Characterization of Sulfhydryl Groups of Actin*

C. J. Lusty† and H. Fasold

ABSTRACT: Actin contains 5 cysteine residues for 44,000 daltons. The number of sulfhydryls was determined with silver nitrate and back-titration of excess silver with glutathione. On treatment of native and urea-denatured actin with a new reagent, 2,2′-dicarboxy-4′-iodoacetamidoazobenzene, three and four sulfhydryl groups, respectively, were found to react. The corresponding sulfhydryl peptides were located by the fingerprint method. They were isolated and their amino acid compositions were determined. Since actin with the azo dye coupled to its three surface sulfhydryl groups can still polymerize it seems that these sulfhydryl groups are probably not directly involved in the polymerization reaction. However, two of these sulfhydryl groups are covered in the course of polymerization. G-actin binds three molecules of salyrgan (*O*-carboxymethylsalicyl[3-oxymercuri-2-methoxypropyl]amide-^{203}Hg). This organomercurial inhibits polymerization. Evidence is presented that salyrgan binds to the same three surface SH groups to which the azo dye binds. In F-actin only one of these three sulfhydryls reacts with the iodoacetamide derivative. This single sulfhydryl group becomes inaccessible in the course of actomyosin formation, but plays no role in the binding of actin to myosin. F-actin containing 0.87 mole of azo dye/mole of actin is still reactive with myosin, even with the azo dye bound to this cysteine residue. Furthermore the complex formed with the derived F-actin undergoes the same viscosity changes as native actomyosin.

Kuschinsky and Turba (1951) demonstrated that certain sulfhydryl reagents inhibit the polymerization of G-actin to F-actin. Since then several attempts were made to determine and to characterize the SH groups of this protein. Comparing the results obtained with various methods (Katz and Mommaerts, 1962; Tonomura and Yoshimura, 1962; Strohman and Samorodin, 1962), Carstens (1963, 1966) arrives at a value of 6.7 moles of cysteine/60,000 daltons of actin. Using the newer molecular weight of 46,000 (Adelstein *et al.*, 1963; Rees and Young, 1967) or 44,500 (Johnson and Perry, 1968), the number of SH groups would be 5 per mole of actin.

With *N*-ethylmaleimide Katz and Mommaerts (1962) found one to two easily accessible SH groups, presumably on the surface of the protein. Martonosi (1968) determined the amino acid sequence around the "fast-reacting" cysteine labeled with [^{14}C]*N*-ethylmaleimide. Iodoacetamide alkylates several cysteines per mole, while iodoacetic acid reacts more slowly (Martonosi, 1968). The reactions of SH groups with alkylating agents did not inhibit polymerization, but the precise relationship between the amount of reagent introduced and the number of cysteines alkylated completely is not known. It is believed, therefore, that organomercurials such as *p*-mercuribenzoate, which inhibit polymerization, must react with other cysteines or with a greater number of them.

It is known that one SH group per mole of G-actin is located on the surface of F-actin. In order to determine more precisely than before the number of all the SH groups which are accessible in native G-actin we used a new reagent, 3,2-dicarboxy-4-iodoacetamidoazobenzene. This reagent introduces a label into the protein that can be easily determined. Its binding to native and urea-denatured G-actin and to F-actin was studied and an attempt was made to compare the reactivity of the new reagent with that of organomercurials, which are known to inhibit polymerization.

Methods

Reagents. All solutions were prepared with doubly distilled water. Urea solutions were deionized shortly before use by passing the solutions through a mixed-bed ion-exchange resin (Servolit MB, Serva, Heidelberg). *N*-Ethylmaleimide was purified by sublimation. Salyrgan-^{203}Hg (*O*-carboxymethylsalicyl-[3-oxymercuri-2-methoxypropyl]amide-^{203}Hg) was obtained from Farbwerke Hoechst, Frankfurt, and iodoacetyl chloride from Schuchardt Co., Munich. All other reagents, except as stated otherwise, were of analytical grade and were used without further purification.

