The Effect of Partial Extraction of Troponin C on the Elementary Steps of the Cross-Bridge Cycle in Rabbit Psoas Muscle Fibers

Yan Zhao, P. M. Gangadhara Swamy, Karen A. Humphries, and Masataka Kawai
Department of Anatomy, College of Medicine, The University of Iowa, Iowa City, Iowa 52242, USA

ABSTRACT The elementary steps of the cross-bridge cycle in which troponin C (TnC) was partially extracted were investigated by sinusoidal analysis in rabbit psoas muscle fibers. The effects of MgATP and phosphate on the rate constants of exponential processes were studied at 200 mM ionic strength, pCa 4.20, pH 7.00, and at 20°C. The results were analyzed with the following cross-bridge scheme:

$$\mathrm{AM} \underset{\mathrm{S}}{\overset{K_1}{\rightleftharpoons}} \mathrm{AM^*S} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \begin{bmatrix} \mathrm{AMS} & \rightleftharpoons & \mathrm{AMDP} \\ \updownarrow & & \updownarrow \\ \mathrm{MS} & \rightleftharpoons & \mathrm{MDP} \end{bmatrix} \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} \mathrm{AM^*DP} \underset{K_5}{\overset{\mathrm{P}}{\rightleftharpoons}} \mathrm{AM^*D} \xrightarrow{k_6}$$

where A is actin, M is myosin, S is MgATP, D is MgADP, and P is phosphate (P_i). When TnC was extracted so that the average remaining tension was 11% (range 8–15%), K_1 (MgATP association constant) increased to 7×, k_2 (rate constant of cross-bridge detachment) increased to 1.55×, k_{-2} (reversal of detachment) decreased to 0.27×, and K_2 (= k_2/k_{-2}: equilibrium constant of cross-bridge detachment) increased to 6.6×. k_4 (rate constant of force generation) decreased to 0.4×, k_{-4} (reversal of force generation) increased to 2×, K_4 (= k_4/k_{-4}) decreased to 0.17×, and K_5 (P_i association constant) did not change. The activation factor α, which represents the fraction of cross-bridges participating in the cycling, decreased from 1 to 0.14 with TnC extraction. The fact that K_1 increased with TnC extraction implies that the condition of the thin filament modifies the contour of the substrate binding site on the myosin head and is consistent with the Fenn effect. The fact that α decreased to 0.14 is consistent with the steric blocking mechanism (recruitment hypothesis) and indicates that some of the cross-bridges disappear from the active cycling pool. The fact that the equilibrium constants changed is consistent with the cooperative activation mechanism (graded activation hypothesis) among thin-filament regulatory units that consist of troponin (TnC, TnI, TnT), tropomyosin, and seven actin molecules, and possibly include cross-bridges.

INTRODUCTION

It has been known for some time that muscle contraction involves the interaction of two sets of interdigitating filaments (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). The thick filament contains a macromolecule myosin, and its N-terminus head portion projects out toward the thin filament to make a cyclic contact. It is this myosin cross-bridge that hydrolyzes ATP and converts its free energy into useful work. The catalytic function of the myosin cross-bridge requires the thin-filament proteins actin (A), tropomyosin (Tm), and troponin (Tn). Therefore it is not surprising that contractile regulation in striated muscle is predominantly accomplished by thin-filament proteins. Troponin (Ebashi and Endo, 1968) is the center of the regulation, and it has three subunits: TnC (for Ca^{2+} binding), TnI (inhibition), and TnT (Tm binding) (Greaser and Gergely, 1973). A thin filament's "regulatory unit" is made up of the Tn-Tm complex with seven actin monomers. Structural studies suggest that the thin filaments consist of two strands of actin with two regulatory units that wind together into a double helix (Hanson and Lowy, 1963). When Ca^{2+} binds to two low-affinity sites on TnC, this signal is relayed to Tm via TnI and TnT (Potter and Gergely, 1975; Holroyde et al., 1980). Tm in turn exposes the active site on actin to enable interaction with myosin cross-bridges. This is known as the steric blocking mechanism (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973), and it is a mechanism of regulation in skeletal and cardiac muscle contraction.

There is increasing evidence that each regulatory unit is not independent, and the neighboring regulatory units cooperate on Ca^{2+} activation based on both physiological and biochemical studies. This cooperativity can be seen by the steep slope of the pCa/tension relationship in skinned fibers (Brandt et al., 1980, 1982, 1984; Moss et al., 1983, 1985) and by the steep pCa/ATPase relationship in isolated actomyosin subfragment 1 (acto-S1) systems (Murray and Weber, 1980; Grabarek et al., 1983). These studies suggested that the steep slope is due to extended cooperativity between neighboring regulatory units (Hill et al., 1980; Hill, 1983; Brandt et al., 1984, 1987; Tobacman, 1996). Cooperativity may not be limited to thin-filament proteins. Güth and Potter (1987) and Hannon et al. (1992) observed that the

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Address reprint requests to Dr. Masataka Kawai, Department of Anatomy, University of Iowa, Bowen Science Building, 1-670, Iowa City, IA 52242. Tel.: 319-335-8101; Fax: 319-335-7198; E-mail: mkawai@blue.weeg.uiowa.edu.

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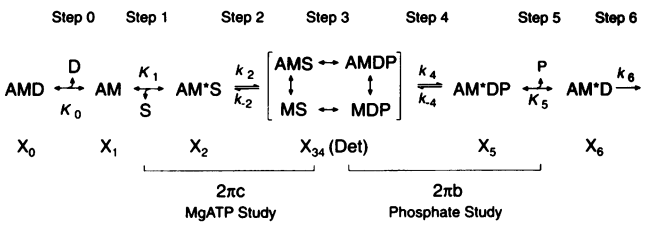
activation level of TnC was increased by cross-bridge attachement, possibly via an increase in the Ca^{2+} affinity of TnC. Moss et al. (1983, 1985) and Metzger and Moss (1991) suggested that the steepness in the pCa/tension relationship may also be due to the result of direct interaction of Ca^{2+} with myosin cross-bridges.

Historically, two hypotheses were proposed for the mechanism of the Ca^{2+} activation of muscle contraction. In the recruitment hypothesis, as suggested by Podolsky and Teichholz (1970), cross-bridges are activated in an all-or-none manner when Ca^{2+} binds to the thin filament. Julian (1969) suggested a graded activation hypothesis, assuming that the rate constant of the cross-bridge attachment is proportional to the number of regulatory sites occupied by Ca^{2+}. Podolsky and Teichholz (1970) and Gulati and Podolsky (1978) observed that the maximum velocity of shortening (V_{max}) did not change with Ca^{2+} concentration, supporting the recruitment mechanism. However, Julian (1971) and Julian and Moss (1981) observed that the V_{max} increased with an increase in the Ca^{2+} concentration, supporting the graded activation mechanism. A difficulty with the V_{max} measurement is that because the length change must be large, parallel elements to cross-bridges such as connectin-titin, collagen, and damaged cross-bridges would modify the results.

With sinusoidal length changes, we observed that the rate constant of process (B) did not change with the Ca^{2+} concentration, from which we concluded that the recruitment hypothesis is the predominant mechanism of Ca regulation of contraction (Kawai et al., 1981, 1984). We further observed the presence of a slower exponential process (B') during partial Ca activation, from which we inferred that the thin filament exists in two activated forms, a low level of activation and a high level of activation (Kawai et al., 1981, 1984). With photolysis of caged P_i, which increases the P_i concentration in a stepwise fashion, Millar and Homsher (1990) observed that the rate constant of the tension transient is not affected by the Ca^{2+} concentration, whereas Walker et al. (1992) observed that the rate constant was affected by the Ca^{2+} concentration. With a rapid decrease in the pressure, which presumably modifies the force generating step, Fortune et al. (1994) observed that the fast phase of tension transient was not affected by the Ca^{2+} concentration. All of these studies were carried out in rabbit psoas fibers. These conflicting results justify a detailed analysis of the elementary steps of the cross-bridge cycle in terms of the effect of the Ca^{2+} concentration. However, a complication with experiments that changes the Ca^{2+} concentration is that if Ca^{2+} has an additional role in myosin or in other proteins associated with thick filaments (Moss et al., 1983, 1985; Metzger and Moss, 1991), it then becomes more difficult to substantiate the role of Ca binding to TnC. A simplified experimental system in which the role of the regulatory strands can be studied independently is needed. A preparation with TnC partially extracted satisfies this criterion, because when activated by full Ca^{2+}, all possible sites on the thick filament are occupied by Ca^{2+}, yet one can experimentally vary the number of active regulatory units by changing the degree of TnC extraction (Metzger and Moss, 1991).

Recently we succeeded in resolving six to seven cross-bridge states and elementary steps in muscle fiber systems by using sinusoidal analysis (Kawai and Halvorson, 1989, 1991; Kawai and Zhao, 1993; Zhao and Kawai, 1996). This analysis method requires small length changes to be applied, and hence the parallel elements can be separated from the cross-bridge's contribution. The following scheme summarizes our results:



Scheme 1

where A is actin, M is myosin, S is MgATP (substrate), D is MgADP, P is phosphate (P_i), and the asterisk (*) indicates a second conformation. The four states in [ ] are combined together and called the detached (Det) state; they are weakly attached states and truly detached states. X_i represents the probability of each cross-bridge state. The purpose of the current study is to resolve the elementary step(s) that is modulated by a partial TnC extraction. This characterization requires MgATP and phosphate effects to be studied as indicated above under Scheme 1. The characterization could not be performed on partially Ca-activated fibers, because both MgATP and P_i are known to shift pCa/tension relationships (Brandt et al., 1982), and hence a change in the MgATP or P_i concentration would change the degree of activation. Interestingly, our results indicate that with TnC extraction, the substrate binding step (K_1) and the cross-bridge detachment step (K_2) are accelerated, whereas the force generation step (K_4) is diminished. Preliminary accounts of the present results have been published (Zhao et al., 1994; Kawai and Zhao, 1995).

