Structural and Functional Reconstitution of Thin Filaments in the Contractile Apparatus of Cardiac Muscle

Hideaki Fujita*, Kenji Yasuda,# Shigehiko Niitsu,* Takashi Funatsu,§ and Shin'ichi Ishiwata*¶

*Department of Physics, School of Science and Engineering, Waseda University, Tokyo 169; #Advanced Research Laboratory, Hitachi, Hatoyama, Saitama 350-03; §Yanagida BioMotron Project, Exploratory Research for Advanced Technology, Research Development Corporation of Japan, Mino, Osaka 562; and ¶Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169, Japan

ABSTRACT The muscle contractile apparatus has a highly ordered liquid crystalline structure. The molecular mechanism underlying the formation of this apparatus remains, however, to be elucidated. Selective removal and reconstitution of the components are useful means of examining this mechanism. In addition, this approach is a powerful technique for examining the structure and function of a specific component of the contractile system. In this study we have achieved the structural and functional reconstitution of thin filaments in the cardiac contractile apparatus. First, all thin filaments other than short fragments at the Z line were removed by treatment with gelsolin. Under these conditions no active tension could be generated. By incorporating exogenous actin into these thin filament-free fibers, actin filaments were reconstituted, and active tension, which was insensitive to Ca^{2+}, was restored. The active tension after the reconstitution of thin filaments reached 135 ± 64% of the original level. The augmentation of tension was attributable to the elongation of reconstituted filaments. As another possibility for augmented tension generation, we suggest the presence of an inhibitory system that was not reconstituted. In any case, the thin filaments of the cardiac contractile apparatus are considered to be assembled so as not to develop the highest degree of tension. Incorporation of the tropomyosin-troponin complex fully restored Ca^{2+} sensitivity without affecting maximum tension. The present results indicate that a muscle contractile apparatus with a higher order structure and function can be constructed by the self-assembly of constituent proteins.

INTRODUCTION

Starting with the tobacco mosaic virus (Fraenkel-Conrat and Williams, 1955), it has been demonstrated that biological supramolecular structures, such as ribosomes (Nomura, 1974), flagella, actin filaments (Oosawa and Asakura, 1975), and so on, can be reconstituted in vitro from exogenous components according to self-assembly mechanisms. Thus a question arises as to whether a higher order biological structure, such as the muscle contractile apparatus, can also be constructed via a similar self-assembly mechanism. The muscle contractile apparatus is composed mainly of two distinct filaments (thick and thin filaments) and the elastic framework that maintains the lattice structure. Muscle contraction is based on mutual sliding between thick and thin filaments, which are arranged in liquid-crystalline arrays (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954).

Actin filaments can be reconstituted by spontaneous polymerization of monomers under appropriate conditions in vitro, but their length distribution is broad and of an exponential type at equilibrium (Oosawa and Asakura, 1975). Thin filaments, which have regulatory systems, can be reconstituted in vitro by incubating the reconstituted actin filaments with tropomyosin and troponin under appropriate conditions (Ishiwata, 1973; Ishiwata and Kondo, 1978). The duration of the incubation period required for reconstitution depends on temperature. Thick filaments can also be reconstituted from myosin molecules under appropriate conditions in vitro, and their length distribution and average length are similar to those in vivo (see review by Katsura and Noda, 1973). Because selective removal of thick filaments can easily be achieved by treatment with a high-salt solution (Huxley and Hanson, 1954), several attempts were made to reconstitute thick filaments using "ghost" fibers or fibrils (Tawada et al., 1976; Taniguchi and Ishikawa, 1982; Maw and Rawe, 1986). Unlike the case with thick filaments, it is difficult to remove thin filaments selectively only by controlling ionic conditions. Recently we succeeded in selectively removing thin filaments in skeletal and cardiac muscle fibers using calf plasma gelsolin, an actin filament-severing protein (Funatsu et al., 1990, 1993; Yasuda et al., 1995). We also succeeded in reconstituting thin filaments by using thin filament-free skeletal muscle fibers and fibrils, but the maximum tension of the reconstituted fibers generated was approximately 20% that of intact fibers (Funatsu et al., 1994). The number density of thin filaments in reconstituted skeletal muscle fibers, based on thin-section electron microscopy, was also about 20% that of intact fibers.

In this investigation we selectively removed and reconstituted thin filaments in the cardiac contractile apparatus. The removal and reconstitution of thin filaments were first examined functionally, according to the ability of the fibers to generate active tension. Reconstitution of regulatory proteins was examined by the pCa-tension relation of the reconstituted fibers. In addition, the removal and reconsti-

Received for publication 31 May 1996 and in final form 16 August 1996.

Address reprint requests to Dr. Shin'ichi Ishiwata, Department of Physics, School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169, Japan. Tel.: 81-3-5286-3437; Fax: 81-3-3200-2567; E-mail: ishiwata@cfi.waseda.ac.jp.

0006-3495/96/11/2307/12 $2.00

tution of thin filaments were examined structurally by laser scanning confocal microscopy after the thin filaments were selectively stained with a fluorescent dye. The reconstituted fibers were observed by thin-section electron microscopy to determine the length, number, and location of reconstituted thin filaments.

The technique and approach described herein are expected to be useful for examining, as an example, the characteristics of genetically altered actin, tropomyosin, and troponin, provided that the thin filaments can be reconstituted by employing these molecules. This technique should also prove useful for clarifying the molecular basis of muscle contraction and its regulation, by incorporating fluorescence-labeled or spin-labeled molecules into the reconstituted thin filaments.

MATERIALS AND METHODS

Muscle fibers and proteins

Bovine cardiac papillary muscle fibers (approximately 5 mm in diameter) or rabbit psoas muscle fibers (3–5 mm in diameter) were tied to a glass rod and incubated in glycerol solution containing 50% (v/v) glycerol, 0.5 mM $NaHCO_3$, 5 mM EGTA, and 2 mM leupeptin at 0°C overnight. Fibers were then stored in fresh glycerol solution at −20°C. Glycerinated fibers were used within 2–8 weeks after storage. Bovine plasma gelsolin was prepared according to the method of Kurokawa et al. (1990); during this preparation, we shortened the incubation time of gelsolin in Ca^{2+}-free buffer as much as possible because the severing activity appeared to be lowered by prolonged incubation in Ca^{2+}-free buffer. Actin was extracted from acetone powder (Kondo and Ishiwata, 1976) of bovine cardiac muscle according to the method of Spudich and Watt (1971). Purified G-actin was stored at 0°C and used within 2 weeks after extraction. The tropomyosin (Tm)-troponin (Tn) complex (nTm) was prepared from bovine cardiac muscle and from rabbit skeletal muscle according to the method of Ebashi and colleagues (1968).

Solutions

The solutions used were: rigor solution, 0.17 M KCl, 1 mM $MgCl_2$, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0), 1 mM EGTA; relaxing solution, 117 mM KCl, 5 mM $MgCl_2$, 4 mM ATP, 10 mM MOPS (pH 7.0), 1 mM EGTA, 20 mM 2,3-butanedione 2-monoxime (BDM); contracting solution, 117 mM KCl, 4.25 mM $MgCl_2$, 2.2 mM ATP, 1.9 mM $CaCl_2$, 20 mM MOPS (pH 7.0), 2 mM EGTA. EGTA and MOPS were purchased from Dojindo Laboratories (Kumamoto, Japan); dithiothreitol (DTT) was from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of reagent grade.

Selective removal of thin filaments

A glycerinated cardiac muscle fiber, a small bundle of several cardiac cells (~60 μm in width and ~2 mm in length), was stripped from the glycerinated muscle in a cold glycerol solution (Fukuda et al., 1996), and both ends were tied to a thin tungsten wire with enamel, one end of which was attached to a tension transducer (AE-801; SensoNor a.s, Horten, Norway). The volume of the measurement cell was approximately 200 μl. Fibers were chemically skinned with rigor solution containing 1% (v/v) Triton X-100 for 30 min. After a 15-min wash in rigor solution and then in relaxing solution, active tension was measured in contracting solution. Fibers were then immersed in contracting solution containing 20 mM BDM, 2 mM leupeptin, 2 mM diisopropyl fluorophosphate (DFP), and 0.3 mg/ml gelsolin at 0°C (gelsolin treatment). After a 10-min gelsolin treatment, the fibers were washed with relaxing solution and tension was measured to confirm that active tension was reduced upon the removal of thin filaments. The gelsolin treatment and tension measurement were then repeated. All procedures were carried out at 0 ± 2°C; tension was measured at room temperature (25 ± 2°C). Thus the thin-filament-free fibers used for reconstitution experiments were routinely prepared by continuous gelsolin treatment for 80 min at 0°C.

