THE CROSSLINKING OF ACTIN AND OF TROPOMYOSIN BY GLUTARALDEHYDE[1]

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Received June 26,1972; Revised July 17,1972

SUMMARY Glutaraldehyde treatment of actin results in intermolecular crosslinked oligomers for the fibrous form (F-actin) but only monomers containing intramolecular crosslinks for the globular form (G-actin). Fluorescence studies on solutions which were dialyzed to remove salt, indicated that both modified actins maintained their native structure and were stabilized against denaturation by EDTA. Glutaraldehyde crosslinks tropomyosin producing oligomers with molecular weights n x 68,000. In the presence of F-actin in 0.1M KCl, tropomyosin is crosslinked end to end giving rise to a polymer of molecular weight > 500,000, indicating the close proximity of tropomyosin molecules on the F-actin filament. Glutaraldehyde modification thus appears to be useful for the study of muscle and other protein interactions.

The bifunctional reagent, glutaraldehyde, has been extensively used as a fixative in electron microscopy and in the preparation and study of insoluble enzymes (1-3). Only a few studies appear to have been directed toward its use as a probe of protein subunit proximity (4) or toward the chemical and functional consequences of the modification (5-9). These studies suggest that lysine residues are the principal site of glutaraldehyde reaction. Despite the lack of knowledge regarding the precise side chain modification, considerable information regarding the proximity and stoichiometry of systems of soluble interacting protein subunits and of the resulting conformational and functional changes produced by reaction with glutaraldehyde can be obtained. In the studies reported below such information has been obtained by reacting glutaraldehyde with rabbit muscle actin alone and in combination

[1]This work was supported by grants from the National Institutes of Health (ROl AM11677) and the National Science Foundation (GB-24316). Inquiries should be addressed to: Boston Biomedical Research Institute, 20 Staniford St., Boston, Mass. 02114

with tropomyosin.[2]

METHODS Oxygen free sealed vials of 8% or 10% glutaraldehyde were obtained from Polysciences, Inc. or Electron Microscopy Sciences, Inc. and used within a few weeks after opening. During this time glutaraldehyde was stored under nitrogen in a closed container at 4°C. Actin was prepared by the method of Spudich and Watt (10). Tropomyosin was prepared by the Bailey method as modified by Greaser and Gergely (11). Glutaraldehyde was incubated with the protein solutions under conditions described in the figure legends. The reaction was quenched by the addition of 0.2M $NaHSO_3$ in 0.1M phosphate buffer, pH 7.0. Excess reagents were removed by dialysis, gel filtration on Sephadex G-25, or, in the case of F-actin, by decanting the supernatant after centrifugation at 100,000 x g, which pelleted the cross-linked F-actin. SDS[3] polyacrylamide gel electrophoresis was performed as outlined by Weber and Osborne (12). Fluorescence studies were carried out as described previously (13).

RESULTS AND DISCUSSION Reaction of Actin with Glutaraldehyde. G-actin consists of a single polypeptide chain of molecular weight 45,000 and can be polymerized in the presence of 0.1M KCl into fibrous actin (F-actin) of molecular weight $> 10^6$. In the latter, the monomeric units are in a double-helical arrangement, each unit making contact with four other monomers (14). The molecular weight distribution of the glutaraldehyde treated G-actin (GaG) and F-actin (GaF) was examined by SDS polyacrylamide electrophoresis on 5% gels (Fig.1). As expected, the unmodified F-actin (F) shows only one band since the SDS treatment produces unfolded single poly-

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[2]For a review of muscle proteins, see Young, Ann.Rev.Biochem. 38 913 1969.

[3]Abbreviations include: SDS=sodium dodecyl sulfate, EDTA=sodium ethylene diaminetetraacetate, ATP=adenosine triphosphate, Hepes= N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid

peptide chains (12). The same result is obtained for G-actin. GaG shows a somewhat spread out single band suggesting that heterogeneous intramolecular crosslinks were formed. If the observed GaF bands are numbered from n=1 to n=6 or 7 with decreasing mobility, a plot of log n versus the observed relative mobilities gives a straight line. This shows that the bands correspond to covalently crosslinked oligomers due to the linear relationship between log (molecular weight) and mobility (12). Thus, intermolecular crosslinks are only produced in the case of F-actin in agreement with the known subunit proximity of F-actin and the monomeric properties of G-actin.

