A Modulatory Role for the Troponin T Tail Domain in Thin Filament Regulation

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In striated muscle the force generating acto-myosin interaction is sterically regulated by the thin filament proteins tropomyosin and troponin (Tn), with the position of tropomyosin modulated by calcium binding to troponin. Troponin itself consists of three subunits, TnI, TnC, and TnT, widely characterized as being responsible for separate aspects of the regulatory process. TnI, the inhibitory unit is released from actin upon calcium binding to TnC, while TnT performs a structural role forming a globular head region with the regulatory TnI-TnC complex with a tail anchoring it within the thin filament. We have examined the properties of TnT and the TnT1 tail fragment (residues 1–158) upon reconstituted actin-tropomyosin filaments. Their regulatory effects have been characterized in both myosin S1 ATPase and S1 kinetic and equilibrium binding experiments. We show that both inhibit the actin-tropomyosin-activated S1 ATPase with TnT, producing a greater inhibitory effect. The S1 binding data show that this inhibition is not caused by the formation of the blocked B-state but by significant stabilization of the closed C-state with a 10-fold reduction in the C- to M-state equilibrium, K_C, for TnT1. This suggests TnT has a modulatory as well as structural role, providing an explanation for its large number of alternative isoforms.

In striated muscle, the troponin (Tn)-tropomyosin (Tm) protein complex functions as a calcium-dependent molecular switch that regulates the force-generating interaction between actin and myosin filaments. Early studies have provided a role for each of the components of Tn, TnI, TnC, and TnT (reviewed Refs. 1 and 2). TnI has been shown to be responsible for the inhibition of ATPase due to its binding to actin (3). This inhibition is relieved by Ca²⁺ binding to TnC, while TnT performs a structural role forming a globular head region with the regulatory TnI-TnC complex with a tail anchoring it within the thin filament. We have examined the properties of TnT and the TnT1 tail fragment (residues 1–158) upon reconstituted actin-tropomyosin filaments. Their regulatory effects have been characterized in both myosin S1 ATPase and S1 kinetic and equilibrium binding experiments. We show that both inhibit the actin-tropomyosin-activated S1 ATPase with TnT, producing a greater inhibitory effect. The S1 binding data show that this inhibition is not caused by the formation of the blocked B-state but by significant stabilization of the closed C-state with a 10-fold reduction in the C- to M-state equilibrium, K_C, for TnT1. This suggests TnT has a modulatory as well as structural role, providing an explanation for its large number of alternative isoforms.

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† The abbreviations used are: Tn, troponin; Tm, tropomyosin; MOPS, 4-morpholinepropanesulfonic acid.

MATERIALS AND METHODS

Isolation of Proteins from Rabbit Skeletal Muscle—Troponin and TnT were purified according to the methods of Greaser and Gergely (3, 5). TnT1 was obtained by chymotryptic digestion of TnT (15).

Tropomyosin was isolated according to Smillie (16). Myosin subfragment 1 (S1) was prepared by cryotryptic digestion of rabbit myosin, as described by Weeds and Taylor (17). Its molar concentration was calculated from absorbance measurements at 280 nm using an E₅₀₀ of 7.9 cm⁻¹ and a molecular mass of 115,000 Da. Actin was purified from rabbit skeletal muscle according to Spudich and Watt (18) and then labeled at Cys-374 with N-(1-pyrene)iodoacetamide according to Cridde et al. (19). Phalloidin (Roche Molecular Biochemicals)-stabilized F-actin was made by incubating a solution of 10 μM pyrene-actin with 10 μM phalloidin overnight in experimental buffer (20 mM MOPS, pH 7.0, 200 mM KCl, 5 mM MgCl₂) at 4 °C.

