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Localization and elasticity of connectin (titin) filaments in skinned frog muscle fibres subjected to partial depolymerization of thick filaments

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Summary

The localization and elasticity of connectin (titin) filaments in skinned fibres of frog skeletal muscle were examined for changes in the localization of connectin and in resting tension during partial depolymerization of thick filaments with a relaxing solution containing increased KCl concentrations. Immunoelectron microscopic studies revealed that deposites of antibodies against connectin at a sarcomere length of 3.0 μm remained at about 0.8 μm from the M-line, until the thick filament was depolymerized to the length of approximately 0.4 μm. On further depolymerization, the bound antibodies were found to move towards the Z-line and, on complete depolymerization, were observed to be within 0.3 μm of the Z-line; a marked decrease in resting tension accompanied this further depolymerization. These results suggest that connectin filament starts from the Z-line, extends to the M-line, and contributes to resting tension. After partial depolymerization of thick filaments, the distances between the anti-connectin deposits and the Z-line and between anti-connectin deposits and the M-line increased with sarcomere length, suggesting that connectin filaments are elastic along their entire length.

Introduction

Every striated muscle has an ordered structure. This ordered structure is maintained not only by thick and thin filaments but also by an elastic structure (Higuchi & Umazume, 1985). Maruyama and colleagues purified this elastic protein known as 'connectin' (also called 'titin') (Wang, 1985; Maruyama, 1986). The localization and elasticity of connectin in muscle fibres were examined by immunoelectron microscopy; deposits of anti-connectin were found to be located from the Z-line to near the M-line, and it was found that movement of the deposits in the I-band is correlated with sarcomere length (Maruyama *et al.*, 1985; Fürst *et al.*, 1988; Itoh *et al.*, 1988; Horowits *et al.*, 1989; Whiting *et al.*, 1989). Connectin filaments linking the ends of thick filaments to the Z-line were directly observed under electron microscopy, after the selective removal of thin filaments with plasma gelsolin (Funatsu *et al.*, 1990).

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The selective digestion of connectin results in a drop in resting tension, discontinuity of sarcomeres and swelling of the myofilament lattice (Yoshioka *et al.*, 1986; Higuchi, 1987; Funatsu *et al.*, 1990). Higuchi and Umazume (1985) showed that the resting tension in skinned fibres of frog skeletal muscle at sarcomere lengths above 3.5 μm was reduced with depolymerization of the A-band from its two ends. These observations suggest that the connectin filaments linking the overall length of the A-band to the Z-line are responsible for the passive elasticity and the maintenance of sarcomere structure.

Connectin filament extend from the Z-line into the A-band but the question remains whether they end at the M-line or somewhere else. Connectin filaments are elastic in the I-band (Fürst *et al.*, 1988; Itoh *et al.*, 1988; Horowits *et al.*, 1989; Funatsu *et al.*, 1990); however, it is not known whether the part of the connectin filament bound to the thick filament is elastic or not. In the present study, we addressed these questions by examining changes in the localization of anti-connectin, as well as resting tension, that accompany the depolymerization of thick

filaments in frog skinned fibres (Higuchi & Umazume, 1985). The results obtained in the present study indicate that the connectin filaments start from the Z-line, extend nearly up to the M-line, and have elasticity along their entire length.

Materials and methods

Fibre preparation

The fibre preparation and experimental procedure were essentially the same as described by Higuchi and Umazume (1985), with slight modifications. Mechanically-skinned fibres (Natori, 1954) were prepared in a skinning solution (90 mM KCl, 5.2 mM $MgCl_2$, 4.3 mM Na_2ATP, 4 mM EGTA, 10 mM piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES), pH 7.0, ionic strength of 0.15 M) from semitendinosus muscle just after bull frogs (*Rana catesbeiana*) were killed. Single skinned fibre segments, 100–150 μm in diameter and 3–5 mm long, were mounted horizontally in a chamber containing the relaxing solution, to hooks from a tension transducer and a micromanipulator. Most fibres were treated with a skinning solution containing 0.5% Brij-58 for 15 min to destroy the sarcoplasmic reticulum, and were subsequently immersed in a relaxing solution similar to the skinning solution, but at 0.14 M KCl. Sarcomere length was then adjusted employing the optical diffraction method. The depolymerization of thick filaments was initiated by exchanging the relaxing solution with that containing total concentrations of KCl of 0.225–0.54 M. Experiments were performed at 20° C.

Immunoelectron microscopy

Electron and immunoelectron microscopic studies were carried out as reported previously (Maruyama *et al.*, 1985; Higuchi *et al.*, 1988). Before and after the depolymerization of thick filaments, the single skinned fibres were fixed for 10 min in a relaxing solution containing 2% formaldehyde, or a relaxing solution containing concentrations of KCl > 0.29 M and 2% formaldehyde at about 20° C. Fibres were then washed in a phosphate solution containing 0.1 M Na-phosphate and 1 mM EGTA at pH 7.0. Both ends of fixed fibres were tied to platinum sticks, 0.3 mm in diameter and 10 mm long. For electron microscopy, tied fibres were fixed for 1 h each in a phosphate solution containing 2.5% glutaraldehyde and then 1% OsO_4. For immunoelectron microscopic study, tied fibres were treated for 24 h with antiserum and a monoclonal antibody 3B9, in the phosphate solution, raised against β-connectin from chicken breast muscle, followed by treatments with secondary antibodies for 24 h at 5° C. Specificity of the antibodies has been shown elsewhere (Maruyama *et al.*, 1985; Itoh *et al.*, 1988). The fibres were then fixed for 24 h with phosphate solution containing 2.5% glutaraldehyde at 5° C, and then 1% OsO_4 for 1 h at room temperature. Post-fixed fibres were dehydrated at room tmeperature and embedded in Epon 812.