Reconstitution of thin filaments

To reconstitute actin filaments, the thin filament-free fibers were immersed in actin-polymerizing solution (80 mM KI, 4 mM $MgCl_2$, 4 mM ATP, 4 mM EGTA, 20 mM BDM, and 10 mM K-phosphate, pH 7.0) containing 1 mg/ml of purified G-actin that had been mixed just before use. The polymerizing solutions were newly prepared and exchanged every 7 min and kept at 0°C to prevent the effect of spontaneous formation of nuclei. To reconstitute actin filaments associated with regulatory proteins (i.e., thin filaments), the actin filament-reconstituted fibers were immersed in relaxing solution containing tropomyosin and troponin complexes (total protein concentration of 3 mg/ml) for 12 h at 0°C. The active tension of reconstituted fibers was measured at room temperature (25 ± 2°C, whereas the fibers were relaxed at 0°C).

Laser scanning confocal microscopy

Fibers were treated with 3% formaldehyde in relaxing solution for 30 min, for the purpose of chemical fixation, before and after the reconstitution of actin filaments and were mounted on a glass slide. Fibers were then treated with relaxing solution containing 8 μM rhodamine-phalloidin (RhPh) (Molecular Probes, Eugene, OR) overnight at 0°C, and then free RhPh was washed out with relaxing solution containing 100 mM DTT before microscopic observation. Preparations were observed by laser scanning confocal microscopy (LSM-GB200; Olympus Co., Tokyo). The light source was a 25-mW Ar laser of 488 nm. A 550-nm long-pass filter was used to detect the fluorescence of RhPh. A PlanApo 60 × 1.00 W LSM objective lens was used. The scanning time to take one picture was 40 s. The fluorescence intensity was recorded in a 1024 × 768 frame memory with an intensity range of 256 steps. The intensity profile was obtained by scanning along a line with a width of five pixels in the *x*-*y* plane; the depth of the *x*-*y* plane was changed along the *z* axis by moving the sample with a piezo stage.

Thin-section electron microscopy

The fibers at each step of reconstitution were tied to a platinum wire (0.3 mm in diameter) and chemically fixed in solution containing 2.5% glutaraldehyde and 100 mM sodium cacodylate (pH 7.2) overnight at 2°C. The fibers were then immersed in a solution containing 0.5% tannic acid for 30 min. After washing with 100 mM sodium cacodylate (pH 7.2), the preparations were postfixed with 1% OsO_4 in the same buffer for 2 h at 2°C, dehydrated with ethanol and acetone, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Thin sections were stained sequentially with saturated uranyl acetate and 2.6% lead citrate at 20°C. Electron micrographs were taken with a JEM 1200EX electron microscope (operating voltage, 100 kV; JEOL, Tokyo).

Sodium dodecyl sulfate gel electrophoresis

Cardiac muscle fibers (~60 μm thick, ~1 mm long) removed from the tungsten wires either before or after reconstitution were dissolved in lysis solution (7.5% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM DTT, and 10 mM Tris-HCl, pH 6.8) and heated for 3 min at 90°C. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970), with 13% polyacrylamide as the running gel and 5% polyacrylamide as the stacking gel. Protein was stained with silver (Silver Stain II Kit; Wako Pure Chemical Industries).

RESULTS

Removal and reconstitution of thin filaments

Fig. 1 schematically illustrates the filamentous structure of a sarcomere after the removal and reconstitution of thin filaments. Thin-filament-free fibers were prepared from glycerinated bovine cardiac muscle fibers (Fig. 1 *a*) by treatment with plasma gelsolin (Fig. 1 *b*). The fibers were then immersed in a solution containing purified G-actin under polymerization conditions. Actin monomers were po-

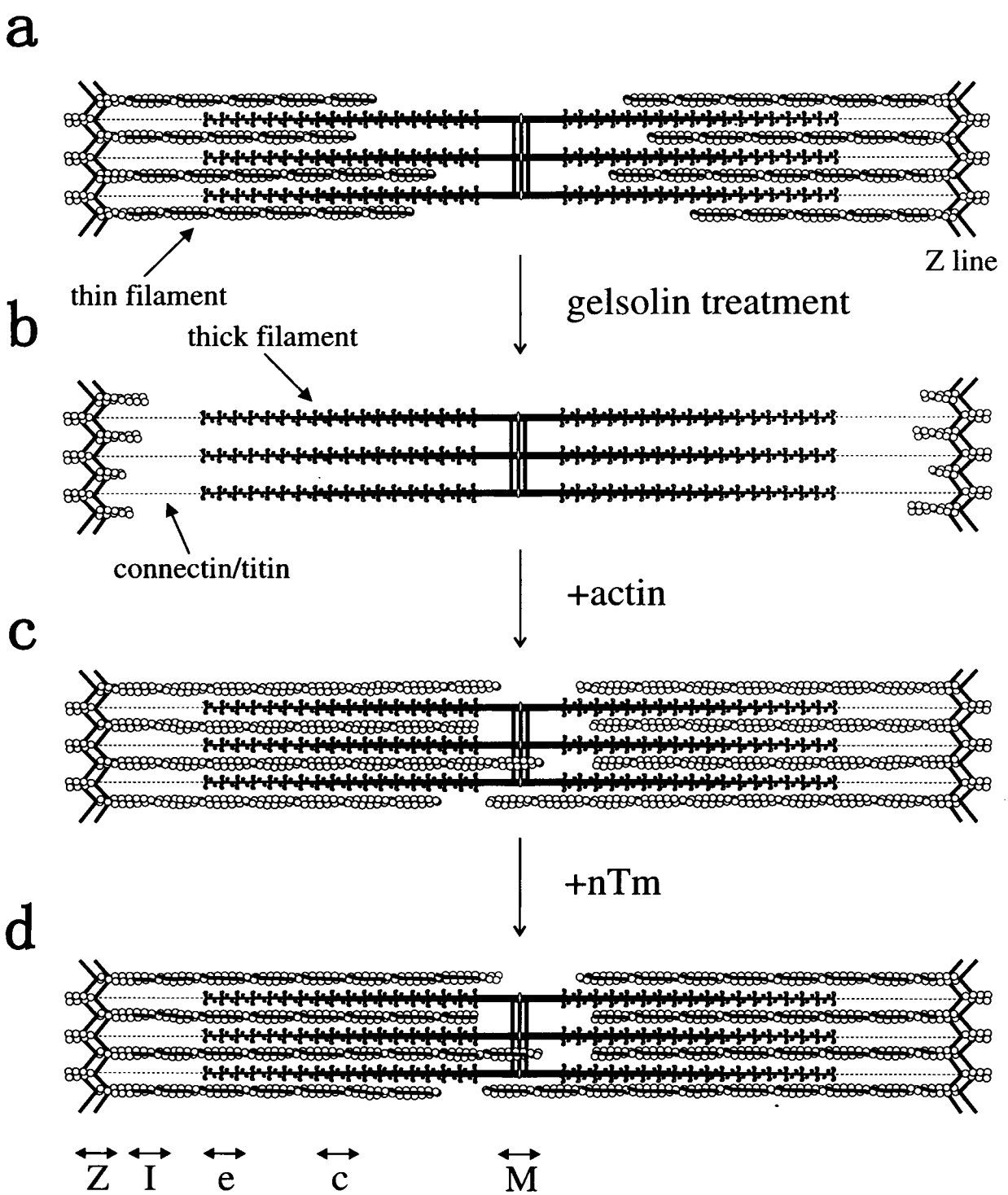

FIGURE 1 A schematic diagram illustrating the removal and reconstitution of thin filaments in a sarcomere. (*a*) Untreated cardiac sarcomere; (*b*) after gelsolin treatment; (*c*) after reconstitution of actin filaments; (*d*) after reconstitution of thin filaments with the addition of nTm. The double-headed arrows labeled Z, I, e, c, and M indicate the regions at which the numbers of thin and thick filaments were counted on electron micrographs (cf. Figs. 4 and 5).

lymerized onto the actin fragments remaining at the Z line (Fig. 1 *c*). To minimize the effects of spontaneous nucleation of actin outside sarcomeres, the fresh actin solution was exchanged every 7 min at 0°C, and a low concentration of KI was used instead of KCl because KI is known to delay nucleus formation (Funatsu et al., 1994). To reconstitute thin filaments, the fibers were immersed in relaxing solution containing tropomyosin-troponin complexes (nTm), and Ca^{2+} sensitivity was measured (Fig. 1 *d*).

