Selective Purification of the Thiol Peptides of Myosin

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1. A method for selective purification of thiol peptides is described. Thiol groups in a protein are treated with radioactive cystine by disulphide-thiol interchange. The labelled cystine peptides in a digest can then be fractionated for peptide 'maps'. Performic acid oxidation of paper strips containing the radioactive peptides followed by further ionophoresis yields the purified cysteic acid peptides. 2. The thiol peptides in a peptic digest of cystine-exchanged myosin were purified in this way, and their amino acid sequences were determined. 3. The conclusion that myosin contains at least 16, and probably between 20 and 22, unique thiol sequences indicates that the molecule consists of two chemically equivalent components.

The diagonal ionophoretic technique of Brown & Hartley (1966) allows the selective purification of peptides containing disulphide bridges, and thereby provides a convenient way of exploring the amino acid sequence in these portions of a protein. Most proteins, however, contain cysteine rather than cystine residues, and the diagonal technique is not readily applicable because of oxidation and random disulphide interchange during proteolysis and fractionation of the peptides. This is unfortunate, since the sequence around thiol groups is often of particular interest because of their role in the catalytic activity of many enzymes. We have therefore developed a technique whereby thiol groups of a protein are converted into disulphides by interchange with a large excess of radioactive cystine. This protects the peptide during proteolysis and fractionation and provides a radioactive label to assist in the separation of the cystine peptides on paper. When the paper strips are exposed to performic acid, cysteic acid peptides and free cysteic acid are produced, and these can be purified from all contaminants by the diagonal procedure of Brown & Hartley (1966).

We chose to apply this procedure to rabbit skeletal-muscle myosin since this is a very large protein of molecular weight about 500 000 (Tonomura, Appel & Morales, 1966) containing about 42 thiol groups. About 330 peptides would be present in a tryptic digest of this protein, and selective purification techniques are almost essential in fractionating such a complex mixture. In a preliminary report of this work (Weeds & Hartley, 1967) we have reported the sequence around 16 thiol groups of myosin. Together with the results of Kimura & Kielley (1966)

this indicated that the molecule contained about 22 unique thiol groups and suggested that the molecule was composed of two identical chains rather than three identical chains (Woods, Himmelfarb & Harrington, 1963). This paper describes the experimental technique, which may be widely applicable to thiol proteins, and documents the evidence for the sequence around the thiol groups of myosin.

MATERIALS

DL-[³⁵S]Cystine was obtained from The Radiochemical Centre, Amersham, Bucks. The radiochemical purity of the [³⁵S]cystine was checked after ionophoresis of the carrier-free [³⁵S]cystine at pH 2, or chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol.). Over 99% of the counts had the mobility of cystine in these systems, and the other counts appeared to be in oxidation products, since performic acid oxidation converted these into cysteic acid. Similar results were obtained when carrier was added. Guanidine hydrochloride was prepared from A.R. grade guanidine carbonate, by using conc. HCl. The salt was recrystallized after rotary evaporation of the solution at 30° to the point where crystallization began, and the crystals were dried over CaCl₂. All other reagents were A.R. grade.

Enzymes. Pepsin (twice crystallized; batch P.M. 692), trypsin (twice crystallized, salt-free), α -chymotrypsin (three-times crystallized), papain (twice crystallized) and carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and Zeo-Karb 225 (X2) (SRC 8) from The Permutit Co. Ltd., London, W. 4.

METHODS

Myosin preparation. Adult male rabbits of the New Zealand White strain were used in all the experiments described in this work. The animals were anaesthetized

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with Nembutal and bled out. The myosin was prepared from the back and leg muscles essentially by the method of Perry (1955).

All operations were carried out at 0–2° and all solutions were made up in glass-distilled water containing 1 mM-EDTA. The minced muscle was stirred for 15 min. with 0.3 M-KCl–0.1 M-KH₂PO₄–0.05 M-K₂HPO₄ (final concentrations) and centrifuged at 1000g for 15 min. The supernatant was filtered slowly through a 1 in. pad of paper pulp, and crude myosin was precipitated by the slow addition of 10 vol. of ice-cold water. After settling, the precipitate was centrifuged at 1000g for 30 min. and redissolved in 0.5 M-KCl. Actomyosin was precipitated by adjusting the pH to 6.6 with solid NaHCO₃ and slowly lowering the ionic strength to 0.30 M. The precipitate was removed by centrifugation at 10000g for 15 min. To remove ribonucleoprotein (cf. Takahashi, Hashimoto & Tonomura, 1963), the supernatant was stirred for 2 hr. at pH 7.6 with DEAE-Sephadex A-50 that had been equilibrated with 0.2 M-KCl–0.1 M-tris-HCl, pH 7.6. The mixture was filtered gently through a sintered-glass funnel and the filtrate was adjusted to 0.3 M-KCl, pH 6.6, by adding KCl and phosphate buffer. The myosin was reprecipitated by slowly diluting this solution to 0.04 M-KCl, allowed to settle and centrifuged at 1000g for 15 min. It was redissolved in 0.5 M-KCl, pH 6.6, and again precipitated at 0.04 M-KCl. Finally the solution in 0.5 M-KCl was centrifuged at 40000g for 1 hr. Exchange experiments between the purified myosin and [³⁵S]cystine were carried out within 3 days of the killing of the rabbit.

Protein concentrations. Measurements of the extinction of myosin solutions at 280 m μ were corrected for turbidity by applying a fourth-power-law correction to measurements at 320 m μ (Mihalyi & Rowe, 1966). $E_{1\text{ cm}}^{1\%}$ at 280 m μ was taken as 5.60 (Small, Harrington & Kielley, 1961), and this figure was confirmed for each batch of myosin by the modified biuret procedure of Rowe (1960). Cystine-exchanged myosin was found to have the same extinction coefficient as myosin.

Amino acid analysis. Samples (about 1 mg.) of protein were hydrolysed in 0.2 ml. of constant-boiling HCl in evacuated tubes at 105° for 24–100 hr. The hydrolysates were evaporated *in vacuo* at room temperature and analysed with a Beckman Spinco model 120B amino acid analyser.

The following procedure was adopted for the cysteic acid analyses. Myosin solutions were freed from salt by exhaustive dialysis against 1% (v/v) formic acid. Samples of the solution were digested with 1% (w/w) pepsin for 6 hr. in 5% (v/v) formic acid. After being dried *in vacuo* the digest was dissolved in 50 μ l. of anhydrous formic acid, to which was then added 300 μ l. of performic acid [1 part of 30-volume hydrogen peroxide to 20 parts (by vol.) of formic acid], and the solution was incubated at room temperature for 1–2 hr. The performic acid was removed in a vacuum desiccator over NaOH pellets, and the digest was redissolved in 100 μ l. of water and then dried again *in vacuo*. Although oxidation at room temperature resulted in significant losses of tyrosine, this method was preferred because of the difficulty encountered in removing performic acid by freeze-drying after oxidation at 0°. Because of the importance attached to the cysteic acid recovery in particular, losses during the above oxidation procedure and subsequent acid hydrolysis were corrected for by a parallel analysis under identical conditions of a standard protein, either insulin or chymotrypsinogen, both of which contain a relatively high percentage of this amino acid. Recoveries

of 95.2 $\pm$ 3.0% were found for cysteic acid in these proteins.

Disulphid-ethiol interchange reaction. The reaction was carried out under conditions similar to those described by Stracher (1964) and Gaetjens, Therattil-Antony & Barany (1964). The incubation medium was 0.5 M-KCl–10 mM-EDTA–0.02 M-tris-HCl, pH 9.6, and contained myosin at a concentration of 4–6 mg./ml. and a 100-fold molar excess of cystine over all the thiol groups present. The [³⁵S]cystine was dissolved in 1.0 N-HCl and mixed with a calculated weight of unlabelled cystine in 1.0 N-HCl to give a radioactivity of approx. 1.4 mc/m-mole. The pH was then adjusted to 9.8 with 2.0 N-KOH, and in this way the cystine remained soluble. (Cystine is difficult to dissolve below pH 10 and it was considered unwise to take the pH much above this value because of the risk of alkali-catalysed cleavage of the disulphide bond.) After addition of the tris-HCl and EDTA to give the final required concentrations (0.02 M and 1 mM respectively) and adjustment of the KCl concentration to 0.5 M, the solution was cooled to 0° and reaction was initiated by addition of the myosin solution, also at 0°.

Samples were removed at regular intervals and acidified with 0.1 N-HCl to stop the reaction, and then dialysed against 0.1 N-HCl exhaustively at 0° to remove the excess of reagent. The incorporation of counts was measured as a function of time and related to the extinction at 280 m μ of the protein solution. When large batches of labelled myosin were prepared, the pH of the solution was lowered to 7.0 at the end of the reaction, and the excess of cystine was allowed to sediment; it could then be removed by centrifugation and recovered for further use if necessary. Acidification followed by exhaustive dialysis over periods of about 4 days against 0.1 N-HCl removed the last traces of the excess of cystine.