MATERIALS AND METHODS

Chemicals and solutions

Creatine phosphate (Na_2CP), adenosine 5′-triphosphate (Na_2H_2ATP), 3-[*N*-morpholino]propane sulfonic acid (MOPS), and H_4EGTA were purchased from Sigma Chemical Co. (St. Louis, MO); $CaCO_3$, MgO, NaOH, KOH, KH_2PO_4, K_2HPO_4, propionic (Prop) acid, and $AgNO_3$ were from Fisher Scientific Co. (Itasca, IL); creatine kinase (CK) was from Boehringer Mannheim (Indianapolis, IN); sodium dodecyl sulfate (SDS), polyacrylamide, bis-acrylamide, and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were from Bio-Rad (Chicago, IL). All chemicals were of analytical grade.

The compositions of the solutions are shown in Table 1. Experimental solutions are designated by *m*Sr*n*P, where *m* represents the millimolar

TABLE 1 Experimental solutions

Solutions	K_2EGTA	$K_2CaEGTA$	$CaPr_2$	Na_2MgATP	$MgPr_2$	$Na_2K_{1.7}ATP$	Na_2CP	$K_{1.5}P_i$	KPr	NaPr	MOPS
Relaxing	6	—	—	2.0		5.0	—	8	48	62	10
Washing	—	—	—	0.5		—	—	8	102	75	10
Control	—	6	—	5.79	—	1.35	15	8	73	11	10
Experimental											
0S8P	—	6	0.049	—	0.845	—	15	8	88	25	10
5S8P	—	6	0.354	5.79	—	1.56	15	8	72	10	10
5S0P	—	6	0.358	5.82	—	1.56	15	—	91	10	10
5S32P	—	6	0.343	5.70	—	1.56	15	32	16	11	10

Total concentrations added are shown in mM. The ionic strength of all solutions is 200 mM, and pH is adjusted to 7.00 ± 0.01. An experimental solution is designated by *m*S*n*P, in which *m* and *n*, respectively, indicate mM concentrations of $MgATP^{2-}$ and P_i (e.g., 5S8P includes 5 mM $MgATP^{2-}$ and 8 mM P_i). The pCa of all experimental solutions is 4.20, the Mg^{2+} concentration is 0.5 mM, total Na is 55 mM, and the solutions contain 320 units/ml CK. The control solution is similar to 5S8P, but pCa = 4.66, and CK = 160 units/ml. Pr, propionate; CP, creatine phosphate; MOPS, 3-[*N*-morpholino]propane sulfonic acid.

concentration of $MgATP^{2-}$ (S), and *n* represents that of phosphate (P): e.g., the 5S8P solution contained 5 mM $MgATP^{2-}$ and 8 mM P_i. The pCa of all experimental solutions was 4.20, pH was adjusted to 7.00 ± 0.01, the Mg^{2+} concentration was 0.5 mM, the total Na was 55 mM, and the experimental solutions contained 320 units/ml CK. The control activating solution was similar to the 5S8P solution, except that pCa was 4.66, and CK was 160 U/ml. All fibers prepared were activated with the control activating solution, and isometric tension and complex modulus were compared among different experiments and reports to ensure that all preparations behaved similarly. The data from control activation were also used for normalization. When the effects of MgATP and P_i were studied, their concentrations were increased in one group of experiments and decreased in the other group, so that the averaged results contained about half of each group. In this way systematic errors due to possible progressive deterioration of the fibers can be minimized. EGTA, CaEGTA, and P_i were added as neutral K salts; MgATP and CP as neutral Na salts; and free ATP was added as $Na_2K_{1.7}ATP$ (neutral salt). We assumed multiple equilibria, and individual concentrations of multivalent ionic species were calculated with our computer program using the following apparent association constants (log values at pH 7.00): CaEGTA, 6.28; MgEGTA, 1.61; CaATP, 3.70; MgATP, 4.00; CaCP, 1.15; MgCP, 1.30. All experiments were performed at 20.0 ± 0.2°C.

Fiber preparations

New Zealand white rabbits (3–5 kg body weight) were sacrificed by injecting Na-pentobarbital (150 mg/kg) intravenously. Psoas muscles were exposed and cooled immediately from the front by filling the peritoneal cavity with crushed ice. The skin from the back area was removed, and the muscles were cooled. After 30 min in ice, the fiber bundles (~60 mm in length and 3 mm in diameter) from psoas muscle were tied to bamboo sticks at body length with silk threads and then excised. The bundles were chemically skinned in a solution containing (mM) 5 EGTA, 2 MgATP, 5 ATP, 132 NaProp, and 6 imidazole (pH 7.0) at 0–2°C. After 48 h in skinning solution the bundles were transferred to a storage solution containing (mM) 5 EGTA, 2 MgATP, 5 free ATP, 132 KProp, 6 imidazole (pH 7.0), and ~6 M glycerol (50%, v/v); bundles were kept at −20°C without freezing. The procedure of cooling the muscles and the use of the Na skinning solution helped in obtaining good fiber preparations (Zhao and Kawai, 1994b). Single or double fibers about 20 mm in length were dissected from the stock bundle and used for the experiments.

We used two fish-hook-shaped muscle clamps made of tungsten wire (diameter 125 μm); one was attached to the length driver, and the other to the force transducer. Each end of the muscle fiber was doubly knotted, and the straight portion toward the center was inserted into the eyes of the hooks. The fiber was stretched slightly, the sarcomere length was adjusted to 2.5 μm by optical diffraction using a He-Ne laser (Spectra Physics, Mountain View, CA), and the fiber length (L_0) was determined. The knots served as stoppers when the muscle was in tension. The cross-sectional area of an individual fiber was estimated by measuring the diameter with an ocular micrometer and by assuming a circular shape. In a typical experiment, a preparation was tested with the control activating solution (500 μl) and then relaxed. At this point, complex modulus data were collected to record the baseline. The saline was changed with the wash solution, followed by two full-volume changes of the experimental solution. The tension started to rise quickly and reached a plateau in a few seconds. The complex modulus data were then collected for the active response, and the fiber was relaxed. This procedure was repeated for a series of experimental solutions.

Transient analysis by sinusoidal length changes and deduction of kinetic constants

The sinusoidal waveform was digitally synthesized in a 386 CPU PC (Industrial Computer Source, San Diego, CA) that controlled the length driver via a 14-bit digital-to-analog converter. This oscillated the length of the fiber at a small peak-to-peak amplitude (0.25% L_0) and at 18 discrete frequencies (f) ranging from 0.25 to 350 Hz. Length and tension signals were simultaneously digitized by two 16-bit analog-to-digital converters (AD676; Analog Device), and the complex modulus data $Y(f)$ were calculated. The complex modulus is the ratio of the stress change to the strain change represented in the frequency domain. The complex modulus data collected during relaxation were subtracted from those during activation to correct for the extraneous pickup that is unavoidable at higher frequencies.

The frequency profile of the complex modulus data was resolved into three exponential processes (A, B, and C) by fitting the data to Eq. 1:

$$\text{Process A} \qquad \text{Process B} \qquad \text{Process C}$$

$$Y(f) = H + A/(1 + a/fi) - B/(1 + b/fi) + C/(1 + c/fi), \tag{1}$$

where $i = \sqrt{-1}$. Lowercase letters *a*, *b*, and *c* represent the characteristic frequencies of the respective processes, and uppercase letters *A*, *B*, and *C* represent respective magnitudes. Process A is an exponential advance and the slowest process ($a \approx 1$ Hz), where the muscle absorbs work from the oscillating length driver. Process B is a medium-frequency exponential delay ($b \approx 17$ Hz) at which muscle generates oscillatory work on the length driver. Process C is a fast exponential advance ($c \approx 60$ Hz) at which the muscle absorbs work. Processes A, B, and C correspond, respectively, to phase 4, phase 3, and phase 2 of tension transients in response to a step length change (Huxley and Simmons, 1971; Heinl et al., 1974). In relaxed and rigor muscle fibers three exponential processes are absent. $2\pi a$, $2\pi b$, and $2\pi c$ are the apparent rate constants of the processes. Details of the sinusoidal analysis technique have been published previously (Kawai and Brandt, 1980).

Extraction of troponin C from skinned fibers

Two methods, I and II, were employed to extract TnC from skinned psoas fibers. In method I, adapted from Cox et al. (1981), TnC was extracted by exposing the muscle fibers to 5 mM EDTA, 20 mM Tris, pH 7.8 at 20°C for 0–30 min. In method II, adapted from Brandt et al. (1987), TnC was extracted by exposing the fibers to 5 mM EDTA, 10 mM MOPS, pH 7.2 at 20°C for 0–30 min. Both methods were used for biochemical analysis, but only method I was used for sinusoidal analysis (MgATP and P_i studies). The solution for bathing skinned fibers was gently stirred during extraction in both methods.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed under the reducing condition according to the method of Laemmli (1971), with an acrylamide-to-bisacrylamide ratio of 37.5. The gels were stained with silver according to the method of Giulian et al. (1983), a modified procedure of Oakley et al. (1980), except that glutaraldehyde fixation time was extended to 12 h (Moss et al., 1985). The density of the stained and dried gels was scanned by video camera (Hamamatsu Newvicon video, Japan) connected through a camera controller (Hamamatsu C2400, Japan) to a Micro Vax II computer (DEC), and the density profiles were digitized and displayed on the computer screen. The relative areas under the peaks were integrated. A common baseline that is very close to the base of the peaks was used to subtract background from the peaks. Most of the background was due to the gel matrix. The amount of TnC present in the fiber before and after extraction was determined as the ratio of the area of the peak corresponding to TnC to that corresponding to TnI.