Depending upon the reaction conditions, a small fraction of the glutaraldehyde treated F-actin was not sedimentable after two hours of ultracentrifugation at 100,000 x g. In these cases, SDS gel electrophoresis showed that monomeric actin was mainly present in the supernatant, whereas higher oligomers were present in the pellet. It is possible that the introduction of intramolecular crosslinks caused this depolymerization. It is also interesting to note that glutaraldehyde treated G-actin was observed to be no longer polymerizable. That is, addition of salt to G-actin previously reacted with 0.04M glutaraldehyde for 20 minutes at 25° does not produce flow birefringence when viewed between crossed polaroids and little actin is pelleted by ultracentrifugation at 100,000 x g for two hours. This latter observation has also been reported by Cooke and Morales (15).

Native G-actin contains a tightly bound Ca^{++} or Mg^{++} and an ATP. The loss of these cofactors by heat or EDTA treatment is associated with an appreciable change in spectral parameters (16,13). A significant red shift of the intrinsic fluorescence spectrum of G-actin accompanies EDTA denaturation, indicating an average

FIGURE 1.

Molecular weight distribution of glutaraldehyde reacted actin and tropomyosin solutions on SDS 5% polyacrylamide gels. Actin at 1 mg/ml in 5 mM Hepes containing 0.2 mM ATP was reacted in the G or F state in the presence and absence of tropomyosin, for 30 min. at 25° with 0.02M glutaraldehyde. The reaction was quenched with 0.2M $NaHSO_3$ as described in the methods section and dialyzed overnight at room temperature against 10 mM sodium phosphate buffer, pH 7.0, 1% SDS and 1% β-mercaptoethanol, and 20 ug was applied to each gel. The sumbols on each gel refer to the state of the actin (G or F) during reaction with glutaraldehyde (Ga). Tm=tropomyosin, M=myosin marker (200,000 daltons), F=control unreacted actin (45,000 daltons). The weight ratios of actin to tropomyosin are indicated. Mobilities increase from top to bottom.

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increased exposure of tryptophyl sidechains to the solvent (13). No further change in the spectrum occurs on heating at 60° for 5 minutes, a procedure which results in denaturation in the absence of EDTA. The rate of this irreversible denaturation can convenientl be studied by following the fluorescence change with time after

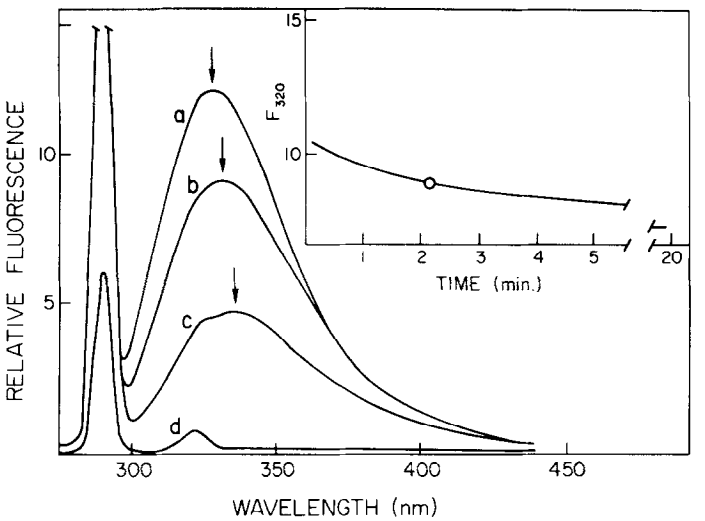

FIGURE 2.

Fluorescence spectra of glutaraldehyde treated F-actin before and after EDTA and heat treatment. F-actin at 1 mg/ml was treated with 0.04M glutaraldehyde for 15 min. at 0°, quenched with 0.2M $NaHSO_3$ and dialyzed overnight against 0.5mM ATP, 0.1mM $CaCl_2$, 5mM Hepes, pH 7.5. (a) Spectrum of GaF diluted 20X in 5mM Hepes, pH 7.5; (b) Spectrum taken after the changes produced by the addition of 1mM EDTA were complete; (c) Spectrum after heating at 60° for 5 min. Insert: The kinetics of the EDTA denaturation. The circle indicates $t_{1/2}$.

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the addition of EDTA and a semilog plot of data for G-actin gives a straight line showing a first order denaturation rate in agreement with Maruyama's results (17). Thus, information regarding the state of a given modification of actin can be obtained by studying the fluorescence spectrum and the changes produced by the addition of EDTA.

