Damage to Actin Filaments by Glutaraldehyde: Protection by Tropomyosin

SHERWIN S. LEHRER

Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Reaction of F-actin and the F-actin-tropomyosin complex with 20 mM glutaraldehyde for 19–22 h at 0°C and 25°C results in extensively cross-linked filaments, as judged by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Electron micrographs show shorter, more irregular filaments for glutaraldehyde-treated F-actin in the absence of tropomyosin as compared to the presence of tropomyosin or untreated controls. There was a 40% drop in viscosity of glutaraldehyde-treated F-actin solutions but a 90% increase in viscosity for the glutaraldehyde-trerated F-actin-tropomyosin complex in solution, as compared to the untreated controls, indicating different effects of cross-linking.

SDS gels indicate that intrasubunit cross-links are introduced into F-actin and that when tropomyosin is present, intramolecular cross-link formation is inhibited. Inhibition of the salt-induced G → F polymerization results when intramolecular cross-links are introduced into G-actin under similar or milder reaction conditions. These data indicate that, under conditions for which extensive F-actin filament cross-linking (fixing) occurs, the filaments become damaged due to the concurrent formation of intrasubunit cross-links that cause local depolymerization and distortion and that tropomyosin protects against this damage.

Early studies of the effect of glutaraldehyde treatment of solutions of F-actin and G-actin showed that covalent cross-links were introduced between actin subunits for F-actin but only within the actin subunit in the case of G-actin, in agreement with the known subunit proximity within the F-actin filament and the monomeric properties of G-actin (5). The more extensive cross-linking of the F-actin filament observed in the presence of tropomyosin was also consistent with the known head-to-tail interaction of the tropomyosin chains and the close interaction between tropomyosin and actin subunits in the groove of the actin helix (5). Damaging effects during glutaraldehyde treatment of F-actin in solution were noted by losses in the characteristic high viscosity. This was not the result of denaturation because the cross-linking process actually stabilized G- and F-actin against heat- and EDTA-induced denaturation (5, 6). Because the G → F polymerization process was strongly inhibited when G-actin was treated with glutaraldehyde, it was suggested that the viscosity loss of F-actin was due to local depolymerization caused by cross-linking within a subunit in or near the subunit-subunit interaction site (5, 6).

In view of the many observations of actin filaments in a variety of cell types, we decided to further characterize the consequences of glutaraldehyde reaction with F-actin filaments with the aim of finding conditions which might minimize possible artifacts. Electron micrographs of negatively stained filaments showed a large degree of damage under conditions for which considerable *inter*subunit cross-linking occurs within F-actin filaments. This damage appears to be the result of localized depolymerization and distortion associated with the formation of *intra*subunit cross-links. When tropomyosin was bound to F-actin, however, optimum intersubunit cross-linking occurred at a glutaraldehyde concentration an order of magnitude lower, and no filament damage was observed in electron micrographs.

MATERIALS AND METHODS

Actin and tropomyosin (Tm) were prepared as outlined previously (5). Heavy meromyosin (HMM) was prepared by the method of Lowey and Cohen (9) using a 400:1 ratio of myosin:trypsin and a 4-min incubation at 25°C. G-actin was prepared by homogenizing a pellet of F-actin in G-buffer (5 mM HEPES, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$) containing 5 mM dithiothreitol (DTT) and dialyzing vs. buffer without DTT. After determining the concentration with $E^{1\,mg/ml}_{290\,nm} = 0.63$ (7), it was diluted to 0.5–1 mg/ml in F-buffer (G-buffer + 0.05 M NaCl, 5 mM $MgCl_2$ or G-buffer + 0.1 M NaCl) to polymerize. In the case of the HMM-decorated filaments, F-actin was made by polymerizing G-actin with the addition of NaCl to 0.1 M. The F-actin+Tm complex was prepared by adding Tm to the F-actin solution to give a final F:Tm weight ratio of 4:1.

Small volumes of 8% (800 mM) glutaraldehyde (Polysciences, Inc., Warrington, Pa., sealed under N_2) were added to test tubes to yield the desired final concentration; the protein solutions were added and mixed, reacted at the temperature and times indicated in the figures, and quenched by the addition of 0.8 M triglycine solution (pH 8.4), or by addition of 1 M Tris buffer (pH 8.4), to final concentrations of 0.1 M, and dialyzed to remove excess reagents.

Decorated actin+HMM filaments were prepared from the cross-linked samples and controls after dialyzing vs. 0.1 M NaCl, 0.1 mM $CaCl_2$ + 5 mM HEPES buffer (pH 7.5) to remove excess ATP. Excess HMM was added and the solutions were incubated at 0°C overnight before applying to carbon-coated grids for electron microscopy.

