Fragmin Induces Tension Reduction of Actomyosin Threads in the Presence of Micromolar Levels of Ca^{2+}

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ABSTRACT Fragmin was able to reduce the isometric tension of *Physarum* actomyosin threads to 15–30% of the control tension at the Ca^{2+} concentrations $>10^{-6}$ M. However, fragmin had no effect on the tension of threads when the Ca^{2+} concentration was lowered below 10^{-7} M. The tension once reduced by fragmin could not be recovered by the removal of Ca^{2+}. The remaining tension was shown to be still active from the experiment with quick release or stretch of the thread. This tension reduction is parallel to the decrease in viscosity of F-actin solution by fragmin. Electron microscopy showed that F-actin filaments became shorter in the thread after the tension was reduced by fragmin. Therefore, the severing of F-actin by fragmin in micromolar concentration of calcium resulted in the relaxation of tension by actomyosin threads.

The contractile proteins, actin and myosin, have been found in a variety of eucaryotic cells. Evidence from biochemistry, immunology, and electron microscopy has indicated that actin and myosin are involved in cell motility such as amoeboid movement, cytoplasmic streaming, and cytokinesis (6, 7, 10, 28, 29). The microfilaments composed of actin and myosin are dynamically assembled and disassembled, or change their higher order organizations between bundles and dispersed filaments during the mitotic cycle of nonmuscle cells (6, 15, 18). The organized states of actin in vivo would be regulated by various kinds of proteins: filamin (31), actin-binding protein (8), actinogelin (23), fascin (26), nerve growth factor (4), profilin (5), DNase I (19), fragmin (9), gelsolin (33), villin (3), and so on. These proteins affect the assembly of F-actin filaments, the length of F-actin filaments, and/or the polymerizability of G-actin in vitro.

Among these regulatory proteins, fragmin was isolated from plasmodia of *Physarum polycephalum* and found to be a Ca^{2+}-sensitive regulator of the length of F-actin filaments (9). Fragmin does not affect the state of actin at pCa ≥ 7 but interacts with both F- and G-actins in the presence of micromolar Ca^{2+}, producing short F-actin filaments. How fragmin is involved in the molecular events relating to cell motility is the subject of current investigation.

Physarum plasmodia exhibit vigorous cytoplasmic movement called "shuttle streaming" (28). Kamiya (16) demonstrated by the double-chamber method that the stream of endoplasm is produced by a pressure gradient in the plasmodium. The pressure difference is apparently generated by contraction of ectoplasm at one end of the plasmodium and by relaxation at the other end. Alternate contraction and relaxation at two ends cause shuttle streaming of endoplasm. Fibrillar structures consisting of actin and myosin have been found in the peripheral ectoplasmic layer (1, 24, 25, 32). Since fragmin has been shown to interact with actin filaments, it is possible that fragmin may be involved in the regulation of contractility of actomyosin in vivo.

Recently, by using *Physarum* actomyosin, we succeeded in constructing synthetic actomyosin threads which generated an active tension reproducibly (21). Using this approach, we report that fragmin reduces the tension generated by the actomyosin thread in the presence of micromolar Ca^{2+}.

MATERIALS AND METHODS

Physarum myosin B was prepared by the method of Hatano and Tazawa (11). Dithiothreitol (DTT) and imidazole/HCl buffer, pH 7.0, were added to all solutions for preparation of myosin B (22). *Physarum* myosin was prepared from myosin B by the method of Matsumura and Hatano (22); Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for gel filtration instead of Sepharose 4B because it gave a better yield of myosin. *Physarum* actin was prepared from myosin B with heat treatment according to the method of Hatano and Owaribe (12), but ultracentrifugation in the presence of 1 M urea was omitted. Fragmin was purified by the procedure of Hasegawa et al. (9).