The reaction was carried out under conditions where the protein retained its native configuration. Attempts to exchange myosin with cystine in 8 M-urea or 5 M-guanidine hydrochloride were less successful, perhaps because some thiol groups became oxidized during the disaggregation.

Peptic digestion of cystine-exchanged myosin. The cystine-exchanged myosin in 0.1 N-HCl was finally dialysed against 1% (v/v) formic acid for 24 hr. before proteolysis. Peptic digestion was carried out in 5% (v/v) formic acid at 37° for 15 hr., with substrate concentrations of about 1 mg./ml. and an enzyme/substrate ratio of 1:40 (w/w). Higher substrate concentrations and shorter digestion times were found to give variable amounts of radioactive 'core', whereas digestions carried out under the described conditions yielded less than 1% insoluble counts at pH 3.1, and very little radioactive material was observed at the origin after two-dimensional ionophoresis of the peptide digest at pH 6.5 and 1.9. After proteolysis, the digest was freeze-dried and then redissolved in 1% (v/v) formic acid at a concentration of about 15 mg./ml.

Group fractionation of peptides on Zeo-Karb 225. Zeo-Karb 225 (X2) (H⁺ form) was equilibrated with 0.1 M-pyridine formate, pH 3.1, before the column was poured. Of the peptic digest, 2.0 g. in 1% (v/v) formic acid was applied to a column of dimensions 65 cm. $\times$ 3.0 cm., and stepwise elution was carried out with pyridine formate buffers (see Fig. 2). All the pyridine formate buffers were made by adjusting pyridine solutions to the appropriate pH with formic acid. Fractions (26 ml.) were collected and 50 μ l. samples were taken from each tube for counting.

Concentration of column fractions. The fractions from the column were concentrated by rotary evaporation at temperatures of less than 30°. In many cases the final product was an oily liquid, which was diluted with water and further evaporated to remove the pyridine and formic acid. Although some of the buffer remained at this stage, it was not found to interfere in the subsequent paper ionophoresis at pH 6.5. Freeze-drying was not a successful method for removal of the oil.

Counting of radioactive samples. Radioactive samples were counted by a Nuclear-Chicago gas-flow proportional counter. The [³⁵S]cystine was diluted with carrier to give between 6×10^5 and 8×10^5 counts/min./ μ mole, i.e. about 1.4 mc/ μ mole. The protein samples were plated out on planchets at loadings of less than 30 μ g./cm.², and the counts measured were between 4000 and 10000 at a rate of not less than 1000 counts/min., this figure being 50 times the background rate of the counter. Since measurements were carried out over periods of several days, corrections were made for the decay of the nuclide and these were checked by counting a standard sample of [³⁵S]cystine in parallel with the other samples. This was also used to check the efficiency of the counter during the assay. Where incorporation of counts was being measured, the background counts was taken by using an equal sample of the diffusate from the final stage of the dialysis. To obtain a measurement of the counts/min./ μ mole of cystine used in the reaction, the carrier-free cystine was counted at rates of up to 15000 counts/min. for between 40000 and 100000 counts. Additions of carrier-free cystine to the unlabelled cystine were made by weight and not by volume, thus ensuring that the dilution factors were as accurate as possible.

High-voltage paper ionophoresis. Peptides were applied as wide bands to Whatman no. 3MM paper with internal fluorescent markers (Brown & Hartley, 1966) at loadings of about 1 mg./cm. After drying in a stream of cold air, the papers were wetted with the appropriate buffers and the bands sharpened. Buffers used were: pH 6.5 (pyridine-acetic acid-water, 25:1:225, by vol.); pH 3.5 (pyridine-acetic acid-water, 1:10:89, by vol.); pH 2 (formic acid-acetic acid-water, 1:4:45, by vol.); pH 9 [1% (w/v) (NH₄)₂CO₃]. Ionophoresis was carried out at 60 v/cm. in solvent-cooled tanks (Michl, 1951). The mobilities of the peptides at pH 6.5 (abbreviated *m*) were expressed relative to aspartic acid = 1.0 (Offord, 1966).

Peptide 'maps'. Samples of the digest were applied as a band to the middle of Whatman no. 3MM paper at a loading of approx. 1 mg./cm. and submitted to ionophoresis at pH 6.5. The neutral band was cut from these sheets, stitched 15 cm. from the anode end of another full sheet of paper and submitted to ionophoresis at pH 2 or pH 3.5.

A two-dimensional 'map' of the peptides anionic at pH 6.5 was prepared as follows. An 8 cm. band was cut, parallel to the direction of ionophoresis, from the anionic section of the pH 6.5 paper and stitched across another sheet of paper parallel to and with mid-point 15 cm. from the anode end. The peptides were eluted to the mid-point by applying pH 2 buffer on each side of the strip, and ionophoresis was carried out at pH 2 at right angles to the strip.

In a similar way, an 8 cm. strip was cut from the cationic section of the pH 6.5 paper. The peptides were eluted to the mid-point of this strip, by using pH 2 buffer. When dry, the strip was stitched 15 cm. from the top of another sheet

of paper and submitted to descending chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:10:12, by vol.) (Waley & Watson, 1953).

Finally, an 8 cm. strip was cut from the pH 3.5 ionophoresis paper of the peptides neutral at pH 6.5, and this was stitched to another sheet and submitted to descending chromatography as described for the cationic peptides. Three two-dimensional peptide 'maps' were thus obtained: a pH 6.5/chromatography 'map' of the basic peptides, a pH 3.5/chromatography 'map' of the neutral peptides (or occasionally a pH 2/chromatography 'map') and a pH 6.5/pH 2 'map' of the acidic peptides.

Performic acid oxidation of the paper strips. High-voltage paper ionophoresis (see above) was used to separate the radioactive cystine peptides from each other. They were, however, considerably contaminated with unlabelled peptides, and the procedure of Brown & Hartley (1966) was used to separate cysteic acid peptides from these contaminants. Strips containing the radioactive bands were exposed to performic acid vapour and then submitted to further ionophoresis at right angles at pH 6.5. A guide strip was stained with cadmium-ninhydrin reagent (Heilmann, Barollier & Watzke, 1957) to reveal the positions of the 'diagonal' contaminants, of free radioactive cysteic acid and of the cysteic acid peptide with altered mobility. Further purification of the band of cysteic acid peptide was frequently necessary, particularly where this became neutral at pH 6.5. This was achieved by stitching the strip to a new sheet of paper for further ionophoresis at pH 2, pH 3.5 or pH 9. The peptides were then eluted with 0.05 N-NH₃.

Sequence methods. The *N*-terminal groups of peptides were determined by their reaction with DNS* chloride by the method of Gray & Hartley (1963a). After hydrolysis, the DNS-amino acids were identified by ionophoresis at pH 4.4 [0.4% pyridine and 0.8% acetic acid (v/v) in water] in a flat-plate apparatus. Further separation of those DNS-amino acids whose mobilities were equivocal was achieved by stitching the strips to a new sheet of paper for ionophoresis at pH 2 or chromatography with light petroleum (b.p. 100–120°)-acetic acid-water (10:9:1, by vol.), only the organic phase being used (Boulton & Bush, 1964).

The 'dansyl'-Edman procedure of Gray & Hartley (1963b) was the main sequence method. Peptides were treated with phenyl isothiocyanate at 37° for 2 hr. Anhydrous trifluoroacetic acid was used for the cyclization step. It was occasionally found that yields of DNS-amino acid diminished drastically after several cycles of the Edman reaction, perhaps because of the accumulation of oxidation products of pyridine, which interfere with the DNS chloride reaction. In these circumstances, the degraded peptide could be purified by ionophoresis and eluted, and the procedure continued. The pyridine, phenyl isothiocyanate and trifluoroacetic acid were redistilled before use in these reactions.

Further digestion of cysteic acid peptides. (a) Enzyme digests with trypsin (abbreviated T in the peptide nomenclature), chymotrypsin (abbreviated C) and leucine aminopeptidase were carried out in 0.5% (w/v) NH₄HCO₃

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; in amino acid sequences: Cya, cysteic acid; Mso, methionine sulphone; Asx, asparagine or aspartic acid; Glx, glutamine or glutamic acid.

Fig. 1. Incorporation of $[^{35}\text{S}]$ cystine into myosin. The Figure shows the average results for four different myosin preparations, with the standard deviation for each point. A, Value at the final incorporation time, which varied from 50 to 92 hr.; B, value after further reaction in 5M-guanidine hydrochloride (see the text).

in water, with enzyme/substrate ratios between 1:50 and 1:100 (w/w), for periods of either 4 hr. at 37° or 16 hr. at room temperature.

(b) Digestions with papain (abbreviated N in the peptide nomenclature) were carried out in 0.5M-pyridine acetate buffer, pH 6.5, containing 0.5% (v/v) of β -mercaptoethanol. The enzyme/substrate ratio was about 1:10 (w/w), and digestion was for 16 hr. at room temperature.

(c) Carboxypeptidase A suspensions were dissolved by the method of Fraenkel-Conrat, Harris & Levy (1955), and digestions were carried out in 0.1M-N-ethylmorpholine acetate at pH 8.0 for 5 hr. at 37° with about 0.01 mg. of enzyme and 0.02–0.05 μ mole of peptide.