Electron micrographs were obtained with a Philips EM300 instrument on samples applied to carbon-coated grids and negatively stained with 1% uranyl acetate. In the case of undecorated filaments, the grid was treated successively with one drop of actin solution diluted to 0.1 mg/ml with F-buffer, rinsed with several drops of F-buffer, and several drops of uranyl acetate, and drained by touching to a piece of filter paper. Decorated filaments were similarly applied to grids using a 5 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl diluted threefold for dilution and rinsing.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed by the Weber-Osborn technique, as outlined previously (5), on samples which were reduced with DTT, and carboxymethylated with iodoacetamide in the presence of 1% SDS, and run on 5% polyacrylamide tube gels in the presence or absence of 4 M urea (11). The viscosity studies were performed with Cannon viscometers (Cannon Instruments, Co., State College, Pa.) at 25°C (water flow time, 30 s).

RESULTS

Pattern of Cross-linked Species

The cross-linking of actin by glutaraldehyde was followed by gel electrophoresis in SDS. Intrasubunit cross-linking increased the mobility of the monomer and intersubunit cross-linking generated a series of oligomers that are multiples of 43,000 dalton or very high molecular weight complexes which do not enter the gel (Fig. 1). Low glutaraldehyde:protein concentration ratios were used for cross-linking actin filaments to increase the probability that glutaraldehyde will react in a bifunctional and specific manner. This necessitated relatively long reaction times. Under conditions where the F-actin+tropomyosin filaments are extensively cross-linked, as evidenced by the loss of the actin and tropomyosin monomer bands and the presence of a very high molecular weight cross-linked species at the top of the 5% gel (glutaraldehyde-treated F-actin+tropomyosin [Ga(F+Tm)]), F-actin is only partially cross-linked, as shown by the oligomeric protein band pattern (GaF). Similar patterns were obtained at lower protein concentrations. A glutaraldehyde concentration of 10–20 mM is necessary to effectively cross-link F-actin in the absence of tropomyosin (Tm) at room temperature (Fig. 2). The same pattern of cross-linked species is observed for reaction at 0°C, but the reaction rate is slowed down considerably. A comparison of the patterns at both temperatures indicates that similar degrees of cross-linking are obtained at room temperature with ~1/10 the glutaraldehyde concentration used at 0°C.

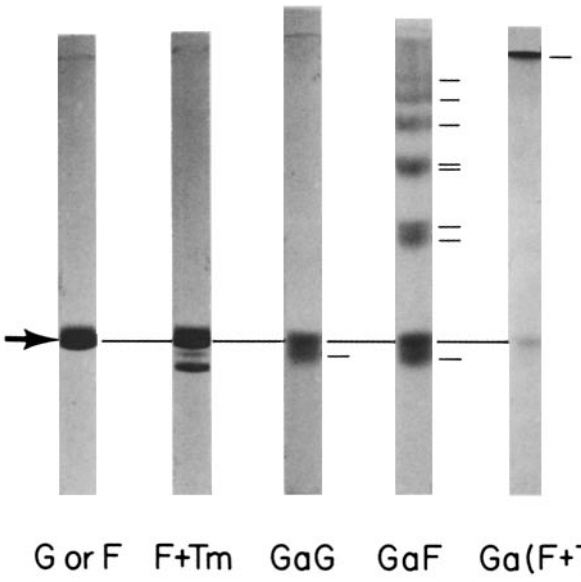


FIGURE 1 Cross-linking of actin and the actin-tropomyosin complex by 2 mM glutaraldehyde (*Ga*) for 19 h at 25°C, visualized by SDS PAGE. Actin concentration, 1 mg/ml; tropomyosin concentration, 0.25 mg/ml. Buffer is 5 mM HEPES, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$. F-actin (*F*) and F-actin+tropomyosin (*F+Tm*) also contained 0.1 M NaCl. Arrow indicates position of unmodified actin monomer. Amount applied, 10 μl.

Treatment of G-actin with glutaraldehyde resulted in species with slightly greater mobility on SDS gels than unreacted controls. As the glutaraldehyde concentration was increased, additional bands in gels were observed. For example, at 2 mM glutaraldehyde, three bands appear to be present (Fig. 3). Because it has been shown that an increase in protein mobility on SDS gels is a result of intrasubunit cross-linking (3), it appears that the diffuse bands of higher mobility observed for glutaraldehyde-treated G-actin represent a distribution of cross-linked G-actin species. Additional protein bands of higher mobility are also seen in the gel pattern of glutaraldehyde-reacted F-actin, particularly resolvable in the monomer and dimer regions. This indicates that glutaraldehyde produces intrasubunit cross-links as well as intersubunit cross-links in the case of F-actin. The possibility that the additional bands of higher mobility are due to proteolytic cleavage does not seem likely because the fraction of material in the faster moving bands increases with glutaraldehyde treatment, and they were not present in the untreated control.

Reaction of F-actin with increased concentrations of glutaraldehyde causes a shift of the oligomeric pattern to higher molecular weight (Fig. 2). At 10–20 mM for an overnight reaction at 25°C, essentially all of the material is present near the top of the 5% gel, indicating that the molecular weight is at least 5×10^5. A similar shift of the oligomeric pattern to higher molecular weight was also seen at constant glutaraldehyde concentration (20 mM) for increasing reaction time (data not shown). Thus, the intracrosslinked subunits of F-actin formed intersubunit cross-links at later stages in the reaction with glutaraldehyde.

