

# The effect of tropomyosin on force and elementary steps of the cross-bridge cycle in reconstituted bovine myocardium

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**The role of tropomyosin (Tm) in the elementary steps of the cross-bridge cycle in bovine myocardium was investigated. The thin filament was selectively removed using gelsolin (thin filament severing protein), and the actin filament was reconstituted from G-actin. Tm was further reconstituted without troponin (Tn), and the kinetic constants of the elementary steps of the cross-bridge cycle were deduced using sinusoidal analysis at  $pCa \leq 4.66$ , pH 7.00, and 25°C. The association constant of MgATP to cross-bridges ( $K_1$ ) after reconstitution of Tm was  $20.7 \pm 2.3 \text{ mM}^{-1}$ , which was about  $2 \times$  the control (untreated) myocardium ( $9.1 \pm 1.3 \text{ mM}^{-1}$ ). Following reconstitution of Tm, the equilibrium constant of the cross-bridge detachment step ( $K_2$ ), the phosphate ( $P_i$ ) association constant ( $K_5$ ) and the equilibrium constant of the force-generation step ( $K_4$ ), which significantly changed in the actin filament-reconstituted myocardium, recovered to those of the control myocardium. Active tension after reconstitution of Tm was  $0.69 \times$  the control myocardium, a value between the control ( $1.00 \times$ ) and the actin filament-reconstituted myocardium ( $0.59 \times$ ). Tm-reconstituted myocardium was further reconstituted with Tn, and the effect of MgATP on the rate constants ( $K_1$ ,  $K_2$ ) was studied. Following reconstitution with Tn, the myocardium regained the  $Ca^{2+}$  sensitivity and the active tension became  $0.83 \times$  the control myocardium. In addition,  $K_1$  recovered to the value of the control myocardium with Tn reconstitution. These results indicate that both Tm and Tn enhance the force generated by each cross-bridge, and that Tm is primarily responsible for the change in the kinetic constants of the elementary steps of the cross-bridge cycle.**

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It has been known for some time that force generation in muscle is accomplished by a cyclic interaction between the myosin molecule, the main component of the thick filament, and the actin molecule, the main component of the thin filament. Myosin and actin convert the chemical energy stored in ATP to mechanical work, resulting in a sliding motion between the thick and thin filaments (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954). In skeletal and cardiac muscles, the  $Ca^{2+}$ -dependent regulatory switch, the tropomyosin (Tm)–troponin (Tn) system, is located on the thin filament (Ebashi & Endo, 1968). Tm is a filamentous dimeric protein which spans seven actin monomers along the thin filament with one Tn complex attached to the Tm dimer. In the steric blocking mechanism, Tm blocks the myosin binding site on the actin

filament when  $Ca^{2+}$  is absent, resulting in an inhibition of the actomyosin interaction (Haselgrove, 1973; Huxley, 1973). The binding of  $Ca^{2+}$  to Tn removes this inhibition by azimuthal movement of Tm, allowing myosin to interact freely with actin (Lehman *et al.* 1994, 1995).

Although the steric blocking mechanism is supported by many lines of evidence, the regulation of actomyosin interaction by a simple movement of Tm from a blocked position to an open position cannot explain some of the biochemical observations (McKillop & Geeves, 1993; Tobacman & Butters, 2000). While it has been shown in regulated actin that the ATP hydrolysis rate is inhibited by 96% in the absence of  $Ca^{2+}$ , the association constant of myosin subfragment 1 (S1) to actin is almost unchanged regardless of the presence of  $Ca^{2+}$  (Chalovich *et al.*

1981; Chalovich & Eisenberg, 1982). Covalently cross-linked acto-S1 still possesses  $\text{Ca}^{2+}$  regulation of the ATP hydrolysis rate when Tm and Tn are present (King & Greene, 1985). These findings indicate that the Tm–Tn system does not act just as a shield between actin and myosin, but serves a more complex role that alters actomyosin interaction and concomitant cross-bridge kinetics. Furthermore, a partial extraction of TnC is known to alter the kinetics of the cross-bridge cycle by promoting ATP binding and the rate of cross-bridge detachment and suppressing the rate of force generation (Zhao *et al.* 1996). Actomyosin ATPase activity is known to increase when Tm is added (Bremel *et al.* 1972; Murray *et al.* 1980). These observations also indicate that the Tm–Tn system modulates muscle contraction via a more complex mechanism.

Recently, we have demonstrated, using the thin filament-reconstitution method, that the Tm–Tn system alters the kinetic constants of the elementary steps of the cross-bridge cycle and increases the force supported by each cross-bridge in bovine myocardium (Fujita *et al.* 2002). In our experiments, the thin filament in bovine myocardium was selectively removed by plasma protein gelsolin, and the actin filament was reconstituted by adding purified G-actin in a polymerizing condition (Fujita *et al.* 1996). The actin filament-reconstituted myocardium generates active tension in a  $\text{Ca}^{2+}$ -insensitive manner because of the lack of regulatory proteins. This tension was about 2/3 of the control myocardium. The thin filament with full  $\text{Ca}^{2+}$  sensitivity can be reconstituted by adding native tropomyosin (nTm: a complex of Tm and Tn) to the actin filament-reconstituted myocardium. This reconstitution resulted in a larger tension similar to that of control myocardium (Fujita *et al.* 2002). Because in our previous studies, the regulatory system was reconstituted as nTm, it remained to be seen whether the effect was caused by Tm, Tn, or both. In this study, we reconstituted Tm and Tn sequentially after reconstitution of the actin filament. We found that Tm is primarily responsible for the change in the kinetic constants that we observed using nTm (except for the MgATP association constant), and that both Tm and Tn contribute to an increase of force supported by each cross-bridge.

## Methods

### Solutions

The relaxing solution (Rx) contained (mM): 6 EGTA, 2.2 MgATP, 5 ATP, 8  $\text{P}_i$ , 41 NaProp (Prop = propionate), 75 KProp, 10 Mops (3-(*N*-morpholino) propane sulphonic acid), and 40 BDM (2,3-butanedione 2-

monoxime). The rigor solution (Rg) contained 8  $\text{P}_i$ , 55 NaProp, 122 KProp, and 10 Mops. Experimental solutions are indicated by *mSnP*, where *m* represents the millimolar concentration of MgATP<sup>2-</sup>, and *n* represents the millimolar concentration of phosphate ( $\text{P}_i$ ). The 5S0P solution contained (mM): 6 CaEGTA, 5.83 MgATP, 1.36 ATP, 15 phosphocreatine (CP), 1 NaProp, 92 KProp, 10  $\text{NaN}_3$ , and 10 Mops. The 5S32P solution contained (mM): 6 CaEGTA, 5.7 MgATP, 1.36 ATP, 15 CP, 32  $\text{P}_i$ , 1 NaProp, 17 KProp, 10  $\text{NaN}_3$ , and 10 Mops. The 0S8P solution contained (mM): 6 CaEGTA, 0.85 MgProp<sub>2</sub>, 15 CP, 8  $\text{P}_i$ , 15 NaProp, 88 KProp, 10  $\text{NaN}_3$ , and 10 Mops. The 5S8P solution contained (mM): 6 CaEGTA, 5.8 MgATP, 1.36 ATP, 15 CP, 8  $\text{P}_i$ , 1 NaProp, 73 KProp, 10  $\text{NaN}_3$ , and 10 Mops. The –Ca solution (5S8P solution without Ca) contained (mM): 6 EGTA, 5.9 MgATP, 1.25 ATP, 15 CP, 8  $\text{P}_i$ , 1 NaProp, 73 KProp, 10  $\text{NaN}_3$ , and 10 Mops. The 5S8P solution is also called the standard activating solution. The pCa of all activating solutions was  $\leq 4.66$ , pH was adjusted to 7.00 by KOH, the  $\text{Mg}^{2+}$  concentration was 0.5 mM, the total sodium concentration was 55 mM, ionic strength was adjusted to 200 mM by NaProp and KProp, and all activating solutions contained 320 units  $\text{ml}^{-1}$  (=0.64  $\text{mg ml}^{-1}$ ) creatine kinase (CK). All solution components were added from neutral stock solutions: EGTA as  $\text{K}_2\text{H}_2\text{EGTA}$ , CaEGTA as  $\text{K}_2\text{CaEGTA}$ ,  $\text{P}_i$  as  $\text{K}_{1.5}\text{H}_{1.5}\text{PO}_4$ , MgATP as  $\text{Na}_2\text{MgATP}$ , ATP as  $\text{Na}_2\text{K}_{1.7}\text{H}_{0.3}\text{ATP}$ , and CP as  $\text{Na}_2\text{CP}$ . Multiple equilibria were assumed, and individual concentrations of multivalent ionic species were calculated using our computer program with the following apparent association constants at pH 7.0 (log values): CaEGTA, 6.28; MgEGTA, 1.61; CaATP, 3.70; MgATP, 4.00; CaCP, 1.15; MgCP, 1.30. The experiments were performed at 25°C.

