The Actin of Muscle and Fibroblasts

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The isolation and quantification of an 18-residue peptide from the *N*-terminal region of chicken actin was used to quantify the amount of actin in acetone-dried powders of chicken breast muscle and chicken-embryo fibroblasts. Either isotope dilution or double labelling can be used for peptide quantification. About 17% of the protein of chicken breast muscle was estimated to be actin. However, only 0.25% of the protein of chicken-embryo fibroblasts was determined to be actin by quantification of this peptide. The actin content of fibroblasts may be low or the amino acid sequences of muscle and fibroblast actin may differ in the *N*-terminal region. The methodology used can be extended to examine whether other regions of muscle actin sequence are present in fibroblasts or other cell types.

Proteins with properties similar to those of the contractile proteins actin and myosin have been found in a wide variety of cells and it has been proposed that these proteins be called cytoplasmic actin and myosin (Pollard & Weihing, 1974). Although there is no direct evidence, it is thought that these proteins are involved in many types of cell motility, such as amoeboid movement and chromosomal movement (Pollard & Weihing, 1974; Sanger, 1975). An important preliminary to the understanding of the function of cytoplasmic actin and myosin in cell processes is the determination of their nature and amount. For most proteins this is done by purifying the particular protein and examining its amino acid sequence and by determining the amount of protein present by measuring its catalytic properties.

Cytoplasmic actin presents problems for the application of both of these procedures. Although there are indications that actin comprises a large amount of the protein of many cells, only very small amounts of pure protein with the properties of actin have been obtained from non-muscle cells. For example, only 0.2% of the protein of *Acanthamoeba* (Pollard & Weihing, 1974) and chicken-embryo fibroblasts (Yang & Perdue, 1972) and 0.1% of the protein of rabbit alveolar macrophages (Hartwig & Stossel, 1975) is recovered as purified protein with the properties of actin. Such low yields complicate amino acid-sequence analysis.

The quantification of actin is difficult because actin has no known enzymic properties. No reliable method for the measurement of actin on the basis of the properties used for its identification, such as molecular weight, electrophoretic mobility, ultrastructure, reversible globular-fibrous transformation and binding to and modulation of the ATPase (adenosine triphosphatase) activity of myosin is

apparent. It would therefore be of value if a sensitive method were available for the examination of the structure and amount of actin in tissues which would give a reliable indication of both the structure and the amount of cytoplasmic actin.

Procedures for the quantification of actin have been developed by Bray & Thomas (1975). One method measures the amount of protein having the same motility as muscle actin on electrophoresis on sodium dodecyl sulphate polyacrylamide gels by densitometry. Although evidence was obtained that a large proportion of the protein with this mobility was similar to actin, no estimate of the degree of purity was possible. In addition such a method is subject to inaccuracies introduced by the variable staining of proteins by Coomassie Brilliant Blue (Fishbein, 1972). A second method involves growing cells in [³⁵S]methionine. The fraction of radioactivity in protein with the same mobility on polyacrylamide gels as muscle actin is then determined. This method is handicapped by the possibility of contamination by proteins having the same electrophoretic motility as actin and by the variable methionine content of proteins.

A method which may provide an accurate estimate of actin content of cells and at the same time indicate the primary structure can be developed from a procedure that has been used to estimate the amount of individual proteins in mixtures (Anderson, 1974). In this procedure the isolation of a portion of the amino acid sequence of a particular protein is used to identify the protein. Since the random occurrence of an amino acid sequence is 1 in 20^n , where n is the number of amino acids in the sequence, a relatively small proportion of the sequence of a protein is required to define uniquely the protein from which it originates. For quantification of an individual

protein in mixtures, the protein to be measured is first purified and certain of its functional groups are quantitatively modified by using a radioactive reagent. This protein is then added to a mixture of proteins in which the same functional groups have been modified by a non-radioactive form of the reagent. Peptides containing the modified functional groups are then generated and isolated. From the dilution of specific radioactivity, the amount of peptide derived from the mixture of proteins and hence the amount of the particular protein in the mixture is determined. The degree of homology between the purified protein and the protein in the mixture can be obtained by isolating a variety of peptides.