In the case of the F-actin+tropomyosin complex, a different pattern of cross-linked bands in gels is observed as the reaction proceeds (Figs. 2 and 4). First, the tropomyosin monomer bands are lost with little or no loss in the actin monomer band. This indicates that tropomyosin molecules are cross-linked to each other while on the actin filament before being cross-linked to actin subunits (5). At later stages in the cross-linking reaction, the actin monomer band is lost with the corresponding appearance of high molecular weight cross-linked species excluded from the top of the gel. During the course of the cross-linking, essentially one actin monomer and no oligomer bands were observed in gels, further indicating that actin-tropomyosin cross-links were formed more readily than actin-actin intersubunit cross-links and actin intrasubunit cross-links. The observation of a small degree of oligomeric actin bands in gels in the initial study (5) was due to the cross-linking of excess actin not complexed to tropomyosin since a 5:1 actin:tropomyosin weight ratio was used in that work.

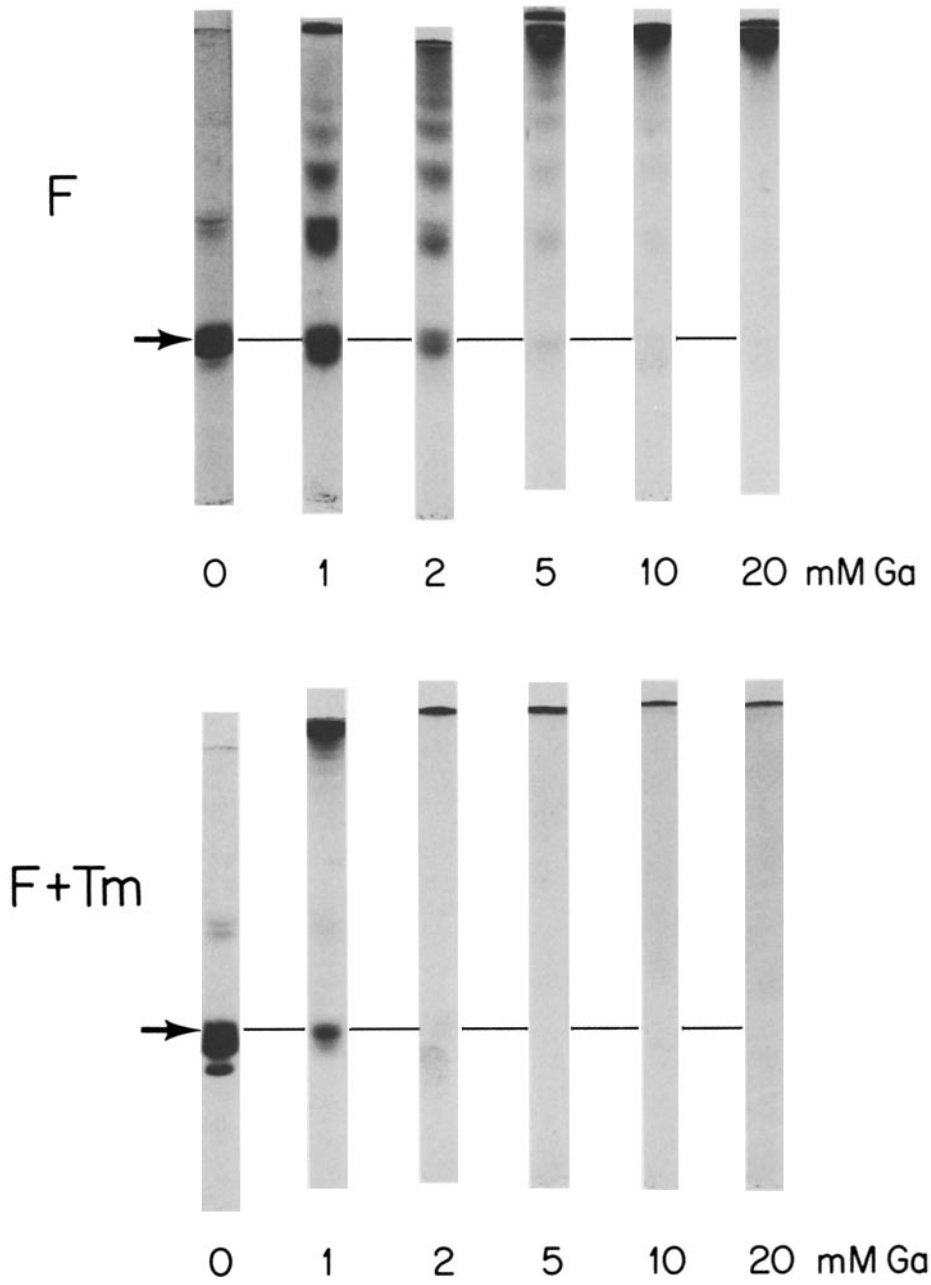


FIGURE 2 Dependence of the cross-linking of F and F+Tm on glutaraldehyde concentration. Conditions as for Fig. 1. Amount applied, 20 μg.

