The role of the inhibitory region of troponin (Tn) I in the regulation of skeletal muscle contraction was studied with three deletion mutants of its inhibitory region: 1) complete (TnI-(Δ96–116)), 2) the COOH-terminal domain (TnI-(Δ105–115)), and 3) the NH2-terminal domain (TnI-(Δ95–106)). Measurements of Ca2+-regulated force and relaxation were performed in skinned skeletal muscle fibers whose endogenous TnI (along with TnT and TnC) was displaced with high concentrations of added troponin T. Reconstitution of the Tn-displaced fibers with a TnI-TnC complex restored the Ca2+ sensitivity of force; however, the levels of relaxation and force development varied. Relaxation of the fibers (pCa 8) was drastically impaired with two of the inhibitory region deletion mutants, TnI-(Δ96–116)/TnC and TnI-(Δ105–115)/TnC. The TnI-(Δ95–106)/TnC mutant retained ~55% relaxation when reconstituted in the Tn-displaced fibers. Activation in skinned skeletal muscle fibers was enhanced with all TnI mutants compared with wild-type TnI. Interestingly, all three mutants of TnI increased the Ca2+ sensitivity of contraction. None of the TnI deletion mutants, when reconstituted into Tn, could inhibit actin-tropomyosin-activated myosin ATPase in the absence of Ca2+, and two of them (TnI-(Δ96–116) and TnI-(Δ105–115)) gave significant activation in the absence of Ca2+. These results suggest that the COOH terminus of the inhibitory region of TnI (residues 105–115) is much more critical for the biological activity of TnI than the NH2-terminal region, consisting of residues 95–106. Presumably, the COOH-terminal domain of the inhibitory region of TnI is a part of the Ca2+-sensitive molecular switch during muscle contraction.

Contraction of skeletal muscle is initiated by the binding of Ca2+ to the regulatory sites of troponin (Tn)3 C, the Ca2+-binding subunit of Tn, which causes an interaction with TnI, the inhibitory subunit of Tn, releasing its inhibition of actomyosin ATPase activity. For full biological function, Tn requires a third subunit, TnT, which anchors the TnI-TnC complex to the actin-tropomyosin (Tm) filaments and also plays a role in the Ca2+-mediated activation of actomyosin ATPase activity and/or contraction (1–5).

The inhibitory function of Tn has been studied in many different ways, yet the structure-function of the inhibitory region of TnI, responsible for this, is still under investigation. The role of the putative inhibitory region of TnI, which consists of 21 amino acids (residues 96–116), has been studied previously utilizing synthetic peptides as well as proteolytic and recombinant fragments of TnI. The cyanogen bromide fragment (CN4) of TnI (residues 96–116) was originally found to possess all of the inhibitory properties of intact TnI (6). Studies with synthetic peptides have demonstrated that residues 105–114 represent the minimal sequence necessary to produce inhibition of actomyosin ATPase activity and to retain TnC binding (7–10); however, the CN4 fragment (TnI-(96–116)) has been shown to have an 8-fold higher affinity for TnC compared with TnI-(104–115) (11).

Several regions of TnI, including its inhibitory region (residues 96–116), have been identified as interacting with actin-Tm and TnC (12–16). Sequence 104–115 of TnI has been shown to share both the actin-Tm- and TnC-binding sites. Studies of Tripet et al. (17) suggest that the region of TnI that follows the inhibitory sequence (residues 96–116) contains additional actin-Tm- and TnC-binding sites. A synthetic peptide consisting of residues 128–148 was able to bind specifically to the actin-Tm filament and could induce a weak inhibitory activity on its own. Truncation of residues 140–148 completely abolished the inhibitory effect of this region when compared with TnI-(96–115), suggesting that region 140–148 of TnI presumably contains the second actin-Tm-binding site (17). This is in agreement with Farah et al. (18), who demonstrated that residues 116–156 are important for the Ca2+ regulation of actomyosin ATPase activity. The recombinant fragment TnI-(1–116) failed to inhibit ATPase activity in the absence of Ca2+ compared with other fragments (TnI-(1–156) and TnI-(103–182)) that were able to regulate actomyosin ATPase activity in a Ca2+-dependent manner (18). Furthermore, Tripet et al. (17) demonstrated that residues 116–131 are not important for inhibition, but are critical for the interaction with TnC and designated this region to be the second TnC-binding site (17). Several studies have shown that residues 96–116 of TnI are primarily responsible for the binding to the COOH-terminal domain of TnC and residues 117–148 for the binding of TnI to the NH2-terminal domain of TnC (19, 20). Reconstituted TnI fragments containing the inhibitory region of TnI (residues 96–116) plus either the NH2-terminal (TnI-(1–116)) or COOH-terminal (TnI-(96–148)) region of TnI were shown to be responsible for either maintaining the maximal level of actomyosin ATPase activity or the Ca2+ dependence of ATPase, respectively (20). In summary, the regulatory complex containing TnT, TnC, and TnI-(96–148) retained all of the full regulatory