Actomyosin threads (AM-threads), in which the molar ratio of actin to myosin was 1 to 1, were formed as described in Matsumura et al. (21), and the isometric tension developed by the threads was measured by a tensiometer constructed by Kamiya (17). In the present work, we used AM-threads of 5–15 mm in length and ~0.2 mm in diameter containing 16–28.5 mg/ml of actomyosin. With increasing protein concentration of actomyosin, the thread showed a higher active tension and was less fragile during tension measurement. Two free ends of a

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thread were glued to thin glass hooks of the tensiometer with a rapid set adhesive, Aron Alpha (for surgical use; made by Sankyo, Japan). The thread was immersed in an open-surface chamber filled with ~8 ml of buffer A (20 mM imidazole/HCl, pH 7.0, 30 mM KCl, 5 mM $MgCl_2$, and 2 mM Ca/EGTA buffer with varied pCa between 4–8), and then the thread was straightened by moderate stretching. Isometric tension was generated by perfusing 15 ml of buffer B (buffer A containing 0.1 mM ATP) with or without fragmin using a peristaltic pump. All measurements of the tension were performed at 24–26°C. To examine whether the thread was actively generating tension or not, we stretched or released the thread quickly by a few percent of its original length and observed the recovery of the tension.

When calcium concentrations of the bathing buffer were changed by the addition of $CaCl_2$ in some experiments (see Fig. 3), pH of the buffer was measured. The decrease in pH from 7 to 6.75 was observed as calcium concentrations were raised up to 1 mM. The pCa was calculated based on the measured pH using the apparent dissociation constants between EGTA and Ca^{2+} (2). Such small changes in pH from 7.0 to 6.75 of the bathing buffer had little effect on the tension generation by AM-threads or on the activity of fragmin (unpublished results).

AM-threads were also formed with myosin and short F-actin which had been severed by fragmin. *Physarum* F-actin was mixed with fragmin at a molar ratio of 5 to 1 (respectively) in a solution containing 20 mM imidazole/HCl (pH 7.0), 30 mM KCl, 0.1 mM EDTA, 0.2 mM DTT, and 1 mM Ca/EGTA buffer (pCa 5 at pH 7.0), and the mixture was incubated for 5 min at 0°C. During this incubation, fragmin severed F-actin into short fragments. Then, myosin in the same buffer solution was added to obtain a molar ratio of actin to myosin to fragmin of 5 to 5 to 1. From this actomyosin solution, actomyosin threads containing fragmin (AMF-threads) were formed in the same way as AM-threads (21).

To observe the lengths of actin filaments in the threads by electron microscopy, we dissolved some threads into a solution containing 20 mM imidazole/HCl (pH 7.0), 0.5 M KCl, 5 mM $MgCl_2$, and 5 mM ATP to disperse filaments. The samples were mounted on grids covered with carbon-coated collodion film and stained with 1% (wt/vol) aqueous uranyl acetate. Electron micrographs were taken with a JEM 100-CX electron microscope at an accelerating voltage of 80 kV.

Protein concentrations were determined by the method of Lowry et al. (20) using bovine serum albumin as the protein standard.

RESULTS

The effect of fragmin and Ca^{2+} on tension generation by AM-threads was examined in two ways: (*a*) fragmin was added to the ambient media of AM-threads at various Ca^{2+} concentrations, (*b*) fragmin was first incorporated into AM-threads at pCa 5 (AMF-threads), and then concentrations of Ca^{2+} were changed.

Effect of Fragmin and Ca^{2+} in the Ambient Media on the Tension of AM-Threads

Fragmin showed no effect on the isometric tension developed by AM-threads at pCa ≥ 7. When the threads (16 mg/ml of actomyosin) were perfused with buffer B containing 40 μg/ml of fragmin at pCa 6.9, they showed the steady tension level of 2.5–3.0 g/cm^2 within 1 min after perfusion. This tension level was almost the same as that developed by threads in the absence of fragmin (Fig. 1). The tension, however, was markedly reduced by fragmin at pCa ≤ 5.5. Unlike the steady tension level shown in the control, the tension profile of the experiment showed three phases sequentially (Fig. 1): in the first phase, the tension increased to a maximum of 1.3–1.7 g/cm^2 within a few minutes; in the second phase, it decreased gradually over 30 min; and it reached a steady value of ~0.5 g/cm^2 in the last phase. The steady tension in the last phase was ~20% of that in the absence of fragmin (Fig. 2). If quick release or stretch was given to the thread, the steady tension was recovered within 5–10 min (Fig. 1). This result suggested that the remaining tension was actively generated by the thread.

