Chemical Studies on the Cysteine and Terminal Peptides in Tryptic Digests of Actin

By P. JOHNSON AND S. V. PERRY

Department of Biochemistry, University of Birmingham

(Received 17 June 1968)

1. On exhaustive digestion of carboxymethylated actin in 6M-urea solutions with carboxypeptidase A, 1 mole of phenylalanine was liberated/43 000 g. of protein. At a lower urea concentration and in the absence of urea, carboxymethyl-cysteine (CMCys) was also liberated. 2. Three cysteine-containing peptides were identified by the study of peptide 'maps' of tryptic digests of actin treated with thiol reagents. 3. The three peptides, each containing one residue of CMCys, were isolated from tryptic digests of carboxymethylated actin by ion-exchange chromatography. 4. One of these peptides was possibly the *N*-terminal peptide and contained about 17–18 residues; another was CMCys-Asp-Ile-Asp-Ile-Arg; the other, CMCys-Phe, was the *C*-terminal tryptic peptide. 5. The chemical evidence suggests that the actin molecule consists of a single polypeptide chain of molecular weight about 44 000.

It is now well established that native actin possesses intact thiol groups that differ in reactivity with thiol reagents. On the basis of such studies different thiol groups have been postulated as involved in the polymerization of G-actin (Kuschinsky & Turba, 1951; Barany, 1956; Drabikowski & Gergely, 1963; cf. Poglazov & Baev, 1961), nucleotide binding (Martonosi & Gouvea, 1961; Strohmman & Samorodin, 1962; Tonomura & Yoshimura, 1961; Katz & Mommaerts, 1962; Katz, 1963) and myosin combination (Perry & Cotterill, 1964; Bailin & Barany, 1967). Analytical data from several Laboratories indicate that there are approx. 6.7 moles of cysteine residues/60 000 g. of actin, but the precise number per mole has been difficult to determine because of ambiguities about the molecular weight of actin, though recent chemical and physical investigations suggest that it may be close to 45 000 (Johnson, Harris & Perry, 1967*a,b*; Asatoor & Armstrong, 1967; Rees & Young, 1967). Studies on isolated radioactively labelled peptides from actin treated with ^{14}C -labelled thiol reagents have not resolved this problem, for Martonosi (1968) reported evidence for eight different cysteine-containing peptides whereas Carsten (1966) could identify only five cysteine-containing peptides per molecule, and both workers suggested that the actin molecule consists of two non-identical polypeptide chains.

The present study was undertaken to define more precisely the sub-unit structure of the actin molecule, with particular reference to the regions

containing cysteine and their role in the biological activity of the molecule. This paper, which is confined largely to the structural aspects, provides evidence that the actin molecule consists of a single polypeptide chain of molecular weight about 44 000. In addition, three cysteine-containing peptides, one of which is the *C*-terminal and another is possibly the *N*-terminal peptide, were isolated from tryptic digests, and the sequences of two of these peptides were determined.

Some aspects of this work were briefly reported by Johnson *et al.* (1967*a*).

MATERIALS AND METHODS

Materials. Carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) and di-isopropyl phosphorofluoridate were supplied by the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Carboxypeptidase B was obtained from the Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and trypsin (twice recrystallized, freeze-dried, salt-free) from Seravac Laboratories (Maidenhead, Berks.).

Protein preparations. The preparation of actin and its carboxymethylated and aminoethylated derivatives were described by Johnson *et al.* (1967*b*).

Enzymic digestion. For the *C*-terminal amino acid determinations the suspension of di-isopropyl phosphorofluoridate-treated carboxypeptidase A (50 mg./ml.) was solubilized immediately before digestion by the addition of 1 vol. of 1% (w/v) NaHCO_3 and 1.5 vol. of 0.1 N-NaOH at 0°. Immediately before use the carboxypeptidase B (10 mg./ml.) was incubated at 0° for 1 hr. with 0.3 vol. of 0.1 M-tris-HCl buffer, pH 7.6, and 0.7 vol. of 0.1 M-di-isopropyl phosphorofluoridate in anhydrous propan-2-ol to inhibit contaminating

chymotrypsin. Suitable samples of the enzyme solutions were added to solutions (6–8 mg./ml.) of the appropriate actin derivatives, and samples containing about 6 mg. of actin were removed from these solutions at selected time-intervals and pipetted into equal volumes of 15% (w/v) trichloroacetic acid containing 0.1 μ mole of norleucine. After centrifugation to remove the precipitated protein, the supernatants were extracted six times with equal volumes of ether, dried by rotary evaporation and then redissolved in 1 ml. of 0.1N-HCl for analysis. Control experiments were also performed in which suitable samples of actin and the carboxypeptidases were analysed for free amino acids.