After digestion with these enzymes, the digests were submitted to ionophoresis at pH 6.5. The neutral bands from these ionograms were submitted to further ionophoresis at either pH 3.5 (tryptic, chymotryptic and papain digests) or pH 2 (carboxypeptidase A digests). Peptides were identified by staining with the cadmium-ninhydrin reagent (Heilmann *et al.* 1957), and amino acids were stained with collidine-ninhydrin (Levy & Chung, 1953). After elution with 0.05N-NH₃, the peptide sequences were determined as described above.

RESULTS

Cystine exchange of myosin

Fig. 1 shows the rate of half-cystine incorporation into myosin, expressed as a percentage of the theoretical maximum. The curve represents averaged results from four different myosin preparations, and the standard deviation is given for each point on the Figure. The final incubation times differed

in the four experiments, ranging from 50 to 92 hr., but the results did not show significantly increased incorporation of half-cystine after 28 hr. Experiments with even longer reaction times showed little further incorporation of counts. The theoretical maximum was taken as 8.36 moles/ 10^5 g., this being the cysteic acid recovery in amino acid analyses of performic acid-oxidized myosin (Table 1), and it is assumed that all the half-cystine occurring in myosin is in the free thiol form. To ensure that all the excess of cystine had been removed by dialysis, a control experiment was carried out in which the myosin and cystine were mixed at acid pH and then dialysed in parallel with the treated samples, and this showed that no radioactivity remained bound to the protein. Thus these experiments showed an average incorporation of about 75% of the theoretical value, with the best results slightly in excess of 80% of the theoretical value.

Other experiments in which the molar excess of reagent was varied showed that with a 200-fold molar excess of cystine it was possible to increase the labelling to up to 90%, whereas with a 20-fold molar excess the reaction reached only 50–60% of the theoretical value. Thus for preparative experiments to isolate the cystine-containing peptides of myosin a 100-fold excess of cystine was used, and, in the particular experiment described, 2g. of myosin were treated under these conditions, giving a final incorporation of counts equivalent to 75% of the theoretical value. However, Fig. 1 also shows that, as described below, by using 5M-guanidine hydrochloride in the last phase of the interchange reaction, it is possible to increase the half-cystine incorporation to well over 90% of the theoretical value.

Purification of the cystine peptides

The cystine-exchanged protein was digested with pepsin and a group fractionation of the peptides was carried out by chromatography on Zeo-Karb 225 (X2), as described in the Methods section. Fig. 2 shows the elution profile of radioactive peptides from the Zeo-Karb 225 (X2) column. The peptic digest of 2g. of cystine-exchanged myosin in 135 ml. of 1% (v/v) formic acid was applied to the column equilibrated with 0.1M-pyridine formate, pH 3.1, and the peptides were eluted with a series of buffers: 0.2M-pyridine formate, pH 3.5; 0.4M-pyridine formate, pH 4.0; 0.8M-pyridine formate, pH 4.5; 2.0M-pyridine formate, pH 5.5; 1.0M-ammonium acetate, pH 8.1 (in all cases the molarity is that of the cation). Less than 1% of the digest was insoluble in the 1% formic acid, and the recovery of counts in the 17 fractions was about 82% of those applied to the column.

The fractions were pooled, concentrated and submitted to ionophoresis at pH 6.5 as described in

Table 1. *Amino acid analyses of performic acid-oxidized myosin*

The analytical results are average values based on the following hydrolysates of oxidized samples: myosin 8, three 24 hr. hydrolysates and one 100 hr. hydrolysate; myosin 10, two 24 hr. hydrolysates and two 78 hr. hydrolysates; myosin 15, two 24 hr. hydrolysates. This last preparation had been purified by chromatography on cellulose phosphate (Harris & Suelter, 1967) and DEAE-Sephadex (Richards, Chung, Menzel & Olcott, 1967). Threonine and serine values were corrected for destruction by extrapolation to zero hydrolysis time, and the valine and isoleucine values were corrected to infinite hydrolysis time. For each series of analyses the cysteic acid recovery was corrected for losses during oxidation and hydrolysis, by analysis of a sample of chymotrypsinogen or insulin treated under identical conditions, as described in the text. The tyrosine values were based on single analyses of unoxidized samples. Since pepsin was used in the oxidation procedure for myosin 8 and myosin 10, these values have been corrected for a 1% contamination by pepsin. The standard deviations are shown from the mean of the analytical results for each preparation and from the mean of the average composition of the three preparations. Tryptophan content is omitted from these calculations, but was estimated from the spectrum of the protein to be $4.9 \text{ moles}/10^5 \text{ g.}$

Amino acid	Amino acid composition (moles/ 10^5 g.)				(moles/ $500\,000 \text{ g.}$) Mean
	Myosin 8	Myosin 10	Myosin 15	Mean	
Lys	88.3 ± 2.7	86.9 ± 1.6	92.0 ± 2.2	89.1 ± 2.1	445
His	15.5 ± 0.4	15.9 ± 0.2	15.8 ± 0.3	15.7 ± 0.2	79
Arg	41.3 ± 1.7	40.5 ± 0.4	42.6 ± 1.6	41.5 ± 0.9	208
Cys	8.48 ± 0.20	8.35 ± 0.22	8.24 ± 0.08	8.36 ± 0.10	42
Asp	88.1 ± 0.4	88.1 ± 1.6	86.0 ± 2.1	87.4 ± 1.0	437
Met	24.1 ± 0.8	25.0 ± 0.7	24.4 ± 0.2	24.5 ± 0.4	123
Thr	46.1	45.5	$45.0 \pm 0.9^*$	45.8	229
Ser	45.6	45.6	$41.2 \pm 0.1^*$	45.6	228
Glu	170.3 ± 5.8	170.8 ± 1.3	176.8 ± 2.3	172.6 ± 3.0	863
Pro	23.3 ± 0.5	23.7 ± 0.9	20.6 ± 0.4	22.5 ± 1.4	113
Gly	41.9 ± 1.3	41.9 ± 0.6	38.6 ± 1.4	40.8 ± 1.5	204
Ala	80.8 ± 2.4	77.4 ± 1.3	75.1 ± 1.0	77.8 ± 2.0	389
Val	45.2	46.2	$41.6 \pm 0.8^*$	45.7 ± 0.5	229
Ile	42.5	43.8	$41.1 \pm 0.4^*$	43.2 ± 0.7	216
Leu	82.5 ± 2.1	82.3 ± 0.6	84.6 ± 0.7	83.1 ± 1.0	416
Tyr	19.0	17.9	18.3	18.4 ± 0.5	92
Phe	29.7 ± 0.4	29.9 ± 0.3	27.5	29.0 ± 1.1	145

* These uncorrected results were omitted from the mean values.

the Methods section. Fig. 3 shows a drawing of the positions of the radioactive bands in each of the fractions and indicates their intensity. The percentage of counts in each of the fractions is given at the head of each column. The neutral bands from each pH 6.5 ionophoresis were submitted to further ionophoresis at pH 2, and Fig. 4 shows the radioactive bands thus separated. Attempts were made to purify all those peptides that are numbered in these Figures. The homogeneity of the radioactive peptide bands was first checked by submitting small strips to further ionophoresis at pH 3.5 or pH 2 or both pH values. In many cases a single radioactive band was found, and the cysteic acid peptide was isolated directly from this by performic acid oxidation of the strip followed by ionophoresis at pH 6.5. In other cases two cystine peptides were present, but these could be resolved as cysteic acid peptides after oxidation of the strip, either directly

(e.g. peptides 4a1 and 4a2) or by a subsequent ionophoresis at pH 3.5 (e.g. peptides 3a1 and 3a2). Occasionally the cystine peptide had to be purified before oxidation (e.g. peptide 10a1). In cases where an apparently major radioactive band at pH 6.5 fragmented into several minor components on further purification (e.g. peptide 7b), the isolation was abandoned.

Table 2 summarizes the purification procedure for the cysteic acid peptides derived from the radioactive bands shown in Fig. 2. The mobilities of the cystine peptides at pH 6.5 are indicated, and the number of components seen after ionophoresis at pH 2, with a qualitative estimate of their relative intensities. The cysteic acid peptides were isolated from these bands after performic acid oxidation by the purification procedure indicated, and the last two columns show their nomenclature and electrophoretic mobility at pH 6.5.

Fig. 2. Fractionation of $[^{35}\text{S}]$ cysteine-containing peptides on Zeo-Karb 225 (X2) resin. Stepwise elution was with the following buffers: A, 0.2M-pyridine formate, pH 3.5; B, 0.4M-pyridine formate, pH 4.0; C, 0.8M-pyridine formate, pH 4.5; D, 2.0M-pyridine formate, pH 5.5; E, 1.0M-ammonium acetate, pH 8.1. The fractions (1-17) were taken as shown by the horizontal arrows.

Fig. 3. Radioactive peptide bands obtained after ionophoresis at pH 6.5 of the 17 fractions from Fig. 2. At the head of each column the counts eluted in that fraction are given as a percentage of the total counts eluted in the 17 fractions. The density of the shading indicates the relative densities of the radioautographs. The letters show bands that were purified, as in Table 2.

