Studies on Actin-Azomercurial Complexes*

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ABSTRACT: G-Actin free of tropomyosin reacts with six equivalents of 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate. Two thiol groups react rapidly and the adduct polymerizes to F-actin. Two more thiol groups react rapidly but the adduct loses bound ATP slowly and polymerizes slowly and incompletely to F-actin. The remaining two thiols react sluggishly and product does not polymerize.

The pK_a of the axomercurial linked to β-mercaptoethylamine is 7.95, while when linked to G-actin it is 10.3. When the G-actin adduct is polymerized to F-actin, the pK_a is 9.7. Upon depolymerization the pK_a returns to 10.3. In D_2O the pK_a of the F-actin-azomercurial is 11.2. When G-actin is inactivated by removal of bound nucleotide or reaction with *p*-mercuribenzoate, the pK_a of the azomercurial adduct is lowered. The pK_a shifts of the actin-azomercurial adducts may indicate changes in the structure of the water surround which accompany G-F transformation and inactivation.

The sulfhydryl groups of actin play an important role in the maintenance of its structural and functional integrity. Kuschinsky and Turba (1951) first observed that G-actin lost its ability to polymerize after treatment with a number of sulfhydryl reagents, among them the organic mercurial, Salyrgan. Straub and Feuer (1950) and Laki *et al.* (1950) demonstrated that the ATP[1] bound to G-actin is hydrolyzed to ADP in the course of polymerization to F-actin, that the bound ATP is regenerated during depolymerization, and that G-actin which had lost its ATP was not able to polymerize. Maruyama and Gergely (1961) found that G-actin from which the bound calcium had been removed also was unable to polymerize. In addition to loss of the ability to polymerize, treatment of G-actin with organic mercurials also results in loss of protein-bound nucleotide (Martonosi and Gouvea, 1961; Barany *et al.*, 1961), loss of bound calcium (Barany *et al.*, 1962), and changes in optical rotatory dispersion (Tonomura and Yoshimura, 1962). It should be noted that there are differences in the relative effects of several mercurials. Thus bound calcium is removed from F-actin by *p*-mercuribenzoate but not by Salyrgan nor methylmercurinitrate (Barany *et al.*, 1962), and although methylmercurihydroxide causes a parallel loss of bound nucleotide and ability to polymerize, the ability to polymerize is lost much more rapidly after reaction with *p*-mercuribenzoate or Salyrgan than is bound nucleotide (Drabikowski and Gergely, 1963). Except in the experiments of Tonomura and Yoshimura (1962), the mercaptide formed with actin was not quantified nor related to the changes observed.

Martonosi (1962) recently reported a procedure for preparing actin free of the large amounts of tropomyosin that contaminate the usual actin preparations (Laki and Cairns, 1959). Different physical measurements have been obtained with highly purified actin, e.g., appreciably lower viscosity and molecular weight (Drabikowski and Gergely, 1962; Lewis *et al.*, 1963), particularly at low ionic strength where tropomyosin forms long polymers.

Recently, we reported a method (Zak *et al.*, 1965) of assessing sulfhydryl groups in proteins using an azomercurial dye, 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate. The mercaptide formed is estimated, and the method is particularly suitable for sequential determinations. In view of the importance of the sulfhydryl groups and their different reactivity with various reagents and the different characteristics of highly purified actin, it seemed worthwhile to study the reaction of the azomercurial with pure actin. The azomercurial dye possesses an ionizable phenolic hydroxyl group relatively far removed from the point of attachment to the protein. When ionized, the absorption maximum of the azomercurial is 434 mμ, while the absorption maximum of the nonionized form is 348 mμ. This ionizable group can be titrated independently by following the change in maximum absorbance of the azomercurial.

Klotz and Ayers (1957) found that the acidity constant of an ionizable group of a similar azomercurial dye was much lower when attached to bovine plasma albumin than when linked to cysteine. The anomalous acidity constant of the titratable group of the azomercurial attached to bovine plasma albumin was explained by

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[1] Abbreviations used in this work: ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; HAPM, 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate.

the conjecture that it was embedded in an envelope of highly ordered water surrounding the native protein which hindered its ionization. Upon denaturation of the protein with 8 M urea, the pK_a of the protein-azomercurial complex was nearly the same as the pK_a of the cysteine-azomercurial adduct, indicating a disintegration of the structured water envelope. While the precise interpretation of these pK_a changes is still uncertain, a study of the actin-azomercurial mercaptide provides valuable information about the environment of the actin molecule and the changes which accompany polymerization.

