ACTIN CROSSLINKED WITH GLUTARALDEHYDE: EVIDENCE TO SUGGEST AN ACTIVE ROLE FOR ACTIN IN THE REGULATORY MECHANISM

W-J. Poo and D. J. Hartshorne

Departments of Biological Sciences and Chemistry
Carnegie-Mellon University, Pittsburgh, Pa. 15213

eceived February 10, 1976

MMARY: Actin crosslinked with glutaraldehyde retains the ability to activate the $^{2+}$-ATPase activity of heavy meromyosin subfragment 1, but the resultant ATPase activity not controlled by the regulatory proteins, troponin and tropomyosin. Fluorescent ergy transfer measurements imply that the crosslinked actin is frozen in the active ate. These results indicate that the conformation of actin is important in the regutory mechanism, and suggest that actin plays a more active role in this mechanism than ought previously.

NTRODUCTION: In the last few years a model has been developed to explain the regulatory echanism in skeletal muscle. It originated in observations made by x-ray diffraction, id it was suggested that the position of tropomyosin in the relaxed and active states as different (1,2). This is the key feature of the model, which may be stated as follows: the presence of Ca^{2+} the position of tropomyosin does not hinder the actin-myosin interction and it is thought that the tropomyosin strands lie in a more central position in e groove of the actin filament; at low Ca^{2+} concentrations (relaxed state) the tropomyosin moves to a more peripheral position and physically prevents the interaction of e myosin heads with actin. The importance of troponin is that it recognizes the intracellular Ca^{2+} level and in some manner stabilizes the position of tropomyosin in either e relaxed or contracted mode.

It was our intention to test this model and to pose the question whether or not e movement of tropomyosin was the only significant factor? The original hypothesis s to prevent, or at least hinder, the movement of tropomyosin using chemical crossnking, and then to test for the regulatory function. During this work we obtained idence that suggested that the conformation of actin was important to the regulatory chanism and this is presented below.

MATERIALS AND METHODS: Protein preparations. The following methods were used for the preparation of proteins: tropomyosin and the complex of troponin and tropomyosin (3), troponin (3,4) heavy meromyosin subfragment-1, S-1 (5); actin (6), the F-actin was purified in 0.6 M KCl (7).

Crosslinking. Oxygen free vials of 10% glutaraldehyde were obtained from Electron Microscopy Sciences, Inc. The pH of this solution was adjusted to 7.0 before mixing with the protein solutions. Crosslinking was carried out at 4°C in a solution containing 1.0 mg/ml F-actin, 1% glutaraldehyde, 0.1 M KCl, 20 mM K_2HPO_4/KH_2PO_4 (pH 7.0). Crosslinking was terminated by the addition of 1 M KCNS (50 µl/ml). The samples were dialyzed exhaustively against 0.1 M KCl, 10 mM tris-HCl (pH 7.6).

Carboxymethylation. Proteins were reduced by dialysis against 1 mM dithiothreitol, 0.1 M KCl, 50 mM tris-HCl (pH 8.5). The dithiothreitol was removed by dialysis under nitrogen using this solvent without dithiothreitol. Carboxymethylation was carried out with a 50 fold molar sulfhydryl excess of [^{14}C] iodoacetamide in 0.1 M KCl, 50 mM tris-HCl (pH 8.5). Samples were dialyzed exhaustively against 0.1 M KCl, 10 mM tris-HCl (pH 7.6).

Binding of tropomyosin and tropomyosin plus troponin to actin. Tropomyosin and the complex of troponin and tropomyosin were labelled with [^{14}C] iodoacetamide. To a solution of F-actin (about 1 mg/ml) in 0.1 M KCl, 10 mM tris-HCl (pH 7.6) was added different amounts of either tropomyosin or the troponin-tropomyosin complex. These were mixed well and centrifuged at 82,000 x g for 4 hours. These experiments were performed with both normal and crosslinked actins. The extent of binding to the sedimented actin was determined from the ^{14}C-labelled protein that remained in the supernatant. In a solution containing only actin it was estimated that approximately 90% of the actin (control and crosslinked) was sedimented. Thus it was necessary to apply a slight correction factor to the binding data.