Sequences of the cysteic acid peptides

Amino acid analyses and *N*-terminal analyses were made on each of the cysteic acid peptides shown in Table 2. The compositions and *N*-terminal residues thus determined are listed below, together with other sequence studies on these peptides. The ionophoretic mobilities of the peptides at pH 6.5, both in the cystine form and in the cysteic acid

Fig. 4. Ionophoresis at pH 2 of the neutral bands from Fig. 3. The numbers indicate radioactive bands that were further purified by pH 2 ionophoresis before oxidation with performic acid.

form, were used to determine the total number of charged groups in the peptides and hence the amide content. Neutral peptides clearly have acidic

Table 2. Purification of cysteic acid peptides

The first two columns show the radioactive bands indicated in Fig. 2, with their ionophoretic mobilities at pH 6.5 (Offord, 1966). The relative intensities of radioactive components of these bands were detected after ionophoresis of test strips at pH 2. The cysteic acid peptides were generally purified by oxidation of the paper strips followed by ionophoresis at pH 6.5 (Ox; 6.5) or at pH 2 (Ox; 2) but occasionally a preliminary purification at pH 2 preceded the oxidation (2; Ox; 6.5). Further purification of the cysteic acid peptides was by ionophoresis at pH 3.5 or pH 9. The final column gives the nomenclature of the peptides that were eluted.

Cystine peptides			'Diagonal' purification procedure	Cysteic acid peptides		
Band at pH 6.5		Number of components at pH 2 and intensity		Mobility at pH 6.5	Further purification	No.
No.	Mobility					
2a	0.88	++	Ox; 6.5	1.17	3.5	2a
3a	0.26	{+++ +++}	{Ox; 6.5	{0.66 0.60	— 3.5	3a1 3a2
4a	0.43	{++ ++}	{Ox; 6.5	{0.81 0.66	— —	4a1 4a2
5a	0.81	Trace	Ox; 6.5	1.10	—	5a
5b	0.62	+	Ox; 6.5	0.84	—	5b
5c	0.44	++	Ox; 6.5	0.65	—	5c
5d	0.32	+	Ox; 6.5	0.65	—	5d
6a	0.65	++	Ox; 6.5	0.80	—	6a
6b	0.11	+	Ox; 6.5	0.53	—	6b
7a	0.54	+	Ox; 6.5	0.72	—	7a
7b	0.20	3 Trace	—	—	—	—
7c	0.11	+++	Ox; 6.5	0.53	—	7c
8a	0.53	+	Ox; 6.5	0.72	—	8a
8b	0.11	+++	Ox; 6.5	0.51	—	8b
9a	0.18	2 Trace	—	—	—	—
9b	0.11	Trace	Ox; 6.5	0.51	—	9b
9c	0.00	{+ Trace}	Ox; 6.5	0.26	—	9c
9d	−0.37	++	Ox; 2	0.00	3.5	9d
10a	0.00	{+ Trace}	2; Ox; 6.5	0.44	3.5	10a1
11a	0.16	3 Trace	—	—	—	—
11b	0.10	4 Trace	—	—	—	—
11c	0.00	{++ Trace}	2; Ox; 6.5	0.25	9	11c1a and 11c1b
12a	0.00	{++ +}	Ox; 6.5	0.24	3.5	12a1a 12a1b
12b	−0.19	++	Ox; 2	0.00	3.5	12b
12c	−0.32	++	Ox; 2	0.00	3.5	12c
13a	0.00	{+++ Trace Trace}	2; Ox; 6.5	0.14 0.11	— —	13a1 13a2
13b	−0.19	+	Ox; 2	0.00	3.5	13b
13c	−0.34	++	Ox; 2	0.00	3.5	13c
14a	0.00	{++ Trace}	Ox; 6.5	0.26	3.5	14a
14b	−0.16	{+++ Trace	Ox; 6.5	0.00	3.5	14b
16a	−0.24	{+ Trace}	Ox; 6.5	0.00	3.5	16a
17a	−0.30	3 Trace (at pH 3.5)	—	—	—	—

groups equal to the number of lysine and arginine residues. Since an extra negative charge is introduced into a cystine peptide by oxidation, and the weight change is negligible, it follows that:

$$\frac{m(\text{before oxidation})}{m'(\text{after oxidation})} = \frac{e}{(e + 1)} \times \text{constant}$$

where *e* is the net negative charge before oxidation,

and the constant is related to the molecular weight of the peptide (Offord, 1966). For example, the cystine peptide 2a had mol. wt. 779 and m 0.88; after oxidation it had mol. wt. 734 and m' 1.17. Thus m/m' is 0.75 [i.e. $e/(e+1) = \frac{3}{4}$], which indicates that this cystine peptide had three negative charges and hence no amide groups (see below).

The location of amide groups in peptides containing both amide and free acid residues was determined by mobility measurements after ionophoresis of the peptides during the progress of the Edman degradation. The mobility of peptides containing lysine can be equivocal, since the acid-cyclization step of the Edman reaction may not fully remove the ϵ -phenylthiocarbamoyl group, and multiple bands may be found. However, under our conditions (trifluoroacetic acid for 1 hr. at 37°) good yields of ϵ -DNS-Lys were detected after reaction with DNS chloride at each step of the degradation, indicating that the ϵ -amino group in the peptide was free.

Peptide 2a. Analysis gave: Cya (0.9), Mso (0.9), Glu (3.0); *N*-terminus, Glx. This peptide was resistant to carboxypeptidase and was not attacked by either subtilisin or papain. Its mobility indicated three glutamic acid residues and one cysteic acid residue as described above. The sequence was determined by the 'dansyl'-Edman method, as shown below. (The symbol -Cya- represents an unequivocal identification of the indicated DNS-amino acid at the appropriate step of the Edman degradation. Doubtful or ambiguous results are discussed in the text. The symbol -Thr-Asp indicates amino acids released by carboxypeptidase, as discussed in the text.) At each step of the Edman degradation the mobility of the peptide was determined, and in each case a decrease of mobility was observed, indicating that a negative charge had been lost. After removal of cysteic acid, the remaining material was subjected to ionophoresis and gave only free methionine sulphone, confirming that this was the *C*-terminal residue. Hence we may write the sequence:

Peptide 5a. Analysis gave: Cya (+), Mso (+), Glu (+++), Phe (?); *N*-terminus, Glx. This peptide was recovered in very poor yield and only a qualitative amino acid analysis was made. The presence of phenylalanine was equivocal, it being masked by the large amount of glutamic acid in the pH2 ionophoretic separation. However, the mobility results suggested a peptide with four negative charges, slightly larger than peptide 2a, and so peptide 5a was probably Glu-Glu-Glu-Cya-Mso-Phe.

Peptide 5b. Analysis gave: Cya (0.9), Asp (0.9), Mso (0.9), Thr (1.0), Glu (3.2), Ala (1.0), Phe (0.5), Lys (0.9); *N*-terminus, Glx.

Peptide 6a. Analysis gave: Cya (1.0), Asp (1.0), Mso (0.9), Thr (0.9), Glu (3.1), Ala (1.1), Phe (1.1), Lys (1.0); *N*-terminus, Glx. The compositions, *N*-termini and mobilities suggest that peptides 5b and 6a were identical. Carboxypeptidase released alanine, threonine and aspartic acid from both, in the proportions (from peptide 6a) Ala (1.0), Thr (1.3), Asp (1.0).

A papain digest of peptide 6a was fractionated by ionophoresis at pH 6.5 and pH 3.5 to yield the fragments shown in Table 3. The sequence of peptide 6aN2 was determined by the 'dansyl'-Edman technique, as shown below. Hence we may write the sequence of peptide 6a and its fragments as:

Peptides 7a and 8a. Analysis gave: Cya (0.9), Asp (1.1), Mso (0.7), Thr (1.5), Ser (1.0), Glu (3.1), Ala (1.2), Phe (1.5), Lys (1.0); *N*-terminus, Glx. These peptides had similar compositions, mobilities and *N*-termini and were pooled for sequence determination. Carboxypeptidase gave

Table 3. *Papain fragments from peptide 6a*

Amino acid compositions were obtained by ion-exchange techniques where quantitative results are shown, otherwise by ionophoresis at pH 2. Ratios are uncorrected for hydrolysis losses.