Cosedimentation and Quantitative Electrophoresis—Cosedimentation assays were performed at 20 °C by mixing actin with binding proteins (Tm, TnT, and TnT1) at relevant concentrations in the standard assay buffer (20 mM MOPS, pH 7.0, 200 mM KCl, 5 mM MgCl₂) to a total volume of 200 μl. The actin was then pelleted (along with any bound proteins) by centrifugation at 100,000 × g for 20 min (Beckman Instruments, TL100A). Equivalent samples of pellet and supernatant were then separated by SDS-PAGE. SDS-PAGE was performed according to Laemmli (20) using 13.5% acrylamide gels and stained with Coomassie Blue G-250. Quantification of proteins was carried out by using a Umax Powerlook III scanner with transparency adapter attached to a PC. The scanner was calibrated using a Kodak neutral density filter.
density step tablet, and scanned images were analyzed using the Image-PC program (Scion Corp., based upon NIHImage).

Stopped-flow and Fluorescence Titrations—Stopped-flow experiments were performed at 20 °C with either a Hi-Tech Scientific SF-61 or SF-61DX2 spectrophotometer in fluorescence mode. Pyrene fluorescence was excited at 365 nm and emission detected at right angles using a KV 389-nm cut-off filter (Schott, Mainz). Data were stored and analyzed using the Kinetasyt software provided with the instrument. Transients shown are the average of 3–5 shots of the machine. In these experiments the concentrations quoted are those after mixing the final concentrations being half of the stock solutions in the syringes. Binding is monitored by the ~70% quenching of the pyrene-actin fluorescence upon binding of the S1 to actin (21). The occupancy of the blocked state (KB) has been determined using the kinetic method of McKillop and Geeves (8). The rate of binding of S1 to reconstituted filaments is measured under excess actin conditions such that the ratio of S1 to actin is 1:10. Under these conditions the rate of binding of S1 to actin is pseudo-first order and dependent upon the available actin concentration. The occupancy of the blocked state reduces the available actin concentration and can therefore be detected by the reduction in the observed rate constant of S1 binding to thin filaments reconstituted with regulatory proteins compared with filaments only containing actin.

Fluorescence titrations were measured at 20 °C using a PerkinElmer Life Sciences 50B spectrophotometer with excitation at 365 nm with a 10-nm bandwidth and measuring emission at 405 nm with a 15-nm bandwidth. A total working volume of 2 ml was used in a 10 × 10-mm cell constantly stirred using a magnetic stirrer below the light path of the instrument. Autotitrations were made by the continuous addition of a concentrated S1 stock solution using a Harvard Apparatus Syringe Infusion Pump 22 driving a 100-µl glass syringe (Hamilton) as described previously (9). Data were acquired over a period of 250 s, with data points being collected every 0.5 s, using an integration time of 0.45 s. Buffer solutions for the titrations were filtered using a 0.22-µm disposable syringe filter to remove dust particles that can produce significant noise in the stirred cell at the low levels of sample fluorescence used. Mixing and equilibration of the reaction in the cell were checked as detailed previously (9).

ATPase assays were performed either by the colorimetric detection of phosphate described earlier (22) or by the continuous coupled enzyme assay system (23) EnzCheck (Molecular Probes).

Interpretation of Stopped-flow and Titration Data—Cooperative binding curves from titrations can be fitted using a version of the McKillop and Geeves model (Equation 1) with a varying cooperative unit size (9).

\[
\frac{\alpha}{K_{PB} + \left(1 + K_\alpha\right)P^{Q + 1} + Q^{1 + Q}} = \frac{F_0 - F}{F_0 - F_\infty}
\]  

(Eq. 2)

Parameter estimates from fitting a single data set produce meaningless standard errors of significantly less than 5% in the fitted parameters. The fitting procedure of systematic variation of n and examination of the sum of squares as detailed previously (9) gives a better estimate of standard errors of around 10–20% for K and K, with n approximated to the nearest whole number. K is determined by the stopped-flow experiments described. The blocked state reduces the available concentration of actin for rapid binding, which can be measured as a reduction in the observed rate (k) compared with that for unregulated actin (k) (Equation 3).

\[
k = k_{\text{act}} - k_{\text{act}} K_0 \frac{F_0 - F}{F_0 - F_\infty}
\]

(Eq. 3)

The data show that occupancy of the blocked state is very low apart from Tm-Tn-Ca, and hence the 1/K term is small and can be disregarded for fitting. The value of K is assumed to be 200 from previous work (21), and all other parameters can then be fitted.