Preparation of 3,2′-Dicarboxy-4′-iodoacetamidoazobenzene. *m*-Aminobenzoic acid (27.4 g) was diazotized in 53 ml of 5 N HCl at 4° by addition of 16.2 g of $NaNO_2$ in 32 ml of water. The pH of the solution was brought to 5.5–6.0 by addition of 4 N NaOH. The temperature was kept below 15°. The yellow fluid was then added to a solution of 46.2 g of sulfonylmethyl-*m*-aminobenzoic acid in 80 ml of water at around pH 6 (Bucherer and Schwalbe, 1906). The pH of the mixture was raised to 8.0 and the reaction mixture was kept at this pH for 2 hr. The sulfonylmethyl group was removed by adding solid NaOH to a final concentration of 0.2 M and by boiling the solution for 2 hr. The clear solution was extracted with ethyl acetate. The azo dye was precipitated from the solution on acidification (20 ml of glacial acetic acid was added per 100 ml). The dye (2 g) was dissolved in 100 ml of 1 N ammonia and 200 ml of methanol or isopropyl alcohol was added, prior to purification by chromatography on an alumina column (2.5 × 100 cm). The column was filled with a slurry of alumina and methanol,

* From the Department of Physiological Chemistry, The University of Wuerzburg, School of Medicine, Wuerzburg, Germany. *Received December 23, 1968.*

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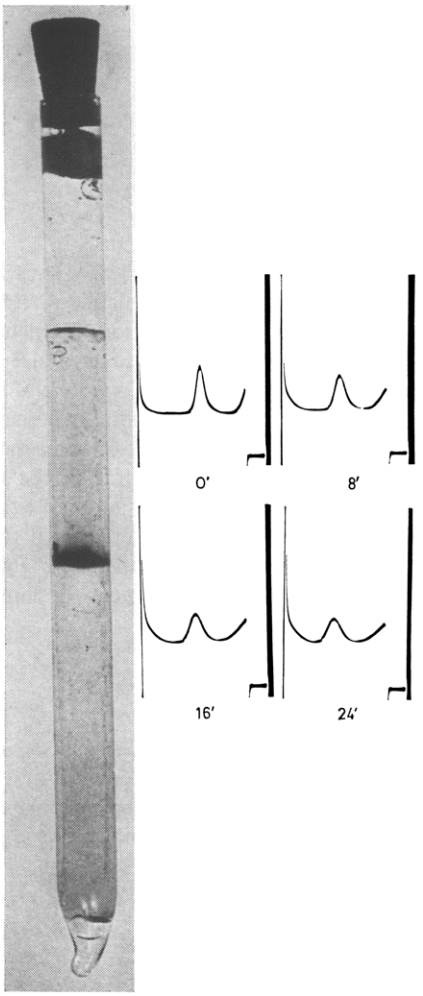

FIGURE 1: Polyacrylamide gel electrophoresis and ultracentrifugal sedimentation velocity measurements of actin. (a, left) A sample of actin was subjected to gel electrophoresis as described in Methods and stained with Amino Black. (b, right) Protein concentration was 1% in 0.2 mM ATP–0.2 mM ascorbate–1 mM Tris-HCl buffer (pH 7.4). The rotor speed was 59,700 rpm. An artificial boundary cell was used. The first picture was taken after the rotor had reached maximal speed. This was taken as 0 min. Sedimentation was from right to left.

and the dye was eluted with a mixture of methanol–1 N ammonia (2:1, v/v). The fractions were analyzed by electrophoresis on paper at pH 6.5. The purified dye was precipitated by acidification, centrifuged, and dried.

The amino group was acylated as follows. The dye (500 mg) was dissolved in 50 ml of 2 N NaOH. Iodoacetyl chloride was added under shaking in an ice bath. When a brown precipitate, without reddish tinge, formed in a test sample upon dilution of the reaction mixture with concentrated HCl, the whole solution was acidified by adding 10 ml of glacial acetic acid. The solution was diluted threefold with 1 M phosphate buffer (pH 7.0), filtered, and then adjusted to pH 2. The precipitate was washed several times with water and collected by centrifugation. The dye was redissolved at pH 6.5 and reprecipitated. It was dissolved again at slightly alkaline pH and the pH was brought to 5.0 before the solution was applied to a column (3 × 40 cm) of cellulose powder, equilibrated with 0.1 N sodium acetate buffer (pH 5.0). A bright yellow zone moved down the column in front of some brown material. The eluted yellow dye was precipitated, washed with water, centrifuged, and finally lyophilized. *Anal.* Calcd: C, 42.38; H, 2.65; I, 28.0; N, 9.27. Found: C, 42.25; H, 2.60; I, 27.88; N, 9.40.

Preparation of Azo-^{14}C Dye. Iodoacetic-^{14}C acid (0.2 mCi) was diluted by addition of 80 mg of cold iodoacetic acid. Thionyl chloride (20 g) was added, and the mixture was heated to 55° for 3 hr under reflux and exclusion of water. After removal of the excess thionyl chloride by evaporation *in vacuo* at 25°, the radioactive iodoacetyl chloride was diluted by addition of 150 mg of cold iodoacetyl chloride. This material was used for the acylation of 100 mg of azo dye by the procedure described above. In order to complete the acylation, cold iodoacetyl chloride was added. The purified azo dye had a specific activity of 1.65 μCi/μmole.

