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Troponin Is a Potential Regulator for Actomyosin Interactions

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Troponin extracted from rabbit skeletal muscle directly binds to an actin filament in a molar ratio of 1:1 even in the absence of tropomyosin. An actin filament decorated with troponin did not exhibit significant difference from pure actin filaments in the maximum rate of actomyosin ATP hydrolysis and the sliding velocity of the filament examined by means of an *in vitro* motility assay. However, the relative number of troponin-bound actin filaments moving in the absence of calcium ions decreased to half that in their presence. The amount of HMM bound to the filaments was less than 4% of actin monomers in the presence of TNs. In addition, actin filaments could not move when Tn molecules were bound in the molar ratio of about 1:1 although they sufficiently bind to myosin heads. These results indicate that troponin can transform an actin monomer within a filament into an Off-state without sterically blocking of the myosin-binding sites with tropomyosin molecules.

Key words: actin filament, ATPase, motility, sliding, troponin.

Abbreviations: HMM, heavy meromyosin; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; TN, troponin; acto-TN, actin filament complexed with TN.

Troponin (TN) and tropomyosin (TM) have been considered to regulate the contraction of skeletal muscle in a calcium-dependent manner (*1*–*3*). They regulate the interaction between actin filaments and myosin heads (*4*, *5*). TN complexes are considered to be calcium-binding and inhibitory units (*6*), and TMs to be allosteric effectors (*7*, *8*). Recently, the inhibitory component of TN (TN-I) and the TM-binding component (TN-T) together were reported to abolish the motility of actomyosin complexes in the absence of TM (*9*, *10*).

In the present paper, we report that an acto-TN filament, a TN-decorated actin filament without TM, could regulate the sliding movement in a calcium-dependent manner at low ionic strength although enhancement of the ATP hydrolysis rate of myosin could not. The results should indicate that TN could induce the conformational change of actin monomers into Off (or Closed)-state, but, owing to the strong co-operativity of acto-TN filaments, the sliding activity of the filaments was regulated in a calcium-dependent manner. The potential property of TN, with respect to the role of TM, in the regulation of the actomyosin interaction should be reconsidered.

MATERIALS AND METHODS

Proteins, Reagents and Buffers—ATP, imidazole and EGTA were purchased from Boehringer Mannheim, Sigma and Dojin Chemicals (Kumamoto), respectively. Other reagents were from Wako Pure Chemicals (Osaka), and of special reagent grade. Rabbit skeletal muscle proteins were used. Actins were purified according to Spudich and Watt (*11*), and myosins were prepared by the method of Perry (*12*). Troponins were obtained by the method of Ebashi *et al.* (*13*) followed by ion-exchange chromatography (*14*). HMM was prepared as reported by Okamoto and Sekine (*15*). Purified HMM and TN were dissolved at concentrations of 20 mg/ml and 5 mg/ml, respectively, in 0.6 M KCl, 10 mM DTT, 10 mM potassium phosphate buffer (pH 7.0), and then stored in liquid nitrogen, and thawed and dialyzed before use. The experimental conditions we adopted throughout the experiments were as follows, unless especially mentioned, 25 mM imidazole-HCl buffer (pH = 7.4), 25 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM $CaCl_2$, 0.1 mg/ml G-actin, and 0–0.8 mg/ml troponin.

Binding Assay—The binding of troponin molecules to actin filaments was determined under the following conditions, 25 mM KCl, 25 mM imidazole-HCl (pH 7.4), 5 mM $MgCl_2$, 4 mM EGTA, 4.07 or 0.45 mM $CaCl_2$ (pCa 4 or 8), 1/200 (v/v) 2-Me, and 2.38×10^{-7} M F-actin. A mixture of actin and Tn molecules was subjected to centrifugation (BECKMAN XL-70 ultra centrifuge, 70.1Ti rotor, 40,000 rpm, 90 min, 25°C), followed by SDS-PAGE at 15% according to Laemmli *et al.* The ratio of Tn subunits to actin molecules was estimated by densitometric analysis of the CBB-stained gel patterms.