Experimental Procedure

Preparation of Actin. About 600 g of ground muscle from the backs and legs of rabbits, killed by intravenous injection of 25% $MgSO_4$ and then exsanguinated, was extracted with 2 liters of buffer, *p*H 6.3, containing 0.3 M KCl, 0.05 M K_2HPO_4, and 0.10 M KH_2PO_4 at 0° for 30 minutes. Water (8 liters) was added and the suspension was filtered through several layers of cheesecloth. The residue was extracted at room temperature for 5 minutes with 1 liter buffer, *p*H 9.0, containing 0.6 M KCl, 0.04 M $NaHCO_3$, and 0.01 M Na_2CO_3. Water (1 liter) was added and the suspension was filtered. The residue was then extracted with 3 liters of 0.4% $NaHCO_3$ at room temperature for 30 minutes and filtered. The latter residue was washed with 6 liters of water and chilled. It was then extracted twice with 450-ml portions of ice-cold 1-butanol in a Waring Blendor, followed by successive extraction with two batches of 450 ml ice-cold acetone in a Waring Blendor. The acetone-extracted powder was dried in the cold room and stored in a deep freeze.

The entire procedure of actin extraction was carried out in the cold room at 0–2° to avoid extraction of tropomyosin (Drabikowski and Gergely, 1962). Acetone-dried powder (40 g) was extracted with 600 ml 1 mM Tris buffer, *p*H 7.8, containing 0.2 mM ATP and 0.2 mM ascorbic acid for 30 minutes. Ascorbate was used in all solutions to minimize oxidation (Straub and Feuer, 1950). The solution was filtered through several layers of cheesecloth and the residue was extracted with an additional 400 ml buffer for 30 minutes. The filtrates were combined and clarified by centrifugation at 60,000 $\times$ *g* for 1 hour. KCl and $MgCl_2$ were added to the clarified filtrates to give final concentrations of 0.1 M and 1.0 mM, respectively. After standing in the cold room overnight, the F-actin was sedimented in the preparative ultracentrifuge, and the pellets were washed with cold buffer and suspended in cold buffer with the aid of a Teflon homogenizer to give a 0.3% suspension. This suspension was dialyzed against several changes of Tris-ATP-ascorbate buffer for 48 hours. The solution was clarified by centrifugation at 60,000 $\times$ *g* and partially polymerized by the addition of $MgCl_2$ to give a final concentration of 0.7 mM (Martonosi, 1962). After standing for 12 hours, the mixture was centrifuged. The pellets of F-actin were washed, resuspended, and dialyzed against Tris-ATP-ascorbate buffer as described.