The fibers were observed under a laser scanning confocal microscope after staining of the actin filaments with fluorescent RhPh before (Fig. 2 *a*) and after (Fig. 2 *b*) gelsolin treatment and after the reconstitution of thin filaments (Fig. 2 *c*). Fig. 2 *b* shows that all thin filaments, other than short fragments at the Z line, were removed by gelsolin treatment. Fig. 2 *c* shows that the thin filaments were reconstituted at the original position by the addition of exogenous actin. The fluorescence intensity distribution of the confocal images demonstrated the reconstitution of thin filaments around the I band, over the entire region of the fibers. The intensity profile of the thin-filament-reconstituted fiber resembles that of an intact fiber, indicating that the length distribution of thin filaments in reconstituted fibers is similar to that of intact fibers.

SDS-PAGE showed that actin was gradually removed during the course of gelsolin treatment (Fig. 3, lanes 1 and 2) and had essentially been eliminated by 80 min (Fig. 3, lane 3). The extent to which the regulatory proteins were removed together with actin was not clear because of the small quantity of muscle fibers, but the removal of TN-I was detectable; on the other hand, LC1, LC2 and the heavy chain of myosin were confirmed to have remained. In addition, we confirmed that the actin level had recovered after 42 min of reconstitution (7 min × 6) (Fig. 3, lane 4).

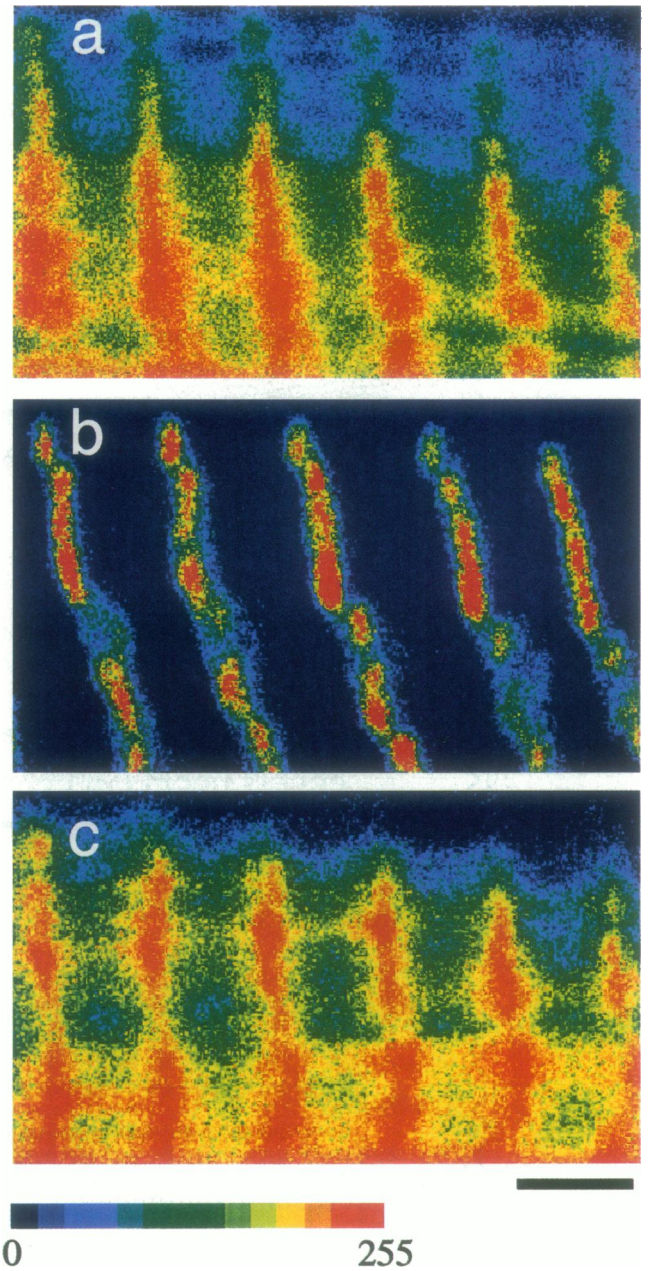

FIGURE 2 Confocal fluorescence images of cardiac muscle at each step in the reconstitution process. (*a*) Untreated cardiac muscle; (*b*) after gelsolin treatment; (*c*) after reconstitution of actin filaments. The images have been pseudocolored using a linear scale of fluorescence intensity of RhPh. The lines of highest intensity (red lines) correspond to the Z lines. Scale bar, 2 μm.

Internal structure of muscle fibers before and after reconstitution of thin filaments

To investigate the postreconstruction internal structure, fibers were observed by thin-section electron microscopy (EM) (Fig. 4). The EM images showed that almost all thin filaments had been removed from the fiber by the gelsolin treatment (Fig. 4 *b, b'*). EM images of longitudinal sections, after reconstitution, revealed the thin filaments to have been reconstituted from the Z line, and cross-sectional images revealed the reconstituted thin filaments to be located inside the hexagonal lattice of thick filaments.

The numbers of thick and thin filaments were counted from cross-sectional EM images. As summarized in Fig. 5, the number densities of thin filaments in untreated and reconstituted fibers were the same at the regions of the Z line (Z) and the I band (I), whereas those in reconstituted fibers were larger than those in untreated fibers at the A-I overlap region (e, c) and the M line (M). This indicates the elongation of thin filaments without a change in the number density of the filaments. The ratio of the numbers of thin and thick filaments at the center of the A-I overlap region (c) was 1.5 in untreated fibers but was increased to 2.0 by reconstitution. The number density of thick filaments in reconstituted fibers was low at the e region, probably because the edges of the thick filaments were misaligned due to slight tension development during reconstitution. The lattice constant (average distance between thick filaments assuming a hexagonal lattice) was decreased from 39 nm to 36 nm by the removal of thin filaments, but was restored to

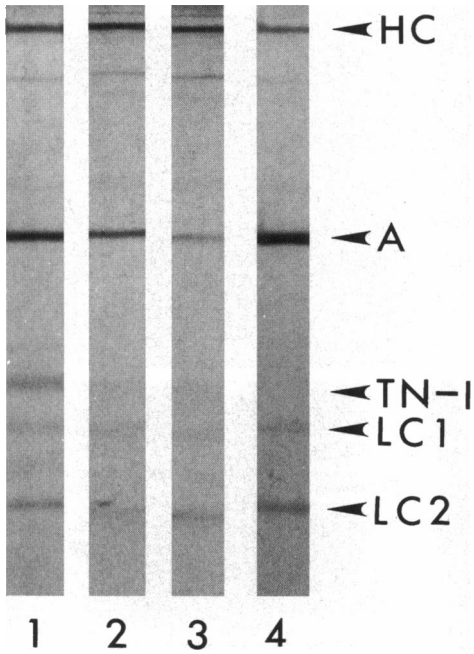

FIGURE 3 SDS-PAGE pattern of cardiac muscle fibers. Lane 1, control muscle fibers. Lanes 2 and 3, muscle fibers treated with 0.3 mg/ml of gelsolin in BDM-contracting solution for 30 min and 80 min, respectively. Lane 4, After gelsolin treatment, the muscle fiber was incubated with 1 mg/ml of actin in the process of polymerization in 80 mM KI, 4 mM $MgCl_2$, 4 mM ATP, 4 mM EGTA, 20 mM BDM, and 10 mM K-phosphate (pH 7.0) for 42 min (7 min × 6). Unbound actin molecules were washed out with relaxing solution containing 20 mM BDM. Arrowheads labeled HC, A, TN-I, LC1 and LC2 indicate the electrophoretic bands of myosin heavy chain, actin, troponin-I, and light chain 1 and light chain 2 of myosin, respectively.