Effects of Cross-linking

The effect of glutaraldehyde on the inhibition of the G-actin → F-actin (G → F) polymerization (5) was studied further. It was found that overnight reaction of G-actin with 1 mM glutaraldehyde completely inhibited the salt-induced polymerization to F-actin for at least 40 min, whereas the control G-actin was fully polymerized in 5 min (Fig. 5). The possibility that G-actin was denatured by the glutaraldehyde treatment was eliminated in the earlier study (5) and verified here. Thus, glutaraldehyde-treated G-actin is actually stabilized against EDTA and heat denaturation, presumably due to the intrasubunit cross-linking. The most likely reason, therefore, for the loss of polymerizability is that the intramolecular cross-links that are formed are localized in the polymerization site and therefore sterically interfere with proper subunit-subunit interaction.

Effects of cross-linking F-actin and F-actin+tropomyosin were monitored by measuring changes in the viscosity of each solution during reaction with 20 mM glutaraldehyde. A time-dependent decrease in viscosity of F-actin solutions and a time-dependent increase in viscosity of F-actin+tropomyosin solutions were observed during reaction, reaching the final value shown in Table I for a typical example. The viscosity decrease is not the result of denaturation because, as mentioned above, glutaraldehyde-treated F- or G-actin has even greater stability toward denaturation (5). Thus, the cross-linking reaction affects the structure of F and F-actin+tropomyosin in solution in different ways. The decrease in viscosity for F-actin suggests that glutaraldehyde cross-linking results in damage to actin

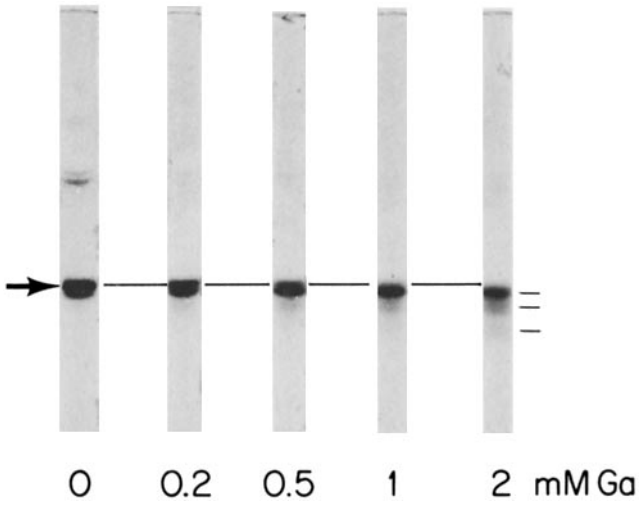


FIGURE 3 Dependence of the cross-linking of G-actin on *Ga* concentration, for 19 h at 25°C. Actin concentration, 1 mg/ml in 5 mM HEPES, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$. Arrow indicates position of monomer. Amount applied, 5 μg.

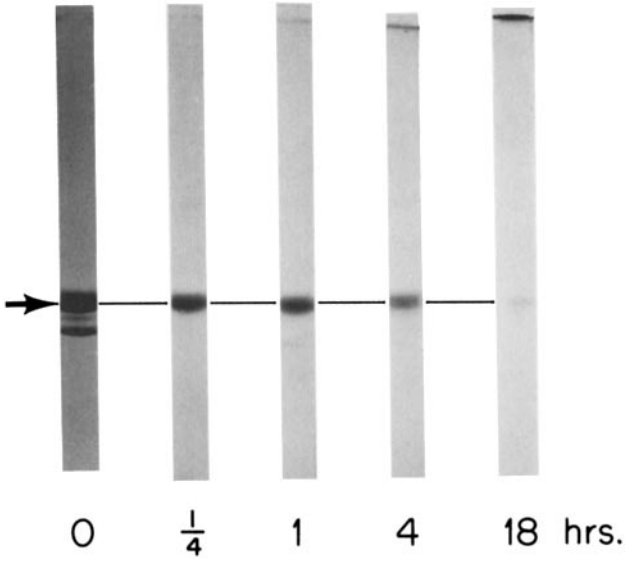


FIGURE 4 Dependence of the cross-linking of *F+Tm* on reaction time. Conditions as for Fig. 1. Amount applied, 10 μg.

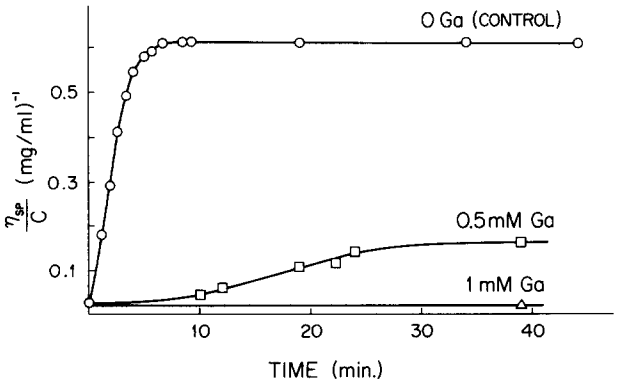


FIGURE 5 Inhibition of polymerization of G-actin at 1 mg/ml, due to reaction with *Ga* at the indicated concentrations for 19 h at 25°C. Conditions as for Fig. 3. Viscometry was used to measure formation of *F* after addition of 0.1 M NaCl at t = 0.