RESULTS

The effects of TnT and TnT on the regulation of actin-activated S1 ATPase are shown in Figs. 2 and 3. Fig. 2A shows the continuous measurement of the liberated Pi using a coupled assay. Under the conditions used (see figure legend), actin caused an ~10-fold increase in the ATPase rate over the basal S1 level. Addition of a saturating level of Tm under these conditions was inhibitory, resulting in an ~20% reduction in the actin-activated ATPase rate. The addition of TnT to actin-Tm produced an additional inhibition with the effect saturating at a 1:1 ratio of TnT to Tm.

Fig. 2B shows the effects of increasing concentrations of TnT and TnT on the activation of actin-Tn-Tm plotted as S1 ATPase rate against concentration of TnT or TnT added. Both show inhibition with saturation at an ~1:1 ratio of TnT or TnT to Tm indicating stoichiometric binding, with TnT producing a slightly greater inhibition than TnT but with a less clear saturation point. Thus, in the presence of Tm, TnT and TnT inhibit actin-activated S1 ATPase by ~60% under these conditions.

Fig. 3 shows representative data for the effects of TnT and TnT on the concentration dependence of the actin-activated S1 ATPase. This demonstrates several important points. First, it can be seen that in the presence of all Tn-containing systems, there are dual effects on actin-activated S1 ATPase due to the induced cooperativity that causes sigmoidal activation with S1 concentration. Second, both TnT and TnT cause an inhibition of the S1 ATPase relative to actin-Tm across the S1 concentration range studied, although less than that produced by the whole Tn complex in the absence of calcium under
similar conditions (24). Finally, the relative inhibition of S1 actin-Tm ATPase produced by the addition of the TnT 1 fragment is greater than that for whole TnT across the S1 concentration range. It should be noted that the ATPase rates of Figs. 2 and 3 are not directly comparable because lower actin and salt concentrations were used to produce reasonable ATPase rates across the range of S1 concentrations studied. In several similar experiments to that shown in Fig. 3, the exact degree of inhibition of the actin-Tm S1 ATPase by TnT and TnT 1 varied from 10 to 50% depending on the conditions used, but qualitatively similar plots were produced in all cases.

A cosedimentation assay was used to confirm the formation of a stoichiometric complex of TnT or TnT 1 with Tm and to assess the effect of TnT and TnT 1 upon Tm binding to actin under the solution conditions used in the following titration experiments (see “Materials and Methods” for details). Binding curves to actin for Tm in the presence or absence of an equal concentration of TnT is shown in Fig. 4. Under the conditions used there was no evidence of precipitation of either TnT or TnT 1 within the concentration range studied. The results for the effect of TnT 1 upon Tm affinity for actin were similar to that of TnT but are not shown on the figure for clarity. Curve fits shown are to the Hill equation commonly used for fitting sigmoidal Tm binding curves which gives a half-saturation point $K_{50\%}$ that can be used as a measure of Tm affinity for actin. Stoichiometry was obtained by comparison of band staining to bands containing known protein concentrations and validated against the known total protein concentrations added in the experiments. The results confirm that a 1:1 complex of TnT or TnT 1 with Tm binds to actin in both cases. Under these conditions TnT or TnT 1 only causes a comparatively small (2–3-fold) increase in the $K_{50\%}$ of Tm for actin similar to that reported for a peptide consisting of residues 70–150 of cardiac TnT (25) and for the effects of TnT 1 and TnT 2 fragments on non-polymerizable Tm (26). This is a far smaller effect than the 2–3 orders of magnitude change seen for the whole Tn complex (27–29).