Microscopic Observation—In order to make the glass slides hydrophobic, we used siliconizer L-25 (Fuji System Co., Ltd.). The *In vitro* motility assay was carried out with fluorescence inverted microscopy under the following buffer conditions, 25 mM KCl, 25 mM imidazole-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP, 4 mM EGTA (in the absence of $[Ca^{2+}]$) or 1 mM $CaCl_2$ (in the presence of $[Ca^{2+}]$), 0.5% v/v 2-Me, 1.19×10^{-8} M rhodamine phalloidin-labeled actin, 0–5.95×10^{-8} M Tn, and 1.47×10^{-7} M HMM. To avoid dissociation of troponin molecules from actin filaments due to the dilution for microscopic observation, unlabeled proteins at the same concentration were mixed with the solution before the observation. The images of

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fluorescent filaments were recorded with a videotape recorder attached to a SIT camera. The velocity of the moving filaments was measured by capturing the microscopic images on a personal computer (*16*). Recorded images were analyzed using NIH image.

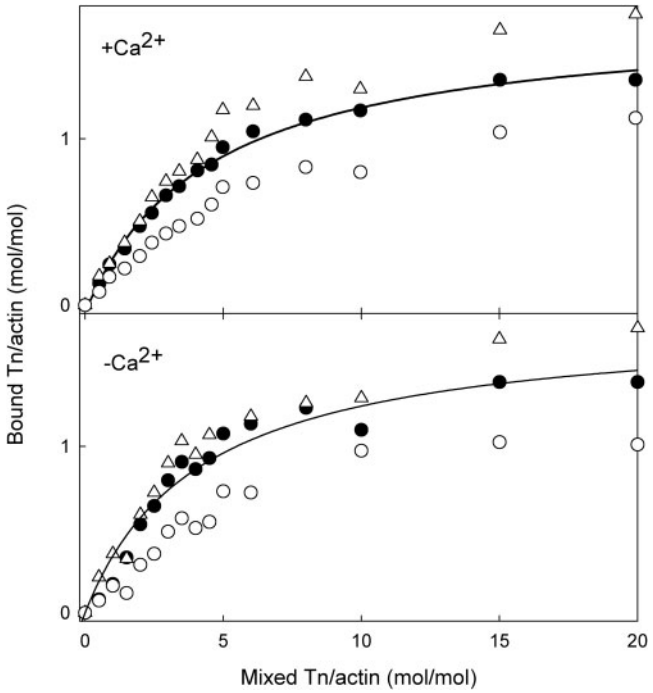

Fig. 1. **Binding of TN to actin filaments in the absence of [Ca^{2+}].** TN was added at the molar ratio of 0 to 3 to actin monomers. The level of binding TN was estimated from the result of densitometric analysis of SDS–polyacryl amide gel electrophoretic pattern obtained for centrifuged pellets at 40,000 rpm for 90 min at 4°C. Open symbols: TnC, filled symbols: TnT, open triangles: TnI. The solid lines were drawn by fitting the filled data with hyperbolic functions.

The velocity of moving filaments was calculated from the displacement in 2 s and was expressed as the average of about 50 independent samples. The ratio of moving filaments was calculated as $N_m/(N_m + N_s)$: (N_m: number of moving filaments, N_s: number of non-motile filaments), where filaments that did not migrate more than 160 nm (2–3 pixels) in 5 s were considered to be non-motile.

ATPase Assay—We assayed actin-activated myosin ATPase of the acto-TN solution under the conditions given above. The reaction was initiated by mixing HMM with an acto-TN solution under water incubation at 25°C. ATP hydrolysis was monitored by measuring the concentration of inorganic phosphate using the Malachite Green method (*17*) after terminating the reaction with perchloric acid at the final concentration of 0.1 M.