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 41, Issue of October 8, pp. 29536–29542, 1999

Printed in U.S.A.
properties of troponin, suggesting that TnI-(96–148) contains the
core sequence of TnI responsible for inhibitory activity
(17, 20). Based upon these experiments, an extended inhibitory
region of TnI has been proposed, containing residues 96–148
(17, 19–22).

This study was undertaken to determine the effect of the
NH2- or COOH-terminal deletions of inhibitory region 96–116
of TnI on the contractile properties of skinned skeletal muscle
fibers. We have expressed and purified wild-type TnI (WTnI)
and three deletion mutants of TnI: TnI-(Δ95–106), TnI-(Δ105–
115), and TnI-(Δ96–116). We have applied the improved
method of Shiraiishi and Yamamoto (23) and Hatakenaka and
Ohtsuki (24) to displace the endogenous TnT complex with high
concentrations of added TnT, followed by functional reconstitution
of the fibers with preformed complexes of TnC and the
TnI mutants. This system allowed us to study the effect of
these deletions on the Ca2+-regulation of force development.

These proteins were also tested for their ability to inhibit
actin-Tm-activated myosin ATPase activity as well as their ability
to regulate ATPase activity when complexed with TnT and
TnC. Our results suggest that the COOH-terminal end of the
inhibitory region of TnI (residues 105–115) is much more
critical for the biological activity of TnI than its NH2-terminal
region (residues 95–106). Presumably, the COOH-terminal
domain of the inhibitory region of TnI is a component of the Ca2+-
sensitive molecular switch that regulates muscle contraction.

MATERIALS AND METHODS

Protein Purification—Rabbit skeletal muscle troponin, tropomyosin,
and actin and myosin were isolated and purified according to Potter
(25), Strzelecka-Golaszewska et al. (26), and Stepkowski et al. (27),
respectively.

Wild-type TnI and the TnI inhibitory region deletion mutants were
expressed in Escherichia coli BL21(DE3) (Novagen) using the protocol
provided by the manufacturer. The expression was checked by SDS-
15% PAGE (28) and Western blotting. The culture for bacterial expres-
sion was collected and centrifuged at 7000 rpm (J-10, Beckman). Bac-
terial pellets were dissolved in a solution containing 6 mM urea, 10 mM
sodium citrate, pH 7.0, 1 mM dithiothreitol, 2 mM EDTA, and 0.01％
NaN3 and sonicated (Sonicator Heat Systems, Inc.) twice at setting 8 for
2 min at 4 °C. After the sonication, the pH of the solution was adjusted
to 5.0. The proteins were eluted with a linear salt gradient of
CaCl2, and 1 mM dithiothreitol, pH 7.0, 20 mM creatine phosphate,
and 15 units/ml creatinine phosphokinase, I = 150 mM. The Ca2+-dependence of force development was tested twice
to make certain that it was stable. Following the initial testing, the fibers
were then incubated in a solution containing 250 mM KCl, 20 mM
MOPS, pH 6.2, 5 mM MgCl2, 5 mM EGTA, 0.5 mM dithiothreitol,
and ~1.5–2 mg/ml rabbit skeletal TnI at 1 °C at room temperature. The fibers
were then washed with the same solution without the protein (10
min at room temperature) and tested for the Ca2+-unregulated force.
Briefly, rabbit psoas skeletal muscle fiber bundles (three to five fibers) were mounted on a force transducer and treated
with a pCa 8 relaxing solution containing 1% Triton X-100 for
20 min. The composition of the pCa 8 solution was 10 -4 × Ca2+, 1 mM
Mg2+, 7 mM EGTA, 5 mM MgATP2-, 20 mM imidazole, pH 7.0, 20 mM
creatine phosphate, and 15 units/ml creatinine phosphokinase, I = 150
mM. The Ca2+-dependence of force development was determined before the TnT treatment and after TnT entry
concentration of the TnI proteins, troponins, and their complexes with actin-Tm
(prior to sedimentation) as well as the supernatants and pellets were
run on SDS-15% polyacrylamide gels (28) and analyzed.