Kinetic measurements of S1 binding to actin-Tm were made to determine whether TnT or TnT 1 can induce occupancy of the blocked B-state in which S1 binding is prevented. Measurements of the rate of S1 binding to reconstituted thin filaments at a 1 to 10 ratio of S1 to actin are shown in Fig. 5A. As detailed under “Materials and Methods,” these experiments measure the fraction of actin available kinetically for S1 to bind accord-
any of the systems is for the presence of whole Tn in the absence of calcium. Thus, there is no evidence from this or previous in vitro data of significant occupancy of the B-state for thin filaments containing Tm alone or accompanied by either TnT or TnT1 (7–9).

Equilibrium binding measurements for S1 to actin-Tm were used to assess the effect of TnT or TnT1 upon the C- to M-state equilibrium. These are shown in Fig. 5B and Table II. It can be clearly seen that both TnT and TnT1 shift the S1 binding curves toward the right, indicating that a higher concentration of S1 is needed to cause the same degree of saturation. The filaments are therefore further switched “off” in comparison to actin-Tm with the shift for TnT1 located at a higher S1 concentration, and hence it is further off than that seen for whole TnT. As the kinetic experiments show no blocked state to be present, these curves can be fitted to a 2-state version of the 3-state model described by Equation 1 ignoring the $K_p$ term (9). The value of $K_s$ (the rigor isomerization) has been shown to be dependent only upon the nucleotide bound to S1 and is hence set at the previously determined value of 200 for nucleotide-free S1 (8). As expected for systems containing the same S1 and actin, fitting produced similar values of $K_1$ (the weak binding) varying from 1.85 to $2.6 \times 10^5$ s$^{-1}$ M$^{-1}$ for all the reconstructed filaments. The shape of the curve is therefore defined by the values of $K_T$ (the C/M equilibrium) and $n$ (the apparent cooperative unit size). In comparison to actin-Tm, the presence of TnT produces a 2-fold change in $K_T$ (0.034) with little effect upon $n$ (7). The TnT1 data show a significantly greater 10-fold reduction in $K_T$ (0.006), along with an increase in the apparent cooperative unit size $n$ (9). The data shown in Fig. 5B are representative curves from experiments performed in triplicate. There is some quantitative variation between the values for $K_1$, $K_T$, and $n$ determined here and those previously published (7, 8) for actin-Tm which showed a slightly lower value of $K_1$ ($1 \times 10^5$ s$^{-1}$ M$^{-1}$) and $K_T$ (0.14) and slightly higher $n$. However, the effects of TnT and TnT1 shown here were highly reproducible between replicates and represent an internally consistent data set.

Fig. 5C shows the titration curves from Fig. 5B plotted with the y axis inverted (corresponding to fraction of actin bound) overlaid with a curve for S1 binding to unregulated actin. In this format the figure can be directly compared with the equivalent ATPase against S1 concentration curves for the same reconstituted filament systems, although at a lower salt concentration, shown in Fig. 3. The correspondence between the two can clearly be seen indicating the cooperativity of S1 binding in both cases. In both plots the three curves for actin-Tm, actin-Tm/TnT, and actin-Tm/TnT1 are offset from left to right, all crossing the actin curve at increasing S1 concentrations in the same order. The difference in salt concentration between the two experiments is not expected to have a substantial effect upon the form of the curves as regulation of S1 binding is similar at the lower salt concentration when sufficient Mg$^{2+}$ (5 mM) is present (21).

