Actin. Its Thiol Groups*

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ABSTRACT: The number of thiol groups of actin was investigated. Seven thiol groups were found in 60,000 g of G-actin. Aged actin solutions showed a loss of thiol groups due to oxidation, but there was no indication of the formation of disulfide bonds or of their participation in aggregation.

Peptide maps of tryptic digests of alkylated actin labeled with iodoacetate-1-^{14}C showed five radioactive peptides with some radioactivity staying at the origin. One of these five peptides did not stain with ninhydrin. Quantitative amino acid analyses for two thiol peptides are given.

Actin, one of the proteins of the myofibril, is unique among the fibrous muscle proteins inasmuch as it polymerizes in the presence of salts and depolymerizes in their absence (Straub, 1943). In the intact muscle fiber actin seems to be present in its fibrous or polymerized form (Perry, 1952). The polymerization of globular actin (G-actin) to the fibrous form (F-actin) is reversible in the presence of adenosine triphosphate (ATP[1]) (Straub and Feuer, 1950). The molecular weight of the monomer is approximately 60,000 (Mommaerts, 1952; Ulbrecht *et al.*, 1960; Lewis *et al.*, 1963; Mihashi, 1964), with 1 mole of ATP bound to 1 mole of G-actin. Since it has been shown that sulfhydryl reagents inhibit the polymerization of actin (Kuschinsky and Turba, 1951), the role of thiol groups of the actin molecule in the polymerization reaction has become accepted, and the number of such groups is of importance.

The determination of the number of sulfhydryl and disulfide groups in proteins has proven to be notoriously difficult with different results obtained according to the methods used (Cecil and McPhee, 1959). In the case of actin, 6.7 cysteic acid groups per mole of G-actin (assuming a molecular weight of 60,000) were obtained by amino acid analysis after performic acid oxidation (Kominz *et al.*, 1954). In a previous publication (Carsten, 1963) we reported 6.7 SH and no S–S groups per mole of G-actin by amino acid analysis following alkylation with iodoacetate. Furthermore, the sum of the SH sulfur and the methionine sulfur agreed well with the value of the sulfur obtained on elemental analysis. On the other hand, values of ~6 were obtained by titration with mercurial and silver reagents or *N*-ethylmaleimide (Katz and Mommaerts, 1962), and 6.1–6.8 using *p*-mercuribenzoate (Tonomura and Yoshimura, 1962).

The determination of the thiol groups of G-actin is further complicated by its instability. Treatment with Dowex 1 to remove free nucleotide from solution in the long run causes dissociation of the bound nucleotide (Asakura, 1961). This change seems to be accompanied by the appearance of several new components in the starch gel electrophoresis pattern and a faster moving boundary in the ultracentrifuge (Carsten and Mommaerts, 1963), interpreted as aggregation (Carsten and Mommaerts, 1963; Katz, 1963). Similar changes occur spontaneously on aging of actin solutions, and it has been suggested that the tendency to aggregate may be due to the presence of some disulfide linkage (Krans *et al.*, 1962, 1965). Because of the discrepancies in the number of thiol groups obtained, it was felt that this problem needed reinvestigation by methods other than those mentioned before. The following paper presents some results, as well as a discussion on the role of thiol or disulfide groups in aggregation of G-actin and analyses of some thiol peptides.

Experimental Section

Actin was extracted from rabbit skeletal muscle and purified by ultracentrifugal isolation of F-actin followed by reversible depolymerization in the presence of ATP and ascorbic acid (Carsten and Mommaerts, 1963). The actin preparations were freeze dried and stored in the cold. These preparations showed one band on starch gel electrophoresis and polymerized upon addition of salt. Quantitative determination of disulfide and thiol groups was carried out with *N,N'*-bis-2,4-dinitrophenyl-L-cystine (DNP-cystine) according to the method of Glazer and Smith (1961). Series of 2-, 4-, 6-, and 9-mg samples were weighed accurately into individual tubes; a series consisted of nine tubes which each contained the actin sample (for example, 2 mg), 1.8 mg of DNP-cystine, and 1.5 ml of 9.6 N HCl. Appropriate blanks (without DNP-cystine) were set up

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[1] Abbreviations used in this work: ATP, adenosine 5'-triphosphate; DNP-cystine, *N,N'*-bis-2,4-dinitrophenyl-L-cystine.