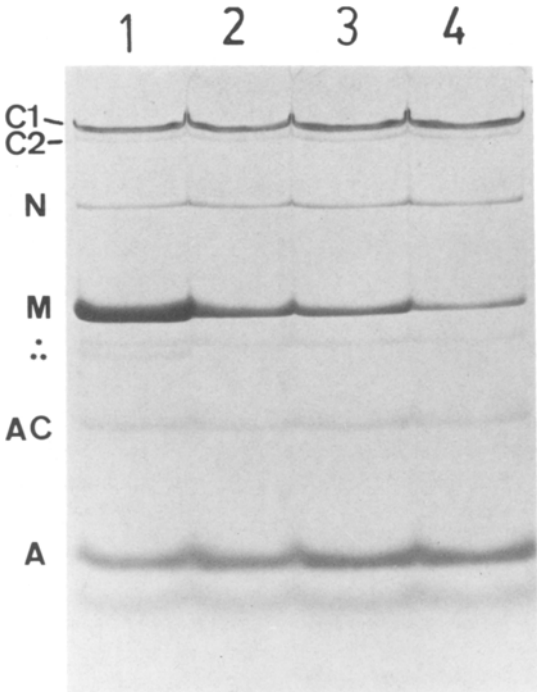

Fig. 1. SDS-PAGE (1–12% gradient gel) patterns of skinned fibres treated with high KCl solutions. Skinned fibres were treated for 10 min with 0.14 M (lane 1), 0.29 M (2), 0.34 M (3) and 0.54 M KCl (4). C1, α-connectin; C2, β-connectin; N, nebulin; M, myosin heavy chain; *, 170 kDa protein, **, 150 kDa protein; AC, α-actinin; A, actin.

Table 1. Relative amounts of proteins in skinned fibres treated with high KCl concentrations

	KCl concentration (M)			
Protein	0.14	0.29	0.34	0.54
α-connectin	0.52	0.48	0.49	0.40
β-connectin	0.040	0.042	0.048	0.032
Nebulin	0.115	0.104	0.100	0.099
Myosin heavy chain	2.98	0.97	0.71	0.25
170 kDa protein	0.051	0.051	0.048	0.036
150 kDa protein	0.056	0.004	0.003	0.000
α-actinin	0.122	0.100	0.107	0.087

Data are represented as the ratio to the amount of actin.

Immunogold staining was performed according to the procedure described by Itoh and colleagues (1988); the fibres, fixed with formaldehyde, were embedded and sectioned, and the thin sections were then treated with anti-connectin and with GAR-5 (Goat anti-rabbit IgG labelled with 5 nm colloidal gold, Jansen Pharmacentica, Holland).

Fig. 2. Electron micrographs of KCl-extracted skinned fibres. (A) Control fibre treated with 0.14 M KCl. Skinned fibres were treated for 10 min with the relaxing solution containing 0.29 M (B); 0.34 M (C); 0.39 M (D); 0.54 M (E); 0.29 M KCl (F) at an initial sarcomere length of 3.0 μm. (F) After treatment with 0.29 M KCl at a sarcomere length of 3.0 μm, the fibre was treated with 0.14 M KCl and slackened. Lower micrographs seen here are higher magnifications of upper ones. Triangles indicate the position of Z-lines. Bar 1 μm for high magnifications and 3 μm for low magnifications.

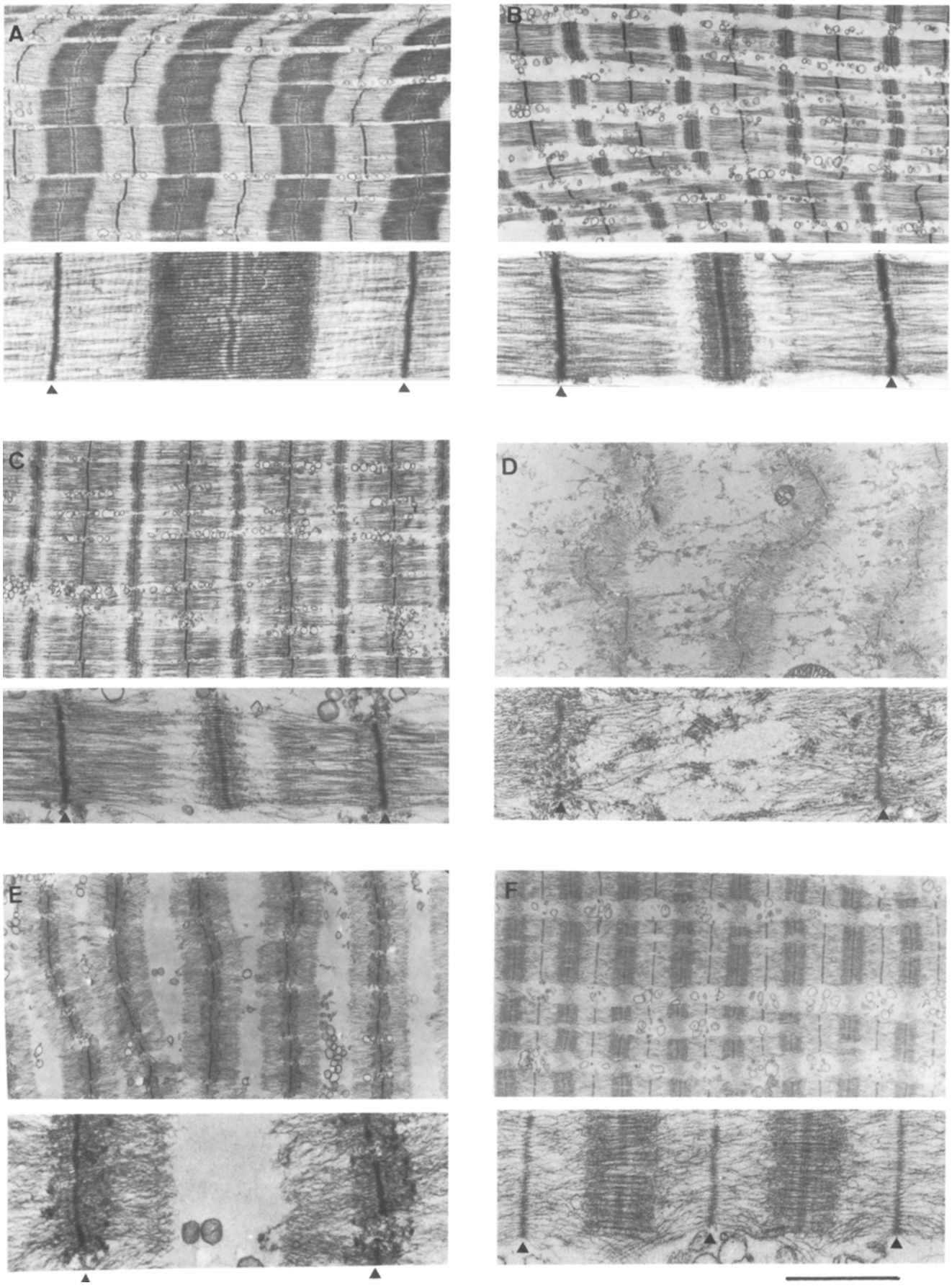

Fig. 2 A–F.

