Does Actin Bind to the Ends
of Thin Filaments in Skeletal Muscle?

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ABSTRACT   We examined whether or not purified actin binds to the ends of thin filaments in rabbit skeletal myofibrils. Phase-contrast, fluorescence, and electron microscopic observations revealed that actin does not bind to the ends of thin filaments of intact myofibrils. However, in I-Z-I brushes prepared by dissolving thick filaments at high ionic strength, marked binding of actin to the free ends, i.e., the pointed ends, of thin filaments was observed when actin was added at an early phase of polymerization. As the polymerization of actin proceeded, the binding efficiency decreased. The critical actin concentration for this binding was higher than that for polymerization in solution. The binding of G-actin was not observed at low ionic strength. On the basis of these results, we suggest that a particular structure suppressing the binding of actin is present at the free ends of thin filaments in intact myofibrils and that a part of the end structure population is eliminated or modified at high ionic strength so that further binding of actin becomes possible. The myofibril and I-Z-I brush appear to be useful systems for studies aimed at elucidating the organizational mechanisms of actin filaments in vivo.

Striated muscle has a well-organized structure, and thick and thin filaments have their own well-defined lengths. Although there is no way at present to measure exactly the number of myosin and actin molecules constituting filaments, it seems reasonable to suppose that the number is strictly determined. On the other hand, if a binding equilibrium between the protein molecules operates, one would expect the length distribution of the filaments to be as broad as in an in vitro system (13, 21, 22). Thus, the question arises, what kind of mechanism does exist in vivo for constructing and maintaining the well-organized structure and fixed size of filaments?

As a first step towards elucidating this problem, we examined whether or not actin molecules can be bound to the free ends (the ends away from the Z line) of thin filaments in a myofibril, i.e., whether some particular structure that suppresses additional binding of actin is present at the free ends of thin filaments. We also examined the possibility of actin binding to the other end (the end at the Z-line) of thin filaments and to the Z line itself.

The free end of thin filaments is called the pointed end, and it has been shown in in vitro experiments that the rate of association of actin to this end is much slower than that to the other end, i.e., the barbed end (8, 15, 37). Furthermore, the binding constant of actin monomer to the pointed end is smaller than that to the barbed end (14, 23, 36). In this

connection, it would be interesting to examine the conditions under which the binding of actin to the pointed end of thin filaments in myofibrils occurs, as well as to measure both the association rate and the association constant and to compare them with those obtained in the in vitro experiments. If the length of thin filaments is determined by a so-called treadmilling mechanism (14, 35), a simple terminator in the above sense may not be present, and actin may be able to bind to the free ends at a concentration higher than the critical one for polymerization. Therefore, studies aimed at detecting a special end structure including a terminator in myofibrils could also cast light on the applicability of the treadmilling mechanism to skeletal muscle.

Since nothing is known about the properties of the end structure of thin filaments in myofibrils or about the mechanism of length determination of thin filaments, it is important as an initial step to determine the conditions under which actin binds to the free ends of thin filaments in myofibrils.

In the present work, we mainly used purified actin molecules labeled with a fluorescent dye as a probe to monitor the location in myofibrils at which added actin molecules were incorporated (cf, reference 30). It was shown that actin does not bind to the free ends of thin filaments in an intact myofibril. However, after the dissolution of thick filaments, it became possible to incorporate actin molecules specifically

at the free ends of thin filaments provided that actin was added during the polymerization process. On the basis of these results, it was suggested that the structure at the free ends of thin filaments in intact myofibrils is different from the rest of the filaments and a part of the end structure population is removed or modified at high ionic strength so that additional binding of actin becomes possible. The properties of actin binding to the free ends of thin filaments in I-Z-I brushes were studied under various conditions. However, the binding of actin to barbed ends of thin filaments was observed only when a myofibril was split in two and the Z line structure was fortuitously absent.

Preliminary reports of parts of this work have been presented at the 19th (1981), 20th (1982), and 21st (1983) annual meetings of the Biophysical Society of Japan. After the submission of our manuscript, Sanger et al. (26) reported results similar to ours (compare Fig. 4, a–e of their paper and Fig. 1, a–d of our paper).

MATERIALS AND METHODS
Proteins

Muscle proteins were prepared from rabbit leg and back white muscle. Acetone powder was prepared according to Straub (29) except that regulatory proteins were removed by washing muscle residue at low ionic strength before the acetone treatment (4). Actin was purified according to Spudich and Watt (28). Actin labeled with fluorescent dye was prepared as follows: F-actin (2.0 mg/ml; 0.1 M KCl, 20 mM $NaHCO_3$, 0.6 mM ATP) was mixed with 50 μM 4-[N-(iodoacetoxy)ethyl-N-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD, SH selective probe; Molecular Probes, Junction City, OR) and stored for 2 h at 0°C. The solution was ultracentrifuged at 100,000 g for 90 min and the pellet was dissolved in 5 mM Tris-HCl buffer (pH 6.8) and 0.1 mM ATP; the resultant solution was dialyzed against the same solvent at 2°C. After sufficiently depolymerizing the actin, we ultracentrifuged the solution again. G-actin thus obtained was used as a labeled G-actin. The polymerizability of labeled actin was essentially the same as that of unlabeled actin (see Fig. 7). We estimated the amount of labeled actin to be ~50% by using an extinction coefficient of 3×10^4 $M^{-1}cm^{-1}$ for IANBD. Although almost all the free dye was removed during the above procedure, Sephadex G25 (fine) was used for final removal of a trace amount of free dye. Free ATP in G-actin solution was removed by treatment with an ion exchange resin, Dowex 1-X1 (mesh 50–100, Cl^- form) (1). Protein concentrations were determined by the biuret method.

Myofibrils

Glycerinated muscle fibers were prepared by storing a thin bundle of rabbit psoas muscle fibers in 50% (vol/vol) glycerol, 0.5 mM $NaHCO_3$, and 5 mM EGTA (pH 7.6 at 25°C) for >3 wk at −20°C. Myofibrils were prepared by homogenizing the glycerinated fibers with a homogenizer (Ultra turrax; IKA-WERK STAUFEN, Staufen, Federal Republic of Germany) in a rigor solution (solution A) containing 60 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-maleate buffer (pH 6.8), and 1 mM EGTA. Sometimes we used myofibrils prepared according to Etlinger and Fischman (5). The results were essentially the same as those obtained with the above myofibrils.

Microscopy

Fluorescence (incident light illumination) and phase-contrast micrographs were taken with a Fluophoto VFD-R (objective lens, CF Plan DM 100×; Nikon, Co., Tokyo, Japan; 20–40- and 0.5-1-s exposure, respectively). Polarizing micrographs were taken with a rectified Apophoto (Nikon Co.; 5-min exposure). The PFM system camera (Nikon Co.) and Kodak Tri-X films were used (developer, Kodak Microdol-X diluted 1:3). Electron micrographs were taken with a JEM 100 CX (JEOL, Tokyo, Japan; operating voltage, 80 kV).

Viscosity Measurement

The specific viscosity of F-actin solution was measured with an Ostwald type viscometer (flow time, ~20 s) at 20 ± 0.5°C.

Method for Examining the Binding of Actin to Thin Filaments

OPTICAL MICROSCOPIC OBSERVATION: All procedures were done on a glass slide or under an optical microscope unless otherwise stated. Labeled actin molecules were added to intact myofibrils or ghost myofibrils, i.e., an array of I-Z-I brushes, under various conditions. Overstretched myofibrils were prepared as follows: myofibrils dispersed in 0.12 M KCl, 4 mM $MgCl_2$, 20 mM Tris-maleate buffer (pH 6.8), and 4 mM EGTA were first relaxed in a test tube by adding 4 mM ATP (relaxing solution) at 0°C and then stretched by slightly moving a coverslip in one direction on a glass slide (11). Contraction of the overstretched myofibrils was not observed at all upon the addition of Ca^{2+} or by replacing the solution with the rigor solution (solution A). Ghost myofibrils were prepared by dissolving thick filaments in 0.5 M KCl, 5 mM $MgCl_2$, 10 mM Tris-maleate buffer (pH 6.8), 10 mM potassium pyrophosphate (PPi),[1] and 1 mM EGTA (12). Association with thin filaments of dissociated myosin molecules was suppressed by the presence of MgPPi at high ionic strength. Ghost myofibrils were prepared in the presence of EGTA because in the presence of Ca^{2+} the fine structure of the I-Z-I brush tended to become disorganized during the dissociation of thick filaments (7). About 5 min after the addition of actin under various conditions, unbound actin molecules were washed out with solution A (or the above relaxing solution; the result was essentially the same as that with solution A) and the region into which labeled actin was incorporated was observed by fluorescence, phase-contrast, and polarizing microscopy in solution A (or the above relaxing solution).