$\text{Na}_2\text{CP}$ ,  $\text{Na}_2\text{H}_2\text{ATP}$ , Mops and  $\text{H}_4\text{EGTA}$  were purchased from Sigma Chemical Co. (St Louis, MO, USA);  $\text{CaCO}_3$ , MgO, NaOH, KOH,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaN}_3$  and propionic acid were purchased from Fisher Scientific Co. (Itasca, IL, USA); creatine kinase was purchased from Boehringer Mannheim (Indianapolis, IN, USA).

### Muscle fibres and proteins

Bovine hearts were obtained from a slaughterhouse within 15 min of death, and immediately cooled with ice. The muscle bundles (~2 mm in diameter, ~10 mm in length) were excised from a straight portion of the ventricular papillary muscles and incubated in the sodium-skinning solution (mM): 2 DTT (dithiothreitol), 30 BDM, 10 EGTA, 5 ATP, 2 MgATP, 122 NaProp and 10 Mops (pH 7.0) for 3 h at 0°C. The sodium-skinning solution was used to

minimize contraction. For further skinning, the solution was replaced with potassium-skinning solution containing (mM): 2 DTT, 30 BDM, 10 EGTA, 5 ATP, 2 MgATP, 122 KProp and 10 Mops (pH 7.0), and stored overnight at 0°C. BDM and EGTA were used to minimize force generation. The solution was further replaced with one containing 50% (v/v) glycerol and (mM): 2 DTT, 30 BDM, 10 EGTA, 5 ATP, 2 MgATP, 122 KProp and 10 Mops (pH 7.0) and stored at 0°C. The solution was replaced once again the next morning, and the muscle bundles were stored at a low temperature (-20°C).

G-actin was extracted and purified from acetone powder (Kondo & Ishiwata, 1976) of rabbit white skeletal muscles as described by Spudich & Watt (1971). G-actin was stored at 0°C and used within 2 weeks of extraction. Tm and Tn were prepared from bovine cardiac muscle as described by Ebashi *et al.* (1968) and purified using DEAE Sephadex A-25 (Pharmacia, Sweden). Tm was further purified as described by Yamaguchi *et al.* (1974). Bovine plasma gelsolin was prepared as described by Kurokawa *et al.* (1990).

Selective removal and reconstitution of actin filament were performed as reported by Fujita *et al.* (1996, 2002). Tm and Tn were reconstituted as reported by Fujita & Ishiwata, (1999).

**Experimental procedure and deduction of kinetic constants**

A strip of myocardium (90–160 μm in width and 2 mm in length) was dissected from a skinned muscle bundle. One end of the myocardium was connected to a tension transducer via a stainless steel wire (210 μm in diameter), and the other end was connected to a length driver via another stainless steel wire. Nail polish was used to glue the ends of myocardium to the wires. The myocardium was stretched until a small passive tension was observed, when the length (L<sub>0</sub>) of the myocardium was determined. The width was measured under a dissecting microscope (20 ×) and the cross-sectional area was estimated assuming

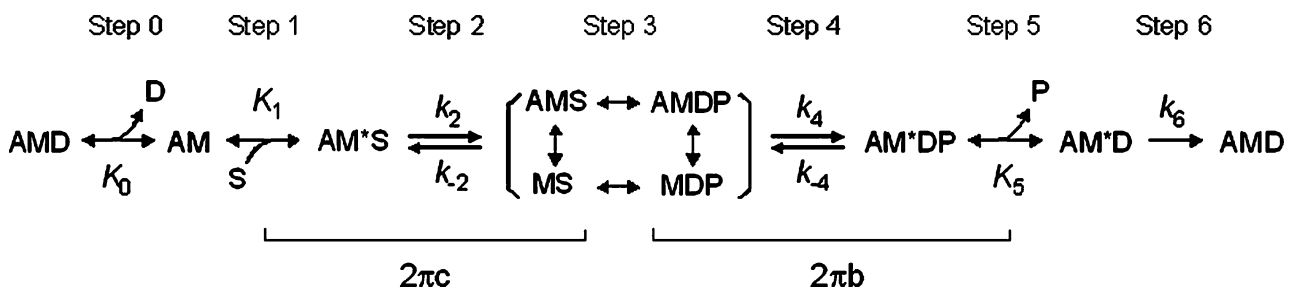
a circular cross-section. At this stage, the myocardium was chemically skinned further in the relaxing solution containing 1% (v/v) Triton X-100 for 20 min.

Two muscle models (Tm-reconstituted and Tm–Tn-reconstituted myocardium) were maximally activated in the presence of saturating Ca<sup>2+</sup> in a bath in which the temperature was controlled at 25°C. A 0.25% L<sub>0</sub> peak-to-peak sinusoidal waveform at 18 discrete frequencies between 0.13 and 100 Hz was digitally synthesized in a 386 CPU PC (Industrial Computer Source, San Diego, CA, USA) that controlled the length driver via a 14-bit DAC. Tension and length signals were simultaneously sampled by two 16-bit A/D converters and complex modulus data Y(f) were calculated as the ratio of the force change to the length change in the frequency domain. The complex modulus data observed during relaxation was subtracted. The complex modulus data were resolved into two exponential processes (B and C) by fitting the data to eqn (1) by minimizing the sum of modulus squares (Kawai & Brandt, 1980; Wannenburg *et al.* 2000):

$$Y(f) = H - \frac{B}{(1 + b/fi)} + \frac{C}{(1 + c/fi)} \quad (1)$$

where  $i = \sqrt{-1}$ . Uppercase letters B and C represent their respective magnitudes (amplitudes) and lower case letters b and c represent the characteristic frequencies of the respective processes. 2πb and 2πc are the apparent rate constants of the respective processes. Process B corresponds to phase 3, process C corresponds to phase 2, and Y(∞) corresponds to phase 1 of tension transients of step analysis (Huxley & Simmons, 1971). In relaxed and rigor muscle fibres, these exponential processes were absent, indicating that these processes are signatures of cycling cross-bridges. Y(∞) is referred to ‘stiffness’ in this paper. The complex modulus data were corrected by those of the rigor condition.