TABLE I

Viscosity of F-actin and F-actin+tropomyosin solutions (1 mg/ml) at 24°C before and after Reaction with Glutaraldehyde (20 mM, 19 h, 25°C)

Sample	η_{sp}/c Before reaction	η_{sp}/c After reaction
	ml/mg	
F-actin	0.67	0.40
F-actin+tropomyosin	0.71	1.32

filaments when tropomyosin is not present. The increase of viscosity of F-actin solutions due to the binding of tropomyosin had been observed (1) and is probably caused by a decrease in flexibility of the filament (4). The very large increase in viscosity after glutaraldehyde treatment can be explained by a further decrease in flexibility due to the presence of covalent cross-links.

Electron micrographs of negatively stained actin filament preparations provided clear evidence that glutaraldehyde damages F-actin filaments. Micrographs of F-actin and F-actin+tropomyosin reacted with 10 and 20 mM glutaraldehyde for 22 h at 0°C are shown in Fig. 6. The glutaraldehyde-treated F-actin+tropomyosin filaments reacted with 20 mM glutaraldehyde, as well as the control F-actin and F-actin+tropomyosin filaments, are long and relatively straight, whereas the glutaraldehyde-treated F-actin filaments, reacted with only 10 mM glutaraldehyde, are shorter with many twists and bends. An estimation of the change in length caused by glutaraldehyde was obtained by measuring the lengths of 60–180 filaments in each case. It was found that the percentage of filaments with lengths <0.5 μm was <30% for F, F-actin+tropomyosin, and glutaraldehyde-treated F-actin+tropomyosin filaments, whereas 90% of the glutaraldehyde-treated F-actin filaments were shorter than 0.5 μm. Similar effects were observed by others using electron microscopy on nondecorated actin filaments at higher concentrations of glutaraldehyde than used here (2). These effects are seen more dramatically for HMM-decorated actin filaments (Fig. 7). Thus, these micrographs show clearly that treatment of F-actin with glutaraldehyde in the absence of tropomyosin results in shorter, more distorted filaments. Either of these effects could cause the observed decrease in viscosity. Despite this damage and the presence of cross-links, HMM can still bind to F-actin-treated F-actin and F-actin+tropomyosin filaments to produce the familiar arrowhead structures.

Further evidence for the protective effect of tropomyosin was obtained by comparing micrographs of samples of glutaraldehyde-treated F-actin and F-actin+tropomyosin after the removal of salt by dialysis (Fig. 8). This dialysis completely depolymerized the F-actin to G-actin in the case of the F-actin and F-actin+tropomyosin controls, as expected, and no filaments were observed in micrographs. In contrast, filaments were observed in micrographs of the glutaraldehyde cross-linked samples, despite the depolymerizing conditions; i.e., the cross-links keep the actin subunits linked together. The resulting glutaraldehyde-treated F-actin+tropomyosin filaments appear much straighter and wider, but also shorter than control samples at normal salt concentrations, probably due to charge repulsion and incomplete cross-linking, respectively. Micrographs of a sample similarly treated in the absence of tropo-

