Research Article

Tropomyosin Period 3 Is Essential for Enhancement of Isometric Tension in Thin Filament-Reconstituted Bovine Myocardium

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Tropomyosin (Tm) consists of 7 quasiequivalent repeats known as “periods,” and its specific function may be associated with these periods. To test the hypothesis that either period 2 or 3 promotes force generation by inducing a positive allosteric effect on actin, we reconstituted the thin filament with mutant Tm in which either period 2 (Δ2Tm) or period 3 (Δ3Tm) was deleted. We then studied: isometric tension, stiffness, 6 kinetic constants, and the pCa-tension relationship. N-terminal acetylation of Tm did not cause any differences. The isometric tension in Δ2Tm remained unchanged, and was reduced to ∼60% in Δ3Tm. Although the kinetic constants underwent small changes, the occupancy of strongly attached cross-bridges was not much different. The Hill factor (cooperativity) did not differ significantly between Δ2Tm (1.79 ± 0.19) and the control (1.73 ± 0.21), or Δ3Tm (1.35 ± 0.22) and the control. In contrast, pCa50 decreased slightly in Δ2Tm (5.11 ± 0.07), and increased significantly in Δ3Tm (5.57 ± 0.09) compared to the control (5.28 ± 0.04). These results demonstrate that, when ions are present at physiological concentrations in the muscle fiber system, period 3 (but not period 2) is essential for the positive allosteric effect that enhances the interaction between actin and myosin, and increases isometric force of each cross-bridge.

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1. Introduction

The tropomyosin (Tm) gene is the product of gene duplication [1] and its sequence reflects the presence of a 7-fold repeat that has been postulated to relate to the binding sites for the 7 actin monomers along the length of a Tm molecule [1–3]. This molecule is a coiled-coil dimeric protein, and together with the troponin complex (Tn), it regulates thin-filament activation in striated muscles. This event is initiated by Ca2+ binding to TnC, and enhanced by myosin binding to actin. The reactions are cooperative and allosteric, as shown in solution studies [4–6], in vitro motility assays [7–10], and in muscle fiber studies [11–14]. Analyses of the mechanism that underlies thin-filament activation have indicated a role for the 7 internal quasiequivalent repeats, called “periods,” in this activation process [15–19] as well as a role for the head-to-tail association of adjacent Tm molecules [20].

Further analysis of mutants with deletions of one or more of the periodic repeats—by solution studies, in vitro motility assays, and muscle-fiber analysis—has revealed the importance of the internal periods for allosteric regulation of actin-filament activation. Specifically, an actomyosin ATPase study and in vitro motility assays have shown that the calcium ion poorly activates actin filaments that are reconstituted with Tn plus a Tm harboring a period 3 deletion (either individually or in combination with the deletion of another period) [16, 19]. Later reports showed that the same deletions reduce the activity in the in vitro motility assay [10, 17] as well as the isometric tension [13], by ∼50%.

Experiments using skinned fibers are important, because force can be measured in these preparations and the measurements can be performed in solutions that are at physiological ionic strength. Extraction of the thin filaments from strips of bovine myocardium, followed by structural and stoichiometric reconstitution of the muscle with natural or recombinant proteins, generates a suitable model for such experiments [11, 12, 21–23]. The advantage of this
isometric tension in a myocardial model reconstituted with
mally involved in the actomyosin interaction. Specifically, the
quasirepeat regions. The N-terminal amino acid residue for each
terminus of nfTm is the full-length protein and was used as a control. The N-
region(s) deleted in the respective mutants.

Reconstitution was performed as described in [11, 13, 21]
[13, 14] have indicated that a truncated Tm mutant,
Myocardium. The technique of thin-filament extraction and
2. Methods

2.1. Thin-Filament Extraction and Reconstitution in
Myocardium. The technique of thin-filament extraction and
reconstitution was performed as described in [11, 13, 21]
and reviewed recently in [23]. In brief, strips of bovine
myocardium (length ∼ 3 mm, diameter 90–110 μm) are
mounted between a length driver and a force transducer,
and treated in relaxing solution that contained 1% Triton
X100 at 0°C for 20 minutes. The thin filament is then
removed by treatment with gelsolin (previously called brevin
[26]) for 45–120 min, which results in fibers bare of thin
filament, except in the immediate vicinity of the Z-line [21].
Connectin (titin), which anchors the thick filament to the
Z-line, is not disturbed by this technique. The actin filament
is then reconstituted by adding exogenous G-actin under
polymerizing conditions. Thin-filament reconstitution
is completed by reconstituting both Tm and Tn at the same
time. Throughout the extraction/reconstitution procedure,
2,3-butanedione 2-monoxime (BDM) is used to inhibit the
actomyosin interaction [27–29]. Modifications in this study
are the use of (1) 56 mM, rather than 80 mM, KI during
actin polymerization, and (2) the incorporation of a 5th
solution change [11]. Consequently, the duration of the
actin polymerization procedure was 35 minutes instead of
28 minutes.

The Tm molecules used for reconstitution included
nfTm, Δ2Tm, Δ3Tm, and acetyl Tm (Figure 1). The recom-
binant TmS are encoded by the chicken α-Tm gene (TPM1). The
sequence of nfTm is that of striated α-Tm (the same
sequence for both skeletal and cardiac muscles), Δ2Tm is
the same but lacks the period 2-encoding sequence (residues
47–88) [30], and Δ3Tm is the same but lacks the period 3-
encoding region (residues 89–123) [15]. nf stands for non-
fusion peptide, and it does not have N-terminal acetylation
or substitution with Ala-Ser N-terminal fusion peptide. The
bovine and chicken striated muscle α-Tms are 95% identical
in sequence. There are 9 amino acid differences located
throughout the sequence. The differences are generally
conservative in nature: D to E, A to S, S to T. Extensive
analysis has shown that there are minor differences in
affinity and stability between the isoforms, but no qualitative
differences were reported. The previously published deletion
mutant, in which periods 2 and 3 were deleted, was made
in both chicken and rat, and had very similar regulatory
properties [15, 16], even when used with skeletal chicken
versus cardiac bovine Tn. Therefore, the species difference
should be of minor consequence. The recombinant proteins
are chicken, because the original mutants were prepared in a
chicken cDNA. nfTm, Δ2Tm and Δ3Tm were expressed in E.
coli and purified as described [19], and thus their N-terminal
Met residues are unacetylated. Acetyl Tm was purified from
bovine myocardium; most of the data on acetyl Tm were
reported previously [13] and are incorporated in both the
Tables and Figure 3(c) for the sake of comparison.

Actin was purified from acetone powder, a gift from Dr.
Shin’ichi Ishiwata of Waseda University in Tokyo and had
been isolated from New Zealand white rabbit’s fast twitch
(white) muscles. Tn was a gift from Dr. Larry S. Tobacman
of The University of Illinois at Chicago and had been purified
from bovine heart as reported in [31].