RESULTS

Troponin Binding to Actin Filaments—The main question regarding the direct interaction of troponin molecules with actin filaments in our experiments must be whether such interactions are physiological or not. We first examined the binding of three subunits (T, I, and C) to actin filaments. TNs bound to actin filaments in both the presence and absence of [Ca^{2+}], but the filaments formed bundles. This bundling made the solution turbid. In order to avoid the formation of bundles, we mixed ATP at the concentration of 5 mM with the acto-TN solution (*18*). The addition of ATP did not affect the binding of TN to actin filaments. The results of the binding assay in the cases of both in the presence and absence of [Ca^{2+}] are shown in Fig. 1. Taking into account some experimental error, three subunits of troponin bound to actin filaments in the molar ratio of one to one or more when a sufficient amount of TN molecules was used. The amount of bound troponin was saturated at about 1.35 mol/mol actin. Here, we should mention that the binding could only be realized under low ionic strength, as far as we examined, *i.e.*, not under physiological conditions. Hereafter, we prepared

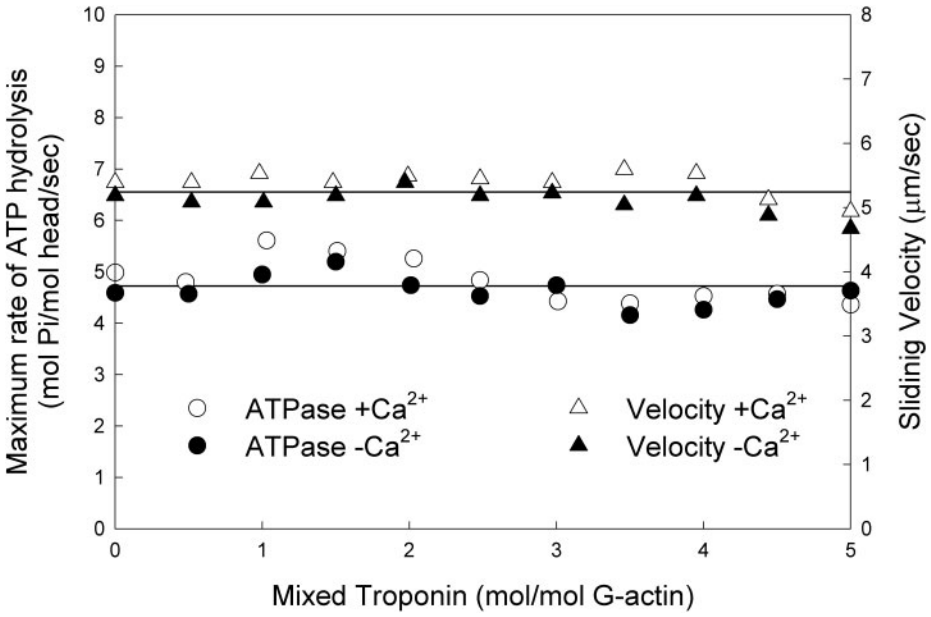

Fig. 2. **Effects of TN-binding to actin filaments on the maximum rate of ATP hydrolysis and the sliding velocity.** The maximum rate of ATP hydrolysis (circles) was calculated by extrapolation of the values obtained with various amounts of acto-TN (data not shown). Average velocity of the sliding (triangles) was obtained for 50 moving filaments in microscopic images.

the TN-actin filaments (filaments bound with TN) by mixing the two in the molar ratio of 1 to 5, which allowed stoichiometric binding of TN-actin.

HMM-ATPase Activation and Sliding Velocity of Acto-TN Filaments—Acto-TN filaments were subjected to both ATPase measurements *in vitro* and sliding assays under a microscope. For quantitative expression, we measured the velocity of sliding filaments under a microscope and the rate of ATP hydrolysis in the bulk solution *in vitro* (as shown in Fig. 2). Regardless of the amount of TN molecules mixed with actins, no appreciable difference was detected in either the sliding velocity or the rate of ATP hydrolysis (Fig. 2). In addition, neither value showed appreciable difference between in the presence and absence of [Ca^{2+}] (open and filled symbols). This may suggest that the non-physiological binding of TN to actin is just an artificial phenomenon and that the functional capacity of TN may be lost. In order to exclude this possibility, we determined the number of filaments moving within the microscope field. The results are presented as the ratio of moving filaments in Fig. 3. In comparison with those in the presence of [Ca^{2+}], the number of moving filaments was found to decrease to about 30% in the absence of [Ca^{2+}].