**DISCUSSION**

This study shows that the binding of either TnT or TnT1 to actin-Tm inhibits both S1 binding to actin and actin-activated S1 ATPase rates. In neither case is the inhibition due to the

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**Table I**

|          | Actin | Actin/Tm | Actin/Tm/TnT | Actin/Tm/TnT1 | Actin/Tm/TnT1 –Ca |
|----------|-------|----------|--------------|---------------|------------------|
| Rate (s$^{-1}$) | 10.9  | 7.8      | 11           | 10.4          | 3.3              |

**Fig. 5.** Effect of regulatory proteins in the kinetics and equilibrium binding of S1 to actin. A, kinetics of S1 binding to actin. Binding curves shown are averages of 4–6 experiments and were fitted to a single exponential with the $k_{obs}$ values shown in Table I. Conditions are as follows: 1 μM S1 mixed with 5 μM actin plus 2 μM Tm/Tn/Tn as marked; 20 mM MOPS, pH 7.0, 200 mM KCl, 5 mM MgCl$_2$ plus 2 mM EGTA for the −Ca$^{2+}$ experiment. B, equilibrium binding of myosin S1 to 50 nM phallloidin-stabilized pyrene-actin plus 2 μM Tm plus 2 μM TnT or TnT$_1$ as marked. The data are shown superimposed with fitted curves to the cooperative binding model with values shown in Table II. C, equilibrium binding data from B replotted along with data for unregulated actin. The y axis is inverted and plotted with decreasing values to allow for easy comparison with Fig. 3.
formation of a blocked B-state, whereby a fraction of the actin is unavailable for binding but purely through the C- to M-state equilibrium being biased further toward the C-state. The data show that the TnT1 fragment of TnT causes a much greater inhibitory effect than whole TnT. We suggest the TnT1 region has a significant role in modulating thin filament regulation. The difference in apparent cooperative unit size between TnT and TnT1 suggests further interactions of the TnT1 "head" region of TnT. These results and their implications will now be discussed further.

**TnT/TnT1 Binding to Actin-Tm**—The combination of the ATPase and cosedimentation data indicate that both TnT and TnT1 interact with actin-Tm specifically to cause the formation of a stoichiometric A7TmTnT complex presumably resembling the native holocomplex. The cosedimentation data also show that both TnT and TnT1 have a relatively small effect on Tm affinity. Tm binding to actin has been described as a polymerization process controlled by two interactions, the single site affinity of Tm to actin which causes the initial binding and the Tm-Tm end-to-end interactions that drive polymerization (28, 30). The former will tend to govern the initial concentration at which binding starts, whereas the latter controls the cooperativity of the binding process once nucleation has occurred. There is some conflicting evidence for the effects of TnT and TnT1 upon the Tm-Tm interaction. It has been shown that both whole Tn, TnT, and TnT1 increase the cooperativity of S1 binding to actin-Tm (this work and Refs. 7 and 9). However, in contrast there appears to be a decrease in the cooperativity of binding of the regulatory complex to actin in these systems (28, 29, 31). It is therefore important to note that cooperativity of Tm and Tn binding to actin does not necessarily reflect the cooperativity of the regulation of S1-actin interactions. Hence the sigmoidal binding curves of Tm and Tn with Tn to actin are measuring a different form of cooperativity to the sigmoidal S1 ATPase and S1 binding curves.

The small effect upon Tm affinity to actin indicates that neither TnT nor TnT1 interact strongly with actin to stabilize the actin-Tm complex. This should be contrasted to the fact that whole Tn increases Tm affinity by 2–3 orders of magnitude (27, 28, 31, 32). This is caused by interactions in the head region of Tn, notably by the known strong TnI interaction with actin in the absence of calcium, which causes a 2–10-fold tighter binding than in the presence of calcium and also by secondary Tn interactions even when calcium is present. There is still some debate about the exact nature and strength of the interactions between Tn, Tm, and actin in these studies.