ELECTRON MICROSCOPIC OBSERVATION: Isolated I-Z-I brushes were prepared by homogenizing myofibrils in a solution containing 0.1 M KCl, 10 mM $MgCl_2$, 5 mM ATP, 5 mM Na-phosphate buffer (pH 6.8), and 0.5 mM EGTA at 0°C (11, 20). An I-Z-I brush placed on a grid was washed first with a high salt solution containing 0.5 M KCl, 5 mM $MgCl_2$, 10 mM Tris-maleate buffer (pH 6.8), 10 mM PPi, and 1 mM EGTA and subsequently with the rigor solution (solution A). (The high salt treatment was not performed for a control experiment.) Then, 0.3 mg/ml of unlabeled actin was added in 20 mM KCl, 0.1 mM ATP, 0.1 mM $CaCl_2$, and 5 mM Tris-HCl buffer (pH 6.8). About 5 min after the addition of actin, unbound actin molecules were washed away with solution A. Then, I-Z-I brushes were negatively stained with 1% uranyl acetate.

RESULTS
Binding of Actin to Intact Myofibrils

We examined whether actin binds to the ends of thin filaments in intact myofibrils under various conditions. First, we examined the binding of actin in the absence of ATP to suppress the contraction of myofibrils. Fig. 1 shows that labeled actin was bound mainly to the central H zone of the sarcomere and partly to the I band including Z line (Figs. 1, a–d), but the former was removed when the thick filaments were dissociated (Fig. 1, e and f). However, only after removal of the thick filaments could actin bind to the free ends of thin filaments as described in detail below, and this bound actin was not removed even by washing with a high salt solution; note the increase of density and the appearance of fluoresence intensity in the gap region between I-Z-I brushes on phase-contrast (Fig. 1g) and fluorescence (Fig. 1h) micrographs, respectively. Also, it should be noted that the actin binding observed on the H zone in intact myofibrils (Fig. 1, a and b) decreased at shorter sarcomere lengths (Fig. 1, c and d). These results can be interpreted as indicating that actin does not bind to the free ends of intact thin filaments but binds to myosin heads that are not associated with thin filaments. After the dissociation of thick filaments, the binding of actin to the free ends of thin filaments becomes possible.

It should be mentioned here that we could not use the same sample in these experiments because the thick filaments became undissociable after having been observed under a fluorescence microscope, probably due to cross-link formation by

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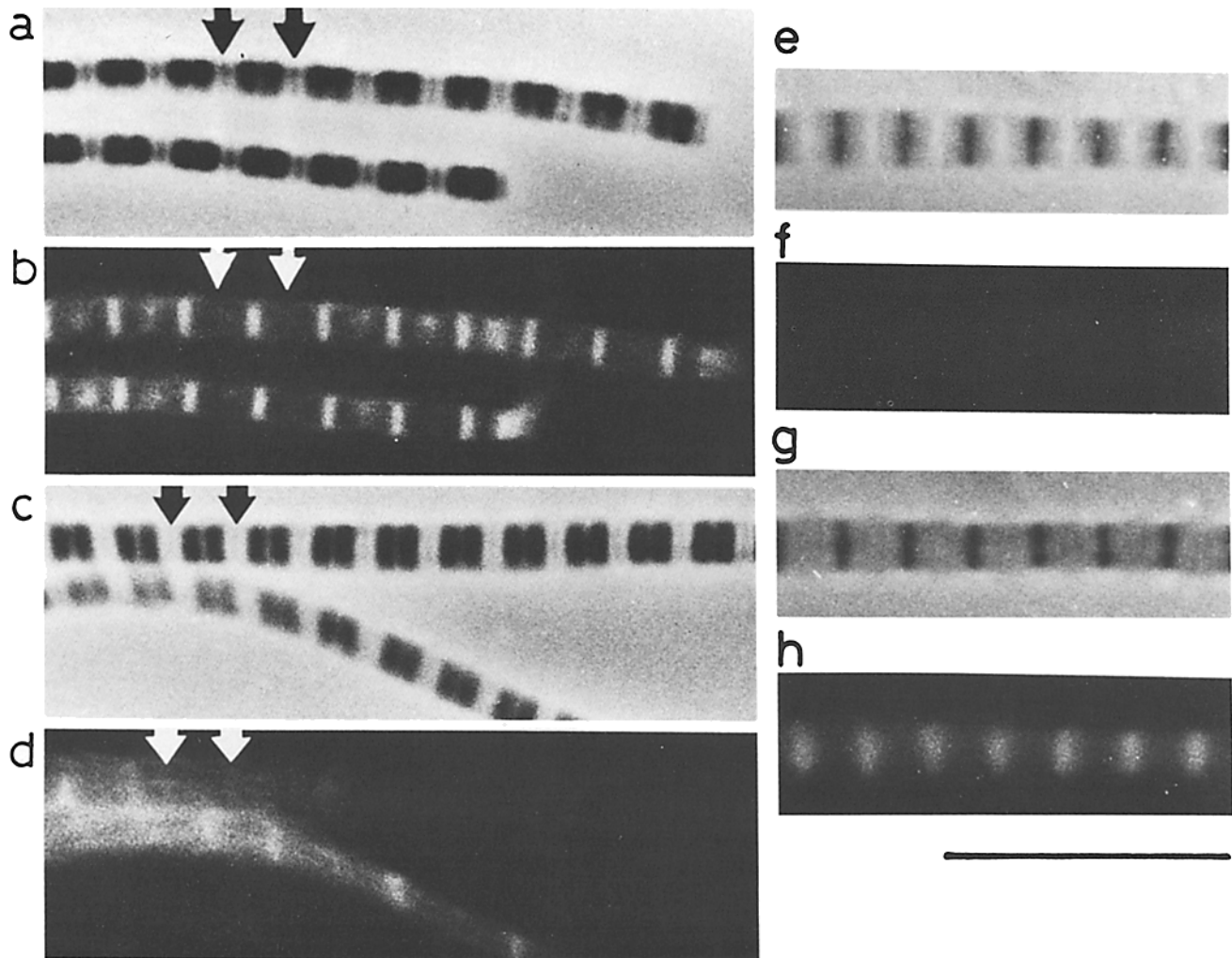[1] *Abbreviation used in this paper:* PPi, pyrophosphate.

FIGURE 1 Phase-contrast and fluorescence micrographs showing the binding of actin to an intact myofibril. (a) Intact myofibril. (b) 1.0 mg/ml of labeled actin preserved at 0°C was added just after the addition of salt solution without ATP (see Fig. 7; final solvent composition, 20 mM KCl, 5 mM Tris-HCl buffer, pH 6.8, and 0.1 mM CaCl$_2$; the CaCl$_2$ was added to extend the polymerization phase). About 5 min after the addition of actin, unbound actin molecules were washed away with the rigor solution (solution A). (c and d) The same as in a and b except that a sarcomere length is ~2.2 µm, that is, a little shorter than that in a and b, ~2.4 µm. (e and f) After the treatment described in b, thick filaments were dissolved by high salt solution containing 0.5 M KCl, 5 mM MgCl$_2$, 10 mM PPi, 10 mM Tris-maleate buffer (pH 6.8), and 1 mM EGTA, and I-Z-I brushes thus obtained were washed with solution A. (g and h) 1.0 mg/ml of labeled actin without ATP prepared as described above was added again to the above sample and the sample was washed with the above high salt solution. In e-h, a myofibril different from that used in a and b (or c and d) was unavoidably examined because thick filaments became undissociable after observation under a fluorescence microscope. (a, c, e, and g) Phase-contrast micrographs; (b, d, f, and h) fluorescence micrographs. Arrows indicate the positions of Z lines. Bar, 10 µm. × 4,000.

absorption of light as described below.