Ratio of Moving Filaments vs. *Calcium Ions*—Figure 4 shows the percentages of moving filaments observed in microscope fields. We have confirmed the calcium-dependent changes in the ratio of moving filaments under the same conditions. In the presence of [Ca^{2+}], the relative number of moving filaments remained constant at the level of about 85%. This value was independent of the amount of mixed TN molecules. Nevertheless, the number decreased to about 30% in the absence of [Ca^{2+}]. This change occurred at the physiological calcium ion concentration (at pCa = 6) in a sigmoidal manner, suggesting the change was caused by an intrinsic property of TN molecules.

DISCUSSION

Signal transduction through muscle contraction is initiated by the binding of [Ca^{2+}] to the troponin C subunit.

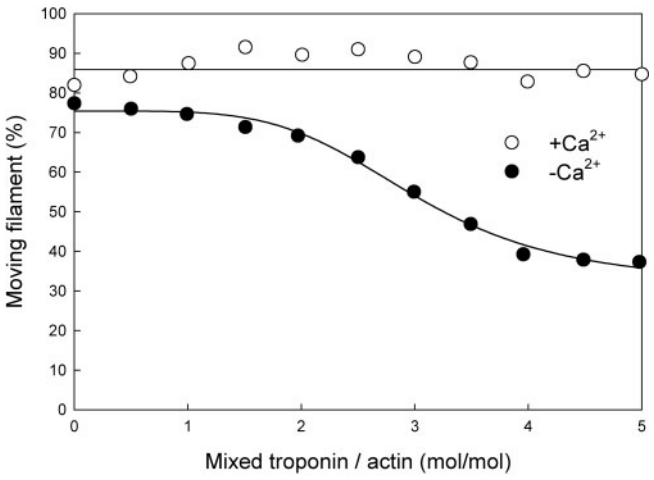

Fig. 3. **Change in the percent ratio of moving filaments as a function of TN added to the microscopic specimens.** The calculation methods for the data are described in the text.

The signal from TN-C would induce all the conformational changes of thin filaments, the troponin-tropomyosin complex and actin filaments. This signal is transmitted to tropomyosin molecules, followed by steric blocking of the myosin-binding site or the conformational change into the "Off"-state of actin molecules (*25*). In a recent paper, the signals that primarily affect the conformation of actin molecules and then the position of tropomyosin molecules within the thin filaments were reported (*27*). The TN did not bind to actin filament under physiological

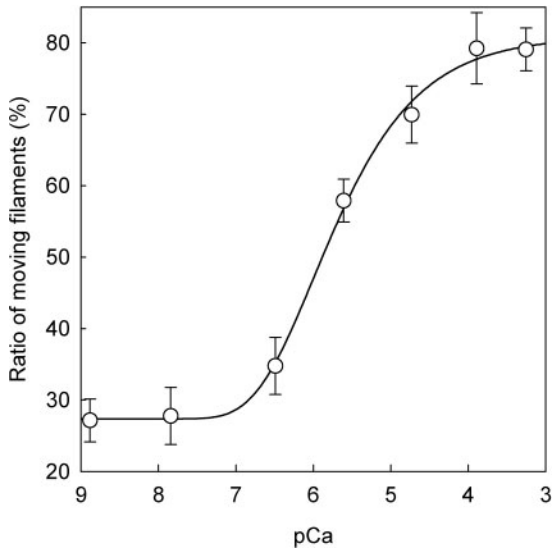

Fig. 4. **The ratio of moving filaments within a fluorescence microscope field.** Bars represent experimental error for five independent experiments.