39 nm by reconstitution, as it has been demonstrated in skeletal muscle (Funatsu et al., 1994).

Tension recovery of reconstituted muscle fibers

Active tension decreased exponentially with an increasing duration of gelsolin treatment and was completely absent at 80 min (Fig. 6). To minimize muscle fiber damage during gelsolin treatment, active tension development was suppressed by adding 20 mM BDM, an inhibitor of actin-myosin interactions (Li et al., 1985; Horiuti et al., 1988), and the temperature was kept at 0°C. We confirmed that the resting tension was not affected by gelsolin treatment, showing that connectin/titin remained intact.

During the course of actin filament reconstitution treatment, active tension recovered sigmoidally and reached a plateau after six incubations in actin polymerization solution (Fig. 7). Each tension measurement revealed a 5–10% decrease in tension (cf. Fig. 8 *a*, iii–v, vi, viii), such that the 60% recovery of tension achieved in this experiment suggests more than 100% recovery. To reach a plateau of tension recovery, 42 min (= 7 min × 6) of reconstitution treatment was needed. In the experiment shown in Fig. 7, approximately 7% of active tension persisted after the gelsolin treatment. However, because the magnitude of tension recovery after reconstitution was not affected by the residual tension persisting after the gelsolin treatment (data not shown), we consider the time course of tension recovery to be the same regardless of whether the tension in the initial fiber had been completely abolished by the gelsolin treatment. An example of the tension record at each reconstitution step is shown in Fig. 8 *a*. Active tension became undetectable after 80 min of gelsolin treatment (Fig. 8 *a*, ii). The tension, once abolished, was restored after 42 min (7 min × 6) of reconstitution with pure actin (Fig. 8 *a*, iii). The restored tension was 135 ± 64% (mean ± SD, n = 30) of the original level and ranged from 38% to 248%, as summarized in Fig. 8 *b*.

As a control, we confirmed that the tension in untreated fibers was not affected by consecutive treatments with contracting solution containing BDM without gelsolin and KI solution without actin (data not shown).

Reconstitution of thin filaments with tropomyosin and troponin

Actin filament-reconstituted fibers generated tension in a Ca^{2+}-insensitive manner because of the absence of nTm (Fig. 8 *a*, iii–v). Relaxation was obtained only by adding BDM. After incorporating nTm, however, the actin filament-reconstituted fibers recovered Ca^{2+} sensitivity (Fig. 8 *a*, vi–viii); the Ca^{2+}-insensitive tension decreased with incubation time in the nTm solution and had almost disappeared by 12 h (Fig. 9). The maximum tension in the presence of Ca^{2+} was not appreciably affected by the addition of nTm (compare Fig. 8 *a*, iii–v and vi, viii).

The thin-filament-reconstituted fibers fully regained the original pCa-tension relation (solid lines in Fig. 10). When nTm of skeletal muscle was used instead of cardiac nTm (thick dashed line in Fig. 10), fibers showed a pCa-tension relation resembling that of a skeletal muscle (thin dashed line in Fig. 10). The n_H and pCa_{50} values of the Hill equation were, respectively, 2.5 and 5.6 for cardiac nTm, but 3.5 and 5.9 for skeletal nTm.

DISCUSSION

Structural and functional reconstitution of actin filaments

In the present investigation, full reconstitution of actin filaments in the contractile apparatus was achieved for the first time. In a previous investigation, removal of thin filaments was performed in two steps, that is, the removal of thin filaments at the I band by gelsolin treatment under rigor conditions and then full removal of thin filaments (except the fragments at the Z line) by gelsolin treatment under contracting conditions, because severing of thin filaments at the overlapping region occurs under contracting conditions. Thus tension development during treatment should be minimized to suppress the damage of the sarcomere structure

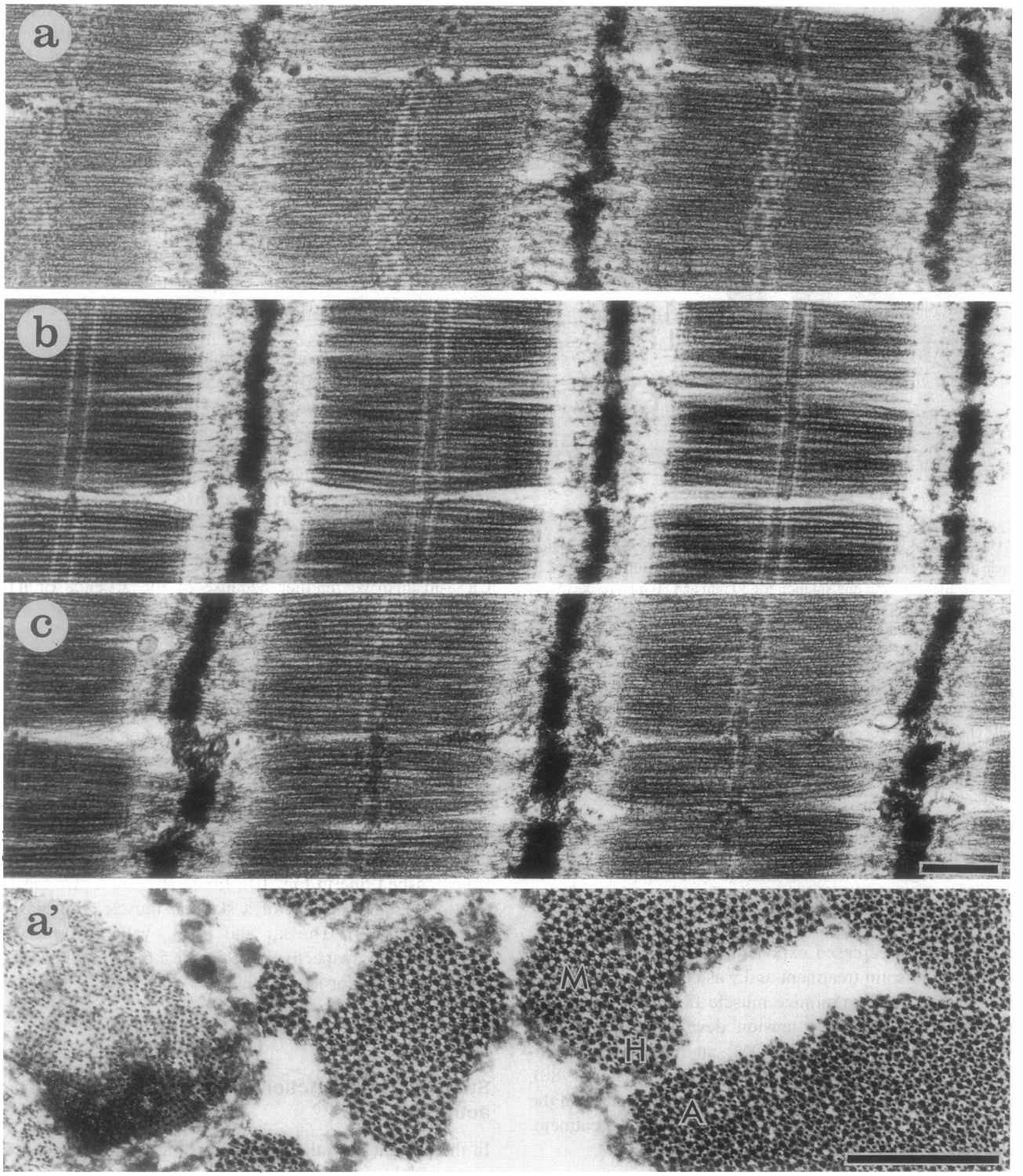

FIGURE 4 Thin-section EM images (a–c, longitudinal section; a'–c', cross section) of cardiac muscle at each step in the reconstitution process. (a and a') Untreated cardiac muscle. (b and b') After gelsolin treatment. (c and c') After reconstitution of thin filaments. M, H, and A, respectively, indicate the cross-sectional areas at the M line; at the pseudo H zone, where myosin heads are absent; and at the A band, where myosin heads are present. Scale bars, 0.5 μm.