Isometric force was measured at the completion of each
step of the extraction/reconstitution procedure, following
brief stimulation (application of the standard activating
solution at 25°C) and immediate relaxation (application of
the relaxing solution at 0°C). The data on force, stiffness
and rigor stiffness that were subsequently gathered, were
normalized to the isometric force (Tis) of the preparation

![Figure 1: Tropomyosins used for reconstitution. The structure of E. coli-synthesized Tm used for our study, highlighting the 7 quasirepeat regions. The N-terminal amino acid residue for each region is shown underneath nfTm. Yellow color represents the region(s) deleted in the respective mutants. Δ23Tm was previously studied [13, 14] and is included here for the sake of comparison. nFm is the full-length protein and was used as a control. The N-terminus of Δ23Tm has the Ala-Ser substitution, whereas the N-termini of Δ2Tm, Δ3Tm, and nFm do not have this substitution.](image-url)
the cooperativity of relaxation, the MgATP concentration at half relaxation, $Y_{m50}$, were studied with 4 series of solutions that changed (1) pCa, (2) $\log \text{MgATP}^{-2}$, (3) $\log [\text{MgATP}^2]$− , and (4) $\log [\text{Pi}^{1-6}]$. (2) was performed in the absence of Ca$^{2+}$; (3) and (4) were performed in the presence of saturating Ca$^{2+}$. Towards the end, the fibers were activated by the standard solution changes. All activations including that of rigor were performed at 25°C, except for those that were part of the temperature study. All relaxation was performed at 0°C. At the termination of each mechanical experiment, the preparation was removed from the experimental apparatus, dissolved in sample dilution buffer, and pooled for SDS-PAGE analysis.

For statistical analysis, a paired t-test was performed using ANOVA and assuming unequal variances, and the results were compared to those of the nfTm-reconstituted group (control).

2.3. pCa-Tension and pCa-Stiffness Studies. The reconstituted fibers were first studied with 13 different Ca$^{2+}$ concentrations (pCa: 7.0, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.6, 4.3, 4.0, 3.5) at the fixed [MgATP] (5 mM) and [Pi] (8 mM). The tension data were then fitted to the Hill equation:

$$T = \frac{T_m}{1 + \left( \frac{\text{Ca}^{2+}}{\text{Ca}_{50}} \right)^h} + T_b,$$

where $T$ is isometric tension, $T_m$ is the maximum tension at saturating Ca$^{2+}$, and $T_b$ is the baseline tension. $\text{Ca}_{50}$ is the dissociation constant of Ca$^{2+}$, which represents [Ca$^{2+}$] at half saturation. $p\text{Ca}_{50} = -\log \text{Ca}_{50}$ is called the Ca$^{2+}$ sensitivity. $h$ is the Hill factor and represents the cooperativity of activation. The data are plotted after subtraction of $T_b$ and normalization to $T_m$. The stiffness data were analyzed with the equation similar to (1).

2.4. pS-Stiffness and pS-Tension Studies. The effect of pS ($= -\log [\text{S}], \text{where S = MgATP}$) on stiffness and tension was studied at every 0.2 pS unit where stiffness and tension change quickly, else, studied less frequently. This experiment was performed in the absence of Ca$^{2+}$ and in the presence of 6 mM K$_2$EGTA. Stiffness was measured at 100 Hz. The data were fitted to (2), which is similar (but not identical) to (1):

$$Y_{100} = \frac{Y_{m100}}{1 + \left( \frac{\text{S}}{\text{S}_{50}} \right)^g} + Y_{b100},$$

where $S_{50}$ is the dissociation constant of MgATP, which is the MgATP concentration at half relaxation, $g$ represents the cooperativity of relaxation, $Y_{100}$ is stiffness measured at 100 Hz, $Y_{m100}$ is the maximum stiffness in the absence of MgATP, and $Y_{b100}$ is the baseline stiffness at high MgATP concentration. The data were plotted after the subtraction of the baseline stiffness ($Y_{b100}$) and normalization to $Y_{m100}$. The tension data are fitted to an equation similar to (2), where only the data to the right of the peak are used.

2.5. Sinusoidal Analysis. At each activation, the computer was triggered and sinusoidal length changes were applied at a small amplitude (0.125%) and 18 different frequencies in the range 0.13 and 100 Hz. Isometric force and force transients were recorded using the same computer, signal averaged, and the complex modulus $Y(f)$ was calculated. $Y(f)$ is a frequency ($f$) response function, which is the ratio of the stress change to the strain change expressed in the frequency domain. $Y(f)$ was displayed on the computer screen immediately after the measurements were taken; it was further fitted to (3), which incorporates three exponential processes, A, B, and C [32]:

$$Y(f) = H + \frac{Afi}{a + fi} - \frac{Bfi}{b + fi} + \frac{Cfi}{c + fi},$$

where $i = \sqrt{-1}$; $2\pi a, 2\pi b$, and $2\pi c$, respectively correspond to the apparent rate constants of processes A, B, and C; $A$, $B$, and $C$ are their respective magnitudes (amplitudes). In cardiac muscle fibers, process A is absent ($A = 0$) at $\leq 25$°C [11, 33, 34], but it plays a role at temperatures $\geq 30$°C [14]. From (3), we have

$$Y_\infty = Y(\infty) = H + A - B + C$$

$Y_\infty$ is the complex modulus extrapolated to the infinite frequency. $Y_\infty$ corresponds to phase 1, process C corresponds to phase 2, process B to phase 3, and process A to phase 4 of step analysis [32].

2.6. The Effect of MgATP on the Apparent Rate Constant $2\pi c$. The preparations were studied with 7 different MgATP concentrations (0.05, 0.1, 0.2, 0.5, 1, 5, 10 mM) at the fixed [Pi] (8 mM) in the presence of saturating Ca$^{2+}$. To deduce the rate and association constants of steps 1 and 2 (Scheme 1), the effect of [MgATP] on the apparent rate constant $2\pi c$ was fitted to the following [35]:

$$2\pi c = \frac{K_S}{1 + K_1 S} k_2 + k_{-2},$$

where $S = [\text{MgATP}]$, $K_1$ is the association constant of MgATP to the myosin head, $k_2$ is the rate constant of cross-bridge detachment step, and $k_{-2}$ is that of its reversal step.

2.7. The Effect of Pi on the Apparent Rate Constant $2\pi b$. The effect of phosphate (Pi) was studied at 6 different Pi concentrations (0, 2, 4, 8, 16, 32 mM: added concentrations) in the presence of the fixed [MgATP] (5 mM) and saturating Ca$^{2+}$. To deduce the rate and association constants of steps 4 and 5 (Scheme 1), the effect of [Pi] on the apparent rate constant $2\pi b$ was fitted to the following [35]:

$$2\pi b = \sigma k_4 + \frac{K_3 P}{1 + K_2 P} k_{-4},$$
were then heated to 100 $^\circ$C.

