Muscular contraction is triggered by an increase in calcium concentration, which is transmitted to the contractile proteins by the troponin complex. The interactions among the components of the troponin complex (troponins T, C, and I) are essential to understanding the regulation of muscle contraction. While the structure of TnC is well known, and a model for the binary TnC-TnI complex has been recently published (Tung, C.-S., Wall, M. E., Gallagher, S. C., and Trewhella, J. (2000) *Protein Sci.* 9, 1312–1326), very little is known about TnT. Using non-denaturing gels and NMR spectroscopy, we have analyzed the interactions between TnC and five peptides from TnT as well as how three TnI peptides affect these interactions. Rabbit fast skeletal muscle peptide TnT-(160–193) binds to TnC with a dissociation constant of 30 ± 6 μM. This binding still occurs in the presence of TnI-(1–40) but is prevented by the presence of TnI-(56–115) or TnI-(96–139), both containing the primary inhibitory region of TnI. TnT-(228–260) also binds TnC. The binding site for TnT-(160–193) is located on the C-terminal domain of TnC and was mapped to the surface of TnC using NMR chemical shift mapping techniques. In the context of the model for the TnC-TnT complex, we discuss the interactions between TnT and the other troponin subunits.

Skeletal muscle contraction is regulated by troponin and tropomyosin located in the thin filament. Contraction begins with the binding of Ca\(^{2+}\) to the troponin complex, triggering conformational changes that are propagated to tropomyosin and actin. The troponin complex is constituted by a Ca\(^{2+}\)-binding subunit, troponin C (TnC), an inhibitory subunit, troponin I (TnI), and a tropomyosin-binding subunit, troponin T (TnT). When the muscle is relaxed, TnI binds inhibitory myosin ATPase activity. The smallest region of TnI capable of this inhibition is known as the inhibitory region, or the inhibitory peptide (residues 96–115). Upon binding of Ca\(^{2+}\), TnC undergoes conformational changes and removes the inhibition by TnI. TnT binds to TnI, TnC, and tropomyosin, anchoring the troponin complex to the thin filament and propagating the conformational changes (for reviews, see Refs. 1–3). The interactions among the three components in the troponin complex as well as how they are affected by the presence of calcium are essential for understanding the regulation of muscle contraction.

The structure of skeletal TnC is well known and has been determined by both x-ray crystallography (4–6) and nuclear magnetic resonance (NMR) spectroscopy (7). NMR and crystallographic data provided some structural information on the interaction between TnI and fragments of TnI (8–14). A model for the structure of the skeletal binary TnC-TnI complex was recently built based on a number of binding and activity assays such as cross-linking, fluorescent resonance energy transfer (FRET), and neutron scattering data as well as the available structural information (15).

Despite the amount of information on the interactions between TnC and TnI that has emerged in the last few years, not much is known about the structure of TnT in either isolated or interacting with another troponin subunit. Rabbit skeletal TnT yields two fragments when digested by chymotrypsin. The N-terminal fragment, known as T1, binds to tropomyosin, while the C-terminal fragment, T2, binds TnI and TnC (for reviews, see Refs. 3 and 16). Although TnI by itself inhibits myosin ATPase and TnC can remove the inhibition, TnT is necessary for the Ca\(^{2+}\)-dependent regulation of myosin ATPase by the troponin complex. Direct interaction of TnT with TnC is responsible for this calcium-sensitizing effect (17).

Herein we show that two TnT peptides, containing residues 160–193 and 228–260, bind to TnC and that other peptides in the T2 region do not bind TnC. We also show that two TnI peptides containing the inhibitory region compete with TnT-(160–193) for TnC, while the first 40 residues of TnI bind to TnC simultaneously with TnT-(160–193). Using two-dimensional \(^{1}H,^{15}N\) NMR spectroscopy to follow the titration of TnC with the peptide TnT-(160–193), we calculated the binding constant for this peptide and mapped the binding region on the surface of TnC. We show this mapping on the model of the binary TnC-TnI complex and discuss the competition results in this context.
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RREAEEK-NH2, TnI-(56–115), acetyl-SMAEVQELCKQLHAKIDAAAEEKIDMKVQKSSKELEDNQLFDLRLGGKPRPPRRLVRV-NH2, TnI-(96–139), acetyl-NQLKFLRLGGKPRPPRRLVRVMSADMLKALLGSKHRVCDMLRAN-NH2. Proteins and peptide solutions were lyophilized and resuspended in NMR buffer or non-denaturing gel electrophoresis buffer in appropriate concentrations. All protein and peptide concentrations were determined by amino acid analysis in duplicate.