Tryptic digestion and peptide 'mapping' of carboxymethylated actin were performed as described by Johnson *et al.* (1967b). In some experiments, carboxymethylated actin that had been incubated with carboxypeptidase A for 3 hr. at 25° in 50 mM-potassium phosphate buffer, pH 7.8, containing 6 M-urea, was precipitated in 7.5% (w/v) trichloroacetic acid, redissolved after centrifugation and then digested with trypsin.

Amino acid analysis. In the C-terminal amino acid studies, solutions were analysed for free amino acids by the standard 20 hr. Technicon autoanalyser system or by the extended basic amino acid column of the Beckman 120B amino acid analyser (Johnson *et al.* 1967b). All results were corrected for losses during mechanical transfer by the determination of norleucine added as an internal standard.

Duplicate samples of protein were hydrolysed for 24, 48, 72 and 96 hr. at 110° *in vacuo* to correct for mechanical and hydrolytic losses according to the methods of Bargetzi, Sampath Kumar, Cox, Walsh & Neurath (1963). Single samples of peptides were hydrolysed for 29, 48 and 60 hr. at 110° *in vacuo* and corrections for destructive losses of amino acids were made. All samples were dried by rotary evaporation after hydrolysis, and the protein hydrolysates were analysed on the Beckman 120B analyser and the peptide hydrolysates on the Technicon autoanalyser.

Tryptophan was estimated by the method of Spies & Chambers (1948, 1949), as modified by Harrison & Hofmann (1961).

Hydrazinolysis. Hydrazinolysis of carboxymethylated actin was performed by the method of Bradbury (1956). After extraction of the amino acid hydrazides, the aqueous solutions were dried by rotary evaporation, redissolved in citrate buffer (0.2N with respect to Na⁺), pH 2.2, and analysed on the Beckman amino acid analyser.

Dinitrophenylation. Dinitrophenylation and hydrolysis of the ninhydrin-negative peptide were performed by the methods of Sanger & Thompson (1953) and chromatography of the ether-soluble fractions was carried out on Whatman 3MM paper in the 'toluene' [toluene-pyridine-ethylene monochlorhydrin-0.8N-NH₃ (5:1:3:3, by vol.) prepared as described by Biserte & Osteux (1951)] and in the phenol-3-methylbutan-1-ol-water (1:1:1, by vol.) solvent system of Biserte & Osteux (1951).

The acid-soluble fractions were chromatographed in the butan-1-ol-acetic acid-water (4:1:5, by vol.) solvent system of Sanger & Tuppy (1951), and electrophoresis on paper at pH 6.5 was carried out as described by Johnson *et al.* (1967b).

ϵ -DNP-lysine was prepared by the method of Porter & Sanger (1948).

Amino acid sequence determination. The Edman degradation method of Konigsberg & Hill (1962) as modified by Smyth, Stein & Moore (1963) was used. The subtractive

method of analysis was preferred to identification of phenylthiohydantoin derivatives to deduce amino acid sequences. Amino acid analyses on approx. 0.1 μ mole of peptide were performed on the Technicon autoanalyser. Samples were hydrolysed at 110° for 20 hr. *in vacuo*, and no corrections were made for incomplete hydrolysis.

Protein determinations. Accurate determinations of the protein contents of actin solutions were made by the method of Strauch (1965), which involved nesslerization after ashing. Where necessary, urea was removed from the protein solutions by dialysis against 4000 vol. of 50 mM-potassium phosphate buffer, pH 9.0, with three changes of the external solution. Control determinations showed the absence of detectable nitrogen from the final diffusates.

RESULTS

C-Terminal amino acid determinations

To study the progress of the release of the C-terminal amino acids by carboxypeptidase A, digestion of carboxymethylated actin was performed in the absence and presence of urea. Under these conditions phenylalanine was the main amino acid released and the amounts obtained did not increase very much after 30 min. incubation, though digestion was continued for several hours. The only other amino acid liberated in detectable amounts was CMCys;* this was released in smaller molar amounts than phenylalanine (Fig. 1). The amounts of isoleucine and serine present after digestion were not significantly greater than the

* Abbreviation: CMCys, *S*-carboxymethylcysteine.

Fig. 1. Digestion of carboxymethylated actin by carboxypeptidase A in the presence of 2M-urea. Carboxymethylated actin (6.1 mg./ml.) in 50 mM-potassium phosphate buffer, pH 7.8, containing 2M-urea was digested at 25° at enzyme/substrate molar ratio 1:30. ●, Phenylalanine; ○, CMCys.

amounts present in control experiments, and it was therefore concluded that tropomyosin was absent from the preparation (cf. Locker, 1954; Laki & Standaert, 1960; Kominz, Saad, Gladner & Laki, 1957).