Thin sections of the embedded fibres were cut on the microtome and then stained with saturated uranyl acetate and 2.6% lead citrate. The stained sections were observed under a JEOL 1200EX electron microscope.

SDS-PAGE

Single skinned fibres, in which the thick filaments were depolymerized for 10 min with high KCl (0.29 to 0.54 M) relaxing solutions, were dissolved in 25 μl of a lysis solution (5.5% SDS, 10 mM Tris HCl, pH 6.8, 10% glycerol and 1 mM DTT). After samples were incubated for 1 min at 90° C, they were loaded on a 1–12% gradient polyacrylamide gel and electrophoresed (Fairbanks *et al.*, 1971). Proteins were stained with Coomassie Brilliant Blue. The absorbance at 560 nm of each band was measured with a chromatoscanner (CS-9000; Shimazu Co. Kyoto, Japan).

Results

Extraction of proteins from skinned fibres with high concentration of KCl

The selective depolymerization of thick filaments by high concentrations of KCl and their disappearance from skinned fibres were investigated employing SDS-PAGE (Fig. 1), and the main protein bands were analysed quantitatively using the chromatoscanner (Table 1). Because actin is not appreciably extracted with high KCl (Higuchi & Umazume, 1985), actin was used as a calibration standard against which the other protein bands were calibrated. Other components of thick filaments, such as myosin heavy chain and approximately 150 kDa protein presumably C-protein (Offer *et al.*, 1973), noticeably disappeared when the KCl concentration was increased above 0.29 M. The α and β-connectin (titin 1 and 2, respectively) and approximately 170 kDa protein, presumably M protein (Masaki & Takaiti, 1974), were not appreciably removed from skinned fibres at 0.29 and 0.34 M KCl; however, about 30% of the initial amount of these proteins was removed at 0.54 M KCl. About 20% of nebulin and α-actinin were removed at concentration of KCl > 0.29 M.

Electron microscopy

We observed, under electron microscopy, the structure of the sarcomeres of skinned fibres that had been exposed to elevated concentrations of KCl. Thick filaments depolymerized from both ends with almost equal velocity in all the sarcomeres in each fibre (Fig. 2B and C). The lengths of thick filaments, measured from electron micrographs of the fibres treated for 10 min at 0.29 M and 0.34 M KCl, were 0.42 ± 0.06 μm and 0.27 ± 0.05 μm, respectively (mean ± SD; 40–70 sarcomeres in four fibres were measured at a sarcomere length of 3.0 μm). At 0.39 M KCl (Fig. 2D), several thick filaments of about 0.2 μm length were scattered between the ends of thin filaments and in the I-band. At 0.54 M KCl (Fig. 2E), thick filaments were completely depolymerized.

The sarcomere lengths in the fibres were uniform at KCl concentrations below 0.34 M (Fig. 2A–C), but were disordered above 0.39 M KCl (Fig. 2D and E). Disordering was also observed in skinned fibres under a differential interference microscope, and was not prevented by fixing the fibres in formaldehyde. Therefore, the disorder was not produced artificially in the process of preparing samples for electron microscopy.

Longitudinally, fine filaments appeared in the region between the ends of thin filaments and partially-depolymerized thick filaments (Fig. 2B and C). The number of fine filaments was markedly reduced at 0.39 M KCl (Fig. 2D); at 0.54 M KCl fine filaments completely disappeared from the region between the ends of thin filaments (Fig. 2E). Instead, the region around 0.3 μm from the Z-line became dense (Fig. 2E).

Electron microscopy of slackened fibres after KCl extraction

The fine structure of fibres that had been slackened after the partial depolymerization of thick filaments was observed by electron microscopy. Slack sarcomere length, which was measured after slackening the fibre, decreased uniformly in the fibre to about 1.5 μm (Fig. 2F, low magnification) after depolymerization of thick filaments with 0.29 M KCl; the slack length before depolymerization was 2.1 μm. Sarcomere length, monitored by the laser diffraction method, did not change during fixation. Highly-magnified micrographs showed that the thin filaments overlapped at the centre of sarcomeres and appeared wavy.

Immunoelectron microscopy of KCl-extracted skinned fibres using polyclonal antibodies

The skinned fibres were treated with antiserum to connectin to examine the changes occurring in the localization of connectin filaments after partial depolymerization of thick filaments. Treatment with connectin antibodies resulted in the formation of three pairs of distinct stripes per sarcomere (Fig. 3A). The three stripes were located symmetrically to the M-line in each sarcomere. The distances between the stripes and the M-line were 0.68, 0.75 and 0.84 μm, respectively. In sarcomeres with partially depolymerized thick filaments, the position of the three stripes formed by antibodies barely changed (Fig. 3B). Connectin antibodies were also deposited between the ends of thick and thin filaments, in addition to the three stripes. Immunogold staining also showed the presence of anti-connectin antibodies at the ends of thick filaments and the region 0.4–0.9 μm from the M-line (Fig. 3C and D). Gold-labelled antibodies were not within 0.7 μm from the Z-line at 0.29 M KCl (Fig. 3C), but were scattered within 0.7 μm at 0.34 M KCl (Fig. 3D). The stripes formed by anti-connectin disappeared at 0.54 M KCl (Fig. 3E); instead, there were deposits of antibodies found within 0.3 μm from the Z-line.