Preparation of G-Actin. An acetone powder was prepared from back and leg muscles of rabbits according to the procedure of Carstens and Mommaerts (1963). The extraction of myosin was omitted. The acetone powder was dried *in vacuo* at 22° for 6 hr and stored at −20° in a tightly closed container until use. Immediately prior to use, 65 g of the powder was extracted twice at 0° with 400 ml of 0.2 mM ATP–0.2 mM ascorbate–1 mM Tris-HCl buffer (pH 7.5; ATP–ascorbate–Tris). The extract was filtered through several layers of cheese cloth. Thereafter, the purification followed the procedure of Carstens and Mommaerts (1963). The G-actin preparation was carried through three polymerization cycles. After the last centrifugation step the precipitate was homogenized in ATP–ascorbate–Tris and dialyzed for 24 hr against several changes of the same buffer solution. Following dialysis the G-actin was centrifuged for 1 hr at 30,000 rpm in the cold and lyophilized to a fine fluffy material, which could easily be redissolved in ATP–ascorbate–Tris. The lyophilized material could be stored at −20° for up to several months, without measurable loss of SH groups by oxidation (as determined by amperometric titration). The stored material was also unchanged with respect to its behavior in the polymerization reaction.

G-actin, prepared as described above, gave a single peak on chromatography on Sephadex G-200 (200 mg in a 4 × 74 cm column) with ATP–ascorbate–Tris (pH 7.0). A single band was also observed on polyacrylamide gel electrophoresis at pH 8.5 (Figure 1a). G-actin was homogeneous as judged by its behavior in the analytical ultracentrifuge (Figure 1b). The same criteria apply to G-actin, purified by the procedure of Rees and Young (1967).

Preparation of Myosin. Rabbit muscle was ground with a meat grinder and extracted by mechanical stirring for exactly 20 min with cold 0.3 M KCl–0.15 M potassium phosphate phosphate buffer (pH 6.5). These steps of the preparation were carried out in the cold room. Myosin was purified by the method of Kielley and Bradley (1956). After fractionation with ammonium sulfate between 40 and 50% saturation, the protein solution was dialyzed for several hours against water and for 12 hr against several changes of 0.6 M KCl.

Preparation of Actomyosin. Actomyosin was extracted from rabbit muscle mince with Weber–Edsall solution (0.6 M KCl–0.04 M $KHCO_3$–0.01 M K_2CO_3) for 24 hr. The protein was precipitated three times by tenfold dilution with distilled water (Kuschinsky and Turba, 1951). After the final precipitation, the protein was taken up in 0.6 M KCl.

Protein Determinations. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine

serum albumin and lyophilized actin as reference standards. The values obtained with this method were in good agreement with results of micro-Kjeldahl analysis (*cf.* Kabat and Mayer, 1961).

Polyacrylamide Gel Electrophoresis. This procedure was carried out by standard techniques (Ornstein, 1964). Gels were made with 5% Cyanogum (British Drug House) in 0.02 M Tris-glycine chloride buffer (pH 8.5). The gels were subjected to electrophoresis prior to use for 3 hr to remove traces of persulfate and ferricyanide. Electrophoresis was carried out at 4° with 5 mA for 3–7 hr in Tris-glycine chloride–2 mM ATP buffer (pH 8.5). Samples of approximately 100 μg of actin were used.

Viscosity Measurements. Viscosity changes were followed with an Ostwald-type viscosimeter (Schott, Mainz, G 20) at 25°. The average time of flow of water through the viscosimeter was 80 sec. The concentration of actin was 9 mg in a total volume of 3 ml of ATP–ascorbate–Tris (pH 7.5). Polymerization was initiated by addition of 0.15 ml of 2 M KCl–2 mM $MgCl_2$ solution.

Hg^{2+} Analyses. Protein-bound salyrgan-^{203}Hg was determined by its radioactivity. Nonradioactive organomercurials (5–50 μg) were determined chemically, by the method of Jacobs and Singerman (1962) and Jacobs *et al.* (1960).

Titration of SH Groups. The amperometric method of Benesch *et al.* (1955) was used. Actin (3–10 mg) was added to 10 ml of 0.02 M Tris-nitrate buffer (pH 7.4) containing an excess of Ag^+ (0.165–0.18 mole/mg of protein). With native actin, incubation was overnight at 4° in the dark. The reaction with urea-denatured protein was complete in 2 hr. After reaction of actin with Ag^+, 2 ml of 1 M Tris, 1.7 ml of 1 M HNO_2, 0.15 ml of 1 M KCl, and 0.2 ml of 0.005 M EDTA were added and the excess Ag^+ was titrated with standardized glutathione. Titration of glutathione gave values for SH groups which were within 2% of theory. These values and those from direct titration with Ag^+ were in good agreement. Direct titration of the actin SH groups was not possible, however, for reasons given by Katz and Mommaerts (1962).