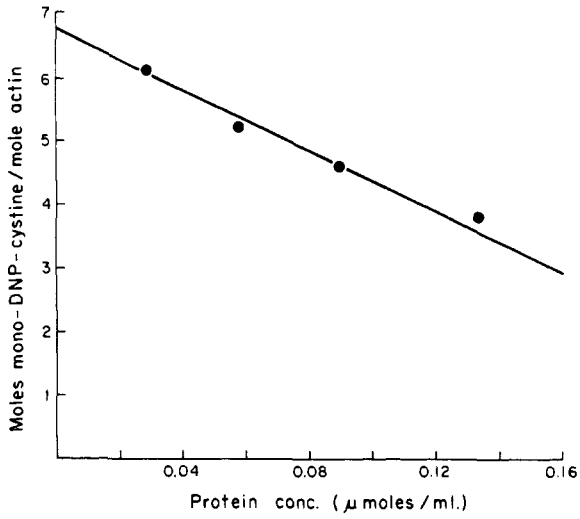

FIGURE 1: Formation of mono-DNP-cystine as a function of actin concentration. Actin concentration corrected for moisture.

simultaneously. The tubes containing the reaction mixtures were sealed in order to avoid loss by evaporation during prolonged equilibration at 39°; tubes were opened at specified time intervals and immediately analyzed. Equilibration was usually complete on opening the second tube in each series, *i.e.*, after 96 hr. The reaction was allowed to continue in the remaining tubes, one tube being opened every 3 or 4 days, the last being opened after 792 hr. The results obtained after reaching equilibrium were averaged, usually eight analyses being averaged. Separate moisture determinations were performed[2] on the samples used, and the weight of samples was corrected for moisture content.

In order to see whether aggregation was brought about by disulfide bond formation, amino acid analyses (Carsten, 1963) were performed of actin solutions first treated with Dowex 1 (Carsten and Mommaerts, 1963) to remove free ATP then aged 4 hr at room temperature and alkylated with iodoacetate in 6 M urea (Carsten, 1963; Carsten and Katz, 1964). In addition, aliquots of the same actin preparations which had been treated with Dowex 1 and aged 4 hr were reduced with mercaptoethanol (Anfinsen and Haber, 1961) or sodium borohydride (Carsten and Pierce, 1963), and then alkylated and analyzed for amino acid composition (Carsten, 1963).

G-Actin alkylated with iodoacetate-1-^{14}C in 6 M urea was precipitated (Carsten and Katz, 1964), dried, and digested with trypsin as previously described (Katz and Carsten, 1963) except that digestion was carried out in an automatic titrater (TTT1b, Radiometer, Copenhagen) at pH 8 for 90 min, at which time digestion seemed to be complete. Two-dimensional chromatography and high-voltage electrophoresis was carried out (Carsten and Katz, 1964; Katz and Carsten, 1963). The peptide maps were stained with ninhydrin (0.1% in absolute alcohol). Radioautographs were obtained after 4-weeks' exposure to Kodak No-Screen X-ray film. For amino acid analysis eight peptide maps were chromatographed simultaneously; electrophoresis was carried out singly. The areas of the SH peptides were cut out, washed with acetone, eluted with 30% acetic acid, taken to dryness, and hydrolyzed with 6 N HCl in sealed evacuated tubes for 20 hr at 110°; identical peptides from the eight papers were pooled and quantitative amino acid analyses performed.

Results

Attempts to label G-actin in the SH groups with iodoacetate-1-^{14}C and to derive quantitatively the number of SH groups from the counts per mole of iodoacetate and the counts in the labeled protein failed. The commercial iodoacetate-1-^{14}C appeared to be partially decomposed with the liberation of iodine, and thus contained labeled acetate.

The method finally employed was that of Glazer and Smith (1961), based on the interchange reaction of DNP-cystine in concentrated acid solution with disulfide and thiol groups of proteins. The half-cystine content, calculated according to this method as moles of mono-DNP-cystine per mole of protein (assuming a molecular weight of 60,000), is given in Figure 1 as a function of protein concentration. Each point in Figure 1 is the average value for eight determinations. The extrapolated value for mono-DNP-cystine per mole of actin, derived by the method of least squares, was 6.81, in very good agreement with our previous result of 6.7 SH groups on amino acid analysis of the alkylated protein (Carsten, 1963).