**ATPase Data**—The ATPase data show the following two important facts: 1) under the conditions in Fig. 2, TnT and TnT1 both cause an inhibition of the S1 ATPase relative to actin-Tm; 2) as shown in Fig. 3, the S1 ATPase can be either inhibited or "potentiated" by Tm and TnT or TnT1, depending on the S1 and actin concentration used. This point has often caused confusion in the literature when interpreting S1 ATPase data. There is no true "potentiation" of the S1 ATPase by the regulatory proteins, and the $V_{\text{max}}$ value at saturating S1 concentrations is similar in both the presence and absence of regulatory proteins (33). If there were no effect upon S1 affinity, then the sigmoidal S1 concentration-dependent S1 ATPase activation curve for a regulated filament would always produce an ATPase rate lower than that seen for an unregulated filament as regulation reduces the amount of actin that is fully available for binding. However, the presence of regulatory proteins, or more specifically vertebrate Tms, causes a 4–7-fold increase in the affinity of S1 for actin (34–37). This increased affinity results in crossing over of the sigmoidal S1-ATPase curves in the presence of regulatory proteins with the hyperbolic curve of unregulated actin (24) (Fig. 3).

This dual effect of the regulatory proteins on ATPase explains why it has been reported previously (38, 39) that Tn acts to "potentiate" the S1 ATPase and is responsible for "activation" of the filament. The comparative effect of regulatory proteins upon the S1 ATPase is dependent upon the conditions used, which govern the filament activation and hence where the system is relative to an S1 ATPase versus S1 curve of the form shown in Fig. 3. To interpret correctly the effect of regulatory proteins using ATPase assays, one therefore needs to understand the effect of the assay conditions upon the observed results, i.e. which part of the sigmoid activation curve is being studied.

The activation of S1 ATPase by bound S1 is demonstrated by close examination of the ATPase data in Fig. 2A. At the end of the traces of ATP versus time, it can be seen that the lines are not actually straight but slightly curved upward. This is due to the fact that at the end of the assay the ATP level is reduced so that the rate of ATP binding rather than hydrolysis becomes limiting. Under these no longer steady-state conditions, more S1 binds to the filament causing a short period where bound S1 increases activation of the filament more than compensating for the decreased ATP concentration causing a short burst in the ATPase rate just before all the ATP is consumed. This is related to the potentiation that was observed in early studies at low [ATP] (40, 41).

**Regulation of S1 Binding**—The effects of TnT and TnT1 upon S1 binding to actin-Tm are measured directly in the kinetic and equilibrium binding measurements. The blocked B-state is expected to be absent from previous data showing that it is specifically caused by the effects of the strong binding of TnI to actin (8, 42) and is not significantly occupied under any other conditions so far identified (7, 11, 37).

Data fitting shows that the difference between the titration curves is caused primarily by effects upon $K_r$, the C to M equilibrium. Whereas TnT produces a 2-fold reduction $K_r$ (0.034), TnT1 produces a 10-fold reduction in $K_r$ (0.006) showing that the filament is more greatly biased toward the C-state than any previous measurements of regulated filaments without showing any significant occupancy of the B-state. Thus, the inhibition of ATPase is due to a strengthening of the closed C-state rather than inhibition of S1 binding due to the blocked B-state.

There appears to be good agreement with the results from the binding assays with those from the ATPase measurements. This would be expected if, as reviewed by Gordon et al. (2), it is accepted that the dominant control of S1-activated ATPase is by regulation of S1 binding to actin. TnT, causes a much greater inhibition than whole TnT, almost comparable with that produced by whole Tn in the absence of calcium. However, unlike whole Tn in the absence of calcium, TnT1 does not produce any significant occupancy of the B-state but has a much higher occupancy of the C-state.