The results were analysed based on scheme 1, where A = actin, M = myosin, S = MgATP, D = MgADP, and



**Scheme 1.**  
**Elementary steps of the cross-bridge cycle**

$P = P_i$  = phosphate. The kinetic constants of elementary step 1 and 2 of the cross-bridge cycle were determined by fitting the MgATP dependence of  $2\pi c$  to eqn (2) by minimizing the sum of squares (Kawai & Halvorson, 1989).

$$2\pi c = k_2 K_1 S / (1 + K_1 S) + k_{-2} \quad (2)$$

The kinetic constants of elementary steps 4 and 5 of the cross-bridge cycle were determined by fitting the  $P_i$  dependence of  $2\pi b$  to eqn (3) by minimizing the sum of squares (Kawai & Halvorson, 1991).

$$2\pi b = \sigma k_4 + k_{-4} K_5 P / (1 + K_5 P) \quad (3)$$

where

$$\sigma = K_2 K_1 S / \{1 + (1 + K_2) K_1 S\} \quad (4)$$

In these equations, S and P indicate their respective concentrations:  $S = [\text{MgATP}]$  and  $P = [P_i]$ .  $K_1$  and  $K_2$  obtained from the MgATP study and  $S = 5 \text{ mM}$  were used for calculation of  $\sigma$  in eqn (4). Details of the sinusoidal analysis technique have been published (Kawai & Brandt, 1980).

### SDS-gel electrophoresis

Approximately 10 muscle fibres, each similar in size to those for mechanical measurements, were placed in the corner ( $500 \mu\text{l}$ ) of a small, tilted petri dish and followed through the extraction and reconstitution protocol. Five petri dishes (50 fibres) corresponding to 5 different kinds of treatment were used. They were then solubilized and electrophoresed at 20 mA for about 90 min at  $22^\circ\text{C}$  using acrylamide gradient (8–16%) ready gels (Tris-HCl, 15 wells, Cat. No. 161-1223, Bio-Rad), as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250.

### Confocal fluorescence microscopy

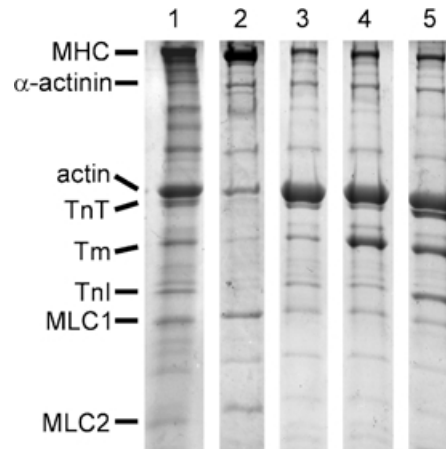
Tetramethyl rhodamine-5-iodoacetamide (Rh-IA) and fluorescein phalloidin (Fl-Ph) were purchased from Molecular Probes (Eugene, OR, USA). Rh-IA-labelled Tm was prepared according to the method of Ishii & Lehrer (1990). The thin filament was first removed by treatment with gelsolin for 80 min, and then the actin filament was reconstituted for a total of 28 min ( $7 \text{ min} \times 4$ ). Then, the myocardium was incubated in relaxing solution containing Rh-IA-labelled Tm for 12 h. Next, the myocardium was fixed with relaxing solution containing 1% formaldehyde for 30 min and stained with  $6.6 \mu\text{M}$  Fl-Ph in relaxing solution for 5 h. For observation, the myocardium was mounted on a cover slip and washed with relaxing solution containing  $4.5 \text{ mg ml}^{-1}$  glucose,  $0.22 \text{ mg}$

$\text{ml}^{-1}$  glucose oxidase,  $0.036 \text{ mg ml}^{-1}$  catalase, and  $10 \text{ mM}$  DTT. This preparation was observed under a laser scanning confocal microscope equipped with 25 mW Ar laser at 488 nm (Fluoview-IX/AR; Olympus Co., Tokyo). No crossover between the fluorescence images of rhodamine ( $> 610 \text{ nm}$ ) and fluorescein (510–540 nm) was detected.

## Results

### Removal and reconstitution of the thin filament

Figure 1 shows SDS-PAGE of myocardium at each step of reconstitution. In control myocardium (lane 1), thick filament proteins, myosin heavy chain and myosin light chains 1 and 2, can be seen. Also seen are thin filament proteins, actin, TnT, Tm and TnI. TnC could not be stained with the method we used. In gelsolin-treated myocardium (lane 2), the amount of thin filament proteins decreased significantly, but the amount of MLC1 and MLC2 remained about the same. In actin filament-reconstituted myocardium (lane 3), the amount of actin increased significantly. In Tm-reconstituted myocardium (lane 4), the amount of Tm increased significantly. In Tm–Tn-reconstituted myocardium (lane 5), the amount



**Figure 1. SDS-PAGE of myocardium at each step of reconstitution**

Lane 1, control myocardium; lane 2, gelsolin-treated myocardium; lane 3, actin filament-reconstituted myocardium; lane 4, Tm-reconstituted myocardium; lane 5, Tm–Tn-reconstituted myocardium. The Tm-reconstituted myocardium (lane 4) was first treated with gelsolin, then actin filament was reconstituted before Tm was reconstituted. The Tm–Tn-reconstituted myocardium (lane 5) was first treated with gelsolin, then actin filament was reconstituted, followed by sequential reconstitution of Tm and Tn. In lane 2, gelsolin is visible just below the  $\alpha$ -actinin band. Lane 1 is from one gel, and lanes 2–5 are from another gel with a different order. Abbreviations: MHC, myosin heavy chain; Tm, tropomyosin; TnT, troponin T; TnI, troponin I; MLC1, myosin light chain 1; MLC2, myosin light chain 2.

of TnT and TnI increased significantly, hence all thin filament proteins were observed here. In lane 2, some residual actin (~20% of lane 1) can be seen, although a similarly treated preparation did not develop tension (Fig. 3B). This residual actin is presumably located within and/or near to the Z-line, but does not overlap with the thick filament. This amount of actin is essential for a successful reconstitution of the actin filament (Fujita *et al.* 2002).

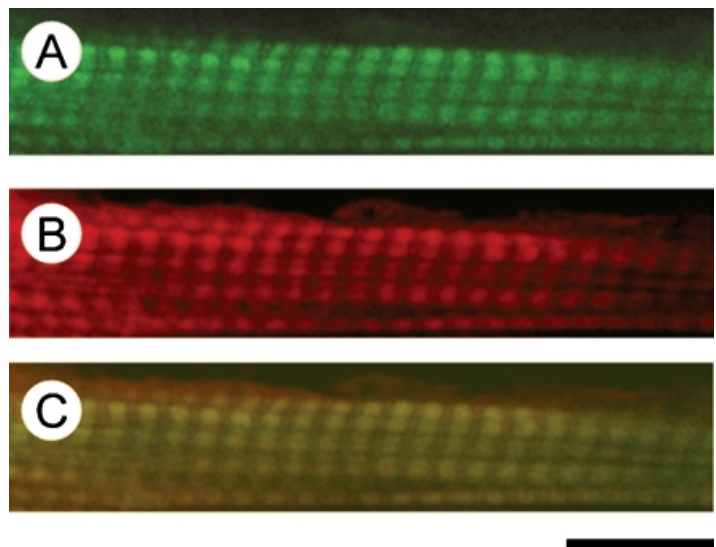
Figure 2 shows confocal fluorescent microscope images of myocardium reconstituted with Rh-IA-labelled Tm and stained with Fl-Ph. The distribution of Fl fluorescence (green), indicating the actin filament, shows a repetitive pattern of bright and dark bands (Fig. 2A). The centre of the bright band corresponds to the Z-line. The distribution of Rh fluorescence (red), indicating Tm, similarly shows a repetitive pattern (Fig. 2B), and the banding pattern appears to be identical to that of the actin filament. To demonstrate colocalization of Tm and the actin filament, both images were superimposed in Fig. 2C, which shows that Tm was homogeneously incorporated onto the actin filament.