The maximum amounts of phenylalanine liberated in 2M-urea by carboxypeptidase A approached 1.2 moles/60 000 g. of protein, but by increasing the enzyme concentration relative to the amount of actin and carrying out the digestion in 6M-urea (Hasley & Neurath, 1955) the yield was increased to 1.4 moles/60 000 g. This value was not exceeded on prolonged digestion at either 25° or 37° (see Table 1). In 6M-urea the liberation of CMCys was suppressed and could not be detected in significant amounts.

The liberation of CMCys at the lower urea concentrations implied that cysteine was either the penultimate amino acid residue of a polypeptide chain terminated by phenylalanine or the *C*-terminal residue of a different chain in the native protein. A less likely explanation, in view of the initial studies on the purity of actin and the amounts of CMCys liberated, was that this residue was derived from a protein impurity in the preparation that possessed cysteine as its *C*-terminal residue.

The rate of liberation of CMCys compared with that of phenylalanine suggested that it was the penultimate residue. To confirm this point aminoethylated actin was digested with carboxypeptidase B in 0.06M-sodium dodecyl sulphate-0.2M-potassium phosphate buffer, pH 7.6 (Guidotti, 1960), with a enzyme/substrate molar ratio 1:30. After periods of digestion up to 3 hr. no aminoethylcysteine, lysine, arginine or indeed any other amino acids were released. As *C*-terminal aminoethylcysteine has been shown to be susceptible to release by carboxypeptidase B (Tietze, Gladner & Folk, 1957) it was concluded that cysteine is not *C*-terminal in actin. Likewise it could be concluded that chains with *C*-terminal lysine or arginine were not present.

To eliminate the possibility of a *C*-terminal proline residue in actin, carboxymethylated actin was subjected to hydrazinolysis. Under these conditions phenylalanine was the only amino acid released, although the yield (0.7 mole/60 000 g.) was lower than that obtained with carboxypeptidase A.

On the assumption that all the *C*-terminal phenylalanine is released by carboxypeptidase A in 6M-urea with a high enzyme/substrate ratio, the analytical data suggest that actin consists of a single polypeptide chain of molecular weight 43 000. From the amino acid analysis of actin (Table 2), assuming a unique polypeptide sequence, a molecule of this size should produce on complete tryptic digestion about 36–38 peptides, one of which should be the *C*-terminal peptide. Evidence for about 29 ninhydrin-staining spots was obtained from peptide-'mapping' studies and from the examination of peptide fractions obtained by Dowex 1 (X2) chromatography (Johnson *et al.* 1967*b*) of tryptic digests of carboxymethylated actin (cf. Carsten & Katz, 1964).

To confirm that only one *C*-terminal peptide was present, the tryptic peptide 'map' of carboxymethylated actin was compared with the tryptic peptide 'map' obtained after the digestion of carboxymethylated actin with carboxypeptidase A in 6M-urea for 3 hr. at 25° (Figs. 2*a* and 2*b*). The 'maps' differed only in one detail. The peptide marked *C*, consistently observed in digests of carboxymethylated actin, was replaced by a ninhydrin-staining spot marked *C'* in tryptic digests of carboxymethylated actin that had been initially treated with carboxypeptidase A. It was concluded therefore that peptide *C* was *C*-terminal in actin and that peptide *C'* represented this peptide from which phenylalanine had been removed. As the *C*-terminal peptide was shown to be CMCys-Phe (see below), peptide *C'* would be expected to be CMCys, for in 6M-urea carboxypeptidase A removed only phenylalanine from

Table 1. *Yields of phenylalanine and CMCys obtained on digestion of carboxymethylated actin by carboxypeptidase A*

All digestions were performed in 50mM-potassium phosphate buffer, pH 7.8. The results are the averages of two or more separate experiments, with the exception of the digestion at 37°.

Digestion conditions			Phenylalanine liberated (moles/60 000 g.)	CMCys liberated (moles/60 000 g.)
Concn. of urea (M)	Enzyme/substrate molar ratio	Temp.		
0	1:30	25°	1.00	0.25
2	1:30	25	1.19	0.52
6	1:20	25	1.27	0
6	1:10	25	1.40	0
6	1:7	37	1.40	0

Table 2. *Amino acid content of carboxymethylated actin*

The present results are the means $\pm$ S.D. of analyses of four different actin preparations.