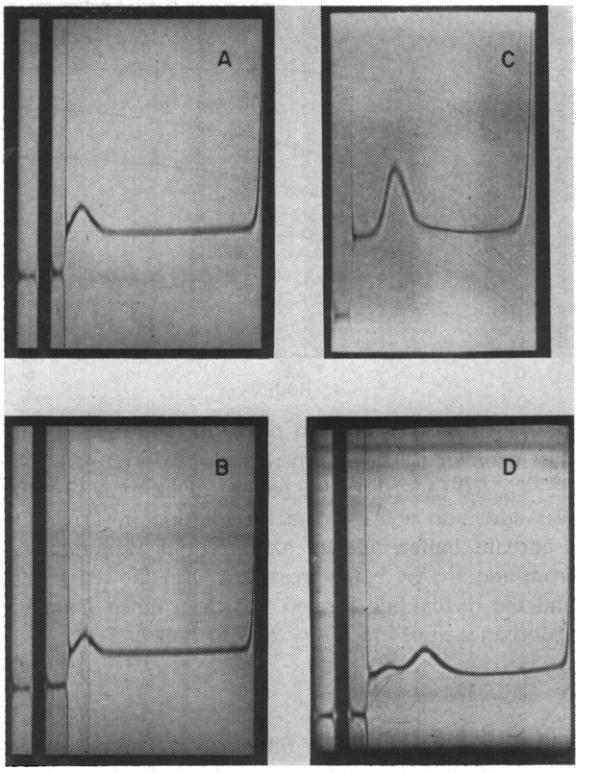

FIGURE 1: Ultracentrifuge diagrams of G-actin. (A) 0.35% G-actin purified by partial polymerization, in Tris-ATP-ascorbate buffer (see methods), *p*H 7.8, 25.0°, 37 minutes, $s_{20,w}$ = 2.64 S. (B) 0.35% G-actin purified by partial polymerization coupled with 2 moles azomercurial at 0°, in Tris-ATP-ascorbate buffer, *p*H 7.8, 25.0°, 37 minutes, $s_{20,w}$ = 2.54 S. (C) 0.6% G-actin prepared by classical procedures (contaminated with tropomyosin), in 10^{-4} M ATP, *p*H 7.0, 25.0°, 101 minutes. (D) 0.38% G-actin prepared by classical procedures coupled with 2 moles azomercurial at 20°, in 10^{-4} M ATP, *p*H 7.6, 37 minutes.

The G-actin solution was concentrated in an LKB Ultrafilter until the protein concentration was greater than 0.3%.

Upon fractionation with ammonium sulfate of these G-actin preparations purified by partial polymerization, no precipitate was obtained between 0.45 and 0.70 saturation. The reduced viscosity was not concentration dependent over the range 1.5–6.0 mg/ml, and the intrinsic viscosity was less than 0.1 dl/g. The addition of KI, final concentration 0.6 M, did not alter the viscosity immediately nor after 6 hours (Figure 3). A single, symmetrical peak, $s_{20,w}$ = 3.04, was obtained in the ultracentrifuge (Figure 1). A molecular weight of 57,200 was adopted for the monomer (Lewis *et al.*, 1963).

Formation of the Mercaptide. 4-(*p*-Hydroxybenzeneazo)phenylmercuriacetate (HAPM) was prepared as described previously (Zak *et al.*, 1965). Approximately 25 mg HAPM was titurated in 25 ml Tris-ATP-ascorbate buffer in a Potter-Elvejhem homogenizer for 30 minutes

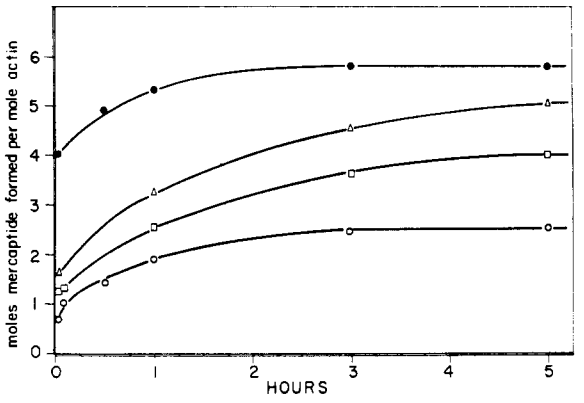

FIGURE 2: Rate of G-actin mercaptide formation with the azomercurial. Azomercurial, 2.5 (○—○), 4.0 (□—□), 6.0 (△—△), or 9.5 (●—●) moles/mole G-actin, was added to a 0.1% G-actin solution in Tris-ATP-ascorbate buffer. Excess azomercurial was removed from aliquots by batch treatment with Dowex 1 (Cl), and the mercaptide formed was determined from the absorbance at 434 mμ ($\epsilon = 2.73 \times 10^4$).

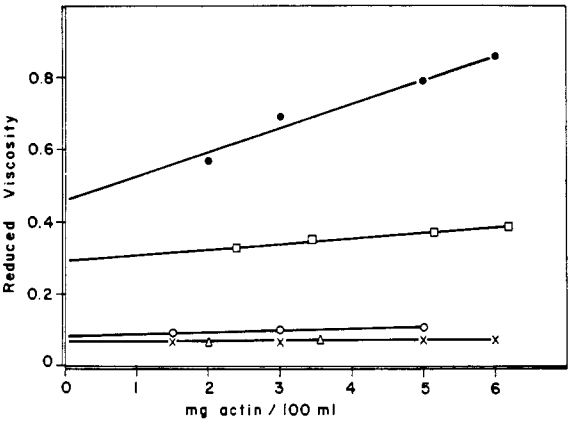

FIGURE 3: Viscosity of G-actin. G-actin extracted in the cold and purified by ultracentrifugation (●—●); same in 0.6 M KI (○—○); G-actin purified by the method of Ulbrecht *et al.* (1960) (□—□); tropomyosin-free G-actin prepared by partial polymerization (Martonosi, 1962) (×—×); same in 0.6 M KI (△—△).