Figure 3A–E shows slow pen traces of isometric tension at each step of the thin filament extraction and reconstitution. The myocardium was initially activated with the 5S8P solution and the standard active tension was measured at 25°C (Fig. 3A). Then the myocardium was treated with gelsolin for 100 min (Fig. 3B, Ge) at 2°C in the solution that contained (mM): 117 KCl, 4.25 MgCl<sub>2</sub> (2.2 mM free Mg<sup>2+</sup>), 2.2 ATP (2.0 MgATP<sup>2-</sup>), 2.0 EGTA, 20 Mops (pH 7.0), 2 CaCl<sub>2</sub>, 40 BDM, and 0.3 mg ml<sup>-1</sup> gelsolin. BDM was used to suppress tension development during the gelsolin treatment, because Ca<sup>2+</sup> is a cofactor

for this protein and needed for the extraction. After the gelsolin treatment, active tension did not develop in the standard activating solution (Fig. 3B), demonstrating a removal of the thin filament.

The myocardium was then immersed in the actin-polymerizing solution containing (mM): 80 KI, 4 MgCl<sub>2</sub>, 4 ATP, 4 EGTA, 40 BDM, 20 KP<sub>i</sub> (pH 7.0), and 1.0 mg ml<sup>-1</sup> G-actin at 2°C to reconstitute the actin filament (Fig. 3C, Ac). The actin-polymerizing solution was replaced every 7 min to avoid spontaneous nucleation in the muscle chamber. Similarly, KI instead of KCl was used to deter the nucleation (Funatsu *et al.* 1994). Actin polymerization was performed for a total of 28 min (=7 min × 4). Subsequently, the myocardium was tested with the -Ca solution (5S8P solution without Ca<sup>2+</sup>), and then with the Ca<sup>2+</sup> solution (5S8P) at 25°C (Fig. 3C). Because of the lack of regulatory proteins, this myocardium developed the same tension regardless of the presence of Ca<sup>2+</sup>. Active tension after reconstitution of actin filament was 59 ± 2% (± s.e.m., *n* = 17) of the control myocardium when tested by the standard activating solution (5S8P) that had 8 mM P<sub>i</sub>. Relaxation was achieved by including 40 mM BDM in the relaxing solution.

To reconstitute Tm, actin filament-reconstituted myocardium was immersed in the relaxing solution containing 1.2 mg ml<sup>-1</sup> Tm for 12 h at 2°C (Fig. 3D, Tm). Tm-reconstituted myocardium developed active tension regardless of the presence of Ca<sup>2+</sup> (Fig. 3D). At 25°C, this tension was generally greater than that of actin filament-reconstituted myocardium: active tension after Tm reconstitution was 69 ± 6% (*n* = 17) of control myocardium, an increase of 10% (=69%-59%) over the actin filament-reconstituted myocardium.



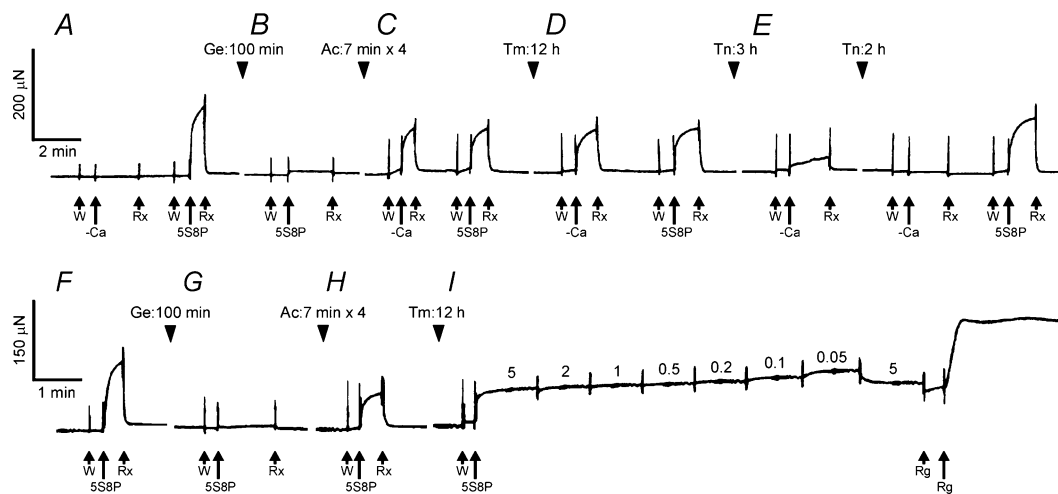
**Figure 2. Confocal fluorescence micrographs of bovine myocardium reconstituted with Rh-IA-labelled Tm and labelled with Fl-Ph**

The thin filament was first removed by gelsolin, and sequentially reconstituted with actin, followed by Rh-IA-labelled Tm. The preparation was then treated with Fl-conjugated phalloidin to label the actin filament. A, Fl fluorescence showing the distribution of the actin filament; B, Rh fluorescence showing the distribution of Tm; C, colocalization of the actin filament and Tm. Calibration bar, 10 μm.

The myocardium was further reconstituted with Tn by immersing it in the relaxing solution containing  $1.0 \text{ mg ml}^{-1}$  Tn at  $2^\circ\text{C}$  (Fig. 3E, Tn). As shown in this figure, 3 h reconstitution with Tn was not adequate, because  $\sim 1/3$  of  $\text{Ca}^{2+}$ -insensitive tension developed, and an additional 2 h was needed to fully restore the  $\text{Ca}^{2+}$  sensitivity; the Tm–Tn-reconstituted myocardium did not develop active tension when  $\text{Ca}^{2+}$  was absent, but it did develop active tension when  $\text{Ca}^{2+}$  was present (Fig. 3E). This observation indicates that Tm and Tn were fully reconstituted. With the reconstitution of Tn, active tension increased to  $83 \pm 9\%$  ( $n = 7$ ) of control myocardium, an increase of  $14\%$  ( $=83\% - 69\%$ ) over the Tm-reconstituted myocardium.

### Effect of MgATP on the exponential process C

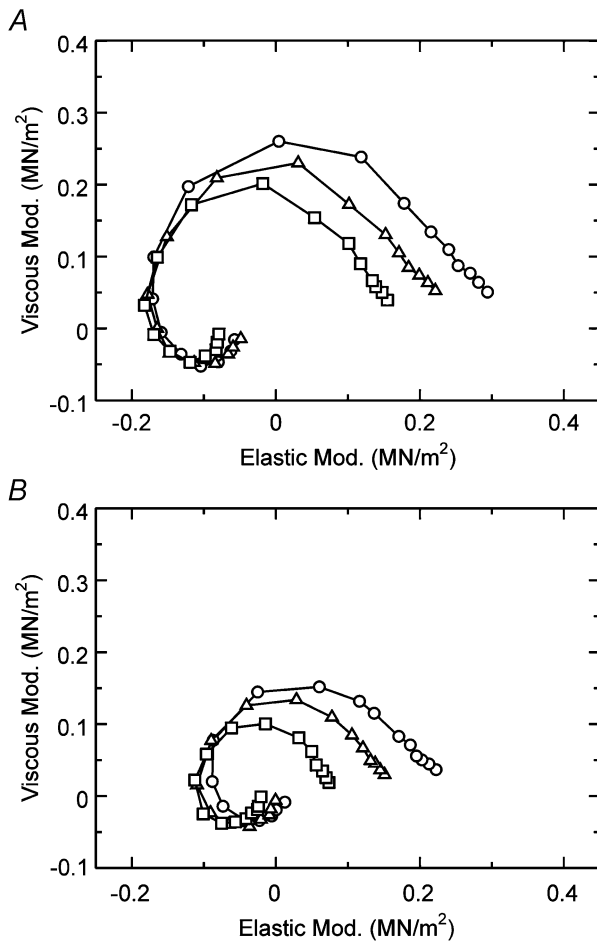
Figure 3F–I shows isometric tension of another myocardium as the thin filament was extracted and reconstituted. In this series of experiments, the reconstitution was only through Tm, and the MgATP and the  $\text{P}_i$  studies were performed. The purpose of these experiments was to investigate whether the larger force observed in the presence of Tm than in its absence is related to a larger force per cross-bridge or to a larger number of force-generating cross-bridges. The effect of MgATP on exponential process C on Tm-reconstituted and Tm–Tn-reconstituted myocardium was studied in the