Peptide	Composition	<i>m</i>	<i>N</i> -Terminus
6aN1	Asp (+)	1.00	Asx
6aN2	Cya (1.0), Mso (1.0), Glu (3.0), Phe (0.9), Lys (1.0)	0.77	Glx
6aN3	Cya (+), Mso (+), Glu (++), Ala (+), Phe (+), Lys (+)	0.73	Glx
6aN4	Asp (+), Thr (+)	0.67	Thr
6aN5	Asp (+), Thr (+), Ala (+)	0.54	Ala
6aN6	Thr (+), Ala (+)	0.00	Ala

phenylalanine and a trace of serine. A tryptic digest of peptide 7a was fractionated at pH 6.5 to give peptide 7aT1 [Cya (0.9), Mso (1.0), Glu (3.0), Phe (1.0), Lys (1.0); *m* 0.77; *N*-terminus, Glx] and peptide 7aT2 [Asp (1.0), Thr (2.0), Ser (1.2), Ala (1.1), Phe (1.0); *m* 0.37; *N*-terminus, Ala]. The sequences of both these peptides were determined by the 'dansyl'-Edman technique, and so we may write the sequence of peptide 7a or peptide 8a and its fragments as:

Glu-Glu-Glu-Cya-Mso-Phe-Lys-Ala-Thr-Asp-Thr-Ser-Phe

Peptide 3a1. Analysis gave: Cya (0.8), Glu (1.0), Ala (1.7), Ile (0.9), Leu (1.1); *N*-terminus, Ala. Carboxypeptidase released only leucine, and subtilisin digestion released some alanine. The mobility of the parent cystine peptide 3a (Table 2) showed that the peptide contained glutamic acid. The sequence was determined by the 'dansyl'-Edman method:

Peptide 4a1. Analysis gave: Cya (0.8), Glu (1.3), Ile (1.0), Leu (1.0); *N*-terminus, Cya. Carboxypeptidase released only leucine, and the 'dansyl'-Edman method confirmed that this peptide was related to peptide 3a1, i.e.:

Peptide 3a2. Analysis gave: Cya (0.9), Asp (2.9), Ser (1.0), Gly (1.0), Ile (1.0), Tyr (0.8); *N*-terminus, Asx. Tyrosine alone was released with carboxypeptidase, and the sequence was partially determined by the 'dansyl'-Edman method as follows:

The mobility change on oxidation indicated that peptide 3a2 contained two amide groups. Thus it was necessary to determine which one of the three aspartic acid residues was in the carboxyl form. The mobility change after removal of the *N*-terminal residue was from 0.61 to 0.33, suggesting that an acidic residue had been removed. Thus the charge assignment was as given in the sequence.

Peptide 4a2. Analysis gave: Cya (0.9), Asp (2.9), Mso (0.3), Ser (1.0), Glu (2.2), Gly (2.0), Ala (1.1),

Ile (1.0), Tyr (0.6); *N*-terminus, Ala, with a trace of Mso.

Peptide 5c. Analysis gave: Cya (0.8), Asp (3.0), Mso (0.8), Ser (1.0), Glu (2.3), Gly (1.9), Ala (1.1), Ile (1.0), Tyr (0.4); *N*-terminus, Mso. The action of carboxypeptidase on both peptides 4a2 and 5c was to release tyrosine, and the similarity of composition and mobility suggested that they might be related. This was confirmed by comparison of the peptide 'maps' after digestion with papain. The only significant difference between these ionograms was that peptide 5cN5, which gave the amino acid sequence Mso-Ala-Gly by the 'dansyl'-Edman method, had only a weak counterpart in the digest of peptide 4a2 (probably representing contamination of peptide 4a2 by peptide 5c). This could be explained, since peptide 5cN5 was the *N*-terminal sequence of peptide 5c, whereas the *N*-terminal sequence of 4a2 was Ala-Gly. Table 4 shows the compositions, mobilities and *N*-terminal residues of the major papain fragments from peptide 5c.

Peptide 5cN6 was shown to be Ala-Gly by the 'dansyl'-Edman method, and the presence of free methionine sulphone in the digest suggested a further papain cleavage of peptide 5cN5.

Peptide 5cN4 gave the sequence Cya-Ile-Asn-Tyr,

Table 4. *Papain fragments from peptide 5c*

Amino acid compositions were obtained by ion-exchange techniques where quantitative results are shown, otherwise by ionophoresis at pH 2. Ratios are uncorrected for hydrolysis losses.

Peptide	Composition	<i>m</i>	<i>N</i> -Terminus
5cN1	Cya (0.4), Asp (2.2), Ser (0.9), Glu (1.9), Gly (1.0), Ile (0.4)	0.75	Glx
5cN2	Cya (0.9), Asp (3.0), Ser (1.0), Glu (2.2), Gly (1.2), Ile (1.0), Tyr (0.4)	0.72	Glx
5cN3	Asp (2.2), Ser (0.9), Glu (0.4), Gly (1.2)	0.56	Asx
5cN4	Cya (1.0), Asp (1.2), Ile (1.0), Tyr (0.9)	0.48	Cya
5cN5	Mso (+), Gly (+), Ala (+)	0.11	Mso
5cN6	Gly (+), Ala (+)	0.00	Ala

Peptide 9c. Analysis gave: Cya (0.9), Asp (1.0), Thr (0.7), Ser (0.4), Glu (1.2), Ala (3.7), Val (0.9), Leu (1.0), Arg (0.9); *N*-terminus, Val. Carboxypeptidase released leucine and a little alamine. Although very little of this peptide was available, the 'dansyl'-Edman analysis appeared unequivocal up to the stage of removal of the eighth residue (Cya). After this step, the remaining peptide was treated with DNS chloride and the product purified by ionophoresis at pH 6.5 in a flat-plate apparatus. Hydrolysis of this DNS-peptide gave DNS-Ala plus approximately equimolar amounts of alanine and leucine, which were detected with cadmium-

ninhydrin reagent after pH 2 ionophoresis of the hydrolysate. Hence peptide 9c was:

Since the cystine precursor was neutral, the peptide must contain one amide group. This ambiguity was resolved by the sequence of peptides derived from tryptic digests of light meromyosin that had been exchanged with cystine or cystamine (Weeds, 1967a): Thr-Asn-Ala-Ala-cystamine, Thr-Asn-Ala-Ala-Cya-Ala-Ala-Leu-Asp-Lys-Lys, and Ser-Asn-Ala-Ala-Cya-Ala-Ala-Leu-Asp-Lys-Lys. The last two peptides appeared to be variants of the same cystine sequence, and the composition of peptide 9c shown above suggested that here too a minor component might be present in which serine replaced threonine.

In a previous paper (Weeds & Hartley, 1967) we made an error in locating the amide group in peptide 9c, based on a wrong interpretation of the mobility of the DNS derivative. However, the evidence presented by Weeds (1967a) made it certain that the sequence of peptide 9c was Val-Glu-Arg-Thr(or Ser)-Asn-Ala-Ala-Cya-Ala-Leu.

Peptide 9d. Analysis gave: Cya (1.0), Ser (1.3), Ala (2.1), Leu (1.0), Lys (0.9); *N*-terminus, Ala. Carboxypeptidase confirmed that leucine was *C*-terminal, and the peptide sequence was determined by the 'dansyl'-Edman method as:

Peptide 10a1. Analysis gave: Cya (1.0), Glu (1.4). Only 0.02 μ mole of this peptide was recovered from the purification, so the sequence had to rely solely on evidence from the 'dansyl'-Edman method. The unoxidized peptide was neutral, indicating that the glutamic acid was charged, and the sequence was determined as:

Peptide 11c1a. Analysis gave: Cya (1.0), Asp (1.0), Thr (1.3), Glu (1.2), Val (0.6), Ile (0.5), Leu (1.0), Phe (1.0), Lys (0.3), Arg (0.9); *N*-terminus, Leu (++), Val (+). Peptide 11c was clearly heterogeneous even though it gave a single band at both pH 3.5 and pH 2; ionophoresis at pH 9 resolved two bands of which peptide 11c1a was the major. The analysis and *N*-termini suggested that it was still contaminated, but the 'dansyl'-Edman analysis was relatively unambiguous and indicated a major peptide of sequence Leu-Thr-Cya-Glx-Ile-Asx-Arg-Phe. Since the cystine precursor (peptide 11c) was neutral, there must be one amide group. After five steps of Edman degradation, the DNS-peptide, DNS-Asx-Arg-Phe, was acidic at pH 6.5, suggesting aspartic acid rather than asparagine.

Hence peptide 11c1a was probably Leu-Thr-Cya-Gln-Ile-Asp-Arg-Phe.

A clue to the identity of the contaminant, peptide 11c1b, in peptide 11c1a was obtained from a tryptic digest of another sample of peptide 11c1 that had not been purified at pH 9, in which the yield of the contaminant was presumably higher. Peptide 11c1a did not appear to have been appreciably split in this digest, since the mobility of the major band was identical and only traces of free phenylalanine were found. This is understandable, since the arginine was next to an acidic residue and close to the *C*-terminus. But two other peptides were observed in pH 6.5 ionophoresis of this digest: a basic dipeptide whose sequence was found to be Val-Arg, and an acidic peptide of mobility 0.73 at pH 6.5 [Cya (0.9), Asp (1.1), Glu (1.2), Pro (0.9), Ile (1.0)]. The mobility suggested two negative charges and hence either aspartic acid or glutamic acid. The 'dansyl'-Edman method gave the sequence Cya-Ile-Ile-Pro-Asx-Glx. Hence peptide

11c1b was almost certainly Val-Arg-Cya-Ile-Ile-Pro-Asn-Glu, which is a peptic fragment of peptide 12b1 (see below). The low value for isoleucine in the analysis was almost certainly due to the resistance of the Ile-Ile bond to hydrolysis. Cleavage of about 50% in 20hr. has been observed with similar sequences in this Laboratory.

