Structural Studies on Rabbit Skeletal Actin. I. Isolation and Characterization of the Peptides Produced by Cyanogen Bromide Cleavage*

Robert S. Adelstein and W. Michael Kuehl

ABSTRACT: Seventeen cyanogen bromide peptides, including the carboxyl- and blocked amino-terminal peptides, have been isolated from rabbit skeletal actin. The sum of the amino acid compositions of these peptides accounts for the amino acid composition of the entire molecule. These results are consistent with a single polypeptide chain of molecular weight 45,000 for globular actin. 3-Methylhistidine is present in a single cyanogen bromide peptide. There is no evidence for microheterogeneity of methylation of histidine. Reduction of methionine sulfoxide to methionine was shown to be essential for achieving greater than 99% conversion of methionine into homoserine during the cyanogen bromide reaction.

G-Actin isolated from rabbit skeletal muscle has a molecular weight of approximately 47,000 (Adelstein *et al.*, 1963; Rees and Young, 1967) and is thought to be composed of a single polypeptide chain (Rees and Young, 1967). Previous studies on the primary structure of this protein have reported: (1) the amino acid composition (Carsten, 1963; Laki *et al.*, 1962; Johnson and Perry, 1968); (2) identification of the amino-terminal (Gaetjens and Barany, 1966; Alving and Laki, 1966) and carboxyl-terminal (Johnson and Perry, 1968; Laki and Standaert, 1960) residues; (3) isolation of the amino- and carboxyl-terminal tryptic peptides as well as some of the other half-cystine-containing tryptic peptides (Johnson and Perry, 1968); and (4) isolation of four half-cystine-containing chymotryptic peptides (Lusty and Fasold, 1969). Recently the unusual amino acid 3-methylhistidine was found to be present both in actin (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967) and myosin (Johnson *et al.*, 1967). Moreover, Johnson *et al.* (1967) inferred from a study of the tryptic peptides of actin that one unique histidine appeared to be methylated per 47,600 g of actin.

In this paper the composition of 17 cyanogen bromide peptides accounting for the entire amino acid composition of actin is presented. The amino-terminal and carboxyl-terminal cyanogen bromide peptides as well as the one unique peptide containing 3-methylhistidine are identified.

In the course of these investigations it became known that Dr. M. Elzinga was independently working on complementary aspects of this problem (Elzinga, 1970). Through mutual agreement among the authors it was decided to publish the initial results from both laboratories concurrently.

Experimental Procedure

Preparation of G-Actin. An acetone powder of rabbit back and leg muscles was prepared essentially as described by Straub (1943). The dried powder was treated with 5 volumes of chloroform three times and air dried. G-Actin was extracted from the acetone powder for 30 min at 5° using a solution of 0.5 mM ATP, 0.1 mM $CaCl_2$, and 0.75 mM 2-mercaptoethanol. The actin was then polymerized at room temperature using 0.1 M KCl and 1 mM $CaCl_2$. The F-actin pellet collected by centrifugation was depolymerized by dialysis after it has been suspended in a hand homogenizer. The depolymerized actin was further purified using Sephadex G-200 filtration on a 5 × 80 cm column equilibrated with the extraction solution (Adelstein *et al.*, 1963; Rees and Young, 1967). The sample size varied from 100 to 200 ml and the elution profile was monitored spectrophotometrically at 280 and 290 mμ. This procedure yielded between 300 and 400 mg of actin in the major peak. The actin solution was then lyophilized prior to further use.

Tritiation. In order to provide a convenient method for detection and quantitation of peptides, 5% (about 20–30 mg) of the lyophilized actin was tritiated by Dr. F. H. White, Jr., using the method of White *et al.* (1969). The lyophilized material, after being evacuated in a glass tube, was exposed to an electrical discharge from an externally placed Tesla coil. This procedure creates a population of free radicals so that subsequent addition of tritiated H_2S results in nonexchangeable tritium being bound to C atoms. Exchangeable tritium (attached to O, N, and S atoms) was removed by repeated lyophilization from water. The quantity of nonexchangeable tritium introduced corresponded approximately to 0.5 μCi of tritium/mg of protein. The tritiated actin was then added back to the previously lyophilized actin prior to reduction and carboxymethylation.

Reduction and Alkylation. For reduction and alkylation approximately 10 μmoles of actin was dissolved in 100 ml of 8–10 M urea and the procedure used was that of Craven *et al.* (1965) except that only a 15-fold molar excess of 2-mercaptoethanol was used. [1-^{14}C]Iodoacetamide which had been dried from water on a rotary evaporator was diluted with nonradioactive recrystallized iodoacetamide, and a four–fivefold molar excess of this solution over the 2-mercaptoethanol was used for alkylation. Excess reagents were removed either by exhaustive

* From the Section on Cellular Physiology, Laboratory of Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received October 22, 1969.* A preliminary report of this work was presented at the 13th Annual Meeting of the Biophysical Society, Los Angeles, Calif., Feb 1969, and at the 53rd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1969.

dialysis or by passing the material over Sephadex G-10 equilibrated with 10% formic acid.

Determination of Radioactivity. A Beckman Model LS-250 scintillation counter was used for detection of ^{14}C and ^{3}H using 4% PPO–toluene as a scintillant and 0.5 ml of NCS solubilizer (Amersham/Searle). Necessary corrections were made for simultaneous determination of ^{3}H and ^{14}C.

Cyanogen Bromide Cleavage. The procedure used was essentially that outlined by Steers *et al.* (1965). A 1% solution of actin was prepared by solubilizing lyophilized actin in 100% formic acid and then diluting this solution to 70% formic acid with deionized water. A 50-fold molar excess of CNBr over methionine residues was used and reaction proceeded at room temperature for 20–24 hr.

Gel Filtration of the Cyanogen Bromide Peptides. Sephadex G-50 fine was used for initial fractionation of the peptides. Gel filtration was carried out at either 5° or at room temperature. Columns run at 5° were eluted with 5% formic acid while those at room temperature were eluted with 10, 20, or 35% formic acid and 0.1% 2-mercaptoethanol. Column sizes are indicated under the appropriate figures. In some instances (*e.g.*, see Figure 2) Sephadex fractions isolated at 5° in 5% formic acid were rechromatographed at room temperature in 20 or 35% formic acid.

Separation of Peptides on Paper. Peptide maps were performed essentially as described by Katz *et al.* (1959). Either 1-butanol–pyridine–acetic acid–water (10:15:3:12), or 1-butanol–acetic acid–water (4:1:5), was used for descending chromatography. A pH 3.6 pyridine–acetic acid–water (1:10:289) system was used for electrophoresis. Phenol red and/or a standard amino acid mixture were used as markers. Peptides were located with the ninhydrin, Sakaguchi, Pauly, Ehrlich, and tyrosine reactions as described by Easley (1965). CMCys and methionine containing peptides were identified with the modified iodoplatinate reagent (Bennett, 1967). When peptides were isolated from a two-dimensional map, light staining with 0.025% ninhydrin–acetone at room temperature was used to identify peptides prior to elution. For preparation of larger amounts of purified peptide, 0.2–3.0 μmoles of peptide mixture was applied as a wide band to Whatman No. 3MM paper. Following chromatography or electrophoresis, components were located by staining a guide strip and eluted with acetic or formic acid.

