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POSSIBLE UNCOUPLING OF THE MECHANOCHEMICAL PROCESS IN THE ACTOMYOSIN SYSTEM BY COVALENT CROSSLINKING OF F-ACTIN

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SUMMARY

It has been speculated that F-actin depolymerizes during muscle contraction. In order to test this speculation, G-actin in F-actins were immobilized by crosslinking. While this did not affect activation of myosin ATPase, superprecipitation could be abolished. Sedimentation, light scattering, electron microscope, electrophoresis and homodyne spectra measurements seem to indicate that the average size of crosslinked segments increases with time of treatment with the crosslinking agent. At the same time, the amounts of dimers and trimers decreased. It appears that crosslinking makes F-actin filaments less flexible. The possible reasons for the loss of the capability to exhibit superprecipitation are discussed.

INTRODUCTION

The main component of the thin filaments of muscle is F-actin, which is a double helical polymer of G-actin globules. De-polymerization and re-polymerization may easily be caused by the action of nucleotides, divalent cations, myosin and tropomyosin, all of which are involved in muscular contraction [1]. Thirty years ago Albert Szent-Györgyi [2] proposed that F-actin depolymerizes during contraction.

On the basis of X-ray studies, the length of the thin filaments does not practically change upon activating a relaxed muscle [3, 4]. Such measurements would not, however, detect local and transient changes due to the cyclic interaction with myosin.

It occurred to us that by covalently binding the G-actins in F-actin transient ruptures might become impossible. Since an actomyosin gel shrinks dramatically by the action of ATP during superprecipitation, this process is considered to represent muscular contraction, i.e. that the occurrence of superprecipitation is taken as an indication for the preservation of the mechanochemical coupling. We therefore studied superprecipitation in the presence of crosslinked F-actin. It was surprising to find out that even though actin activation of myosin ATPase was not impaired by crosslinking the actin under the proper conditions, superprecipitation could be abolished. This would then mean that temporary dissociation of G–G native bonds is

required for the mechanochemical coupling between ATP splitting and motility. The exact nature of the process taking place during superprecipitation is, however, not yet clear. If the reason for the "contraction" of actomyocin during superprecipitation is not the outcome of sliding of the two kinds of filaments (which probably takes place earlier) but rather represents a crystallization process which is accompanied by the loss of water (while during muscular contraction the volume of the shortening myofibrils remains constant), then our results might suggest other changes to be responsible for the loss of the capability to exhibit superprecipitation. Thus a proper alignment of the filaments might be a pre-requisite for crystallization to take place and for this purpose the filaments should be flexible enough [1]. This second possibility was checked by studying the effect of crosslinking on the rigidity of F-actin by following changes in laser light scattering. It was found that crosslinking led to an increase in rigidity.

EXPERIMENTAL

Actin and myosin were prepared according to Lehrer and Kerwar [5] and to Azuma and Watanabe [6], respectively. The proteins were used within two weeks from the date of their preparation. Their concentrations were determined by the Lowry method [7].

As a crosslinking agent we used the well-known fixative glutaraldehyde (product of Polyscience, U.S.A.). Crosslinking was carried out in the cold (5 °C) in a solution containing 0.5 mg/ml actin, 20 mM phosphate buffer pH 7.0 and 1 % glutaraldehyde. Under these conditions the protein did not precipitate and activation of myosin ATPase was not affected (at higher concentrations of either actin or glutaraldehyde the protein precipitated probably due to interfilamental crosslinking). Glutaraldehyde supplied by BDH was later found to be more effective so that we had to use lower concentrations of both actin and glutaraldehyde in order to prevent precipitation and inactivation. It has been reported [8] that the behavior of glutaraldehyde solutions was affected by aging, concentration etc. and varied according to source. Samples were taken after different times. Each sample was divided into two: one was dialyzed against 50 vol. of a 2 mM Tris buffer, pH 7.6 (depolymerizing conditions) and the other against 50 vol. of a solution containing 50 mM KCl and 10 mM Tris–HCl buffer, pH 7.6 (ionic strength which favors F-actin). Hereafter, we shall refer to these solutions as "low ionic strength" and "high-ionic strength", respectively. Dialysis was repeated 4 times within 24–48 h. The addition of 50 μl KCNS per ml [9] (from a stock solution of 1 M KCNS) to the samples just before dialysis in order to destroy remaining active aldehyde groups did not affect the reslts. Dialysis against a low ionic strength was carried out in order to cause the dissociation of G-actin molecules which did not undergo crosslinking.

