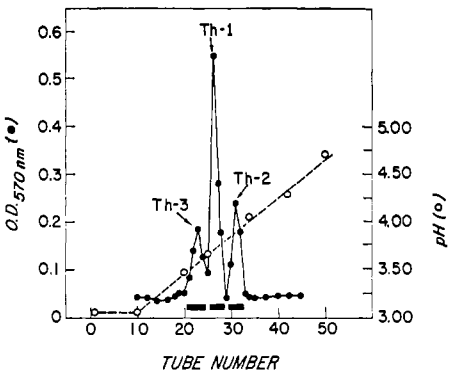

FIGURE 6: Dowex 50-X2 chromatography of a thermolysin digest of CB-5. Fractions (4 ml) were collected, and the tubes pooled for each peak are indicated by the heavy bars.

1: **Phe, 1.07**; Glx, 1.21; Thr, 0.95; step 2: Phe, 0.99; **Glx, 0.27**; Thr, 1.09; step 3: Phe, 1.00; Glx; 0.29; **Thr, 0.27**.

Peptide CB-4-C-2 was eluted at pH 3.20 (yield, 28%). The results of amino acid analyses were as follows: peptide: Asx, 0.94; Val, 1.03; Pro, 0.93; Ala, 1.02; Hse, 0.74; step 1: **Asx, 0.20**; Val, 0.94; Pro, 1.01; Ala, 1.04; Hse, –; step 2: Asx, 0.08; **Val, 0.22**; Pro, 0.98; Ala, 1.00; Hse, –; step 3: Asx, 0.07; Val, 0.21; **Pro, 0.28**; Ala, 1.00; Hse, –. Aminopeptidase M digestion of CB-4 revealed the presence of asparagine and glutamic acid. Peptide CB-4-C-2 was placed at the carboxyl terminus of CB-4 because it contained homoserine. Based upon the above data, the sequence of CB-4 must be Phe-Glu-Thr-Phe-Asn-Val-Pro-Ala-Hse.

CB-5. This peptide proved to be resistant both to Edman degradation and aminopeptidase M digestion, suggesting the presence of pyrrolidonecarboxylic acid at the amino terminus. Tryptic digestion (enzyme:substrate, 1:50) followed by chromatography of the digest on Dowex 50-X2 yielded two peptides, designated CB-5-T-1 and CB-5-T-2. CB-5-T-1 was eluted at pH 3.6 (yield, 45%) and its composition was: Glx, 1.02; Lys, 0.98. The absence of homoserine places this peptide at the amino terminus of CB-5, and from the specificity of trypsin and the suggestion (see above) of pyrrolidonecarboxylic acid at the amino terminus of CB-5, the sequence of CB-5-T-1 was assumed to be pyrrolidonecarboxylic acid–Lys. CB-5-T-2 was eluted at pH 3.25 (yield 53%). Amino acid analysis of the peptide and of six steps of Edman degradation gave as follows: peptide: Glx, 1.00; Ile, 0.94; Thr, 2.12; Ala, 2.05; Leu, 1.12; Pro, 1.00; Ser, 1.12; Hse, 0.97; step 1: **Glx, 0.14**; Ile, 0.93; Thr, 2.01; Ala, 1.97; Leu, 1.00; Pro, 0.97; Ser, 1.05; Hse, –; step 2: Glx, 0.24; **Ile, 0.15**; Thr, 2.01; Ala, 1.94; Leu, 0.94; Pro, 0.96; Ser, 1.05; Hse, –; step 3: Glx, 0.23; Ile, 0.12; **Thr, 1.16**; Ala, 1.87; Leu, 0.89; Pro, 1.06; Ser, 0.92; Hse, –; step 4: Glx, 0.21; Ile, 0.14; Thr, 1.14; **Ala, 1.28**; Leu, 0.89; Pro, 0.95; Ser, 0.90; Hse, –; step 5: Glx, 0.16; Ile, 0.13; Thr, 1.12; Ala, 1.29; **Leu, 0.54**; Pro, 1.02; Ser, 0.98; Hse, –; step 6: Glx, 0.16; Ile, 0.13; Thr, 1.14; **Ala, 0.68**; Leu, 0.35; Pro, 1.07; Ser, 0.89; Hse, –.