(Funatsu et al., 1994). In this study, by adding an inhibitor of actin-myosin interactions, BDM, to the gelsolin solution, the removal of thin filaments was achieved in a single step. BDM was also included in the actin-polymerizing solution to suppress the damage of the sarcomere structure, due to contraction, as fully as possible. Observation by laser scan-

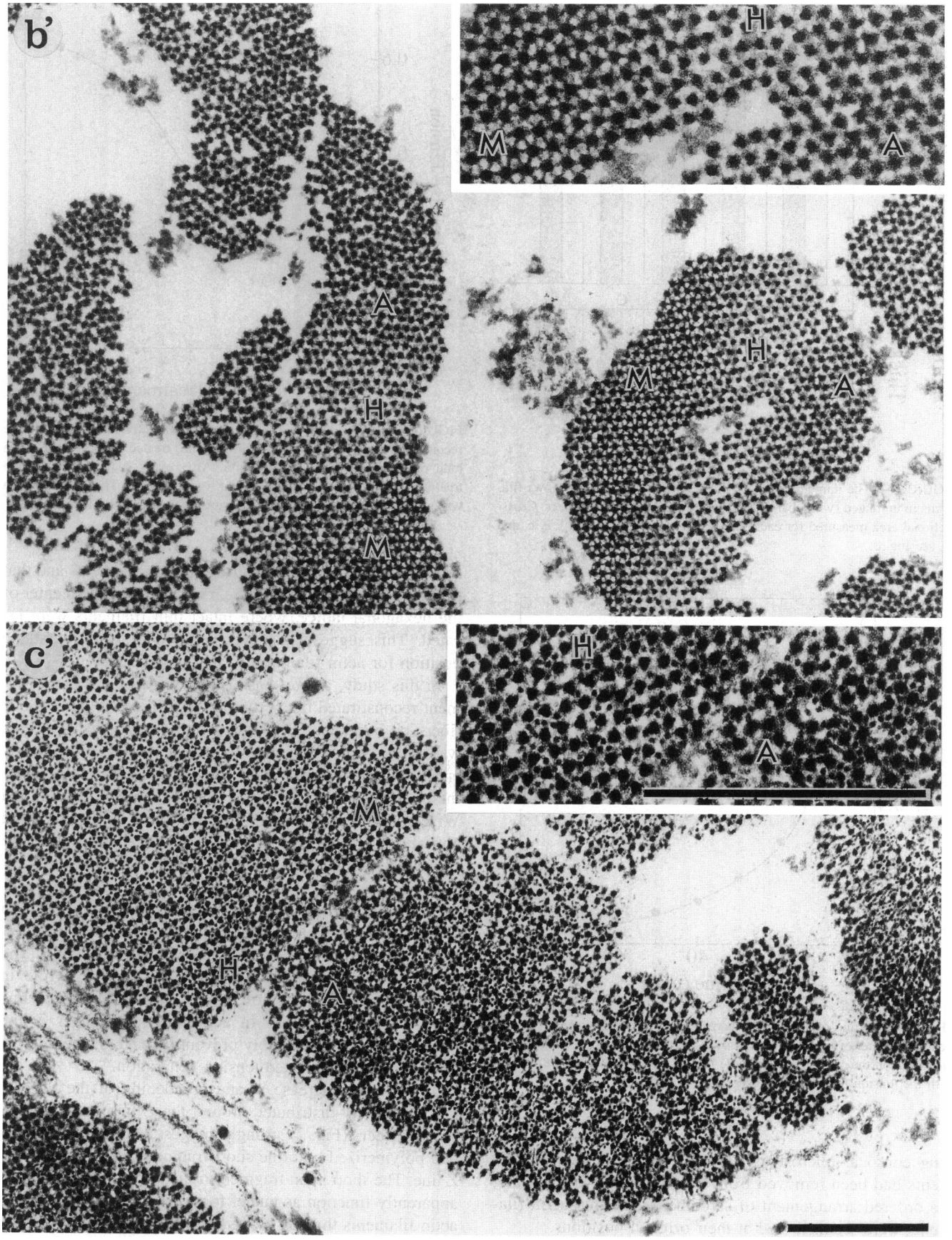

FIGURE 4 *Continued*

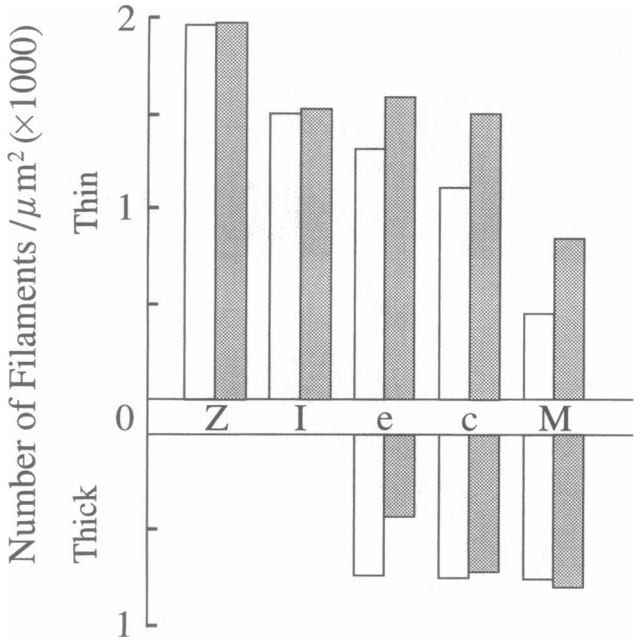

FIGURE 5 The number density of thin (above) and thick (below) filaments in untreated (white bars) and reconstituted (gray bars) fibers. Cross-sectional area measured for each region larger than 1 μm^2. Z, I, e, c, and M, see Fig. 1.

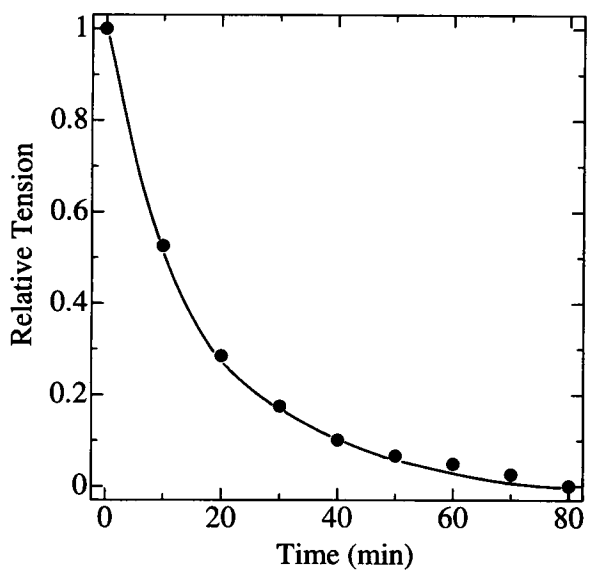

FIGURE 6 Time course of the decrease in active tension with gelsolin treatment. After every 10 min of gelsolin treatment, the active tension was measured on the same muscle bundle. The active tension was normalized with the tension measured before gelsolin treatment.

ning confocal microscopy (Fig. 2) showed that thin filaments had been removed from the fiber without disturbing the ordered arrangement of sarcomeres and that actin filaments were reconstituted at their original positions.

The cross-sectional image on thin-section electron microscopy revealed that actin filaments had been reconstituted inside the hexagonal lattice of thick filaments and that most of the thin filaments were reconstituted at the center of the hexagonal lattice, where intact thin filaments were located. This suggests that this position is the most stable position for actin filaments in the filament lattice.