Amino acid	Amino acid content			
	Present work		Carsten (1963)	
	(moles/100 000 g.)	(moles/45 000 g.)	(moles/100 000 g.)	(moles/45 000 g.)
Lys	43.6 $\pm$ 0.7	19.6	42 $\pm$ 0.8	18.9
His	16.3 $\pm$ 0.4	7.4	16.8 $\pm$ 0.3	7.6
NH ₃	61.3	27.6	74 $\pm$ 8.5	33.3
Arg	38.4 $\pm$ 0.7	17.3	40.6 $\pm$ 0.3	18.3
Asp	72.7 $\pm$ 0.9	32.7	78.1 $\pm$ 1.3	35.2
Thr	55.6	25.0	62.9 $\pm$ 1.5	28.2
Ser	47.5	21.4	53.8 $\pm$ 2.5	24.2
Glu	91.2 $\pm$ 1.3	41.0	91.3 $\pm$ 2.1	41.0
Pro	39.8 $\pm$ 1.3	17.8	42 $\pm$ 1.7	18.8
Gly	60.3 $\pm$ 0.7	27.2	62.3 $\pm$ 2.5	28.0
Ala	64.1 $\pm$ 0.9	28.9	67.5 $\pm$ 0.5	30.4
Cys	0	0	0	0
Val	43.3 $\pm$ 0.5	19.5	41.6 $\pm$ 1.0	18.7
Met	33.0 $\pm$ 0.8	14.9	36.3 $\pm$ 0.3	16.3
Ile	61.6 $\pm$ 0.5	27.7	61.1 $\pm$ 2.7	27.5
Leu	56.5 $\pm$ 0.6	25.4	57.5 $\pm$ 1.2	25.9
Tyr	32.5 $\pm$ 0.5	14.6	35.9 $\pm$ 0.3	16.1
Phe	25.3 $\pm$ 0.4	11.4	26.2 $\pm$ 0.5	11.8
CMCys	11.2 $\pm$ 0.1	5.04	11.2 $\pm$ 0.3	5.04
Trp	9.8 $\pm$ 0.2	4.4	8.5 $\pm$ 1.7	3.8
3-MethylHis	2.1 $\pm$ 0.04	0.95	—	—

Fig. 2. Peptide 'maps' of tryptic digests of actin: (a) carboxymethylated actin; (b) carboxymethylated actin after carboxypeptidase A digestion in 6 M-urea. The open spots represent peptides present in the tryptic digests of carboxymethylated actin. Spots A (ninhhydrin-negative), B and C were CMCys-containing peptides. Spot C' was a peptide spot replacing C when carboxymethylated actin was treated with carboxypeptidase A before tryptic digestion. After application of approx. 1 mg. of peptide material at O-O', electrophoresis was performed in pH 6.5 buffer [5% (v/v) pyridine-0.25% (v/v) acetic acid] with a potential gradient of 100 v/in. along the axis D-E. Descending chromatography with pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.) solvent was then performed along the axis D-F.

carboxymethylated actin. Experiments in which an authentic sample of CMCys was chromatographed with the tryptic digest showed that it had

electrophoretic and chromatographic mobilities identical with those of peptide C', which was therefore provisionally identified as CMCys.

Cysteine-containing peptides of actin

Confirmation that peptide *C* contained CMCys was obtained by comparing the peptide 'maps' of tryptic digests of carboxymethylated and carboxyamidomethylated actin. The 'maps' obtained by ninhydrin staining were identical except for the positions of two peptides. Two relatively acidic peptides present in carboxymethylated actin, represented by spot *C*, previously identified as the *C*-terminal peptide, and spot *B* (Fig. 2*a*), were absent from the carboxyamidomethylated derivative. In tryptic digests of the latter derivative, two new ninhydrin-staining spots *Y* and *Z* (Fig. 3), which were not present in carboxymethylated actin, were present.

In addition to the ninhydrin-staining peptides, there was also present in actin digests one peptide that was ninhydrin-negative but starch-iodide-positive (Rydon & Smith, 1952). This peptide, labelled *A* in Figs. 2(*a*) and 2(*b*), was relatively acidic in carboxymethylated actin, but in carboxyamidomethylated actin it was replaced by the slightly less acidic peptide *X*, which was also ninhydrin-negative (Fig. 3).

This preliminary study suggested that the tryptic peptides *A*, *B* and *C* obtained from carboxymethylated actin contained CMCys, in view of their changed mobility in the carboxyamidomethylated protein. It seemed likely that peptides *X*, *Y* and *Z* represented the carboxyamidomethylated forms of peptides *A*, *B* and *C* respectively, but though the latter were all more basic than the former, as would be expected, peptides *Y* and *Z* seemed more basic than would be predicted from their known composition by simple replacement of CMCys by *S*-carboxyamidomethylcysteine.

Separation and composition of CMCys-containing peptides

In an attempt to clarify the apparent discrepancy between the number of CMCys peptides recognized in the tryptic digest and those expected from analysis, a study was made of the distribution and recovery of CMCys in the peptides obtained by fractionation on Dowex 1 (X2) of the tryptic digest of carboxymethylated actin as described by Johnson *et al.* (1967*b*). To minimize the decomposition of CMCys all buffers contained 0.2% (v/v) thiodiglycol (Li & Vallee, 1964). Under these conditions, on average 56% of the total amount of CMCys in peptide form applied to the column was recovered in the separated fractions. Of the total CMCys recovered from the column, about 80% was present in fractions XVIII and XX (Fig. 4 and Table 3).