Peptide Ion-Exchange Chromatography. All ion-exchange materials were washed with alternating cycles of deionized water, 2 N NaOH, and 2 N HCl prior to equilibration with the starting buffer. Dowex AG 50W-X2 (200–400 mesh) chromatography was performed essentially as described by Schroeder (1967). Phosphocellulose (Bio-Rad Cellex P, 0.82 mequiv/g and DEAE-cellulose (Bio-Rad Cellex D, 0.43 mequiv/g) chromatography were performed in jacketed columns, *ca.* 1 × 20 cm, at 40–55°, with the use of a pump to maintain constant flow rates. Both volatile buffer systems (*e.g.*, pyridine–acetate) and fixed buffers with linear NaCl gradients were used. In some instances cellulose chromatography was performed in the presence of 8 M deionized urea, in which case column temperatures were kept at 30–35°. In all cases, the final gradient was terminated by a step elution with approximately 3 N HCl, ammonia, or NaOH. Chromatographic columns were monitored by various methods depending on the nature of the eluent. For peptide detection, these methods included removal of aliquots for measurement of ultraviolet absorption (A_{230}, A_{280}, A_{288}), quantitation of ^{3}H and/or ^{14}C, and measurement of A_{570} after an alkaline hydrolysis reaction using a Technicon peptide analyzer. Gradients were followed by removal of aliquots for pH and/or conductivity measurements. When necessary peptides were desalted on columns of Sepahdex G-10. Further details of chromatography will be described in the Results.

Amino Acid Analysis. Samples (usually 0.02–0.10 μmol) for routine analysis were mixed with 1 ml of 6 N HCl[1] in tubes which were flushed three times with nitrogen and evacuated, sealed, and hydrolyzed for 18–22 hr at 106–110°. Following hydrolysis, HCl was removed by rotary evaporation or over NaOH pellets in a vacuum desiccator. The hydrolysates were then taken up in water, alkalinized to pH 11–12 with NaOH (in order to quantitatively convert homoserine lactone into homoserine and also to facilitate removal of ammonia), and again dried. Just prior to application to the analyzer column, the samples were taken up in water and adjusted to *ca.* pH 2.2 with HCl. Amino acid analyses were performed by the method of Spackman *et al.* (1958) on a Spinco Model 120 amino acid analyzer equipped with a 4- to 5-mV resistor for high sensitivity and an Infotronics Model CRA-10A integrator. A G.E. time-sharing computer, utilizing the integrator output, provided quantitation and normalization (to homoserine = 1.00) of each amino acid residue. In addition to an 8-cm column for basic amino acids, a 20-cm column was also used with the pH 5.28 0.35 N sodium citrate buffer system to resolve tryptophan and/or 3-methylhistidine, the latter eluting approximately midway between histidine and ammonia. Half-cystine was determined as CMCys or as cysteic acid following performic acid oxidation (Hirs, 1967). Methionine sulfoxide and tryptophan were determined after a 48–72 hr hydrolysis with 5 N NaOH or 4 N $Ba(OH)_2$ (Noltmann *et al.*, 1962; Ray and Koshland, 1962). Alternatively, tryptophan was determined from ultraviolet spectral studies, but using 5–50% formic acid instead of guanidine as the solvent (Edelhoch, 1967).

Amino-Terminal Residues Determinations. Dansylated amino-terminal residues (Gray, 1967) were determined by polyamide layer chromatography essentially as described by Woods and Wang (1967) except for modification of the solvents (R. Hartley, personal communication).[2]

Enzymic Digestions. Whole actin and cyanogen bromide peptides were incubated in 0.1 M NH_4HCO_3, pH 8.5, with TPCK-trypsin[3] (2% by weight of substrate) at room temperature for 18 hr. CB13 (0.05 μmole) was incubated with 0.01 mg of pronase for 4 hr at room temperature in 0.02 M sodium phosphate, pH 7.5.

Materials. Cellophane dialysis tubing (Visking) was boiled for 15 min in 5% $NaHCO_3$. Urea solutions were prepared with SBR Ultra Pure Biological Grade urea from Schwarz, and passed through a column of AG 501-X8(D) for deionization immediately before use. Iodoacetamide was recrystallized from ethyl acetate and washed with cold anhydrous ether. TPCK-

[1] More recently 6 N HCl–0.15% phenol was used for hydrolysis because of observed hydrolytic losses of tyrosine for peptides eluted from paper (Sanger and Thompson, 1963).

[2] Solvent 1 was ammonia–water (1:100) and solvent 2 was heptane–butanol–99% formic acid (10:10:1).

[3] The following abbreviations are used: HS, homoserine; CM-actin, carboxymethylated actin; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; 3MH, 3-methylhistidine.

trypsin was prepared from Worthington trypsin (Schoellmann and Shaw, 1963). Grade B pronase (*Streptomyces griseus* protease) was obtained from Calbiochemicals. All chemicals were reagent grade unless otherwise specified.

Results

Introduction of ^{3}H and ^{14}C into Actin. Tritiation has been shown not to alter the molecular structure of other proteins (White and Riesz, 1968). The cyanogen bromide peptides of tritium labeled actin, which never constituted more than 5% of the total actin used in any preparation, were found to chromatograph in a manner similar to the nontritiated peptides. Evidence for this can be seen in Figures 1A and B where the tritium profile closely follows the profile obtained using the peptide analyzer. For peptides of 20–40 residues in size the specific activity was approximately 30,000–50,000 cpm/μmole.

Alkylation with [1-^{14}C]iodoacetamide allowed for monitoring and quantitating the CMCys containing peptides. In general, incorporation of ^{14}C paralleled the results obtained by amino acid analysis.

Amino Acid Analysis of Actin. The amino acid composition of carboxymethylated actin before and after cyanogen bromide cleavage is given in Table I. The values are expressed as residues per molecule assuming a molecular weight of 45,000. The column on the far right, expressed in the same manner as the first two, is taken from Johnson and Perry (1968). In general the agreement before and after cyanogen bromide cleavage and with Johnson and Perry is excellent.

As indicated in Table I alkylation with iodoacetamide yielded approximately five residues of CMCys. In contrast performic acid oxidation yielded values for cysteic acid of about six residues.

The average value for methionine obtained prior to cyanogen bromide cleavage (16.9) agreed quite well with the value obtained for homoserine plus residual methionine after cleavage (16.4), and was consistent with 94% conversion of methionine into homoserine. These values would be consistent with either 17 or 18 cyanogen bromide fragments.

Tryptophan was determined by ultraviolet spectroscopy and $Ba(OH)_2$ hydrolysis, and gave values of 4.2 and 4.4 residues, respectively. This suggested that either 4 or 5 unique tryptophan residues would be isolated from actin.

The amino acid analysis agrees with the findings of Johnson *et al.* (1967) that 1 mole of 3-methylhistidine is present per mole of actin.

Cyanogen Bromide Cleavage. The cyanogen bromide reaction was 90–95% complete in 18 hr as judged by amino acid analysis for residual methionine and the appearance of homoserine (see Table I). Neither repetition of the cyanogen bromide reaction for 18 hr nor extension of the time of the initial reaction from 18 to 40 hr appeared to significantly alter the degree of conversion.