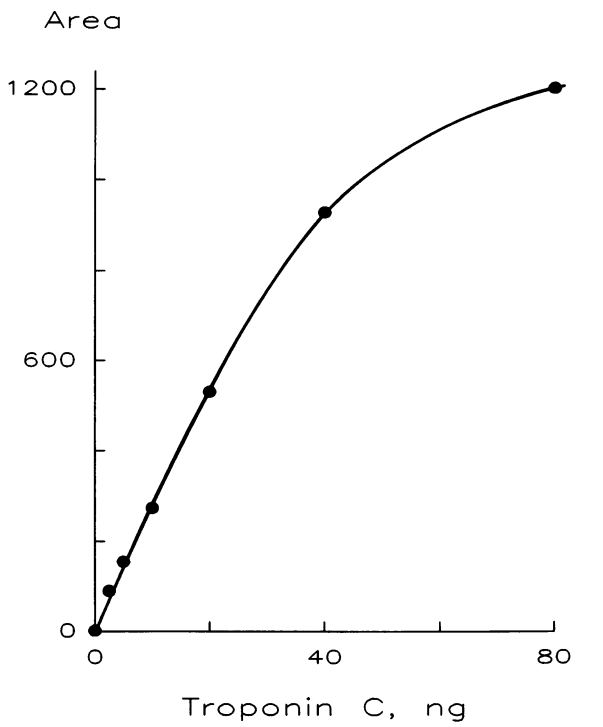


FIGURE 1 Calibration curve of TnC. The specified amount (shown in the abscissa) of purified TnC was loaded onto each lane of the gel, SDS-polyacrylamide gel electrophoresis was performed, and the gel was silver stained. Densitometric trace was then obtained, and the staining intensity of the TnC band was calculated by integrating the area under the TnC peak. Area values are expressed in arbitrary units.

RESULTS

Proportionality between the TnC content and the staining intensity

To establish the proportionality between the TnC content and the intensity of silver staining, SDS-PAGE was performed on purified TnC. Skeletal TnC was purified from rabbit psoas muscle according to the method of Potter (1982). Purified and lyophilized TnC (800 μg) was dissolved in 100 μl of water. Twenty microliters of this solution was serially diluted with 20 μl of water each time, and 10-μl aliquots from each serially diluted sample were loaded onto the gel. SDS-PAGE was then performed and the gel was stained by silver as described. A typical result is plotted in Fig. 1. As seen in this figure, the TnC content and the staining intensity were approximately proportional up to 40 ng/lane, demonstrating the high reliability of this method of quantitating the TnC content. We avoided experiments in which loading exceeded 40 ng/lane, as the proportional relationship did not hold beyond this point (Fig. 1).

Stoichiometry of TnC

The stoichiometry of TnC was tested against TnI by SDS-PAGE with silver stain on fibers in which TnC was not extracted. In determining the stoichiometry we made a correction for the difference in the staining intensity of TnI and TnC according to the method of Sperling et al. (1979), who reported that TnI stains 1.15 times more than TnC. We assumed a molecular weight of 21,162 for TnI (Sheng et al., 1992) and 17,846 for TnC (Potter, 1974; Collins et al., 1977). The results are shown in Table 4. As is seen in this table, the ratio of TnC:TnI was 1.02 ± 0.04, and a good 1:1 stoichiomery TnC:TnI was observed.

Extraction of troponin C from skinned fibers and reconstitution

The psoas fibers were first activated by the control activating solution to test for isometric tension, and sinusoidal analysis was performed. The fibers were relaxed and TnC was extracted by method I or II (see Materials and Methods) for up to 30 min. The extracted fibers were activated again with the control activating solution to test for remaining tension. The fibers were then subjected to sinusoidal analysis, and the MgATP and P_i effects were studied to determine the kinetic constants of the elementary steps. After sinusoidal analysis, the fibers were removed from the apparatus and subjected to SDS-PAGE analysis to quantify the remaining TnC and to determine whether other protein components were lost. The SDS-PAGE analysis of the fibers before and after extraction of TnC is shown in Fig. 2. The densitometric profiles of the proteins are shown in Fig.

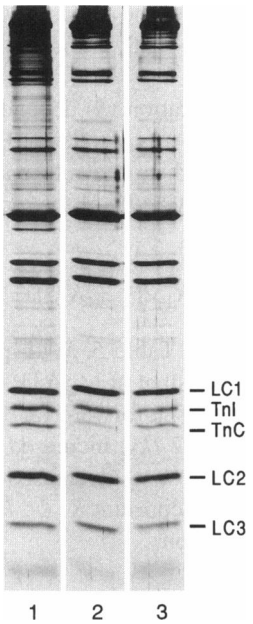


FIGURE 2 SDS-PAGE of single fiber before extraction of TnC (*Lane 1*), after partial extraction of TnC (*Lane 2*), and after reconstitution with TnC (*Lane 3*). The electrophoresis was carried out in 10.5% polyacrylamide gels (Laemmli, 1971). Gels were stained with silver.

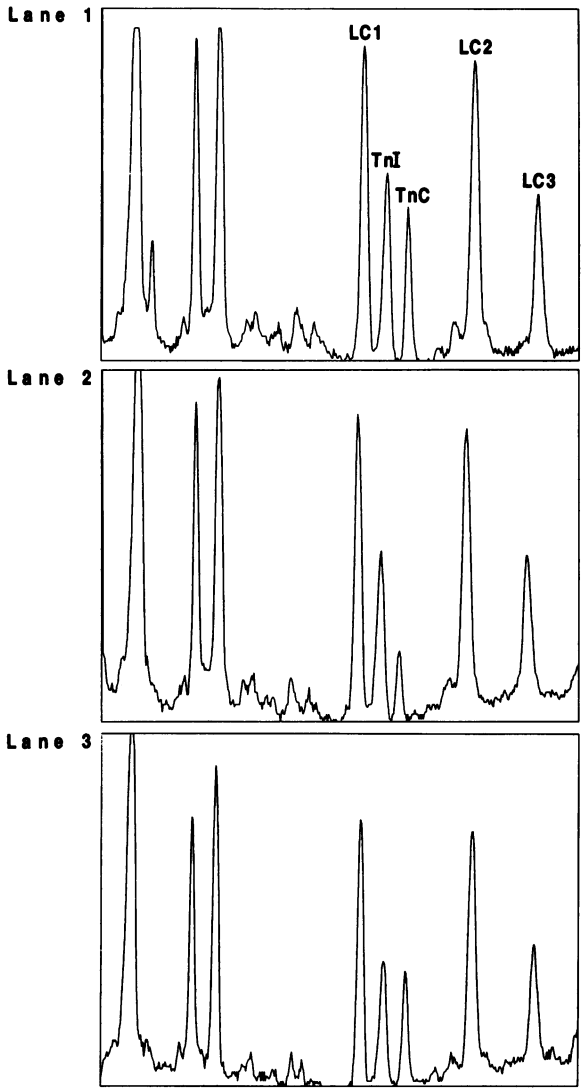


FIGURE 3 Densitometric profiles of the proteins in the corresponding lanes from Fig. 2.

TABLE 2 The area values (in arbitrary units) of the protein components before extraction of TnC, after extraction of TnC, and after reconstitution with TnC corresponding to Lane 1, Lane 2, and Lane 3, respectively, of Figs. 2 and 3

Protein components	Lane 1	Lane 2	Lane 3
LC1	1115	1044	779
LC2	1401	1320	1070
LC3	750	919	693
TnI	691	628	400
TnC	438	181	310

The data presented here are from one experiment. We obtained similar data for all the other TnC-extracted fibers (n = 12) in which the degree of TnC extraction was varied. Note that because of the variation in the sample volume loaded in each lane, the area values for LCs and TnI varied.

3, and the respective area values of the protein components are listed in Table 2.

To test the integrity of fibers after TnC extraction, we reconstituted the fibers with purified TnC (1 mg/ml) in relaxing solution for 30 min and tested for control tension (Fig. 4). As seen in Fig. 4, the isometric tension decreased to 6% of maximum tension after extraction of TnC by method II, and increased to 89% of maximum tension after reconstitution with purified TnC. Table 3 shows tension recovered after reconstitution with purified TnC for each fiber. The tension returned to 87–114% of the control level (i.e., tension before extraction) in seven experiments. From these results we infer that the extraction procedure we employed extracted only TnC (Moss et al., 1985), and it did not affect the structural integrity of the fibers. The fact that tension reproducibility exceeded 100% in three preparations (1, 2, and 6) suggests that TnC may have been lost during the skinning and/or storage procedure.