Furthermore, the binding and polymerization of actin on the Z line (upper right in Fig. 1 b, compare with Fig. 1 a where a dense line corresponding to the Z line is observed) or at the barbed ends of thin filaments (lower right in Fig. 1 b, compare with Fig. 1 a where the Z line has apparently been removed) were unexpectedly observed in Fig. 1 b. These bindings may be explained by the fortuitous splitting of I-Z-I brushes into two.

We next carried out the same experiment by using over-stretched myofibrils. The sarcomere was lengthened to ~5.5 µm, so that the separation between the edges of thick and thin filaments became ~1 µm. This length was sufficient to determine whether or not actin was bound to the free ends of thin filaments. Fig. 2 shows that actin binds to thick filaments but does not bind to the free ends of thin filaments. Bound

actin filaments were protruded from the edge of the A band but did not contact the edge of thin filaments. These bound actin filaments were removed during the dissociation of thick filaments in a high salt solution, as in the first experiment (Fig. 1, e and f). This result suggests that the reason why actin could not bind to the free ends of thin filaments in an intact myofibril (Fig. 1) is not the steric hindrance by thick filaments but the intrinsic character of the end structure of thin filaments.

We carried out the same experiments as above in the relaxing solution (see Materials and Methods) at 0.1–1.0 mg/ml of labeled actin using stretched and unstretched myofibrils. (The critical concentration for the polymerization of labeled actin in the relaxing solution was <0.1 mg/ml.) Under this condition, the binding of actin to thick filaments and also to the free ends of thin filaments was not observed, although the
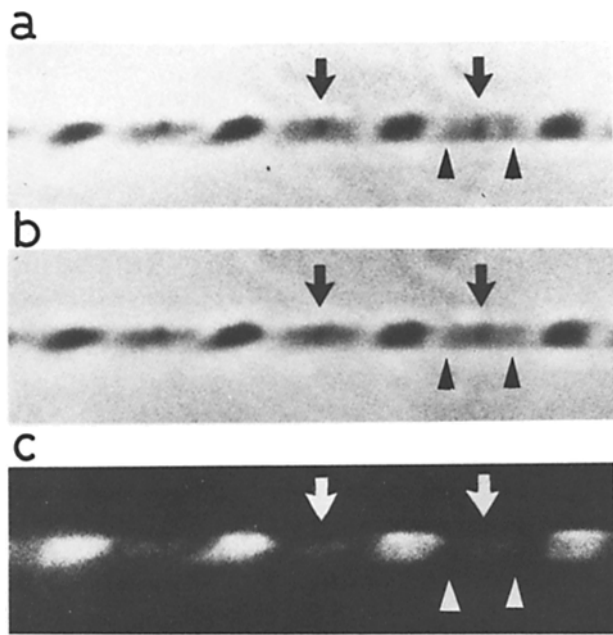
FIGURE 2   Binding of actin to an overstretched intact myofibril. (a) Overstretched intact myofibril in the rigor solution (solution A) (see Materials and Methods); (b and c) after the addition of 1.0 mg/ml of labeled actin in the presence of 20 mM KCl, 5 mM Tris-HCl buffer (pH 6.8), 0.1 mM CaCl₂, and 0.1 mM ATP. (a and b) Phase-contrast micrographs; (c) fluorescence micrograph. Other conditions were the same as in Fig. 1. Arrows indicate the positions of Z lines. Arrowheads indicate the positions of the free ends of thin filaments. Bar, 10 μm. × 3,900.

latter binding occurred after removal of thick filaments as in the above experiments (Fig. 4). These results indicate that the binding of actin to the free ends of thin filaments in intact myofibrils does not occur irrespective of the solvent composition and it occurs only after the dissolution of thick filaments.

Actin Binds to the Free Ends of I-Z-I Brushes Prepared by Dissolution of Thick Filaments

The typical optical micrographs shown in Fig. 3 reveal that labeled actin is incorporated into the ends of the I-Z-I brushes, i.e., the free ends of thin filaments. An increase of density in the gap region between I-Z-I brushes and on both sides of an isolated or tilted I-Z-I brush was observed on a phase-contrast micrograph (Fig. 3a). The labeled actin was seen to be localized in these regions on a fluorescence micrograph (Fig. 3b). The fluorescence intensity in the gap region between I-Z-I brushes was larger than that on both sides of an isolated or tilted I-Z-I brush. This shows that in the former region actin filaments bound to two adjacent I-Z-I brushes were overlapped, so that the fluorescence intensity was nearly double that in the latter region (cf. Fig. 3b). Moreover, the fluorescence observed in the gap region was largely restricted to the original H zone. This suggests that it is difficult for actin filaments to enter the adjacent I-Z-I brush, probably because of the high density of thin filaments.

The binding of actin to the free ends of thin filaments in I-Z-I brushes was also observed in a physiological solution (Fig. 4). Under this condition, however, the binding of actin to the whole I-Z-I brush was also noticeable (Fig. 4, d and h). These
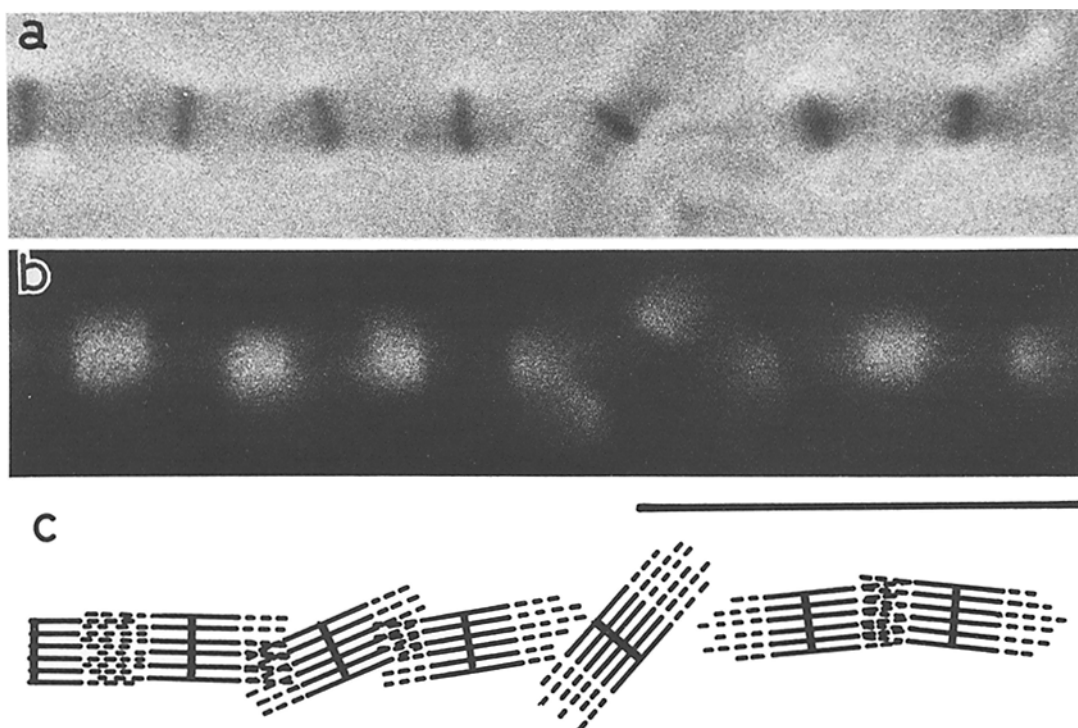