Since neither methionine sulfoxide nor methionine sulfone is converted to homoserine by cyanogen bromide (Gross, 1967), it is possible that the presence of these residues accounted for part or all of the residual methionine. Methionine sulfoxide cannot be quantitated following acid hydrolysis but the sulfoxide and the sulfone can be quantitated after alkaline hydrolysis (Ray and Koshland, 1962). Amino acid analysis following alkaline hydrolysis revealed 0.6–0.8 residue of methionine sulfoxide—both before and after the initial cyanogen bromide reaction—but no methionine sulfone (see Table II). Thus essentially all of the residual methionine (as determined by acid hydrolysis) present after the initial

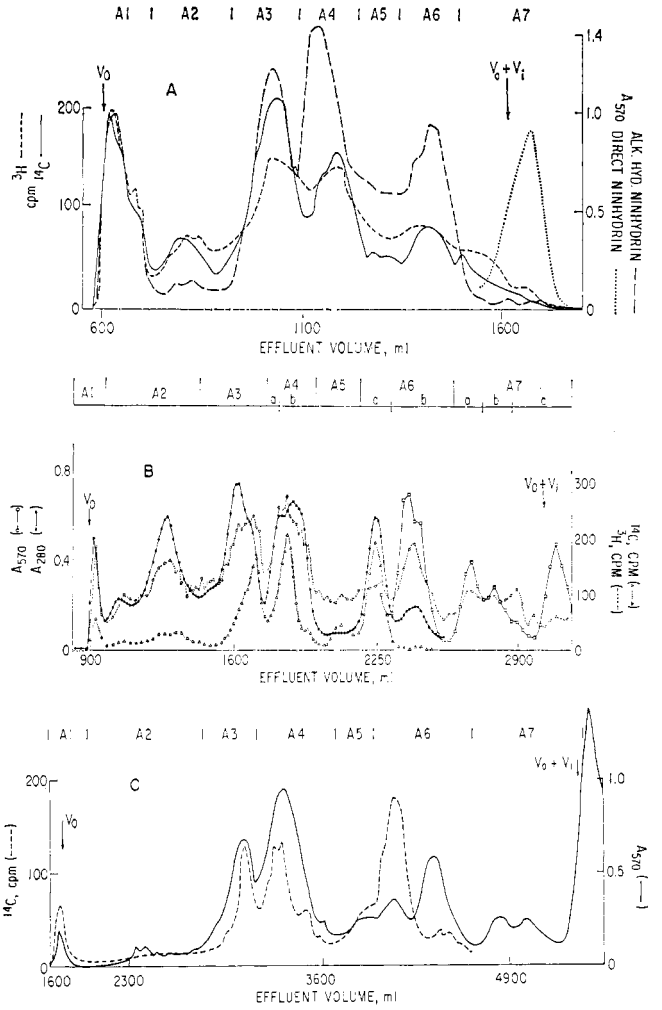

FIGURE 1: Profiles of Sephadex G-50 fine gel filtration of cyanogen bromide peptides. (A) The procedure was carried out in 5% formic acid at 5° utilizing one 5 × 88 cm column. A 396-mg sample was applied in 25 ml of 30% formic acid to a Sephadex column equilibrated with 5% formic acid. The column was eluted with 5% formic acid at a flow rate of 75 ml/hr. Fractions of 10 ml were collected, of which 0.5-ml aliquots were used for ninhydrin reaction before and after alkaline hydrolysis and 0.1-ml aliquots were used for ^{14}C and ^{3}H determination. Fractions were pooled as indicated. (B) The procedure was carried out in 20% formic acid at room temperature utilizing two 5 × 88 cm columns in tandem. A 450-mg sample was applied in 30 ml of 50% formic acid to Sephadex columns equilibrated with 20% formic acid and 0.1% 2-mercaptoethanol. The column was eluted with the latter solvent at a flow rate of 25 ml/hr. Fractions of 13 ml were collected, of which 1-ml aliquots were used for ultraviolet spectroscopy at 280 mμ, 0.5-ml aliquots were used for the peptide analyzer, and 0.05-ml aliquots were used for ^{14}C and ^{3}H determination. (C) The procedure was carried out with CNBr peptides containing less than 0.1 residue of methionine in 20% formic acid at room temperature, utilizing three 5 × 88 cm columns in tandem. A 350-mg sample was applied in 30 ml of 70% formic acid to Sephadex columns equilibrated with 20% formic acid and 0.1% 2-mercaptoethanol. The column was eluted with the latter solvent at a flow rate of 46 ml/hr. Fractions of 12 ml were collected, of which 0.5-ml aliquots were used for the peptide analyzer and 0.05-ml aliquots were used for determination of ^{14}C.

TABLE I: Amino Acid Composition of Carboxymethylated Actin.[a]

Residue[f]	Before[b] CNBr	After[b] CNBr	Johnson and Perry (1968)[c]
Lysine	21.0	20.8	21.8
Histidine	8.1	8.6	8.2
3-Methylhistidine	0.97	1.15	1.06
Arginine	20.7	20.9	19.3
CMCys[d]	5.3	5.1	5.6
Aspartic acid	38.6	38.4	36.4
Threonine	27.9	27.9	27.8
Serine	23.3	23.0	23.8
Glutamic acid	44.5	44.3	45.6
Proline	20.9	21.6	19.8
Glycine	31.5	31.8	30.3
Alanine	32.3	32.8	32.2
Valine	21.8	21.4	21.7
Methionine	16.9	1.0	16.6
Isoleucine	28.6	28.8	30.8
Leucine	29.2	29.2	28.3
Tyrosine	17.0	16.4	16.2
Phenylalanine	13.7	13.7	12.7
Homoserine	0.1	15.4	
Tryptophan[e]	4.3		4.9
NH_3			30.7

[a] Values for amino acids are expressed as residues per molecule, assuming a molecular weight of 45,000. In each case compositions are determined from the actual or corrected recovery of 45,000 g of protein. [b] Average of 5 or more 18–22-hr acid hydrolysates without correction for hydrolytic losses or incomplete hydrolysis. [c] Corrected for hydrolytic losses as described. [d] Includes sum of CMCys + cysteic acid + half-cystine when necessary. [e] Determined from ultraviolet spectrum (4.2) and barium hydroxide hydrolysis (4.4). See text. [f] There was no evidence by amino acid analysis for significant carboxymethylation of residues other than half-cystine.

cyanogen bromide reaction is accounted for by the methionine sulfoxide.

In order to achieve 100% conversion of methionine into homoserine a number of methods for the reduction of methionine sulfoxide to methionine were investigated. 2-Mercaptoethanol (25%) (Jori *et al.*, 1968), 4% mercaptoacetic acid (Hofmann *et al.*, 1966), and 0.1 M dithiothreitol were employed as reducing agents at both room temperature and 45°. 2-Mercaptoethanol (25%) converted 99% of the free methionine sulfoxide into methionine in 0.2 M NH_4HCO_3, pH 7.0, 45° in 22 hr. It converted 91%, when 50% formic acid was used as a solvent, in 42 hr at the same temperature. Neither redistilled mercaptoacetic acid nor dithiothreitol reduced methionine sulfoxide as efficiently as 2-mercaptoethanol under these conditions. Formic acid (50%) was employed as a solvent because some of the larger cyanogen bromide fragments are markedly insoluble except when high concentrations of formic acid are used.

TABLE II: The State of Methionine in Actin and Its Effect on the Cyanogen Bromide Reaction.[a]

	HS	Met	Met Sulfoxide	Total	% Met + Met Sulfoxide
CM-Actin	0	16.0	0.8	16.8	100.0
Post CNBr (1)	15.5	<0.1	0.8	16.3	4.9
Post reduction	14.5	0.7	<0.1	15.2	4.6
Post CNBr (2)	15.5	<0.1	<0.1	15.5	<0.7

[a] The results summarized in this table are expressed in residues per 45,000 g. The cyanogen bromide reactions [CNBr (1) and CNBr (2)] were performed as outlined in the text. Reduction was carried out in 30% 2-mercaptoethanol in 50% formic acid for 48 hr at 37°. Homoserine and methionine were determined after acid hydrolysis, and methionine sulfoxide was determined after alkaline hydrolysis in 5 N NaOH.