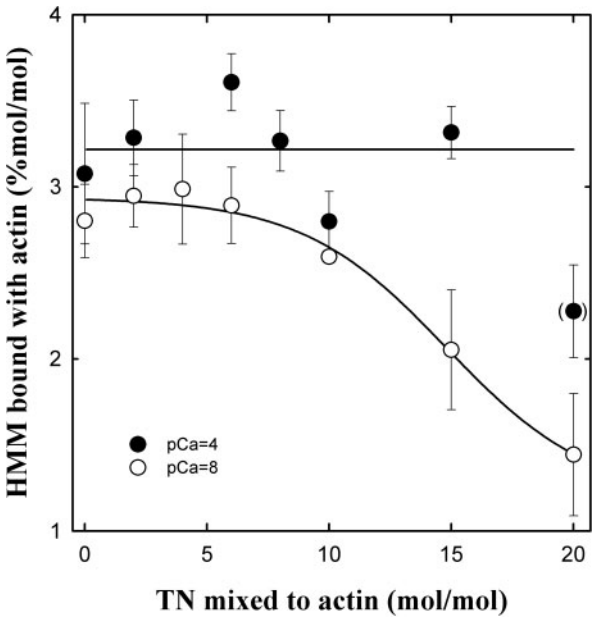

Fig. 5. **The amount of HMM bound to troponin-actin filaments.** The ratio of HMM bound to actin molecules was plotted against the amount of actin. Filled symbols show the ratio in the absence of [Ca^{2+}] and open ones that in its absence. The bracketed datum may include some experimental artifacts due to the extremely large amount of Tn molecules.

conditions throughout this process, however, TN-T subunits have been reported to have at least two actin-binding sites (*22*, *23*). This binding is thought to prevent the movement of tropomyosin in the perpendicular coordinate of the filaments (*24*). We have been interested in whether this binding of TN to actin could be incorporated into the signal transduction pathways for inhibition of the sliding movement of actomyosin.

Our binding assay of TN and actin filaments (Fig. 1) revealed that the amount of bound troponin was saturated at the level of about 1.35. However, TN-C exhibited slightly less affinity in the absence of [Ca^{2+}]. The binding assay results coincide with the release of TN-C from the I subunit in physiological processes (*4*), implying that the direct binding of TN to actin is physiological. In spite of this expectation, the TN-actin did not inhibit either ATPase activation of myosin heads or the velocity of sliding filaments in vitro (Fig. 2). These results might mean that the binding was not sufficient enough to inhibit these activities or to block the interaction between actin and myosin.

As shown in Fig. 3, the number of moving filaments decreased with the addition of TN at low [Ca^{2+}]. In our study, about 85% of the filaments exhibit the sliding movement at high [Ca^{2+}] and the value decreased to about 30% at low [Ca^{2+}]. This phenomenon was reversible, *i.e.*, immobilized acto-TN filaments began to move as [Ca^{2+}] increased, and vice versa. Why could acto-TN filaments move even at low [Ca^{2+}]? The question must be what factors determine the motility of a certain filament. We have settled two possibilities; (i) there was an appreciable number of "bare" actin molecules that could bind and drive the filament sliding. (ii) TN-binding to a filament could be in rapid equilibrium so that some actin molecules happen to interact with myosin to drive the entire filament.

No intermittent movement, however, was detected during the observation. Considering the results in Fig. 1, it is hard to consider that no TN molecules remained bound to actins within a half pitch of filaments, 14 monomers, throughout the observation period. The fact that the acto-TN binding was saturated at a molar ratio of about 1.3 strongly indicated that TN binds to the filaments in a overlapping manner. As the smallest unit that could move actin filaments in vitro was reported to be a half pitch of the actin helix (*26*), we could easily reach the following conclusion; if On-state actin molecules happen to continued as long as a single half pitch within a single filament, the filament could plausibly have the ability to move along the myosin molecule. If not, the filament must stop.

We have determined the amount of myosin heads using HMMs under the same experimental conditions as those we used previously. As shown in Fig. 5, the amount of actin filaments binding with HMM remarkably decreased as the amount of TN increased in the absence of [Ca^{2+}]. When an extremely large amount of Tn was added, 1:20 (!), the amount of HMM apparently decreased due to some experimental artifacts (bracketed datum in figure). These results and those for the ratio of moving filaments were plotted against the actual amount of Tn attached to the actin filaments, and re-plotted in Fig. 6. In the range of attached Tn, an appreciable number of filament stopped the active sliding movement, however, myosin heads did bind to the filaments at the same level as in the absence of TN. With such TN-actin filaments, actin molecules should be transformed into an off, or closed, state, and thus myosin heads could easily gain access to actin filaments but could not generate sliding movement. It may be more important that the phenomenon could occur in the absence of tropomyoisn. For this reason also, troponin might be a potent switch for the actomyosin sliding movement.