Displacement of the Endogenous Troponin Complex in Skinned Skeletal Muscle Fibers with TnT: Steady-state Force and the Ca2+-Sensitivity of Force Development—Displacement of the endogenous Tn complex in skinned skeletal muscle fibers was performed according to method of Shiraiishi and Yamamoto (23) and Hatakenaka and Ohtsuki (24).

We have slightly modified this method (described below) to achieve complete
Tn displacement, as judged by SDS-PAGE, and the measurements of
Ca2+-unregulated force. Briefly, rabbit psoas skeletal muscle fiber bundles (three to five fibers) were mounted on a force transducer and treated
with a pCa 8 relaxing solution containing 1% Triton X-100 for
20 min. The composition of the pCa 8 solution was 10 -4 × Ca2+, 1 mM
Mg2+, 7 mM EGTA, 5 mM MgATP2-, 20 mM imidazole, pH 7.0, 20 mM
creatine phosphate, and 15 units/ml creatinine phosphokinase, I = 150
mM. The Ca2+-dependence of force development was tested twice
to make certain that it was stable. Following the initial testing, the fibers
were then incubated in a solution containing 250 mM KCl, 20 mM
MOPS, pH 6.2, 5 mM MgCl2, 5 mM EGTA, 0.5 mM dithiothreitol,
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creatine phosphate, and 15 units/ml creatinine phosphokinase, I = 150
mM. The Ca2+-dependence of force development was tested twice
to make certain that it was stable. Following the initial testing, the fibers
were then incubated in a solution containing 250 mM KCl, 20 mM
MOPS, pH 6.2, 5 mM MgCl2, 5 mM EGTA, 0.5 mM dithiothreitol,
and ~1.5–2 mg/ml rabbit skeletal TnI at 1 °C at room temperature. The fibers
were then washed with the same solution without the protein (10
min at room temperature) and tested for the Ca2+-unregulated force.

RESULTS

To identify domains of physiological significance in inhibitory
region 96–116 of TnI, we generated WTnI and three inhibitory
region deletion mutants: an NH2-terminal deletion mutant,
TnI-(Δ95–106); a COOH-terminal deletion mutant, TnI-(Δ105–115); and a deletion of the complete inhibitory
region, TnI-(Δ96–116). Fig. 1 shows amino acid sequences of the inhibitory region of TnI that have been deleted.

Inhibition of Actin-Tm-activated Myosin ATPase Activity by TnI and Its Inhibitory Region Deletion Mutants—The inhibitory properties of rabbit skeletal TnI, WTnI, and its deletion mutants are presented in Fig. 2. Actin-Tm-activated myosin ATPase activity was measured as a function of increasing concentra-
tions of the TnI proteins. As illustrated, recombinant TnI (WTnI) had essentially no effect on actin-Tm
T1, and both inhibited ~90–95% of the ATPase activity. The NH2-
terminal inhibitory deletion mutant, TnI-(Δ95–106), only partially
inhibited actin-Tm-activated myosin ATPase activity,
and ~40–50% inhibition occurred at a 3–4-fold molar excess of
TnI-(Δ95–106) over actin. The COOH-terminal deletion mu-

FIG. 1. Amino acid sequences of the inhibitory region of the TnI deletion mutants. TnI-(Δ96–116) has the entire inhibitory region deleted. TnI-(Δ95–106) has residues 95–106 deleted. TnI-(Δ105–115) has residues 105–115 deleted. Amino acids that have been deleted are boxed.
tant, TnI-(Δ105–115), and the entire inhibitory region deletion mutant, TnI-(Δ96–116), completely lost the ability to inhibit ATPase activity. The latter also gave a slight activation. In summary, the TnI mutants alone could not inhibit actin-Tm-activated myosin ATPase activity when the COOH-terminal region or the entire inhibitory region was deleted.