Having ascertained the optimal conditions for reduction of methionine sulfoxide, cyanogen bromide fragments containing 0.8 residue of methionine sulfoxide were dissolved in 50% formic acid and reduced in 30% 2-mercaptoethanol at 37° for 48 hr. This procedure quantitatively reduced the methionine sulfoxide to methionine, as judged by amino acid analysis following alkaline hydrolysis (see Table II).

Most of the mercaptoethanol was removed by repeated lyophilization and rotary evaporation and the cyanogen bromide reaction was then repeated.[4] In contrast to repetition of the cyanogen bromide reaction without prior reduction, this time the amount of residual methionine and methionine sulfoxide was reduced to less than 0.1 residue, corresponding to a net conversion of methionine into homoserine in excess of 99%.

Gel Filtration of the Cyanogen Bromide Peptides. Sephadex G-50 fine gel filtration was used for the initial separation of the cyanogen bromide fragments. As shown in Figure 1A, when 5% formic acid was employed as eluent at 5° a substantial amount (23%) of material was eluted in the void volume. In contrast when a similar preparation was eluted in 20% formic acid at room temperature (Figure 1B) markedly less material (5.1%) was eluted in the void volume. This suggested that aggregation was occurring under the former conditions, and advantage was taken of this phenomenon in isolating three peptides which were otherwise difficult to obtain.

An initial fractionation was made in 5% formic acid at 5° and the material eluted in the combined A1 and A2 fractions was rerun at room temperature in 20% formic acid (Figure 2). Whereas P1 through P3 were complex mixtures of a number of previously identified peptides (CB10, CB11, CB13; see Table

[4] Rotary evaporation and lyophilization proved to be inefficient for quantitatively removing residual 2-mercaptoethanol. Gel filtration or dialysis have subsequently proven to be the methods of choice. The presence of large amounts of 2-mercaptoethanol (or its products) apparently leads to destruction of selected amino acids, mainly proline, tyrosine, and lysine, during acid hydrolysis. Once 2-mercaptoethanol was completely removed recovery of these amino acid became quantitative again.

TABLE III: Distribution of 3-Methylhistidine Among the Cyanogen Bromide Fractions.[a]

	A1	A2	A3	A4	A5,6	A7
3-Methyl-histidine	0	1.10	<0.05	4.16	0.21	0
Homoserine	1.6	3.25	13.55	20.5	31.7	32.1
Methionine	0.02	0.14	0.15	0.22	0.02	0

[a] The results are expressed in μmoles of amino acid. The fractions referred to are shown in Figure 1C.

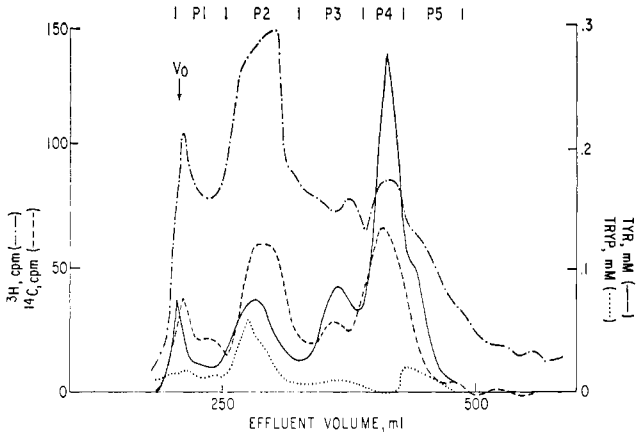

FIGURE 2: Profile of Sephadex G-50 fine gel filtration of cyanogen bromide peptides eluted in fraction Al and A2 at 5° and rerun at room temperature. The equivalent of fraction Al and A2 (from Figure 1A) was applied in 10 ml of 50% formic acid and 0.1% 2-mercaptoethanol to two 2.5 × 88 cm Sephadex columns connected in tandem, and equilibrated with 20% formic acid and 0.1% 2-mercaptoethanol. The columns were eluted with the latter solvent at a rate of 29 ml/hr. Fractions of 4.8 ml were collected, of which 1-ml aliquots were used for determination of tyrosine and tyrptophan by ultraviolet spectroscopy and 0.1-ml aliquots were used for determination of ^{14}C and ^3H.

IV below), P4 clearly contained only two peptides (CB15, CB17) and P5 contained one peptide (CB16). All three of these peptides had been previously isolated, but only with difficulty, using ion exchange chromatography (see section on peptide separation).

The gel filtration experiments shown in Figures 1B and 1C were performed under similar conditions, but the total residual methionine was less than 1% in the latter as opposed to 8% in the former. Except for the quantity of material eluted in fractions A1 and A2 similar profiles were obtained from Sephadex fractionation (see Figures 1A–C). Whereas the quantity of material in fraction A1 appeared to be most dependent on the temperature and/or concentration of formic acid the amount of material in fraction A2 correlated with the quantity of residual methionine found in that fraction.

The large ninhydrin peak present at the salt boundary in Figures 1A–C was obtained from direct ninhydrin determination (Figure 1A) or by using the peptide analyzer (Figures 1B, C), but disappeared when alkaline hydrolysis was performed in open test tubes prior to the ninhydrin reaction. It was attributed to NH_4HCO_3 which was employed as a buffer during reduction and alkylation.

The recovery of material from Sephadex chromatography was monitored by radioactivity which gave total yields in excess of 95% for ^{14}C but slightly less for ^3H. Amino acid analyses of the various fractions, when added together, indicated greater than 95% recovery of the starting material for all amino acids except CMCys which was slightly lower.

Of particular importance was the distribution of residual methionine and of the unusual amino acid 3-methylhistidine among the various fractions. In the experiment in which reduction preceded repetition of the cyanogen bromide reaction, the amount of methionine in each of the fractions was negligible (Table III). In other experiments where the amount of conversion varied between 90 and 95% most of the residual methionine was distributed in fractions A1 and A2.

3-Methylhistidine was present in significant amounts in two clearly separated fractions, A2 and A4, the amount in A2 varying from 15 to 55%. The presence of 3-methylhistidine in fraction A2, which contained no significant residual methionine, demonstrated that its presence in this fraction was not due to failure of conversion of methionine into homoserine (see Table III). The evidence that the 3-methylhistidine in both fractions is derived from the same peptide is as follows: a tryptic digest of CB10, the 3-methylhistidine-containing peptide (see Table IV) isolated from fraction A4, yielded 6 major peptides which accounted for all the amino acids. A tryptic map of fraction A2 yielded the same pattern but with additional spots attributed to other peptides that had also been identified as belonging to non-3-methylhistidine-containing peptides. The presence of the same peptide in two widely separated Sephadex fractions was therefore attributed to aggregation and/or the failure of a methionine derivative (*e.g.*, carboxymethylated methionine or homoserine) to be fully cleaved (see Discussion).

Purification of the Cyanogen Bromide Peptides of Actin. Following the initial fractionation on Sephadex, the various cyanogen bromide fragments were further purified using ion exchange chromatography and peptide mapping. Table IV provides a summary of the amino acid compositions, amino-terminal residues, and minimum overall yields of the purified cyanogen bromide peptides of actin. In addition, identification of the G-50 fine Sephadex fraction(s) (see Figures 1A, B, and C) from which each peptide was purified are included in this table.