After removal of free ATP with Dowex 1 and 4-hr aging, alkylation and amino acid analysis yielded 5.1, 4.2, 3.5, and 4.3 residues of *S*-carboxymethylcysteine per mole of actin in four independent experiments. In the last two cases additional 1.5 and 0.4 moles of cysteic acid were present. Upon reduction of aliquots of the same preparations before alkylation 4.6, 3.4, 5.6, and 5.7 moles of *S*-carboxymethylcysteine were found. The first sample was reduced with mercaptoethanol (Anfinsen and Haber, 1961), the last three with sodium borohydride (Carsten and Pierce, 1963). In no case was there any cystine on amino acid analysis. Reduction thus resulted in disappearance of the cysteic acid with increased recovery of *S*-carboxymethylcysteine; in the absence of cysteic acid no increase in *S*-carboxymethylcysteine was obtained on reduction. These results suggest that, once oxidation has proceeded to a level of oxidation higher than cysteic acid, it is no longer reversible. Although complete quantitative amino acid analyses were obtained in all experiments, they are not given here since they agreed essentially with previous analyses (Carsten, 1963; Carsten and Katz, 1964), and their presentation does not seem to add materially to the data presented above.

Peptide maps of tryptic digests of alkylated actin labeled in the thiol groups with ^{14}C showed the same number of ninhydrin-staining peptides as previously obtained (Carsten and Katz, 1964). After 4-weeks'

[2] Elek Micro Analytical Laboratories, Torrance, Calif. 90505.

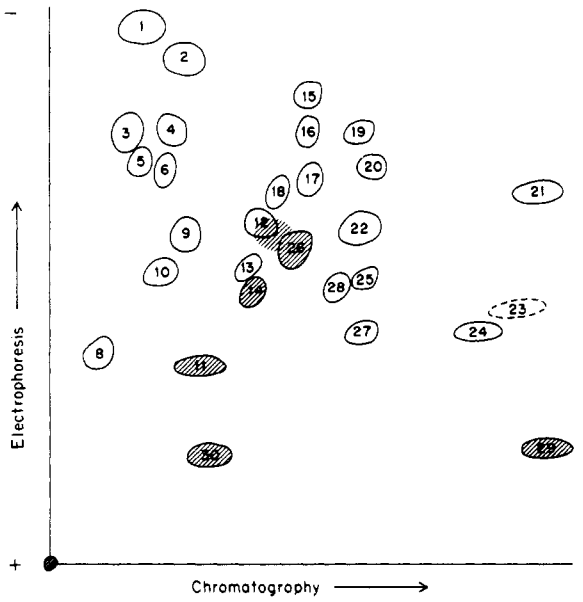

FIGURE 2: Peptide map of tryptic digest of alkylated actin. Cross-hatched areas represent thiol peptides shown on radioautograph.

TABLE I: Amino Acid Composition of Peptides 29 and 30 of Tryptic Digest.

	Moles Amino Acid/Mole Lysine			
	29		30	
Amino Acid	1	2	1	2
Lysine	1.0	1.0	1.0	1.0
S-Carboxymethyl-cysteine	Trace	2.2	—	0.4
Phenylalanine	2.9	4.0	—	—
Serine	2.7	3.1	2.5	2.0
Glycine	2.5	3.3	3.8	3.8
Glutamic acid	2.0	3.3	3.4	3.2
Alanine	1.8	1.9	2.1	1.7
Aspartic acid	1.7	1.4	4.0	3.5
Isoleucine	1.3	0.9	1.2	0.7
Threonine	1.2	1.5	2.9	2.3
Leucine	1.1	1.0	2.1	1.8
Valine	0.8	0.5	2.1	2.1

exposure to Kodak No-Screen X-ray film, radio autographs showed five radioactive spots, with some radioactivity remaining at the origin. One of the radioactive spots, no. 30, did not stain with ninhydrin; the others were identified with peptides 29, 11, 14, and either 12 or 26 (Carsten and Katz, 1964), as shown in Figure 2. Quantitative amino acid analyses calculated relative to 1 mole of lysine for peptides 29 and 30 from two separate digests are given in Table I. It is seen that agreement between duplicate analyses was generally good. In initial analyses no *S*-carboxymethylcysteine was recovered, indicating decomposition, as observed by others in similar experiments (Li and Vallee, 1964). However, with 0.02 M mercaptoethanol present during chromatography and electrophoresis in experiment 2, some *S*-carboxymethylcysteine was recovered in both peptides; peptide 29 must have contained at least two cysteine residues. The other three eluted peptides showed ratios of lysine:arginine of 1:1.5–2, 1:2, and 1.5–2:1, respectively, indicating heterogeneity of the material. Their analyses are not given as they need further purification.