In conclusion, troponin molecules could bind to actin filaments in a molar ratio of about 1:1.3 and inhibit the sliding activity of the filaments even in the absence of TM. For this inhibition, steric blocking of TM is not necessary. Troponin molecules could regulate the sliding

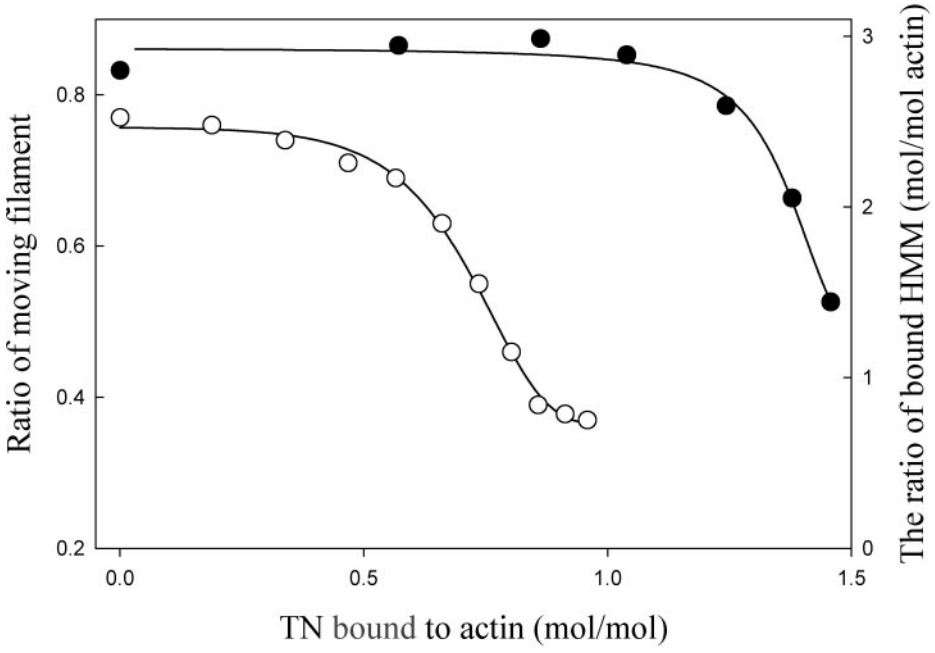

Fig. 6. **Ratio of moving filaments and bound HMM plotted against the amount of actually binding troponin molecules.** The points on the horizontal axis were calculated from the binding assay data in Fig. 1, and the other one, *i.e.*, the ratio of moving filaments and the amount of HMM, were re-plotted from Figs 3 and 5, respectively. This graph clearly demonstrates that, when Tn is binding at the molar ratio of 1:1, HMM can bind to Tn-actin filaments but can not move.

movement of actomyosin filaments in a calcium-dependent manner as an intrinsic property even in the absence of tropomyosin.