Chymotryptic Digestions. Solutions of azoprotein (5–10 mg/ml) in 2% ammonium carbonate (pH 7.8) were digested overnight with six-times-recrystallized chymotrypsin at a concentration of 0.05–0.1 mg/ml. The digest was concentrated by evaporation, lyophilized, dissolved in 0.1 M ammonium acetate buffer (pH 3.5), and chromatographed on talcum.

Preparation of Talcum for Chromatography. Talcum (400 ml; Merck, Darmstadt) was refluxed for several hours in a solution of 500 ml of ethanol, 100 ml of concentrated HCl, and 200 ml of water (Tuppy and Witter, 1960). The talcum was washed with water until neutral. The talcum fines were removed by decantation.

Peptide Maps. The azopeptides (1–3 mg) were applied to Whatman No. 3MM paper (29 × 62 cm) and separated by high-voltage electrophoresis in 0.1 M pyridine acetate (pH 6.5) at 7000 V for 60 min. Electrophoretic separation was also carried out in 2 M acetic–formic acid buffer (pH 1.9) at 500 V for 10 hr. Following electrophoresis separation in the second dimension was carried out by ascending paper chromatography in 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v).

Amino Acid Analyses. The peptides were hydrolyzed with 6 N HCl for 22.5 hr. Hydrolysis was carried out in tubes sealed *in vacuo*. Amino acid analyses were carried out according to the method of Spackman *et al.* (1958).

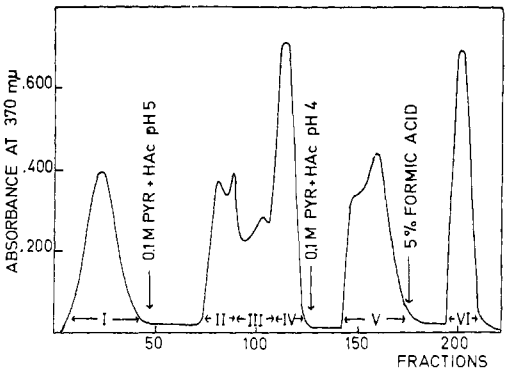

FIGURE 2: Elution pattern of a mixture of azopeptides from a DEAE-Sephadex A-25 column. Experimental details are in the text.

Radioactivity measurements were carried out by liquid scintillation spectrometry. An aliquot of the protein solution was brought to dryness *in vacuo* and dissolved by addition of 0.5–1 ml of hyamine hydroxide solution. Toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene solution (Ott, 1958) was used as scintillator. Quenching was corrected with internal standards. All samples counted had more than tenfold background activity.

Isolation of the Azopeptides. The lyophilized chymotryptic digest of azo–actin (800 mg) was dissolved in 0.1 M ammonium acetate buffer (pH 3.5) and applied to a talcum column (2.5 × 20 cm), equilibrated with the same buffer. The column was washed with several hundred milliliters of 0.1 M ammonium acetate, buffer, and water. About 90% of extraneous, ninhydrin-positive peptides was removed by this procedure. The azopeptides remained adsorbed on the column. They were eluted with 0.1 N ammonia. Since peptide 1 was bound more strongly than the other peptides, elution had to be continued until this peptide also came off the column. The eluted azopeptides were lyophilized, and finally chromatographed on DEAE-Sephadex A-25 columns (2.2 × 20 cm), equilibrated with 0.1 M pyridine acetate buffer (pH 6.0). For elution the pH of the buffer was stepwise lowered to pH 4. Chromatography was continued with 5% (v/v) formic acid. An elution profile is shown in Figure 2. Fraction I consisted mainly of peptide 1. It was further separated from contaminating ninhydrin-positive peptides by repeated chromatography on paper with 1-butanol–water–acetic acid (5:4:1, v/v) and finally with the solvents used for the peptide maps. Fractions II and III contained peptide 2 and varying amounts of peptide 3. Fraction II was rechromatographed on DEAE-Sephadex and eluted with a linear pH gradient from 6.0 to 4.9. Final purification was achieved by electrophoresis at pH 1.9. Fraction IV contained peptide 3. Impurities were removed by electrophoresis and chromatography. Fraction V contained 3,2′-dicarboxy-4′-aminoazobenzene which was split off from the peptides during eluation of the talcum columns with dilute ammonia. Fraction VI contained a single peptide, 4. The amino acid compositions of the isolated azopeptides are in Table I.