CB1–CB4. These peptides occurred in the A7 fraction from G-50 Sephadex in all instances studied. Figure 3 shows a peptide map of the A7 fraction from Figure 1A. Four peptides are well separated. Preparative descending paper chromatography of A7 produced enough of each peptide for determination of its amino acid composition and amino-terminal residue. This result was confirmed by Dowex 50 chromatography of A7 (not shown), which also afforded separation of CB1–CB4 in good yields. In addition the G-50 Sephadex fractionation on a 176-cm column (shown in Figure 1B) directly yielded subfractions of A7. On the basis of amino acid compositions of CB1–CB4 as eluted from paper (see above) and of the subfractions of A7, it was possible to identify and quantitate the peptide content of each subfraction: A7a contained predominantly CB3 and CB4, as well as a small amount of CB2; A7b contained primarily CB2; and

TABLE IV: Amino Acid Compositions of the Cyanogen Bromide Peptides of Actin.[a]

	CB1	CB2	CB3	CB4	CB5	CB6	CB7	CB8	CB9
HS	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0
CMCys[b]								0.7 (1)	0.3 (1)[a]
Asp		0.2	1.0 (1)	1.1 (1)		1.1 (1)	3.2 (3)	4.7 (5)	1.2 (1)
Thr		1.7 (2)		1.0 (1)	1.9 (2)	1.9 (2)	1.0 (1)		1.1 (1)
Ser		0.6 (1)			1.0 (1)	2.0 (2)			1.4 (1)
Glu				1.0 (1)	2.0 (2)	2.1 (2)	0.2 (0)		3.4 (3)
Pro			1.1 (1)	0.9 (1)	1.1 (1)				1.4 (1)
Gly	0.9 (1)	2.0 (2)	1.0 (1)	0.3 (0)	0.2 (0)	1.1 (1)	1.1 (1)		1.0 (1)
Ala			1.0 (1)	1.0 (1)	1.9 (2)	1.0 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Val	0.9 (1)			1.0 (1)				0.9 (1)	0.9 (1)
Ile			1.0 (1)		0.9 (1)	1.7 (2)		1.8 (2)	2.0 (2)
Leu					0.9 (1)		3.8 (4)	0.9 (1)	
Tyr			0.9 (1)			0.7 (1)	1.0 (1)	0.8 (1)	0.9 (1)
Phe				1.7 (2)					1.2 (1)
Lys					0.7 (1)		0.2 (0)	1.9 (2)	2.0 (2)
His						0.7 (1)			1.0 (1)
3MH									
Arg			0.8 (1)				1.7 (2)	0.8 (1)	1.0 (1)
Trp[c]									1.0 (1)[d]
No. of residues	3	6	8	9	12	14	14	16	20
Yield[e] (%)	75	72	79	90	35	44	43	62	43
Sephadex fraction	A7[f,g]	A7[f,g]	A7[f,g]	A7[f,g]	A5–6[f,g]	A5–6[f,g]	A5–6[f,g]	A5–6[f,g]	A5–6[f,g]
N-Terminal residue	Val	Ser	Tyr	Phe	Glx	Glx	Arg	Lys	Trp[m]

	CB10	CB11	CB12	CB13	CB14	CB15	CB16	CB17	CB1–CB17 Total	Total Expected[h]
HS	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	(16)	16.6
CMCys[b]			0.8 (1)	0.8 (1)				0.8 (1)	(5)	5.2
Asp	5.0 (5)	2.9 (3)	2.3 (2)	6.1 (6)	2.8 (3)	3.5 (3)	1.1 (1)	4.1 (4)	(39)	38.5
Thr	2.0 (2)	2.8 (3)	2.7 (3)	1.9 (2)	1.8 (2)	3.3 (3)	1.7 (2)	3.4 (3)	(29)	27.9
Ser	2.0 (2)	0.2 (0)	5.0 (5)	1.8 (2)	1.4 (2)	3.2 (3)	4.2 (4)	1.3 (1)	(24)	23.0
Glu	4.1 (4)	5.5 (6)	6.0 (6)	2.9 (3)	3.6 (3)	2.5 (3)	3.5 (4)	7.0 (7)	(44)	44.4
Pro	1.0 (1)	3.5 (4)	3.0 (3)	2.7 (3)	1.7 (2)	2.2 (2)	2.2 (2)	0.3 (0)	(21)	21.3
Gly	4.0 (4)	0.5 (0)	3.0 (3)	5.8 (6)	2.3 (2)	5.2 (5)	3.0 (3)	1.4 (1)	(31)	31.6
Ala	1.0 (1)	3.0 (3)	3.0 (3)	4.9 (5)	2.5 (3)	5.4 (5)	3.1 (3)	2.6 (3)	(34)	32.5
Val	0.8 (1)	1.0 (1)	0.7 (1)	4.2 (5)[i]	1.6 (2)	4.5 (4)	1.9 (2)	3.0 (3)	(23)	21.6
Ile	3.0 (4)[i]	1.5 (1)	2.7 (3)	0.8 (1)	2.0 (2)	3.6 (4)	4.3 (4)	3.1 (3)	(30)	28.7
Leu	1.9 (2)	3.7 (4)	3.0 (3)	1.8 (2)	2.4 (2)	4.3 (4)	2.9 (3)	4.1 (4)	(30)	29.2
Tyr	1.8 (2)	1.0 (1)	1.0 (1)		0.9 (1)	3.7 (4)	1.7 (1)[j]	2.1 (2)	(17)	16.7
Phe		1.0 (1)	2.6 (3)	1.7 (2)	0.9 (1)	0.2 (0)	1.0 (1)	2.1 (2)	(13)	13.7
Lys	3.1 (3)	3.2 (3)	1.2 (1)	0.9 (1)	1.6 (2)	0.3 (0)	2.0 (2)	3.0 (3)	(20)	20.9
His		2.4 (3)[k]		0.8 (1)	0.7 (1)	2.0 (2)	0.3 (0)	0.2 (0)	(9)	8.3
3MH	0.8 (1)								(1)	1.06
Arg	0.9 (1)	1.8 (2)	2.0 (2)	2.7 (3)	2.0 (2)	1.2 (1)	1.2 (1)	3.1 (3)	(20)	20.8
Trp[c]	0.9 (1)	0.7 (1)					1.1 (1)		(4)	4.3
No. of residues	35	37	41	44	31	44	35	41	(410)	406.3
Yield[e] (%)	60	60	60	53	20	17	21	20		
Sephadex fraction	A4[f,g,l]	A3[f,g,l]	A4[f,g,l]	A3[f,g,l]	A3[g,l]	A1[f] A4[g]	A1[f] A4[g]	A4[g] A1[f,l]		
N-Terminal residue	Gly	Glx	Ala	Blocked	Ala	Tyr	Lys	Lys		

[a] Results of representative analyses are expressed to the nearest tenth of a residue after normalization so that homoserine (or histidine in CB9) equals 1.00; values less than 0.15 residue are omitted. The integral values in parentheses are based on the results shown plus analyses of the purified peptide from other preparations. In the case of CMCys in CB9 the specific activity of the [^{14}C]CMCys group was considered in assigning the value shown in parentheses. [b] Sum of cysteic plus CMCys. [c] From ultraviolet spectral measurements. [d] From acid hydrolysis. [e] Minimum overall yield relative to starting material prior to carboxymethylation. [f] From profile shown in Figure 1A. [g] From profile shown in Figure 1B. [h] Residues per 45,000 g of actin from first two columns of Table I. [i] Values in parentheses are from 96-hr hydrolysates (M. Elzinga, personal communication). [j] CB16 is probably contaminated by CB15 accounting for the high tyrosine value observed. [k] Three unique histidines were isolated from tryptic peptides of CB11. [l] From profile shown in Figure 1C. [m] M. Elzinga, personal communication.