At an intermediate Ca^{2+} concentration, pCa 6.2, fragmin reduced the tension less effectively; the maximum tension was 2.9 g/cm^2 in the first phase, and the tension dropped more slowly to a steady level of 1.1 g/cm^2.

When fragmin was absent, the values of tension at steady state decreased by ~20% when pCa was decreased from 8 to 4.4 in buffer B (Fig. 2). The values of the tension at steady state were plotted against pCa in the presence or absence of 40 μg/ml of fragmin (Fig. 2).

The dependence of fragmin concentration on the tension reduction was examined at pCa 4.4. Up to the concentration of 10 μg/ml of fragmin, reduction of the tension was not observed; however, with increasing concentration to 40 μg/ml, both the maximum tension in the first phase and the steady tension in the last phase became lower, and the rate of tension drop in the second phase became faster. Further increases in the concentration of fragmin up to 80 μg/ml produced no significant change.

The above results were obtained by using new AM-threads for each experiment with changing the concentration of Ca^{2+} or fragmin. To confirm the above results, we also examined the effect of fragmin by using the same thread throughout the

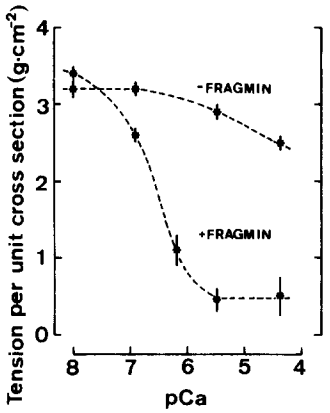


FIGURE 2 Free Ca^{2+} concentration dependence of tension generation. The results of the experiments in Fig. 1 were summarized. Final steady levels of tension per unit cross-section were plotted against pCa. +FRAGMIN, measurements in buffer B (20 mM imidazole/HCl, 30 mM KCl, 5 mM $MgCl_2$, 2 mM Ca/EGTA buffer, 0.1 mM ATP, pH 7.0) containing 40 μg/ml of fragmin. −FRAGMIN, measurements in buffer B without fragmin.

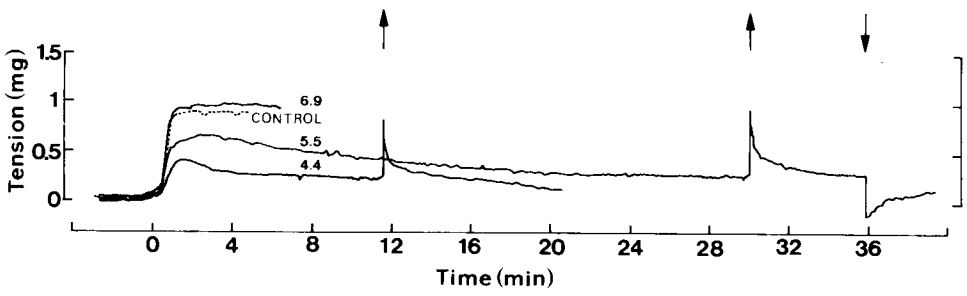


FIGURE 1 Tension generation in the presence of fragmin. *Physarum* AM-threads were 5–15 mm in length and 0.2 mm in diameter, and protein concentration of the thread was 16 mg/ml of actomyosin (molar ratio of actin to myosin, 1:1). Isometric tension was generated by perfusing buffer B (20 mM imidazole/HCl, 30 mM KCl, 5 mM $MgCl_2$, 2 mM Ca/EGTA buffer, 0.1 mM ATP, pH 7.0) containing 40 μg/ml of fragmin. Numbers on each tension curve show Ca^{2+} concentrations expressed in pCa-unit. Arrows indicate quick release or stretch of the threads by a few percent of their original lengths (upward arrow, stretch; downward arrow, release). CONTROL (a dotted line) was measured in the absence of fragmin at pCa 4.4.