Peptide 12a1a. Analysis gave: Cya (1.0), Asp (2.0), Val (0.9), Ile (0.9), Tyr (1.9), Lys (1.0); *N*-terminus, Val. Since this peptide was neutral before oxidation it must contain one aspartic acid residue and one asparagine residue. Carboxypeptidase liberated only tyrosine, and the 'dansyl'-Edman method gave Val-Asx-Lys-Asx-Ile-Cya-Tyr-Tyr. The mobility of the DNS-peptide increased after removal of the first Asx residue in the Edman procedure, suggesting the sequence:

Peptide 12a1b. Analysis gave: [Cya (1.0), Asp (2.2), Val (0.6), Ile (0.8), Tyr (1.7), Lys (+); *N*-terminus, Asx. This peptide was difficult to separate from peptide 12a1a and was still contaminated by the latter. It was distinguishable by the yellow colour with cadmium-ninhydrin (consistent with *N*-terminal asparagine rather than aspartic acid), compared with the slowly developing red colour of peptide 12a1a. A qualitative analysis of a hydrolysate by ionophoresis at pH 2 showed that lysine was present in approximately the same amount as cysteic acid and valine, but because of the limited amount of material the basic amino acids were not analysed quantitatively. Carboxypeptidase gave only tyrosine, so we may conclude that peptide 12a1b was related to peptide peptide 12a1a, i.e. Asn-(Lys,Asp,Ile,Cya,Tyr)-Tyr.

Peptides 12b and 13b. Analysis gave: Cya (0.9), Mso (0.9), Asp (1.1), Thr (1.8), Glu (1.1), Pro (1.2), Gly (1.0), Ala (1.0), Val (0.8), Ile (1.1), Lys (1.0), Arg (0.7); *N*-terminus, Val.

These peptides were pooled for sequence study, being similar in composition, mobility and *N*-terminus. The peptide was resistant to carboxypeptidase. A tryptic digest was fractionated by ionophoresis at pH 6.5 and pH 3.5 to give the following products.

Peptide 12bT1. Analysis gave: Cya (0.9), Asp (1.0), Thr (0.8), Glu (1.0), Pro (1.1), Ile (1.2), Lys (1.0); *m* 0.34; *N*-terminus, Cya. The 'dansyl'-Edman method gave the partial sequence Cya-Ile-Ile-Pro-Asx-Glx-(Thr,Lys). A very low yield of DNS-Pro was found at the fourth position, but this DNS-amino acid is recovered in only 20% yield after 18 hr. hydrolysis (Gray, 1964).

Peptide 12bT2. Analysis gave: Mso (1.0), Thr (1.0), Pro (1.0), Gly (1.1), Ala (1.1); *m* 0.00; *N*-terminus, Thr. The 'dansyl'-Edman method gave Thr-Pro-Gly-Ala-Mso.

Peptide 12bT3. Analysis gave: Val (1.0), Arg (1.1); *m* -0.55; *N*-terminus, Val. Since valine was *N*-terminal in the whole peptide, and assuming that lysine was *C*-terminal in peptide 12bT1 because of the specificity of trypsin, we may write the sequence of peptide 12b:

Since the oxidized peptide was neutral at pH 6.5, the peptide must contain one amide group.

Peptide 13a1. Analysis gave: Cya (0.9), Asp (1.2), Mso (1.0), Thr (1.8), Glu (3.4), Pro (1.9), Gly

(1.1), Ala (1.2), Val (0.9), Ile (1.5), Leu (0.6), Lys (1.2), His (1.0), Arg (0.9), after 72 hr. hydrolysis; *N*-terminus, Val. Tryptic digestion of this peptide gave five peptides, which were separated by ionophoresis at pH 6.5 and by further ionophoresis of the neutral band at pH 3.5. The resultant peptides are shown in Table 5 together with compositions, mobilities and *N*-terminal groups.

Peptide 13a1T5 was Val-Arg, the same basic dipeptide as found in peptide 12b. Peptide 13a1T4 had the sequence Glx-Thr-Lys by the 'dansyl'-Edman method and was neutral at pH 6.5, indicating glutamic acid. Peptide 13a1T1 gave the sequence Cya-Ile-Ile-Pro-Asx, but its mobility was

consistent with a single-charged pentapeptide; thus the aspartic acid was in the amide form. Peptides 13a1T1 and 13a1T4 probably arose by an unusual but not unprecedented minor chymotryptic cleavage at the Asn-Glu bond, catalysed by impurities in the trypsin. The other tryptic product containing cysteic acid, peptide 13a1T2, was Cya-Ile-Ile-Pro-Asx-(Glx,Thr,Lys), overlapping

peptides 13a1T1 and 13a1T4.

Peptide 13a1T3 had a mobility consistent with both the glutamic acid residues carrying charge, and

Table 5. *Tryptic fragments from peptide 13a1*

Amino acid compositions were obtained by ion-exchange techniques where quantitative results are shown, otherwise by ionophoresis at pH 2. Ratios are uncorrected for hydrolysis losses.

Peptide	Composition	<i>m</i>	<i>N</i> -Terminus
13a1T1	Cya (1.0), Asp (1.0), Pro (1.0), Ile (1.3)	0.49	Cya
13a1T2	Cya (0.8), Asp (1.0), Thr (0.9), Glu (1.2), Pro (0.9), Ile (1.1), Leu (0.4), Lys (0.9)	0.34	Cya
13a1T3	Mso (1.0), Thr (1.0), Glu (2.0), Pro (0.9), Gly (1.0), Ala (1.0), His (1.2)	0.31	Thr
13a1T4	Thr (1.0), Glu (1.0), Lys (1.0)	0.00	Glx
13a1T5	Val (+ +), Arg (+ +)	-0.57	Val

Table 6. *Tryptic fragments from peptides 13a2 and 14b*

Amino acid compositions were obtained by ion-exchange techniques where quantitative results are shown, otherwise by ionophoresis at pH 2. Ratios are uncorrected for hydrolysis losses.

Peptide	Composition	<i>m</i>	N-Terminus
13a2T1	Asp (+), Thr (+), Glu (+ +), Leu (+)	0.70	—
14bT1	Asp (0.3), Thr (0.9), Glu (1.2), Leu (1.0)	0.49	Thr
14bT2	Asp (0.9), Thr (0.6), Glu (1.2), Leu (1.0)	0.47	Leu
13a2T5	Cya (+), Mso (+), Arg (+)	0.11	Mso
14bT3	Cya (1.0), Mso (0.9), Arg (1.2)	0.12	Mso
13a2T7a	Thr (+), Ser (+), Glu (+), Val (+), Lys (+)	0.00	—
14bT4a	Thr (1.1), Ser (1.4), Glu (1.0), Val (1.3), Lys (0.9)	0.00	Glx
13a2T7b	Asp (+), Glu (+), Gly (+), Leu (+), Lys (+)	0.00	—
14bT4b	Asp (1.0), Glu (1.1), Gly (0.7), Leu (0.9), Lys (1.0)	0.00	Gly
14bT5	Glu (1.0), Ala (1.0), Leu (0.9), Arg (1.3)	−0.38	Leu
13a2T8	Leu (+), Lys (+)	−0.53	Leu
14bT6	Leu (1.0), Lys (1.0)	−0.53	Leu

Peptide 12c. Analysis gave: Cya (0.9), Asp (2.0), Glu (2.5), Ile (2.0), Leu (1.8), Tyr (0.9), Lys (0.8), Arg (0.9); *N*-terminus, Lys. This peptide was very difficult to purify from contaminants and was finally recovered in poor yield after an additional ionophoresis at pH 3.5. Carboxypeptidase released glutamine, leucine and tyrosine in approximately equimolar amounts, and the 'dansyl'-Edman method gave the *N*-terminal sequence Lys-Asx-Ile-Cya-Arg-. Hence the partial sequence of peptide 12c was probably Lys-Asx-Ile-Cya-Arg-(Asx₁₋₂,Ile,Leu)-(Gln,Leu,Tyr).

Peptides 13a2 and 14b. Both these peptides were finally isolated in rather poor yield, and their amino acid analyses indicated contamination but suggested that the peptides might be related in sequence. This conclusion was confirmed by carrying out tryptic digests of these peptides and fractionating the products by ionophoresis at pH 6.5 with further separation of the neutral bands at pH 3.5. The mobilities of several peptides were identical and their compositions, mobilities and *N*-terminal residues are shown in Table 6.

Peptides 13a2T5 and 14bT3 were the only cysteine acid peptides and gave the sequence Mso-Cya-Arg by the 'dansyl'-Edman method. The limited amount of material made it impossible to determine unequivocally the sequences of the other fragments or any overlaps between them.

Peptide 13c. Analysis gave: Cya (1.0), Asp (1.0), Gly (1.0), Val (0.9), Leu (2.0), Arg (1.1); *N*-terminus, Arg. The 'dansyl'-Edman analysis suggested the *N*-terminal sequence Arg-Cya-Asx-. The cystine precursor was basic at pH 6.5; hence the peptide contained asparagine and not aspartic acid. The partial sequence must therefore be Arg-Cya-Asn-(Gly,Val,Leu₂).