Discussion

The large number of methods for the determination of thiol groups in proteins is proof for the need of such methods and for the difficulties encountered in obtaining quantitative results in all proteins. In G-actin, generally speaking, titration methods seem to give lower results than methods based on amino acid analyses. However, one must suppose that during hydrolysis decomposition and some loss of sulfhydryl derivative occur, though one tries to make correction for this. In the method used here no hydrolysis is involved; therefore this method was thought to be of value in re-evaluating previous results. An estimation of the sum of the half-cystine plus cysteine content was obtained, but since in previous work it was established that actin contains no cystine (Carsten, 1963) it can be said that 6.8 thiol groups were found per mole of G-actin of 60,000 molecular weight. This agrees very well with the previously obtained value of 6.7 and should be interpreted to mean that G-actin contains at least seven thiol groups per mole.

In the analysis of actin for cysteine by alkylation and amino acid analysis, considerable variation has been encountered, more than for any other amino acid (Carsten and Katz, 1964). This variation is obviously due to the instability of cysteine, partly when in the alkylated form during hydrolysis but probably even more so in the original preparation. The reason for this may have to be sought in the method of preparation of actin, the time taken for preparing and purifying actin, the age of the preparation, the pH, and a variety of unknown factors beyond experimental control. Structural changes in alkylated actin and in spontaneously aged actin, manifested by several new slow-moving components in the starch gel electrophoresis and by an increased sedimentation constant in alkylated actin, were observed previously (Carsten and Mommaerts, 1963; Katz, 1963). It was reasoned that these changes are accompanied by loss of bound ATP, a process which is accelerated after removal of free ATP (Asakura, 1961), and that they were due to aggregation. Recently, it has been suggested that disulfide bonds play a role in aggregation (Krans *et al.*, 1962, 1965). It is now shown that the analytically obtained values for thiols decrease from 6.7 to 4 or 5, but no cystine could be

found. One is forced to conclude that indeed aggregation is accompanied by oxidation but not by disulfide bond formation to a measurable extent. Rather, oxidation proceeded to higher oxidation products than cystine and was irreversible when it reached stages beyond cysteic acid. It is not clear from the data whether upon reduction cysteic acid was reduced, or other oxidation products, or both. Probably a variety of oxidative products was present in amounts too small to be detected. Also, some further air oxidation must have occurred during the reduction process in spite of precautions taken, such as having the reaction mixture under a stream of nitrogen. Furthermore, since preparations aged 4 hr in the absence of free ATP as those used here still are able to polymerize completely upon addition of KCl, $MgCl_2$, and ATP, as evidenced by the starch gel electrophoresis pattern (Carsten and Mommaerts, 1963), it appears that not all thiol groups are necessary for polymerization in agreement with previous investigations (Katz and Mommaerts, 1962).

The peptide maps of tryptic digests were in good agreement with previous results (Carsten and Katz, 1964), but only five radioactive peptide spots were obtained. If the thiol groups were distributed along the peptide chain so that there would be not more than one in any one tryptic peptide, seven radioactive spots should have shown. As we see, however, from amino acid analysis peptide 29 contained two SH groups. Furthermore, some radioactivity stayed at the origin, probably in a large peptide or undigested core. The number of lysine plus arginine residues is close to twice that of the number of peptides obtained, indicating either subunits of approximately half size or a large undigested core. Actin has indeed been split into subunits of approximately half the molecular weight by treatment with guanidine hydrochloride (Adelstein *et al.*, 1963; Mihashi and Ooi, 1965). The finding of five different thiol peptides upon tryptic digestion suggests, assuming digestion is complete, that the two subunits are not identical.

A rather interesting discovery was peptide 30 which did not stain with ninhydrin. The reason for this is not entirely clear at present. Possibilities to consider are that the ϵ-amino group of lysine is bound and that the α-amino group could belong to an amino acid which decomposes easily, such as glutamic acid or one which stains poorly with ninhydrin, such as isoleucine. Alternatively, the possibility that this peptide might be the N-terminal peptide with an acetylated N-terminal group has to be taken into account, particularly in view of the fact that we could not find any free N-terminal amino group in actin or alkylated actin using Sanger's fluorodinitrobenzene method (as described by Fraenkel-Conrat *et al.*, 1955). The absence of free amino groups together with the high aspartic acid content would explain the low mobility of peptide 30.

In view of the difficulties in accurately spotting and eluting peptides from paper and of the large number of eluates pooled, agreement between duplicate amino acid analyses of peptides 29 and 30 is probably as good as can be obtained. *S*-Carboxymethylcysteine was recovered only on second analysis when precautions against decomposition due to oxidation were taken. Even under those conditions, recovery was only partial in peptide 30. So far only two of the sulfhydryl peptides were obtained in relatively pure form. Nevertheless, their analysis may contribute to the final elucidation of the structural basis for the essential role of thiol groups in the polymerization of actin.

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