and filtered on glass-fiber filter paper. The concentration of HAPM in the filtrate was determined by adding 4 volumes of 0.2 M glycine buffer, *p*H 10.6, to an aliquot and measuring the absorbance at 434 mμ using a molar absorbtivity of 2.73×10^4. Desired amounts of this solution were added to known actin solutions. The mercaptide was determined after removing the unreacted HAPM in an aliquot by absorption to Dowex 1, using approximately 25 mg resin/mg protein (Zak *et al.*, 1965). Four volumes of 0.2 M glycine buffer, *p*H 10.6, was then added and the absorbance was measured at 434 mu.

Analytical Methods. Ultracentrifuge sedimentations were carried out in a Spinco Model E analytical ultracentrifuge. Viscosity measurements were obtained with a modified Ostwald viscometer with a spiral capillary (Ulbrecht *et al.*, 1960) having an outflow time of about 60 seconds at 25° for water. Optical rotation was measured in a Rudolph Model 80 photoelectric polarimeter with an oscillating polarizer, using sodium and mercury lamps as light sources. Measurements were made at 25° in 10-cm cells at 589, 578, 546, 435, 405, and 365 mμ.

Reactive sulfhydryl groups on actin were determined using HAPM (Zak *et al.*, 1965), *p*-mercuribenzoate (Boyer, 1954), and amperometric titration with silver nitrate (Benesch *et al.*, 1955). After removal of free nucleotides by the addition of 15 mg Dowex 1 (chloride cycle) per ml solution, actin-bound nucleotides were determined by making a cold 2% (final concentration) perchloric acid extract and measuring the absorbance at 260 mμ using a molar extinction coefficient of 1.40×10^4. The presence of impurities other than adenine nucleotides was assessed by measuring the absorbance at 230 mμ. Total protein was determined with the biuret method using standard samples calibrated by Kjeldahl nitrogen determinations.

The acidity constants, pK_a, for the conjugate acid of the azomercurials were calculated from a plot of absorbance against *p*H by the procedure of Klotz and Ayers (1957) using a Beckman Model B spectrophotometer modified by Instrument Development Products Co., Chicago, to permit simultaneous measurements of absorbance and *p*H. A Leeds and Northrup Model 7664 a-c-operated *p*H meter was used for all *p*H measurements.

Reagents. ATP was purchased from Pabst Laboratories, *p*-mercuribenzoate from the California Corp. for Biochemical Research, Tris from Sigma Chemical Co., and all other reagents were reagent-grade commercial products.

Results

Mercaptide Formation. The reactivity of G-actin thiol groups in forming an azomercurial mercaptide is depicted in Figure 2. At 20°, 4.05 moles of mercaptide per mole of G-actin were formed within 5 minutes of the addition of a slight molar excess of HAPM, and an additional 1.86 moles (5.86 moles total) reacted within 3 hours. When 4 moles of HAPM or less per mole of G-actin was added, approximately one-third of the azomercurial reacted within 5 minutes and all reacted within 3 hours. When 6.0 moles HAPM/57,200 g G-actin was added, 1.89 moles of mercaptide was formed in 5 minutes and 5.19 moles was formed after 5 hours. Upon amperometric titration with silver nitrate 5.74 thiol groups/57,200 g G-actin were found and 5.91 moles SH/57,200 g G-actin was found with *p*-mercuribenzoate.

Physical Properties of the Mercaptide. The reduced viscosity of G-actin purified by partial polymerization (Figure 3) was not concentration dependent over the range 1.5–6.0 mg/ml, and the intrinsic viscosity was

TABLE I: Effect of Azomercurial on Some Properties of Actin.

Moles of Azomercurial per 5.72×10^4 g of Actin	η_{red}	Per Cent Polymerization of G-Actin[a]	Per Cent Depolymerization of F-Actin[b]	Nucleotide Content[c]	Optical Rotation		
					$[\alpha]_D$	λ_c	Per Cent Helix
0	0.095	100	0	0.993	44	247	25
1.5		88.5		1.005			
2.0	0.100	38.6	28		43	244	24
4.0		8.3		0.628			
5.0		0.0					
6.0	0.086	0.0	100	0.562	75		

[a] The η_{sp} of actin alone 4 hours after addition of KCl and $MgCl_2$ to final concentration; 0.1 M and 0.1 mM was used as 100%. [b] Expressed as per cent drop in η_{sp} of F-actin 20 hours after addition of azomercurial; η_{sp} of F-actin was used as 100%. [c] Moles of nucleotide per 5.72×10^4 g actin.