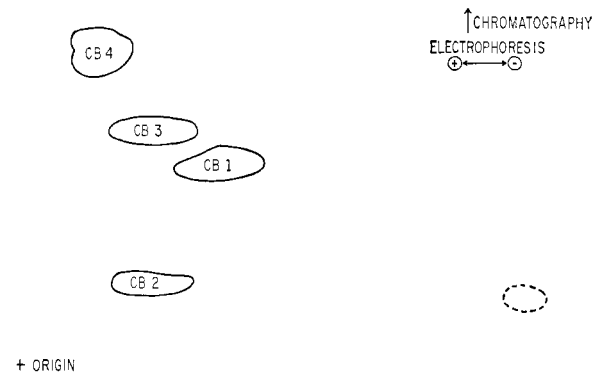

FIGURE 3: Peptide map of G-50 fine Sephadex fraction A7. A portion of A7 (from Figure 1A) containing approximately 0.25 μmole of homoserine was applied at the origin. Descending paper chromatography with the butanol–acetic acid–water solvent was terminated after 16 hr, and followed by electrophoresis at 3000 V for 45 min with pH 3.6 pyridine acetate. Peptides were located by dipping the paper in 0.3% ninhydrin–acetone.

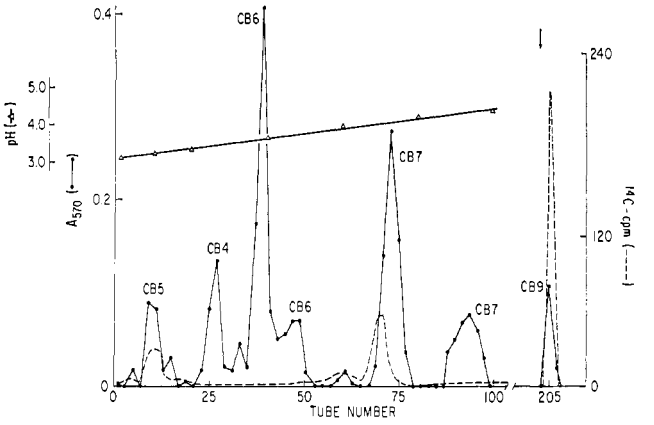

FIGURE 4: Ion-exchange chromatography of G-50 fine Sephadex fraction A6. One-third of A6 (from Figure 1A) at pH 2 was applied to a 0.9 × 60 cm column of AG 50W-X2 equilibrated with starting buffer, *i.e.*, 0.2 M, pH 3.1, pyridine acetate, at 50°. Elution was performed with a gradient of 200 ml of starting buffer *vs.* 400 ml of 2.0 M, pH 5.0, pyridine acetate until tube 200, when the column was stripped with 0.1 M NaOH and 0.1 M NaCl. Fractions of 3.5 ml were collected at a flow rate of 17 ml/hr; 0.5-ml aliquots were removed for the peptide analyzer; and 0.1-ml aliquots for determination of radioactivity. Identification of the peptides is noted adjacent to the peaks (see Table IV).

A7c contained essentially pure CB1. The overall yields for CB1–CB4 shown in Table IV were estimated from the latter chromatographic separation.

CB5–CB9. In all cases these peptides occurred in G-50 Sephadex fractions A5 and A6. Figure 4 shows a Dowex 50 chromatographic separation of G-50 Sephadex fraction A6 (from Figure 1A). As shown, small amounts of CB4 as well as CB5, CB6, CB7, and CB9 were clearly separated. The small ^{14}C peak on the leading shoulder of CB7 was identified by amino acid composition as a small amount of CB8. A Dowex 50 chromatographic fractionation (not shown) of Sephadex fraction A5 (also from Figure 1A) yielded larger amounts of pure CB8 and CB5 in the same positions shown in Figure 4. CB9 (Figure 4) is an example of a peptide which is eluted only with a step up to strong base following the limit 2.0 M, pH 5, pyridine acetate buffer.

With fractionation on a 176-cm column of G-50 Sephadex (see Figure 1B), subfractions of A5 and A6 could be directly resolved as follows: A6b contained CB5, CB6, and CB7 as well as small amounts of CB3 and CB4; A6a contained primarily CB8 and CB9; and A5 contained a small amount of CB9.

Unlike the remaining peptides (31–44 residues), CB1 (3 residues) to CB9 (20 residues) separated on Sephadex G-50 essentially as expected on the basis of size.

CB10 and CB12. These peptides always eluted in the G-50 Sephadex fraction A4, the former being somewhat more retarded although still overlapping the latter. Figure 5 shows separation from A4 (see Figure 1A), of these two peptides, plus small amounts of a peptide mixture (predominantly CB8) eluting between them, on a column of phosphocellulose at 50° with a pH 3.6 acetate–NaCl gradient. In this instance each peptide elutes as an essentially symmetrical binodal peak, although each half of the double peak had an amino acid composition identical with the other half. Since the double peaking occurs both with a carboxymethylated peptide (CB12) and a noncarboxymethylated peptide (CB10), this phenomenon cannot be attributed to variation in the chemical state of the carboxymethylated cysteine. More probably it is due to an equilibrium between the homoserine and homoserine lactone forms of the peptide at the elevated temperature (50°) used with this buffer system. In contrast, a pyridine acetate gradient at 55° (*cf.* Figure 6) applied to this fraction (A4) on phosphocellulose produced the same pattern of elution of peptides CB10 and CB12 but double peaking was not evident. This difference may occur because the peptides were eluted less sharply in the latter system, or, alternatively, the difference in buffer systems may have resulted in a shift of the homoserine–homoserine lactone equilibrium. This difference between the two types of eluents was reproducible and not affected by alkalinization of the fraction prior to chromatography. DEAE-cellulose chromatography also afforded a good separation of these peptides, but with smaller yields.

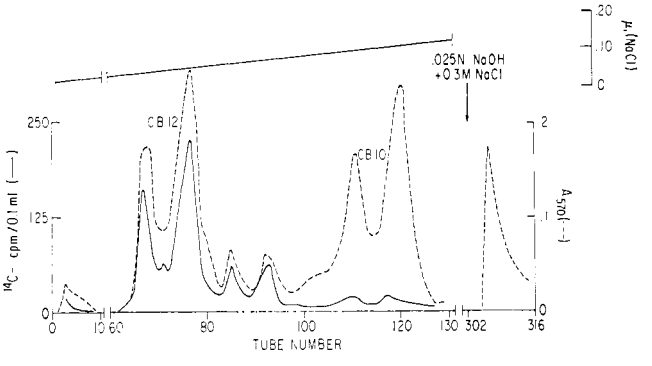

FIGURE 5: Phosphocellulose chromatography of G-50 fine Sephadex fraction A4. One-third of A4 (from Figure 1A) in 10% formic acid was applied to a 0.9 × 22 cm column of phosphocellulose, equilibrated with 0.01 M sodium acetate, pH 3.6, at 50°. Elution was performed with a linear NaCl gradient in 0.01 M sodium acetate, pH 3.6. The arrow indicates a final step elution with 0.25 M NaOH plus 0.3 M NaCl. Fractions of 5 ml were collected at a flow of 0.5 ml/min; 0.5-ml aliquots were used for the peptide analyzer and 0.1-ml aliquots were used for determination of ^{14}C.