Sinusoidal analysis was performed before and after TnC extraction (see modulation in tension trace in Fig. 4). The sinusoidal time courses were collected for a minimum of 0.4 s per each frequency for fibers without TnC extraction and 1.6 s for fibers with TnC extraction. This difference was intended to increase the signal-to-noise ratio for TnC-extracted fibers, which gave only 1/10 isometric tension. The complex modulus data before and after TnC extraction are compared in Figs. 5 and 6 for the same set of preparations. Figs. 5 *A* and 6 *A* show the plots of the elastic modulus versus frequency, and Figs. 5 *B* and 6 *B* show the plots of viscous modulus versus frequency. Figs. 5 *C* and 6 *C* show the Nyquist plots, which are plots of elastic modulus in the x axis versus viscous modulus in the y axis. It is clear that the Nyquist plot shrank significantly with extraction compared to those before extraction (Fig. 5 *C*) (notice a

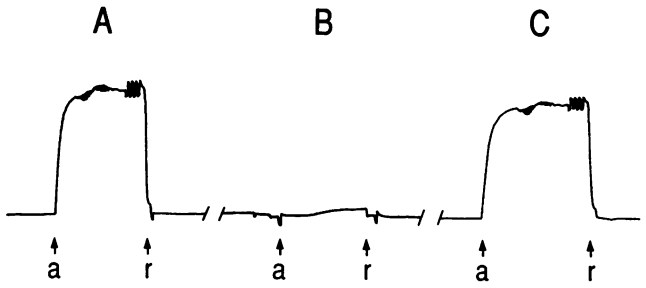


FIGURE 4 Record of isometric tension developed by double fibers before extraction of TnC (*A*), after partial extraction of TnC (*B*), and after reconstitution with TnC (*C*). a, fiber activated by the control activating solution. r, fiber relaxed by the relaxing solution.

difference in scales in Figs. 5 and 6). The diameters of the arcs indicate the magnitudes of the respective processes and are approximately scaled with tension. As seen in Figs. 5 *C* and 6 *C*, the relative magnitudes of A and B decreased and the relative magnitude of C increased with extraction.

Effect of MgATP on exponential process C

The effect of $MgATP^{2-}$ on exponential process C on fibers without TnC extraction was studied in the range of 0.1–5 mM, and on fibers with partial TnC extraction in the range of 0.05–2 mM. In both conditions, muscle fibers were activated with a saturating Ca^{2+} (pCa 4.20) concentration, and the kinetic constants associated with elementary steps 1 and 2 (Scheme 1) were determined. Two extreme MgATP solutions (0S8P and 5S8P, Table 1) were prepared and solutions with intermediate concentrations were made by mixing appropriate volumes of these two solutions without significantly affecting the other ionic constituents. The complex modulus data obtained before and after extraction of TnC were fitted to Eq. 1 to deduce the apparent rate constant $2\pi c$. $2\pi c$ was then averaged and plotted against the MgATP concentration with SEM error bars (Fig. 7). Squares in Fig. 7 are averaged results over eight experiments on preparations in which no TnC was extracted (tension 100%); solid circles are averaged results over six experiments on TnC-extracted preparations in which the remaining tension was 9–14% (average 11%). As seen in Fig. 7, $2\pi c$ increased significantly with an increase in the MgATP concentration in the range of 0.05–1 mM and saturated with a further increase in the MgATP concentration. The plot (see Eq. 2 below) is convenient because the intercept to the ordinate (0 mM MgATP) represents the rate constant of the reversal of step 2 (k_{-2}); the asymptote to a high MgATP concentration represents the sum of the forward and reversal rate constants of step 2 ($k_2 + k_{-2}$); and the reciprocal of the $MgATP^{2-}$ concentration at the half-saturation point represents the association constant (K_1) of the $MgATP^{2-}$ molecule to the cross-bridges (step 1). As seen in Fig. 7, it is apparent that the MgATP concentration needed for half-saturation is lower in fibers with TnC extraction (*circles*) than those without TnC extraction (*squares*). The intercept to the ordinate (k_{-2}) decreases with extraction, whereas the final saturation value ($k_2 + k_{-2}$) appears to not change.

The MgATP concentration (S) dependence of the apparent rate constant $2\pi c$ was fitted to Eq. 2 (Kawai and Halvorson, 1989) to deduce the rate constants k_2 and k_{-2} and the association constant K_1 of the elementary steps (see Scheme 1):

$$2\pi c = k_2K_1S/(1 + K_1S) + k_{-2}. \quad (2)$$

The results are listed in Table 5. With partial TnC extraction, the association constant of MgATP to cross-bridges K_1 increased to about 7×. The forward rate constants of cross-bridge detachment step 2 (k_2) increased to 1.55×, and the backward rate constant (k_{-2}) decreased to 0.27×. Consequently, the equilibrium constant K_2 ($= k_2/k_{-2}$) increased to 6.6× with TnC extraction.

Effect of phosphate on exponential process B

To determine the kinetic constants associated with elementary steps 4 and 5, we studied the effect of P_i on exponential process B in the range of 0–32 mM with saturating Ca^{2+} (pCa 4.20). The experiments were carried out in the presence of a high MgATP concentration (5 mM) because cross-bridges are more plentiful around the force-generating transition (step 4). Two extreme P_i solutions (5S0P and 5S32P, Table 1) were prepared and solutions with intermediate concentrations were made by mixing appropriate volumes of these two solutions. The complex modulus data obtained before and after TnC extraction were fitted to Eq. 1 to obtain the apparent rate constant $2\pi b$. $2\pi b$ was then plotted against the P_i concentration with SEM error bars (Fig. 8). Squares are the averaged results of eight experiments on preparations from which no TnC was extracted (tension 100%); solid circles are the averaged results of eight experiments on preparations with TnC extraction in which the remaining tension was 8–15% (average 12%). In both conditions $2\pi b$ increased with an increase in the P_i concentration. Each plot is curved upward, and $2\pi b$ approaches saturation at higher P_i concentrations. This plot is convenient (see Eq. 3 below) because the intercept to the ordinate (0 mM P_i) represents the product of the forward rate constant (k_4) of the cross-bridge attachment step 4 and a constant factor σ defined in Eq. 4 below; the asymptote to a large P_i concentration represents a linear combination ($\sigma k_4 + k_{-4}$) of the forward and backward rate constants of step 4; and the P_i at the half-saturation point represents the dissociation constant ($1/K_5$) of P_i ions from cross-bridges (step 5). See Scheme 1 for the definition of the rate and association constants.

The data of Fig. 8 were fitted to Eq. 3 (Kawai and Halvorson, 1991) to obtain the rate constants k_4 and k_{-4} and the association constant K_5 of the elementary steps:

$$2\pi b = \sigma k_4 + k_{-4}K_5P/(1 + K_5P), \quad (3)$$

TABLE 3 Summary of tension and the TnC content after extraction of TnC and reconstitution

Preparation no.	Extraction method	Tension after extraction* (%)	Tension after reconstitution* (%)	TnC content after reconstitution# (%)
1	II	14.3	114	—
2	II	5.0	109	106
3	I	5.8	96	—
4	II	5.6	89	90
5	II	5.8	87	—
6	I	27.9	113	—
7	I	12.7	92	—

—, Fibers were not saved for SDS-PAGE analysis.
* Measured in the control-activating solution; the results are expressed with respect to tension before extraction.
# Measured with respect to TnI.

where

$$\sigma = K_2K_1S/\{1 + (1 + K_2)K_1S\}. \quad (4)$$

K_1 and K_2 obtained from the MgATP study and $S = 5$ mM were used for the calculation of the constant σ, and then k_4 was deduced. The results are listed in Table 5. The rate constant k_4 decreased with the extraction of TnC (to 0.4×) when the remaining tension was an average of 12%. Because σ increased (to 1.6×) at the same time, the decrease in the intercept in Fig. 8 is somewhat less (to 0.6×). The rate constant k_{-4} increased (to 2×), which resulted in a significant decrease (to 0.17×) in the equilibrium constant K_4 (= k_4/k_{-4}) with TnC extraction (Table 5). The association constant K_5 did not change with TnC extraction.

The effect of TnC extraction on cross-bridge distribution

We calculated the steady-state distribution (probability) of cross-bridges among various states before and after TnC extraction based on Eqs. 5–10 (Fig. 9) (Kawai and Halvorson, 1991):

$$X_1 \equiv (\text{AM}) = K_5P/M \quad (5)$$

$$X_2 \equiv (\text{AM*S}) = K_1SK_5P/M \quad (6)$$

$$X_{34} \equiv (\text{Det}) = K_1SK_2K_5P/M \quad (7)$$

$$X_5 \equiv (\text{AM*DP}) = K_1SK_2K_4K_5P/M \quad (8)$$

$$X_6 \equiv (\text{AM*D}) = K_1SK_2K_4/M, \quad (9)$$

where

$$M \equiv K_1SK_2K_4 + K_5P[1 + K_1S(1 + K_2 + K_2K_4)], \quad (10)$$

and X_i is the probability of a cross-bridge in the respective state, as shown in Scheme 1. In particular,

$$X_1 + X_2 + X_{34} + X_5 + X_6 = 1. \quad (11)$$

The calculation was based on the equilibrium constants we obtained (Table 5) and our standard activating condition, which included 5 mM MgATP and 8 mM P_i. In Fig. 9 the results without TnC extraction are shown with dark bars, and the results with TnC extraction are shown with cross-hatched bars. From Fig. 9 it is clear that the number of all attached cross-bridges decreases by about one-half, and the number of detached cross-bridges increases about 4× after extraction of TnC.

X_0 (probability of the AMD sate) was excluded from analysis, because D is on the order of 0.01 mM in the presence of CP/CK (Meyer et al., 1985); therefore, $X_0 < 0.1\%$ and negligible. This is shown as follows. Without TnC extraction, $K_0 = 2.8\ \text{mM}^{-1} \times 2.5$ (Kawai and Halvorson, 1989) and X_1 (AM) = 1.17% (Fig. 9); therefore, $X_0 = K_0DX_1 = 0.082\%$. After TnC extraction, even if K_0 increased by 10-fold, $X_0 = K_0DX_1 = 0.063\%$, because $X_1 = 0.09\%$ (Fig. 9).