ATPase was measured following Fiske and SubbaRow [10] under the following conditions: 0.07 mg/ml F-actin (either native or crosslinked), 0.2 mg/ml myosin, 0.1 mM Ca^{2+}, 2.5 mM ATP, 4 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl buffer, pH 7.6 [11]. The extent of superprecipitation was followed by measuring turbidity in a Zeiss spectrophotometer at 660 nm, 15 min after the addition of ATP to a solution, the final composition of which was 0.07 mg/ml actin, 0.2 mg/ml myosin, 0.125 mM Mg^{2+}–ATP, 50 mM KCl [12].

Light-scattering measurements were carried out in a spectrofluorometer at $\lambda = 550$ nm and 90 °.

Electron microscopic pictures were taken with a Philips 300 electron microscope, following Huxley [13]. A drop of actin solution (0.1 mg/ml), after different times of treatment with glutaraldehyde (dialyzed against high ionic strength), was put on a carbon-covered grid. Negative staining was performed with 1 % uranyl acetate for 4 min, followed by drying.

Homodyne spectra [14] were obtained for all samples. The output of an ITT FW 130 photomultiplier was amplified and fed into a digital autocorrelator (Digital Correlator, Precision Devices and Systems Ltd, Malvern, England). Normalized autocorrelation functions [15] were obtained for various angles, and a fit for a single exponent was computed for each of them.

RESULTS

Solutions of glutaraldehyde-treated F-actin which were dialyzed against a high ionic strength were checked for superprecipitation and for ATPase activation. Superprecipitation occurred in samples taken after 0–5 h of treatments (reading in the spectrophotometer varied in an inconsistent manner in the range 0.12–0.16, as compared to 0.05–0.07 for the actomyosin before the addition of ATP). Samples taken after 24 h did not show any superprecipitation (reading: 0.06). Solutions containing 0.2 mg/ml of actin (instead of 0.5 mg/ml) did not exhibit superprecipitation when checked after 48 and 72 h of treatment. On the other hand, the ATPase was not affected and it was the same whether superprecipitation took place or not (0.56 ± 0.06 μmole P_i per mg myosin per min). These experiments were repeated many times, always giving the same results. The transition from a state in which superprecipitation occurs to one in which it is absent is quite sharp and due to the variation from one glutaraldehyde solution to another it was difficult to "catch" an intermediate time or glutaraldehyde concentration.

The light scattering of glutaraldehyde-treated actin after dialysis at low ionic strength was compared to that of the solutions dialyzed against the relatively higher ionic strength. As can be seen, from Table I, light scattering increased with time of

TABLE I

LIGHT SCATTERING OF DIALYZED ACTIN SOLUTIONS

The reading, in V, is proportional to the light scattered.

Time of treatment with aldehyde (h)	Low ionic strength	High ionic strength
Blank (no aldehyde added)	0.8	5.9
1	1.7	3.3
2	1.9	3.5
3	2.3	2.6
4	2.6	3.3
5	2.1	3.9
24	2.6	3.9

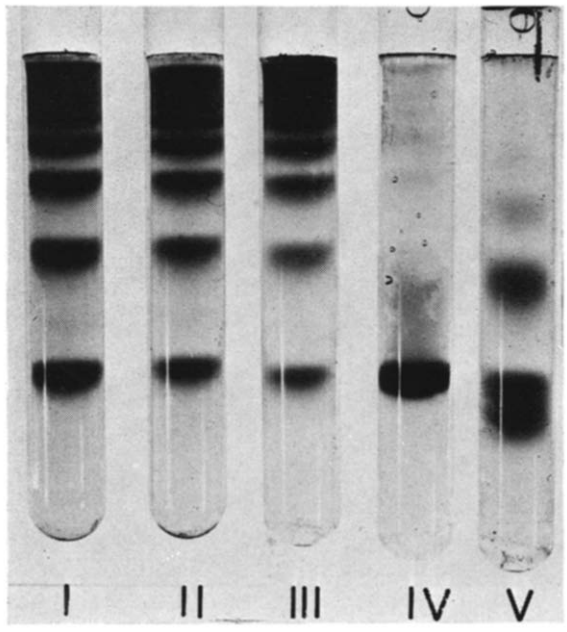


Fig. 1. Gel electrophoresis of glutaraldehyde-treated F-actin. 5 % polyacrylamide, 0.1 % sodium dodecyl sulfate, 0.1 M phosphate buffer, pH 7.0. The samples were pretreated by heating at 100 °C for 2 min in a medium containing 1 % sodium dodecyl sulfate, 5 mM dithiothreitol, 10 % sucrose, 0.1 M phosphate buffer, pH 7.0. Excess glutaraldehyde was removed from F-actin by dialysis against a high ionic strength. I, 1 h treatment; II, 5 h; III, 24 h; IV, untreated F-actin; V, G-actin treated with 1 % glutaraldehyde for 1 h.