The K-skinning solution (Bio-Rad, Cat. No. 161–1223), was then performed. The an 8–16% acrylamide gradient gel (with a 4% stacking gel: acetic), 10 MOPS, and 30 BDM. The K-skinning solution

Solution

MgADP, $S = $MgADP, and $P = $Pi = phosphate.

where

\[ \sigma = \frac{K_1SK_2}{1 + K_1S(1 + K_2)}, \]  

and $P = [P_i]$. $k_4$ is the rate constant of the step 4 (force generation step), $k_{-4}$ is that of its reversal step, and $K_2$ is the association constant of the Pi molecule to the myosin head. $\sigma$ of (7) was calculated from $K_1$ and $K_2$ obtained from the MgATP study (5) with $S = 5$ mM.

2.8. Biochemical Analysis of Extracted and Reconstituted Myocardium. To prepare samples for SDS-PAGE, which requires a large quantity of muscle tissue relative to mechanical studies, an additional group of 10–15 fibers/mutant was suspended between two stainless steel (ss) wires (diameter 500 $\mu$m, length 15 mm, 4 mm apart) after being glued to them with nail polish (Figure 10). A 3rd ss wire was placed in the center to prevent fibers from breaking when they cross the meniscus (the fibers were not glued to this central ss wire). These muscle fibers were placed into Eppendorf tubes and subjected to the same extraction and reconstitution procedures as the fibers that were used for mechanical analysis. Each solution (200 $\mu$l) was added at 0 $^\circ$C. Once reconstituted, the fibers were dissolved in the sample dilution buffer together with fibers used for mechanical analysis and were then heated to 100 $^\circ$C for 5 minutes. SDS-PAGE, using an 8–16% acrylamide gradient gel (with a 4% stacking gel: Bio-Rad, Cat. No. 161–1223), was then performed. The samples were electrophoresed at 45 mA ($\sim$ 200 V) for about 40 min, and stained with Coomassie brilliant blue R250 as reported [36]. The gels were photographed using a high-resolution digital camera, and the intensity of each band was measured using the UN-SCAN-IT (version 5.1) program.  

2.9. Solutions. The Na-skinning solution contained (mM) 10 K$_2$EGTA, 2 Na$_2$MgATP, 5 Na$_2$K$_2$ATP, 122 NaAc (Ac = acetate), 10 MOPS, and 30 BDM. The K-skinning solution contained 10 K$_2$EGTA, 2 Na$_2$MgATP, 5 Na$_2$K$_2$ATP, 122 KAc, 10 MOPS, and 30 BDM. The storage solution contained 10 K$_2$EGTA, 2 Na$_2$MgATP, 5 Na$_2$K$_2$ATP, 122 KAc, 10 MOPS, 30 BDM, and 6 M glycerol. The relaxing solution contained 6 EGTA, 2.2 Na$_2$MgATP, 5 Na$_2$K$_2$ATP, 8 K$_3$Pi, 41 NaProp, 75 KProp, 10 MOPS, and 40 BDM. The thin filament extraction solution contained 2 CaEGTA, 2.2 Na$_2$K$_2$ATP, 121 KCl, 4.25 MgCl$_2$, 2 leupeptin, 2 diisopropyl fluorophosphate (DFP), 40 BDM, 20 MOPS, and ~ 0.3 mg/ml purified gelsolin from bovine plasma. The actin filament reconstitution solution contained (mM) 4 EGTA, 4 Na$_2$MgATP, 32 KCl, 56 KI, 40 BDM, 20 K$_{1.5}$Pi, and 1 mg/ml purified G-actin from rabbit back muscles. The $T_m/T_n$ reconstitution solution contained ~ 0.6 mg/ml Tm and ~ 0.6 mg/ml bovine cardiac Tn in the relaxing solution. The standard activating solution contained 6 CaEGTA, 5.8 Na$_2$MgATP, 1.36 Na$_2$K$_2$ATP, 15 creatine phosphate (Na$_3$CP), 4 KH$_2$PO$_4$, 4 K$_2$HPO$_4$, 1 NaProp, 73 KProp, 10 NaN$_3$, 10 MOPS, and 320 U/ml CK (pCa 4.66, total Na 55 mM, ionic strength 200 mM). Any other activating solution was a variant of the standard activating solution, in which ionic strength was maintained at 200 mM and the total [Na] at 55 mM. The rigor solution contained 55 NaProp, 4 KH$_2$PO$_4$, 4 K$_2$HPO$_4$, 122 KProp, and 10 MOPS. The pH of each solution was adjusted to 7.00. The concentrations of multivalent ionic species were calculated using our computer program ME, based on the following apparent association constants (log values at pH 7.00): CaEGTA = 6.28, MgEGTA = 1.61, CaATP = 3.70, MgATP = 4, CaCP = 1.15, and MgCP = 1.30.

3. Results

3.1. Mechanical Studies of Thin Filament-Reconstituted Fibers. To compare fiber mechanics between mutant and control Tms, and to establish the structure-function relationship for periods 2 and 3 of this protein, we dissolved bovine myocardium thin filaments with gelsolin, and then reconstituted them with component proteins as described in [11, 21]. The effects of fiber reconstitution on isometric tension are illustrated in Figure 2. Panel D shows control activation of the native myocardium with the standard activating solution, and E shows the consequences of gelsolin treatment (for 60–120 min) for the same activation. In Figure 2E tension was not generated because the thin filament had been removed. Figure 2F shows that, after actin filament reconstitution, isometric tension was restored to 50–70% of that in the control [11]. Figure 2G shows the consequences of thin-filament reconstitution with nTm (Figure 2A), $\Delta T_m$ (Figure 2B), and $\Delta T^*_m$ (Figure 2C). In the cases of reconstitution with nTm and $\Delta T^*_m$, tension was greater than that attributable to actin-filament tension (Figure 2F), and comparable to that generated in response to initial activation in Figure 2D. However, in the case of reconstitution with $\Delta T^*_m$ (Figure 2CG), tension was significantly lower than that of actin-filament reconstituted preparations (Figure 2CF).
3.3. pCa-Tension and pCa-Stiffness Studies. Whether the cooperative activation mechanism and the Ca\(^{2+}\) sensitivity of the myocardium are altered by the Tm mutants was determined by studying tension and stiffness of the thin filament-reconstituted fibers as functions of pCa. The results obtained for filaments reconstituted with nfTm, Δ2Tm, and Δ3Tm are shown in Figure 3(a) (tension) and in Figure 3(b) (stiffness). The data were fitted to (1) and the results are summarized in Table 2. Maximal tension (\(T_m\)) at saturating Ca\(^{2+}\) and stiffness (\(Y_\infty\)) are entered in Table 4. This table demonstrates that \(T_m\) hardly differs between the nfTm and Δ2Tm muscle models, but the \(T_m\) of the Δ3Tm muscle model is only 60 ± 12% that of the nfTm model. Hence we conclude that isometric tension is significantly reduced in this muscle model. Table 2 also demonstrates that pCa\(_{50}\) is slightly decreased (by 0.17 ± 0.08 units) in the case of Δ2Tm but substantially increased (by 0.23 ± 0.09 units) in Δ3Tm. Also, cooperativity (\(h\)) does not differ significantly between the nfTm and Δ2Tm models, but is slightly reduced in the Δ3Tm model. A similar trend can be seen for stiffness (\(Y_\infty\)) (Table 2). Thus, the large difference in maximal tension and stiffness of the Δ3Tm mutant cannot be explained by a difference in the Ca\(^{2+}\) sensitivity.