An example of the spectral changes produced by EDTA and by subsequent heating is shown in Fig. 2 for an actin solution which was treated with 0.04M glutaraldehyde for 15 min. at 0° in the fibrous form and dialyzed overnight to remove excess reagents. The spectrum obtained before EDTA addition (a) is almost identical to that given by unmodified native actin (λmax=329nm). After

EDTA addition, the spectrum shifts to the red (b) (λmax=332nm) but not to the extent of the EDTA induced spectral shift of an unmodified actin (λmax=335-6nm) (13). In order to produce a shift to λmax=336nm, the solution had to be heated (c). The fluorescence changes with time after the addition of EDTA as shown in Fig.2 insert indicated first order kinetics with $t_{1/2}$= 2.2 min. as compared to $t_{1/2}$=1.0 min. for an unmodified actin measured under the same conditions. The spectral change was not reversed on addition of excess Ca^{++}. Thus, both the spectral and rate changes indicate that glutaraldehyde treated actin is stabilized against denaturation. A comparison of fluorescence data for G-actin and F-actin treated with glutaraldehyde at a higher temperature and for a longer time than the Fig. 1 data is shown in Table I. Results for unmodified actin obtained under the same conditions (G) is also shown.

All the solutions were exhaustively dialyzed to remove excess reagents and salts. F-actin is depolymerized to G-actin at low ionic strength resulting in the tightly bound Ca^{++} or Mg^{++} becoming accessible to EDTA. Stabilization against denaturation is possible at low ionic strength by preventing the disorganization of backbone structure associated with the loss of the cofactors (18) both by inter- and intramolecular cross links. As seen in Table I, both GaG and GaF were stabilized against denaturation to about the same extent, and both of the EDTA treated solutions had to be heated to shift the spectrum to that characteristic of unmodified denatured actin. Thus, under these conditions it appears that intra- rather than intermolecular cross links mainly contribute to the structure stabilization.

Reaction of Glutaraldehyde with Actin-Tropomyosin Mixtures. Tropomyosin consists of two similar α-helical polypeptide chains

TABLE I.

Comparison of Fluorescence Spectra and EDTA Denaturation Half-times of Untreated (G) and Glutaraldehyde Treated G-actin (GaG) and F-actin (GaF)

Species	$\lambda_{max}^{Initial}$ (nm)	λ_{max}^{EDTA} (nm)	λ_{max}^{Heat} (nm)	$t_{1/2}^{EDTA}$ * (min)
G	329	336	336	1.18
GaG	330	334	336	5.4
GaF	329	332	336	5.2

F-actin or G-actin at 1 mg/ml with 0.2 mM ATP in 5mM Hepes buffer, pH 7.5, was treated with 0.05M glutaraldehyde for 60 min. at 25° and quenched by the addition of 0.2M $NaHSO_3$. At that time GaF and GaG did not exhibit flow birefringence.[3] The solutions were then dialyzed for 43 hours against 0.2mM ATP, 5mM Hepes, pH 7.5. The fluorescence was studied at 0.03 mg/ml before and after the change produced by the addition of 1mM EDTA was complete and after subsequent heating at 60° for 5 min.
* the half-time of the first order rate of EDTA denaturation.

strongly bound non-covalently (19) in a coiled-coil configuration. It binds to F-actin in the groove of the double helix (14), one molecule of tropomyosin interacting with about 6-7 actin monomers. This large degree of interaction suggested a high probability of intermolecular crosslinking between F-actin and tropomyosin. The results of Fig. 1 in which the molecular weight distribution of the cross-linked species is shown indicates that this does not in fact happen. A comparison of the unreacted controls of F-actin (F) and F+tropomyosin in the weight ratio of 5:1 (F+Tm) shows the tropomyosin band in the expected place since the molecular weight of the reduced SDS treated molecule is 34,000. The reaction of glutaraldehyde with a 5:1 weight ratio mixture of F-actin to tropomyosin under optimum conditions of interaction (0.1M KCl) (20) produced an SDS gel pattern (GaFTm) very similar to that of GaF except for the presence of a new band in the excluded part of the

gel (top) corresponding to a molecular weight > 500,000. The original tropomyosin band is absent and no bands corresponding to mixed oligomers of actin and tropomyosin were produced. Thus, actin acts as a template on which tropomyosin molecules are intermolecularly crosslinked end to end.