0.095 dl/g. The addition of 2.0 moles HAPM/57,200 g G-actin resulted in a mercaptide with the same viscosity. The adduct formed upon the addition of 6.0 moles HAPM/57,200 g G-actin had an intrinsic viscosity of 0.086 dl/g. No effect upon viscosity was observed after the addition of KI, 0.6 M final concentration. On the other hand, the reduced viscosity of tropomyosin-contaminated G-actin was concentration dependent, and the intrinsic viscosity ranged from 0.28 to 0.46 dl/g in various preparations. The reduced viscosity progressively fell as more HAPM reacted with G-actin, and was still concentration dependent. The intrinsic viscosity of the adduct formed upon the addition of 6.0 moles HAPM/57,200 g G-actin had an intrinsic viscosity of 0.17 dl/g. The addition of KI, 0.6 M final concentration, to tropomyosin-contaminated G-actin or any of its adducts with HAPM resulted in a fall of intrinsic viscosity to 0.10 dl/g. Similar results were obtained when *p*-mercuribenzoate was used.

When the adduct of 2 moles or less HAPM with G-actin purified by partial polymerization was sedimented in the ultracentrifuge, a single, broad, symmetrical peak was observed similar to that obtained by Martonosi (1962), and in these experiments for tropomyosin-free G-actin (Figure 1). When more than 2 moles of HAPM reacted with G-actin, a very broad, asymmetrical peak was observed. Using G-actin prepared by classical procedures (Mommaerts, 1952), the adduct with 2.0 moles HAPM or 2.0 moles *p*-mercuribenzoate gave two well-separated peaks.

The optical rotatory dispersion for G-actin in Tris-ATP-ascorbate buffer was evaluated using the simple, one-term Drude equation. A value of $\lambda_c = 247$ was obtained, corresponding to a helical content of 25% calculated by the method of Yang and Doty (1957). These values are similar to those of Nagy and Jencks (1962), who used tropomyosin-free actin; but in previous studies where the actin was prepared by classical methods, higher values were obtained for the helical content (Tonomura and Yoshimura, 1962; Standaert and Laki, 1962). A value of $\lambda_c = 244$ and a helical content of 24% was obtained for the adduct of 2.0 moles HAPM/57,200 g G-actin. Optical rotation could not be determined at all wavelengths when more than 2 moles of HAPM was coupled to actin because of the strong absorption of the azomercurial adduct. However, the values for $[\alpha]_D$ were $-44°$, $-43°$, and $-75°$, respectively, for G-actin and adducts containing 2.0 moles and 6.0 moles of HAPM, indicating a lower helical content when all thiol groups had been coupled (Table I).

No decrease in bound nucleotide was found in the adduct with 1.5 moles HAPM/57,200 g G-actin while 37 and 44% of the tightly bound nucleotide was released when 4.0 and 6.0 moles HAPM, respectively, were coupled.

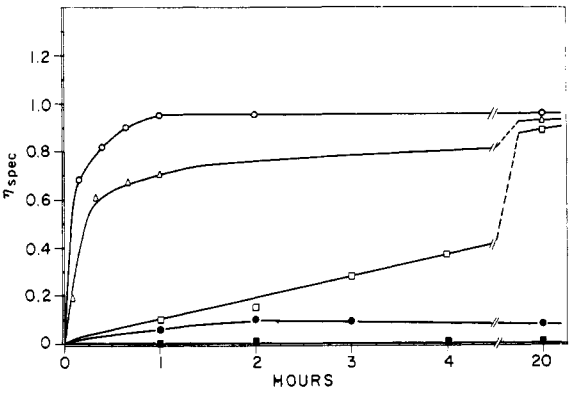

FIGURE 4: Polymerization of G-actin-azomercurial adducts. Viscosity of G-actin (○—○) or G-actin which had been reacted with 1.5 (△—△), 2.0 (□—□), 4.0 (●—●), or 4.5 (■—■) moles azomercurial/mole G-actin; 0.2% protein in Tris-ATP-ascorbate buffer at 25°.