Since actin can be obtained in pure form and its amino acid sequence is known (Collins & Elzinga, 1975), the application of a procedure based on peptide isolation to the study of the structure and amount of cytoplasmic actin appeared possible. Muscle actins are very highly conserved proteins (Elzinga & Collins, 1973). No differences were found in tryptic peptide 'maps' of mammalian and avian actins (Carsten & Katz, 1964). It was therefore assumed that the amino acid sequences of rabbit and chicken actin are very similar. As it is often difficult to obtain large amounts of material from cells grown in culture, a more sensitive method for measurement than isotope dilution was desirable. A method using double labelling with ^3H and ^{14}C for measurement was therefore developed and applied to the determination of actin in acetone-dried powders of muscle and chicken-embryo fibroblasts.

Experimental

Materials

Actin was prepared from chicken muscle (Seraydarian *et al.*, 1967) and freed of tropomyosin by the addition of 0.6M-KCl after polymerization (Spudich & Watt, 1971). The resulting preparation was free of contamination as judged by electrophoresis on sodium dodecyl sulphate-containing polyacrylamide gels (Weber & Osborn, 1969). Iodo[2- ^{14}C]-acetic acid was obtained from Amersham-Searle, Oakville, Ont., Canada, and diluted with carrier iodoacetic acid to a specific radioactivity of 1mCi/mmol before use. Iodo[2- ^3H]-acetic acid was obtained from the same supplier and was used at a specific radioactivity of 70mCi/mmol. Trypsin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and elastase was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Sequanol-grade reagents from Pierce Chemical Co., Rockford, IL, U.S.A., were used whenever possible. All other reagents were of the best grade available.

Cultures were derived from 10-day chicken embryos. Cells were grown in 75cm² Falcon flasks

containing 25ml of Medium 199. The medium was supplemented with 10% foetal calf serum and 2% chicken-embryo extract and contained 100 units of penicillin/ml and 100 μg of streptomycin/ml. These materials were obtained from Flow Laboratories, Rockville, MD, U.S.A. Confluent cultures were subcultured at a split ratio of 1:4. Cells used in actin studies had gone through at least four subcultivations. These were then harvested by treatment with trypsin and washed three times in 10 vol. of Ca^{2+} , Mg^{2+} -free phosphate-buffered saline (Dulbecco & Voght, 1954) and frozen at -20°C .

Acetone-dried powders

Acetone-dried powders of chicken breast muscle and of chicken-embryo fibroblasts were prepared by homogenization in 10 vol. of acetone at -15°C twice. Samples were left to air-dry for 2h and then were further dried under vacuum.

Chemical modification (carboxymethylation) of thiol groups

Samples for carboxymethylation were dissolved to protein concentrations of 10mg/ml in 6M-guanidine hydrochloride containing 100mM-Tris/HCl, pH8.0, and 2.5mM-dithiothreitol. A glass homogenizer was used to ensure complete dispersal of material. Actin and the acetone-dried powders were completely solubilized by this procedure. The samples were then dialysed for 2h against 10 vol. of the same buffer. Iodoacetic acid (0.1 vol. of 100mM) was then added and the mixtures were sealed and left to react in the dark at room temperature for 40min. Reactions were then stopped by the addition of excess of dithiothreitol and the samples were purified by dialysis against water and freeze-dried.

Peptide generation and isolation

CNBr-cleaved peptides were generated and separated on a column of Sephadex G-50 (superfine grade) as previously described (Anderson & Randall, 1975) with 0.5% NH_4HCO_3 as the eluting buffer. Digestions with trypsin and elastase were carried out in 0.5% NH_4HCO_3 at 37°C for 2h at an enzyme/substrate ratio of about 1:50 (w/w). The resulting peptides were isolated by paper chromatography and high-voltage paper electrophoresis as previously described (Anderson *et al.*, 1969). Radioactive peptides were located in the effluent of Sephadex G-50 columns by liquid-scintillation counting of 0.1ml portions in 10ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.) and on paper by radioautography. N-Terminal amino acid residues were identified by a subtractive Edman procedure (Anderson & Randall, 1975).