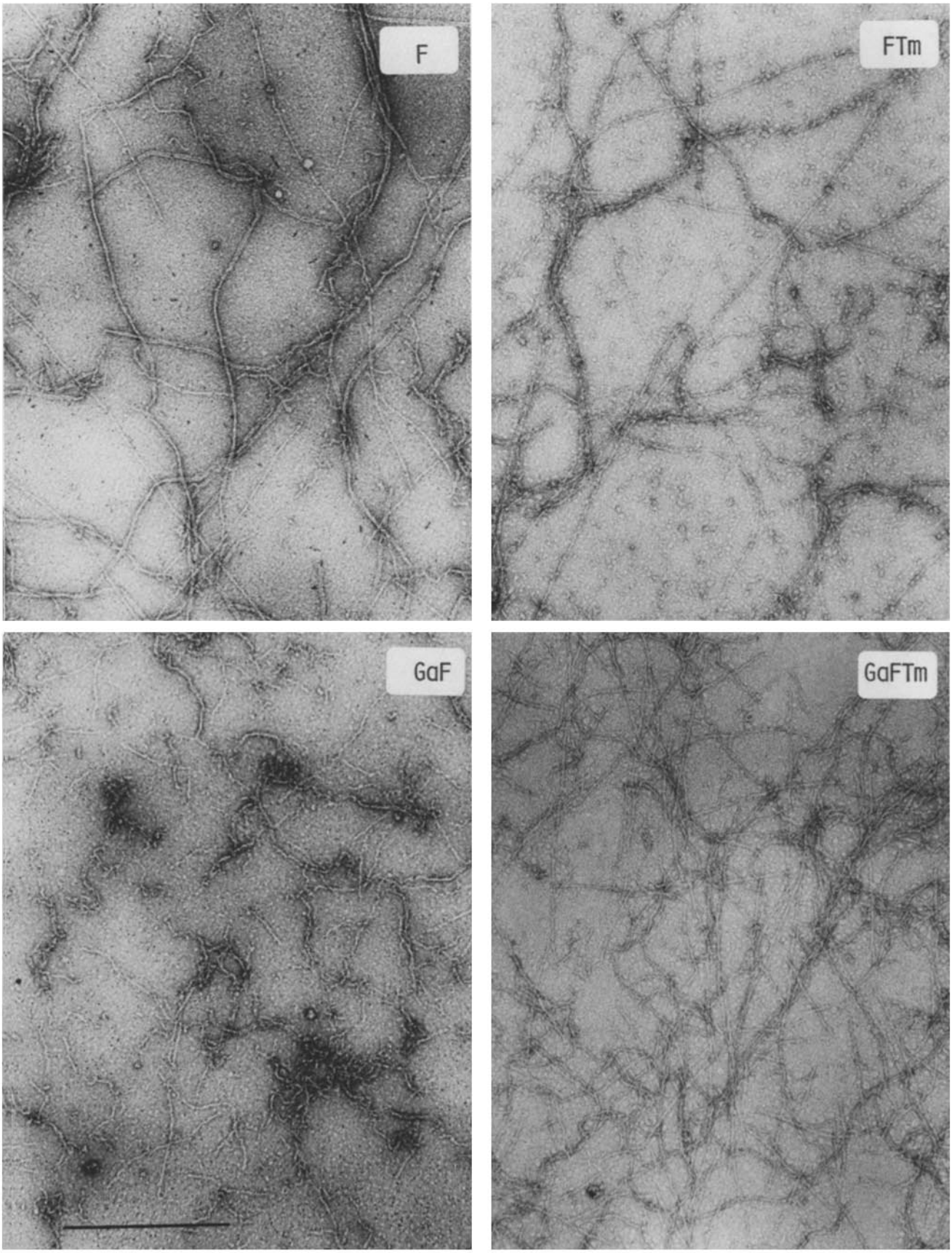


FIGURE 6 Electron micrographs of *Ga*-treated *F* and F-actin+tropomyosin (*FTm*). Treatment conditions as for Fig. 3; [*Ga*] = 10 mM for *GaF* and 20 mM for *GaFTm*. Bar, ½ μm. × 66,000.