Fluorescence studies. Tropomyosin and actin were labelled with N-methyl-2-anilino-6-naphthalene sulfonyl chloride (mans) and fluorescein isothiocyanate (FITC), respectively, using the method of Rinderknecht (9). Approximately 1 molecule of each fluorescent probe was incorporated per mole of protein. Spectra were recorded at 25°C with a Farrand spectrofluorometer Mark I equipped with corrected excitation and emission. Transfer efficiency was determined by the quenching of donor (mans) fluorescence at 450 nm in the presence of the acceptor (FITC). In order to avoid artifacts the A_{334nm} of each sample was <0.08.

ATPase assays. These were carried out as described earlier (3).

SDS gel electrophoresis. The method of Fairbanks et al. (8) was used.

RESULTS: SDS gels of F-actin crosslinked with glutaraldehyde (under the conditions given in Materials and Methods) indicated that after 10 minutes approximately 25% of the 42,000 dalton component remained. Most of the crosslinked-protein did not enter the polyacrylamide matrix, although small amounts of dimers and trimers were detected. With longer periods of crosslinking the percentage of monomeric actin decreased slightly and after 24 hours approximately 20% of the protein was visible in the 42,000 dalton band. The formation of insoluble polymers of actin did not occur even after prolonged exposure to glutaraldehyde.

The crosslinked actin retained the ability to activate the Mg^{2+} ATPase activity of

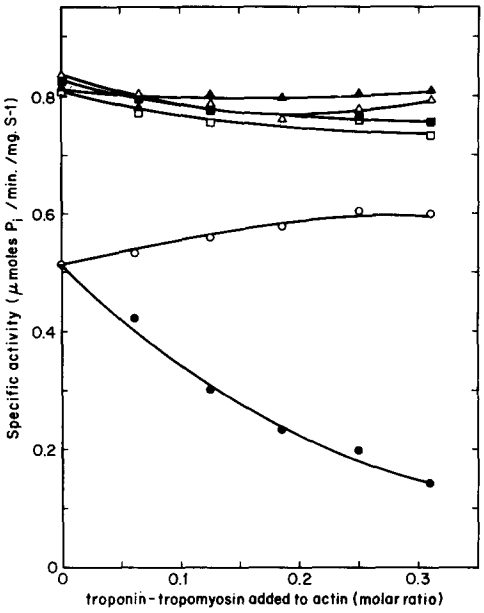

Fig. 1. The effect of the troponin-tropomyosin complex on the Mg^{2+} activated ATPase activity of acto-heavy meromyosin subfragment 1 (S-1) using control and crosslinked actin. Assay conditions: 2.5 mM ATP, 2.5 mM $MgCl_2$, 25 mM tris-HCl (pH 7.6), S-1, 0.17 mg/ml, actin 0.20 mg/ml. Open symbols indicate absence of EGTA, closed symbols indicate the presence of 1 mM EGTA. (○,●) control actin; (△,▲) actin crosslinked with glutaraldehyde for 1 hour; (□,■) actin crosslinked with glutaraldehyde for 24 hours. For conditions of crosslinking see Materials and Methods.

-1, as shown in Fig. 1. In fact, the specific activity of acto-S-1 was slightly higher hen crosslinked actin was used, approximately 0.8 μmoles P_i/min/mg S-1 as compared to bout 0.5 μmoles P_i/min/mg S-1 using control actin. The most striking difference, owever, between the crosslinked and the control actins was seen when the regulatory roteins, troponin and tropomyosin were added. The Mg^{2+} ATPase activity of the control cto-S-1 mixture was inhibited in the absence of Ca^{2+} whereas no significant inhibition as observed when crosslinked actin was used. The time of exposure of actin under crossinking conditions did not alter this response and it was similar between the time limits hat were tested (10 mins. to 24 hours). The inhibition of Mg^{2+} ATPase activity of cto-S-1 by troponin I plus tropomyosin (1:1 molar ratio) was also eliminated. Thus, lthough the crosslinked actin was capable of activating the Mg^{2+} ATPase of S-1, the ɔility of troponin and tropomyosin to regulate this activity was lost.