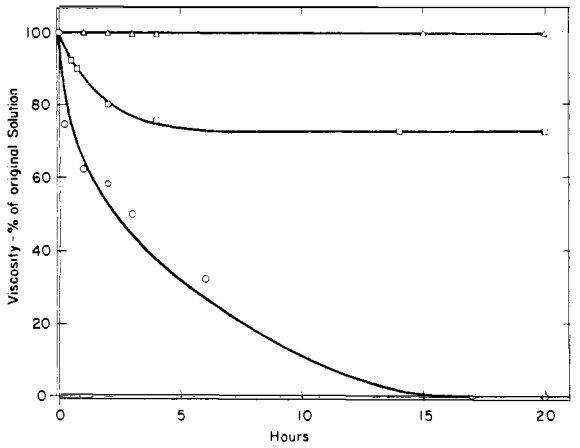

FIGURE 5: Depolymerization of F-actin by azomercurial. Viscosity of F-actin (as per cent of original solution) alone (△—△) or after the addition of 2.0 (□—□) or 6.0 (○—○) moles azomercurial/mole G-actin monomer; 0.2% protein in Tris-ATP-ascorbate buffer containing 0.1 M KCl and 0.1 mM $MgCl_2$.

Polymerization of the Mercaptide. The rate of polymerization of G-actin and several azomercurial adducts is shown in Figure 4. At 25°, the maximum viscosity of a 0.2% actin solution was reached within an hour after the addition of 0.1 M KCl and 0.1 mM $MgCl_2$ final concentrations. The mercaptides containing 1.5 moles and 2.0 moles HAPM polymerized more slowly but essentially the same maximum viscosity was finally reached. With the adduct containing 4.0 moles HAPM, the maximum viscosity was reached after 2 hours and was 12.5% of the maximum viscosity obtained with pure actin. Later the viscosity fell slightly.

When 2.0 moles HAPM/57,200 g actin was added to 0.35% F-actin in Tris-ATP-ascorbate buffer containing 0.1 M KCl and 0.1 mM $MgCl_2$ at 25°, the viscosity fell to 72% of the initial value within 5 hours and remained constant thereafter (Figure 5). When 6.0 moles HAPM/57,200 g actin was added to F-actin, the viscosity fell to 72% of the initial value in 15 minutes, to 59% in 2 hours, and after 17 hours the F-actin was completely depolymerized. The viscosity of F-actin itself did not change at 25° over a 20-hour period.

Acidity Constants of the Titratable Group on the Azomercurial. The acidity constants (pK_a values) of HAPM linked to β-mercaptoethylamine, β-lactoglobulin, or actin in a variety of conditions are listed in Table II. When linked to β-mercaptoethylamine, a thiol of low molecular weight which forms a soluble mercaptide with HAPM, the pK_a of the OH group was 7.95 in water. Protein-HAPM adducts have higher pK_a values, e.g., the β-lactoglobulin adduct has a pK_a of 9.26 in water. The actin-HAPM adduct has an even higher acidity constant. A pK_a of 9.67 was obtained for the adduct formed with F-actin containing approximately 0.5 mole HAPM/57,200 g actin. Upon depolymerization of the F-actin adduct in Tris-ATP-ascorbate buffer, the pK_a was 10.38. When the G-actin adduct was polymerized again to an F-actin adduct in 0.1 M KCl, the pK_a was 9.70, and when the G-actin adduct was subsequently reformed in Tris-ATP-ascorbate buffer, the pK_a was 10.28. Similar results were obtained when HAPM reacted with G-actin. Regardless of whether mercaptide formation was carried out with F-actin or G-actin, the HAPM adduct (containing less than 1 mole HAPM/57,200 g actin) had a pK_a in the neighborhood of 10.3 when in the monomer form and a pK_a in the neighborhood of 9.7 when polymerized.

When polymerized in D_2O, the F-actin-azomercurial had a pK_a of 11.20. The pK_a of the G-actin-azomercurial in D_2O was higher than that of the F-actin-azomercurial, but because of denaturation of the G-actin at the high *p*H, the values obtained were not thought to be reliable.

When two SH groups of G-actin were first blocked by reaction with *p*-mercuribenzoate and an adduct then formed with 0.5 mole HAPM/57,200 g G-actin, the pK_a was 10.02. A similar adduct, formed after first blocking four SH groups of G-actin, had a pK_a of 9.76.