Amino acid analysis and peptide quantification

Peptide hydrolysis and amino acid analyses were carried out as previously described (Anderson & Randall, 1975), with norleucine added as an internal standard. The radioactivity of peptides was determined by adding 10 μ l portions to 10 ml of Aquasol in glass scintillation vials and counting in a Nuclear-Chicago Isocap/300 liquid-scintillation counter. For samples containing both ^3H and ^{14}C , corrections for spill-over were made by counting 10 μ l samples of each radioisotope in Aquasol.

Results

Fig. 1 shows the elution profile from Sephadex G-50 (superfine grade) obtained when 20 mg of chicken muscle actin that had been carboxymethylated with iodo[2- ^{14}C]acetic acid and treated with CNBr was applied to the column. Peptides were solubilized by treatment with citraconic anhydride before application (Gibbons *et al.*, 1970). The major radioactive peak was eluted between fractions 36 and 44. Previous work with aldolase peptides on this column indicated that peptides of mol.wt. approx. 5000 would be eluted in this region. The 44-residue CNBr peptide CB 13 (Collins & Elzinga, 1975) would be expected to be eluted in this position. Inspection of the amino acid sequence of this peptide indicates that a very acidic peptide containing carboxymethylcysteine (Ac-Asp-Glu-Thr-Glu-Asp-Thr-Ala-Leu-Val-CmCys-Asp-Asp-Gly-Ser-Gly-Leu-Val-Lys; Ac-Asp, *N*-acetylaspartic acid; CmCys, carboxymethylcysteine) should be generated from it by treatment with trypsin. This peptide would be expected to be excellent for the identification of actin, since it is large enough (18 residues) to make it very unlikely that it would be found in any other protein and because its highly acidic nature should facilitate purification by high-voltage electrophoresis. Accordingly, fractions 36–44 from the Sephadex G-50 (superfine grade) column were pooled and freeze-dried. Citraconyl groups were removed by treatment overnight with 0.1M-acetic acid, and after freeze-drying, the material was digested with trypsin. A radioactive peptide with a mobility 0.9 that of aspartic acid was found after high-voltage paper electrophoresis of the tryptic digest at pH 6.5. This peptide was further purified by chromatography in butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) and high-voltage paper electrophoresis at pH 3.5 and 2.1. The peptide proved to be ninhydrin negative, and no *N*-terminal amino acid could be detected. After hydrolysis in 6M-HCl the amino acid composition of the peptide was determined to be: CmCys (0.8), Asp (3.9), Thr (1.8), Ser (1.0), Glu (2.0), Gly (2.0), Ala (1.0), Val (2.0), Leu (2.0), Lys (0.9). This composition is identical with that of the [^{14}C]-CmCys-containing peptide which would be expected

from tryptic digestion of peptide CB 13. As further evidence that this peptide was identical in amino acid sequence with the expected peptide, digestion of the purified peptide with elastase was carried out. A peptide with an amino acid composition CmCys (0.8), Asp (2.0), Ser (0.9), Gly (2.1), Leu (1.0), Val (1.0), Lys (0.9) and an *N*-terminal CmCys residue was subsequently isolated. This is compatible with a peptide that could be produced by digestion of peptide CB 13 with trypsin and elastase and indicated that the desired peptide had been isolated. The tryptic peptide was designated peptide X2-T-A.

The potential usefulness of this peptide in the determination of actin was first examined by using isotope dilution. An acetone-dried powder (70 mg) of

Fig. 1. Elution profile of actin peptides from Sephadex G-50 (superfine grade)

Chicken muscle actin (about 20 mg) was carboxymethylated with iodo[^{14}C]acetic acid and peptides were generated with CNBr. After treatment with citraconic anhydride the peptides were applied to a 2.5 cm × 90 cm column, eluted with 0.5% NH_4HCO_3 , and 5.5 ml fractions were collected.

Table 1. Dilution of actin peptide X2-T-A by chicken breast-muscle acetone-dried powder

Peptide X2-T-A was isolated from carboxy[^{14}C]methylated pure actin and from a mixture of 5 mg of carboxy[^{14}C]methylated pure actin and 70 mg of carboxymethylated muscle acetone-dried powder after treatment with CNBr and trypsin.