Sequential Labeling of Actin with N-Ethylmaleimide and Azo Dye. G-actin (300 mg), at a concentration of 7 mg/ml, was incubated at pH 6.0 for 1 hr with a twofold molar excess of *N*-ethylmaleimide over SH groups. The azo dye (400 μmoles) was added, and the pH was raised to 8. After 60 min of reac-

TABLE I: Amino Acid Compositions of Peptides 1–4.[a]

Amino Acid	Peptide No. 1	2	3	4
Lysine	97	137		
Histidine			Trace	
Arginine	90	63	75	
Aspartic acid		198		351
Threonine			85	
Serine			148	168
Glutamic acid		Trace	79	37
Proline			90	
Glycine	Trace			354
CM-cysteine[b]	85	58	75	120
Valine				158
Isoleucine		140		
Leucine		65	90	184
Phenylalanine	100			

[a] Millimicromoles of amino acids. [b] Cysteine residues appear as CM-cysteine.

tion, the protein was polymerized by addition of KCl and $MgCl_2$. Following centrifugation and washing, the F-actin (265 mg) was digested.

Sequential Labeling of G-actin with Salyrgan and Azo Dye. G-actin (300 mg) was incubated with 107 μmoles of salyrgan in 100 ml of ATP–ascorbate–Tris (pH 7.5). The reaction mixture was passed over Sephadex G-50 (4 × 70 cm) to remove excess mercurial. Viscosity measurements showed complete inhibition of polymerization. To one-half of the protein effluent from the Sephadex column 40 mg of the azo dye, dissolved in 15 ml of water and adjusted to pH 8.0 with NaOH, was added. After 30-min reaction, 20 ml of 0.1 M cysteine (pH 7.0) was added; 5 min later, polymerization was induced by addition of KCl and $MgCl_2$. In the other half of the protein effluent the organomercurial was removed from the protein by first adding the cysteine solution. The mixture was then passed over a Sephadex G-50 column prior to polymerization. In both cases, the F-actin formed (70–75%) was collected by centrifugation at 30,000 rpm for 2 hr in the cold. They were washed once with 70 ml of ATP–ascorbate–Tris, containing 0.1 M KCl (pH 7.0) and centrifuged again. After depolymerization, the protein from the second sample was treated with the azo dye. The reaction was terminated by a second polymerization step. This sample of F-actin was again centrifuged and washed before final depolymerization. Aliquots from the two protein solutions were counted to determine azo-^{14}C dye. In addition aliquots were diluted with an equal volume of 4 N NaOH, and hydrolyzed for 3 hr in an oil bath at 100°. These samples were used for absorbancy measurements at 370 mμ.

Results

SH Titrations. From the slopes of the back-titration curves for actin with Ag^+ and glutathione standard one calculates 4.8–5.0 SH groups/44,000 daltons. The same method was used to determine the loss of cysteine residues by reaction of actin with the azo dye, in the presence and in the absence of 8 M urea. As is seen from Figure 3, after 40-min reaction 3 of the SH groups of native G-actin were completely labeled. At this time the curve levels off. With urea-denatured actin about 3.8–4.0 SH groups were alkylated in 60 min, while the remaining SH group reacted much more slowly, if at all.

Amount of Azo Dye Bound. Urea-denatured actin was reacted with ^{14}C-labeled azo dye for 30 min under the conditions given in the legend of Figure 3. The protein was separated from excess reagent and urea by chromatography on Sephadex G-50 columns, equilibrated with ATP–ascorbate–Tris (pH 8.0). Native G-actin was reacted for 40 min and separated from free dye by the same procedure. The F-actin formed on polymerization (75% of the starting amount) was collected by centrifugation and depolymerized. F-actin, a viscous solution in ATP–ascorbate–Tris (pH 8.0) 0.1 M KCl, 0.001 M $MgCl_2$, was labeled for 35 min with the azo dye under identical conditions. The solution was centrifuged in the cold at 30,000 rpm for 2.5 hr. The pale yellow precipitate was washed twice with the same buffer. After depolymerization, radioactivity was measured. The amounts of bound azo-^{14}C dye are given in Table II.

Initially, the extinction of the protein-bound azo dye was measured at 370 mμ. For this purpose, solutions of the protein were diluted with the same volume of 2 N NaOH. The slightly opalescent solution was heated in a boiling-water bath for 10 min, before readings were taken. The reaction of the azo dye with glutathione served as reference standard. Although the absorbancy measurements are less accurate, they gave on the average results similar to those from radioactivity measurements. However, the absorbancy measurements varied much more (see Table II). Because of the variability of the absorbancy measurements, the reaction of the protein with ^{14}C-labeled dye was the preferred method.