Peptide *B*. This peptide was eluted, together with another peptide that did not contain CMCys, in

fraction XVIII after chromatography on Dowex 1 (X2) (Fig. 4). Separation of these two peptides was achieved by further chromatography on Dowex 50 (X2) as shown in Fig. 5. Paper electrophoresis and chromatography showed that the first peak eluted represented peptide *B*.

Amino acid analysis indicated that peptide *B* was a hexapeptide with presumably a *C*-terminal arginine residue (Table 4). The sequence deduced by a single series of Edman degradations (Table 5) was CMCys-Asp-Ile-Asp-Ile-Arg.

Peptide *A*. Fraction XX eluted from Dowex 1 (X2) (Fig. 4) contained peptides *A* and *C* only. On

Fig. 3. Peptide 'map' of tryptic digest of carboxyamidomethylated actin. Peptides at spots *X*, *Y* and *Z* replaced the spots *A*, *B* and *C* seen in digests of carboxymethylated actin (Fig. 2). For experimental details, see Fig. 2.

Table 3. Distribution of CMCys in the peptide fractions obtained by Dowex 1 (X2) column chromatography of tryptic digests of carboxymethylated actin (Fig. 4)

A 500mg. sample of the peptide mixture was applied to the column, which was developed as described by Johnson *et al.* (1967*b*). Samples of peptide fractions were hydrolysed in 6*N*-HCl *in vacuo* at 110° for 20hr. before amino acid analysis. See Fig. 4 for the fraction numbers.

CMCys in peptide fraction (% of total amount of CMCys applied to the column)

Fraction no.	Run (i) Run (ii)	
	Run (i)	Run (ii)
IX	1	1
XIII	2	3
XIV	1	0
XVI	2	2
XVIII	12	18
XIX	2	4
XX	35	31

Fig. 4. Chromatography of peptides in a tryptic digest of carboxymethylated actin. A 500mg. sample of digest was applied to a Dowex 1 (X2) column, which was developed as described by Johnson *et al.* (1967b). The ordinate represents the ninhydrin colour after alkaline hydrolysis. [Fraction XIII, reported by Johnson *et al.* (1967b) to contain 3-methylhistidine, is identical with fraction XV in this Figure.]

Fig. 5. Chromatography of fraction XVIII (Fig. 4) on Dowex 50 W (X2; 200-400 mesh). Approx. 3mg. of peptide mixture was applied to a column (55cm. $\times$ 1cm.) equilibrated with 0.1M-pyridine-acetic acid buffer, pH 3.5. Elution was carried out with a linear gradient system of 100ml. of 0.1M-pyridine-acetic acid buffer, pH 3.5, and 100ml. of 1.0M-pyridine-acetic acid buffer, pH 5.5. Both buffers contained 0.2% (v/v) thiodiglycol. The flow rate was 20ml./hr., fractions (2ml.) being collected. Samples (0.2ml.) were taken for ninhydrin-colour measurement after alkaline hydrolysis (Hirs, Moore & Stein, 1956). The first peak eluted contained peptide B.

Table 4. *Amino acid composition of CMCys-containing peptides isolated from tryptic digests of actin*

The results are given as molar ratios relative to lysine for peptide A, to isoleucine for peptide B and to phenylalanine for peptide C.

Amino acid	Peptide ...	Amino acid composition (molar ratios)		
		A	B	C
CMCys		0.98	1.07	0.95
Asp		3.6	2.0	0
Thr		2.2	0	0
Ser		0.9	0	0
Glu		2.0	0	0
Gly		2.1	0	0
Ala		1.0	0	0
Val		2.0	0	0
Ile		0	2.0	0
Leu		2.3	0	0
Phe		0	0	1.0
Lys		1.0	0	0
Arg		0	0.95	0

further chromatography of fraction XX on Dowex 50 (X2), separation was obtained into two peaks of which the first eluted corresponded to peptide A and the second to peptide C (Fig. 6).

Table 5. *Sequential Edman degradation of peptide B*

The results are given as molar ratios relative to isoleucine in the original peptide *B* and to arginine after successive degradations.

Amino acid	Peptide <i>B</i>	Amino acid composition (molar ratios)				
		Step no. ...	Edman degradation products			
			1	2	3	4
CMCys	1.07	0	0	0	0	0
Asp	2.0	2.0	0.9	1.3	0.2	
Ile	2.0	2.1	1.9	1.2	1.2	
Arg	0.95	1.0	1.0	1.0	1.0

Fig. 6. Chromatography of fraction XX (Fig. 4) on Dowex 50 W (X2; 200–400 mesh). Approx. 4mg. of peptide mixture was applied to a column (50 cm. $\times$ 1 cm.) equilibrated with 0.2M-pyridine-acetic acid buffer, pH 3.1, and elution was carried out with 60 ml. of the same buffer followed by 0.4M-pyridine-acetic acid buffer, pH 4.4. Other conditions were as for Fig. 5. The first peak eluted was peptide *A* and the other peak contained peptide *C*.