Source of peptide X2-T-A	Specific radioactivity (c.p.m./nmol)
Carboxy[^{14}C]methylated actin	1745
Mixture of carboxy[^{14}C]methylated actin and carboxymethylated muscle acetone-dried powder	506

Table 2. *Measurement of actin content of protein mixtures by using double labelling*

Known amounts of carboxy[^{14}C]methylated pure actin were mixed with protein mixtures that had been carboxy[^3H]methylated. Peptides were generated by using CNBr and trypsin, and peptide X2-T-A was isolated.

Source of peptide X2-T-A	$^3\text{H}/^{14}\text{C}$ ratio	Actin (nmol)	Actin (%, w/w)
10mg of carboxy[^3H]methylated foetal calf serum protein	No ^3H counts	—	—
10mg of carboxy[^3H]methylated foetal calf serum protein containing 1 nmol of added actin	0.33	1.0	—
10mg of carboxy[^3H]methylated foetal calf serum protein containing 2 nmol of added actin	0.68	2.0	—
10mg of carboxy[^3H]methylated chicken muscle acetone-dried powder	12.60	38.2	16.1
20mg of carboxy[^3H]methylated chicken-embryo fibroblast acetone-dried powder	0.36	1.1	0.23

chicken breast muscle that had been carboxymethylated with non-radioactive iodoacetic acid in 6M-guanidine hydrochloride was mixed with 5mg of purified chicken muscle actin that had been carboxymethylated with iodo[2- ^{14}C]acetic acid. The mixture was dissolved in 70% (v/v) formic acid, in which it was completely soluble, and CNBr peptides were generated and separated on Sephadex G-50 (superfine grade). The second radioactive peak in the effluent (Fig. 1) was digested with trypsin and the peptide X2-T-A was isolated in very pure form, as indicated by amino acid analysis. Table 1 shows the specific radioactivities of peptide X2-T-A before and after mixing with chicken breast-muscle acetone-dried powder. It can be calculated from these results that 17.5% by wt. of chicken breast-muscle acetone-dried powder is actin.

To make the procedure more sensitive, carboxymethylation of acetone-dried powders with iodo[^3H]acetic acid was carried out. Samples (about 10mg) were carboxymethylated in 6M-guanidine hydrochloride. To the carboxy[^3H]methylated acetone-dried powders, 6mg of carboxy[^{14}C]methylated pure actin was added. The mixtures were dissolved in 70% (v/v) formic acid and CNBr peptides and peptide X2-T-A were generated and isolated as described above. To test if the method was quantitative, known amounts of purified actin were added to 10mg samples of foetal calf serum protein and were carboxymethylated with iodo[^3H]acetic acid. Peptide X2-T-A was then generated and isolated after the addition of carboxy[^{14}C]methylated purified actin as described above. Table 2 summarizes the results of these experiments. As would be expected if the method were quantitative, the ratio of $^3\text{H}/^{14}\text{C}$ in the samples to which known amounts of actin were added is directly related to the amount of actin added. When the peptide X2-T-A was isolated from samples of foetal calf serum protein to which no actin had been added before carboxymethylation with iodo[2- ^3H]acetic acid, no ^3H was detected in the peptide. From the $^3\text{H}/^{14}\text{C}$ ratio given by known amounts of

actin and assuming a mol.wt. of 42000 for actin, the amount of actin in the acetone-dried powders given in Table 2 was calculated. As a check of the purity of the X2-T-A peptides, the isolated peptides were digested with elastase and the CmCys-containing peptide produced by this procedure was isolated. The $^3\text{H}/^{14}\text{C}$ ratio of peptides produced from X2-T-A peptides by this procedure was unaltered. The possibility that the low actin content of the fibroblast acetone-dried powder may have been due to proteolysis during carboxymethylation or subsequently was examined by adding known amounts of bovine pancreatic ribonuclease to the acetone-dried powder before carboxymethylation with iodo[2- ^3H]acetic acid. Bovine pancreatic ribonuclease carboxymethylated with iodo[2- ^{14}C]acetic acid was then added and after treatment with CNBr the peptide Asp-Ser-Ser-Thr-Ser-Ala-Ala-Ser-Ser-Ser-Asn-Tyr-CmCys-Asn-Gln-Hsr (Hsr, homoserine) was isolated and identified by its composition and *N*-terminal residue. The $^3\text{H}/^{14}\text{C}$ ratio of this peptide indicated that no significant proteolysis of ribonuclease by the chicken-embryo fibroblast acetone-dried powder took place.