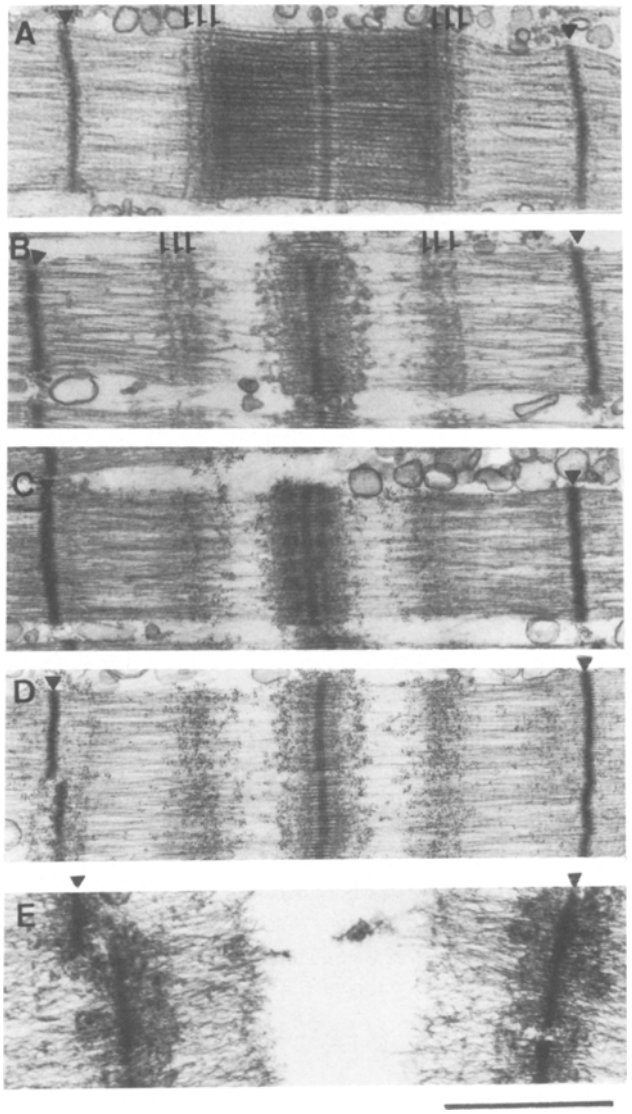

Fig. 3. Immunoelectron micrographs of skinned fibres treated with high KCl concentration. Skinned fibres were treated with connectin antiserum. (A) Control fibre at 0.14 M KCl; treated for 10 min with 0.29 M (B and C); 0.34 M (D); 0.54 M KCl (E). (C and D) Treated with anti-rabbit IgG labelled with 5 nm colloidal gold. Arrows show the stripes resulting from the binding of anti-connectin; triangles indicate the position of Z-lines. Bar = 1 μm.

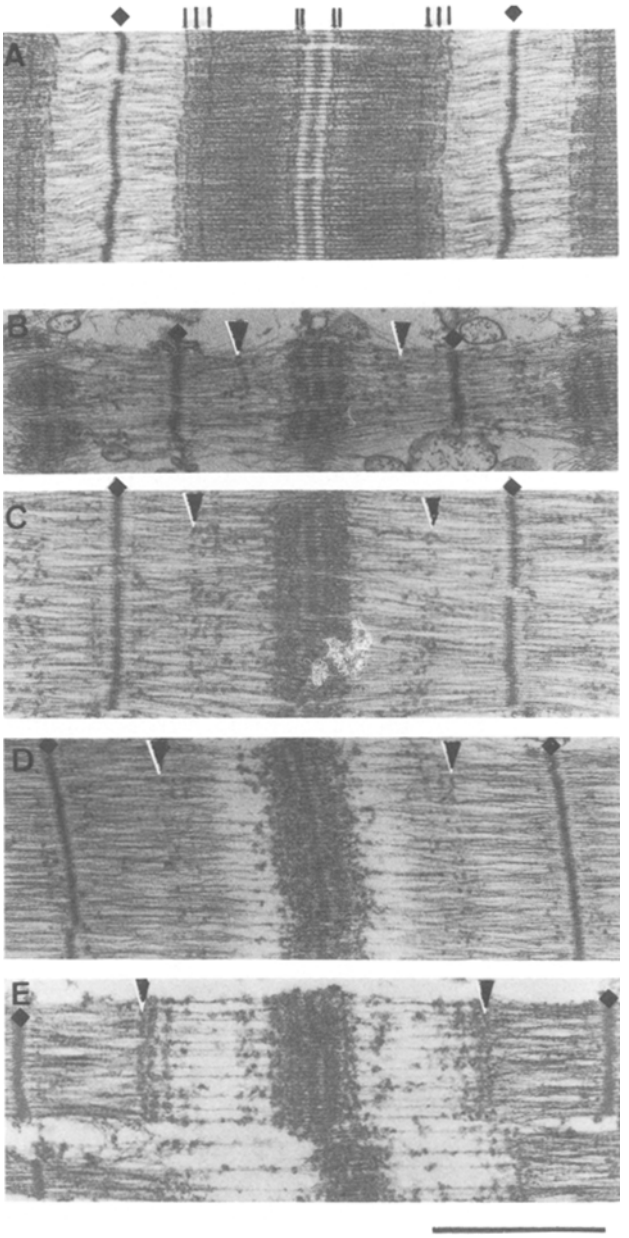

Fig. 4. Immunoelectron micrographs of KCl-extracted skinned fibres reacted with the monoclonal antibody 3B9. (A) Control fibre. Vertical bars show the stripes resulting from binding of an antibody. (B–E) Treated with 0.29 M KCl for 10 min at sarcomere length of 3.0 μm and then the sarcomere length changed. Arrowheads indicate the ends of stripes formed by antibodies. Diamonds indicate the position of the Z-lines. Bar = 1 μm.