Because E. coli-synthesized Tm lacks acetylation at the N-terminal Met, and this modification is thought to be important for the head-to-tail association of the Tm molecule [37, 38], we compared the pCa-tension plots for nfTm and acetyl Tm purified from bovine myocardium (fitted parameters were reported previously [13]). The results are plotted in Figure 3(c) and are compared in Table 2. They demonstrate that there is hardly any difference in the pCa-tension plot between nfTm and acetyl Tm: ΔpCa\(_{50}\) was 0.12 ± 0.09, and Δ\(h\) was 0.01 ± 0.31. Therefore, we conclude that after unacetylated Tm is incorporated in the thin filament, it behaves as the normal acetylated Tm does. We further conclude that the difference in Ca\(^{2+}\) sensitivity or cooperativity associated with thin-filament activation in the presence of Δ3Tm is not a consequence of the lack of acetylation of the N-terminal Met.

3.4. pS-Stiffness and pS-Tension Studies. To examine whether the cooperativity with respect to relaxation from the rigor condition is altered when mutant Tms are present, both Ca\(^{2+}\) and MgATP\(^{2-}\) were deleted from the standard activating solution to produce fibers in the “high-rigor state” [39] with large tension. In the absence of Ca\(^{2+}\), MgATP\(^{2-}\) was added to the rigor preparation incrementally, and both tension and stiffness at 100 Hz were studied as functions of pS. The results are shown in Figure 4(a) (pS-tension plot) and in Figure 4(b) (pS-stiffness plot), which compare the effects of nfTm, Δ2Tm, and Δ3Tm. As shown in Figure 4(a), increasing [MgATP] led to increased tension, which peaked at around pS = 5.4 ~ 5.6, and declined quickly over higher MgATP concentrations [39, 40]. The increase in tension was due to rigor activation of the thin filament (owing to thin-filament activation by attached cross-bridges [41]) and increased availability of MgATP to generate higher tension [39]. The stiffness (Figure 4(b)) stayed about the same in the pS range between 6.5–5.6; this indicates that the maximum number of strongly attached cross-bridges has been attained and does not change appreciably in the pS range between 6.5–5.6. The tension and stiffness then declined towards the higher MgATP concentrations, as had been demonstrated earlier using crayfish walking-leg muscle fibers [39, 40]. The decrease in tension and stiffness was due to an increase in [MgATP], which results in the detachment of myosin cross-bridges. Because this detachment disrupts cooperative activation, the relaxation was abrupt, as shown in Figure 4. The stiffness data were fitted to (2), and the results are

3.2. Resting Tension and Stiffness, and the Ability to Turn Off the Actomyosin Interaction at pCa 7. It is possible that the actomyosin interaction may not be completely turned off in some of the mutant Tms used for reconstitution. For this reason, tension at pCa7.0 was measured at 25°C as the incremental tension from the relaxed level that exists at 0°C in the relaxing solution that contains 40 mM BDM. All the measured parameters were normalized to \(T_{ac}\) averaged, and listed in Table 1 for three muscle models. The results from acetyl Tm are also included. Similarly, stiffness values at relaxation (0°C) and at pCa 7 (25°C) are listed in Table 1. As this table demonstrates, all the parameters measured are not any different from those of the nfTm control, indicating that the actomyosin interactions were indeed turned off at pH 7 with any of the Tms used.
Table 1: Resting tension and stiffness. The fibers were relaxed at 0°C in the relaxing solution that contains 40 mM BDM. Tension at pCa7.0 was measured at 25°C as the incremental tension from the relaxed level. All the measured parameters were normalized to $T_{ac}$, the active tension of the actin filament reconstituted fibers at 25°C. Compared to the ntim control, all $P$ values are > .05, hence there are no significant differences from ntim in three parameters measured.

|                        | ntim (control) | Δ2Tm   | Δ3Tm   | Acetyl Tm† | Units |
|------------------------|----------------|--------|--------|------------|-------|
| Number of expts.       | 17             | 18     | 18     | 8          |       |
| Tension at pCa 7       | 0.065 ± 0.113  | 0.109 ± 0.033 | 0.196 ± 0.047 | 0.168 ± 0.052 | $T_{ac}$ |
| Stiffness at pCa 7     | 0.044 ± 0.015  | 0.039 ± 0.004 | 0.040 ± 0.004 | 0.038 ± 0.011 | $T_{ac}$ |
| Stiffness at relaxation| 0.055 ± 0.022  | 0.049 ± 0.005 | 0.044 ± 0.005 | 0.048 ± 0.015 | $T_{ac}$ |

†Data obtained together with [13] but were not published.

Figure 3: pCa-tension curves. (a) pCa-tension plot comparing Δ2Tm-, Δ3Tm-, and ntim-reconstituted muscle fibers. The tension data were fitted to (1), and the continuous lines are drawn based on best-fit parameters (Table 2). Error bars represent SEM. $N = 14$ for ntim, $N = 15$ for Δ2Tm and Δ3Tm, and $N = 9$ for acetyl Tm. For acetyl Tm, the fitted parameters were reported in Lu et al. [13]. (b) pCa-stiffness plot, fitted to an equation similar to (1). (c) pCa-tension plot comparing ntim and acetyl Tm.
The complex modulus data $Y(f)$ resulting from the application of this methodology to the three muscle models are plotted in Figure 5, where each point represents the frequency of oscillation. The complex modulus data all show a typical cardiac response [33, 34]: the dynamic modulus (Figure 5(a)) is $\nu$-shaped, with the minimal value occurring at $\sim 1.4$ Hz ($= f_{\text{min}}$), and it increases to a plateau both in the low-frequency and high-frequency ranges. The average value of $2\pi f_{\text{min}}$ is entered in Table 4. The phase shift (Figure 5(b)) is $s$-shaped; its minimum occurs at $\sim 0.5$ Hz and its maximum at $\sim 3$ Hz. The Nyquist plot (Figure 5(c)) for the cardiac preparations is in the form of two semicircles; its low-frequency component is in the 4th quadrant, whereas its high-frequency component is in the 1st. The general shape of the plots does not differ significantly among the three muscle models, except that in $\Delta 3$Tm the amplitude of the dynamic modulus is about 66% that of others (Figure 5(a)). Consequently, the diameter of the semicircles in the $\Delta 3$Tm muscle model is about 66% that of the other two models (Figure 5(c)). The highest frequency used (100 Hz; time resolution 1.6 milliseconds) is adequate to characterize complex modulus in cardiac muscle fibers, because both dynamic modulus (Figure 5(a)) and phase shift (Figure 5(b)) approach steady values towards 100 Hz, the exponential process B centers at around 1 Hz ($= b$), and exponential process C centers at around 5 Hz ($= c$).