FIGURE 3   Binding of actin to the free ends of thin filaments in I-Z-I brushes. (a) Phase-contrast and (b) fluorescence micrographs; and (c) schematic illustration of the arrangement of thin filaments (thin lines) and bound actin filaments (dashed lines). A transverse thick line represents the Z line. Ghost myofibrils were prepared by dissolving thick filaments in the presence of high concentrations of KCl and MgPPi (see Materials and Methods) and by replacing the high salt solution with the rigor solution (solution A). A tilted I-Z-I brush was obtained by chance. Then 1.0 mg/ml of labeled actin preserved at 0°C was added just after the addition of salt solution (final solvent composition, 20 mM KCl, 0.1 mM ATP, 0.1 mM CaCl₂, and 5 mM Tris-HCl buffer, pH 6.8). About 5 min after the addition of actin, unbound actin molecules were washed away with solution A. For other conditions, see Materials and Methods. Bar, 10 μm. × 6,000.
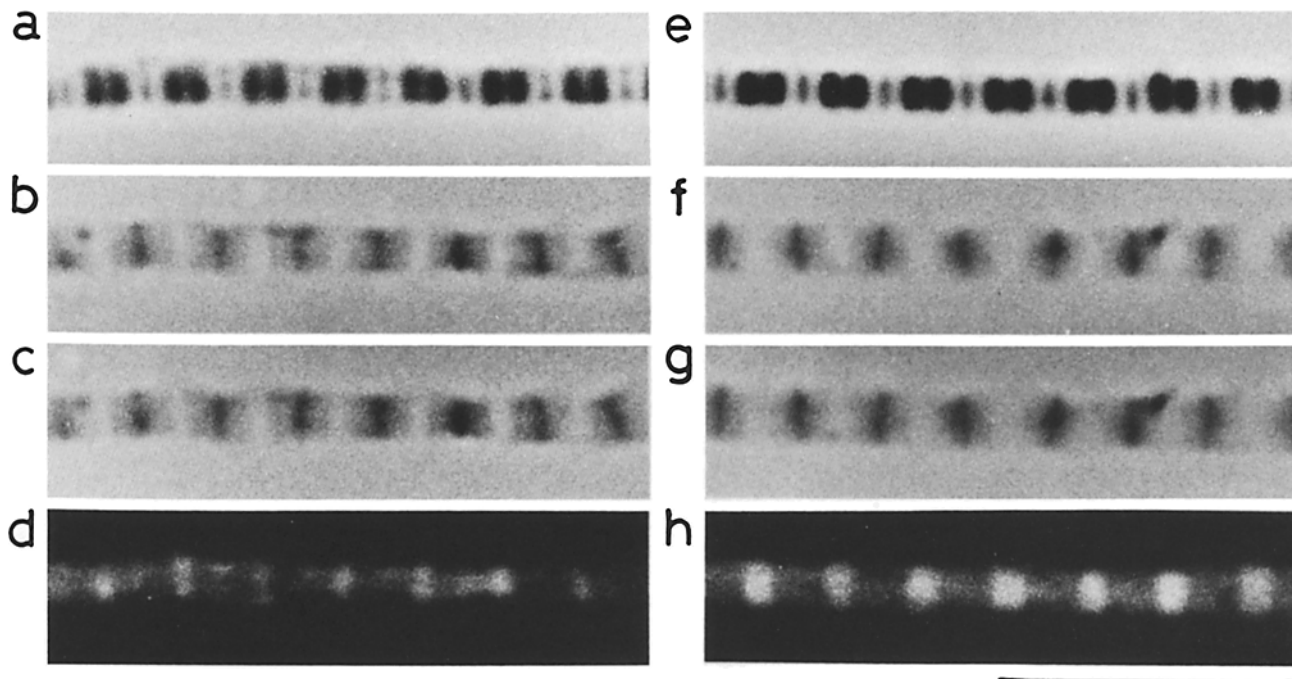
FIGURE 4   Binding of actin to the free ends of thin filaments in a relaxing solution. (a) Intact myofibril; (b) I-Z-I brushes obtained by dissolving thick filaments in a high salt solution (cf. Fig. 1). (c and d) I-Z-I brushes were washed with the relaxing solution (0.12 M KCl, 4 mM MgCl₂, 4 mM ATP, 20 mM Tris-maleate buffer, pH 6.8, and 4 mM EGTA). Then 0.1 mg/ml of labeled actin preserved at 0°C was added just after the addition of salt solution (final solvent composition was the same as the relaxing solution). About 5 min after the addition of actin, unbound actin molecules were washed away with the relaxing solution. (e–h) The same as a–d except that 1.0 mg/ml of labeled actin was added. Photographs were taken in the relaxing solution. (a–c and e–g) Phase-contrast micrographs; (d and h) fluorescence micrographs. Bar, 10 μm. × 4,000.

results together with Fig. 3 show that the binding of actin to the free ends of thin filaments in I-Z-I brushes occurs irrespective of the solvent composition, although its efficiency changes with the experimental condition. The experiments in Fig. 4 excluded the possibility that the relocation of dissociated myosin molecules may have some role on the binding of actin, because the association between actin and myosin was inhibited in the relaxing solution.

The binding of actin to the free ends of thin filaments in an I-Z-I brush was observed when actin was added just after the addition of salt, that is, during the polymerization process. As the polymerization proceeded (during several hours after the addition of salt under the condition in Fig. 3), the efficiency of binding to the free ends of thin filaments became very low, and the binding to the whole I-Z-I brush increased somewhat. This latter binding appears to be nonspecific in contrast with the specific binding to the ends of the I-Z-I brush. Under the conditions at which the polymerization of actin occurs rapidly, e.g., at physiological solvent conditions, the proportion of the nonspecific binding tended to be higher (Fig. 4). Therefore, we mostly used 20 mM KCl, 0.1 mM CaCl₂ (slowing down the rate of polymerization; cf. reference 22), 5 mM Tris-HCl buffer (pH 6.8), and 0.1 mM ATP as a solvent to induce specific binding of actin to the free ends of thin filaments (Fig. 3). As the polymerization of actin proceeded further and polymerization equilibrium was attained (for example, after storing F-actin in the above low salt solution with 1 mM NaN₃ for a few days at room temperature), the binding efficiency increased again, although it was lower than that at the early phase of polymerization. On the other hand, the binding of G-actin (0.1–1.0 mg/ml) was not observed at low ionic strength (5 mM Tris-HCl buffer, pH

6.8, 0.1 mM ATP), nor was binding of actin to the Z line in an I-Z-I brush.

Under a fluorescence microscope, it is possible to observe the time course of the increase of fluorescence intensity due to the binding of labeled actin to the free ends. Especially, in an isolated or tilted I-Z-I brush, the increase of the fluorescent region accompanying the growth of actin filaments can be seen. The average rate of growth was thus estimated. In Fig. 3, thin filaments were elongated by 1—1.5 μm within 4 min, and the elongation gradually slowed down.

On the other hand, in the case of I-Z-I brushes having a length shorter than the intact one and showing high density at both ends under a phase-contrast microscope, the binding of actin to the ends of thin filaments was hardly observed. In such I-Z-I brushes the ends of thin filaments may be entangled or folded, so that the efficiency of actin binding is very low.

Polarizing Micrograph

Fig. 5 shows that actin bound to an I-Z-I brush had positive birefringence, although the resolution of the micrograph is low compared with that of crab myofibrils (16), because the sarcomere length of rabbit skeletal muscle is shorter than that of crab muscle. This result suggests that incorporated actin filaments are oriented along the long axis of myofibrils.