The amino acid composition (Table 4) shows that peptide *A* was the largest of the three CMCys peptides isolated and probably contained about 17 or 18 residues.

After treatment of peptide *A* with fluorodinitrobenzene and removal of excess of reagents, no free lysine was detectable in the acid hydrolysate. The acid-soluble fraction remaining after ether extraction contained only one yellow component, which had the same R_F value in butanol-acetic acid-water and electrophoretic mobility at pH 6.5 as ϵ -DNP-lysine. The ether-soluble fraction of the acid

hydrolysate contained only dinitrophenol and traces of two DNP derivatives, the amounts of which in comparison with the yield of ϵ -DNP-lysine were insignificant; these were ignored.

It was concluded, because of its failure to react with ninhydrin and to yield significant amounts of an α -DNP-amino acid, that peptide *A* had a blocked *N*-terminal residue and that lysine was almost certainly the *C*-terminal amino acid. In view of the reports of Gaetjens & Barany (1966) and Alving & Laki (1966) that the *N*-terminal residue of actin is *N*-acetylaspartic acid it seemed possible that peptide *A* represented the *N*-terminal tryptic peptide.

Peptide *C*. After chromatography of Fraction XX on Dowex 50 (X2), peptide *C* was eluted in a pure form and shown by analysis to contain CMCys and phenylalanine in equimolar amounts (Table 4). In view of the absence of arginine and lysine and the results of the *C*-terminal analysis with carboxypeptidase described above it was almost certainly the *C*-terminal tryptic peptide. Edman degradation confirmed the structure as CMCys-Phe, as the phenylalanine/CMCys ratio was greater than 10:1 after a single Edman degradation.

Paper and column chromatography of the peptide fractions that were eluted immediately before and after fractions XVIII and XX showed that these fractions contained small but significant amounts of the peptides present in fractions XVIII and XX that had already been shown to contain CMCys. It therefore seemed likely that the major part of the total recovered CMCys not present in fractions XVIII and XX was due to contamination of other peptide fractions by peptides *A*, *B* and *C*. Thus it was concluded that only three CMCys-containing peptides were eluted from the Dowex 1 (X2) column.

As virtually all the CMCys present in the peptides eluted from Dowex 1 appeared to be accounted for by peptides *A*, *B* and *C*, it was necessary to determine whether additional CMCys-containing peptide material was present in the undigested core remaining after tryptic digestion of actin. Usually

about 20–25% of the original actin nitrogen was precipitated from tryptic digests by the deproteinization procedure used before chromatography. This precipitate contained CMCys but in a similar proportion to the original carboxymethylated actin (11.1 moles of CMCys residues/100 000 g.). No evidence was obtained for the existence of peptides other than those present in the soluble actin digest when this partially digested material was redigested with trypsin. Although the core could not be completely solubilized by redigestion with trypsin the insoluble material could be decreased to 14% of the original actin. Further treatment of the core with chymotrypsin produced a peptide mixture that on electrophoresis at pH 6.5 gave an electrophoretogram very similar in appearance and relative intensity of the ninhydrin-staining spots to that obtained from the chymotryptic digest of carboxymethylated actin not previously digested with trypsin.

DISCUSSION

There seems little doubt that phenylalanine is the C-terminal residue of actin, for this residue was reported earlier to be liberated by carboxypeptidase (Locker, 1954; Laki & Standaert, 1960). The latter workers also reported the liberation of small amounts of serine and isoleucine, which were almost certainly due to traces of tropomyosin in the actin preparation, for in tropomyosin-free actin serine and isoleucine cannot be detected as C-terminal amino acid residues. The failure of earlier workers to detect cysteine in C-terminal studies was probably due to the fact that their digestions were carried out on actin in which the cysteine residues had not been modified. Cysteine is known to be resistant to carboxypeptidase A (Bailey, 1962), but the present studies with carboxymethylated actin indicate that a penultimate CMCys residue can be liberated by carboxypeptidase A.

The yield of phenylalanine liberated by carboxypeptidase A was higher than in earlier studies, probably because the digestion was carried out in urea. The yield suggests a molecular weight of

43 000, compared with values obtained by similar earlier studies of 70 000 (Locker, 1954) and 56 000 (Laki & Standaert, 1960). A lower value for the minimum molecular weight is also derived from the 3-methylhistidine content (Johnson *et al.* 1967b; Asatoor & Armstrong, 1967). It is significant that a value of 44 000–45 000 corresponds closely to integral numbers of residues of two other amino acids that are present in small amounts, namely cysteine and tryptophan (Table 6). Earlier hydrodynamic studies gave values ranging from 47 000 to 62 000 (Adelstein, Godfrey & Kielley, 1963; Lewis, Maruyama, Carroll, Kominz & Laki, 1963; Mihashi, 1964; Krans, Van Eijk & Westenbrink, 1965), but a recent study in which particular care was taken to ensure that G-actin preparations were fully depolymerized gave a value of 46 000 (Rees & Young, 1967). Thus recent chemical and hydrodynamic methods show good agreement. The chemical evidence strongly suggests that the actin molecule consists of a single polypeptide chain and provides no evidence for the presence of sub-units as was suggested by Adelstein *et al.* (1963) and Mihashi & Ooi (1965). In this respect also it is in good agreement with the results of Rees & Young (1967), who found the molecular weight unchanged by guanidine hydrochloride treatment.