**Figure 3. Slow pen traces of isometric tension at each step of reconstitution**

A–E, a slow pen trace of isometric tension at each step of removal and reconstitution of the thin filament in one myocardium. In A, a control (untreated) myocardium was first tested with a solution without  $\text{Ca}^{2+}$  (–Ca) to demonstrate that active tension did not develop without  $\text{Ca}^{2+}$ . Then the myocardium was treated with the standard activating solution (5S8P, pCa 4.66) at  $25^\circ\text{C}$ . Before activation, the myocardium was immersed in the same 5S8P solution at  $0^\circ\text{C}$  (labelled W) to wash out BDM, which was present in the Rx solution. The myocardium did not develop active tension in W because of the low temperature. In B, the myocardium was treated by gelsolin (Ge) for 100 min, and then immersed in the 5S8P solution at  $25^\circ\text{C}$  with the result that no tension developed, thus demonstrating that the thin filament was sufficiently removed so that there is no overlap between the thick and the thin filaments. In C, the actin filament was reconstituted for 28 min over four sessions (Ac), then the myocardium was immersed in the solution without  $\text{Ca}^{2+}$  (–Ca) followed by the solution with  $\text{Ca}^{2+}$  (5S8P) at  $25^\circ\text{C}$ . Both treatments resulted in the same active tension development, demonstrating the absence of the regulatory system. In D, Tm was reconstituted for 12 h (Tm). Once again, the active tension developed irrespective of  $\text{Ca}^{2+}$ . In E, reconstitution with Tn was first performed for 3 h; this turned out to be insufficient, as can be seen by the  $\text{Ca}^{2+}$ -insensitive tension. An additional reconstitution for 2 h was necessary to fully restore the  $\text{Ca}^{2+}$  sensitivity, as represented by no tension in the absence of  $\text{Ca}^{2+}$ , and tension levels close to the original tension (A) in the presence of  $\text{Ca}^{2+}$ . F–I, another myocardium was similarly treated as in A–D, resulting in reconstitution of the actin filament with Tm. In this myocardium, the complex modulus data were collected at 7 different MgATP concentrations (0.05–5 mM), as indicated in I. The standard activation at 5 mM MgATP (5S8P) was repeated to detect any decrease in active tension; the preparation was discarded if more than 20% of tension was lost. This activation was followed by an induction of the rigor state with the Rg solution. In all experiments, relaxation was obtained in the solution containing 40 mM BDM (Rx) at  $0^\circ\text{C}$ . All activations including rigor (Rg) were performed at  $25^\circ\text{C}$ .

range of 0.05–5 mM in the presence of 8 mM  $P_i$  under the maximal  $Ca^{2+}$ -activating condition ( $pCa \leq 4.66$ ) and as shown in Fig. 3I. This study characterizes the elementary steps of the MgATP binding step 1 and subsequent cross-bridge detachment step 2 in Scheme 1. Figure 4A shows the Nyquist plot of the complex modulus  $Y(f)$  in the Tm-reconstituted myocardium activated at three different MgATP concentrations (0.1 mM, 0.5 mM, 5 mM). These plots and the MgATP effect are similar to other myocardial systems reported earlier (Kawai *et al.* 1993; Zhao & Kawai, 1996; Wannenburg *et al.* 2000; Fujita *et al.* 2002).

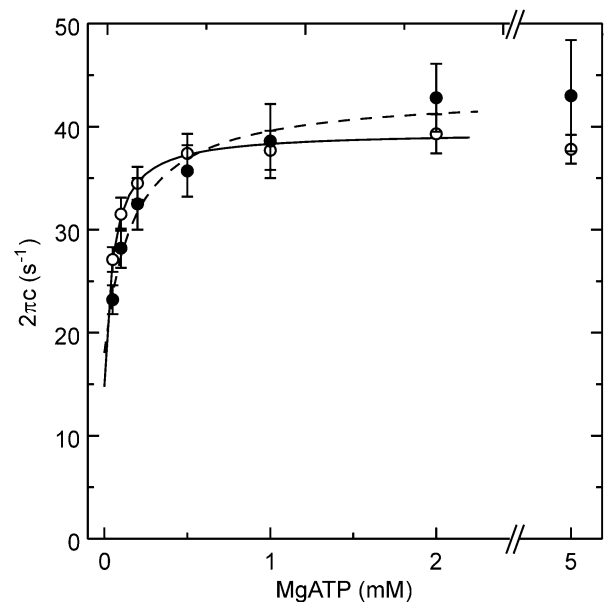


**Figure 4. Nyquist plots of the complex modulus of Tm-reconstituted myocardium**  
 A, the effect of MgATP on the complex modulus  $Y(f)$  in Tm-reconstituted myocardium (average of 9 data).  $\circ$ , 0.1 mM MgATP;  $\Delta$ , 0.5 mM MgATP;  $\square$ , 5 mM MgATP. The phosphate concentration was fixed at 8 mM. B, the effect of  $P_i$  on the complex modulus  $Y(f)$  in Tm-reconstituted myocardium (average of 11 data).  $\circ$ , 2 mM  $P_i$ ;  $\Delta$ , 8 mM  $P_i$ ;  $\square$ , 32 mM  $P_i$ . The MgATP concentration was fixed at 5 mM. Peak-to-peak amplitude was 0.25%  $L_0$ . Data are shown in the Nyquist plot, which is a plot of elastic modulus in the abscissa versus viscous modulus in the ordinate. Frequencies used are (clockwise): 0.13, 0.25, 0.35, 0.5, 0.7, 1, 1.4, 2, 3.1, 5, 7.5, 11, 17, 25, 35, 50, 70 and 100 Hz.

The MgATP dependence on  $2\pi c$  of Tm-reconstituted ( $\circ$ ), and Tm-Tn-reconstituted ( $\bullet$ ) myocardium is shown in Fig. 5 with s.e.m. error bars. The kinetic constants of elementary steps 1 and 2 of the cross-bridge cycle were determined by fitting the MgATP dependence of  $2\pi c$  to eqn (2), and the results are summarized in Table 1. In Tm-reconstituted myocardium, the association constant of MgATP to cross-bridges ( $K_1$ ) was  $20.7 \pm 2.3 \text{ mM}^{-1}$  ( $\pm$  s.e.m.,  $n = 9$ ). This value is about twice of that of control bovine myocardium ( $9.1 \text{ mM}^{-1}$ ) reported previously (Fujita *et al.* 2002). The forward rate constant of cross-bridge detachment step 2 ( $k_2$ ) was  $25.7 \pm 2.7 \text{ s}^{-1}$ , the backward rate constant ( $k_{-2}$ ) was  $13.8 \pm 1.8 \text{ s}^{-1}$ , and the equilibrium constant  $K_2 (= k_2/k_{-2})$  was  $2.3 \pm 0.5$ . These values are almost the same as those of control bovine myocardium reported previously ( $k_2 = 26.6, k_{-2} = 12.1$ , and  $K_2 = 2.6$ ; Fujita *et al.* 2002). In Tm-Tn-reconstituted myocardium,  $K_1$  decreased significantly to  $13.3 \pm 1.9 \text{ mM}^{-1}$  ( $n = 7$ ), which is comparable to the control myocardium. The  $k_2, k_{-2}$ , and  $K_2$  values did not change significantly by the reconstitution of Tn.