Since additional information was required to establish the sequence of this peptide, CB-5 was digested with thermolysin, and the peptides were chromatographed on Dowex 50-X2 (Figure 6). Peptide CB-5-Th-1 (yield 64%) had the composition: Glx, 2.11; Lys, 1.00. Since CB-5 has only one lysine and only two glutamic acids, CB-5-Th-1 must contain the peptide CB-5-T-1. Carboxypeptidase A digestion (2 hr) released only glutamic acid. Combining the data on CB-5-Th-1 and CB-5-T-1, one obtains the sequence pyrrolidonecarboxylic acid-Lys-Glu for CB-Th-1.

CB-5-Th-2 (yield 55%) had the composition Ile, 0.95; Thr, 0.90; Ala, 1.00. This peptide must represent the sequence Ile-Thr-Ala, which is part of CB-5-T-2.

The hexapeptide CB-5-Th-3 (yield 30%) contained homoserine, identifying it as the carboxyl end of CB-5. Amino acid analysis and four steps of Edman degradation on CB-5-Th-3 gave the following results: peptide: Leu, 0.92; Ala, 1.00; Pro, 0.99; Ser, 1.10; Thr, 0.97; Hse, 0.90; step 1: **Leu, 0.17**; Ala, 0.94; Pro, 1.04; Ser, 1.11; Thr, 1.04; Hse, –; step 2: Leu, –; **Ala, 0.42**; Pro, 0.98; Ser, 0.92; Thr, 1.07; Hse, –; step 3: Leu, –; Ala, 0.36; **Pro, 0.45**; Ser, 1.00; Thr, 1.00; Hse, –; step 4: Leu, –; Ala, –; Pro, 0.41; **Ser, 0.44**; Thr, 1.00; Hse, –.

Placing CB-5-Th-1 at the amino terminus of CB-5 (see above), CB-5-Th-2 in the center, and CB-5-Th-3 at the carboxyl end, the following sequence for CB-5 can be deduced: pyrrolidonecarboxylic acid-Lys-Glu-Ile-Thr-Ala-Leu-Ala-Pro-Ser-Thr-Hse.

CB-6. Chymotryptic digestion (enzyme:substrate, 1:50; 6 hr) followed by Dowex 50-X2 chromatography yielded two peptides, designated CB-6-C-1 (pH 3.6; 35% yield) and CB-6-C-2 (pH 3.95; 35% yield).

CB-6-C-1 was analyzed and subjected to three steps of Edman degradation with the following results: peptide: Glx, 2.02; Ser, 1.00; Ala, 1.00; Gly, 1.10; Ile, 0.84; His, 0.95; Thr, 2.02; Tyr, 0.90; step 1: **Glx, 1.08**; Ser, 0.90; Ala, 0.94; Gly, 1.08; Ile, 0.76; His, –; Thr, 1.74; Tyr, 0.74; step 2: Glx, 1.23; **Ser, 0.39**; Ala, 1.07; Gly, 1.36; Ile, 0.89; His, –; Thr, 1.86; Tyr, 1.00; step 3: Glx, 1.17; Ser, 0.44; **Ala, 0.45**; Gly, 1.20; Ile, 0.80; His, –; Thr, 1.77; Tyr, 0.90.

Carboxypeptidase A released tyrosine from CB-6-C-1 after 5 min. CB-6-C-1 was digested with thermolysin and the chromatography of this digest is shown in Figure 7. Peptide CB-6-C-1-Th-1 had the composition Glx, 1.00; Ser, 0.97, and aminopeptidase M digestion indicated the presence of glutamic acid. After one step of Edman degradation glutamic acid was lost (**Glx, 0.09**; Ser, 1.00), indicating the sequence Glu-Ser. Since this sequence is found at the amino terminus

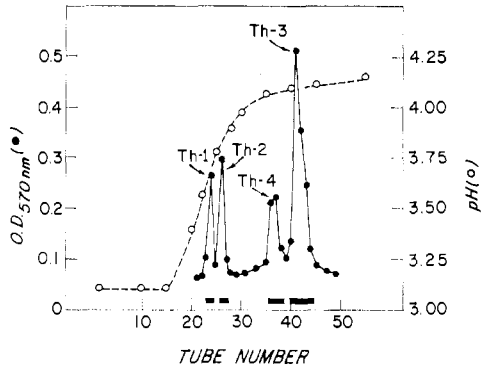

FIGURE 7: Dowex 50-X2 chromatography of a thermolysin digest of CB-6-C-1. Fractions (4 ml) were collected, and the tubes pooled for each peak are indicated by the heavy bars.

of CB-6-C-1, CB-6-C-1-Th-1 is placed at the amino end of CB-6-C-1.