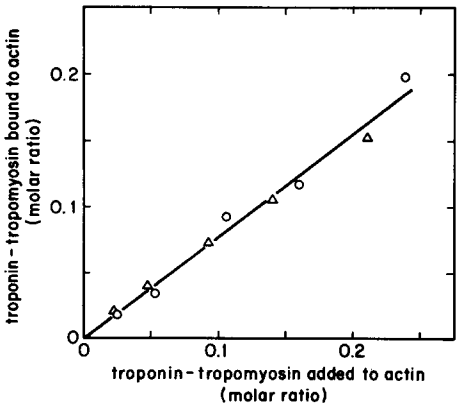

Fig. 2. The binding of the troponin-tropomyosin complex to control and cross-linked actin. (O) control actin; (Δ) actin crosslinked with glutaraldehyde for 1 hour. For conditions and estimation of binding see Materials and Methods.

A possible explanation for this effect could be that the crosslinked actin had lost the ability to bind either tropomyosin or the troponin-tropomyosin complex. This was tested using ^{14}C-labelled proteins (see Materials and Methods). The results obtained using the troponin-tropomyosin complex are shown in Fig. 2. The binding to control and crosslinked actins was identical. Similar results were obtained using ^{14}C-labelled tropomyosin. The crosslinking of actin with glutaraldehyde therefore does not impair its ability to bind with the regulatory proteins.

Another possibility that we wished to check was whether or not the crosslinked actin allowed unrestrained movement of the tropomyosin strands. Our approach was to use energy transfer between two fluorescent probes, one on tropomyosin (the mans group) and the other on actin (the FITC group). These two probes served as a donor (mans) - acceptor (FITC) pair for singlet energy transfer. The two proteins were labelled with their respective fluorophores and added, to control and crosslinked actins. (The reactio of either tropomyosin or actin with the fluorescent probes did not affect their biologica activity [10]). In the presence of troponin the effect of Ca^{2+} was measured. The result are presented in Table I. Consider first the control experiment using non-crosslinked actin. In the contracted state the extent of energy transfer was 27%, and this increased to 40% when Ca^{2+} was removed, i.e. the relaxed state (control samples 2) and 3)). When

Table I

Fluorescence energy transfer between mans-tropomyosin and FITC-actin with control and crosslinked actins using partially and fully reconstituted thin filaments

Sample	Energy transfer efficiency using: control actin	crosslinked actin
1) Mans-tropomyosin, FITC-actin (1:5, respectively)	42%	32%
2) Mans-tropomyosin, FITC-actin, troponin plus Ca^{2+} (1:5:1, respectively)	27%	27%
3) Mans-tropomyosin, FITC-actin, troponin minus Ca^{2+} (1:5:1, respectively)	40%	35%
4) Mans-tropomyosin, FITC-actin, troponin 1 (1:5:1, respectively)	39%	30%

Additions referred to are in molar ratios. Excitation of the mans group was at 334 nm. See Materials and Methods for other details.

rosslinked actin was used, under otherwise identical conditions, a change in energy ransfer was detected although the magnitude of the change was not as large as in the ontrol situation. It is interesting that in the absence of troponin the tropomyosin position" was different when control and crosslinked actins were used. With cross-inked FITC-actin the extent of energy transfer suggested that mans-tropomyosin assumed position closer to the contracted state, whereas the normal arrangement for tropomyosin n the absence of troponin was in the "blocking" or relaxed position (Table I, sample 1). he effect of troponin I on the extent of energy transfer is also consistent with this iew, as the apparent position of tropomyosin on crosslinked actin even in the presence f the inhibitory subunit of troponin remained closer to the "non-blocking" or contracted tate.

ISCUSSION: The ability of crosslinked actin to activate Mg^{2+} ATPase activity of myosin as been reported previously (11), but the loss of control that the crosslinked actin mposes on the activity has not been documented. It was shown that this was not due o an impairment of the binding of tropomyosin to the actin filament, and thus the

effect appears to be centered on the actin molecule itself. Glutaraldehyde reacts primarily with the $-NH_2$ groups of lysine (12) and one possibility was that the effect was due to the chemical modification of lysine residues as opposed to the covalent crosslinking between residues. This is unlikely as the incorporation of up to 10 moles of FITC (a monofunctional reagent which reacts also with lysine residues [13]) per mole of actin, does not impair its normal functioning. Thus we visualize the effect of glutaraldehyde as "freezing" actin in a particular conformation which does not allow the regulation of Mg^{2+} ATPase activity by troponin and tropomyosin.