Fingerprints of Azopeptides. The experiments of Carstens (1963) with carboxymethylated actin and our preliminary experiments with azoactin indicated difficulties in the separation of tryptic peptides by the usual fingerprint methods. For this reason chymotryptic digestion was preferred over tryptic cleavage.

Native G-Actin. The fingerprint of this protein showed a characteristic pattern of three spots after isolation of the chymotryptic azopeptides. The isolation procedure is described in the legend of Figure 4. A weaker spot, which stained red with dilute hydrochloric acid, represents traces of 3,2′-dicarboxy-4′-aminoazobenzene. When G-actin was reacted with the dye for 20 rather than 40 min, peptides 2 and 3 appeared as rather weakly labeled spots, while peptide 1 was unchanged. The latter peptide always showed up as a strongly labeled spot.

Denatured Actin. The fingerprint of denatured actin showed four strongly labeled peptide spots (Figure 4), and in addition, two very weakly labeled spots. When electrophoresis was carried out at pH 6.5 instead of 1.9, the pattern toward the anode was similar, but peptides 3 and 4 tended to split into two spots. This was confirmed by amino acid analysis.

F-actin. Only a single peptide was obtained, peptide 1 (not shown in Figure 4).

Amino Acid Compositions of Peptides 1–4. The results are given in Table I. It seems highly improbable that in the case of peptides 1 and 2, two different peptides could have arisen by different chymotryptic cleavage from one and the same cysteine region. This is more likely perhaps in the case of

TABLE II: Amount of Sulfhydryl Reactants Bound per Mole of Actin (44,000 Daltons).[a]

Reagent Used	Native G-actin	Urea-Denatured G-actin	F-actin	Method Used
Azo dye	3.0	4.0 ± 0.2		Amperometric titration
Azo dye	2.7 ± 0.2	3.9 ± 0.1	0.9 ± 0.1	Radioactivity measurements
Azo dye	2.8 ± 0.5	3.8 ± 0.6	0.9 ± 0.2	Absorbancy measurements at 370 mμ
p-Mercuribenzoate	3.15 ± 0.25			Chemical analysis
Salyrgan	2.46 ± 0.65			Chemical analysis
Salyrgan-^{203}Hg	3.00 ± 0.27			Radioactivity measurements

[a] The values are given as moles of reagent per mole of actin monomer with the exception of the experiments with Ag^+. In these experiments they are given as quantities of (gram-equivalents) Ag^+ per mole of actin monomer. All reactions were carried out until the end point of the reactions was reached. The number of single determinations with each of the different reagents varied between 12 and 18.

peptides 3 and 4. However, it should be recalled that peptide 4 appeared only in fingerprints from denatured actin.

Binding of N-Ethylmaleimide and Mercurials to Actin. *N*-Ethylmaleimide, the azo dye, iodoacetic acid, and iodoacetamide do not inhibit polymerization of actin, whereas organomercurials, such as *p*-mercuribenzoate and salyrgan, block polymerization very effectively. This raises the question whether these two groups of reagents are bound to different sites. In order to obtain information which bears on that point, G-actin was labeled first with *N*-ethylmaleimide and subsequently with the azo dye (see Methods). This material gave only weak spots, corresponding to peptides 2 and 3, and none corresponding to peptide 1, even though the azo dye derivatives were present in sufficiently large amounts, since they were concentrated by chromatography on talcum. This indicated that the same groups (*i.e.*, peptides 1–3) which are reactive with *N*-ethylmaleimide (Martonosi, 1968) are also reactive with the azo dye.

The amount of salyrgan or *p*-mercuribenzoate, bound to native G-actin, was determined after reaction of the protein

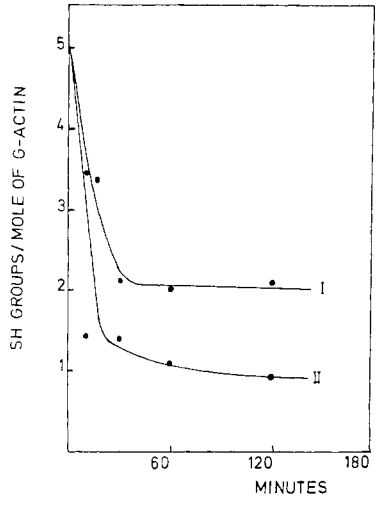

FIGURE 3: Reaction of the azo dye with SH groups of native and urea-denatured actin. The reaction was followed by amperometric titrations. The dye, dissolved in water brought to pH 8 with NaOH, was added to G-actin in ATP–ascorbate–Tris (pH 8.0, 80 μmoles of dye/150 mg of protein). The protein concentration was 6–10 mg/ml. During the reaction, the pH was maintained at 8 by further addition of NaOH. Aliquots (5 ml) were withdrawn at various times and the reaction was stopped by adding 1 ml of cold 0.1 M cysteine solution. The azoprotein was freed from excess reagent and cysteine by gel filtration at 4° on Sephadex G-50 columns (2.5 × 20 cm) equilibrated with ATP–ascorbate–Tris buffer (pH 7.5). The same conditions were used for experiments in 8 M urea. Duplicate samples (5 mg of azoprotein each) were used for amperometric titrations. Curve I: native G-actin; curve II: urea-denatured actin.