Peptide 14a. Analysis gave: Cya (1.0), Asp (1.2), Thr (1.8), Glu (1.3), Leu (1.1), Phe (0.9), Lys (0.9),

Arg (1.1); *N*-terminus, Leu. Carboxypeptidase released only phenylalanine, and the 'dansyl'-Edman analysis gave Leu-Thr-Thr-Glx-Asx- as the *N*-terminal sequence. Since the cystine precursor was neutral it must contain two negatively charged residues. Hence the partial sequence must be:

Peptide 16a. Analysis gave: Cya (1.0), Asp (1.1), Glu (1.2), Pro (0.9), Gly (1.0), Val (2.0), Leu (0.9), Tyr (0.3), Lys (1.0), Arg (1.0); *N*-terminus, Leu. The peptide was resistant to carboxypeptidase. A tryptic digest of this peptide was fractionated by ionophoresis at pH 6.5, and the neutral band was re-run at pH 3.5 and gave the following products.

Peptide 16aT1. Analysis gave: Asp (1.0), Glu (1.0), Gly (1.0), Val (0.9); *m* 0.48 at pH 6.5; *N*-terminus, Val. The 'dansyl'-Edman method gave the sequence Val-Gly-Asx-Glx.

Peptide 16aT2. Analysis gave: Cya (1.0), Pro (0.9), Leu (1.0), Tyr (0.8), Arg (1.1); *m* 0.00 at pH 6.5; *N*-terminus, Leu. The 'dansyl'-Edman method gave the sequence Leu-Cya-Tyr-Pro-Arg.

Peptide 16aT3. Analysis gave: Val (0.9), Lys (1.0); *m* −0.62 at pH 6.5; *N*-terminus, Val. The 'dansyl'-Edman method gave the sequence Val-Lys.

The cystine precursor was basic at pH 6.5, so peptide 16a must contain either aspartic acid and glutamine or glutamic acid and asparagine. Since the *N*-terminus was leucine, peptide 16aT2 must be the *N*-terminal piece, and peptide 16aT1 must be *C*-terminal because it contained no basic residue. Hence the sequences of peptide 16a and its tryptic fragments were:

Fig. 5. Two-dimensional peptide 'maps' of acidic radio-active peptides in peptic digests of cystine-exchanged myosin, separated by ionophoresis at pH 6.5 and pH 2. (a) Native myosin exchanged under the standard conditions (see the Methods section); (b) myosin further exchanged in 5M-guanidine hydrochloride (see the text). The letters indicate spots referred to in Table 7.

Fig. 6. Two-dimensional peptide 'maps' of radioactive peptides in peptic digests of cystine-exchanged myosin, from the neutral band at pH 6.5, separated by ionophoresis at pH 3.5 and chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:10:12, by vol.) (BAWP). (a) Native myosin exchanged under standard conditions (see the Methods section); (b) myosin further exchanged in 5M-guanidine hydrochloride (see the text). Other details are as in Fig. 5.

Accessibility of thiol groups to cystine exchange

The above investigation of the labelled peptides from cystine-exchanged myosin leaves open the possibility that some thiol groups exchanged rapidly with cystine while others were almost immune. We therefore compared peptide 'maps' of similar peptic digests of native myosin that had been exchanged for 2 min., 30 min. or 92 hr. with a 100-fold excess of radioactive cystine under the standard conditions. In each case the total number and positions of the radioactive spots were similar to those shown in Figs. 5(a), 6(a) and 7(a), though the intensities were markedly different in each case. Some of the well-separated spots were cut out from each paper and

Fig. 7. Two-dimensional peptide 'maps' of basic radioactive peptides in peptic digests of cystine-exchanged myosin, separated by ionophoresis at pH 6.5 and chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:10:12, by vol.) (BAWP). (a) Native myosin exchanged under standard conditions (see the Methods section); (b) myosin further exchanged in 5M-guanidine hydrochloride (see the text). Other details are as in Fig. 5.

Table 7. Rate of cystine exchange with some individual thiol groups

The counts in each of the lettered spots from peptide maps of myosin (Figs. 5a, 6a and 7a) exchanged with [^{35}S]cystine for 2 min. and 30 min. are expressed as percentages of the final value obtained after 92 hr. exchange. The assignment of the appropriate cysteine acid sequence (Table 8) has been made on the basis of the mobilities of the cystine peptides at pH 6.5 and pH 2.

Peptide spot	Radioactivity in spot (% of counts after 92 hr.)		Cysteic acid sequence
	2 min. exchange	30 min. exchange	
a	7.3	15.1	3a1
b	7.8	16.2	6a
c	8.7	19.1	7a
d	10.4	18.7	2a
e	14.7	19.8	4a1
f	16.1	28.3	—
g	20.3	65.7	7c and 8b
h	27.7	67.1	3a2
i	29.5	70.5	—
j	32.3	74.7	4a2 and 5c
k	39.3	91.8	—
l	46.5	89.3	—

counted in the scintillation counter. Table 7 shows the number of counts in each spot as a percentage of the final value at 92 hr. The correspondence between each spot and the indicated cysteine acid

peptide was established by investigating the mobilities of the radioactive peptides at pH 6.5 and pH 2. There appeared to be a class of thiol groups that exchanged rapidly with cystine (e.g. spots g-l) and another that reacted much more slowly (e.g. spots a-f). It is also quite likely that there was a third class of thiol groups in myosin that exchanged to an insignificant extent with cystine and that might not have been detected in these radioautographs. The inference that the data in Table 7 reflected accessibility of the appropriate thiol group in the native molecule is supported by the fact that peptic peptides that derived from the same thiol sequence (e.g. spots b, c and d, or spots h and j) showed similar rates of exchange. Our conclusion is similar to that of Rainford, Hotta & Morales (1964), that there were three classes of thiol groups in native myosin that reacted at considerably different rates with thiol reagents.

We tried to investigate the possibility that unfolding the molecule would expose additional thiol groups to exchange with radioactive cystine. Experiments in which the exchange was carried out in 8M-urea or 5M-guanidine solutions resulted in low degrees of incorporation (less than 60%). We therefore examined the effect of an additional cystine exchange in 5M-guanidine hydrochloride on native myosin that had been exchanged for 74 hr. at 0° under the standard conditions to its plateau level of incorporation, in this case 74% (see Fig. 1). After an additional 20 hr. at pH 9.6 and 37° in the presence of 5M-guanidine hydrochloride, 2mM-EDTA and a 100-fold molar excess of radioactive cystine, followed by removal of the excess of cystine in the usual way, the degree of incorporation of counts increased to 93% of the theoretical value. Similar experiments in which the thiol groups of myosin were exchanged with [³⁵S]cystamine (A. G. Weeds, unpublished work) confirmed the conclusion that almost stoichiometric exchange of all the thiol groups can be obtained by this technique of exchange of the native molecule followed by exchange in 5M-guanidine hydrochloride.

The question arises, however, whether the 'buried' groups represent unique sequences or are a random sample of the groups already observed. To attempt an answer, we compared peptide 'maps' of the native form with those of myosin exchanged additionally in denaturing conditions. Figs. 5, 6 and 7 are tracings of the radioautographs in these experiments. Additional peptide spots were clearly visible in the 'maps' from denatured myosin (Figs. 5b, 6b and 7b). Unfortunately, however, this could not be taken as unequivocal evidence for unique 'buried' thiol groups, since certain of the bands in the map from the native cystine-exchanged myosin disappeared or became weaker after the guanidine treatment. This could have been an effect of a

different specificity of pepsin towards acid-denatured cystine-exchanged myosin or the guanidine-denatured protein. On balance, though, we lean to the view that some of the spots in Figs. 5(b), 6(b) and 7(b) represented thiol sequences that had not been exchanged in the native molecule.

DISCUSSION

This work was partly undertaken to test a selective purification procedure for thiol peptides. We originally investigated a diagonal ionophoretic technique in which the thiol groups of a protein were first treated with iodoacetic acid. The carboxymethylated protein was then digested enzymically and the peptides were subjected to paper ionophoresis at pH 3.5. When a strip from this paper was exposed to the vapour of performic acid, the carboxymethylcysteine residue was oxidized to its sulphone with a lower pK for the side-chain carboxyl group. Hence after ionophoresis at right angles at pH 3.5 the peptides containing the sulphone emerged from an undifferentiated diagonal. However, this method was severely limited, since the ionophoretic mobility of the peptides was affected only at pH 3.5, and at this pH minor variations in the ionophoretic conditions can affect the pK of the C-terminal carboxyl group of all peptides and result in a very diffuse 'diagonal'. Moreover, the carboxymethylcysteine sulphone was unstable at high pH, where it appeared to undergo elimination reactions.