CB-6-C-1-Th-2 had the composition Ala, 0.95; Gly, 1.06. After one step of Edman degradation the composition was **Ala, 0.10**; Gly, 1.00, indicating the sequence Ala-Gly. Since the single alanine is at position 3 in CB-6-Cl, CB-6-C-1-Th-2 is placed adjacent to CB-6-C-1-Th-1 (see Figure 8).

The peptide CB-6-C-1-Th-4 had the composition Thr, 1.03; Tyr, 0.97. Since Tyr was shown to be the carboxyl terminus of CB-6-C-1, the sequence of the peptide was deduced to be Thr-Tyr and it was placed at the carboxyl terminus of CB-6-C-1.

The remaining four residues were found in CB-6-C-1-Th-3. The analyses of this peptide and three steps of Edman degradation were as follows: peptide: Ile, 0.90; His, 0.96; Glx, 1.17; Thr, 1.13; step 1: **Ile, 0.00**; His, 1.06; Glx, 1.00; Thr, 0.88; step 2: Ile, –; **His, 0.10**; Glx, 1.12; Thr, 0.88; step 3: Ile, –; His, –; **Glx, 0.54**; Thr, 0.88. Aminopeptidase M digestion indicated the presence of glutamic acid; thus the sequence of this peptide is Ile-His-Glu-Thr. It is placed between CB-6-C-1-Th-2 and CB-6-C-1-Th-4 by difference, since CB-6-C-1-Th-1, CB-6-C-1-Th-2, and CB-6-C-1-Th-4 were unequivocally placed in position (see above).

The composition and sequence of peptide CB-6-C-2 were determined as follows: peptide: Asx, 1.00; Ser, 1.02; Ile, 0.99; Hse, 0.75; step 1: **Asx, 0.10**; Ser, 0.99; Ile, 1.01; Hse, –; step 2: Asx, 0.12; **Ser, 0.37**; Ile, 1.00; Hse, –; step 3: Asx, 0.12; Ser, 0.31; **Ile, 0.49**; Hse, 1.00. Aminopeptidase M digestion indicated asparagine was present; the sequence of CB-6-C-2 is thus Asn-Ser-Ile-Hse. The presence of homoserine places this peptide at the carboxyl end of CB-6. Based on the above data, the sequence of CB-6 is deduced to be Glu-Ser-Ala-Gly-Ile-His-Glu-Thr-Thr-Tyr-Asn-Ser-Ile-Hse.

CB-7. Tryptic digestion of CB-7 followed by chromatography of the digest on Dowex 50-X2 yielded two peptides. CB-7-T-1 was eluted at pH 4.5 (yield, 40%); the composition and results of five steps of Edman degradation were as follows: peptide: Arg, 1.82; Leu, 2.00; Asx, 0.91; Ala, 1.11; Gly, 1.03; step 1: **Arg, 1.24**; Leu, 2.10; Asx, 1.00; Ala, 0.93; Gly, 0.96; step 2: Arg, 1.18; **Leu, 1.26**; Asx, 0.96; Ala, 1.00; Gly, 1.03; step 3: Arg, –; Leu, 1.13; **Asx, 0.22**; Ala, 0.95; Gly, 1.00; step 4: Arg, –; **Leu, 0.25**; Asx, 0.12; Ala, 1.10; Gly, 0.91; step 5: Arg, 1.15; Leu, 0.32; Asx, 0.07; **Ala, 0.55**; Gly, 1.00.

Since CB-7-T-1 is a tryptic peptide the arginine residue remaining after five steps of Edman degradation was assumed to be at the carboxyl terminus, and the glycine was placed between alanine and arginine. This part of the sequence was confirmed by treatment of CB-7-T-1 with a mixture of carboxypeptidases A and B, for 5 min and 1 hr. Arginine was released first, followed by glycine. Aminopeptidase M digestion revealed the presence of aspartic acid. Combining the above data, the sequence of CB-7-T-1 must be Arg-Leu-Asp-Leu-Ala-Gly-Arg.