The calculated rate constants and associated parameters are summarized in Table 4, in which it can be seen that both apparent rate constants ($2\pi b, 2\pi c$) differ very little between the $\text{nTm}$ and $\Delta 2$Tm models. However, they are significantly different in the $\Delta 3$Tm model: $2\pi b$ is $1.4\times$ smaller, and $2\pi c$ is $1.2\times$ larger, than their counterparts in the $\text{nTm}$ model. These facts imply that the cross-bridge kinetics of the $\text{nTm}$ and $\Delta 2$Tm models are similar, but that those of the $\Delta 3$Tm model are significantly different. The magnitudes $B, C$, and $H$ do not differ significantly between the $\text{nTm}$ and $\Delta 2$Tm models but are significantly lower (by 33–66%) in the $\Delta 3$Tm model. In all four muscle models tested, however, $f_{\text{min}}$ is similar and falls between the characteristic frequencies $b$ and $c$ defined in (3).

### 3.5. Sinusoidal Analysis and the Apparent Rate Constants

To characterize the cross-bridge kinetics in the thin-filament reconstituted muscle fiber system, we performed sinusoidal length oscillations during standard activation, and followed tension transients as reported [32]. We prefer this method over step analysis because of its higher resolving power.

### 3.6. Study of Elementary Steps of the Cross-Bridge Cycle

To compare each elementary step of the cross-bridge cycle...
**Figure 4:** pS-tension curves. pS-tension plot ($S = [\text{MgATP}^2-]$) in (a) and pS-stiffness plot in (b) comparing nfTm, Δ2Tm, and Δ3Tm. The stiffness data were fitted to (2), and continuous lines are drawn based on best-fit parameters. The tension data in (a) are fitted to an equation similar to (2), where only the data to the right of the peak are used for fitting. The experiments were carried out at 8 mM Pi, and in the absence of Ca$^{2+}$. Error bars represent SEM. $N = 5$ for nfTm and Δ2Tm, and $N = 4$ for Δ3Tm.

Table 4: Measured parameters of Cross-bridges. *P value ≤ .05 compared to nfTm; **P value ≤ .01 compared to nfTm; else, P value > .05. Other than rigor stiffness, all parameters were measured in the standard activating solution. $T_m$ is the steady-state active tension. Tension and stiffness were normalized against the actin filament tension ($T_{ac}$) in the standard activating solution at 25°C ($T_{ac} = 9.0 \pm 0.9$ kPa, ±SEM, $N = 52$). To deduce $f_{\text{min}}$, 5 frequency points around the minimum $|Y(f)|$ are fitted to the cubic equation with 4 constants. $2\pi b$ corresponds to the rate constant of phase 3, and $2\pi c$ corresponds to that of phase 2 of step analysis [32]. In all experiments, temperature was 25°C, ionic strength 200 mM, and pH 7.00. The rigor stiffness was measured at 100 Hz. The data were individually fitted to respective equations, averaged, and tabulated with ±SEM. †Data based on Lu et al. [13].

|                | nfTm          | Δ2Tm          | Δ3Tm          | Acetyl Tm† | Units |
|----------------|---------------|---------------|---------------|------------|-------|
| Number of expts.| 15            | 17            | 18            | 9          |       |
| Tension ($T_m$) | $1.51 \pm 0.28$ | $1.65 \pm 0.29$ | $0.91 \pm 0.08^*$ | $1.65 \pm 0.24$ | $T_{ac}$ |
| $Y_\infty$     | 103 ± 24      | 103 ± 14      | 71 ± 5        | 89 ± 14    | $T_{ac}$ |
| $T_m/Y_\infty$ | $1.56 \pm 0.08$ | $1.58 \pm 0.14$ | $1.31 \pm 0.10^*$ | $1.91 \pm 0.10^{**}$ | $T_{ac}$ |
| Rigor stiffness | 166 ± 11      | 201 ± 19*     | 249 ± 59      | 159 ± 23   | $T_{ac}$ |
| $2\pi f_{\text{min}}$ | 8.6 ± 0.4    | 7.4 ± 0.4     | 7.2 ± 0.4**   | 7.6 ± 0.6  | s$^{-1}$ |
| $2\pi b$       | 6.2 ± 0.4     | 6.3 ± 0.5     | 4.7 ± 0.4**   | 7.1 ± 1.0  | s$^{-1}$ |
| $2\pi c$       | 28 ± 1        | 28 ± 2        | 36 ± 3**      | 41 ± 4**   | s$^{-1}$ |
| $H$            | 49 ± 11       | 51 ± 9        | 34 ± 4        | 28 ± 6*    | $T_{ac}$ |
| $B$            | 56 ± 20       | 55 ± 13       | 22 ± 2*       | 29 ± 6    | $T_{ac}$ |
| $C$            | 110 ± 34      | 107 ± 17      | 58 ± 3        | 90 ± 13   | $T_{ac}$ |

among the three muscle models, we studied the effects of MgATP and Pi on the apparent rate constants $2\pi b$ and $2\pi c$ in the presence of saturating Ca$^{2+}$. The results for the MgATP study, in which [MgATP] was changed gradually from 0.05 mM to 10 mM while [Pi] was kept at 8 mM, are plotted in Figure 6; those for the Pi study, in which added [Pi] was changed gradually from 0 mM to 32 mM while [MgATP] was kept at 5 mM, are plotted in Figure 7. Results were fitted to a 6-state cross-bridge scheme (Scheme 1) [33, 35, 42]. Together, the rate and association constants of the elementary steps as defined in Scheme 1 are referred to as “kinetic constants.” These are summarized in Table 5, in which it can be seen that the kinetic constants for the nfTm and Δ2Tm models differ minimally, if at all. The largest effects are the 2.3× changes in $k_4$ and $K_5$, which represent the reversal of the force-generation step and the association
constant of Pi, respectively. Some kinetic constants differ significantly between the nfTm and Δ3Tm models, and the largest consequence of this is a 5.2× change in $K_5$.

3.7. Cross-Bridge Distribution. There are two possible reasons for the reduced isometric tension in the Δ3Tm model with respect to that in the nfTm and Δ2Tm models. (1) Fewer force-generating cross-bridges are formed in the Δ3Tm model, and (2) the force generated by each cross-bridge is less in the Δ3Tm model than in the nfTm and Δ2Tm models. To determine which of these scenarios is correct, we calculated the distribution of cross-bridges in each state according to (7)–(13) in [43]. The results are plotted in Figure 8. This plot demonstrates that the number of strongly attached cross-bridges is the same for nfTm and Δ2Tm, and that the number is slightly larger in Δ3Tm. In Δ3Tm, the extra cross-bridges are distributed in the AM and the AM*S states; although there is also some variation in the number of cross bridges in each of the two major force-generating states (AM*DP and AM*D), the sums of the cross-bridges in these states are similar for all three muscle models. These findings lead us to conclude that the force/cross-bridge is less in the Δ3Tm model than in the nfTm and Δ2Tm models.