Changes in the position of monoclonal antibody with sarcomere length in KCl-extracted fibres

The changes in binding positions of monoclonal anti-connectin were observed to examine the elasticity of connectin filaments after the partial depolymerization of thick filaments. We used the monoclonal antibody 3B9, which binds to epitopes in the A-band region and does not move with stretches of the fibre up to a sarcomere length of 3.5 μm (Itoh *et al.*, 1988). The antibody produced five pairs of decorated stripes per sarcomere (Fig. 4A): two pairs located near the M-line and three pairs on and near the ends of the thick filaments. The reason for the monoclonal antibodies forming several lines in half-sarcomeres would be that the connectin filament has several repeated domains of the same, or very similar, amino acid sequences (Itoh *et al.*, 1988; Fürst *et al.*, 1989; Labeit *et al.*, 1990). The distances between the stripes and the centre of the M-line were 0.09, 0.11, 0.63, 0.73 and 0.78 μm, respectively. After the thick filaments were partially depolymerized by treatment of the fibre for 10 min with 0.29 M KCl, the positions of the stripes,

which had been located on and near the ends of thick filaments, changed with the sarcomere length (Fig. 4B–E). The distances between the end of antibody stripes shown by the arrows in Fig. 4 and the centre of the M-line were 0.78 ± 0.03 (Fig. 4A), 0.50 ± 0.07 (B), 0.69 ± 0.05 (C), 0.84 ± 0.06 (D) and 1.01 ± 0.06 μm (E) at sarcomere length of 2.32 ± 0.07 (Fig. 4A), 1.61 ± 0.05 (B), 2.34 ± 0.06 (C), 2.96 ± 0.08 (D) and 3.47 ± 0.08 μm (E), respectively (mean ± SD; 7–10 sarcomeres were measured). The two pairs of antibody stripes near the M-line became unclear after partial depolymerization.

Connectin monoclonal antibody bound to the fine filaments which appeared between the thin filaments and partially depolymerized thick filaments (Fig. 4D and E). Figure 2C and D also showed that the polyclonal antibodies bound to the fine filaments. These results suggest that thick filaments mask the antibody-binding sites of connectin, and that the monoclonal and polyclonal antibodies bind to the part of the connectin filament which is released from the thick filament on depolymerization of the filament.

Changes in resting tension in skinned fibres with partially depolymerized thick filaments

We investigated changes in resting tension and the relation between resting tension and sarcomere length before and after partial depolymerization of thick filaments to estimate the elasticity of elastic filaments, that is, connectin. Part of the measuring procedure of the resting tension is shown in Fig. 5. The skinned fibres were stretched stepwise from slack sarcomere length of 2.1 μm to 3.0 μm (Fig. 5a) or directly to 3.0 μm (Fig. 5b). At

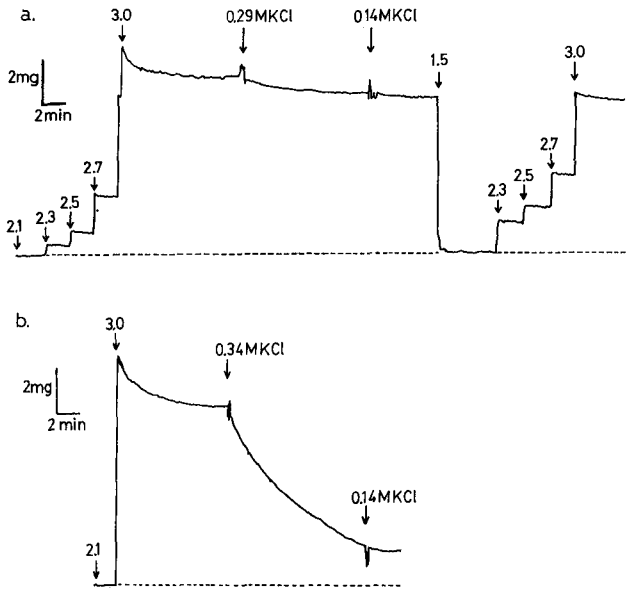

Fig. 5. Resting tension of skinned fibres before and after the partial depolymerization of thick filaments. Numbers = sarcomere lengths; broken line, zero tension level. Thick filaments were depolymerized by relaxing solutions containing 0.29 M KCl and 0.34 M KCl (b).

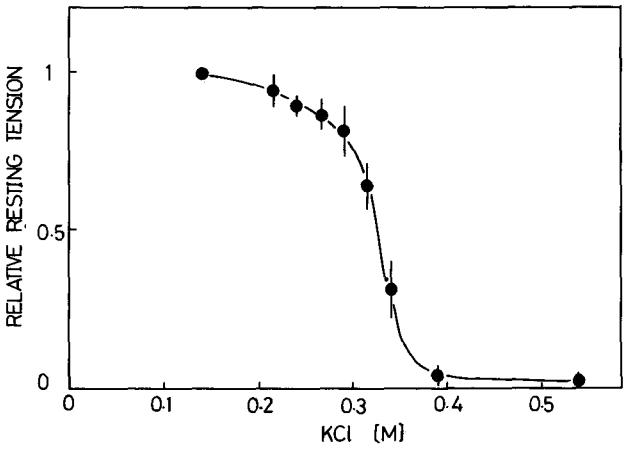

Fig. 6. Changes in the resting tension of skinned fibres at varied KCl concentrations. Skinned fibres were treated for 10 min with relaxing solutions containing 0.14–0.54 M KCl. The resting tension was normalized against that at 0.14 M KCl. Sarcomere lengths, 3.0 μm; mean values ± SD of three to four afibres are shown.

about 10 min after stretching, thick filaments were partially depolymerized at various KCl concentrations. The depolymerization of thick filaments was terminated by replacing the high KCl solution with relaxing solution. The fibre was then allowed to slacken for a few minutes (Fig. 5a), after which it was again stretched stepwise.