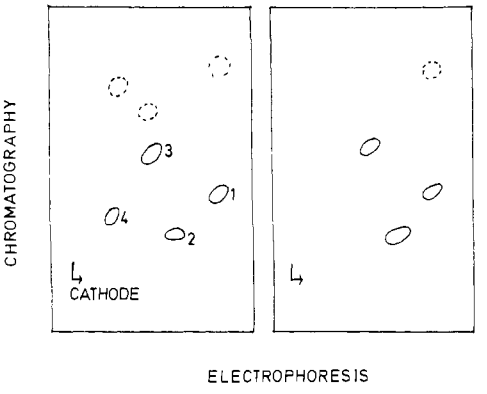

FIGURE 4: Fingerprints of chymotryptic azopeptides. Native G-actin was treated with 10 moles of azo dye/mole of SH at pH 8.0. The protein was separated from excess dye as described in the legend to Figure 3. Prior to chymotryptic digestion, actin was polymerized. The F-actin thus formed was collected by centrifugation and then depolymerized. In order to obtain the azopeptides, the peptide mixture was chromatographed on talcum as described in Methods. Actin was denatured in 8 M urea and treated with azo dye for 60 min. The protein was separated on Sephadex G-50 prior to chymotryptic digestion and chromatography on talcum. The same conditions used for the reaction of native G-actin were used for the reaction of F-actin with azo dye. The usual ATP–ascorbate–Tris buffer in addition contained 0.1 M KCl and 0.1 mM $MgCl_2$. The protein was collected by centrifugation, washed once with 0.1 M KCl–0.1 mM $MgCl_2$, depolymerized, digested with chymotrypsin, and chromatographed on talcum. The fingerprints from denatured actin are shown on the left and those from native G-actin are shown on the right.

(300 mg) at 0° for 1 hr or overnight with a fourfold excess of the organomercurials over SH groups. The unreacted mercurial was removed by gel filtration at 4° on Sephadex G-50, equilibrated with ATP–ascorbate–Tris (pH 7.5). Viscosity measurements indicated complete inhibition of polymerization. Mercury was determined chemically or by radioactivity. The results are summarized in Table II. They show that no more than three molecules of organomercurial are bound to each molecule of actin.

In order to see whether salyrgan and the azo dye react with the same sites, although their effect on polymerization is strikingly different, sequential labeling with salyrgan and the azo dye was carried out (*cf.* Methods). When the reaction with ^{14}C-labeled azo dye was carried out with a G-actin preparation, previously reacted with salyrgan, only 0.15 mole of the dye was bound per mole of G-actin, and less than 0.1 mole/mole of protein was estimated from absorbancy measurements at 370 mμ. However, when the organomercurial was removed by cysteine prior to reaction with the azo dye, 2.8–2.9 moles of azo-^{14}C dye bound per mole were found, and absorbancy measurements indicated 3.3 moles of dye/mole of protein. The fingerprints of this material showed three azopeptides, *i.e.*, 1, 2, and 3.

Finally, G-actin, with 2.7 moles of radioactive azo dye/mole, was incubated with a tenfold excess of salyrgan-^{203}Hg over the SH groups of the unreacted protein. Incubation was for 1 in ATP–ascorbate–Tris (pH 7.0) at a protein concentration of 4 mg/ml. After separation by Sephadex G-50 filtration, 0.84 mole of salyrgan was bound per mole of actin. Polymerization was blocked.

Myosin Binding. Native G-actin was combined with rabbit muscle myosin, as described by Johnson and Landolt (1951). The viscosity of the protein solution dropped in a typical manner on addition of an ATP solution at pH 7.4. When actomyosin was formed under identical conditions by combining myosin with an F-actin preparation which contained 0.94 azo group/44,000 daltons, the viscosity change on addition of ATP was exactly the same as that of native actomyosin. Only azopeptide 1 was found in the chymotryptic digest of the derived F-actin.

In actomyosin the cysteine of peptide 1 is no longer accessible to the azo dye. This SH group seems to be covered in the course of binding of myosin to actin. This confirms the findings of Bailin and Bárány (1967), who found no reactive SH groups in actin bound to myosin.