Isometric tension

Isometric tension as a function of $MgATP^{2-}$ concentration is plotted in Fig. 10 for the same experiments as shown in Fig. 7. The tension was first normalized to the control tension (T_c) that was obtained with the control activating solution in the beginning of the MgATP study series before averaging. The mean control tension was $T_c = 140 \pm 4$ kN/m^2 (±SEM, $N = 30$). As is well known for fibers without TnC extraction, tension decreased as the MgATP concentration was increased. This was also the case for fibers in which TnC was extracted (Fig. 10). Similarly, isometric tension as the function of the P_i concentration is plotted in Fig. 11 for the same experiments as shown in Fig. 8. As is well established for fibers without TnC extraction, tension decreased as the P_i concentration was increased. This was also the case for fibers from which TnC was extracted (Fig. 11).

From equation 5 of Kawai and Zhao (1993), the isometric tension of Scheme 1 is formulated as

$$\text{Tension} = \alpha(T_1X_1 + T_2X_2 + T_5X_5 + T_6X_6). \quad (12)$$

T_i is the tension associated with each cross-bridge state X_i (Kawai and Zhao, 1993); $T_{34} = 0$ because this is the tension associated with weakly attached or detached cross-bridges. The new coefficient α is called the "activation factor," and it represents the fraction of cross-bridges that participate in

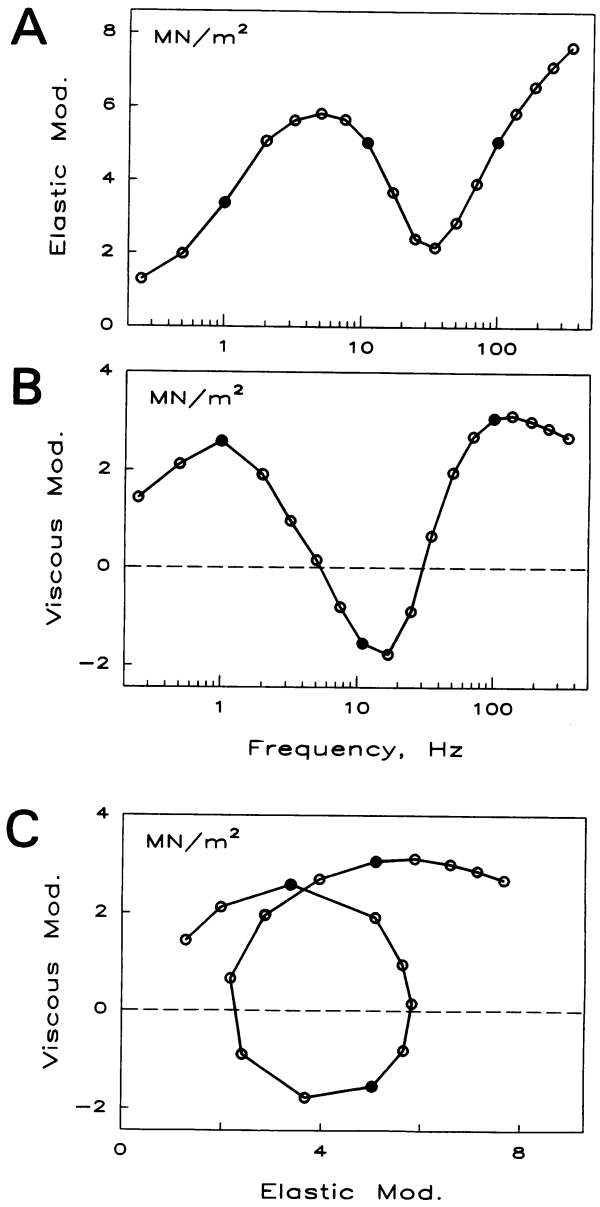


FIGURE 5 Complex modulus $Y(f)$ in control activating solution before TnC extraction. (*A*) Elastic modulus versus frequency. (*B*) Viscous modulus versus frequency. (*C*) Data shown in Nyquist plot, which is a plot of elastic modulus (in abscissa) versus viscous modulus (in ordinate). The frequencies used are 0.25, 0.5, 1, 2, 3.2, 5, 7.5, 11, 17, 25, 35, 50, 70, 100, 135, 187, 250, and 350 Hz; decade frequencies (1, 11, 100 Hz) are shown in filled symbols. Peak-to-peak amplitude: 0.25% L_0. Average of 11 experiments.

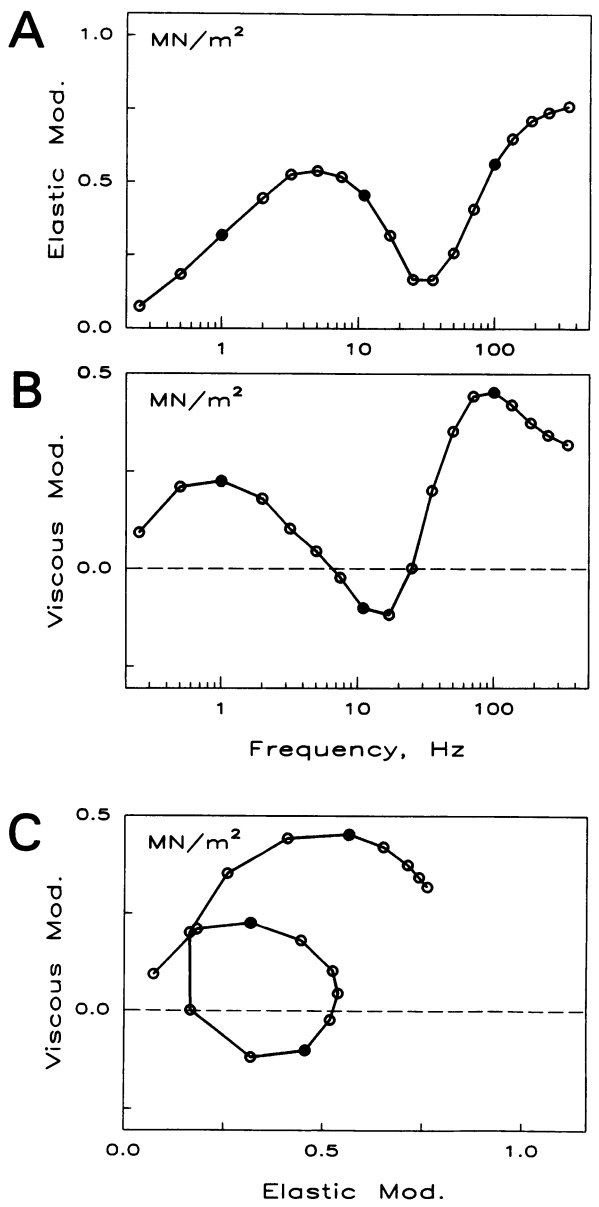


FIGURE 6 Complex modulus $Y(f)$ after TnC extraction. The same set of preparations as in Fig. 5 is used. The remaining tension was 11%. Plotting nomenclatures are the same as in Fig. 5, except that the scales are changed. Average of 11 experiments.

ATP hydrolysis and force generation (cross-bridge cycling). $\alpha = 1$ for full activation by definition (pCa 4.2 and without TnC extraction), and $0 < \alpha < 1$ for partial activation. The data of isometric tension were fitted to Eq. 12 by replacing X_1, X_2, X_5, and X_6 with the actual forms shown in Eqs. 5–10 and by using the equilibrium constants shown in Table 5. T_i and α are fitting parameters. The fitting was performed independently for four sets of data in Figs. 10 and 11, and the best-fit results are shown with solid lines in Figs. 10 and 11. As the figures illustrate, the fitting results were generally satisfactory. The fitted parameters were evaluated to identify common values, isometric tension was recalculated based on the common parameters, and the results were compared with the data in Figs. 10 and 11. The best fit was obtained for $T_1 = T_4 = T_5 = 1.30\ T_c$, and $T_2 = 0$ for all conditions, and $\alpha = 0.14$ for TnC-extracted preparations. Calculated tension for this set of parameters is shown in Figs. 10 and 11 with dashed lines. As illustrated in these figures, the data fit extremely well, except for the case of the MgATP study of TnC-extracted fibers (Fig. 10). The reason for this departure is probably because, in the ATP study, the

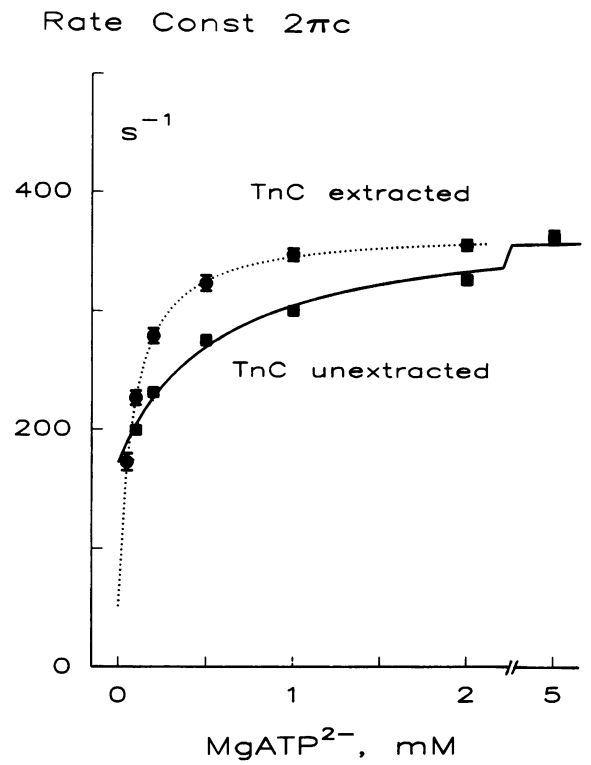


FIGURE 7 The rate constant $2\pi c$ is plotted as a function of MgATP concentration. ■, Without TnC extraction (N = 8); ●, after TnC extraction with remaining tension 9–14% (N = 6). Error bars represent SEM. Error bars smaller than the symbol size cannot be seen. Units of ordinate: s^{-1}. Continuous curves are based on Eq. 2.