3.8. Temperature Study. Because the positive temperature effect on isometric tension depends on the hydrophobic interaction between actin and myosin [24, 43], and this interaction is hypothesized to change depending on the
Table 5: Kinetic constants of Scheme 1. *P value ≤ .05 compared to nfTm; ** P value ≤ .01 compared to nfTm; else, P value > .05. The kinetic constants (for definitions, see Scheme 1) were measured by the sinusoidal length perturbation method at 25°C, ionic strength 200 mM, and pH 7.00. $K_2 = k_2 / k_−2$ and $K_4 = k_4 / k_−4$. The data were individually fitted to the respective equations, averaged, and tabulated with ±SEM. The number of experiments is given in parentheses ( ).

|          | nfTm     | Δ2Tm     | Δ3Tm     | Acetyl Tm | Units |
|----------|----------|----------|----------|-----------|-------|
| $K_1$    | 6.9 ± 1.8 (13) | 6.6 ± 1.8 (7) | 4.6 ± 2.4 (10) | 6.2 ± 1.3 (6) | mM$^{-1}$ |
| $K_2$    | 2.3 ± 1.0 (13) | 1.7 ± 0.5 (7) | 1.0 ± 0.4 (10) | 1.08 ± 0.21 (6) | —     |
| $K_4$    | 0.44 ± 0.08 (10) | 0.61 ± 0.11 (13) | 1.52 ± 0.54 (10)* | 0.61 ± 0.24 (6) | —     |
| $K_5$    | 0.13 ± 0.03 (10) | 0.30 ± 0.07 (13)* | 0.68 ± 0.21 (10)* | 0.11 ± 0.03 (6) | mM$^{-1}$ |
| $k_2$    | 15 ± 2 (13) | 17 ± 2 (7) | 14 ± 3 (10) | 22 ± 4 (6) | s$^{-1}$ |
| $k_−2$   | 13 ± 2 (13) | 14 ± 3 (7) | 22 ± 3 (10)** | 21 ± 2 (6)** | s$^{-1}$ |
| $k_4$    | 3.5 ± 0.3 (10) | 2.8 ± 0.3 (13) | 2.8 ± 0.4 (10) | 5.2 ± 1.1 (6) | s$^{-1}$ |
| $k_−4$   | 14.1 ± 5.8 (10) | 6.0 ± 1.0 (13) | 3.8 ± 1.1 (10)* | 14.5 ± 3.4 (6) | s$^{-1}$ |

Figure 6: The effect of MgATP on the apparent rate constant $2πc$. The data were fitted to (5). The Pi concentration was kept at 8 mM. The continuous lines are based on (5) with best-fit parameters.

Figure 7: The effect of Pi on the apparent rate constant $2πb$. To deduce the rate and association constants of steps 4 and 5, the results of Figure 7 were fitted to (6). The MgATP concentration was kept at 5 mM. The continuous lines are based on (6) with best-fit parameters.

Figure 9(a) shows that tension increased with rising temperature in all muscle models studied. The plots of the data for the nfTm and Δ2Tm muscle models are indistinguishable, but the plot for the Δ3Tm model is distinctly different, with a slope of about half that of the other two models. The results for stiffness (Figure 9(b)) were similar, with the plots for the nfTm and Δ2Tm muscle model being essentially indistinguishable, but that for the Δ3Tm model being distinctly different and having slope that is less pronounced than those of the two other muscle models. However, the fact that stiffness increased with temperature (1.5–3X) in each muscle model revealed a common feature; specifically,
it indicates that more cross-bridges are formed at higher temperatures in all of these models.

3.9. Biochemical Analysis of Component Proteins. Fibers used for the mechanical experiments were pooled and used for SDS-PAGE analysis. Because the amount of protein obtained for those experiments was not sufficient for gel electrophoresis, additional preparations of reconstituted fibers were made from 10–15 additional cardiac preparations per mutant (see Methods for details; see also Figure 10).

Figure 11 shows the results of the SDS-PAGE analysis. Following gelsolin treatment, all thin-filament proteins (actin, Tm, TnT, TnI) were removed, except for the small amount left on the gel (Lane 2; compare to Lane 1, which is native cardiac tissue). The remaining actin was required to nucleate subsequent actin polymerization. The actin filaments were reconstituted (Lane 3), after which Tm and Tn were reconstituted (Lanes 5, 7, 9). TnT is difficult to see because it runs just below the heavy actin band and near 40 kD, as shown in our earlier publications [11, 13]. nTm, Δ2Tm, and Δ3Tm were all incorporated into reconstituted fibers, as were TnT and TnI (Lanes 5, 7, 9). The staining method used does not visualize TnC.

Densitometric analysis of the gels demonstrated that the Tm/(actin+TnT) ratio in myocardium reconstituted with recombinant Tms was within the error range of that in native cardiac myocardium (Results not shown). The amount of TnI may have been somewhat lower in the Δ3Tm-reconstituted fibers, but the error of densitometric measurement is large (∼ 20%), so the significance of this is likely minimal.

4. Discussion

Tropomyosin is well known to enhance actomyosin interaction. This was first demonstrated by Weber’s group [41] who showed that regulatory proteins, troponin and, tropomyosin, in the presence of saturating Ca2+, make function the thin filament cooperative, as measured by the actin-activated ATP hydrolysis rate. Eaton [44] demonstrated that HMM cooperatively increases the affinity of Tm for actin; an effect that is consistent with work of Lehrer and Morris [45] who showed a cooperative effect of actin on the activation of the myosin S1 ATPase in the presence of Tm.

Because the deletion of period 3, but not that of period 2, of Tm’s seven repeats impairs the normal function of reconstituted cardiac muscle fibers (Tables 2–5), we infer a specific role for this constitutively expressed period in thin-filament regulation. We previously reported that thin-filament reconstituted with a Tm mutant harboring deletions of both periods 2 and 3 (Δ23Tm; deletion of residues 47–123) exhibited impaired tension and stiffness-generating capacity as a consequence not of a reduced number of cross-bridges, but rather due to reduced tension and stiffness per cross-bridge [13]. The temperature dependence of isometric tension and cross-bridge kinetics for this mutant were reduced as well, leading to the suggestion that a hydrophobic interaction contributes to the positive allosteric effect of Tm [14]. The results reported in the current study attribute the impaired function of the Δ23 mutant entirely to the loss of period 3, a constitutively expressed region that is encoded by exon 3. We also show that N-terminal acetylation of Tm is not a requirement for normal contractile function (Figure 3(c)). We thus conclude that cooperative actin-filament activation is influenced by specific internal periods of Tm, but not by N-terminal acetylation, which is primarily a determinant of actin affinity [37, 38]. In contrast, the generic ability of Tm to bind to the actin filament and to stiffen it, thereby stabilizing it against severing proteins such as cofilin, has not been ascribed to specific periods.