The resting tension of the fibres was slightly decreased by treatment for 10 min with < 0.29 M KCl; resting tension was markedly decreased above this concentration, reaching nearly zero above 0.39 M KCl (Fig. 6). Reduced

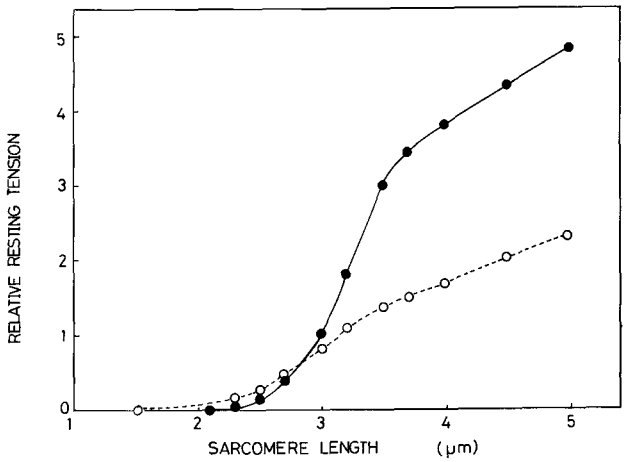

Fig. 7. Relation between sarcomere length and the resting tension of single skinned fibres. ●, measured in relaxing solution before depolymerization of thick filaments, ○, measured in relaxing solution for 10 min after depolymerization at 0.29 M KCl. Each symbol is the means of four fibres. Resting tension was normalized against that at sarcomere length of 3.0 μm before depolymerization. The resting tension at a sarcomere length of 3.0 μm was 8.3 ± 1.7 × 10^3 Nm^{-2} (mean ± SD of four fibres). The cross-sectional area was calculated from the two diameters, assuming that the cross-sectional shape fo the fibre was elliptical.

resting tension barely changed when depolymerization of thick filaments was terminated by reincubation in relaxing solution (Fig. 5). Slack sarcomere length decreased from 2.1 μm before depolymerization of thick filaments to 1.5 μm after partial depolymerization (Figs 5 and 7). Resting tension after partial depolymerization increased at sarcomere lengths below 2.7 μm and decreased at longer sarcomere lengths (Figs 5 and 7). Resting tension at sarcomere lengths above 3.5 μm was about 45% of that before depolymerization.

We further examined whether changes occurring in resting tension and slack length are induced by interaction of actomyosin. Resting tension and slack length before and after partial depolymerization of thick filaments scarcely depended on temperature (0–20° C) or KCl concentration (0.09–0.14 M KCl before depolymerization and 0.14–0.24 M KCl after that), despite the fact that active tension depended on so much of these parameters. Shortening velocities of skinned fibres in relaxing solution from sarcomere length of 2.7 to 2.2 μm before depolymerization and 2.1 to 1.6 μm after the partial polymerization were over 20 μm 5^{-1} per sarcomere at 2° C. This value was much larger than the maximum shortening velocity, about 2 μm s^{-1} per sarcomere, found in active contraction of frog muscles (Horiuti *et al.*, 1988). These results suggested that the changes found in resting tension and the shortening of the slack length seen are not the result of active contraction by actomyosin, but rather to an elastic structure other than actomyosin.

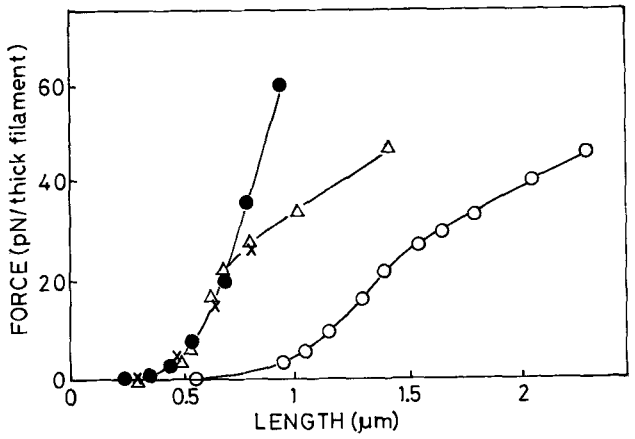

Fig. 8. Length-force relation of connectin filament. Force per connectin filament bound to a thick filament was calculated from the 1,0 lattice spacing, 40.4 nm (Higuchi & Umazume, 1986), the resting tension of skinned fibre at a sarcomere length of 3.0 μm, 8.3×10^3 Nm^{-2} (Fig. 6) and occupation of myofilaments to the cross-sectional area of skinned fibre, 80% (Schönberg, 1980). ●, portion of connectin in the I-band (C_I in Fig. 9); ○, portion of connectin in I-band and portion of connectin detached from thick filament ($C_I + C_A$ in Fig. 9); △, ×, portion of connectin detached from thick filament (C_A in Fig. 9C) by partial depolymerization at 0.29 M KCl for 10 min. The triangles were estimated from the relation between sarcomere length and the resting tension shown in Fig. 7, while the crosses are from electron micrographs shown in Fig. 4.

Length-force relation of connectin filament in skinned fibre

We estimated the length-force relation (Fig. 8) of connectin filament on the basis of resting tension (Fig. 7) before and after partial depolymerization of thick filaments. Before depolymerization, the increase seen in resting tension with sarcomere length should correspond to the increase in the elastic force with the length (L_I μm) of that part of connectin in the I-band (C_I in Fig. 9A) at a sarcomere length below 3.5 μm because anti-connectin binding at the end of thick filaments does not move (Itoh *et al.*, 1988), and because the resting tension in skinned fibres is attributed mainly to connectin (Yoshioka *et al.*, 1986; Higuchi, 1987; Funatsu *et al.*, 1990). Sarcomere length, L(μm), is twice L_I plus the length of the thick filament (1.6 μm); therefore $L = 2 \times L_I + 1.6$. The force per connectin filament bound to a given thick filament (Fig. 8, closed circles) at $L_I = (L - 1.6)/2$ was calculated from the resting tension (Fig. 7, closed circles) per unit area and the number of connectin filaments per unit area (see legend in Fig. 8) at L in the fibre. The length-force relation of