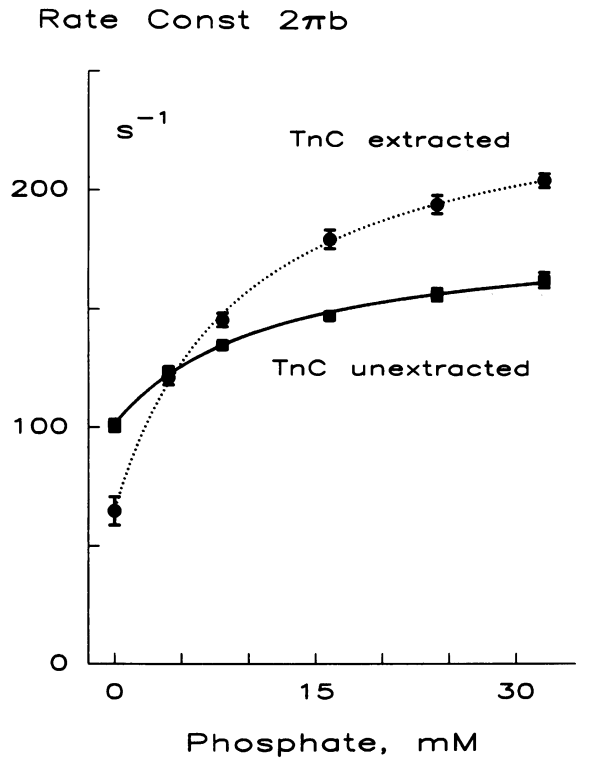


FIGURE 8 The rate constant $2\pi b$ is plotted as a function of P_i concentration. ■, Without TnC extraction (N = 8); ●, after TnC-extraction with remaining tension 8–15% (N = 8). Units of ordinate: s^{-1}. Continuous curves are based on Eq. 3.

fibers after TnC extraction had less average control tension than those in the P_i study. A smaller activation coefficient (α) would have fit the data more satisfactorily.

As discussed above, we found that $\alpha = 0.14$ for preparations with TnC extraction when the remaining tension was 11–12%. Thus, to compare the cross-bridge numbers before and after TnC extraction, the cross-bridge distribution should be multiplied by α, because the data in Fig. 9 were calculated with the restriction of Eq. 11. This multiplication is carried out in Fig. 9 for the case of TnC-extracted fibers (cross-hatched bars), and the results are shown in Fig. 9 with white bars. From $\alpha = 0.14$ we conclude that 86% of cross-bridges are idle, and they disappear from our measurements with TnC extraction.

DISCUSSION

Troponin C stoichiometry and partial TnC extraction

Using SDS-PAGE with silver stain in unextracted fibers, we found that TnC and TnI are present in a 1:1 molar ratio (Table 4). In determining this stoichiometry we made a correction for the difference in the staining intensity of TnI and TnC (Sperling et al., 1979) and used published molecular weights for TnI (Sheng et al., 1992) and TnC (Potter, 1974; Collins et al., 1977). The reported ratio for the TnC: TnI stoichiometry ranges from 0.5 to 1 (Potter, 1974; Sperling et al., 1979).

Previous studies demonstrated the presence of cooperativity among neighboring regulatory units in controlling isometric tension in striated muscle (Hill et al., 1980; Hill, 1983; Brandt et al., 1984, 1987; Moss et al., 1985). However, little is known about the relationship between cooperativity and cross-bridge kinetics, particularly the elementary steps of the cross-bridge cycle. The basic question we are asking is whether the degree of cooperative activation affects cross-bridge kinetics, i.e., whether the degree of activation of the thin filament modifies the elementary steps of the cross-bridge cycle. To answer these questions, we partially extracted TnC from rabbit psoas muscle fibers, activated them with saturating Ca^{2+}, performed MgATP and P_i studies, and deduced the kinetic constants of the elementary steps.

We tried two methods of TnC extraction, designated I (Cox et al., 1981) and II (Brandt et al., 1987). In our experience, method I extracted TnC faster than method II did. In both methods, up to 60% TnC was extracted in 30 min (Figs. 2 and 3), and the least amount of remaining tension was 6%. As expected, a longer extraction time generally resulted in more TnC extraction and lower isometric tension.

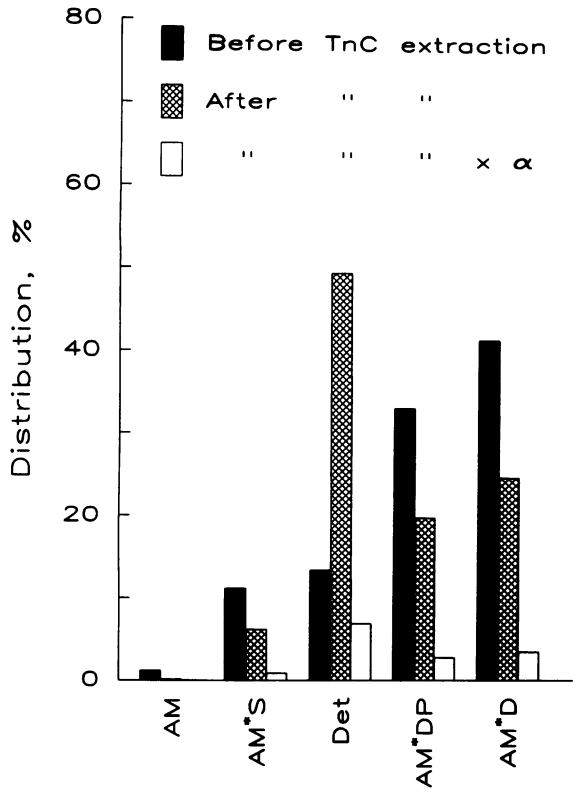


FIGURE 9 Cross-bridge distribution in unextracted (■) and TnC extracted (▩) fibers. The calculation is based on Eqs. 5–10 and on our standard activating condition, which included 5 mM MgATP and 8 mM P_i. White bars (□) represent the result of multiplication to the cross-hatched bars (▩) by $\alpha = 0.14$ (activation factor). This multiplication is necessary to explain isometric tension (11–12%) after TnC extraction.

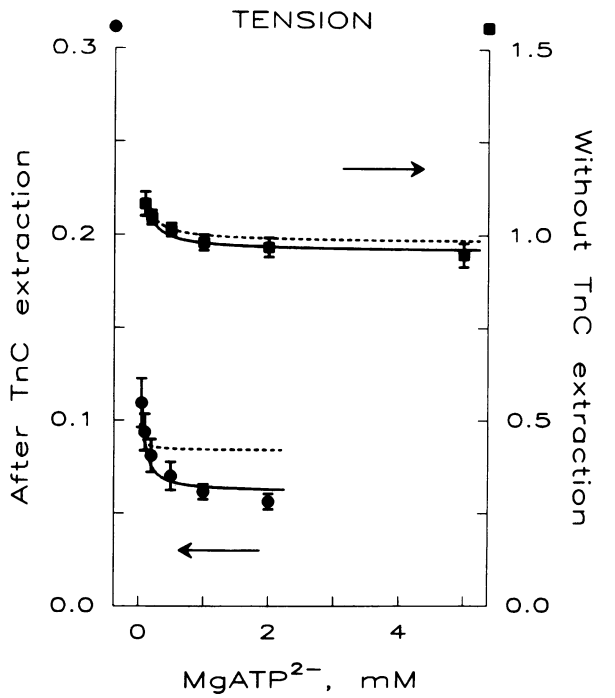


FIGURE 10 Isometric tension is plotted against the MgATP concentration. Tension was normalized to the control tension (T_c); the data were averaged and are shown with SEM error bars. $N = 8$ without TnC extraction (■, right ordinate), and $N = 6$ after TnC extraction (●, left ordinate). The data are based on the same set of experiments as in Fig. 7. From the results of both Figs. 10 and 11, $T_1 = T_4 = T_5 = 1.30\ T_c$, $T_2 = 0$, and $\alpha = 0.14$ were found, and the projected tension is shown by broken lines. Solid lines are the results of individual fits (see text).

MgATP binding and cross-bridge detachment steps

From the MgATP study we deduced the association constant K_1 of MgATP to the AM state and the rate constants of the cross-bridge detachment step (k_2 and k_{-2}). We compared K_1 before and after TnC extraction. Our results showed that K_1 increased sevenfold when the remaining tension was 11% (Table 5). Our observation demonstrates that the affinity of the MgATP binding to the myosin head in the AM state was increased with TnC extraction. This is only possible if the contour of the nucleotide-binding site changes by the degree of activation of the thin-filament regulatory unit; hence the effect must be mediated by the actin-myosin interface. It is now known that the nucleotide-binding site has a pocket-like structure and is mostly localized in the N-terminus 27-kDa domain (Winkelmann and Lowey, 1986; Dan-Goor et al., 1990; Rayment et al., 1993). The main actin-binding site is localized in the 50-kDa domain (Rayment et al., 1993). These two binding domains are separated by 3.5–5 nm, and there is evidence that suggests their interaction (Greene and Eisenberg, 1980; Kodama, 1985). White and Taylor (1976) reported that the binding of MgATP to myosin subfragment 1 (S1) became weaker by a factor of 10^5 when S1 was attached to actin. Similarly, Bremel and Weber (1972) reported (quoted from their unpublished data) that myosin without bound nucleotides has a higher affinity for regulated thin filaments (binding constant $10^7\ M^{-1}$) than nucleotide-bound myosin in the absence of Ca^{2+}. Although it is not evident exactly how the coupling between the nucleotide-binding site and the actin-binding site is achieved, a possible explanation is that when the head is attached to the thin filament under a stressed condition, the contour of the nucleotide binding site is deformed by actin binding, possibly via the myosin cleft that exists between the upper 50-kDa domain and the lower 50-kDa domain of the myosin head (Rayment et al., 1993; Holmes, 1995). In other words, the condition of the regulatory unit on the thin filament may influence the contour of the nucleotide-binding site on the myosin head.