Discussion

The reactivity of SH groups on the surface of native G-actin seems to depend on the chemical structure of the sulfhydryl reagent used. Thus, while *N*-ethylmaleimide reacts very quickly with one cysteine residue and much more slowly with others, the strongly polar iodoacetic acid reacts much more slowly, but preferentially with only one SH group (Martonosi, 1968). Salyrgan, which is bound to three SH sites on the native protein, reacts very rapidly, within a few seconds, with these SH groups. Besides possible chelation with mercury, the strongly hydrophobic aromatic structure of salyrgan might be responsible for its greater reactivity. Similar differences in the reactivity of various SH reagents, which likewise seem to arise from the different chemical structures of the reagents, were noted in the case of the SH groups of skeletal muscle phosphorylase *b* (*cf.* Battell *et al.*, 1968; Kastenschmidt *et al.*, 1968).

Although several reports have been published on the SH groups of G-actin, the number of the surface SH groups of this protein has not yet been clearly defined. A survey of previously published data indicates the existence of one "fast-reacting" SH group and of a varying number of sulfhydryls which are only partially reactive (Bárány *et al.*, 1957; Katz and Mommaerts, 1962; Mihashi and Ooi, 1965; Martonosi, 1968). In the work reported here, a new reagent, 3,2′-dicarboxy-4′-iodoacetamidoazobenzene, was introduced for the first time for the characterization of SH groups. With this reagent the location of three cysteines per mole of native G-actin at or near the surface could be unequivocally established. One of these three sulfhydryls is more quickly alkylated than the other two. A fourth cysteine residue becomes accessible only after denaturation of actin with urea, and the fifth SH group reacts extremely slowly even in the urea-denatured protein. This SH group is probably located within a core of the protein. The corresponding SH-containing chymotryptic peptides were isolated and their amino acid composition was determined.

With *N*-ethylmaleimide, a fast reacting SH group was identified in G-actin. It is the only SH group in F-actin which is accessible to this reagent. It is not certain whether our fast-reacting SH group is the same cysteine which was found to be reactive with *N*-ethylmaleimide by Martonosi (1968). This cannot be decided at present, because the three tryptic peptides isolated so far by Martonosi (1968) and Johnson and Perry (1968) and the chymotryptic peptides isolated by us are not comparable. This fast-reacting SH group is also the only SH group which is accessible in F-actin. However, this again could depend upon the nature of the alkylating agents which are used.

Since alkylation of three accessible cysteine residues with the azo dye does not interfere with polymerization, these SH groups do not seem to participate directly in the polymerization of actin. The results of the sequential labeling experiments with salyrgan and the azo dye strongly suggest that both of these sulfhydryl reagents are bound to the same sites. In any event sulfhydryl groups which are located in the close vicinity of the polymerization site can be alkylated with the azo dye and the polymerization site can still accept a mole of organomercurial. Contrary to the currently held opinion (Kuschinsky and Turba, 1951; Carstens, 1963; Martonosi, 1968; Gerber and Ooi, 1968) this suggests to us that SH groups probably play only a supporting role in the polymerization of actin. It is quite possible that other side chains are part of the polymerization sites.

Gerber and Ooi (1968) have likewise suggested a role for other amino acid side chains, aside from SH groups, in the polymerization of actin. However, their conclusions were based on rather indirect evidence. A total of 20 different amino acid side chains was reacted with dinitrofluobenzene. The dinitrophenylation reaction was only followed spectrophotometrically. Strohman and Samorodin (1962) could show that reaction of G-actin with *p*-mercuribenzoate inhibits the binding of ATP, whereas *N*-ethylmaleimide had no effect. However, the reaction times used in their experiments with *N*-ethylmaleimide were too short to allow the blocking of all three SH groups on the surface of the protein. ATP seemed to protect one sulfhydryl group against reaction with *N*-ethyl-

maleimide, but an additional side chain was thought to be required for the binding of ATP. It could be that the same groups which are involved in the polymerization reaction are also involved in ATP binding.

Two of the three sulfhydryls of G-actin which are accessible to the azo dye are covered in F-actin. This could mean that these two SH groups are situated at or near the contact sites of the actin subunits in the polymer. Aside from changes in quarternary structure, a rearrangement of the ternary structure in the course of the polymerization reaction should also be considered as possible explanation for the unreactivity of these two SH groups in F-actin.

It should be a goal of future experiments to elucidate precisely the nature of the amino acid side chains which form the polymerization sites in G-actin. This might be achieved by several carefully selected side chain modifications, followed by sequence determination of the isolated peptides, and perhaps with the use of a suitable specific inhibitor for the polymerization reaction.

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