The deletion mutant design incorporates the Phillips’ [3] proposed actin binding sites based on the heptapeptide repeat of hydrophobic residues that defines the coiled-coil structure. In his analysis, period 2 has 42 residues while period 3, a constitutively expressed region that is encoded by exon 3. We also show that N-terminal acetylation of Tm is not a requirement for normal contractile function (Figure 3(c)). We thus conclude that cooperative actin-filament activation is influenced by specific internal periods of Tm, but not by N-terminal acetylation, which is primarily a determinant of actin affinity [37, 38]. In contrast, the generic ability of Tm to bind to the actin filament and to stiffen it, thereby stabilizing it against severing proteins such as cofilin, has not been ascribed to specific periods.

The deletion mutant design incorporates the Phillips’ [3] proposed actin binding sites based on the heptapeptide repeat of hydrophobic residues that defines the coiled-coil structure. In his analysis, period 2 has 42 residues while period 3 has 35 residues. Previous work showed that Δ3Tm with 35 or 42 residues deleted has similar conformational stability and actin binding properties [15]. For structural considerations we continued our analyses on Δ3Tm with a 35-residue deletion and reported that it impaired the actin affinity, Ca2+-dependent activation of the regulated actomyosin ATPase, and cooperative myosin S1-induced binding to actin compared to Δ2Tm [19]. The overall stability of the Δ3Tm mutant is similar to that of the Δ23Tm, in which 77 residues were deleted (Δ23Tm binds with higher actin affinity than Δ3Tm [15] but Δ23Tm has altered regulatory function [13, 14] that parallels that of the Δ3Tm mutant.

Because we observed no noticeable tension development at pCa 7.0 in all 4 Tms tested (Table 1), we conclude that the inhibition of the actomyosin interaction is adequate at pCa 7 in any muscle models we studied.
4.1. N-Terminal Acetylation of Tm Does Not Affect Thin-Filament Function in Reconstituted Cardiac Muscle Fibers.

The recombinant rat α-Tms used to reconstitute the thin filaments in the myocardial strips were expressed in E. coli, an organism that lacks the enzyme that carries out post-translational N-terminal acetylation. The acetyl moiety is present in Tms prepared from eukaryotic cells. Because the N-terminal acetylation of Tm is required for the protein’s normal affinity for actin, the head-to-tail association among Tm molecules, tropomodulin binding, and the binding of the Tm N-terminal domain to TnT [37, 38, 46, 47], we compared acetylated and unacetylated Tm in our experiments. We found that Ca²⁺ regulation, as assessed by measurements of pCa₅₀ and cooperativity (h) between neighboring regulatory units and cross-bridges, was essentially the same regardless of acetylation at the N-terminus (Figure 3(c) and Table 2). Furthermore, the kinetic constants of elementary steps of the cross-bridge cycle were not significantly different, except for k₋₂ (Table 5). From these results, we conclude that unacetylated Tm has normal regulatory function after it is incorporated into the muscle fiber system, as it does in an in vitro regulated actomyosin system [37]. Similar results have been reported for Tm with an N-terminal Ala-Ser extension [13, 14]. We presume that the binding of our Tm proteins to actin and to the N-terminal domain of TnT “corrects” the structural deficiency of Tm, or that the primary function of N-terminal acetylation is to ensure assembly with the actin filament without being required for subsequent functions.

4.2. Comparison to Other Reports.

By using Δ₂₃Tm in skeletal myofibrils, a recent report by Siththanandan et al. [48] demonstrated that, at 10°C, pCa₅₀ increased by 0.47 units and cooperativity decreased from 2.92 to 1.44, whereas in this report using Δ₃Tm in myocardium at 25°C, pCa₅₀ increased by 0.23 units and the cooperativity decreased (but insignificantly) from 1.73 to 1.35 (Table 2). Therefore, there is a qualitative agreement between these two reports. Some differences must be based on differences in preparations (skeletal versus cardiac backgrounds), in the reconstitution methods, in the temperature (10°C versus 25°C), and in mutant proteins themselves (ASΔ₂₃Tm versus Δ₃Tm). In addition, low isometric tension in reconstituted preparations with Δ₂₃Tm (20%) [48] may be a concern. With our methods, isometric tension was 42% in Δ₂₃Tm [13] and 60% in Δ₃Tm at 25°C (Table 4).

For cross-bridge kinetics, Siththanandan et al. [48] measured kₛₑ and kᵣₑ, but they did not relate these parameters to
the kinetic constants of the cross-bridge cycle. If we assume that $k_{rel} \sim k_2 + k_{-2}$, and $3k_{act} \sim (k_4 + k_{-4})$, approximate numerical agreements can be seen among control muscle models, and among mutant ($\Delta 2Tm$, $\Delta 3Tm$) muscle models. These agreements may be fortuitous, however, because we know that the myosin isoform can have a major influence on the cross-bridge kinetics [49, 50], and the ambient temperature difference (10°C versus 25°C) affects all rate constants significantly, $k_4$ in particular [24, 43, 51]. Another reason why $k_{act}$ is slow may be related to the fact that cross-bridges cycle several times to stretch series elastic elements on activation, hence the rate-limiting step of the cycle dominates $k_{act}$ [42]. Furthermore, Siththanandan et al. [48] measured the rate constants of Pi transient at 2 different Pi concentrations, whereas we characterized the force generation step 4 and the Pi release step 5 with 6 different Pi concentrations (Figure 7). Because steps 4 and 5 involve at least 3 independent kinetic parameters (Scheme 1) [35, 52], it would not be possible to deduce mechanisms underlying these steps based on 2 experimental points.