**Effects of  $P_i$  on the exponential process B**

To determine the kinetic constants associated with elementary steps 4 and 5 of Scheme 1, we studied the effect of  $P_i$  in the range of 0–32 mM on exponential process B in Tm-reconstituted myocardium. The study



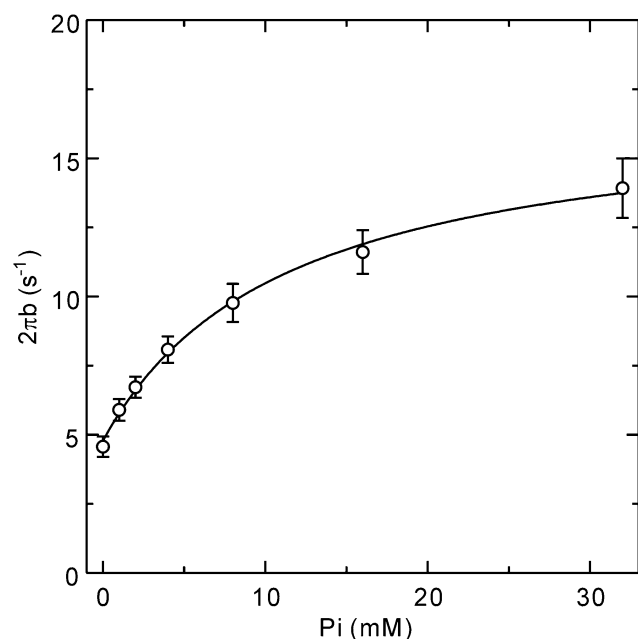
**Figure 5. The rate constant  $2\pi c$  is plotted as a function of the MgATP concentration in Tm- and Tm-Tn-reconstituted myocardium**  
 $\circ$ , Tm-reconstituted myocardium ( $n = 9$ );  $\bullet$ , Tm-Tn-reconstituted myocardium ( $n = 7$ ). Error bars represent s.e.m. Continuous curves are based on eqn (2) with best fit parameters.

**Table 1. The kinetic constants of Tm-reconstituted and Tm-Tn-reconstituted bovine myocardium**

Kinetic constants	Units	Tm-reconstituted	Tm-Tn-reconstituted
$K_1$	$\text{mM}^{-1}$	$20.7 \pm 2.3$ (9)	$13.3 \pm 1.9$ (7)
$k_2$	$\text{s}^{-1}$	$25.7 \pm 2.7$ (9)	$27.6 \pm 3.6$ (7)
$k_{-2}$	$\text{s}^{-1}$	$13.8 \pm 1.8$ (9)	$12.4 \pm 1.6$ (7)
$K_2$	—	$2.32 \pm 0.48$ (9)	$2.70 \pm 0.61$ (7)
$k_4$	$\text{s}^{-1}$	$6.80 \pm 0.60$ (11)	—
$k_{-4}$	$\text{s}^{-1}$	$13.5 \pm 1.70$ (11)	—
$K_4$	—	$0.57 \pm 0.08$ (11)	—
$K_5$	$\text{mM}^{-1}$	$0.13 \pm 0.05$ (11)	—

Values are means  $\pm$  s.e.m. The number of observations is shown in parentheses.

was carried out in the presence of the saturating MgATP concentration (5 mM) under maximal  $\text{Ca}^{2+}$ -activating conditions (pCa 4.66). Figure 4B shows the Nyquist plot of the complex modulus  $Y(f)$  in the Tm-reconstituted myocardium activated at three different  $\text{P}_i$  concentrations (2 mM, 8 mM, 32 mM). The complex modulus data were fitted to eqn (1) to obtain the apparent rate constant  $2\pi b$ .  $2\pi b$  data were then plotted against  $\text{P}_i$  concentration (Fig. 6) and fitted to eqn (3) (Kawai & Halvorson, 1991) to deduce the kinetic constants of elementary steps 4 and 5. The results are summarized in Table 1. In the Tm-reconstituted myocardium, the rate constant of the force generation step ( $k_4$ ) was  $6.8 \pm 0.6 \text{ s}^{-1}$  ( $n = 11$ ) and its reversal step ( $k_{-4}$ ) was  $13.5 \pm 1.7 \text{ s}^{-1}$ . Its equilibrium constant  $K_4$  ( $= k_4/k_{-4}$ ) was  $0.57 \pm 0.08$ , and the  $\text{P}_i$  association constant ( $K_5$ ) was  $0.13 \pm 0.05 \text{ mM}^{-1}$ . These



**Figure 6. The rate constant  $2\pi b$  is plotted as a function of the  $\text{P}_i$  concentration in Tm-reconstituted myocardium**

Error bars represent s.e.m. ( $n = 11$ ). A continuous curve is based on eqn (3) with best fit parameters.

values are qualitatively the same compared to the control myocardium reported earlier ( $k_4 = 7.1$ ,  $k_{-4} = 12.6$ ,  $K_4 = 0.59$ , and  $K_5 = 0.14$ ; Fujita *et al.* 2002).

### Isometric tension and stiffness

Figure 7 shows isometric tension, stiffness, and the tension/stiffness ratio plotted against MgATP concentration in the Tm-reconstituted myocardium. These results are based on the same experiments as shown in Fig. 5. Both isometric tension and stiffness decreased whereas the ratio increased by the increase in the MgATP concentration. This result is in agreement with previous results in myocardium (Kawai *et al.* 1993; Fujita *et al.* 2002) as well as in skeletal muscle fibres (Kawai & Zhao, 1993). Figure 8 shows isometric tension, stiffness, and the tension/stiffness ratio plotted against the  $\text{P}_i$  concentration for the same experiments as shown in Fig. 6 in Tm-reconstituted myocardium. Both isometric tension and stiffness decreased with the increase in the  $\text{P}_i$  concentration, which is in agreement with previous results in myocardium (Nosek *et al.* 1990; Kawai *et al.* 1993; Fujita *et al.* 2002) as well as in skeletal muscle fibres (Dantzig *et al.* 1992; Kawai & Zhao, 1993).

In the presence of 8 mM  $\text{P}_i$  (standard activating condition with 5S8P), isometric tension in the control myocardium was  $15.4 \pm 1.1 \text{ kN m}^{-2}$  ( $n = 17$ ). In the absence of added  $\text{P}_i$  (5S0P), isometric tension in the control myocardium was  $27.6 \pm 3.2 \text{ kN m}^{-2}$  ( $n = 11$ ). Isometric tension after reconstitution of the actin filament decreased to  $61 \pm 5\%$  ( $n = 11$ ) of the control myocardium when tested with the 5S0P solution. After reconstitution of Tm, the active tension increased to  $69 \pm 7\%$  ( $n = 11$ ) of the control myocardium with the 5S0P solution.