This interpretation, if correct, means that the conformation of actin is important to the regulatory mechanism, and suggests that actin plays a more active role in the control process than thought previously. The regulation of actin-myosin interaction solely on the basis of the position of tropomyosin molecule would seem therefore to be an oversimplification. It is significant that Yanagida et al. (14) using ultraviolet dichroism measurements on reconstituted and native thin filaments found evidence for a change in the conformation of actin in the presence and absence of Ca^{2+}. They suggested further that the state of actin in the thin filament in the presence of Ca^{2+} was similar to that of pure F-actin. Our crosslinking results are consistent with this view, as the crosslinked actin is fixed in the "activated" state. Loscalzo et al. (15) recently have also suggested that the cooperative behaviour of the thin filament may not be due entirely to positional changes of the tropomyosin molecules. The activation of Mg^{2+} ATPase activity of actomyosin by tropomyosin (16) or by the troponin-tropomyosin complex (3) is another feature that is difficult to rationalize solely on the basis of the position of the tropomyosin strands.

The results of the fluorescence energy transfer experiments (Table I) in general are consistent with the views proposed above. In the absence of troponin the position of tropomyosin on the non-crosslinked actin lies closer to the relaxed state [17], whereas the tropomyosin position using crosslinked actin appears more similar to the contracted state. This interpretation is only tentative as it is not known if the conformational change on actin causes the FITC probe to move, or, if the different conformation imposes a new binding position for the tropomyosin strands. When troponin was added an alteration

ı the extent of energy transfer in the presence and absence of Ca^{2+} was observed with
›th the crosslinked and control actins. Assuming that the change in energy transfer
ı a reflection of an alteration in the distance separating the two probes (rather than
change in the orientation factor) then this would indicate that tropomyosin movement
ı allowed in both cases. In the case of the crosslinked actin, however, relaxation does
›t occur and one might infer from this that the conformation of actin, frozen in the
›n" state, overrides the steric hindrance of the tropomyosin molecule. It is apparent,
ıerefore, that the movement of tropomyosin is not the only requirement of the regulatory
echanism, and in addition a change in the conformation of actin is necessary. At the
oment it is not possible to decide which of the two is the dominant feature.

CKNOWLEDGEMENTS: This work was supported by grants HL-09544 and GM-46407 from The
ational Institutes of Health.

EFERENCES:

1. Huxley, H.E. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 361-376.
2. Haselgrove, J.C. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 341-352.
3. Hartshorne, D.J. and Mueller, H. (1969) Biochim. Biophys. Acta, 175, 301-319.
4. Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) J. Biochem. 69, 441-445.
5. Margossian, S.S. and Lowey, S. (1973) J. Mol. Biol. 74, 313-330.
6. Bárány, M. and Bárány, K. (1959) Biochim. Biophys. Acta, 35, 293-309.
7. Spudich, J.A. and Watts, S. (1971) J. Biol. Chem. 246, 4866-4871.
8. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry, 10, 2606-2617.
9. Rinderknecht, H. (1962) Nature, 193, 167-168.
.0. Poo, W-J. and Hartshorne, D.J. (1975) J. Cell Biol. 67, A340.
.1. Gadasi, H., Oplatka, A., Lamed, R., Hochberg, A. and Low, W. (1974) Biochim. Biophys. Acta, 333, 161-168.
.2. Habeeb, A.F.S.A. and Hiramoto, R. (1968) Arch. Biochem. Biophys. 126, 16-26.
.3. Fraenkel-Conrat, H. (1957) Methods in Enzymology, 4, 247-269, Academic Press, New York.
.4. Yanagida, T., Taniguchi, M. and Oosawa, F. (1974) J. Mol. Biol. 90, 509-522.
.5. Lascalzo, J., Reed, G.H. and Weber, A. (1975) Proc. Natl. Acad. Sci. 72, 3412-3415.
.6. Katz, A.M. (1964) J. Biol. Chem. 239, 3304-3311.
.7. Eaton, B.L., Kominz, D.R. and Eisenberg, E. (1975) Biochemistry, 14, 2718-2728.