These inhibition effects can be explained by either by an effect of TnT on the conformation of Tm or by additional weak interactions between TnT and actin altering the positioning
of Tm upon actin. The large effect of TnT$_1$ upon both $K_T$ and $n$ shows the Tm-Tm overlap region plays a significant role in effecting the regulatory properties of Tm. This idea is compatible with previous experiments that have shown that altering the sequence of the N terminus of yeast Tm can also substantially affect the closed/open equilibrium (43). TnT seems to have little effect upon regulatory cooperativity with a similar value of $n$ (7) as determined here ($n = 6–7$) and previously ($n = 7–8$) for Tm alone. In the presence of TnT$_1$ there is significant increase in $n$ (9–10) as shown previously by Schaertl et al. (7) using a different assay method. The reduced modulation of regulatory properties caused by whole TnT may be explained by a non-native interaction with actin of the hydrophobic C-terminal region which would normally interact with TnI and TnC. This could result in a 1 per 7 interaction (equal to the structural repeat) of this region with the actin filament. This would explain both the determined value for $n$ being smaller than that for TnT, and equal to the structural repeat and also a smaller effect upon $K_T$.

Our results can be compared with those reported for a similar TnT$_1$ fragment from bovine cardiac troponin by Tobacman et al. (44). Their TnT$_1$ fragment inhibits S1 ATPase, in vitro motility, and S1-ADP binding assays, in agreement with our data. We both agree that this inhibition is caused by a shift away from the open M-state. However, their interpretation differs significantly from ours in suggesting that this inhibition is caused by the occupancy of the blocked B-state rather than the stabilization of the closed C-state as we state. Their evidence comes from EM structural data which indicate that TnT$_1$ causes a shift in skeletal Tm from a position of actin largely corresponding to the C-state to one corresponding to the B-state. However, they also show that cardiac Tm is already located in the structural B-state and hence the presence of TnT$_1$ shows no change. Although generally the structural and biochemical data have corresponded, the cardiac Tm data suggests this is not always the case (11). These differences may be the result of the sequence differences between the TnT$_1$ fragments from the different tissues. However, whether cardiac TnT$_1$ produces a biochemical blocked state as defined by McKillop and Geeves (8) and shown to be absent for skeletal TnT$_1$ remains to be tested.

The results that show that TnT$_1$ inhibits actin-Tn-S1 ATPase are in agreement with previous ATPase measurements by Ohtsuki and colleagues (45, 46) but have to be contrasted with apparently contradictory data by Reinach and co-workers (47, 48) which have suggested that the TnT$_1$ region is responsible for activation of the filament. However, as noted by them, the chicken muscle TnT they used differs from the rabbit skeletal TnT in this and other previous studies. The two sequences are not identical, and the chicken TnT$_1$ sequence also contains an additional 5 residues (49, 50). The different results for the different sequences in both their work and that of Tobacman et al. (44) may therefore support the idea that this region of TnT is responsible for regulatory tuning and demonstrates the importance of further careful investigation of its function. However, it should be pointed out that the free Mg$^{2+}$ concentration used in the Reinach studies was only around 0.5 mM, appreciably lower than used in the ATPase study here (2.5 mM) and in the region where low Mg$^{2+}$ can have a significant effect upon regulation (21, 40).

The effect of the TnT$_1$ region upon $K_T$ shown in this work is complementary to our previous data (9) upon whole Tn regulation in reconstituted thin filaments where we demonstrated a 4-fold decrease in $K_T$ values in the absence of calcium. This is compatible with the description of regulatory action published by Potter et al. (51), in which they state that association of Ca$^{2+}$ with TnC causes a dissociation or weakening in the C-terminal TnT interaction with Tm and hence an activation of the ATPase. This supports the idea that the TnT interaction in the absence of Ca$^{2+}$ is inhibitory and released upon Ca$^{2+}$ binding to TnC.

These results support our view that that the energy differences between the C- to M-states are relatively low and can therefore be influenced by relatively small changes in structure interactions between the regulatory proteins. It also demonstrates a role for the TnT$_1$ region, which is one of significant isomform variation, in the modulation of the regulatory properties of the thin filament via $K_T$ and $n$. This also provides some explanation for the large number of familial hypertrophic cardiomyopathy mutants found in this region of TnT, as if suggested here it has a modulatory role in regulation, it would provide a hot-spot for non-lethal changes in regulatory function.

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