**Regulation of Actin-Tm-activated Myosin ATPase Activity by Tn Containing TnT, TnC, and the TnI Inhibitory Mutants—** Fig. 3 (A and B) illustrates the effect of the TnI inhibitory region deletion mutations on actin-Tm-activated myosin ATPase activity in reconstituted thin filaments. TnI and its deletion mutants were complexed with TnT and TnC, and the actomyosin ATPase activity was measured in the presence (Fig. 3A) or absence (Fig. 3B) of Ca²⁺. As shown in Fig. 3, the troponin complex containing wild-type TnI or rabbit skeletal TnI regulated the ATPase activity in a similar way (+Ca²⁺), suggesting that recombinant TnI is functional. Tn containing the NH₂-terminal inhibitory region deletion mutant, TnI-(Δ95–106), activated the ATPase activity in the presence of Ca²⁺, but its inhibitory function (in the absence of Ca²⁺) was lost. The COOH-terminal inhibitory region deletion mutant, TnI-(Δ105–115), activated the ATPase activity in the presence or absence of Ca²⁺, with ~1.3-fold higher activation in Ca²⁺. A similar, Ca²⁺-independent activation of ATPase activity was observed for the complete inhibitory region deletion mutant, TnI-(Δ96–116); however, the extent of activation was not as high as for TnI-(Δ105–115). In summary, in the presence of Ca²⁺ (Fig. 3A), all the activation curves were not significantly different, whereas the inhibition curves (in the absence of Ca²⁺) were dramatically different among the various TnI mutants (Fig. 3B). TnI-(Δ105–115) as well as TnI-(Δ96–116) not only did not inhibit the ATPase activity in the absence of Ca²⁺, but gave a 1.4-fold activation of the ATPase. TnI-(Δ95–106) did not inhibit the ATPase activity in the absence of Ca²⁺ and also lacked the activation seen with the other mutants (Fig. 3B).

**Sedimentation Studies (Airfuge)—** To examine the binding of TnI proteins alone or complexed with TnT and TnC to actin-Tm, the complexes were Airfuged, and the pellets and supernatants were analyzed by SDS-PAGE. In parallel, the controls of the TnI proteins alone or complexed with TnT and TnC containing TnI proteins alone or complexed with TnC and TnT to actin-Tm, whereas Fig. 4B demonstrates the binding of the TnI complexes in the presence or absence of Ca²⁺. The analysis of the pellets and supernatants indicated that all of the troponin complexes containing either WTnI or the TnI deletion mutants bound well to actin-Tm in the presence or absence of Ca²⁺ (Fig. 4B). Their binding to actin-Tm was weaker for the TnI mutants alone than when they were complexed with TnT and TnC (Fig. 4, A and B).

**Force Activation, Relaxation, and the Ca²⁺ Sensitivity of Force in Skinned Skeletal Muscle Fibers Reconstituted with the TnI Inhibitory Region Deletion Mutants—** The physiological significance of the NH₂- and COOH-terminal domains of the inhibitory region of TnI was examined using rabbit psoas skinned fibers, in which steady-state force, relaxation, and the Ca²⁺ sensitivity of force development were measured. Following Tn displacement, the fibers were reconstituted with preformed complexes of TnC and WTnI and its deletion mutants. Fig. 5 illustrates a typical experiment on the TnT-treated fiber (panel A) compared with the control buffer-treated fiber (panel B), which had been tested in parallel to estimate the time-dependent rundown of the fibers. Fig. 6 illustrates the Tn displacement procedure. As shown, incubation of the fibers with TnT resulted in a complete loss of Ca²⁺ dependence of force, and the fibers became unregulated (Fig. 5A). This is illustrated in Fig. 6 by a transition from step 1 to step 2. When Tn-displaced fibers were incubated with a preformed TnI-TnC complex, dissolved

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**Fig. 2. Effect of the TnI inhibitory region deletion mutants on the inhibition of actin-Tm-activated myosin ATPase activity.** The concentration of actin was 3.5 μM, that of Tm was 1 μM, and that of myosin was 0.6 μM. The error bars represent S.D. values from four to six experiments. RSTnI, rabbit skeletal TnI.