Discussion

In the present paper the actin content of protein mixtures is calculated from the amount of a tryptic peptide that is produced from acetone-dried powders after carboxymethylation with iodoacetic acid. The peptide is generated by treatment with CNBr and trypsin and is large enough (18 residues) that it is highly unlikely that it will be found in any protein other than actin. The amount of the peptide in the acetone-dried powder can be determined by using either isotope dilution or double labelling. When amounts of the peptide to be estimated are present in quantities of 100 nmol or greater, the isotope-dilution method, because it is relatively inexpensive, is the method of choice. When smaller amounts of a protein are present, double labelling can be used. In the present study this method was used to estimate actin

contents in the range of 1 mmol. By using iodo[2-³H]-acetic acid of very high specific radioactivity, the method could easily be made still more sensitive.

Comparison of the amount of actin in chicken breast-muscle acetone-dried powder obtained by the isotope-dilution method (Table 1) and the double-labelling procedure (Table 2) indicates that both methods are in excellent agreement. Previous experiments on the protein content of skeletal muscle (Hanson & Huxley, 1957) indicated that one-third of the protein was myosin, one-third was soluble protein, and that other muscle components such as actin, tropomyosin and connective-tissue proteins constituted the remainder. The actin content of skeletal muscle has been estimated to be 65% of that of myosin (Tregear & Squire, 1973; Potter, 1974). From these results it can be calculated that about 20% of muscle protein is actin. This value is in very good agreement with the results obtained in the present study and indicates that the methods used to obtain them give a reasonable estimate of actin content.

Although the present experiments indicate, as expected, that a large amount of actin is found in muscle, only 0.23% of the protein of chicken-embryo fibroblasts was estimated to be actin by the same procedure. This is much lower than a previous estimate that 8% of the total protein of these cells is actin (Bray & Thomas, 1975), but is similar to the actin content of fibroblasts as calculated from the amount of purified protein (Yang & Perdue, 1972). It seems very unlikely that the estimation of actin content by the present methods would give rise to low values. Although amino acid analysis and digestion of the purified peptides with elastase indicated that the isolated peptides were very pure, the most likely source of error is the presence of contaminating peptides. However, these would give rise to an overestimate of the actin content.

The presence of an actin-like protein with a different amino acid sequence in the region of actin sequence examined in this study would account for the difference in actin content determined in the present study and previously (Bray & Thomas, 1975). Observations noted in the preparation of purified cytoplasmic actin from chicken-embryo fibroblasts (Yang & Perdue, 1972) suggest that this might be so. When acetone-dried powders of glycerinated fibroblasts were extracted with an ATP-containing buffer only 6% of the protein, as estimated by gel electrophoresis, was actin. The most abundant component of this extract was a protein with the mobility of tropomyosin. These findings suggest that an actin-like protein with a solubility different from that of muscle actin may be present in fibroblasts. In this regard, it may also be noted that cytoplasmic and muscle myosins have different solubilities (Pollard & Weihing, 1974). If the solubilities of cytoplasmic and muscle actins are different, it would be expected that they would have

amino acid-sequence differences. In addition, recent results obtained by using peptide 'mapping' (Gruenstein *et al.*, 1975) and peptide isolation (Elzinga *et al.*, 1976) indicate that mouse fibroblast and human platelet actins have different amino acid sequences from those of muscle actin. The use of peptides arising from other regions of the amino acid sequence of muscle actin to determine chicken fibroblast actin may indicate whether an actin-like protein which is not identical with muscle actin, but contains some regions of homologous amino acid sequence, is present in these cells. Thus the methods developed for actin determination may be extended to the study of cytoplasmic actin structure by the isolation of a variety of actin peptides.

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