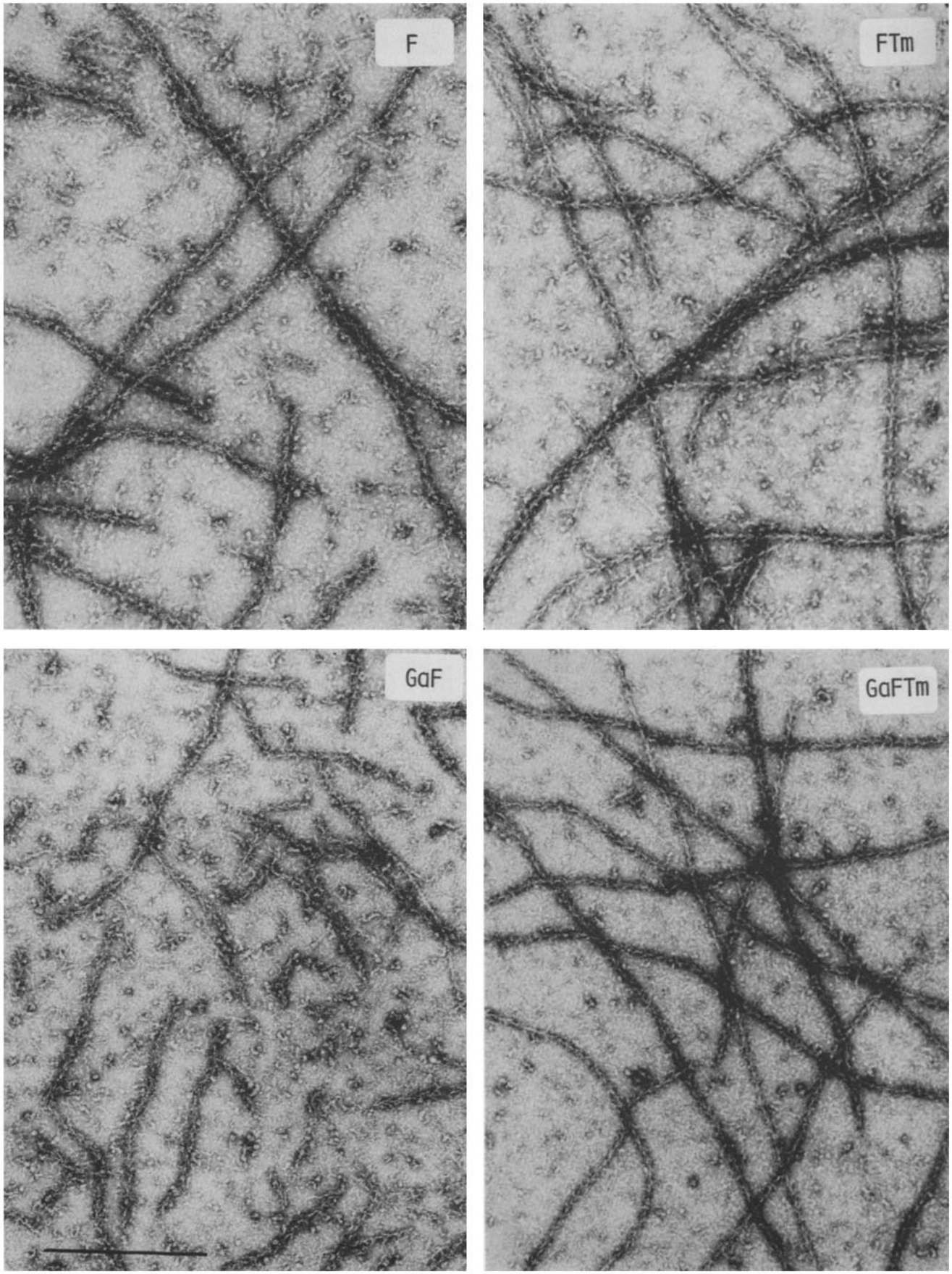
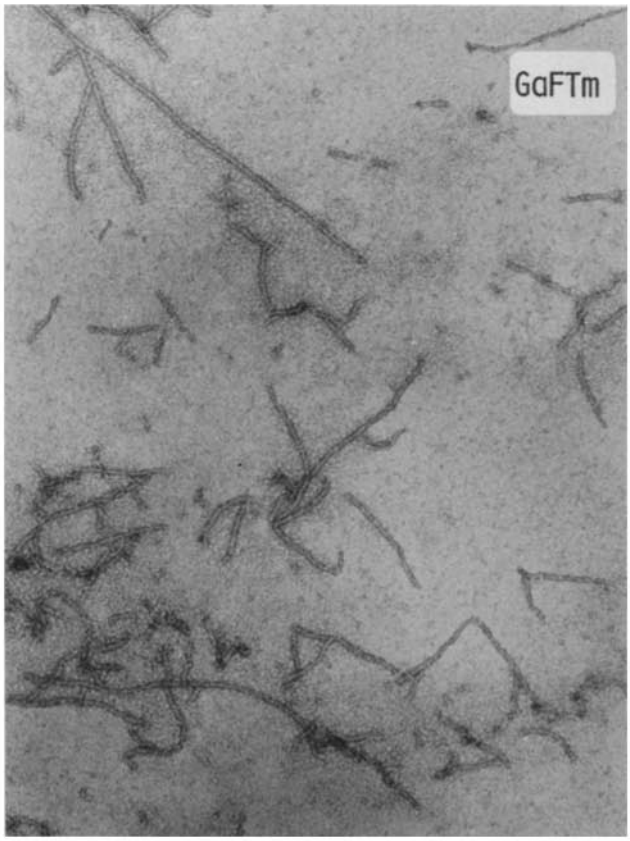


FIGURE 7 Electron micrographs of *Ga*-treated *F* and *FTm* decorated with HMM. Treatment conditions as for Fig. 1; [*Ga*] = 20 mM. Bar, ½ μm. × 66,000.



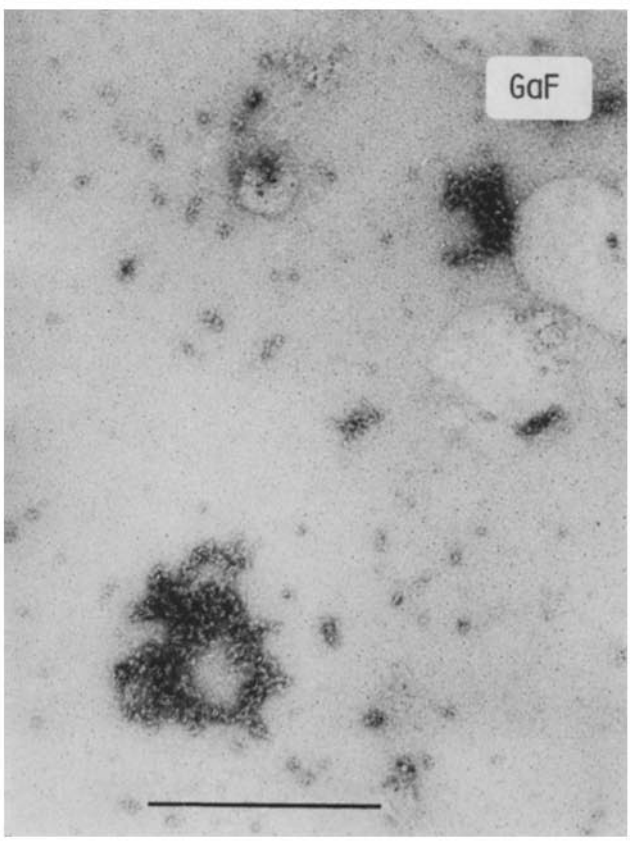


FIGURE 8 Electron micrographs of glutaraldehyde treated F-actin (*GaF*) and F-actin-tropomyosin (*GaFTm*) after dialysis vs. low salt to depolymerize. Treatment conditions as for Fig. 3; [*Ga*] = 10 mM. Bar, ½ μm. × 66,000.