**Fig. 3. Activation (+Ca²⁺) (A) and inhibition (−Ca²⁺) (B) of actin-Tm-activated myosin ATPase activity by the troponin complex containing TnT, TnC, and TnI inhibitory region deletion mutants.** The concentration of actin was 3.5 μM, that of Tm was 1 μM, and that of myosin was 0.6 μM. Data points are the average of five to seven experiments, and the error bars are the S.D.
in the relaxing solution \((p_Ca = 8)\), the fibers underwent a gradual relaxation as the \(Tn\) activity was reconstituted (Fig. 5A). This step (step 3) restored the \(Tn\) complex (Fig. 6), and the fibers became entirely regulated by \(Ca^{2+}\). The level of force relaxation depended on the TnI mutant used for the TnI\(_z\)TnC complex. In Fig. 5A, WTnI or rabbit skeletal TnI was used, and full relaxation in the \(p_Ca = 8\) solution was achieved. Fig. 7 summarizes the effect of the TnI mutations on the level of relaxation and force development following reconstitution of the fibers with preformed TnI\(_z\)TnC complexes. The dashed line in Fig. 7 represents the level of \(Ca^{2+}\)-unregulated force after RSTnT treatment (this step and the level of \(Ca^{2+}\)-unregulated force are also shown in Fig. 5A). Arrows indicate the percentage of force inhibition relative to \(Ca^{2+}\)-unregulated force. As shown, the relaxation in the fibers could be restored upon incubation with TnITnC; however, the level of relaxation varied depending on the TnI mutant utilized in the reconstitution. The NH\(_2\)-terminal inhibitory region deletion mutant, TnI-(\(D\)95–106), inhibited \(\sim 54.9 \pm 6\%\) of the force following reconstitution. On the other hand, the COOH-terminal inhibitory region deletion mutant, TnI-(\(D\)105–115), as well as the complete inhibitory region deletion mutant, TnI-(\(D\)96–116), inhibited only \(28.3 \pm 10\) and \(28.0 \pm 7\%\) of the force, respectively. Interestingly, all three TnI deletion mutants gave an elevated maximal level of force recovery in the high \(Ca^{2+}\) solution (\(p_Ca = 4\)) (Fig. 7). The extent of activation over that recovered with WTnI\(_z\)TnC was \(\sim 114 \pm 16, 116 \pm 15,\) and \(123 \pm 5\%\) for TnI-(\(D\)95–116), TnI-(\(D\)105–115), and TnI-(\(D\)95–106), respectively (Fig. 7). The \(Ca^{2+}\) sensitivity of force development measured after fiber reconstitution is demonstrated in Fig. 8. All three deletion mutants of TnI increased slightly the \(Ca^{2+}\) dependence of force \((\Delta p_Ca_{90} = 0.1–0.2)\) compared with WTnI\(_z\)TnC-reconstituted fibers.
complex and loss of Ca\(^{2+}\) results in the displacement of the fibers with TnT (1 h at room temperature) resulted in the displacement of the thin filament structure and Ca\(^{2+}\)-regulated force. Reconstitution of the Tn-displaced fibers with the TnI-TnC complex resulted in the restoration of the thin filament structure and Ca\(^{2+}\)-regulated force.

**DISCUSSION**

This study characterizes the effect of NH\(_2\)- and COOH-terminal deletions within the inhibitory region of TnI on the Ca\(^{2+}\) regulation of the actin-Tm-activated myosin ATPase activity and force development in skinned skeletal muscle fibers. The lack of ATPase inhibition with some additional activation for the troponins containing TnI-(\(\Delta 105-115\)) or TnI-(\(\Delta 96-116\)) indicates that the inhibitory region of TnI, especially its COOH terminus, is directly involved in the regulation of ATPase activity. Applying the three-state model of muscle regulation (31), one can speculate that this depleted region of TnI is the minimum sequence necessary to maintain the “blocked” state of the thin filament, where the interaction between actin and the myosin heads is highly inhibited. Removing this part of the inhibitory region of TnI (residues 105–115) resulted in a dramatic alteration of the blocked state of the thin filament, facilitating a transition from the blocked to the “closed” and/or “open” state (31). Our ATPase and force development results support the above hypothesis and suggest that the COOH terminus of the inhibitory region of TnI is much more critical for its regulatory function than the NH\(_2\) terminus.