treatment in the low ionic strength media. The ratio of the scatterings at the two ionic strengths for a given sample decreased with time of treatment from 7.4 for the untreated actin to 1.1–1.5 after 3–24 h. These results are consistent with the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the dialyzed samples. As can be seen from Fig. 1, the amount of G-actin (lowest band) decreased with increasing time of treatment with glutaraldehyde. At the same time, the amounts of dimer and trimer decreased while that of longer-chain oligomers (which could not penetrate the gel) increased. The solutions were also centrifuged and the percentage of sedimented protein determined (80 000 $\times g$ for 3.5 h in a Spinco ultracentrifuge). Under these conditions, untreated F-actin was practically fully sedimented while G-actin did not sediment at all. F-actin (0.5 mg/ml) treated with glutaraldehyde for 1–24 h and dialyzed against a low ionic strength gave only 20 % in the sediment. The supernatant was run electrophoretically, giving bands representing G-actin monomers, oligomers up to a hexamer and some other larger oligomers. When glutaraldehyde-treated (1–24 h) F-actin (0.5 mg/ml) was dialyzed against a high ionic strength and sedimented, 50 % of the protein was obtained in the pellet. When this was homogenized and dialyzed against a low ionic strength solution, addition of KCl (final concentration 50 mM) caused an increase in viscosity.

The observed homodyne spectra of samples dialyzed against solutions containing 50 mM KCl could be fairly well fitted by a single exponent. From the depen-

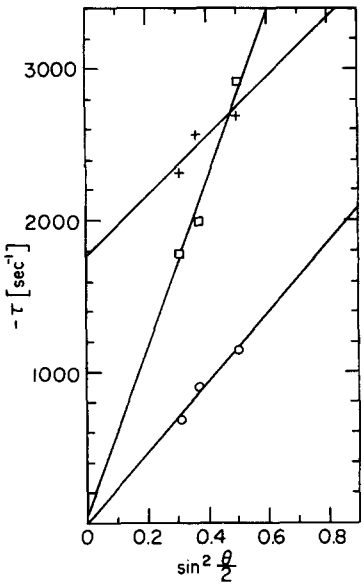


Fig. 2. The exponent, $-\tau$, in the autocorrelation function as a function of $\sin^2 \theta/2$, where θ is the scattering angle. ○, 1 h treatment (0.1 % glutaraldehyde); □, 2 h treatment (1 % glutaraldehyde); +, 24 h treatment (1 % glutaraldehyde).

dence on the scattering angle, the linear diffusion coefficient and the flexibility of the molecules could be computed [16] (Fig. 2).

A sample of F-actin which interacted with 0.1 % glutaraldehyde for 1 h had the same linear diffusion coefficient and rigidity as those of ordinary F-actin. A sample which interacted with 1 % glutaraldehyde for 1 h had a diffusion constant about 2.5 times that of ordinary F-actin, and a similar rigidity. As the time of interaction increased, the diffusion constant decreased and the rigidity increased. The value of the diffusion constant approached that of untreated F-actin; this seems to indicate that the actin which could not superprecipitate with myosin consisted of single F-actin chains and not of nets made of several chains which are crosslinked to each other. The spectra of samples dialyzed against a low ionic strength could not be analyzed in the simple way described above. It appears that glutaraldehyde bonds give rise to a rigid molecule: the higher the percentage of glutaraldehyde bonds, the more rigid the molecule becomes. When the native F-actin bonds are broken by lowering the ionic strength, the resulting fragments of F-actin molecules are probably rod-like, rigid molecules. In such a case, the rotational motion of the molecules should be taken into account when analyzing the homodyne spectrum. We are currently trying to do this by using a wider range of angles and by carrying out further analyses.