TABLE II: Acidity Constants of 4-(*p*-Hydroxybenzeneazo)phenylmercury Adducts.

Derivative and Solvent	pK_a
β-Mercaptoethylamine in water	7.95
β-Mercaptoethylamine in 0.1 M KCl	8.04
β-Mercaptoethylamine in 8 M urea	8.72 (8.12)[a]
β-Mercaptoethylamine in 8 M urea, 0.1 M KCl	8.52 (8.02)[a]
β-Lactoglobulin in water	9.26
β-Lactoglobulin in 0.1 M KCl	9.04
β-Lactoglobulin in 8 M urea	9.21 (8.61)[a]
β-Lactoglobulin in 8 M urea, 0.1 M KCl	9.20 (8.71)[a]
F-Actin in 0.1 M KCl[b]	9.67
↓	
G-Actin + 2 moles *p*-mercuribenzoate	10.02
G-Actin + 4 moles *p*-mercuribenzoate	9.76
G-Actin in 8 M urea	9.10 (8.50)[a]
G-Actin in 0.01 M EDTA, 0.1 M KCl	9.80
G-Actin in 1.0 mM Tris, 0.2 mM ATP, 0.2 mM ascorbate	10.38
F-Actin in 0.1 M KCl in D_2O	11.20
F-Actin in 0.1 M KCl	9.70
G-Actin in 0.2 mM ATP, 0.2 mM ascorbate	10.28
G-Actin, charcoal treated, water	10.10
↓	
F-Actin, charcoal treated, 0.1 M KCl	10.52

[a] The pK_a values "corrected" for the presence of 8 M urea (Donovan and Scheraga, 1959). [b] Less than 1 equivalent of azomercurial reacted with the F-actin preparation. The actin-azomercurial adduct was depolymerized to G-actin, then polymerized again to F-actin, and finally again depolymerized to G-actin.

The pK_a of the G-actin-azomercurial was 9.10 in 8 M urea and 9.80 in 0.01 M EDTA + 0.1 M KCl. The pK_a of the F-actin-azomercurial was 9.13 in 8 M urea. The pK_a values of phenolic OH groups are anomalous in 8 M urea (Donovan and Scheraga, 1959); the corrected pK_a values are approximately 8.5. The acidity constant of the charcoal-treated G-actin adduct was 10.10 while that of the F-actin adduct treated with charcoal in 0.1 M KCl was 10.52. Treatment with charcoal may have effects on the actin molecule in addition to removal of nucleotides (Barany *et al.*, 1961).

Discussion

The six thiol groups of G-actin, as previous investigators have observed (Tonomura and Yoshimura, 1962; Katz and Mommaerts, 1962; Drabikowski and Gergely, 1963), can be divided into three pairs which differ in several characteristics. On the basis of their reactivity with the azomercurial dye used in this study, one pair of thiol groups reacts very rapidly, and although the mercaptide formed polymerizes somewhat less rapidly than pure G-actin, the F-actin-azomercurial adduct finally formed has the same viscosity as pure F-actin. The second pair of thiols reacts rapidly, too, with HAPM, but the mercaptide formed does not polymerize completely. These observations are similar to those of Tonomura and Yoshimura (1962), who used *p*-mercuribenzoate, but unlike the results of Katz and Mommaerts (1962), who found that four thiol groups could be reacted with *p*-mercuribenzoate without affecting the ability of G-actin to polymerize. The third pair of thiol groups reacts quite sluggishly and the mercaptide formed does not polymerize, although F-actin does not appear to contain any disulfide linkages.

It is worth noting that the actin used in this study was extracted from the acetone powder at 0° with Tris-ATP-ascorbate buffer and purified by partial polymerization (Martonosi, 1962). This actin was thought to be tropomyosin free, as judged by an intrinsic viscosity of approximately 0.1 dl/g for this preparation and a reduced viscosity which was not concentration dependent nor lowered by the addition of KI. In contrast to actin contaminated with tropomyosin, which gives an asymmetrical peak in the ultracentrifuge, the actin used here gave the symmetrical peak characteristic of tropomyosin-free actin (Martonosi, 1962). It was difficult to obtain an actin preparation free of tropomyosin when ascorbate was omitted during the extraction from the acetone powder.