The inhibitory activity of TnI-TnC-depleted fibers also suggest that the inhibitory region of TnI (residues 96–116) is not the only interaction site for actin-Tm and that the other TnI site may be involved in the interaction of TnI with TnC. Even removing the entire inhibitory region of TnI did not eliminate the binding of the TnI-(\(\Delta 96-116\)) mutant to actin-Tm. The binding experiments of the TnI inhibitory region mutants to actin-Tm fibers and to TnI-TnC-depleted fibers also suggest that the inhibitory region of TnI (residues 96–116) is not the only interaction site for actin-Tm and that the other TnI site may be involved in the interaction of TnI with TnC. These results are in accord with studies of Tripet et al. (17) that suggest that the second site on TnI for actin is located in the region COOH-terminal to inhibitory sequence 96–116, somewhere between residues 140 and 148. These authors also suggested that this region is involved in conferring an inhibitory activity to the thin filaments. Interestingly, the region containing residues 116–131, adjacent to sequence 96–116 and preceding region 140–148, was found not to be important for actin-Tm binding, but significant for the Ca\(^{2+}\)-dependent TnI-TnC interaction (17). Moreover, the studies of Pearlstone et al. (19) and Van Eyk et al. (20) demonstrated that the inhibitory residues (positions 96–116) of TnI are primarily responsible for the binding to the COOH-terminal domain of TnC (and to actin-Tm), whereas residues 117–148 of TnI are responsible for the binding to the NH\(_2\)-terminal regulatory domain of TnC.

Our results suggest that the COOH terminus of the inhibitory region of TnI is functional and that the COOH terminus of TnI is directly involved in the regulation of ATPase activity. Applying the three-state model of muscle regulation (31), one can speculate that this depleted region of TnI is the minimum sequence necessary to maintain the “blocked” state of the thin filament, where the interaction between actin and the myosin heads is highly inhibited. Removing this part of the inhibitory region of TnI (residues 105–115) resulted in a dramatic alteration of the blocked state of the thin filament, facilitating a transition from the blocked to the “closed” and/or “open” state (31). Our ATPase and force development results support the above hypothesis and suggest that the COOH terminus of the inhibitory region of TnI is much more critical for its regulatory function than the NH\(_2\) terminus.

The binding experiments of the TnI inhibitory region mutants to actin-Tm fibers and to TnI-TnC-depleted fibers also suggest that the inhibitory region of TnI (residues 96–116) is not the only interaction site for actin-Tm and that the other TnI site may be involved in the interaction of TnI with TnC. Even removing the entire inhibitory region of TnI did not eliminate the binding of the TnI-(\(\Delta 96-116\)) mutant to actin-Tm or the TnI-TnC-depleted fibers.

These results are in accord with studies of Tripet et al. (17) that suggest that the second site on TnI for actin is located in the region COOH-terminal to inhibitory sequence 96–116, somewhere between residues 140 and 148. These authors also suggested that this region is involved in conferring an inhibitory activity to the thin filaments. Interestingly, the region containing residues 116–131, adjacent to sequence 96–116 and preceding region 140–148, was found not to be important for actin-Tm binding, but significant for the Ca\(^{2+}\)-dependent TnI-TnC interaction (17). Moreover, the studies of Pearlstone et al. (19) and Van Eyk et al. (20) demonstrated that the inhibitory residues (positions 96–116) of TnI are primarily responsible for the binding to the COOH-terminal domain of TnC (and to actin-Tm), whereas residues 117–148 of TnI are responsible for the binding to the NH\(_2\)-terminal regulatory domain of TnC.
Fig. 8. Ca2+ sensitivity of force development for WTnI and TnI inhibitory region deletion mutants when reconstituted with TnC in Tn-displaced skinned skeletal muscle fibers. Data points (mean ± S.D. of three to five experiments) were fitted to the Hill equation, and the pCa50 and Hill coefficient (nH) values of the respective curves are presented in the graph.