Electron Microscopy

We directly observed under an electron microscope how actin filaments were elongated from the free ends of thin filaments in an I-Z-I brush. Fig. 6, a and b shows that actin molecules are bound to and elongated from the free ends of thin filaments in an I-Z-I brush obtained after washing with
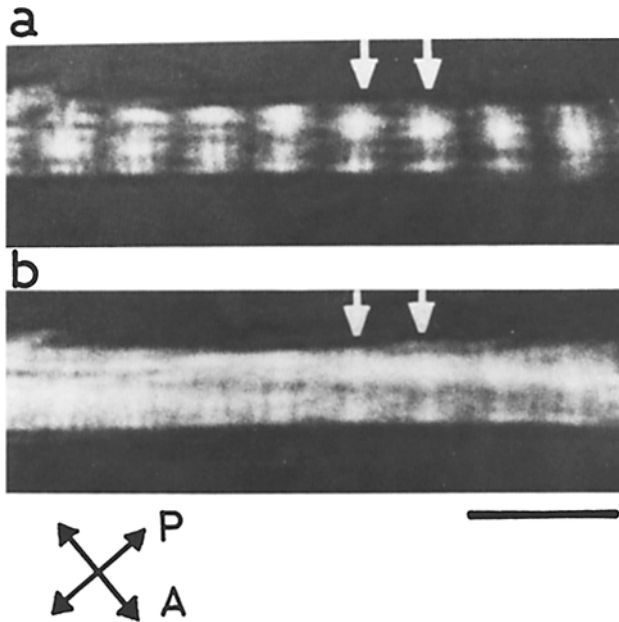
FIGURE 5 Polarizing micrographs of I-Z-I brushes before and after the binding of actin filaments. (a) Before and (b) after the addition of actin. Unlabeled actin (1.0 mg/ml) was added at 20 mM KCl, 0.1 mM ATP, 0.1 mM CaCl$_2$, and 5 mM Tris-HCl buffer (pH 6.8). Photographs were taken after washing away unbound actin molecules with the rigor solution (solution A). Arrows indicate the positions of Z lines. The polarizer (P) and analyzer (A) were set perpendicular to each other. For other conditions, see Materials and Methods. Bar, 5 μm. × 4,000.

a high salt solution, although it is difficult to strictly determine the position of the free end of each native thin filament. It should be noted here that two classes of thin filaments were observed in the same I-Z-I brush (Fig. 6b): in one class, the length of thin filaments was maintained at 1 μm and no binding of actin was observed, whereas in the other class, filaments were elongated by binding of actin and the length distribution appeared to be broad. An elongated thin filament seems to resemble a grafted tree in that the elongated part of the filament looks fragile and is thinner than the intact part of the filament, probably because the intact filaments contain regulatory proteins. When an I-Z-I brush was examined without washing with a high salt solution, we could not identify actin filaments elongated from the free ends of thin filaments, although actin filaments formed spontaneously or bound to thick filaments were observed (Fig. 6c).

Effect of Actin Concentration

First, we obtained the relation between the specific viscosity and total concentration of actin with or without labeling and in the presence or absence of free ATP at low ionic strength. Fig. 7 shows that the polymerizability of actin was not greatly changed by labeling; the critical concentration was ~0.1 and ~0.3 mg/ml in the presence and absence of ATP, respectively. Also, the change by labeling of the polymerization rate was not detected at 1.0 mg/ml of actin (data not shown). On the other hand, the phase-contrast and fluorescence micrographs in Fig. 8 show that in the presence of ATP 0.1 mg/ml of labeled actin was not bound but 0.5 mg/ml of labeled actin could be bound to the free ends of thin filaments. (At a physiological ionic strength the binding of labeled actin could

be observed even at 0.1 mg/ml [Fig. 4].) This observation suggests that the critical concentration for binding to the free ends of thin filaments was >0.1 mg/ml. In the absence of ATP, 0.5 mg/ml of labeled actin was not bound but 1.0 mg/ml of labeled actin could be bound (photographs not shown). Therefore, the critical concentration for binding to the free ends of thin filaments in the absence of ATP is considered to be >0.5 mg/ml. These results show that actin at a concentration higher than the critical concentration for polymerization in the solution is required for binding to the free ends of thin filaments.

Binding of Actin to I-Z-I Brushes Treated with KI

Fig. 9, a–d shows that large amounts of actin bound to the free ends of thin filaments in I-Z-I brushes mildly treated with KI solution. However, when the thin filaments were dissolved at a higher KI concentration, only a small amount of actin was bound to the remaining part of the I-Z-I brushes, i.e., a doublet structure appeared at both sides of the Z-line as seen in Fig. 9, e–h (6). When thin filaments were thoroughly dissolved at a much higher KI concentration, e.g., 0.6 M, actin binding to the remaining doublet structure could no longer be observed (photographs not shown).

Other Observations

As described above, thick filaments became undissociable after the fluorescence microscope observations. The exposure to ultraviolet light probably causes this phenomenon. On the other hand, labeled actin molecules bound to the free ends of thin filaments seemed to be dissociated by light irradiation at high intensity: the density, after being increased due to the binding of labeled actin under a phase-contrast microscope, decreased after the fluorescence microscope observations accompanied by photobleaching (photographs not shown). This phenomenon is related to the absorption of light by the fluorescent dye bound to actin, because unlabeled actin bound to the free ends of thin filaments did not dissociate. These phenomena may represent a kind of photodynamic effect (e.g. reference 24).

DISCUSSION

The length of thin filaments of rabbit skeletal muscle seems to be strictly determined. In contrast, the length distribution of thin filaments reconstructed in vitro, where a dynamic binding equilibrium exists between the filaments and free actin molecules, is very broad (13, 21, 22). This suggests that there is a mechanism for size determination in vivo.

If the length of thin filaments is determined and maintained in a steady state, for example, by a simple treadmilling mechanism as observed in vitro (35), a flow of proteins should occur through association at one end and dissociation at the other end of a filament, no matter how slow the flow rate is. In this case, it is expected that even if the free ends are dissociation ends, actin can bind to the free ends under the condition that the actin concentration is higher than the critical concentration for polymerization. However, we found that this was not the case (cf. Figs. 1 and 2). Actin could bind to the free ends of thin filaments only after the treatment at high ionic strength. From these results, we inferred that some particular structure is present at the free ends of thin filaments and a simple treadmilling process is inhibited.

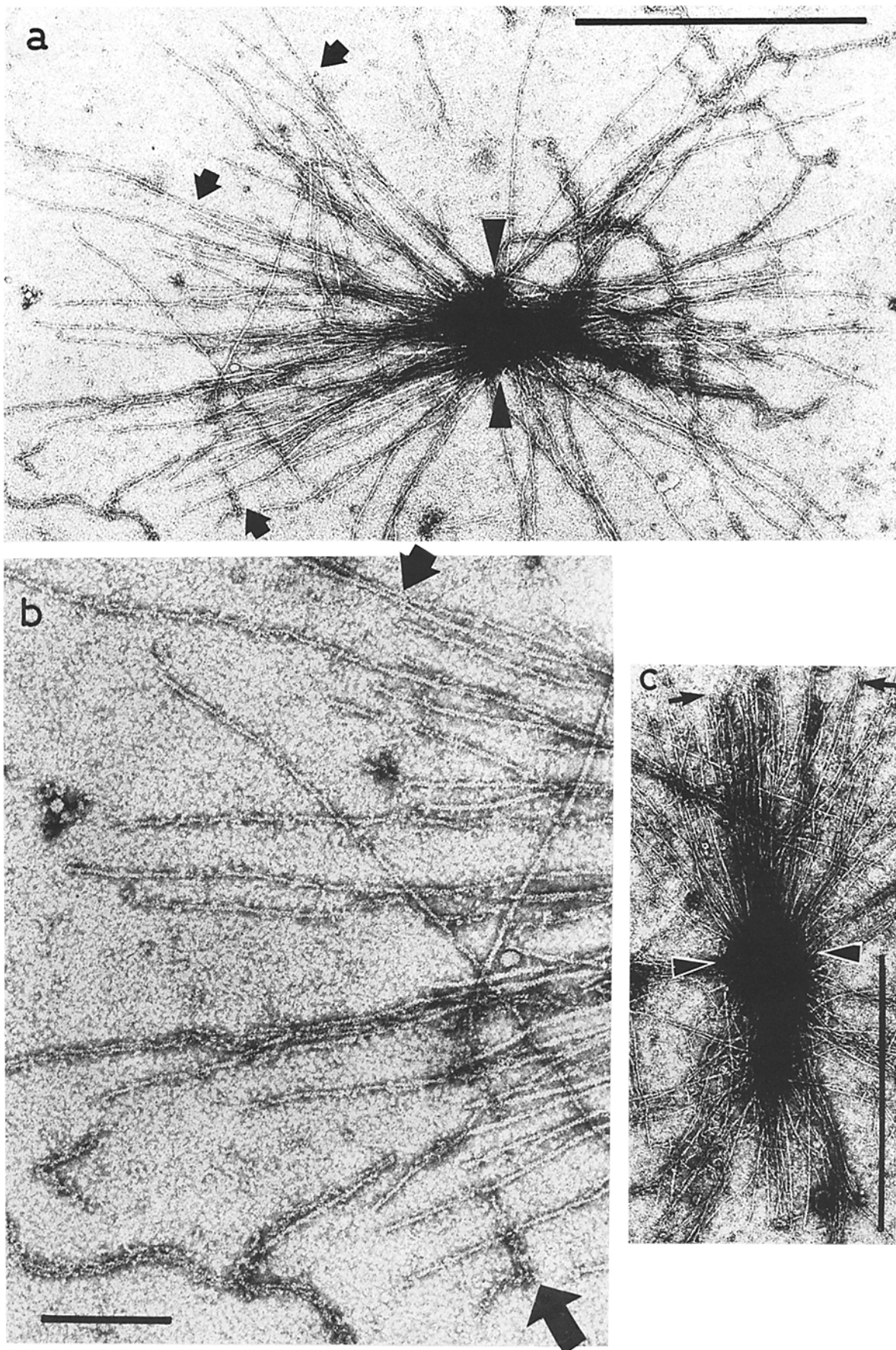The end structure of thin filaments is not open but closed