Peptide CB-7-T-2 was eluted at pH 3.21 (yield, 35%). The amino acid composition and three steps of Edman degradation were as follows: peptide: Asx, 2.00; Leu, 1.87; Thr, 0.97; Tyr, 0.69; Hse, 0.85; step 1: **Asx, 0.95**; Leu, 2.00; Thr, 0.85; Tyr, 1.09; Hse, –; step 2: Asx, 1.00; **Leu, 1.38**; Thr, 0.86; Tyr, 0.95; Hse, –; step 3: Asx, 1.09; Leu, 1.21; **Thr, 0.36**; Tyr, 1.00; Hse, –. Digestion of this peptide using carboxypeptidase A gave the following results: 1 min: Hse, 0.33; Leu, 0.36; Tyr, 0.13; Asp, 0.07; 5 min: Hse, 1.00; Leu, 1.03; Tyr, 0.97; Asp, 0.12; 35 min: Hse, 0.93; Leu, 0.98; Tyr, 1.02; Asp, 0.63. Total digestion with aminopeptidase M indicated the presence of two residues of aspartic acid. Combining the above results, the sequence of CB-7-T-2 was deduced to be Asp-Leu-Thr-Asp-Tyr-Leu-Hse. The presence of homoserine placed CB-7-T-2 at the carboxyl end of CB-7, and the complete sequence of CB-7 is Arg-Leu-Asp-Leu-Ala-Gly-Arg-Asp-Leu-Thr-Asp-Tyr-Leu-Hse.

Discussion

Since its introduction by Gross and Witkop (1962), the use of cyanogen bromide to cleave polypeptide chains at methionine residues has proven to be a valuable technique in the determination of the primary structures of large proteins (Edelman *et al.*, 1969; Drapeau and Yanofsky, 1967; Nomoto *et al.*, 1969). Actin has an unusually high methionine content and it is apparent from this study that the methionines are rather uniformly distributed throughout the actin polypeptide chain. The 13 peptides described here range in size from 3 to 44 residues and account for about 261 amino acid residues. Since actin contains about 420 residues in all, only about 160 residues remain to be accounted for in the 4, or possibly 5, remaining peptides. This suggests that cleavage of actin with cyanogen bromide yields a mixture of peptides which can be purified and sequenced using conventional methods.

Among the 261 amino acid residues in these 13 peptides are many of the amino acids which are potentially suitable for chemical modification studies in the native protein. Among these residues are 4 of the 5 cysteines, 3 of the 5 tryptophans, 6 of the 9 histidines, as well as the single 3-methylhistidine. The procedure described for the isolation of these peptides may thus be useful in defining the sites of modifications in future experiments directed toward alteration of specific groups in actin.

The CNBr cleavage is essentially quantitative, as shown by a 96–99% loss of methionine; thus, the molar amounts of the peptides formed should be equal to the number of moles of protein which are treated with CNBr. If pure peptides or defined mixtures can be obtained after a single quantitative operation such as gel filtration, the ratio between the weight of starting protein and the moles of peptide released can be ascertained. In the Sephadex G-50 chromatography used here, two of the peptides (CB-1 and CB-2) are recovered essentially pure after a single passage of the digest over the columns,