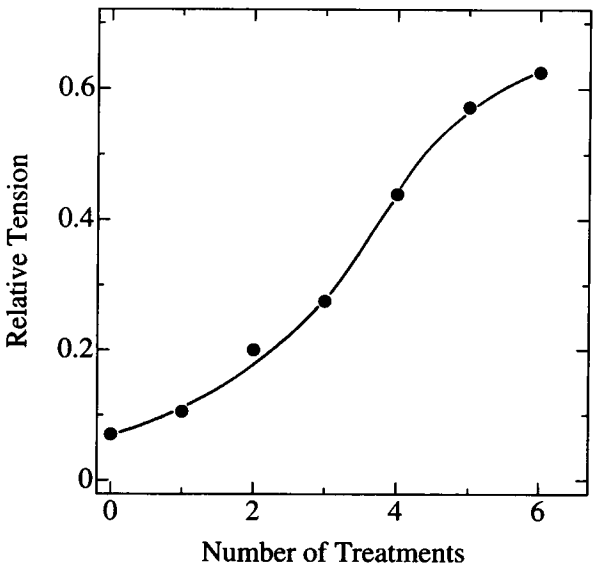

FIGURE 7 Time course of the recovery of active tension with the reconstitution of actin filaments. Fibers were treated with gelsolin for a total of 80 min before reconstitution. After every 7-min reconstitution treatment, the recovered active tension was measured. The active tension was normalized with the tension measured before gelsolin treatment.

In this study, active tension generated by the actin filament-reconstituted fibers reached 135% of that of the intact fibers on average. When reconstitution of actin filaments was performed in skeletal muscle, the active tension recovered was approximately 20% of the tension developed by intact fibers. Cardiac muscle lacks nebulin (Wang and Wright, 1988; Locker and Wild, 1986; Hu et al., 1986) and appears to have a thick, relatively strong, Z-line structure (Yamaguchi et al., 1985); these properties may account for the successful reconstitution of the cardiac contractile apparatus. The entanglement of nebulin at the N2 line, seen in gelsolin-treated skeletal muscle (Funatsu et al., 1990), is thought to disturb the polymerization of actin filaments. The stronger Z-line structure is speculated to play a role in maintaining the number of intact fragments at the Z line after gelsolin treatment. In addition, the stronger Z-line structure would presumably prevent damage of the internal structure due to repeated tension generation.

The gradual increase in and broadening of the fluorescence intensity distributed around the Z line in the reconstituted fibers (Fig. 2) strongly suggest that actin molecules had polymerized onto the short fragments remaining at the Z line. The short actin fragments remaining at the Z line can apparently function as nuclei for actin polymerization, and actin filaments thereby elongate from the Z line. This conclusion is consistent with the observation that tension recovery occurred sigmoidally with an increasing duration of

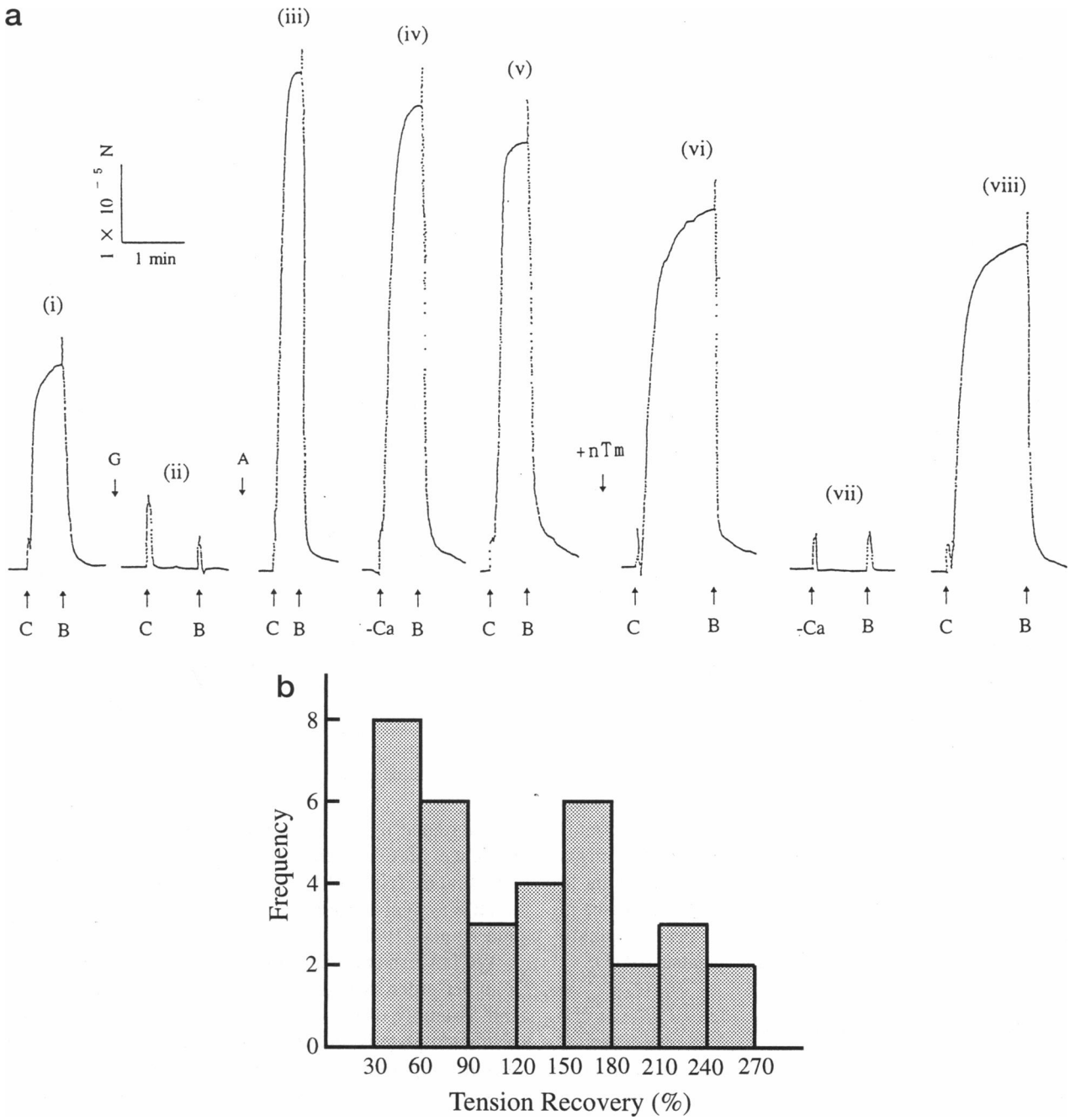

FIGURE 8 Recordings of tension generation at each step of thin filament reconstitution. (*a*) (i) untreated fiber; (ii) after gelsolin treatment; (iii–v) after reconstitution of actin filaments; (vi–viii) after reconstitution of thin filaments (+nTm). All records were taken sequentially from the same fiber. Temperature, 25°C for C, B, and −Ca; 0°C for G, A, and +nTm. Arrows indicate the time at which the solution was exchanged. Spikes observed in the records at each arrow are artifacts due to solution exchange. C, contracting solution; B, relaxing solution; -Ca, contracting solution without Ca^{2+}; G, gelsolin treatment; A, reconstitution of actin filaments; +nTm, addition of the cardiac tropomyosin-troponin complex. (*b*) Distribution of percentages of restored tension in the actin filament-reconstituted fibers. All data shown were obtained from fibers in which active tension had been completely abolished by gelsolin treatment.

actin filament reconstitution treatment (Fig. 7); in the initial lag phase, the reconstituted filaments were probably not long enough to contribute to tension recovery. If the reconstituted actin filaments had attached to connectin/titin, the fluorescent intensity at the I-band region where connectin/titin is exposed must have increased; but this was not the case (Fig. 2).

When actin molecules were directly added to untreated fibers under polymerizing conditions, neither elongation of actin filaments nor tension augmentation was observed

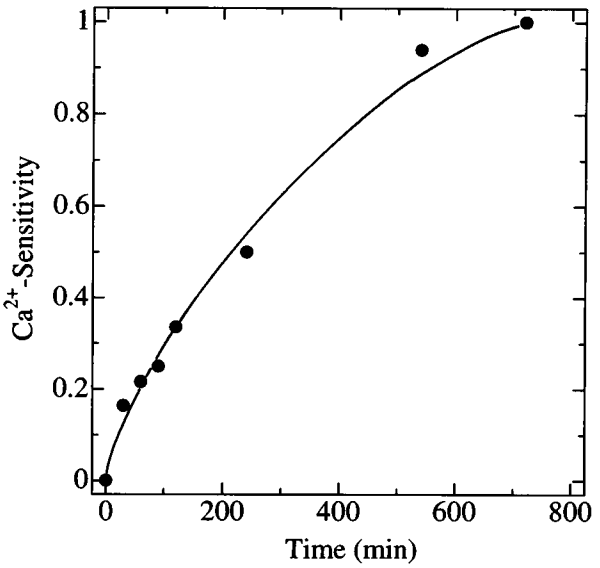

FIGURE 9 Time course of Ca^{2+} sensitivity recovery with incubation of actin filament-reconstituted fibers in relaxing solution containing 3 mg/ml cardiac nTm. Ca^{2+} sensitivity = 1 − (active tension without Ca^{2+})/(active tension with Ca^{2+}).