Fig. 9. Western blotting of TnI-TnC-reconstituted fibers performed with the TnI-specific antibodies. After force measurements, the fibers reconstituted with TnI inhibitory region deletion mutants were run on SDS-12.5% polyacrylamide gel and tested for the presence of the TnI mutants with Western blotting and TnI-specific antibodies (mouse anti-TnI IET and goat anti-mouse IgG-peroxidase). Under these conditions (12.5% gel), there was no difference in the migration of the TnI deletion mutants. Lane 1, control untreated fiber; lane 2, the fiber incubated with RSTnT; not reconstituted with the TnI-TnC complex; lane 3, WTnI standard protein; lane 4, RSTnT-treated fiber reconstituted with the WTnI-TnC complex; lane 5, TnI-(95–106) standard protein; lane 6, TnI-(Δ95–106)TnC-reconstituted fiber; lane 7, TnI-(Δ105–115) standard protein; lane 8, TnI-(Δ105–115)/TnC-reconstituted fiber; lane 9, TnI-(Δ96–116) standard protein; lane 10, TnI-(Δ96–116)/TnC-reconstituted fiber.

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nated myosin ATPase activity in the absence of Ca2+ for TnI-(Δ95–106) suggests that this region is not that critical to maintain the Ca2+-dependent interactions with TnC. These results support the above conclusion that the inhibitory region of TnI is not functionally uniform and give rise to the question of the structural basis for this functional heterogeneity seen in the NH2 and COOH termini of the inhibitory region of TnI.

The structure of the inhibitory peptide (residues 96–115) was presented in a recent study of Hernandez et al. (32). Utilizing CD and NMR spectroscopy, the authors demonstrated a predominantly extended conformation of the free TnI peptide with no significant change on binding to TnC. This is opposite to what was found by Campbell and Sykes (10), who demonstrated that a smaller inhibitory peptide of TnI (residues 104–115) forms two helical regions (residues 104–108 and 112–115) upon binding TnC. Important structural information on the interaction between TnI and TnC comes from the crystal structure of TnC complexed with a TnI peptide comprising the first 47 amino acids of TnI (33). This structure of TnI-(1–47)/TnC confirmed the antiparallel interaction between the COOH-terminal domain of TnC and the NH2-terminal region of TnI and also revealed a more compact organization of TnC in this complex compared with the free state of TnC (34). Based on this newly resolved TnI-(1–47)/TnC structure, atomic interactions were modeled with the free inhibitory region of TnI-(96–127) (TnIreg) and the NH2-terminal lobe of TnC, implying that this region of TnI acts as a Ca2+-sensitive switch in muscle contraction that moves between actin-Tm and the hydrophobic pocket of the NH2-terminal domain of TnC (33).

A similar mechanism was proposed by McKay et al. (22) based upon the NMR structure of the regulatory NH2-terminal domain of TnC and the elongated inhibitory peptide of TnI containing residues 96–148. These authors demonstrated that this region of TnI interacts with the NH2-terminal domain of TnC in a Ca2+-sensitive manner and that this regulatory domain of TnC does not undergo a major structural change upon binding to TnI-(96–148). A large-scale in situ movement of mass within TnI, in response to Ca2+ binding to the regulatory sites of TnC, was observed in the neutron scattering experiments of Stone et al. (35). TnI, when bound to TnC and TnT, underwent a significant conformational change upon binding of Ca2+ to the regulatory Ca2+-specific sites of TnC. Therefore, the TnI molecule was identified as part of the Ca2+-sensitive molecular switch during muscle contraction (35, 4, 5). Modeling of TnI indicated that the rod-like portion of the molecule, containing ~35% of the mass, moved closer to the larger oblate ellipsoid portion (65% of the mass) upon Ca2+ binding (35).

Our actomyosin ATPase results and activation instead of inhibition in the absence of Ca2+ for TnI-TnC-TnI-(Δ105–115) support these interesting findings and suggest that the Ca2+-sensitive molecular switch identified by Stone et al. (35) could be located somewhere in the COOH-terminal domain of the inhibitory sequence of TnI. This region would move between its inhibitory position on actin-Tm in the absence of Ca2+ and activate the ATPase activity in the presence of Ca2+, possibly through an interaction with TnC. Deletion of this region from TnI would result in an activation of the ATPase activity in the absence of Ca2+ or a very impaired relaxation when reconstituted in skinned skeletal muscle fibers. Future studies are planned to investigate the physiological significance of the second interaction site on TnI for actin-Tm and its importance for force development and the Ca2+ regulation of skeletal muscle fibers.

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