Electron microscopic studies seem to support the idea that glutaraldehyde crosslinks F-actin segments end-to-end in a linear manner. In Fig. 3 we present pictures of F-actin and of glutaraldehyde-treated F-actin, after 1, 6 and 24 h (dialyzed against a high ionic strength). It appears that after 24 h, when the actin lost its capability to superprecipitate, the actin still forms linear filaments and not aggregates of

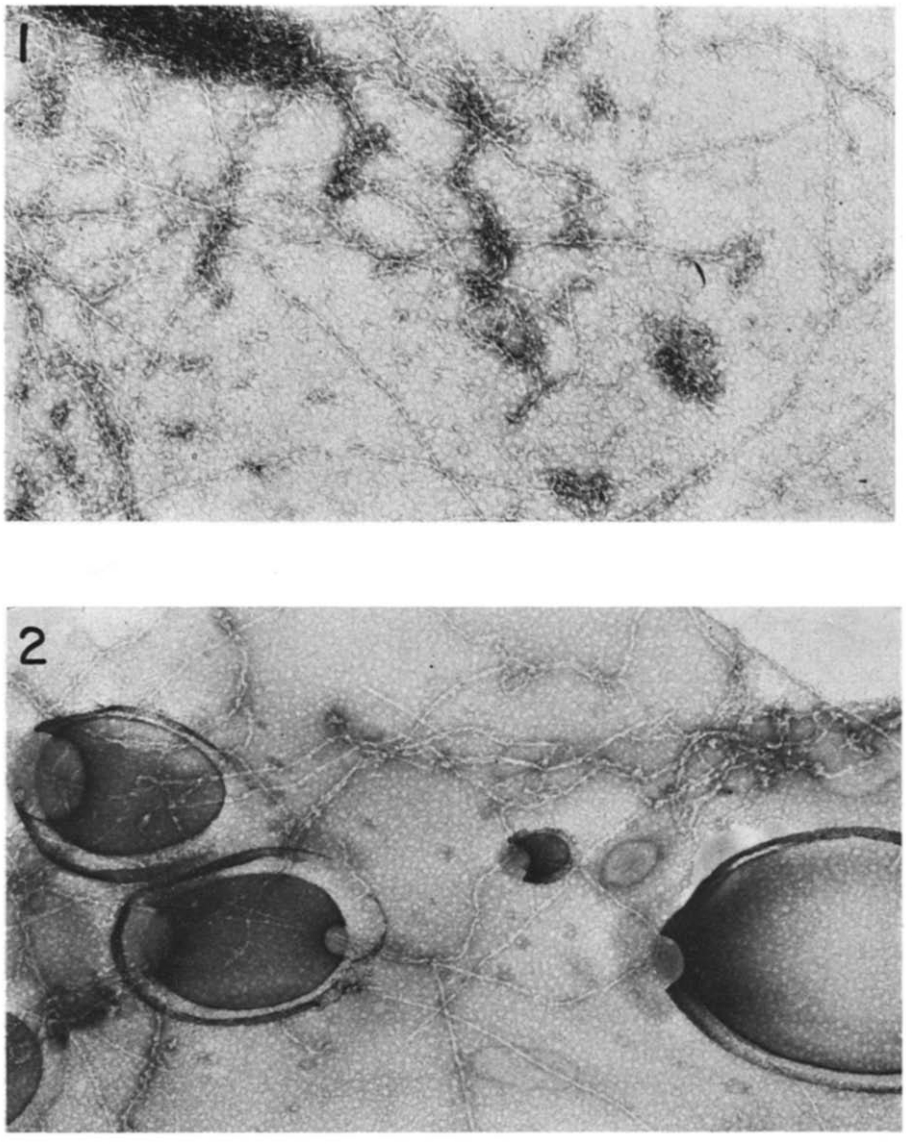
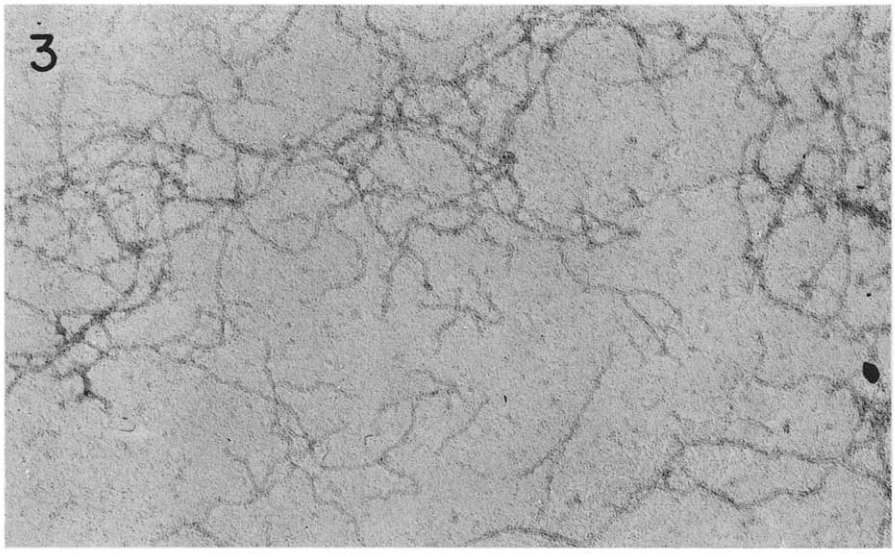
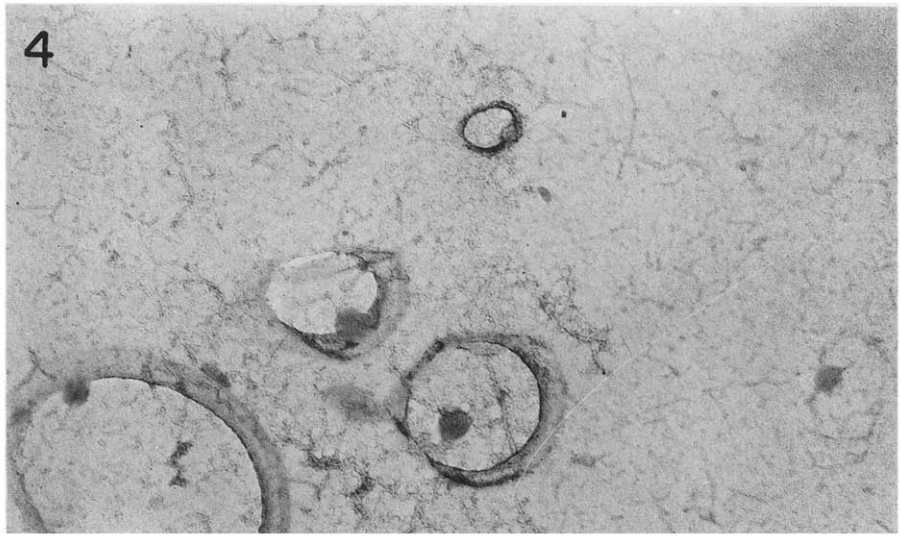