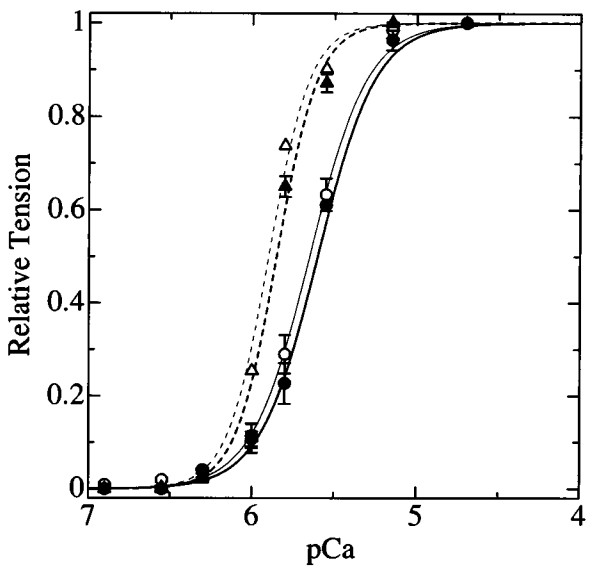

FIGURE 10 pCa-tension relation of intact and thin-filament-reconstituted fibers. ○, thin solid line, untreated cardiac muscle; △, thin dashed line, untreated skeletal muscle; ●, thick solid line, thin filament-reconstituted cardiac muscle fibers using cardiac nTm; ▲, thick dashed line, thin-filament-reconstituted cardiac muscle fibers using skeletal nTm. With the exception of untreated skeletal muscle, SDs of three data points are indicated by vertical bars. Solid and broken lines are fitted, respectively, for cardiac and skeletal nTm's by Hill equations with different Hill coefficients (n_H) and pCa values at half-maximum tension (pCa_{50}). For untreated cardiac muscle, n_H = 2.5, pCa_{50} = 5.6_5; for cardiac muscle reconstituted with cardiac nTm, n_H = 2.5, pCa_{50} = 5.6; for untreated skeletal muscle, n_H = 3.5, pCa_{50} = 5.9; and for cardiac muscle reconstituted with skeletal nTm, n_H = 3.5, pCa_{50} = 5.8_5.

(data not shown). This is attributable to the capping protein tropomodulin (Gregorio et al., 1995), which is located at the pointed ends of thin filaments. It has been reported that the removal of this capping protein results in the ability to bind G-actin to the pointed ends of thin filaments in skeletal muscle (Ishiwata and Funatsu, 1985) and in cardiac myocytes (Gregorio et al., 1995). The capacity of actin filaments to become nuclei for polymerization at their pointed ends does not appear to depend on the length of the fragments remaining at the Z line, because polymerization occurs to the same extent, even at both ends of the I-Z-I brush, in which filament length has been shown to be nearly equal to that of an intact filament (Ishiwata and Funatsu, 1985).

Reconstitution of thin filaments

We have shown that Ca^{2+} sensitivity can be restored only by adding tropomyosin-troponin complexes to the actin filament-reconstituted fibers. As shown in Fig. 10, the cooperativity (n_H) and Ca^{2+} sensitivity (pCa_{50}) of thin-filament-reconstituted fibers were nearly the same as those of intact fibers. The reconstitution of nTm was also confirmed by SDS-PAGE, although we could not estimate the quantity of reconstitution because of a small amount of protein. When nTm from skeletal muscle was used, on the other hand, reconstituted fibers showed a pCa-tension relation resembling that of skeletal muscle fibers. This indicates that the pCa-tension relation is determined only by the type of tropomyosin-troponin complexes used.

In this study, incubation for 12 h in nTm solution was needed to fully restore Ca^{2+} regulation (Fig. 9). This slow reconstitution is attributable mainly to the slow diffusion of nTm in the fiber (Kraft et al., 1995). The recovery time may be shortened by incubation at a higher temperature in the presence of Ca^{2+}, that is, by annealing treatment, at a temperature (e.g., 30°C) at which the functions of the contractile system are not damaged (Ishiwata, 1973; Ishiwata and Kondo, 1978).

We have shown that a muscle contractile apparatus with a higher order structure and function can be constructed by self-assembly of the constituent proteins when the nucleus or the template is located at an appropriate position. It was recently reported that actin filaments appear together with precursors of the Z-line structure in myofibrillogenesis (Rhee et al., 1994; cf. Fischman, 1972). Therefore, the self-assembly of thin filaments as revealed here may play a role in the late stage of I-Z-I brush formation. Furthermore, the splitting and growth of thin filaments may play a role in protein turnover after muscle cell maturation, especially in cardiac muscle, in which nebulin is absent (cf. Imanaka-Yoshida et al., 1993). This absence of nebulin lowers the stability of the thin-filament structure as compared to that of skeletal muscle, which does contain nebulin.

Tension augmentation of thin filament-reconstituted fibers

The present results demonstrate that active tension is not only restored but actually augmented by reconstitution of

actin (thin) filaments. In cardiac muscle, the length distribution of thin filaments is broad and the average length is shorter than that of skeletal muscle (Robinson and Winegrad, 1977; cf. Fig. 5). Therefore, the augmented tension restoration is attributable mainly to the greater length of reconstituted actin filaments; in the case shown in Fig. 5, the number of available cross-bridges is estimated to have increased by 140%, consistent with the degree of tension augmentation.

However, levels of augmentation as high as 250% as shown in Fig. 8 *a* may be at least partially mediated by other mechanisms, including 1) deterioration of tension generation in untreated fibers due to partial dissociation of thin filaments and/or partial removal of TN-C, 2) changes in sarcomere length, and/or 3) the presence of an inhibitory system in untreated fibers that was not restored in the present reconstitution treatment.

As for the first possibility, we confirmed that neither extensive thin-filament dissociation nor TN-C removal occurred during storage in glycerol solution at −20°C, because the tension of untreated glycerinated fibers was estimated to be 86 ± 3 kN/m^2 (mean ± SD, n = 3; the cross-sectional area was determined using confocal images), i.e., it exceeded the average values reported to date (Saeki et al., 1991; Allen and Kentish, 1985). In addition, marked tension augmentation was also observed when fresh fibers, which had been chemically skinned with 1% Triton X-100, were used (data not shown). The second potential mechanism would make a negligible contribution because fully activated tension is minimally affected by sarcomere lengths at around 2.0 μm in skinned cardiac muscle; we confirmed that the tension variation in untreated fibers was only 10% at most between 1.8 and 2.2 μm.

As for the third possibility, an inhibitory system, if one does exist, it must be associated with thin filaments, because other components were observed to be unchanged during the treatments. One possible candidate is nebulette, a recently identified member of the nebulin family. The molecular size of nebulette is, however, small, and it is located near the Z-line (Moncman and Wang, 1995). The cardiac contractile apparatus in vivo is probably specialized so as not to develop the maximum degree of tension.

Application of reconstituted fibers

As an application of this reconstituted system, we examined the effect of RhPh on the tension generation of actin filament-reconstituted fibers without nTm. Recent studies have made it possible to measure the force produced between a single myosin molecule and an actin filament using optical tweezers or a glass microneedle. The technique requires staining of actin filaments with fluorescent dye, usually Rh-Ph, but it is not yet clear whether an actin filament thus stained produces the same amount of force as an unstained filament. Our actin filament-reconstituted system can be applied to measuring both the force produced by an untreated actin filament and that produced after staining with Rh-Ph. Active tension of the actin filament-reconstituted fibers was unaffected by staining with 3.3 μM RhPh in relaxing solution for 2 h at 0°C (data not shown). This result is consistent with that obtained using glycerinated skeletal muscle fibers (Bukatina and Fuchs, 1994).