changes in Ca^{2+} concentrations. In Fig. 3, an AM-thread (28.5 mg/ml of actomyosin) was initially immersed in buffer B containing 120 μg/ml of fragmin at pCa 8. Then, pCa was lowered stepwise to three by the addition of 100 mM $CaCl_2$. The tension was markedly reduced to 30% of the original value at pCa ≤ 4.4. This indicated that fragmin could relax the AM-thread which was generating high tension. On the other hand, in the control (without fragmin), tension was decreased to 65% of the original value with decreasing pCa to 4.4. The small decrease in tension of the control by Ca^{2+} was always observed in *Physarum* AM-threads (see Fig. 2).

The effective Ca^{2+} concentration for tension reduction by fragmin in this experiment (Fig. 3) appeared to be higher than that of the experiment in Fig. 1. This was ascribed to the difference in the incubation time between two experiments. In the experiment in Fig. 3, we did not incubate the AM-thread at each pCa as long as the experiment in Fig. 1, in order to avoid the decrease in the concentration of ATP. Longer incubation, for example, at pCa 5.5 resulted in similar reduction of tension as observed in the experiment in Fig. 1.

The reversibility of the effect of fragmin on the tension was examined. After the tension was reduced by fragmin at pCa 4.4, pCa was increased to 6.4 or 7.4 by adding EGTA (pH 7.0). The tension was not recovered within the experimental period of 30 min.

Electron microscopic investigation was carried out to compare the length of actin filaments between AM-threads showing control tension and reduced tension by fragmin at pCa 4.4. As Fig. 4 *a* and *b* show, much shorter filaments were observed in AM-threads whose tension was reduced to 20% of control by fragmin than those in controls. On the other hand, at a pCa of 8, no significant difference in length of actin filaments was observed between AM-threads in the presence and absence of fragmin. Therefore, these observations confirmed that fragmin severed F-actin filaments into shorter filaments when the tension of the thread was reduced by fragmin in the presence of calcium ions.

Effect of Ca^{2+} on the Tension of AM-Threads Containing Fragmin (AMF-Threads)

Next, we examined whether Ca^{2+} had any effect on the tension generation by AM-threads containing fragmin (AMF-threads). In such AMF-threads, F-actin filaments had already been severed into short filaments by fragmin at pCa 5.

Fig. 5 represents the profile of the tension generation by the AMF-threads in buffer B at pCa 4.4 or 4.0. The AMF-threads did not show any "overshoot" phenomenon in tension development, unlike the tension profile shown by AM-threads in the presence of fragmin and Ca^{2+} (see Fig. 1). Furthermore, the AMF-threads did not show any Ca^{2+}-sensitivity in generation of the tension. The tension developed by the AMF-thread was low at both pCa 4.4 and pCa 8.0 and remained to be ~30% of that generated by the control AM-thread in the same conditions (Fig. 5). The absence of Ca^{2+}-sensitivity of the AMF-threads in tension generation was consistent with the previous observation that the tension of an AM-thread once reduced by fragmin was not recovered by the removal of Ca^{2+} from buffer B.

DISCUSSION

This paper shows that fragmin not only inhibited the generation of tension by AM-threads in the presence of micromolar concentration of Ca^{2+} but also reduced the tension which had been generated by AM-threads. We have concluded that the reduction of tension was caused by severing of F-actin filaments into short filaments by fragmin for the following three reasons: first, both the tension of AM-threads and the viscosity of F-actin solutions showed similar dependence on free Ca^{2+} concentrations in the presence of fragmin (compare Fig. 2 in this paper with Fig. 10 in reference 9). Second, electron microscopy showed that fragmin severed F-actin filaments in AM-threads (Fig. 4 *a*) as has been shown for F-actin solution (9). Third, AMF-threads in which actin filaments had already been severed into short filaments produced low tension (Fig. 5) similar to that generated by AM-threads in the presence of fragmin and Ca^{2+} (Fig. 1).