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REFERENCES

1. Ebashi, S. and Ebashi, F. (1964) A new protein component participating in the superperecipitation of myosin B. *J. Biochem.* **55**, 604–613
2. Ebashi, S., Ohtsuki, I., and Mihashi, K. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 215–224
3. Ebashi, S. and Kodama, A. (1965) A new protein factor promoting aggregation of tropomyosin. *J. Biochem.* **58**, 107–108
4. Ohtsuki, I., Maruyama, K., and Ebashi, S. (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. *Adv. Prot. Chem.* **38**, 1–67
5. Potter, J.D. and Gergely, J. (1974) Troponin, tripomyosin, and actin interactions in the Ca^{2+} ion regulation of muscle contraction. *Biochemistry* **13**, 2697–2703
6. Ebashi, S. (1972) Separation of troponin into its three components. *J. Biochem.* **72**, 787–790
7. Honda, H., Kitano, Y., Hatori, K., and Matsuno, K. (1996) Dual role of tropomyosin on chemically modified actin filaments from skeletal muscle. *FEBS Lett.* **383**, 55–58
8. Lehrer, S.S. and Geeves, M.A. (1998) The muscle thin filament as a classical cooperative/allosteric regulatory system. *J. Mol. Biol.* **277**, 1081–1089
9. Honda, H., Tamura, T., Hatori, K., and Matsuno, K. (1995) Decorating actin filaments with troponin T-I complexes and acceleration of their sliding movement on myosin molecules. *Biochim. Biophys. Acta* **1251**, 43–47
10. Honda, H., Tagami, N., Hatori, K., and Matsuno, K. (1997) Regulated crosslinked actin filaments and the decoupling between their ATPase activity and sliding motility. *J. Biochem.* **121**, 47–49
11. Spudich, J.A. and Watt, S. (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
12. Perry, S.V. (1955) Myosin adenosinetriphosphatase. *Methods Enzymol.* **2**, 582–588
13. Ebashi, S. and Endo, M. (1968) Calcium ion and muscle contraction. *Prog. Biophys. Mol. Biol.* **18**, 123–183
14. Miki, M. (1990) Resonance energy transfer between points in a reconstituted skeletal muscle thin filament. A conformational change of the thin filament in response to a change in Ca^{2+} concentration. *Eur. J. Biochem.* **187**, 155–162
15. Okamoto, Y. and Sekine, T. (1985) A streamlined method of subfragment one preparation from myosin. *J. Biochem.* **98**, 1143–1145
16. Hatori, K., Honda, H., and Matsuno, K. (1996) ATP-dependent fluctuations of single actin filaments in vitro. *Biophys. Chem.* **58**, 267–272
17. Ohno, T. and Kodama, T. (1991) Kinetics of adenosine triphosphate hydrolysis by shortening myofibrils from rabbit psoas muscle. *J. Physiol.* **441**, 685–702
18. Wakabayashi, T. (1970) Doctoral thesis in Tokyo University
19. Ohtsuki, I., Onoyama, Y., and Shiraishi, F. (1988) Electron microscopic study of troponin. *J. Biochem.* **103**, 913–919
20. Tanokura, M., Tawada, Y., Ono, A., and Ohtsuki, I. (1983) Chymotryptic subfragments of troponin T from rabbit skeletal muscle. Interaction with tropomyosin, troponin I and troponin C. *J. Biochem.* **93**, 331–337
21. Squire, J.M. and Morris, E.P. (1988) A new look at thin filament regulation in vertebrate skeletal muscle. *FASEB J.* **12**, 761–771
22. Pearlstone, J.R. and Smillie, L.B. (1982) Binding of troponin-T fragments to several types of tropomyosin (sensitivity to Ca^{2+} in the presence of troponin-C). *J. Biol. Chem.* **257**, 10587–10592
23. Potter, J.D., Sheng, Z., Pan, B.-S., and Zhao, J. (1995) A direct regulatory role for troponin T and a dual role for troponin C in the Ca^{2+} regulation of muscle contraction. *J. Biol. Chem.* **270**, 2557–2562
24. Malnic, B., Farah, C.S., and Reinach, F.C. (1998) Regulatory properties of the NH_2- and COOH-therminal domains of troponin T. *J. Biol. Chem.* **273**, 10594–10601
25. Gordon, A.M., Homsher, E., and Regnier, M. (2000) Regulation of contaction in straiated muscle. *Physiol. Rev.* **80**, 853–924
26. Harada, Y. and Yanagida, T. (1988) Direct observation of molecular motility by light microscopy. *Cell Motil. Cytoskeleton* **10**, 71–76
27. Miki, M., Hai, H., Saeki, K., Shitaka, Y., Sano, K.-I., Maeda, Y., and Wakabayashi, T. (2004) Fluorescence resonance energy transfer between points on actin and the C-terminal region of tropomyosin in skeletal muscle thin filaments. *J. Biochem.* **136**, 39–47