An actin molecule of molecular weight 45 000 would contain five cysteine residues, and if the amino acid sequence was unique and all the cysteine residues were separated by trypsin-sensitive bonds it would give rise to five different cysteine-containing peptides on tryptic digestion. In the present study we have been able to isolate only three peptides from such digests by column chromatography on Dowex 1 and by comparing the mobilities of ninhydrin-staining spots from digests of carboxymethylated and carboxyamidomethylated actins. It should be emphasized that, for two of these peptides, the electrophoretic mobilities of the supposed carboxyamidomethylated forms were somewhat greater at pH 6.5 than would be expected from their amino acid analyses.

Other investigators who reported the presence of larger numbers of cysteine peptides, namely five

Table 6. *Molecular weight of actin based on amino acid-analytical data*

Amino acid	Residues (moles/100 000 g.)	Assumed no. of residues/mol.	Mol. wt.
C-Terminal Phe	2.3	1	43 000
3-MethylHis	2.22*	1	45 000
Cys	11.2†	5	45 000
Trp	9.1†	4	44 000
Mean			44 200

* Mean of analyses of Asatoor & Armstrong (1967) and Johnson *et al.* (1967b).

† Mean of analyses in the present work and those of Carsten (1963); estimated as CMCys.

(Carsten, 1966) or eight (Martonosi, 1968), relied mainly on identification of peptide spots by labelling with radioactive iodoacetate. Most of the peptides that have been so far isolated by other workers for which analyses have been published have been eluted from paper chromatograms. When allowances are made for the inaccuracies of this method they appear to be identical with two of those separated by us on Dowex 1.

It is likely that peptide *A* corresponds to peptide 30 analysed by Carsten (1966). Although the electrophoretic and chromatographic mobilities are very similar the analyses differ considerably, probably due in Carsten's (1966) case to the errors inherent in eluting peptides from paper chromatograms for analyses. The fact that this peptide is the only ninhydrin-negative peptide observed in tryptic digests suggests that it is the *N*-terminus, which has been shown to be acetylated in actin. The *N*-terminal sequence *N*-acetyl-Asp-Glu-Thr-Ala- (Gaetjens & Barany, 1966; Alving & Laki, 1966) could be accommodated in peptide *A* as judged from its amino acid composition. This peptide also appears to be identical with that readily reacting with *N*-ethylmaleimide (Martonosi, 1968).

Peptide *B* has not been previously identified and analysed but, judged from its electrophoretic and chromatographic mobilities, it could correspond to Carsten's (1966) peptide 11 identified simply as a radioactive and ninhydrin-staining spot. It must be located somewhere in the body of the actin polypeptide chain, and it is noteworthy that the cysteine residue is adjacent to a trypsin-sensitive residue, presumably a lysine or arginine residue.

Peptide *C* represents the *C*-terminal dipeptide, and here also the cysteine residue is presumed to be adjacent to a lysine or arginine residue. Its mobilities on electrophoresis and chromatography are similar to Carsten's (1966) peptide 29, but, apart from the fact that the latter contained phenylalanine and CMCys, there appears to be no similarity of composition.

It thus appears that the actin molecule is a single polypeptide chain with cysteine residues close to the *N*- and *C*-termini.

Careful study of the material eluted from Dowex 1 failed to reveal any other peptides with significant amounts of CMCys. It cannot be completely excluded at this stage that additional peptides are specifically held on Dowex 1 and not readily eluted (cf. Weeds & Hartley, 1968). In view of the fact that only about 60% of the CMCys was recovered from the Dowex column, which is normal for this type of fractionation, and because of the well-known lability of CMCys, it is difficult to draw any quantitative conclusions from the yields of peptides obtained and hence to decide whether some are duplicated or

not. It is noteworthy, however, in view of the classification of the thiol groups in actin into three types on the basis of reactivity and requirement for biological activity, that three different CMCys-containing peptides have been identified. A final decision on the additional two cysteine peptides to be expected in the actin molecule of weight 45000 must await further investigation with radioactively labelled thiol reagents.

We thank Mr C. I. Harris for performing the amino acid analyses carried out on the Beckman amino acid analyser. This work was supported in part by a research grant from the Medical Research Council to S. V. P.