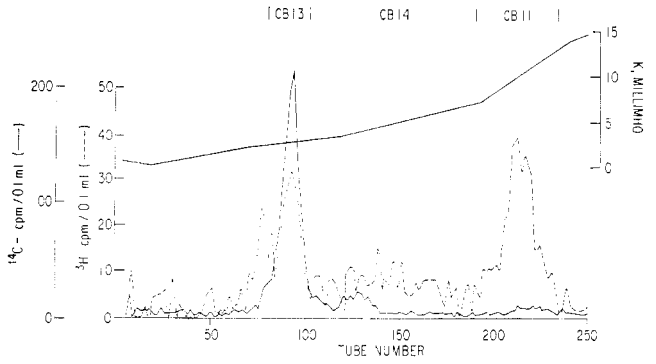

FIGURE 6: Phosphocellulose chromatography of G-50 fine Sephadex fraction A3. One-sixth of A3 (from an experiment comparable to Figure 1B) in 20% formic acid was applied to a 0.9 × 23 cm column of phosphocellulose, equilibrated with 1% acetic acid, at 55°. Elution was performed as follows: (a) tube 1—150 ml of 1% acetic acid *vs.* 150 ml of 0.2 M, pH 3.1, pyridine acetate; (b) tube 82—400 ml of 0.15 M, pH 3.1, *vs.* 400 ml of 0.7 M, pH 5.0, pyridine acetate; (c) tube 183—0.7 M, pH 5.0, pyridine–acetate replaced with 2.0 M, pH 5.0, pyridine acetate; (d) tube 250—a final step with strong base (not shown). Fractions of 3 ml were collected at a flow rate of 0.5 ml/min; 0.1-ml aliquots were used for determination of ^{14}C and ^{3}H.

CB11, 13, and 14. These peptides were always found in G-50 Sephadex fraction A3, and in very small yields in A2. Figure 6 shows a typical fractionation of A3 (from Figure 1B) on phosphocellulose at 55° using a pyridine acetate gradient. Whereas CB11 and CB13 elute as relatively sharp peaks, CB14 elutes between them as a smear and in substantially smaller yields. Indeed CB14 was initially overlooked when the eluted fractions were monitored only with the peptide analyzer rather than by quantitation of tritium. In one instance CB14 eluted as a sharp peak between CB11 and CB13 when a steeper gradient was used but this was not reproducible. Fixed buffer systems with NaCl gradients failed to resolve CB14 from either phospho- or DEAE-cellulose, but CB11 and CB13 were readily identified as discrete components with either type of exchanger.

In order to gain insight into losses of peptide during chromatography, all of the fractions from the chromatographic run shown in Figure 6 were lyophilized and then analyzed for ^{3}H, ^{14}C, and amino acid content. Some of these results are summarized in Table V. ^{3}H, ^{14}C, and most amino acid residues were recovered in 85–97% yields, with the exception of tyrosine. The low yield of this latter residue is probably due to a selective loss of peptides having a relatively high tyrosine content (*e.g.*, CB15 and CB17)—but might also be due to partial destruction of tyrosine during acid hydrolysis. The results shown in Table V suggest that only small amounts of peptides other than CB11, CB13, and CB14 occurred in the A3 fraction, and therefore that no major losses of peptide occurred during chromatography.

CB15–CB17. These peptides were recovered in much smaller yields than CB1-13, and, in addition, varied in position (fractions A1 to A4) on G-50 Sephadex chromatography, depending on the conditions used for elution.

CB15 was purified in about 20% yields from G-50 Sephadex fraction A1 (from Figure 1A) by chromatography on phosphocellulose at 35° using a pH 3.6 acetate–NaCl gradient in the presence of 8 M urea; omission of urea precluded elution of

TABLE V: Recoveries from Phosphocellulose Chromatography of G-50 Sephadex Fraction A3.[a]

	Amount Applied[b]	% Recovered
Isotope		
^{14}C	113,000	95
^{3}H	87,000	97
Amino Acid Residue		
HS	2.5	97
Tyr	3.1	67
Leu	7.6	89
Glu	11.5	86
His	3.3	87

[a] The results summarized here refer to the chromatographic separation shown in Figure 6. Fractions eluting as peaks as well as tubes between peaks were separately pooled and lyophilized, and then aliquots from each pooled fraction were used for quantitation of ^{3}H, ^{14}C, and amino acid content. Results for representative amino acids as well as total ^{14}C and ^{3}H are shown. The middle column shows the amount of material applied to the column whereas the final column lists the total percentage recovered for all fractions eluted. [b] The first two values are given in cpm, all others in μmoles.

CB15 as a discrete component from this fraction. A second method for isolating relatively pure CB15 depended on identification of subfraction A4a (from Figure 1B) which was selected on the basis of a high tyrosine content (determined by monitoring the G-50 Sephadex fractions for A_{280} and A_{288} and then using these data for calculation of the concentrations of tyrosine and tryptophan in each fraction) (Edelhoch, 1967). This tyrosine-rich subfraction was then chromatographed on phosphocellulose at 55° with a pyridine acetate gradient. Following elution of small amounts of CB10 and CB12, CB15 eluted with 2.0 M, pH 5, pyridine acetate as a relatively pure component as judged by fractional content of phenylalanine and lysine (see Table IV). A final method of purification of CB15 was based on an initial isolation of the A1 and A2 fractions from G-50 Sephadex eluted at 5° with 5% formic acid (see Figure 1A). Rechromatography of these combined fractions on G-50 Sephadex at room temperature with a 20% formic acid eluent is shown in Figure 2. P4, selected as a tyrosine rich peak, contained essentially equal amounts (corresponding to about 15% overall yield of each) of CB15 and CB17 as determined by amino acid composition.

CB16 was partially purified in about 10% yields from G-50 Sephadex fraction A1 (Figure 1A) by chromatography on either phosphocellulose or DEAE-cellulose with a fixed buffer–NaCl gradient in the presence of 8 M urea. Better purification with about 20% overall yield was achieved by selecting subfraction A4b (see Figure 1B) just after the tyrosine-rich subfraction (A4a) and rechromatographing this subfraction on phosphocellulose with a pyridine acetate gradient. CB16 eluted with 2.0 M, pH 5, pyridine acetate as did CB15 from the tyrosine-rich fraction (A4a). From its amino

acid composition (Table IV) and pattern of elution, it is likely that CB16 is slightly (about 15–20%) contaminated with CB15. A third method of separation is based on the G-50 Sephadex fractionation shown in Figure 2. Again CB16 (P5), in about 20% yield, trails the tyrosine-rich fraction (P4) but is also recognizable by a tryptophan peak in this instance. The amino acid composition of P5 is consistent with 70–80% purity, the major contaminant being CB15.

CB17 was purified in about 20% overall yield from a combination of G-50 Sephadex fractions A3 and A4 (from Figure 1B) by step elution from phosphocellulose with 3 M ammonia following a pyridine–acetate gradient to remove other peptides. Similar phosphocellulose chromatographic runs of G-50 Sephadex fractions A3, A4, or subfractions from these two fractions (from both Figures 1A and B) also yielded CB17. In these latter cases CB17 was eluted with strong base, as described above for the combined A3 and A4 fractions, but it was usually considerably contaminated by CB15, CB16, and other peptide material. G-50 Sephadex fraction A1 from carboxymethylated actin in which the total residual methionine was less than 0.1 residue (see Figure 1C) was 60–70% pure CB17 and was readily purified to 80–90% purity (judged by amino acid composition) by phosphocellulose chromatography as described above. As noted previously, P4 (from Figure 2) contained approximately equal amounts of CB17 and CB15.