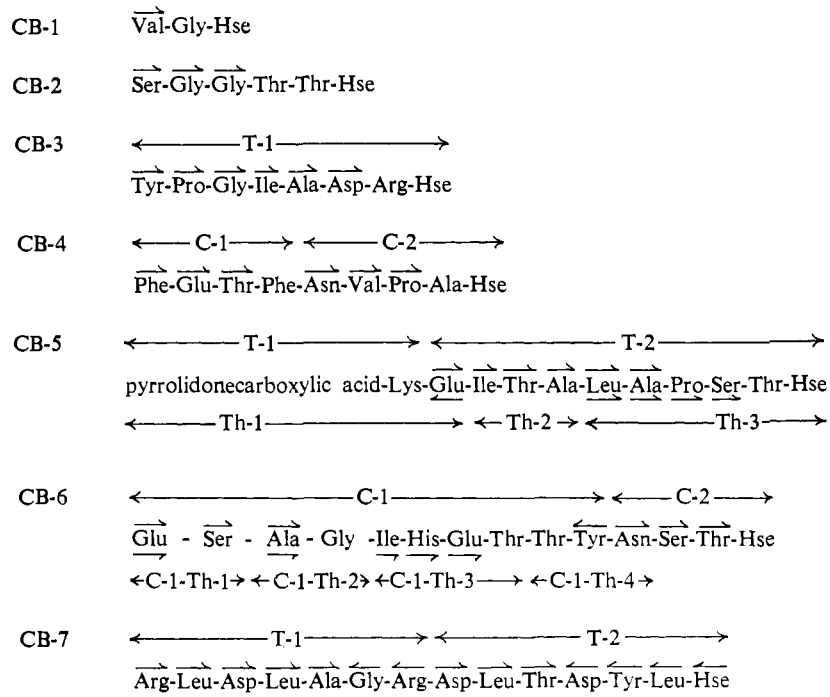

FIGURE 8: Summary of sequence data on peptides CB-1 through CB-7. A half-arrow pointing to the right indicates the residue was placed by Edman degradation; a half-arrow pointing to the left indicates the residue was placed by digestion with carboxypeptidase A or a mixture of carboxypeptidase A and B. Half-arrows above the peptides indicate residues placed in undigested cyanogen bromide peptides, tryptic peptides, or chymotryptic peptides; half-arrows below the peptides indicate residues placed in thermolytic peptides.

while peak CB-3,4 is a mixture of two peptides. The amount of contamination of peak CB-3,4 with other peptides may be estimated from the observed levels of the amino acids, *i.e.*, lysine, histidine, serine, and leucine, which are not present in the purified peptides. As shown in Table II, the amount of contamination appears to be less than 10%. In addition, the calculated number of residues per mole of the CB-3,4 mixture agrees well with the actual number of residues in the two peptides. Based upon the amino acid compositions of these peptides, their recoveries are calculated from the total amino acid analyses of the peaks, and the yields of peptides CB-1, CB-3, and CB-4, are 1 μmole/49–51 mg of protein. Assuming that actin has a single polypeptide chain, its maximum molecular weight is thus approximately 49,000–51,000 g/mole. This is compatible with a lower molecular weight (about 46,000) recently obtained by physical methods (Rees and Young, 1967), but not with earlier estimates of about 60,000 for the molecular weight of actin. The apparent low yield of CB-2 may be due to partial destruction of the peptide during preparation and chromatography of the digest, or possibly incomplete cleavage at one of the methionines.

The seven peptides CB-1 through CB-7, whose sequences are reported here, must all arise from positions other than the ends of the molecule. The amino terminus of actin is acetylaspartate (Alving and Laki, 1966; Gaetjens and Barany, 1966) and this group is not present in any of these peptides. Peptide CB-9 lacks homoserine and must represent the carboxyl terminus of actin.

Determination of the sequences of two oligopeptides, 10 and 12 residues long, was facilitated by digestion of the peptides with the enzyme thermolysin. Since information on the specificity of this enzyme is fragmentary, it may be desirable to review the pattern of digestion (see Figure 8). In peptide CB-5 only two splits were observed, a Gly–Ile bond and an Ala–Leu bond. In CB-6, hydrolysis occurred at a Ser–Ala, a Gly–Ile, and a Thr–Thr bond. All of these cleavages were apparently complete and specific; no overlapping peptides were found. Of the five bonds hydrolyzed, three involve the amino group of leucine or isoleucine. A Ser–Ala was split in CB-6-C-1, but a somewhat similar bond, Thr–Ala, was not split in CB-5. Also, the Thr–Thr bond which was split in CB-6 resembles the Ser–Thr which was not hydrolyzed in CB-5. Undoubtedly neighboring amino acids influence the rate of digestion of a particular bond, and in the case of thermolysin it is likely that the presence of at least two amino acids on each side of the susceptible bond is required. This predicts that free amino acids would not be released by thermolysin. It should be noted that the enzyme:substrate ratio used in the present study (about 1:15) was considerably higher than that used by other workers (Bradshaw, 1969; Ambler and Meadway, 1968; Matsubara *et al.*, 1966). Our results indicate that thermolysin may be used successfully to obtain novel sets of peptides from fragments containing 10–12 amino acid residues.

Recent studies have shown that a variety of intracellular fibers and filaments are assembled from protein units bearing similarities in amino acid composition and physical properties to skeletal muscle actin (reviewed by Adelman *et al.*, 1968). If some of these proteins have evolved from a common precursor there should be similarities in their amino acid sequences. It has been possible to demonstrate homology between bovine carboxypeptidase A and B by comparison

of short segments of the amino acid sequences of these two proteins (Dayhoff, 1969; Bradshaw *et al.*, 1969). Thus, while the sequences of the peptides CB-1 through CB-7 together contain only about 16% of the total residues in actin, they may offer a frame of reference within which the possibility of homologies in amino acid sequence among different actin-like proteins may be experimentally tested.

Sequence studies on the remaining CNBr peptides from actin are in progress; the C-terminal peptide and the 3-methyl histidine containing peptide have been completed (Elzinga, 1969) and will be reported in detail in subsequent publications.

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