REFERENCES

- Adelstein, R. S., Godfrey, J. E. & Kielley, W. W. (1963). *Biochem. biophys. Res. Commun.* **12**, 34.
- Alving, R. E. & Laki, K. (1966). *Fed. Proc.* **25**, 223.
- Asatoor, A. M. & Armstrong, M. D. (1967). *Biochem. biophys. Res. Commun.* **26**, 168.
- Bailey, J. L. (1962). *Techniques in Protein Chemistry*, 1st ed., p. 202. Amsterdam: Elsevier Publishing Co.
- Bailin, G. & Barany, M. (1967). *Biochim. biophys. Acta*, **140**, 208.
- Barany, M. (1956). *Biochim. biophys. Acta*, **19**, 560.
- Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A. & Neurath, H. (1963). *Biochemistry*, **2**, 1468.
- Biserte, G. & Osteux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
- Bradbury, J. H. (1956). *Nature, Lond.*, **178**, 912.
- Carsten, M. E. (1963). *Biochemistry*, **2**, 32.
- Carsten, M. E. (1966). *Biochemistry*, **5**, 297.
- Carsten, M. E. & Katz, A. M. (1964). *Biochim. biophys. Acta*, **90**, 534.
- Drabikowski, W. & Gergely, J. (1963). *J. biol. Chem.* **238**, 640.
- Gaetjens, E. & Barany, M. (1966). *Biochim. biophys. Acta*, **117**, 176.
- Guidotti, G. (1960). *Biochim. biophys. Acta*, **42**, 177.
- Harrison, P. M. & Hofmann, T. (1961). *Biochem. J.* **80**, 38 f.
- Hasley, Y. D. & Neurath, H. (1955). *J. biol. Chem.* **217**, 247.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.
- Johnson, P., Harris, C. I. & Perry, S. V. (1967a). *Proc. Brit. biophys. Soc.*, December Meeting.
- Johnson, P., Harris, C. I. & Perry, S. V. (1967b). *Biochem. J.* **105**, 361.
- Katz, A. M. (1963). *Biochim. biophys. Acta*, **71**, 397.
- Katz, A. M. & Mommaerts, W. F. H. M. (1962). *Biochim. biophys. Acta*, **65**, 82.
- Kominz, D. R., Saad, F., Gladner, J. A. & Laki, K. (1957). *Arch. Biochem. Biophys.* **70**, 16.
- Konigsberg, W. & Hill, R. J. (1962). *J. biol. Chem.* **237**, 2547.
- Krans, H. M. J., Van Eijk, H. G. & Westenbrink, H. G. K. (1965). *Biochim. biophys. Acta*, **100**, 193.
- Kuschinsky, G. & Turba, F. (1951). *Biochim. biophys. Acta*, **6**, 426.
- Laki, K. & Standaert, J. (1960). *Arch. Biochem. Biophys.* **86**, 16.

- Lewis, M. S., Maruyama, K., Carroll, W. R., Kominz, D. R. & Laki, K. (1963). *Biochemistry*, **2**, 34.
- Li, T.-K. & Vallee, B. L. (1964). *Biochemistry*, **3**, 869.
- Locker, R. H. (1954). *Biochim. biophys. Acta*, **14**, 533.
- Martonosi, A. (1968). *Arch. Biochem. Biophys.* **123**, 29.
- Martonosi, A. & Gouvea, M. A. (1961). *J. biol. Chem.* **236**, 1345.
- Mihashi, K. (1964). *Arch. Biochem. Biophys.* **107**, 441.
- Mihashi, K. & Ooi, T. (1965). *Biochemistry*, **4**, 805.
- Perry, S. V. & Cotterill, J. (1964). *Biochem. J.* **92**, 603.
- Poglazov, B. F. & Baev, A. A. (1961). *Biokhimiya (Engl. trans.)*, **26**, 475.
- Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
- Rees, M. K. & Young, M. (1967). *J. biol. Chem.* **242**, 449.
- Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
- Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 353.
- Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
- Smyth, D. G., Stein, W. H. & Moore, S. (1963). *J. biol. Chem.* **238**, 227.
- Spies, J. R. & Chambers, D. C. (1948). *Analyt. Chem.* **20**, 30.
- Spies, J. R. & Chambers, D. C. (1949). *Analyt. Chem.* **21**, 1249.
- Strauch, L. (1965). *Z. klin. Chem.* **5**, 165.
- Strohman, R. C. & Samorodin, A. J. (1962). *J. biol. Chem.* **237**, 363.
- Tietze, F., Gladner, J. A. & Folk, J. E. (1957). *Biochim. biophys. Acta*, **26**, 659.
- Tomomura, Y. & Yoshimura, J. (1961). *J. Biochem., Tokyo*, **50**, 79.
- Weeds, A. G. & Hartley, B. S. (1968). *Biochem. J.* **107**, 531.