Criteria for purity of CB15–CB17 include qualitative amino-terminal analysis, which yielded a single major dansyl derivative in each case, and amino acid composition: CB15 is distinguished by 3.7 Tyr (the only peptide to contain more than two), 0.2 Phe, 0.3 Lys, and 2.0 His (which clearly distinguished it from CB17 with which it coelutes on Sephadex); CB16 by 1.1 Asp, 0.3 His, 1.1 Trp; and CB17 by 0.8 CMCys, 1.3 Ser, 0.3 Pro, and 0.2 His. Independent evidence that CB15–CB17 have been properly identified is obtained from studies of tryptic methionine overlap peptides,[5] which have yielded fragments consistent with all three cyanogen bromide peptides.

The Carboxyl- and Amino-Terminal Cyanogen Bromide Peptides of Actin. CB9 is the only peptide isolated which does not contain homoserine, and therefore it is concluded that it is the carboxyl-terminal cyanogen bromide peptide of actin. This designation is consistent with the results of Johnson and Perry (1968) who showed that the sequence -CMCys-Phe occurs at the carboxyl terminus of actin.

The NH_2-terminal sequence of actin is known to be *N*-acetyl-Asp-Glu-Thr- from previous studies by Gaetjens and Barany (1966), and also by Alving and Laki (1966). The following evidence demonstrated that CB13 contains the blocked NH_2-terminal tripeptide sequence cited above and is indeed the NH_2-terminal cyanogen bromide peptide of actin. CB13 failed to yield an NH_2-terminal residue by the dansyl NH_2-terminal method. Chromatography of a tryptic digest of CB13 on Dowex 50-X2 produced an 18-residue lysine peptide with a composition identical with that of a tryptic peptide previously isolated from actin (Johnson and Perry, 1968; Martonosi, 1968). Following pronase digestion of this tryptic peptide, the resulting peptide mixture was passed through a column of Dowex 50-X2 (hydrogen cycle) at pH 2, and the ninhydrin-negative material which emerged unretarded was shown to contain only stoichiometric amounts of Asp, Thr, and Glu by acid hydrolysis. It is noteworthy that Johnson and Perry (1968) failed to produce an NH_2-terminal dinitrophenyl derivative from the 18-residue tryptic peptide mentioned above, and therefore suggested that this might be the NH_2-terminal tryptic peptide of actin.

The 3-Methylhistidine Cyanogen Bromide Peptide. CB10 was the only purified cyanogen bromide peptide which contained significant amounts (*i.e.*, greater than 0.1 residue) of 3-methylhistidine. Conversely CB10, as isolated, did not contain significant amounts (*i.e.*, greater than 0.1 residue) of histidine. These results are consistent with a unique position for the single residue of 3-methylhistidine in actin.

Discussion

The purpose of the present investigation was to identify all of the peptides produced by cyanogen bromide cleavage of actin. Using gel filtration with formic acid as solvent CB1–CB13 were readily fractionated and subsequently purified with ion-exchange chromatography and peptide mapping. Peptides CB14–CB17 were considerably more difficult to purify.

Two major problems that had to be circumvented were the tendency of some of the peptides to aggregate under the conditions used for gel filtration and the possible failure of cyanogen bromide to cleave quantitatively at a particular methionine residue.

Aggregation was noted when Sephadex fractionation was performed at 5° in 5% formic acid (see Figure 1A). Peptides CB15–CB17, which had previously been difficult to isolate, were fractionated by taking advantage of their tendency to specifically aggregate at low temperature and low formic acid concentration (Figure 1A) and then disaggregate at higher temperature and formic acid concentration (Figure 2).

Since actin contains 16 or 17 methionines, and the extent of conversion of methionine into homoserine varied from 90 to 95% as determined by residual methionine, it is conceivable that resistance of particular methionine residues to cyanogen bromide could result in low yields of one or more peptides. Amino acid analyses after *alkaline* hydrolysis had revealed that essentially all the residual methionine was present as methionine sulfoxide. By reducing the sulfoxide form to methionine, it was possible to achieve greater than 99% conversion of methionine into homoserine.

Although reduction was originally performed using 50% formic acid as the solvent (since some of the cyanogen bromide peptides may be insoluble with lesser concentrations of formic acid), subsequently it has been performed with equal success under the conditions described but using a solvent of dilute Tris buffer at pH 8.5. In the latter case reduction of actin was performed after alkylation but prior to any exposure to cyanogen bromide, since intact CM-actin (unlike the cyanogen bromide peptides of CM-actin), is soluble at pH 8.5. Use of a slightly alkaline pH for reduction should minimize deamidation and peptide hydrolysis, both of which might reasonably be expected when reduction is performed in 50% formic acid at 37° for 48 hr. Using these milder conditions for reduction this method should have general applica-

[5] At present 13 methionine overlaps have been determined from a tryptic digest of actin. These tryptic overlap peptides are consistent with tryptic peptides isolated from the cyanogen bromide peptides presented in Table IV.

tion for other proteins that fail to approach 100% conversion of methionine into homoserine due to the presence of methionine sulfoxide.

Other causes of noncleavage have been documented. These include alkylation of methionine by iodoacetamide (Gross, 1967), and the occurrence of the sequences: -Met-Thr- (Schroeder *et al.*, 1969) and -Met-Ser- (Narita and Titani, 1968), which have been associated in some instances with conversion of methionine into homoserine without subsequent cleavage. In regard to the former, only very small quantities, accounting for less than 0.2 residue of carboxymethylated homocysteine plus homoserine, were noted on amino acid analysis of CM-actin prior to the cyanogen bromide reaction. Nonetheless, it should be noted that accurate quantitation of carboxymethylmethionine by acid hydrolysis is difficult (Gundlach *et al.*, 1959).

The possible failure of -Met-Thr- and -Met-Ser- sequences to cleave appears to be unlikely in that none of the peptides isolated in low yields have a Ser or Thr NH_2-terminal residue (CB2, the only peptide which has a Ser or Thr NH_2-terminal, was recovered in 72% yield; see Table IV).

Evidence that these seventeen peptides account for all the cyanogen bromide peptides is as follows: (1) the amino acid composition of CM-actin is in agreement with the composition of the sum of the cyanogen bromide peptides; (2) the sum of the molecular weights of the 17 cyanogen bromide peptides agrees with the molecular weight of actin, *ca.* 45,000 (Adelstein *et al.*, 1963; Rees and Young, 1967). It is possible that a peptide could have been overlooked but work in progress[5] on the ordering of the cyanogen bromide peptides will resolve this issue.

The number of cysteines in actin is thought to be either five or six (Rees and Young, 1967; Carsten, 1963; Johnson and Perry, 1968; Lusty and Fasold, 1969). Five unique peptides, with similar specific activities and accounting for all of the ^{14}C introduced during alkylation, have been isolated from actin. Included in these five CMCys peptides are the carboxyl-terminal and the amino-terminal cyanogen bromide peptides. Martonosi (1968) had previously isolated a tryptic peptide containing cysteine which readily reacted with *N*-ethylmaleimide without altering the ability of actin to polymerize. By comparing its amino acid composition with the 18-residue tryptic peptide described in the Results, it is quite clear that this tryptic peptide could have come only from the NH_2-terminal cyanogen bromide peptide.

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