myosin showed the presence of large and small aggregates in which individual filaments were difficult to distinguish.

DISCUSSION

From the electron micrographs seen above, it is clear that glutaraldehyde treatment of F-actin in the absence of tropomyosin results in shorter, more distorted cross-linked actin filaments as compared to untreated controls. In the presence of tropomyosin, little or no damage is observed. The shorter cross-linked actin filaments still correspond to molecular weights very much greater than would be excluded from the 5% SDS PAGE gels used above to study the cross-linking process. The gels, therefore, provide information mainly about the early cross-linking process when short covalently cross-linked oligomers are mainly present. With the observation of more than one band of higher mobility at each oligomer position, these gels showed that the subunits of F-actin are intracross-linked early in the reaction and that these modified subunits are able to undergo further intersubunit cross-linking. The observation that the polymerization of G-actin is completely inhibited by mild treatment with glutaraldehyde suggests that localized depolymerization or distortion would result when some of the same sidechains of the subunits of F-actin are modified. Shorter filaments would result as the number of intrasubunit cross-links increases, particularly if local distortion further increases the probability of intracross-linking.

In the presence of tropomyosin, no filament damage is observed in micrographs and little or no intrasubunit actin cross-links are indicated on gels. The protection by tropomyosin could be due to the stabilization of the filament by the tropomyosin-tropomyosin and tropomyosin-actin cross-links which are formed earlier than the destabilizing intra-actin cross-links.

These studies indicate that caution is necessary in the interpretation of micrographs when tissues or other preparations suspected of containing F-actin filaments uncomplexed with tropomyosin are fixed with glutaraldehyde (8). In such cases, it may be possible to add tropomyosin externally before fixation, to protect against the damage. A similar protection by tropomyosin was observed in studies of OsO_4-induced artifacts in the fixation of F-actin filaments (10). In that case, OsO_4 damaged filaments by reacting with certain amino acid residues causing denaturation and peptide bond cleavage. In the case of the glutaraldehyde effect, the damage appears to be caused by local distortions which leave the subunits in a native state.

I would like to express appreciation to Mr. K. Mabuchi for his expert help in the initial electron microscopy preparations and pictures, and to Ms. Mary Ho Eng and Ms. Justine Strungis for their excellent technical assistance.

This work was supported in part by grants from the National Institutes of Health (AM 11677 and HL 22461) and the Muscular Dystrophy Association.

Correspondence should be addressed to Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford St., Boston, Massachusetts 02114.

Received for publication 3 November 1980, and in revised form 6 April 1981.

REFERENCES

1. Drabikowski, W., and E. Nowak. 1968. Studies of the interaction of F-actin with tropomyosin. *Eur. J. Biochem.* 5:376–384.
2. Gadasi, H., A. Oplatka, R. Lamed, A. Hochberg, and W. Low. 1974. Possible uncoupling

of the mechanochemical process in the actomyosin system by covalent crosslinking of F-actin. *Biochim. Biophys. Acta.* 333:161–168.
3. Griffith, I. P. 1972. The effect of cross-links on the mobility of proteins in dodecyl sulphate-polyacrylamide gels. *Biochem. J.* 126:553–560.
4. Ishiwata, S., and S. Fujime. 1971. A dynamic study of F-actin-tropomyosin solutions by quasielastic light scattering. *J. Phys. Soc. Jpn.* 30:302 (Abstr.).
5. Lehrer, S. S. 1972. The crosslinking of actin and tropomyosin by glutaraldehyde. *Biochem. Biophys. Res. Commun.* 48:967–976.
6. Lehrer, S. 1976. Depolymerization of actin filaments by glutaraldehyde: protection by tropomyosin. *Fed. Proc.* 35:1746 (Abstr.).
7. Lehrer, S. S., and G. Kerwar. 1972. Intrinsic fluorescence of actin. *Biochemistry.* 11:1211–1217.
8. Lemanski, C. F. 1979. Role of tropomyosin in actin filament formation in embryonic salamander heart cells. *J. Cell Biol.* 82:227–238.
9. Lowey, S., and C. Cohen. 1962. Studies on the structure of myosin. *J. Mol. Biol.* 4:293–308.
10. Maupin-Szamier, P., and T. D. Pollard. 1978. Actin filament destruction by osmium tetroxide. *J. Cell Biol.* 77:837–852.