Fig. 3. Electron microscopic pictures of glutaraldehyde-treated F-actin. 1, blank; 2, 3, 4, after 1, 6 and 24 h of treatment, respectively.

short filaments. We cannot thus attribute the loss of superprecipitation to aggregate formation. It should be noticed that glutaraldehyde-treated F-actin filaments look shorter than those of un-crosslinked F-actin, in accordance with our previous conclusion.

Monomeric G-actin, treated with 1 % glutaraldehyde for 1 h and dialyzed against a low ionic strength appeared to lose its capability to polymerize upon in-





creasing the ionic strength: its viscosity did not increase and it did not activate myosin ATPase. These observations might explain the fact that upon increasing the ionic strength, the crosslinked segment did not associate to give a polymer of the original F-actin size. As can be seen from Fig. 1, glutaraldehyde treated G-actin is partially crosslinked into a dimer and the monomer band becomes diffuse (probably indicating conformational changes in the monomers due to intramolecular crosslinking).

After this work was practically concluded, our attention was drawn to a paper by Lehrer [17] who also treated F- and G-actin with glutaraldehyde. His main interest

was, however, stabilization against denaturation by EDTA and binding of tropomyosin.

DISCUSSION

The various methods utilized in this study suggest that the average size of crosslinked F-actin filaments increases with time and that such segments are capable of binding to each other in a higher ionic strength medium, the resulting chains appearing to be smaller than the original, untreated F-actin.

It may well be that flexibility of the F-actin is required for both the mechanochemical coupling in muscle and for proper alignment of the filaments in superprecipitation. It is not yet clear whether the conformational changes involved are accompanied by rupture of G–G bonds. A better insight into this problem may be gained by utilizing bi-functional crosslinking agents of different chain lengths. It now appears to us that, in view of our ignorance with respect to the nature of the superprecipitation process, myofibrils may serve as a more appropriate system for investigation.

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