The interaction between fragmin and actin was reported to be reversible (9). However, the process from short F-actin filaments to longer ones by removal of Ca^{2+} was extremely slow (9). This may explain why the AM-threads treated with fragmin did not recover to high values of control tension after removal of Ca^{2+}, and why AMF-threads developed a low tension regardless of Ca^{2+} concentrations in our experiments (Fig. 5).

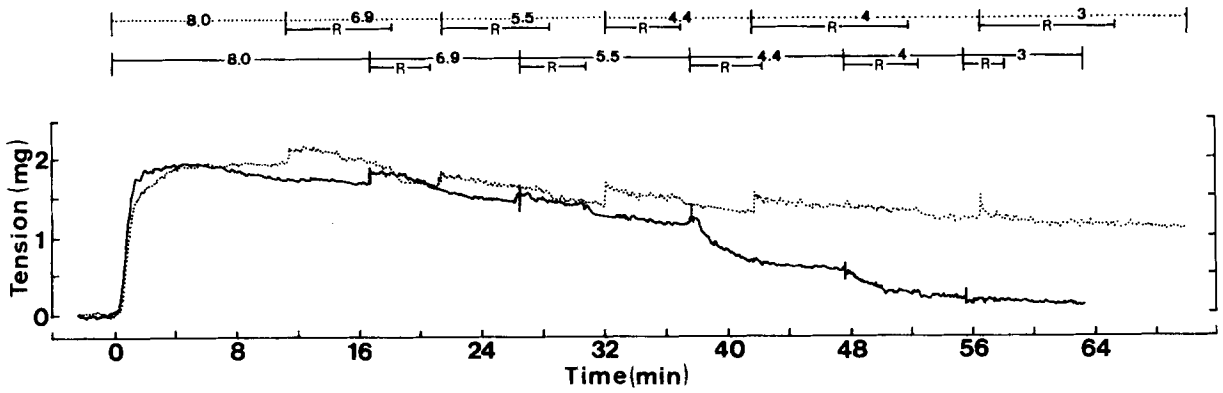


FIGURE 3 Measurement of tension using a single actomyosin thread at various Ca^{2+} concentrations. Protein concentration of the AM-thread was 28.5 mg/ml of actomyosin. Isometric tension was initially generated by perfusing buffer B (20 mM imidazole/HCl, 30 mM KCl, 5 mM $MgCl_2$, 2 mM Ca/EGTA buffer [pCa 8.0], 0.1 mM ATP, pH 7.0) containing 120 μg/ml of fragmin. Then, Ca^{2+} concentration was increased stepwise by adding $CaCl_2$ to the medium. Numbers on the top of the figure represent Ca^{2+} concentrations expressed in pCa-unit, and a couple of bars for each number represents the period when the thread was immersed at each pCa. For the period indicated by "*R*," the medium was circulated with a peristaltic pump to facilitate the mixing of $CaCl_2$ added. Solid line, in the presence of fragmin. Dotted line, in the absence of fragmin.