We therefore turned to the cystine-exchange method. This has the advantage over alkylation techniques of being completely specific for thiol groups, and gives the stable cysteic acid peptide after the diagonal procedure. A disadvantage is that the radioactive label is lost during the performic acid oxidation. Another disadvantage is that cystine is sparingly soluble in the high concentrations necessary for complete substitution of the protein. This difficulty has subsequently been circumvented by using [³⁵S]cystamine instead of cystine in studies with light meromyosin fraction 1 (Weeds, 1967a) with the carbonate subfragment of myosin (Weeds, 1967b) or with myokinase (Weeds & Noda, 1968).

Table 8 summarizes the sequences of the thiol peptides found in this work. They appear to arise from 16 unique thiol residues in the protein. Although we have tried to isolate the cysteic acid peptide from every significant radioactively labelled component in the peptic digest, we believe that the total number of unique thiol sequences will prove to be greater than 16. The broad specificity of pepsin inevitably means that several peptides may arise from the same thiol sequence. If the number of these were large in any one case, it is quite possible

Table 8. *Summary of cysteic acid sequences from cystine-exchanged myosin*

Peptide	Amino acid sequence
7a and 8a	Glu-Glu-Glu-Cya-Mso-Phe-Lys-Ala-Thr-Asp-Thr-Ser-Phe
5b and 6a	—
2a	—
3a1	Ala-Ala-Cya-Ile-Glu-Leu
4a1	—
5c	Mso-Ala-Gly-Gln-Glu-Asp-Ser-Asn-Gly-Cya-Ile-Asn-Tyr
4a2	—
3a2	—
5d	Cya-Ile-Asn-Phe-Thr-Asn-Glu
6b and 7c	—
8b and 9b	Cya-Ile-Gln-Tyr
9c	Val-Glu-Arg-Thr-Asn-Ala-Ala-Cya-Ala-Ala-Leu
9d	Ala-Lys-Cya-Ala-Ser-Leu
10a1	Ala-Glu-Cya-Arg-Leu
11c1a	Leu-Thr-Cya-Gln-Ile-Asp-Arg-Phe
12a1a	Val-Asn-Lys-Asp-Ile-Cya-Tyr-Tyr
13a1	Val-Arg-Cya-Ile-Ile-Pro-Asn-Glu-Thr-Lys-Thr-Pro-Gly-Ala-Mso-Glu-His-Glu
12b and 13b	—
11c1b	—
12c	Lys-Asx-Ile-Cya-Arg-(Asx, Glx ₁₋₂ , Ile, Leu)-(Gln, Leu, Tyr)
14bT3	Mso-Cya-Arg
13c	Arg-Cya-Asn-(Gly, Val, Leu ₂)
14a	Leu-Thr-Thr-Glu-Asp-(Cya, Lys, Arg)-Phe
16a	Leu-Cya-Tyr-Pro-Arg-Val-Lys-Val-Gly-Asx-Glx

Table 9. *Thiol sequences of myosin (Kimura & Kielley, 1966)*

These data were obtained for peptides containing carboxymethylcysteine (Ccm) from a tryptic digest of myosin treated with iodo[¹⁴C]acetic acid.

Peptide	Amino acid sequence	Probable corresponding cysteic acid sequence (Table 8)
B-1-a	(Ccm, Asx, Ser, Glx ₃ , Leu ₂)-Lys-Lys	—
C-1-a	Ccm-Ile-Pro-Asx-Glx-Thr-Lys	13a1
C-2-a	(Ccm, Asx ₂ , Ala ₃ , Leu)-Lys	9c
D-1-a	Ccm-Gly-Asn-Val-Leu-Glu-Gly-Ile-Arg	13c
D-1-b		
D-2-b		
D-2-a	Ccm-Asx-Glx-(Ile, Leu)-Lys	—
D-3-a	Ccm-Ala-Ser-Leu-Glu-Lys	9d
D-4-a	(Ccm, Asx ₃ , Leu ₂)-Arg	—
E-1-a	(Ccm, Ser, Glx ₂ , Ile ₂ , Phe)	—
E-2-a-1	(Ccm, Asx, Thr, Glx, Ala, Ile)-Arg	10a1
E-2-a-2	(Ccm, Asx, Ser, Leu)-Arg	—
E-3-a	Thr-Gln-(Ccm, Ala, Met)-Arg	—
F-1-a	Ccm-Arg	14a
F-2-a	Ile-Ccm-Arg	12c
F-3-a	Met-Ccm-Arg	14bT3
F-4-a	(Ccm, Ala, Pro, Leu)-Arg	16a

that every such representative would have been overlooked as a trace component. Moreover, our purification techniques could select against particularly hydrophobic thiol peptides. The recovery of counts from the Zeo-Karb 225 column (82%) was reasonably high, but the missing margin (18%)

could represent up to three more thiol sequences that were bound extremely tightly and specifically to the column. A third reason why our determination of 16 unique thiol sequences may represent a minimum lies in the incomplete extent of the disulphide interchange. Under the conditions that

we generally used, only 80% of the total thiol groups reacted. If the missing 20% were concentrated into specific 'buried' thiol groups, up to four more unique groups could be involved. The experiments described above, in which the exchange was continued in the presence of 5M-guanidine hydrochloride, lend support to the idea that some unique thiol groups are resistant to exchange with cystine in the native molecule. Hence we estimate that our discovery of 16 unique thiol peptides might indicate a total of over 20 unique thiol sequences in myosin.

This conclusion is supported by consideration of the sequences of some peptides isolated from tryptic digests of carboxymethylated myosin by Kimura & Kielley (1966). Since these authors used both a different labelling method for thiol groups and different digestion and purification methods for the peptides, the chances are increased that they might have discovered some of our 'missing' sequences. Table 9 lists the peptides described by Kimura & Kielley (1966). We have pointed out previously (Weeds & Hartley, 1967) that, even with a pessimistic bias towards the possibility of errors in our sequences or those of Kimura & Kielley (1966), there appears to be a total of 22 unique thiol sequences in myosin. Some recent studies with cystamine-exchanged light meromyosin fraction 1 (Weeds, 1967a) have shown that it contains sequences 9c and 9d (Table 8), plus an additional thiol sequence: His-Asp-Cys-Asp-Leu-Leu-Arg. This thiol sequence was not located in the peptic digest of cystine-exchanged myosin, probably because it was found to react poorly with disulphides in the absence of guanidine hydrochloride (A. G. Weeds, unpublished work). This peptide may be related to peptide D-4-a (Kimura & Kielley, 1966) (Table 9).

In a recent study on heavy meromyosin subfragment 2 (A. G. Weeds, unpublished work), two other peptides have been isolated that were not found in this study: Cys-Asp-Gln-Leu-Ile-Lys and Lys-Leu-Glu-Asp-Glu-Ser-(Cys,Glu)-Leu-Lys-Lys. These correspond to peptides D-2-a and B-1-a (Kimura & Kielley, 1966) (Table 9).

The purity of myosin preparations is always doubtful, and the possibility must be considered that some of the thiol peptides are derived from impurities. Any such peptide would have to be present in roughly stoichiometric amounts with the myosin, and our protein preparations met the accepted criteria of purity. But we are particularly impressed with the observation that myokinase, which is frequently a contaminant of myosin, has two unique thiol sequences (Weeds & Noda, 1968), which are not represented in Table 8. Hence it is unlikely that any less common impurity could have been detected in our peptic peptides.

Therefore we believe that myosin has between 20

and 22 unique thiol groups. Taking the molecular weight as 500 000 (Tonomura *et al.* 1966), our cysteine acid analysis indicates 42 half-cystine residues/mol.; so we have argued (Weeds & Hartley, 1967) that myosin contains two identical chains. Our conclusion is supported by the studies of cystamine-exchanged light meromyosin fraction 1, mentioned above, in which three unique thiol groups and a half-cystine content of 6.7 residues/mol. of mol.wt. 130 000 also point to a structure with two identical chains.

Two of the thiol groups of myosin have been implicated in its adenosine triphosphatase activity. Yamashita *et al.* (1964) treated myosin with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide to the extent of 1.5 moles/200 000 g. of sub-unit, and observed a 12-fold stimulation of the Ca^{2+} -activated adenosine triphosphatase activity, whereas the EDTA-activated adenosine triphosphate activity was decreased to 8% of the control value. They isolated a single labelled peptide whose sequence was Ile-Cys-Arg. This must correspond to our peptide 12c1: Lys-Asx-Ile-Cys-Arg-(Asx,Glx₁₋₂,Ile)-(Gln,Leu,Tyr). Yamashita, Soma, Kobayashi & Sekine (1965) similarly treated myosin with the maleimide reagent in the presence of ATP. In this case the Ca^{2+} -stimulated adenosine triphosphatase activity was inhibited, and a unique labelled peptide, Cys-Asx-Gly, was isolated. This may correspond to our peptide 13c1: Arg-Cys-Asn-(Gly,Val,Leu₂). Both the thiol groups represented in these sequences appeared to exchange poorly with cystine, which supports the conclusion of Stracher (1964) that the thiol groups in myosin related to the adenosine triphosphatase activity do not exchange readily with disulphides.

Determination of the complete amino acid sequence of myosin is clearly an awesome project. However, the thiol sequences listed above may act as useful markers in such efforts, particularly when their distribution among the various known subfragments of the molecule has been established.

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