FIGURE 6   Electron micrographs showing the binding of actin to an I-Z-I brush. The binding of actin was performed on a grid. (a and b, which is an enlargement of the left part of a) Before the addition of actin, an I-Z-I brush was washed with a high salt solution; (c) the I-Z-I brush without washing with a high salt solution. (Thick filaments are seen on the micrograph.) For other conditions, see Materials and Methods. The arrows indicate the positions at which the free ends of thin filaments are located, i.e., 1 μm from the Z line. The position of the Z line is indicated by arrowheads. Bars, (a and c) 1.0 μm; (b) 0.2 μm. × 50,000 (a); × 22,000 (b); × 48,000 (c).

for the additional binding of actin molecules, which is different from the case of reconstituted actin filaments. There are several possibilities regarding this termination mechanism: some special terminator proteins may be present at the free ends of thin filaments or an actin molecule at the end may be chemically modified. It is inferred that at high ionic strength, a part of the end structure population of thin filaments is eliminated or modified accompanying the dissociation of thick filaments. If the end structure is eliminated at high ionic strength, it may be possible to find terminator proteins in the same fraction as dissociated myosin molecules.
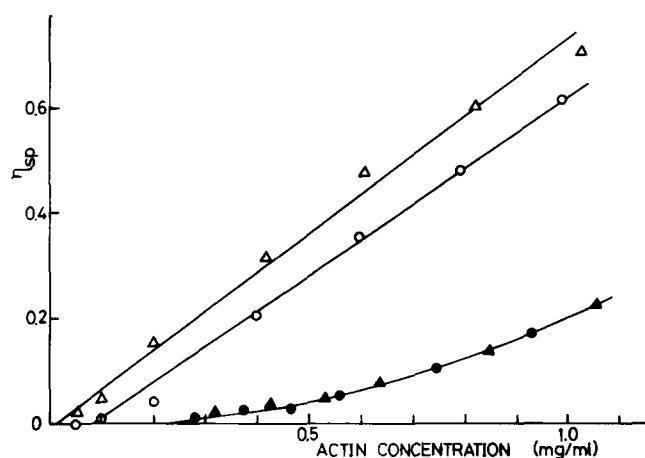
FIGURE 7 Specific viscosity (η_{sp}) of F-actin versus actin concentration in the presence and absence of ATP. Triangles and circles represent unlabeled and labeled actins, respectively. ($\triangle$, $\bigcirc$) 20 mM KCl, 5 mM Tris-HCl buffer (pH 6.8), 0.1 mM $CaCl_2$, and 0.1 mM ATP. ($\blacktriangle$, $\bullet$) Excess ATP was removed from G-actin solution by Dowex treatment and the relationship was examined in the above solvent without ATP. Viscosity was measured ~12 h after the addition of salt at 20 ± 0.5°C.

In any case, the binding of actin to the free ends thus becomes possible (Figs. 3–6, 8, and 9). The elimination or modification of the end structure is thought to occur statistically in an all-or-none fashion, judging from the fact that there are clearly two classes of thin filaments in the same I-Z-I brush (Fig. 6): with or without bound actin filaments. On the other hand, β-actinin is considered to be a strong candidate for the terminator (17). A preliminary study showed that β-actinin obtained by KI extraction according to Maruyama et al. (17) suppressed the binding of actin to the free ends of thin filaments in I-Z-I brushes; however, suppression was not complete under our experimental conditions (T. Funatsu and S. Ishiwata, in preparation). More detailed studies will be required to determine what the termination mechanism is.