4.3. Tm’s Allosteric Effects on Actin. Tm’s role in the cooperative enhancement of actomyosin interactions has led to the hypothesis that Tm in the presence of Tn and Ca$^{2+}$ induces a positive allosteric effect on actin, and possibly also on myosin [11, 53, 54]. We previously reported that actin-filament reconstituted myofibrillar preparations undergo greater force development when Tm and Tn are incorporated, demonstrating the positive allosteric effect [11, 13]. We further showed that the force per cross-bridge is about twofold different. Similarly, the inclusion of Tm and Tn in an in vitro motility assay system increased the sliding speed and force per cross-bridge [7–10, 55]. In the case of reconstitution with the $\Delta 2Tm$ mutant, the force was lower than that produced when the actin filament alone was reconstituted, demonstrating the negative allosteric effect [13, 14]. The similar negative allosteric effect can be seen in $\Delta 3Tm$ in tension (Table 4, average $T_m$ is less than 1). Based on the results of the current study, we can attribute this negative allosteric effect to the absence of period 3, and suggest that period 3, but not period 2, is essential for the positive allosteric effect that nfTm or acetyl Tm has on the thin filament, and for the enhanced hydrophobic interaction between actin and myosin molecules. These results are consistent with the results of solution assays involving the regulated actomyosin ATPase, in which addition of $\Delta 2Tm$ was sufficient to rescue regulation [19, 30], whereas the addition of $\Delta 3Tm$ was not, that is, Ca$^{2+}$ only partially relieved inhibition [19]. While our investigation demonstrates the significance of period 3 for the positive allosteric effect of Tm on actin and excludes period 2 for this effect in the muscle fiber system, it does not rule out the possibility that other periods are also involved in eliciting this positive allosteric effect. Indeed, solution studies have suggested that periods 4 and 5 are involved in this fundamental regulatory function [17, 19, 56], and this possibility needs to be investigated in the muscle fiber system in future studies. It is likely that Tn also plays a role for the allosteric effect and as reported by us [54] as well as others [55].

4.4. The Tension per Cross-Bridge, rather than the Number of Force-Generating Cross-Bridges, is Reduced in the $\Delta 3Tm$ Model. Low tension could be the result of a reduced number of force-generating cross-bridges and/or reduced force per cross-bridge. Based on our analysis of the kinetic constants of the elementary steps of the cross-bridge cycle, we estimate that the $\Delta 3Tm$ model involves the formation of slightly more cross-bridges in the strongly attached force-generating states than in the nfTm or $\Delta 2Tm$ models (Figure 8). Since the isometric tension is reduced in this model, we calculate the force per cross-bridge in the $\Delta 3Tm$ model as about one half that in the $\Delta 2Tm$, nfTm or acetylated Tm model. The
measured isometric tensions in the Δ2Tm and nfTm models are very similar (Table 4); thus we infer that tension/cross-bridge in the Δ2Tm model is unchanged from that in the nfTm model.

The fact that rigor stiffness is similar among the three Tms tested (Table 4) implies that when the rigor state is established, the connection between actin and myosin becomes similar in each of these models. This observation suggests that the configuration of the AM state in Scheme 1 in the presence of ATP may be different from the rigor-state linkage which exists in the absence of ATP.

4.5. Evidence that Tm Period 3 is Responsible for the Increased Hydrophobic Interaction (Allosteric Effect) between Actin and Myosin Molecules. Many studies on skinned cardiac and skeletal muscle fibers have demonstrated that isometric force increases as the ambient temperature increases [43, 51, 57–65]. Two mechanisms have been proposed to explain this increase: (1) an increase in the number of force-generating cross-bridges as temperature rises, and (2) an increase in the force/cross-bridge as temperature rises. The first mechanism is supported by the fact that the equilibrium constant of the force generation step (Kf) increases with temperature [43, 51, 52, 64, 65]. The second mechanism is supported by the fact that stiffness does not change much with temperature [57, 58, 63, 66], and that there is a structural rearrangement in the cross-bridge conformation (such as axial tilting) based on low angle X-ray diffraction studies [67, 68]. Our finding in the current study, that the stiffness increases as much as 4-fold as temperature increases from 5°C to 40°C (Figure 9(b)), supports the first mechanism as the major cause underlying the temperature effect. While we agree that the cross-bridge conformation may cause a small effect on isometric tension and stiffness, it would be difficult to explain the 4-fold change in stiffness and 13-fold change in tension (Figure 9) based on conformational changes.

The reason for the large temperature effect on Kf is that the Van der Waals force between hydrophobic (apolar) amino acid residues of actin and myosin has a significant effect on force generation, as shown by a large increase in enthalpy (∆H°), a large increase in entropy (∆S°), and a large decrease in heat capacity (∆Cp) [43, 51, 64]; reviewed by [24]. When the total number of available cross-bridges is the same, the slope of the temperature-tension plot can be used as an index of the degree of the impact the hydrophobic interaction has on force generation [24]. Our finding that the slope in muscle fibers reconstituted with Δ3Tm instead of nfTm is reduced to half (Figure 9) implies that Δ3Tm leads to an ∼ 50% reduction in the hydrophobic interaction. Our finding that the slope is unchanged when Δ2Tm is used instead of nfTm implies that Δ2Tm does not cause a change in the hydrophobic interaction relative to that due to the presence of nfTm. Thus, the results of the temperature study are consistent with the hypothesis that the hydrophobic interaction between actin and myosin is ∼ 2-fold stronger in fibers reconstituted with nfTm or Δ2Tm than in those reconstituted with Δ3Tm. This observation suggests that period 3 is essential for induction of the positive allosteric effects of Tm on actin, and for an enhanced hydrophobic interaction between actin and myosin molecules.

According to Holmes et al. [69], there are stereospecific and hydrophobic interactions between residues Pro-529, Met-530, Glu-538, Met-541, Phe-542, Pro-543 of myosin (sequence based on chicken skeletal myosin II), and residues Leu-140, Tyr-143, Ile-341, Ile-345, Leu-349, Phe-352 of actin; similar results had been obtained earlier by Rayment et al. [70]. However, these analyses were carried out in the absence of regulatory proteins. Our results imply that the number of hydrophobic amino acid residues at the actin-myosin interface increases as the regulatory proteins are added and as hypothesized earlier [11, 56]. This is because the allosteric interaction between Tm and actin may modify the actin molecule for better stereo optical match with the myosin molecule, and that this increased actin–myosin interaction may be further responsible for the increased slope of the temperature-tension plot. Our results indicate that Tm plays a central role in regulating and directing the stereospecific interactions between actin and myosin molecules that lead to optimal force development.

The interaction between Tm and actin is also postulated to include hydrophobic interactions. However, the source of these hydrophobic amino acid residues is not known at the present time. It has long been recognized that the binding of Tm to actin is weaker at low temperature [71], and this is consistent with the idea that the interaction includes a hydrophobic component. The local instability of Tm appears to be essential for actin binding and for the cooperative myosin S1-induced binding of Tm to actin [72, 73]. Periods that are stable in the cold are partially unfolded at physiological temperatures and may confer the flexibility required for Tm to bind to the helical actin filament in this context. Whereas the overall stability of Δ2Tm is similar to that of nfTm, Δ3Tm is less stable, and its lower affinity for actin may reflect a weaker hydrophobic interaction with actin [19]. Because of the weaker interaction between Δ3Tm and actin, this Tm mutant may not be capable of exerting the same allosteric effect on actin that nfTm would. Indeed, this is consistent with our finding that Δ3Tm has a negative allosteric effect. It is important to emphasize that the results presented here were obtained in an organized system in which force can be generated, and that the experiments were carried out at physiological ionic strength. It would be difficult to interpret the results of an experiment if force was not detected. If the experiments were performed in low ionic strength solutions, then the electrostatic interaction among molecular domains would become the major contributing factor, which might invalidate interpretations.

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