These data are consistent with the suggestion that the tropomyosin molecules overlap on the F-actin filament. However, this cannot be unambiguously concluded from these data alone, since, for example, an actin monomer may serve to crosslink the ends of neighboring tropomyosin molecules. The lack of extensive crosslinking between F-actin subunits and tropomyosin shows that although there is subunit proximity, the appropriate side chain proximity was not present. Reaction of F-actin + tropomyosin at a higher ratio of tropomyosin to actin (GaFTm) (1:1) produces additional covalent oligomeric bands at n x 68,000 daltons superimposed on the actin oligomeric pattern and the band at the top. The n x 68,000 dalton pattern is also seen for the product of the reaction of glutaraldehyde with tropomyosin in the absence of actin at low salt (GaTm). Thus, additional crosslinking between tropomyosin molecules occurs when they are in excess over actin. The lack of molecular species produced with tropomyosin corresponding to odd multiples of 34,000 daltons supports the known stable dimeric structure of tropomyosin chains and suggests that intramolecular crosslinking is quite efficient.

These studies indicate that reaction with glutaraldehyde can be used to obtain information regarding proximity of protein subunits by an analysis of the resulting molecular weight distribution of the products. In the systems described above, all protein subunits that were linked by glutaraldehyde are known to bind to each other. The converse was not true (e.g. tropomyosin-

F-actin), i.e. the lack of heterologous intermolecular crosslinking may not be taken as evidence for lack of binding, since crosslinks are made between side chains of certain amino acid residues (mainly lysine) that are at a favorable distance and orientation with respect to each other.

This work has also shown that under certain conditions, glutaraldehyde causes some depolymerization of F-actin in solution. Electron microscopic studies involving the fixation and visualization of actin-like microfilaments in tissue or in vitro should take this into consideration.

Finally, it should be noted that the oligomeric species of GaF provide convenient molecular weight markers for SDS gel electrophoresis at 45,000 dalton intervals ranging from 45,000 to 360,000 daltons and possibly beyond. Further studies of the consequences of the crosslinking of actin are in progress.

Acknowledgment: The excellent technical assistance of Ms. Mary Ho is appreciated.

REFERENCES

1. Quiocho, F.A. and Richards, F.M., Proc. Nat. Acad. Sci. (U.S.) 52 833 (1964).
2. Habeeb, A.F.S.A., Arch. Biochem. Biophys. 119 264 (1967).
3. Jansen, E.F., Tomimatsu, Y. and Olson, A., Arch. Biochem. Biophys. 144 394 (1971).
4. Steck, T.L., J. Mol. Biol. 66 295 (1972).
5. Habeeb, A.F.S.A. and Hiramoto, R., Arch. Biochem. Biophys. 126 16 (1968).
6. Quiocho, F.A. and Richards, F.M., Biochemistry 5 4062 (1966).
7. Richards, F.M. and Knowles, J.F., J. Mol. Biol. 37 231 (1968).
8. Wang, J. H-C. and Tu, J-I., Biochemistry 8 4403 (1969).
9. Korn, A.H., Feairheller, S.H. and Filachione, F.M., J. Mol. Biol. 65 525 (1972).
10. Spudich, J.A. and Watt, S., J. Biol. Chem. 246 4866 (1971).
11. Greaser, M. and Gergely, J., J. Biol. Chem. 246 4226 (1971).
12. Weber, K. and Osborn, M., J. Biol. Chem. 244 4406 (1969).
13. Lehrer, S.S. and Kerwar, G., Biochemistry 11 1211 (1972).
14. Moore, P.B., Huxley, H.E. and DeRosier, D.J., J. Mol. Biol. 50 279 (1970).
15. Cooke, R. and Morales, M.F., J. Mol. Biol. 60 249 (1971).
16. West, J.J., Nagy, B., and Gergely, J., Biochem.Biophys.Res.Comm. 29 611 (1967).

17. Maruyama, K., Scientific Papers of the College of General Education, University of Tokyo 12 73 (1962).
18. Nagy, B. and Jencks, W.P., Biochemistry 1 987 (1962).
19. Woods, E.F., J. Biol. Chem. 242 2859 (1967).
20. Tanaka, H. and Oosawa, F., Biochim. Biophys. Acta 253 274 (1971).