How does the closed-end structure of thin filaments relate to the length determination of the filaments? We can not clearly answer this question at present. As a length determination mechanism for thin filaments, a so-called vernier mechanism has been proposed (10), where, for example, the difference between the length of tropomyosin molecules and the helical pitch of an actin filament functions as a vernier. In our system presented here, there is a possibility that some of the tropomyosin-troponin complexes that act as a kind of terminator in the vernier mechanism were dissociated together with thick filaments at high ionic strength (although the amount of dissociated tropomyosin-troponin complex was very small as judged by SDS gel electrophoresis). Therefore, we examined whether the addition of tropomyosin-troponin complexes or tropomyosin to I-Z-I brushes suppresses the binding of actin to the free ends of thin filaments. The results (data not shown) showed that the binding of actin to the free ends of thin filaments was somewhat delayed by the addition of tropomyosin-troponin complexes or tropomyosin but not suppressed under the same conditions as used above. Therefore, the length of thin filaments in skeletal muscle may not be determined by a simple vernier mechanism. However, this
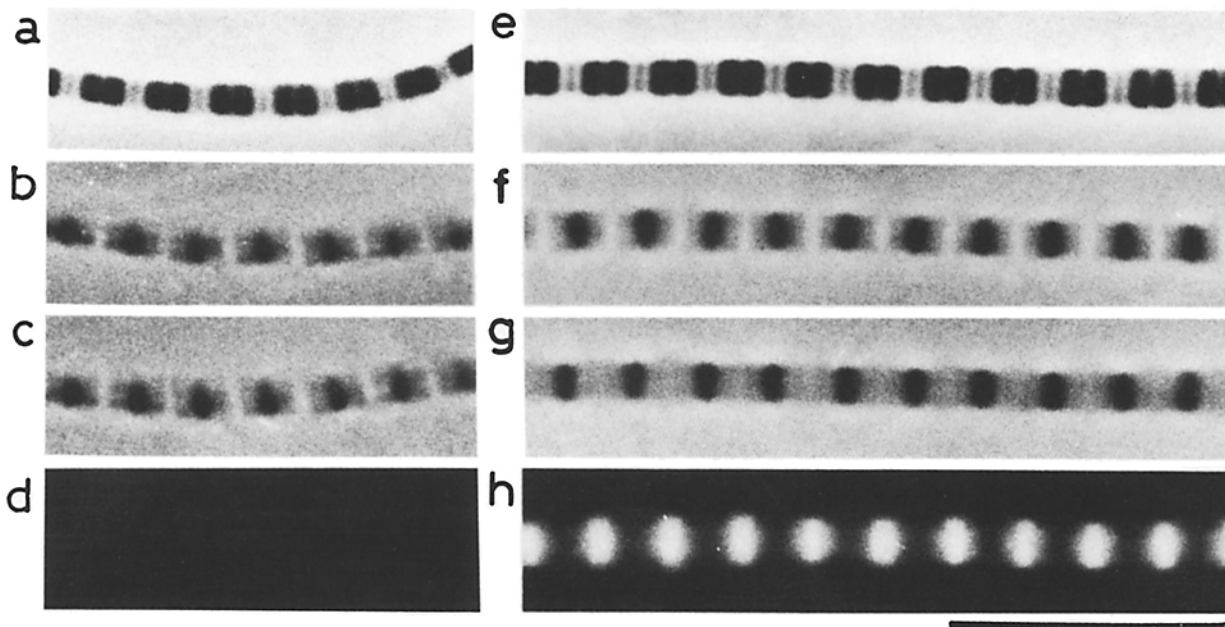
FIGURE 8 Effect of protein concentration on the binding ability of actin to thin filaments in the presence of ATP. (a) Intact myofibril; (b) I-Z-I brushes; (c and d) after the addition of 0.1 mg/ml of labeled actin; (e) intact myofibril; (f) I-Z-I brushes; (g and h) after the addition of 0.5 mg/ml of labeled actin. Solvent composition: the same as in Fig. 7 with ATP (O). All photographs were taken in the rigor solution (solution A). (a–c and e–g) Phase-contrast micrographs; (d and h) fluorescence micrographs. Bar, 10 μm. × 3,800.
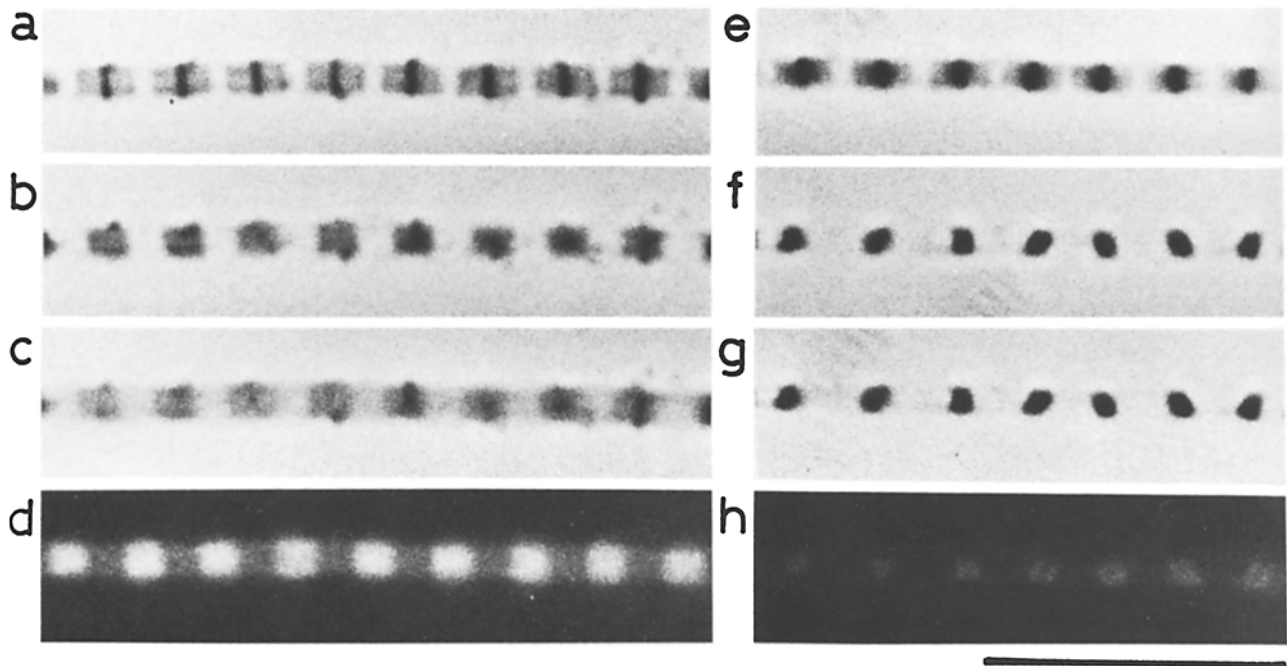
FIGURE 9   Effect of KI treatment on the binding ability of actin to thin filaments in I-Z-I brushes. (a) I-Z-I brushes; (b) after treatment with the rigor solution (solution A) containing 0.2 M KI instead of 60 mM KCl; (c and d) after the addition of 1.0 mg/ml of labeled actin; (e) I-Z-I brushes; (f) after treatment with solution A containing 0.3 M KI instead of 60 mM KCl; (g and h) after the addition of 1.0 mg/ml of labeled actin. Solvent composition: the same as in Fig. 7 with ATP (○). Photographs were taken in solution A. (a-c and e-g) Phase-contrast micrographs; (d and h) fluorescence micrographs. Bar, 10 μm. × 4,000.

does not necessarily mean that a vernier mechanism does not take part in the length determination. For example, a vernier mechanism may determine the location along a thin filament at which a terminator protein can bind. In this case, a terminator protein is directly involved in the length determination mechanism.

We estimated the rate of growth of actin filaments from the free ends of thin filaments in I-Z-I brushes to be 0.2–0.5 μm/min (Fig. 3). This value is comparable to that at the pointed ends in an actin filament bundle of intestinal microvillus core (23) or in an actin fragment decorated with heavy meromyosin (15, 32). There may be no essential difference between the structures of actin filaments in vivo and those of reconstituted actin filaments in vitro except for the presence of the end structure in the former.

The critical concentration for actin binding to the free ends, i.e., pointed ends, of thin filaments in the presence of ATP was higher than that for polymerization in solution (cf. Figs. 7 and 8). This was also the case in the absence of ATP (data not shown). These findings can be ascribed to the fact that the critical concentration at the pointed end is higher than that at the barbed end (14, 36). We inferred that the critical concentrations at pointed ends and barbed ends in solution are, respectively, higher and lower than 0.1 mg/ml, so that the apparent critical concentration obtained in solution becomes 0.1 mg/ml. If the above interpretation is correct, actin polymerization will be occurring in solution at the critical concentration for the free ends of thin filaments. This means that the binding of actin fragments to the free ends of thin filaments, i.e., end-to-end annealing, cannot be ignored (15). Therefore, the association rate estimated above, i.e., 0.5 μm/min, may be overestimated as a net rate of association of actin monomers. In connection with this, it is interesting that the binding efficiency of actin to the free ends of thin filaments

increases again after real polymerization equilibrium is attained (see the discussion of Fig. 3 in the text). A few hours after the addition of salt, the length distribution of actin filaments may be of a Poisson type, while finally (at equilibrium) it is of exponential type (21, 22). The number of short actin filaments that efficiently bind to the ends of thin filaments may be smaller in the Poisson type than in the exponential type distribution. That is, one reason why the binding ability to the free ends of thin filaments increases again on approaching polymerization equilibrium may be that the probability of end-to-end annealing between actin filaments in solution and thin filaments in an I-Z-I brush increases because of the change of length distribution of actin filaments.

When I-Z-I brushes were treated with 0.2 M KI solution, most of the end parts of thin filaments were probably dissociated. This would explain why large amounts of actin were bound to the free ends of such thin filaments (Fig. 9, a-d). However, binding of actin was not observed after overtreatment of the brushes with 0.6 M KI solution, probably because the structure that acts as a nucleus for actin polymerization is disorganized or destroyed. At the same time, this means that actin does not bind to the remaining network, which is composed of connectin, intermediate filaments, and other components (3, 18, 19, 33, 34).

Fig. 1 shows the binding of actin to the H zone in intact myofibrils in the absence of ATP. It appears that actin is bound to free myosin heads present in the H zone as a rigor complex. In the presence of ATP also, the binding of actin to thick filaments seems to occur in some particular condition (Fig. 2). In a relaxing condition, however, the binding of actin to thick filaments hardly occurs (data not shown). Whether or not the binding occurs may depend on the affinity between myosin and actin. The fact that actin filaments were protruded from the edge of the A band (Fig. 2) suggests that directional

growth of actin filaments occurs on thick filaments. The binding mode of actin and thick filaments might be analogous to that observed in the early phase of myofibril assembly (27) and in a reconstituted acto-myosin system (9).