The possibility that stress (tension) on the cross-bridge modifies the nucleotide-binding affinity is strengthened by the following observations (Kawai and Zhao, 1995). We reported in a previous study on rabbit psoas fibers that the addition of 2,3-butanedione monoxime (BDM) (Zhao and Kawai, 1994a) or lowered temperature (Zhao and Kawai, 1994b) resulted in a 7–14× increase in the MgATP binding constant (K_1); both of these procedures resulted in lowered isometric tension. The current observation that K_1 was in-

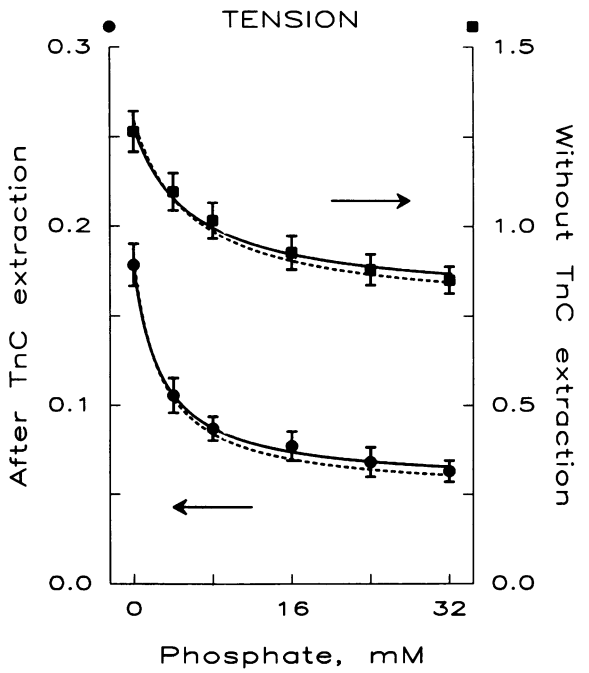


FIGURE 11 Isometric tension is plotted against the phosphate concentration. Tension was normalized to the control tension (T_c); the data were averaged and are shown with SEM error bars. $N = 8$ for both before (■, right ordinate) and after (●, left ordinate) TnC extraction. The data are based on the same set of experiments as in Fig. 8. From the results of both Figs. 10 and 11, $T_1 = T_4 = T_5 = 1.30\ T_c$, $T_2 = 0$, and $\alpha = 0.14$ were found, and the projected tension is shown by broken lines. Solid lines are results of individual fits (see text).

TABLE 4 Molar stoichiometry of TnC:TnI in rabbit skinned psoas fibers, as determined by SDS-PAGE and silver staining

Preparation no.	TnC:TnI
1	1.16
2	1.04
3	1.02
4	1.01
5	0.99
6	0.92
Mean ($n = 6$),	1.02 ± 0.04 (SEM).

creased 7× by TnC extraction when isometric tension was reduced to 11% is additional evidence that nucleotide binding becomes stronger when isometric tension is lowered. We further demonstrated in porcine myocardium that the addition of EMD 53998 [5-(1-(3,4-dimethoxybenzoyl)-1,2,3,4,-tetrahydro-6-chinolyl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one] increased isometric tension and decreased K_1 (Zhao and Kawai, 1996). These observations are summarized in Fig. 12. Kraft et al. (1992) reported that the binding of a nucleotide analog (adenosine 5′-γ-thiophosphate) increased when the Ca^{2+} concentration was decreased in rabbit psoas fibers. Rosenfeld and Taylor (1987) reported that ATP dissociation increased with Ca^{2+} in regulated actin and S1. Kuhn (1981) reported that a stretch-and-release cycle of insect muscle fibers accelerated the nucleotide binding. Our results, shown in Fig. 12, are also consistent with the "Fenn effect" (Fenn, 1924), because a reduction in tension leads to an increase in K_1, which shifts the equilibrium of Scheme 1 to the right, resulting in an increased number of cross-bridges in the AM*D state. This in turn results in increased energy usage, because step 6 is the slowest step in the cross-bridge cycle; hence the ATP hydrolysis rate is proportional to the number of cross-bridges in the AM*D state: ATPase = k_6 [AM*D] (Kawai and Halvorson, 1991; Zhao and Kawai, 1994b).

The fact that $2\pi c$ increased with partial activation (Fig. 7) is consistent with our earlier results, which showed that $2\pi c$ increased when the Ca concentration was lowered to the threshold level without TnC extraction (Kawai et al., 1981) and that the rate constant of the fast portion of phase 2 (equivalent to $2\pi c$ of this report) increased when the Ca concentration was lowered (Martyn and Chase, 1995), both in rabbit psoas fibers. Our interpretation is that the primary cause of this increase in $2\pi c$ is an increase in K_1 (Eq. 2, Fig. 7) with partial activation.

With partial TnC extraction, the rate constant of the cross-bridge detachment step (k_2) increased and its reversal step (k_{-2}) decreased significantly. Consequently, these resulted in a sevenfold increase in the equilibrium constant (K_2). The increase in K_1 and K_2 caused cross-bridges to populate more in Det states (Fig. 9, cross-hatched bars), which is reflected by the relatively large magnitude C in the TnC-extracted fibers (Fig. 6 *C*). This is because the magnitude parameter is proportional to the steady-state probability of the cross-bridge states involved (Kawai and Halvorson, 1991); hence this result is consistent with Scheme 1.

The force generation and phosphate-release steps

From the P_i study, we obtained the rate constant of the force generation step 4 (k_4), its reversal step (k_{-4}), and the P_i association constant (K_5) before and after TnC extraction (Table 5). There is now evidence that indicates force is generated on step 4, and before P_i is released (step 5). Such evidence includes the evaluation of tension as the function of the P_i concentration (Kawai and Halvorson, 1991; Kawai and Zhao, 1993), the results of the pressure-release experiment (Fortune et al., 1991; Geeves, 1992), and the results of tension transient after a photorelease of P_i (Danzig et al., 1992; Walker et al., 1992). Our evidence, that $T_5 = T_6 = 1.30\ T_c$ (Figs. 10 and 11) and $T_{34} = 0$, also demonstrates that tension is generated in step 4, and the same tension is maintained with P_i release (step 5).

Step 4 is an isomerization of the AMDP state that forms the AM*DP state, and this reaction primarily involves hydrophobic interaction (Highsmith, 1977; Rayment et al., 1993; Zhao and Kawai, 1994b). Step 4 may also include the cross-bridge attachment step (A + MDP ↔ AMDP). Our results demonstrate that k_4 became 0.4×, and k_{-4} became 2× when TnC was partially extracted and the average

TABLE 5 The kinetic constants before and after TnC extraction

Kinetic constants	Without TnC extraction	With TnC extraction	Units	With / Without
K_1	1.9 ± 0.2 (8)	13.0 ± 1.2 (6)	mM^{-1}	6.8 ± 0.9
k_2	209 ± 9 (8)	323 ± 6 (6)	s^{-1}	1.55 ± 0.07
k_{-2}	171 ± 5 (8)	47 ± 7 (6)	s^{-1}	0.27 ± 0.04
K_2	1.23 ± 0.08 (8)	8.1 ± 1.5 (6)		6.6 ± 1.3
k_4	192 ± 5 (8)	79 ± 3 (8)	s^{-1}	0.41 ± 0.02
k_{-4}	86 ± 9 (8)	185 ± 9 (8)	s^{-1}	2.2 ± 0.3
K_4	2.5 ± 0.3 (8)	0.43 ± 0.02 (8)		0.17 ± 0.02
K_5	0.101 ± 0.017 (8)	0.101 ± 0.016 (8)	mM^{-1}	1.0 ± 0.2

The mean ± SEM and the number of experiments (in parentheses) are shown.