The azomercurial adduct with two thiol groups of G-actin also had an intrinsic viscosity of about 0.1 dl/g and a reduced viscosity which was not concentration dependent nor lowered by the addition of KI. A single, symmetrical peak in the ultracentrifuge was also obtained when 2 moles of HAPM was reacted with actin not contaminated with tropomyosin. However, the HAPM adduct prepared by classical procedures gave a double peak. Although other evidence has been brought forward for the existence of a G-actin dimer (Oosawa and Kasai, 1962; Lewis *et al.*, 1963), the two peaks observed by Katz (1963) in his *p*-mercuribenzoate-actin adduct may be an artifact resulting from his use of actin contaminated with tropomyosin. The HAPM-actin adduct had the same nucleotide content and optical rotatory dispersion as pure G-actin. These data indicate that the properties and conformation of G-actin change very little, if at all, when two or fewer thiol groups react with HAPM.

When more than two sulfhydryl groups react with HAPM, bound ATP is slowly released and the mercaptide formed polymerizes sluggishly and incompletely to F-actin, and the large differences in optical rotatory dispersion from pure G-actin indicate gross conformational changes in the mercaptide monomer. Similar results were obtained by Tonomura and Yoshimura (1962) for *p*-mercuribenzoate-actin adducts.

It is possible that the changes in acidity constants of the titratable group on the azomercurial attached to actin may result entirely from electrostatic interactions. These electrostatic effects may be generalized or they may be local. For example, the proximity of an amino group on one strand of F-actin to the hydroxyl group of HAPM attached to an adjacent strand of F-actin could possibly result in lowering of the pK_a compared to G-actin. Bar-Eli and Katchalski (1963) observed shifts in the *p*H optima of enzymes enmeshed in a cross-linked plastic. However, Klotz and Elfbaum (1964) compared the pK_a values of a number of azomercurial complexes with proteins with varying content of basic groups and concluded that electrostatic effects did not contribute significantly to the pK_a shifts. It should be remembered that at these *p*H values both F- and G-actin are well on the alkaline side of their isoelectric points. Also, the addition of salt results in a fall in pK_a while an increase in pK_a would be expected if the addition of salt provided a small negative counterion. Studies with acetylated actin may clarify the role of electrostatic effects.

The shift in pK_a might occur as a result of a complex formed by hydrogen bonding between the phenolic OH group of HAPM and a carboxyl group. Such a shift would be analogous to the anomalous acidity constants of some tyrosyl residues of ribonuclease which may form hydrogen-bonded complexes (Cowgill, 1964).

While there are alternative explanations, it is attractive to interpret the changes in pK_a of the titratable group on the actin-azomercurial in terms of an increased organization of the water which surrounds protein molecules, a notion suggested by Klotz (Klotz and Ayers, 1957; Klotz, 1960). Whether clathrate formation (Klotz, 1960) or clusters of hydrogen-bonded water molecules stabilized by the protein (Némethy and Scheraga, 1962) are responsible for the structured water adjacent to protein molecules, the ionization of titratable groups embedded in it is hindered because of the disruptive effect of ionic charges on the structure of the lattice. The higher pK_a of the phenolic hydroxyl of HAPM when attached to protein as compared to the pK_a of HAPM-β-mercaptoethylamine adducts is thought to result from this mechanism; the magnitude of the pK_a shift may reflect the extent and stability of the

structured water envelope in the neighborhood of the dye. On the basis of hydrodynamic data, Lewis *et al.* (1963) suggested that the G-actin molecule contains a solvent-penetrable domain. It may be noted that studies with the dimethylaminoazomercurial used by Klotz were not possible because actin is irreversibly denatured at low *p*H.

In D_2O where the structural order is greater than H_2O (Némethy and Scheraga, 1964), the pK_a of the HAPM-actin adduct is much higher than in water. When the nucleotide is removed from G-actin by treatment with charcoal, the pK_a of the HAPM-actin adduct drops somewhat. Treatment of the F-actin adduct with charcoal also results in depolymerization in addition to loss of nucleotide and the pK_a is the same as that of charcoal-treated G-actin adduct. When a G-actin adduct is formed after blocking the rapidly reacting thiol groups with *p*-mercuribenzoate, the pK_a is also lowered. Thus the pK_a shifts of the HAPM-actin adducts may signify a change in the structured water surrounding the protein which accompanies the transformation from G-actin to F-actin, or the conformational changes of G-actin which follow removal of nucleotide or blocking sulfhydryl groups.

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