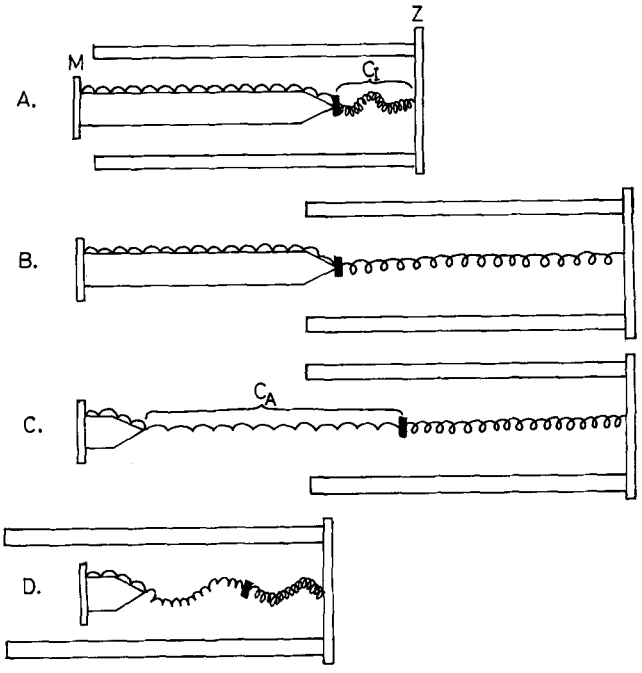

Fig. 9. Schematic model of the connectin filament and its elasticity in half-sarcomeres. M, M-line, Z, Z-line. Thin filaments attach to the Z-line, and thick filaments to the M-line. Connectin filaments bind to almost the whole length of halfthick filament and to the Z-line. Small rectangular form attached to connectin is the monoclonal antibody which binds to the end of thick filaments before the depolymerization (A) and (B): only the portion of connectin in the I-band (C_I) is stretched with sarcomere length before depolymerization. (C and D) After partial depolymerization, the part of connectin (C_A) which detaches from the thick filament is also elongated with sarcomere length. (A) and (D), slack sarcomere lengths of 2.1 and 1.5 μm, respectively; (B) and (C), sarcomere length of 3.5 μm.

the total parts of C_I and connectin filaments in A-band (C_A in Fig. 9) after the partial depolymerization of thick filaments was estimated by the same process as an estimation of the elasticity of C_I. L after the partial depolymerization of thick filaments is twice the length of $C_I + C_A$ and the residual length of thick filaments of 0.4 μm (Fig. 9C and D): $L = 2 \times (L_I + L_A) + 0.4$, where L_A(μm) is the length of C_A. The force posed by C_I and C_A at $(L_I + L_A) = (L + 0.4)/2$ (Fig. 8, open circles) was calculated from the resting tension (Fig. 7, open circles) and the number of connectin filaments per unit area at L after the partial depolymerization of thick filaments. The difference between the length-force relation of C_I and $C_I + C_A$ gives that of C_A (Fig. 8, triangles).

The elasticity of C_A was similar to that of C_I at lengths below 0.7 μm; the value was less than that at increased lengths. We compared the length-force relation of connectin filaments, estimated from the relation between the resting tension and sarcomere length, with that measured from electron microscopy. The length of C_A in electron micrographs was calculated by the half-length of partially depolymerized thick filaments, 0.2 μm, subtracted from the distance between the end of the antibody stripe and the centre of the M-line, as shown in Fig. 4. Forces of C_A in electron microscopy were estimated from the resting tension (Fig. 7, open circles) at sarcomere lengths corresponding to those in electron micrographs. The force-length relation of C_A estimated from electron microscopy (Fig. 8, crosses) agreed with that estimated from the tension-sarcomere length relation (Fig. 8, triangles).

Discussion

To where do the ends of connectin bind?

Immunoelectron microscopy showed that antibodies against connectin form pairs of decorated lines, symmetrical with respect to the M-line, running from near the M-line to theZ-line (Maruyama *et al.*, 1985; Fürst *et al.*, 1988; Whiting *et al.*, 1989). This finding raised three possibilities regarding the localization of connectin. Each connectin filament extends: (1) from the M-line to the Z-line, (2) from the Z-line, through the M-line, to the opposite Z-line, or (3) from the M-line, through the Z-line, to the adjacent M-line. Possibility (2) is discounted by the result obtained in the present study that connectin filaments retract to within 0.3 μm from each Z-line (Fig. 3E) after the dissociation of thick filaments without hydrolysis of connectin (Fig. 1). Moreover, possibility (3) is discounted by the results obtained after using protease. Connectin in skinned fibres was digested from α-connectin to β-connectin by trypsin (Yoshioka *et al.*, 1986; Funatsu *et al.*, 1990). If connectin molecules extend from the M-line to the next M-line, the molecular weight of β-connectin should be less than half that of α-connectin because β-connectin is located in the A-band (Fig. 3A) (Yoshioka *et al.*, 1986) and α-connectin is cut off in the I-band by trypsin treatment (Yoshioka *et al.*, 1986; Fürst *et al.*, 1988; Funatsu *et al.*, 1990). The molecular weight of β-connectin of 2100 kDa is, however, larger than half that of α-connectin of 2800 kDa (Maruyama *et al.*, 1984).

The first possibility mentioned above agrees with the present results that connectin retracted to near the Z-line after dissociation of the thick filaments. Because connectin filaments did not retract towards the Z-line until the thick filament has been depolymerized to a length of 0.27 μm, one origin of the connectin filament should be located at a distance no greater than half of 0.27 μm (0.135 μm) from the centre of the M-line, hence it is located in or near the bare zone of the thick filament. This view is supported by the finding that monoclonal antibody 3B9 binds at a distance of 0.09 μm from the centre of the M-line (Fig. 4). Recently it has been reported that one end of each connectin filament binds to the constituents of the M-line (Nave *et al.*, 1989). The 170 kDa protein, presumably M protein, was extracted from the skinned fibre concomitantly with α-connectin at 0.54 M KCl (Table 1), suggesting the binding of 170 kDa protein to α-connectin. Thus it seems quite plausible that one end of each connectin filament binds to the M-line and the other end to the Z-line.

Connectin filaments bear resting tension

Resting tension decreased markedly in relaxing solutions that contained concentrations of KCl > 0.34 M. As the resting tension in mechanically skinned fibres is attributed mainly to connectin (Yoshioka *et al.*, 1986; Funatsu *et al.*, 1990), the possible causes of the prominent decrease in resting tension seen at moderate KCl concentrations include: (1) the reduction of the number of connectin filaments contributing to resting tension, and/or (2) the reduction of the elasticity of connectin itself. The second cause is unlikely for several reasons. The elasticity of connectin filament depends minimally on ionic strength, as evidenced by the fact that resting tension at a high KCl solution barely changes binding subsequent decreases in KCl concentration. The anti-connectin that bound at a distance of 0.65–0.9 μm from the M-line at 0.29 M KCl failed to move appreciably at 0.34 M KCl (Fig. 3C and D), although the resting tension fell markedly with the increase in KCl concentration from 0.29 M to 0.34 M KCl (Fig. 6). These findings suggest that the elasticity of the connectin filament was not reduced appreciably after the dissociation from 0.29 M to 0.34 M KCl at a sarcomere length of approximately 3.0 μm.