Finally, we would like to stress that our system is potentially useful for studying the structures and functions of mutant actin and regulatory proteins. Furthermore, reconstitution of thin filaments using fluorescent dye-labeled or spin-labeled proteins will make it possible to study the conformational changes in the constituent proteins of thin filaments accompanying muscle contraction and the molecular mechanisms by which these processes are regulated.

We thank Dr. H. Kanbara of the Central Research Laboratory, Hitachi, for the use of a confocal microscope; Dr. S. Umemura of Advanced Research Laboratory, Hitachi, for encouragement; and Drs. Y. Saeki of Tsurumi University and H. Higuchi of Exploratory Research for Advanced Technology for their critical reading of an early version of the manuscript.

H. Fujita is the recipient of a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists. This research was partly supported by Grants-in-Aid for Scientific Research (07680728 and 07558227 to SI) and for Scientific Research on Priority Areas (06213233 to SI) from the Ministry of Education, Science, Sports and Culture of Japan and the Uehara Memorial Foundation.

REFERENCES

Allen, D. G., and J. C. Kentish. 1985. The cellular basis of the length-tension relation in cardiac muscle. *J. Mol. Cell Cardiol.* 17:821–840.

Bukatina, A., and F. Fuchs. 1994. Effect of phalloidin on the ATPase activity of striated muscle myofibrils. *J. Muscle Res. Cell Motil.* 15: 29–36.

Ebashi, S., A. Kodama, and F. Ebashi. 1968. Troponin. I. Preparation and physiological function. *J. Biochem.* 64:465–477.

Fischman, D. A. 1972. Development of striated muscle. *In* The Structure and Function of Muscle, Vol. 1, 2nd Ed. G. H. Bourne, editor. Academic Press, New York. 75–148.

Fraenkel-Conrat, H., and R. C. Williams. 1955. Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc. Natl. Acad. Sci. USA.* 41:690–698.

Fukada, N., H. Fujita, T. Fujita, and S. Ishiwata. 1996. Spontaneous tension oscillation in skinned bovine cardiac muscle. *Pflügers Arch.* In press.

Funatsu, T., T. Anazawa, and S. Ishiwata. 1994. Structural and functional reconstitution of thin filaments in skeletal muscle. *J. Muscle Res. Cell Motil.* 15:158–171.

Funatsu, T., H. Higuchi, and S. Ishiwata. 1990. Elastic filaments in skeletal muscle revealed by selective removal of thin filaments with plasma gelsolin. *J. Cell Biol.* 110:53–62.

Funatsu, T., E. Kono, H. Higuchi, S. Kimura, S. Ishiwata, T. Yoshioka, K. Maruyama, and S. Tsukita. 1993. Elastic filaments in situ in cardiac muscle: deep-etch replica analysis in combination with selective removal of actin and myosin filaments. *J. Cell Biol.* 120:711–724.

Gregorio, C. C., A. Weber, M. Bondad, C. R. Pennise, and V. M. Fowler. 1995. Requirement of pointed-end capping by tropomodulin to maintain actin filament length in embryonic chick cardiac myocytes. *Nature (Lond.).* 377:83–86.

Horiuti, K., H. Higuchi, Y. Umazume, M. Konishi, O. Okazaki, and S. Kurihara. 1988. Mechanism of action of 2,3-butanedione 2-monoxime on contraction of frog skeletal muscle. *J. Muscle Res. Cell Motil.* 9:156–164.

Hu, D. H., S. Kimura, and K. Maruyama. 1986. Sodium dodecyl sulfate gel electrophoresis studies of connectin-like high molecular weight proteins

of various types of vertebrate and invertebrate muscles. *J. Biochem.* 99:1485–1492.

Huxley, A. F., and R. Niedergerke. 1954. Interference microscopy of living muscle fibres. *Nature (Lond.).* 173:971–973.

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.

Imanaka-Yoshida, K., J. M. Sanger, and J. W. Sanger. 1993. Contractile protein dynamics of myofibrils in paired adult rat cardiomyocytes. *Cell Motil. Cytoskeleton.* 26:301–312.

Ishiwata, S. 1973. A study on the F-actin-tropomyosin-troponin complex. I. Gel-filament transformation. *Biochim. Biophys. Acta.* 303:77–89.

Ishiwata, S., and T. Funatsu. 1985. Does actin bind to the ends of thin filaments in skeletal muscle? *J. Cell Biol.* 100:282–291.

Ishiwata, S., and H. Kondo. 1978. Studies on the F-actin·tropomyosin·troponin complex. II. Partial reconstitution of thin filament by F-actin, tropomyosin and the tropomyosin binding component of troponin (TN-T). *Biochim. Biophys. Acta.* 534:341–349.

Katsura, I., and H. Noda. 1973. Assembly of myosin molecules into the structure of thick filaments of muscle. *Adv. Biophys.* 5:177–202.

Kondo, H., and S. Ishiwata. 1976. Uni-directional growth of F-actin. *J. Biochem.* 79:159–171.

Kraft, T., M. Messerli, B. Rothen-Rutishauser, J.-C. Perriard, T. Wallimann, and B. Brenner. 1995. Equilibration and exchange of fluorescently labeled molecules in skinned skeletal muscle fibers visualized by confocal microscopy. *Biophys. J.* 69:1246–1258.

Kurokawa, H., W. Fujii, K. Ohmi, T. Sakurai, and Y. Nonomura. 1990. Simple and rapid purification of brevin. *Biochem. Biophys. Res. Commun.* 168:451–457.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Li, T., N. Sperelakis, R. E. Teneick, and J. Solaro. 1985. Effects of diacetyl monoxime on cardiac excitation-contraction coupling. *J. Pharmacol. Exp. Ther.* 232:688–695.

Locker, R. H., and D. J. C. Wild. 1986. A comparative study of high molecular weight proteins in various types of muscle across the animal kingdom. *J. Biochem.* 99:1473–1484.

Maw, M. C., and D. J. C. Rawe. 1986. The reconstitution of myosin filaments in rabbit psoas muscle from solubilized myosin. *J. Muscle Res. Cell Motil.* 7:1473–1484.

Moncman, C. L., and K. Wang. 1995. Nebulette: a 107 kD nebulin-like protein in cardiac muscle. *Cell Motil. Cytoskeleton.* 32:205–225.

Nomura, M. 1974. Assembly of bacterial ribosomes. *J. Supramol. Struct.* 2:163–165.

Oosawa, F., and S. Asakura. 1975. Thermodynamics of the Polymerization of Protein. Academic Press, New York.

Rhee, D., J. M. Sanger, and J. W. Sanger. 1994. The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskeleton.* 28:1–24.

Robinson, T. F., and S. Winegrad. 1977. Variation of thin filament length in heart muscle. *Nature (Lond.).* 267:74–75.

Saeki, Y., M. Kawai, and Y. Zhao. 1991. Comparison of crossbridge dynamics between intact and skinned myocardium from ferret right ventricles. *Circ. Res.* 68:772–781.

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.

Taniguchi, M., and H. Ishikawa. 1982. In situ reconstitution of myosin filaments within the myosin extracted myofibril in cultured skeletal muscle cells. *J. Cell Biol.* 92:324–332.

Tawada, K., A. Yoshida, and K. Morita. 1976. Myosin-free ghosts of single fibers and an attempt to re-form myosin filaments in the ghost fibers. *J. Biochem.* 80:121–127.

Wang, K., and J. Wright. 1988. Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microscopic evidence that suggests a set of parallel inextensible nebulin filaments anchored at the Z-line. *J. Cell Biol.* 107:2199–2212.

Yamaguchi, M., M. Izumimoto, R. M. Robson, and M. H. Stromer. 1985. Fine structure of wide and narrow vertebrate muscle Z-lines. *J. Mol. Biol.* 184:621–644.

Yasuda, K., T. Anazawa, and S. Ishiwata. 1995. Microscopic analysis of the elastic properties of nebulin in skeletal myofibrils. *Biophys. J.* 68:598–608.