It was recently reported that in some skeletal and heart muscles the length of thin filaments is not well determined and has a broad distribution (25, 31). In these muscles, association and dissociation between thin filaments and free actin monomers might be occurring in the same way as in an in vitro system. It would be interesting to examine whether or not actin binds to the free ends of thin filaments and whether the end structure of thin filaments is open or closed.

Belágyi et al. (2) added spin-labeled G-actin to glycerinated muscle fiber to measure the orientation of actin molecules incorporated. They concluded that incorporated actin molecules were mostly bound to thick filaments, in accordance with our observation (Figs. 1 and 2). If thick and thin filaments could be reconstructed from purified proteins that had been specifically labeled, studies on the interaction between myosin and actin in muscle fiber would progress considerably. In the case of thin filaments, labeled actin molecules could be incorporated into their free ends if specific removal of the end part of thin filaments is possible. At least, it is possible in a ghost fiber to incorporate labeled actin molecules into the free ends of thin filaments according to the procedure described here.

The results of the present study indicate that the free ends of thin filaments in skeletal muscle are terminated in some fashion that prevents the additional binding of actin molecules. A part of the end structure population may be eliminated or modified together with the dissolution of thick filaments under high ionic strength. As a result, the binding of actin to the free ends of thin filaments becomes possible. It is clear that myofibrils and I-Z-I brushes, either intact or partially disorganized, are attractive systems for studying the properties of actin binding to pointed and barbed ends of thin filaments and to other specific parts of the sarcomere.

REFERENCES

1. Asakura, S. 1961. The interaction between G-actin and ATP. *Arch. Biochem. Biophys.* 92:140–149.
2. Belágyi, J., G. Pallai, P. Gróf, and K. Trombitás. 1978. Interaction of heavy meromyosin and actin in myofibrils. A spin label study. *Stud. Biophys.* 73:159–164.
3. dos Remedios, C. G., and D. Gilmour. 1978. Is there a third type of filament in striated muscle? *J. Biochem. (Tokyo).* 84:235–238.
4. Ebashi, S., and K. Maruyama. 1965. Preparation and some properties of α-actinin-free actin. *J. Biochem. (Tokyo).* 58:20–26.
5. Etlinger, J. D., and D. A. Fischman. 1972. M and Z band components and the assembly of myofibrils. *Cold Spring Harbor Symp. Quant. Biol.* 37:511–522.
6. Granger, B. L., and E. Lazarides. 1978. The existence of an insoluble Z disc scaffold in chicken skeletal muscle. *Cell.* 15:1253–1268.
7. Hattori, A., and K. Takahashi. 1982. Calcium-induced weakening of skeletal muscle Z-disks. *J. Biochem. (Tokyo).* 92:381–390.
8. Hayashi, T., and W. Ip. 1976. Polymerization polarity of actin. *J. Mechanochem. Cell Motil.* 3:163–169.
9. Hayashi, T., R. B. Silver, W. Ip, M. L. Cayer, and D. S. Smith. 1977. Actin-myosin interaction. Self-assembly into a bipolar "contractile unit." *J. Mol. Biol.* 111:159–171.
10. Huxley, H. E., and W. Brown. 1967. The low-angle x-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor. *J. Mol. Biol.* 30:383–434.
11. Huxley, H. E., and J. Hanson. 1954. Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature (Lond.).* 173:973–976.
12. Ishiwata, S. 1981. Melting from both ends of an A-band in a myofibril. Observation with a phase-contrast microscope. *J. Biochem. (Tokyo).* 89:1647–1650.
13. Kawamura, M., and K. Maruyama. 1970. Electron microscopic particle length of F-actin polymerized *in vitro. J. Biochem. (Tokyo).* 67:437–457.
14. Kirschner, M. W. 1980. Implication of treadmilling for the stability and polarity of actin and tubulin polymers *in vivo. J. Cell Biol.* 86:330–334.
15. Kondo, H., and S. Ishiwata. 1976. Uni-directional growth of F-actin. *J. Biochem. (Tokyo).* 79:159–171.
16. Maéda, Y. 1978. Birefringence of oriented thin filaments in the I-bands of crab striated muscle and comparison with the flow birefringence of reconstituted thin filaments. *Eur. J. Biochem.* 90:113–121.
17. Maruyama, K., S. Kimura, T. Ishii, M. Kuroda, K. Ohashi, and S. Muramatsu. 1977. β-Actinin, a regulatory protein of muscle. Purification, characterization and function. *J. Biochem. (Tokyo).* 81:215–232.
18. Maruyama, K., S. Matsubara, R. Natori, Y. Nonomura, S. Kimura, K. Ohashi, F. Murakami, S. Handa, and G. Eguchi. 1977. Connectin, an elastic protein of muscle. Characterization and function. *J. Biochem. (Tokyo).* 82:317–337.
19. Maruyama, K., R. Natori, and Y. Nonomura. 1976. New elastic protein from muscle. *Nature (Lond.).* 262:58–60.
20. Ohtsuki, I. 1974. Localization of troponin in thin filament and tropomyosin paracrystal. *J. Biochem. (Tokyo).* 75:753–765.
21. Oosawa, F. 1970. Size distribution of protein polymers. *J. Theor. Biol.* 27:69–86.
22. Oosawa, F., and S. Asakura. 1975. Thermodynamics of the Polymerization of Protein. Academic Press, Inc., London and New York. 204 pp.
23. Pollard, T. D., and M. S. Mooseker. 1981. Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* 88:654–659.
24. Pooler, J. 1972. Photodynamic alteration of sodium currents in lobster axons. *J. Gen. Physiol.* 60:367–387.
25. Robinson, T. F., and S. Winegrad. 1977. Variation in thin filament length in heart muscle. *Nature (Lond.).* 267:74–75.
26. Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Analysis of myofibrillar structure and assembly using fluorescence labeled contractile proteins. *J. Cell Biol.* 98:825–833.
27. Shimada, Y., and T. Obinata. 1977. Polarity of actin filaments at the initial stage of myofibril assembly in myogenic cells *in vitro. J. Cell Biol.* 72:777–785.
28. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
29. Straub, F. B. 1942. Actin. *Studies Inst. Med. Chem. Univ. Szeged.* 2:3–15.
30. Taylor, D. L., and Y-L. Wang. 1980. Fluorescently labelled molecules as probes of the structure and function of living cells. *Nature (Lond.).* 284:405–410.
31. Traeger, L., and M. A. Goldstein. 1983. Thin filaments are not of uniform length in rat skeletal muscle. *J. Cell Biol.* 96:100–103.
32. Tsukita, S., S. Tsukita, and H. Ishikawa. 1984. Bidirectional polymerization of G-actin on the human erythrocyte membrane. *J. Cell Biol.* 98:1102–1109.
33. Wang, K., J. McClure, and A. Tu. 1979. Titin: major myofibrillar component of striated muscle. *Proc. Natl. Acad. Sci. USA.* 76:3698–3702.
34. Wang, K., and R. Ramirez-Mitchell. 1983. A network of transverse and longitudinal intermediate filaments is associated with sarcomeres of adult vertebrate skeletal muscle. *J. Cell Biol.* 96:562–570.
35. Wegner, A. 1976. Head to tail polymerization of actin. *J. Mol. Biol.* 108:139–150.
36. Wegner, A., and G. Isenberg. 1983. 12-Fold difference between the critical monomer concentrations of the two ends of actin filaments in physiological salt conditions. *Proc. Natl. Acad. Sci. USA.* 80:4922–4925.
37. Woodrum, D. T., S. A. Rich, and T. D. Pollard. 1975. Evidence for biased bidirectional polymerization of actin filaments using heavy meromyosin prepared by an improved method. *J. Cell. Biol.* 67:231–237.