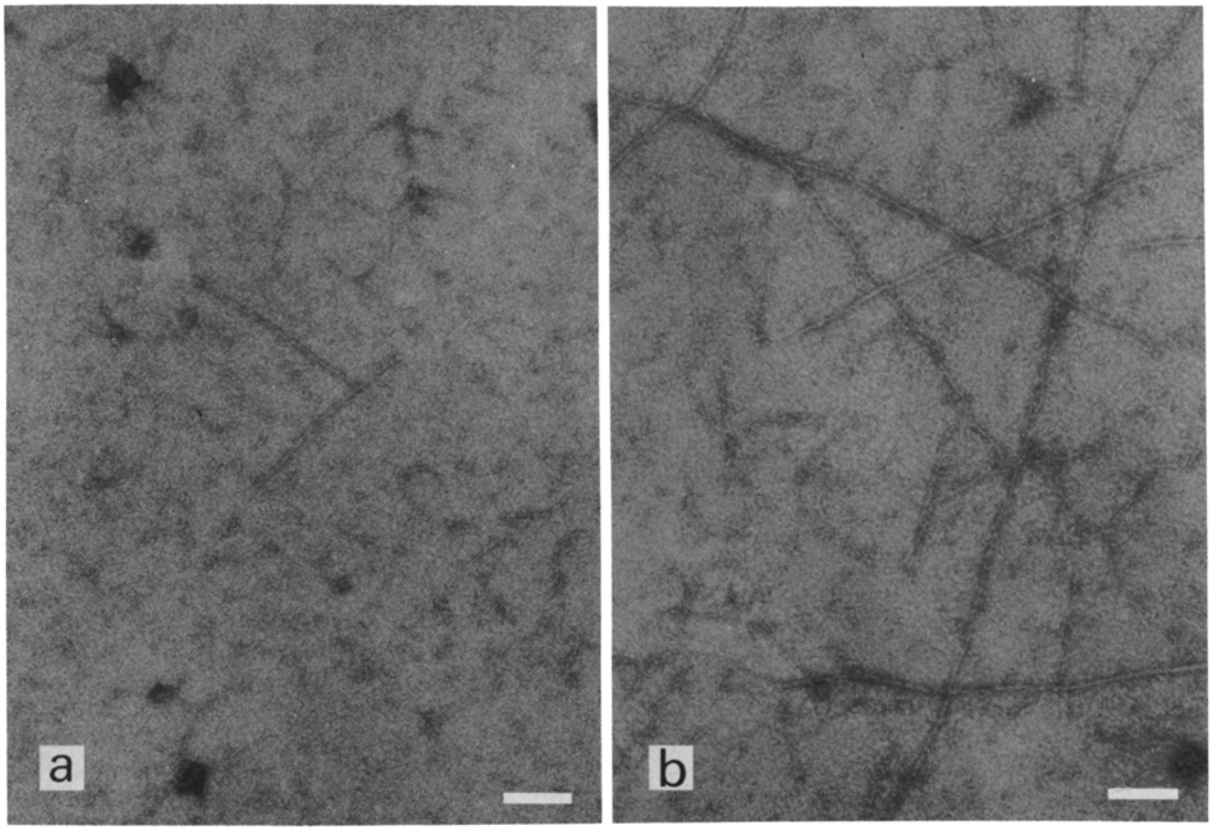
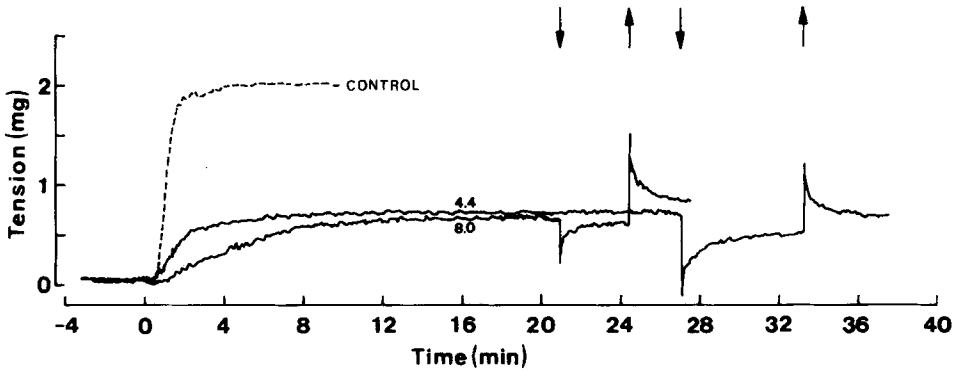


FIGURE 4 Electron micrographs of solubilized actomyosin threads. After the measurement of tension, each thread was dissolved into a solution containing 20 mM imidazole/HCl, 0.5 M KCl, 5 mM $MgCl_2$, and 5 mM ATP, pH 7.0. Specimens were stained with 1% (wt/vol) aqueous uranyl acetate. (*a*) A specimen prepared from the AM-thread which generated a low tension in the presence of fragmin at pCa 4.4. (*b*) A specimen prepared from the AM-thread which generated a control tension in the absence of fragmin at pCa 4.4.