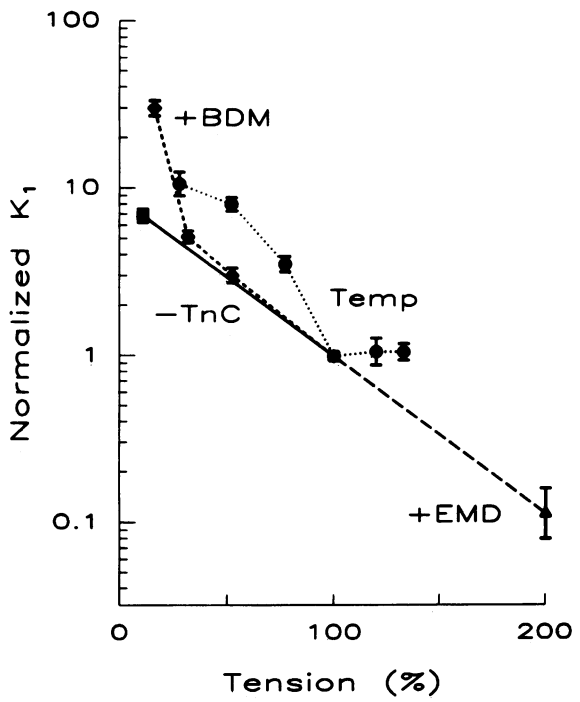


FIGURE 12 The association constant of MgATP to cross-bridges (K_1) is plotted against isometric tension from various reports. +BDM (♦) indicates the addition of BDM (4.5–18 mM) (Zhao and Kawai, 1994a); Temp (●) indicates experiments with altered temperatures (range: 5–30°C) (Zhao and Kawai, 1994b); +EMD (▲) indicates the addition of EMD 53998 (50 μM) (Zhao and Kawai, 1996), and −TnC (■) indicates TnC extraction (this report). Experiments on BDM, temperature change, and TnC extraction were performed on rabbit psoas fibers. The experiment on EMD was performed on porcine myocardium. The data were normalized to the standard condition (20°C, no BDM, no EMD, and no TnC extraction).

remaining tension was 12% (Table 5). Because of the opposing effects of TnC extraction on k_4 and k_{-4}, the overall effect on the equilibrium constant K_4 is a decrease (to 0.17×) with extraction (Table 5). From the effect of TnC extraction on steps 1, 2, and 4, we conclude that partial TnC extraction promotes cross-bridge detachment (Fig. 9, cross-hatched bars). Supposing that the extraction of TnC is analogous to a decreased Ca concentration, it can be concluded that Ca modulates steps 1, 2, and 4. The effect of partial TnC extraction did not change the P_i association constant (K_5). This result suggests that the contour of the P_i-binding site or its release channel is not affected by the condition of the thin-filament regulatory unit.

Recruitment versus graded activation mechanisms

In the recruitment mechanism, originally proposed by Podolsky and Teichholz (1970), cross-bridges are simply turned on or off by Ca^{2+} (switch hypothesis), and an increase in the Ca^{2+} concentration recruits more cross-bridges into the active pool. The property of each cross-bridge does not change. This prediction suggests that in our Scheme 1, none of the kinetic constants will change with the degree of activation, but the activation factor α (Eq. 12) will increase from 0 (no activation) to 1 (full activation). The steric blocking mechanism (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973) is an example of the recruitment mechanism. In the graded activation mechanism, originally proposed by Julian (1969), the attachment rate constant increases with an increase in the Ca^{2+} concentration. The increase in the attachment rate constant in turn results in increased isometric tension. This indicates that, in our Scheme 1, the kinetic constants will change with the degree of activation, but the activation factor α will remain constant ($\alpha = 1$).

Our results demonstrate that α decreased to 0.14 on partial activation (TnC extraction). This is consistent with the recruitment mechanism and thus with the steric blocking mechanism. This is a logical scenario, because a cross-bridge that cannot find an activated thin-filament regulatory unit in its vicinity is idle and disappears from the actively cycling pool. Our results demonstrate, however, that the property of each cross-bridge changes as well, which is shown by the altered kinetic constants with TnC extraction (Table 5). Therefore, our results are also consistent with the graded activation mechanism. Thus we conclude that both the recruitment mechanism and the graded activation mechanism play a role in the activation of the cross-bridge cycle in response to the altered numbers of Ca-TnC complex. The previous disparity of the Ca^{2+} effect as discussed in the Introduction may have arisen because plots in Fig. 8 have a crossover point. Depending on the Pi concentration and the

condition of the fibers, the apparent rate constant may decrease, may not change, or may even increase on partial activation.

Cooperative interaction of thin-filament regulatory units

The next question is, why does K_4 decrease with TnC extraction? As mentioned earlier, step 4 includes the isomerization from a weakly attached state AMDP to a strongly attached state AM*DP (Scheme 1) in which force is generated, and presumably involves a stereospecific and hydrophobic interaction between residues of actin and myosin (Holmes, 1995). One way to alter this interaction is to change the stereospecifity of actin and myosin; namely, to change the conformation of one or both of these molecules. The other way to alter the interaction is to shift the position of tropomyosin, so that the sterospecificity of actin and myosin is modified. Because actin is physically linked to TnC via Tm, TnT, and TnI, it is likely that either the actin molecule changes its conformation or Tm shifts its position in response to the degree of cooperative activation of the regulatory unit. The opposing effects of TnC extraction on k_4 and k_{-4} are both consistent with this mechanism. If actin and myosin bind more strongly with an increased stereospecific interaction, an increase in k_4 or a decrease in k_{-4} is expected. These predictions are consistent with our results (Table 5). Our finding, that k_2 (cross-bridge detachment step) increased and k_{-2} (reversal detachment) decreased with TnC extraction (Table 5), is also consistent with the hypothesis that the stereospecificity of actin-to-myosin cross-bridges is lessened with extraction.

If each regulatory unit (troponin subunits, tropomyosin and 7 actin molecules) works independently, it follows that each unit is turned on or off, depending on the availability of the Ca-TnC complex. In this case there will be only one value each for k_4 and k_{-4}, which could not change with TnC extraction, and the activation factor α takes a value of either 0 (no Ca or no TnC) or 1 (in the presence of Ca and TnC). If, on the other hand, neighboring regulatory units work interactively (cooperatively), k_4 and k_{-4} can gradually change with the degree of TnC extraction. When more regulatory units are activated, these interact with each other to change the actin conformation further, or shift the position of tropomyosin more, so that an increased stereospecific interaction between actin and myosin becomes possible. Our results demonstrating that k_4 and k_{-4} change their values on TnC extraction (Table 5) are consistent with the cooperative activation mechanism (see Tobacman, 1996, for review), which was previously developed based on the steepness of the pCa-tension relationship (Hill et al., 1980; Hill, 1983; Brandt et al., 1980, 1982, 1984, 1987; Moss et al., 1983, 1985) and on the pCa-ATP hydrolysis rate relationship (Murray and Weber, 1980; Grabarek et al., 1983). What is new about our results is that k_{-2} and k_4 increase and k_2 and k_{-4} decrease with increased cooperativity, and these changes are related to an increased stereospecific and hydrophobic interaction between the residues of actin and myosin.

Furthermore, the cooperativity can also be induced by attached cross-bridges (Güth and Potter, 1987; Hannon et al., 1992; Tobacman, 1996). In this case the thin filament can have three states: the "off" state (in the absence of Ca or TnC), the "on" state (in the presence of Ca and TnC), or the "potentiated" state (Ca, TnC, and cross-bridges). Because the number of attached cross-bridges can vary, the cooperativity can be graded. Our results that k_4 and k_{-4} change with TnC extraction are also consistent with the cooperativity mechanism induced by cross-bridges.

Relationship to the model proposed by McKillop and Geeves

Based on both solution and fiber studies, Geeves (1992) and McKillop and Geeves (1993) proposed a model in which the thin filament exists in three states:

$$\text{Blocked} \underset{}{\overset{\text{Step I}\ K_I}{\rightleftharpoons}} \text{Closed} \overset{\text{Step II}\ K_{II}}{\rightleftharpoons} \text{Open.}$$

McKillop and Geeves (1993) reported that Ca^{2+} controls both equilibrium constants (K_I and K_{II}). Actin is in the "blocked" state (also called the "off" state) in the absence of Ca^{2+}, and the equilibrium shifts to the right as the Ca^{2+} concentration is raised. Our result is approximately consistent with this model, with the blocked state increasing from 0% (no TnC extraction, pCa 4.20) to 86% (TnC extraction, pCa 4.20). The "closed" state which allows the "A" state (also called "on" state) to occur corresponds to the Det state, and its biochemical equivalence is shown in [ ] in Scheme 1. The "open" state which allows the rigor state to occur corresponds to the force-generating states, in particular to the AM*DP and AM*D states. Step II of McKillop and Geeves' model corresponds to step 4 of Scheme 1. Force is generated with this transition, which is enhanced by the Ca^{2+}-Tn-Tm system. If we fit our results to the model of McKillop and Geeves (1993), $K_I = 0.080$ for the TnC extracted condition, and $K_I > 10$ for the TnC unextracted condition can be deduced.

Structural evidence for the three states was discussed by Holmes (1995). He showed that in the absence of Ca^{2+}, Tm blocks the actin-myosin interaction altogether (off state); in the presence of Ca^{2+}, Tm allows either a partial interaction (on state) or a full interaction (R state). These interactions are stereospecific and based on hydrophobic amino acid residues of actin and myosin. Structural evidence for two actomyosin states (on and off states) was presented by Ishikawa and Wakabayashi (1994), Lehman et al. (1994), Poole et al. (1994), and Reedy et al. (1994). Thermodynamic evidence for hydrophobic interaction of the force

generation step was presented by Zhao and Kawai (1994b) and Murphy et al. (1996).

CONCLUSION

Our finding that the partial extraction of TnC modifies the rate and association constants of the elementary steps demonstrates that cross-bridge kinetics are under the influence of thin-filament proteins. From the effect of partial extraction of TnC on the equilibrium constants of the elementary steps, we conclude that the extraction promotes cross-bridge detachment and suppresses cross-bridge attachment. The net result is an increase in the number of cross-bridges in detached states, and a decrease in the number of cross-bridges in attached states. At the same time we found that the activation factor α decreases to 0.14 with extraction, implying that some of the cross-bridges are idle, and they disappear from the actively cycling pool because they cannot reach activated regulatory units. Both of these findings explain the suppression of tension in TnC-extracted muscle fibers. The fact that equilibrium constants changed with extraction is consistent with the cooperative activation mechanism among thin-filament regulatory units. This finding further suggests that the stereospecificity of actin and myosin is increased on Ca binding to TnC.

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