Our results support the first of the above-mentioned causes, namely that a reduction in the number of connectin filaments gives rise to the reduction found in resting tension. Electron micrographs show that a total number of fine filament appearing between the ends of thick and thin filaments decreased considerably at 0.39 M KCl and reached zero at 0.54 M KCl (Fig. 2). The binding of connectin antibodies to the fine filaments confirms that these fine filaments contain connectin (Figs 3 and 4).

Anti-connectin was not scattered within 0.7 μm from Z-line at 0.29 M KCl but was so at 0.34 M KCl (Fig. 3). This change in position associated with the increase in KCl concentration from 0.29 to 0.34 M appears not to result from hydrolysis of connectin because the relative amount of α-connectin failed to decrease appreciably as the KCl concentration was increased from 0.29 to 0.34 M (Fig. 1 and Table 1). The decrease found in relative resting tensions above 0.29 M KCl, therefore, results mainly from the reduction in the number of connectin filaments which bind between the thick filaments and the Z-line.

Elasticity of connectin

When muscle fibres are stretched, in relaxing solution, to sarcomere lengths up to 3.5 μm, the anti-connectin epitopes in the A-band region fail to move: however, the anti-connectin epitopes in the I-band region move away from the Z-line (Fürst *et al.*, 1988; Itoh *et al.*, 1988; Horowits *et al.*, 1989). These observations imply that connectin filaments are associated with the rigid thick filaments and consequently have elasticity only in the I-band region. The increment in resting tension with sarcomere length before the dissociation of thick filaments (Fig. 7), therefore, should reflect the elasticity of that part (C_I) of the connectin filament in the I-band (closed circles in Fig. 8).

In slackened fibres, the distance between the M-line and the anti-connectin epitope that is located in control fibres at the A–I junction decreased by 0.28 μm (=0.78 − 0.50) (Fig. 4A and B) on the partial depolymerization of thick filaments. The distance between the Z-line and the anti-connectin epitope was, however, hardly changed on the partial depolymerization; the distances before and after the polymerization were 0.29 (=2.13/2 − 0.78) and 0.31 μm (=1.61/2 − 0.50), respectively, where 2.13/2 and 1.61/2 (μm) were half of slakened sarcomere lengths before and after the depolymerization, respectively. These suggest that the decrease in slack length results from the shortening of the part of the connectin filament (C_A in Fig. 9) that is released from the thick filament on depolymerization of the filament and does not result from shortening of the part of the connectin filament in the I-band (C_I in Fig. 9). The shortening of connectin filaments after depolymerization of the thick filaments is not from active contraction resulting from actomyosin interaction (see Results), but may result from conformational changes on the part of the connectin filament which is released from the thick filaments. Recently, Roos and Brady (1989) reported that slack sarcomere length is shortened by the partial extraction of thick filaments in cardiac muscles at high KCl concentrations. This shortening of slack length may be explained as resulting from shortening of connectin filaments.

The anti-connectin epitopes, whose position in fibres before depolymerization of thick filaments were independent of sarcomere length, moved with changes in sarcomere length after partial depolymerization (Fig. 4). The part of the connectin filament (C_A in Fig. 9) that detached from the thick filaments definitely showed elasticity. We obtained comparable estimations of the length-force relation of this part of connectin from mechanical measurements (filled triangles in Fig. 8) and from immunoelectron microscopic measurements (Fig. 8, open circles) Both estimations indicate that the elasticity of C_A is similar to that of C_I, and thus suggest that the connectin molecule is elastic along its entire length. For highly-stretched fibres, the reduction of resting tension that accompanies the depolymerization of thick filaments at long sarcomere length (Fig. 7; Higuchi & Umazume, 1985) is most reasonably explained by the elasticity of that part of the connectin filament (C_A) that is released from the thick filament and by the consequent elongation of this part.

The slack length, 0.55 μm, of the connectin filaments in skinned fibres (Fig. 8) corresponds not to the traced length of the connectin filaments but to the end-to-end length, because of the crookedness of the compressed connectin filament in slackened fibres (Funatsu *et al.*, 1990). Nave and colleagues (1989) reported that the length of straight, purified β-connectin, oriented by centrifugation, is about 0.9 μm. Thus, the small increment in resting tension when the length of connectin increases from approximately 0.55 μm to approximately 1.0 μm may reflect the straightening of the crooked, compressed connectin. The almost linear increment of the resting tension when the length of connectin increases above approximately 1.0 μm (Fig. 8) may reflect the stretching of connectin filaments to actual traced lengths.

In an effort to understand the molecular properties of connectin, we estimated the force per connectin molecule of a given connectin filament binding to a thick filament (Fig. 8). The slope of the length-force relation for connectin (Fig. 8) between 1.0 and 1.5 μm reveals that the elasticity of a given connectin filament is 48 pN μm^{-1}. The number of connectin molecules per half of a thick filament has been estimated to be three to six (Wang, 1985; Maruyama, 1986). In this case, the elasticity of a connectin molecule is 8–16 pN μm^{-1}. This elasticity is much less than that of the myosin molecule (two cross-bridges) during contraction, 1 pN nm^{-1} (Lombardi and Piazzesi, 1990).

Model of localization and elasticity of connectin filaments

Figure 9 schematically illustrates the localization and elasticity of connectin filaments before and after partial depolymerization of thick filaments. One end of the connectin filaments lies near the M-line; the other lies to the Z-line (Fig. 9A and B). Although connectin filaments may have lateral linkages, as suggested by Higuchi and Umazume (1986) and Funatsu and colleagues (1990), the linkages were omitted from the model to simplify it. The part of the connectin filament in the I-band (C_I) exhibits elasticity. The part of the connectin filament that lies in the A-band (C_A), however, exhibits no appreciable

elasticity and thus probably serves to bind the connectin filament to the thick filament. On partial depolymerization of the thick filament, the released connectin (C_A) evidences elasticity that is comparable to the elasticity of C_I, which causes the reduction seen in slack sarcomeres.

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