FIGURE 5 Tension generation of actomyosin threads reconstituted with myosin and fragmented F-actin (AMF-thread). Protein concentration in the AMF-threads was 26.5 mg/ml of actomyosin-fragmin complex (molar ratio; myosin:actin: fragmin = 5:5:1). Isometric tension was generated by perfusing buffer B (20 mM imidazole/HCl, 30 mM KCl, 5 mM $MgCl_2$, 2 mM Ca/EGTA buffer, and 0.1 mM ATP, pH 7.0). Numbers and arrows are the same as those noted in the legend of Figure 1. CONTROL (a dotted line), tension generation by an AM-thread containing 28.0 mg/ml of actomyosin in buffer B at pCa 8.0.



We could roughly estimate the amounts of fragmin which finally bound to actin in the AM-threads to cause the observed reduction (20% of the control tension) in tension. Since the tension of the AMF-thread was reduced to the similar extent (30% of the control), a molar ratio of fragmin to actin of 1:5 in the AMF-thread would be needed to reduce the tension to such extent. The "overshoot" phenomenon of the tension development shown by AM-threads in the presence of fragmin and Ca^{2+} (Fig. 1) could be explained as follows. The diffusion of fragmin from the ambient media into threads was much slower than the diffusion of ATP. Therefore, the tension was first developed to some extent and then it was decreased gradually as F-actin filaments were severed into shorter filaments by slowly diffused fragmin. When equilibrium of the binding between fragmin and actin was reached, AM-threads showed the low steady state tension. Since F-actin filaments in AMF-threads were already severed into short filaments, AMF-threads did not show "overshoot" phenomenon in the tension development.

Fragmin did not reduce the tension developed by AM-

threads when ATP concentration was lowered to 10 μM with an ATP-regenerating system (64 U/ml creatine phosphokinase, 4 mM phosphocreatine). It was reported that fragmin cannot react with F-actin if F-actin is fully decorated with muscle heavy meromyosin (9). Since these AM-threads were reconstituted with actomyosin at a molar ratio of myosin to actin of 1:1, it is likely that high concentration of ATP (0.1 mM) was necessary to dissociate such actomyosin.

Our results showing that fragmin reduced the tension of AM-threads in the presence of Ca^{2+} suggest that fragmin may play a role in relaxing the actomyosin system of plasmodia in vivo. This idea appears to favor the recent report by Yoshimoto et al. (34). They found that Ca^{2+} efflux oscillated with the same period as the cycle of tension generation in a permeabilized plasmodial strand, but the phase of cyclic changes in Ca^{2+} efflux was opposite to that of tension generation. They interpreted these observations to mean that the intracellular Ca^{2+} concentration increased in the relaxing phases. This interpretation might fit into a fragmin-mediated relaxation mechanism in living plasmodia. However, Hatano (13), Ridgway and Durham (27), and Ueda and Von Olenhusen (30) reported the contradictory evidence that Ca^{2+} acted as a stimulating agent for contraction of living plasmodia. Furthermore, the lack of reversibility of the effect of fragmin on the tension of AM-threads is apparently unfavorable to explain the cyclic tension generation in *Physarum*. It is, however, possible that some unknown factors may promote the slow dissociation between actin and fragmin so that tension may be recovered.

At the present time, it still is an open question whether in a living plasmodium Ca^{2+} acts as a stimulating agent for contraction as in the muscle, or, on the contrary, as an inhibitory agent for streaming as in the *Nitella* cells (35–38). Therefore, possibilities of the in vivo function of fragmin other than that discussed above should be examined. For example, fragmin may slightly disrupt actomyosin gel made by gelation factors in order to permit contraction (solution-contraction coupling) as first suggested by Hellewell and Taylor (14). Another possibility is that fragmin may act on actomyosin in ectoplasm after contraction in order to recycle the actin system. Further in vivo and in vitro experiments are needed to examine these possibilities.

It is very likely that cytoplasmic streaming of *Physarum* is regulated with complex functions of actomyosin-associated proteins other than fragmin. Studies with reconstituted AM-threads containing such associated proteins will be useful to understand in vivo function of fragmin in the contractility of living plasmodia.

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