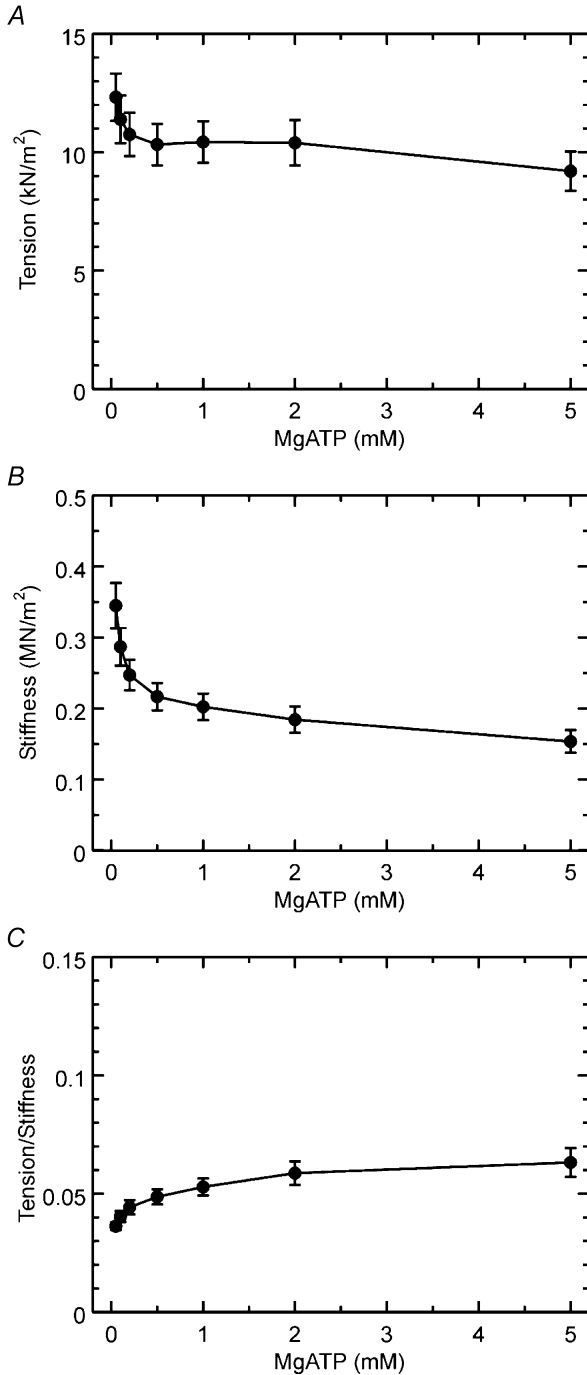
### Cross-bridge distribution

Cross-bridge distribution at the standard activation condition (5S8P) of Tm-reconstituted myocardium was calculated based on eqn (18) of Kawai & Halvorson (1991)



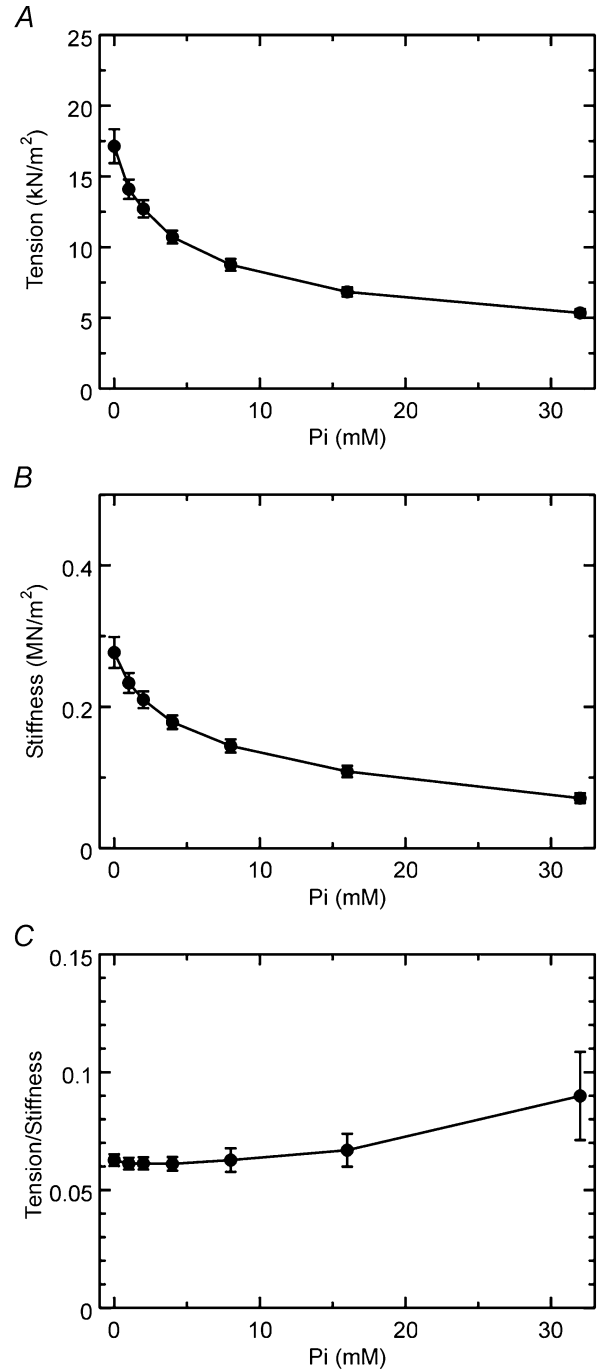
using the equilibrium constants in Table 1, and shown in Fig. 9. The probability of cross-bridges in the AM state was 0.16% and not significantly populated. This is because  $K_1$  is large and a saturating concentration of MgATP was

used. The probability of force-generating states AM\*DP and AM\*D was about 22% each, and the probability of the AM\*S state was about 17%. Cross-bridges were mostly populated in the 'Det' state, which was about 39%. The Det state is a combination of detached states (MS and



**Figure 7. Isometric tension (A), stiffness (B), and the ratio (tension/stiffness) (C) are plotted against MgATP concentration in Tm-reconstituted myocardium**

Error bars represent s.e.m. ( $n = 9$ ). Experiments were performed in the presence of 8 mM  $P_i$ .



**Figure 8. Isometric tension (A), stiffness (B), and the ratio (tension/stiffness) (C) are plotted against the  $P_i$  concentration in Tm-reconstituted myocardium**

Error bars represent s.e.m. ( $n = 11$ ). Experiments were performed in the presence of 5 mM MgATP.

MDP) and weakly attached states (AMS and AMDP) (see Scheme 1). These states cannot be distinguished with our analysis, which depends on strongly attached states. This cross-bridge distribution was not significantly different from the control bovine myocardium or the nTm-reconstituted myocardium reported previously (cf. Fig. 6 of Fujita *et al.* 2002).

## Discussion

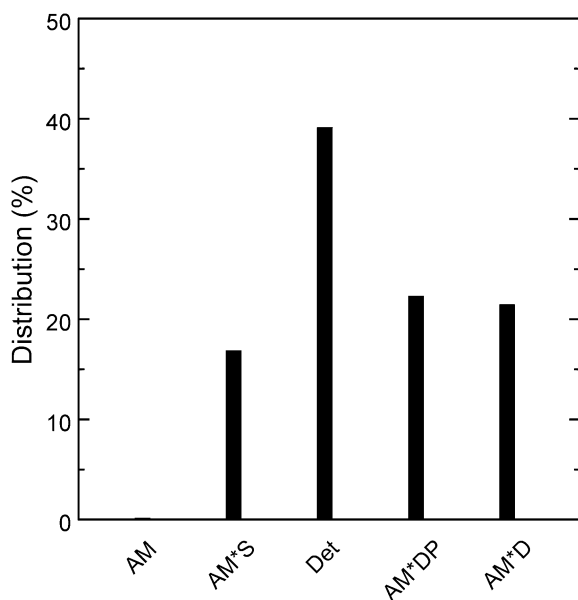
### Reconstitution of Tm and Tn

We have succeeded in selectively removing the thin filament from bovine myocardium, and sequentially reconstituting the myocardium with actin, Tm, and then with Tn. The degree of reconstitution was quantified by SDS-PAGE (Fig. 1), confocal images (Fig. 2), and isometric tension (Fig. 3). The SDS-PAGE demonstrates removal of the thin filament (Fig. 1, lane 2), reconstitution of the actin filament (lane 3), reconstitution of Tm (lane 4), and reconstitution of Tn (lane 5). In lane 5, TnT and TnI can be identified. As expected, the confocal images demonstrate that actin (Fig. 2A) and Tm (Fig. 2B) can be seen colocalized (Fig. 2C), indicating that Tm was incorporated into the actin filament homogeneously. The

functional reconstitution was assessed by isometric tension (Fig. 3). Both actin filament-reconstituted myocardium and Tm-reconstituted myocardium developed tension irrespective of the presence of  $\text{Ca}^{2+}$  (Figs 3C and D). These results demonstrate that the  $\text{Ca}^{2+}$  regulatory system was absent in these preparations as expected. In Tm-Tn-reconstituted myocardium, the  $\text{Ca}^{2+}$  sensitivity returned: the myocardium developed tension when  $\text{Ca}^{2+}$  was added to the activating solution, and the myocardium relaxed when  $\text{Ca}^{2+}$  was removed from the solution (Fig. 3E). These results imply that the  $\text{Ca}^{2+}$  regulatory system was restored by the Tm and Tn reconstitution. Thus, we conclude that the reconstitution of the thin filament-extracted myocardium with actin, Tm and Tn was both structurally and functionally complete.

### Tension augmentation by reconstitution of Tm

Our previous study (Fujita *et al.* 2002) was focused on the effect of native tropomyosin (nTm), which is a complex of Tm and Tn. We noticed enhancement of active tension by 40% (relative to initial control tension) with nTm, from which we concluded that the enhancement was based on an increase in the force supported by each cross-bridge. This conclusion was also supported by our observation that the number of force-generating cross-bridges decreased by about 20% with the nTm reconstitution (Fujita *et al.* 2002). However, at that time we were not able to tell whether this enhancement was based on Tm, Tn or both. To determine which one of these proteins enhances isometric tension, we reconstituted Tm and Tn sequentially in this report. We found that Tm in the absence of Tn enhanced isometric tension by about 10%. This result demonstrates that Tm partially contributes to the enhancement of isometric tension. Previous studies using *in vitro* motility assays on actin and heavy meromyosin (HMM) demonstrated a similar contribution of Tm to force (VanBuren *et al.* 1999; Bing *et al.* 2000). This coincidence of results from two very different methods is remarkable, because fibre experiments are typically carried out at or near physiological ionic strength ( $\sim 200$  mM), whereas *in vitro* motility assays are typically carried out at low ionic strength (50–70 mM). It has been known for some time that the mechanisms of force generation depend on ionic interaction (Sutoh, 1993) as well as hydrophobic interaction (Zhao & Kawai, 1994). The ionic interaction is diminished by an increase in the ionic strength, whereas the hydrophobic interaction is unchanged by ionic strength.



**Figure 9. Calculated cross-bridge distribution in Tm-reconstituted myocardium**

Calculated cross-bridge distribution based on the equilibrium constants shown in Table 1 and under standard activating conditions (5S8P). Det, detached state, which is a combination of weakly attached states (AMS and AMDP) and truly detached states (MS and MDP). Other abbreviations are the same as those in Scheme 1.

### Tension augmentation by reconstitution of Tn

We then added Tn to the Tm-reconstituted myocardium. We found that the isometric tension increased further by 14% when Tn was added to the Tm-reconstituted myocardium in the presence of  $\text{Ca}^{2+}$ . This fact implies that Tn has a further activating role in the actomyosin interaction. This activating role must be mediated by Tm, actin, or both. Such an enhancement of tension by Tn and  $\text{Ca}^{2+}$  was observed previously in single molecule experiments on tension and gliding speed (Gordon *et al.* 1998; Bing *et al.* 2000; Homsher *et al.* 2000) using *in vitro* motility assays. The combined recovery of isometric tension was 24%, which is consistent with our earlier results (Fujita *et al.* 2002) using nTm to reconstitute the regulatory system.

### The effect of Tm on the elementary steps of the cross-bridge cycle

We studied the cross-bridge kinetics of Tm-reconstituted myocardium by sinusoidal analysis, and found that most kinetic constants of the elementary steps resumed those of the control myocardium as soon as Tm was reconstituted. An exception was  $K_1$  (MgATP binding constant) which was about  $2 \times$  the control myocardium. These results contrast with our previous study of actin-filament-reconstituted myocardium, in which  $K_2$ ,  $K_4$  and  $K_5$  became  $0.23 \times$ ,  $4.4 \times$  and  $2.8 \times$  of the control myocardium, respectively (Fujita *et al.* 2002); there was little change in  $K_1$ . Thus, it can be concluded that Tm enhances ATP binding, but otherwise Tm reconstitution recovers the equilibrium constants to the control level. The distribution of cross-bridge states is not very different between Tm-reconstituted (Fig. 9), nTm-reconstituted, and control myocardium (Fig. 6 of Fujita *et al.* 2002). These facts imply that Tm partially modifies actin configuration so as to enhance stereospecific and hydrophobic interaction between actin and myosin molecules. This modification increases the force generated by each cross-bridge. A similar line of evidence was presented by using  $\Delta 23\text{Tm}$ , a Tm mutant (Lu *et al.* 2003). A model that accounts for the regulatory proteins-actin interaction was proposed by Tobacman & Butters (2000).

### The effect of Tn on the elementary steps of the cross-bridge cycle

We found that the kinetic constants simulated those of the control myocardium as soon as Tn was reconstituted and  $\text{Ca}^{2+}$  was included in the activating saline. This fact

implies that the reconstitution was functionally complete. What is important is that isometric tension increased further to  $1.2 \times$  with the Tn reconstitution. The kinetic constants of Tm-reconstituted myocardium and Tm-Tn-reconstituted myocardium were almost the same, except that  $K_1$  decreased following the Tn reconstitution (see above). This decrease, however, does not seriously affect the overall distribution of cross-bridges among the six states; hence the number of force-generating cross-bridges is approximately the same before and after the Tn reconstitution (cf. Fig. 9 and Fig. 6 of Fujita *et al.* 2002). The reason for the tension increase must therefore be based on an increase in cross-bridge force, i.e. each cross-bridge must generate a larger force as Tn is reconstituted. The fact that the addition of Tn enhanced isometric tension by 14% over the Tm-reconstituted myocardium implies that the tension on each cross-bridge increased by  $\sim 14\%$ .

It may be interesting to discuss why  $K_1$  was altered at each step of reconstitution. This observation is consistent with the idea that Tm and Tn affect the microenvironment of the nucleotide-binding site on myosin. This effect must be mediated through chain reactions that include Tm, actin, actomyosin interface and the myosin head. How could this be possible? One possibility is that isometric tension itself can mediate such chain reactions: we have known from previous studies that  $K_1$  becomes larger with smaller tensions in rabbit psoas fibres (Zhao *et al.* 1996). Furthermore, our earlier studies have shown that different muscle types have different  $K_1$  values when the amino acid sequence of the nucleotide-binding site is nearly identical (Wang & Kawai, 2001). This difference may depend on the charge distribution of loop 1 of MHC: if it is more positive, the  $\text{MgATP}^{2-}$  molecule may bind more strongly. It is likely that the positively charged loop 1 readily attracts the negatively charged MgATP molecule. Therefore, the possibility arises that the charge distribution and the position of loop 1 may contribute to the ATP binding affinity (Kelley *et al.* 1993; Rovner *et al.* 1997; Sweeney *et al.* 1998; Wang & Kawai, 2001). In fact, it is known that loop 1 comes close to the nucleotide-binding pocket (Rayment *et al.* 1993); hence loop 1 could serve as a local ATP carrier. This loop exists between the N-terminus 25K and 50K domains of the myosin head, and its position may change depending on the state of the thin filament and/or tension imposed on the myosin head. An interesting hypothesis is that the position of the loop 1 is altered by each step of reconstitution of Tm and Tn on the actin filament, so as to change the binding affinity of the  $\text{MgATP}^{2-}$  molecule.

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