Gel Electrophoresis—TnC complexes with TnT and TnI peptides were visualized on 10% glycerol-polyacrylamide gels (25). Unlabeled recombinant TnC (or the individual domains) and the peptides were resuspended in 20 mM Hepes pH 7.6, 100 mM NaCl, and 5 mM CaCl2. Recombinant TnC (or the individual domains) and the peptides were visualized on 10% glycerol-polyacrylamide gels (25). Unlabeled TnT or TnI peptides were mixed in ratios from 1:1 to 1:4. TnC/TnI/TnT mixtures were made in 1:1:1 ratios. The mixtures were incubated for at least 30 min at room temperature before analysis and then diluted in sample buffer and analyzed in 10% glycerol, 8% polyacrylamide gels as described (26).

TnT-(160–193) Titration of [13C,15N]CTnC—Recombinant TnC uniformly labeled with 13C and 15N and the peptide TnT-(160–193) were resuspended in 100 mM KCl, 10 mM imidazole, 6 mM CaCl2, 0.03% sodium azide, and 0.2 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid in 90% H2O, 10% D2O at pH 6.9. TnC concentration in the initial NMR sample was 0.6 mM, and TnT-(160–193) concentration in the stock solution was 21 and 62 mM, respectively. Titration points of 0.14, 0.29, 0.43, 0.57, 0.71, 0.86, 1, 1.14, 1.29, 1.43, 1.87, and 2.3 mM were added for each point. A last point was acquired later, with 0.54 mM CTnC and 128 mM TnT-(160–193). Titration points of 0.14, 0.29, 0.43, 0.57, 0.71, 0.86, 1, 1.14, 1.29, 1.43, 1.87, and 2.3 mM were added for each point. A last point was acquired later, with 0.54 mM CTnC and 128 mM TnT-(160–193). A stable complex should be seen in the gel. A weak complex would dissociate while migrating in the gel and would not be seen as a band.

TnT peptides described in Fig. 1a (TnT-(151–170), TnT-(160–193), TnT-(181–215), TnT-(209–243), and TnT-(228–260)) were individually mixed with TnC in the presence of Ca2+ and applied to a non-denaturing gel. TnT-(160–193) and TnT-(228–260) are able to displace the TnC band, thus binding to TnC (Fig. 2a). Therefore, TnT-(160–193) and TnT-(228–260) contain the probable sites in TnT responsible for the interaction with TnC. However, when TnT-(228–260) is mixed with TnC in TnC/TnT-(228–260) ratios larger than 1:3, the TnC band cannot be seen in the gel (data not shown). This probably results from the nonspecific binding of multiple copies of TnT-(228–260) to TnC. For this reason, this study focuses on the interactions between TnT-(160–193) and TnC. TnT-(181–215) can be seen as a separate band in the gel in the presence or absence of TnC because of its negative charge and does not affect the TnC band. In the absence of Ca2+ (1 mM EGTA, 10 mM MgCl2), none of the peptides bound TnC (data not shown).

When the TnC-binding peptide TnT-(160–193) is mixed to the individual domains of TnC in the presence of Ca2+, it causes a partial displacement of the C domain (CtChC) band (Fig. 2b) but not of the N domain (NTnC) band. This suggests that the interaction between TnT-(160–193) and TnC occurs through the C domain of the protein. The weakening of the NTnC band suggests some binding to the N domain, but no band for the complex is seen. Given the charges of NTnC and TnT-(160–193), a stable complex should be seen in the gel. A weak complex would dissociate while migrating in the gel and would not be seen as a band.
When TnI-(96–139) is combined with TnC and TnT-(160–193), a different result is obtained. The only displacement seen on the TnC band is the one caused by the binding of TnI-(96–139) (Fig. 2d), which binds to TnC with greater affinity than TnT-(160–193) and prevents it from binding. The greater affinity of TnI-(96–139) can be seen from the complete displacement of the TnC band that it causes, whereas TnT-(160–193) only produces partial displacement.

The same experiment was done with TnI-(56–115) and TnT-(160–193). As with TnI-(96–139), TnI-(56–115) also competes with TnT-(160–193), preventing it from binding TnC (data not shown).

Titration of [15N]TnC and [13C,15N]CTnC with TnT-(160–193)—TnT-(160–193) was titrated into [15N]TnC and [13C,15N]CTnC solutions. The binding was followed by two-dimensional 1H,15N HSQC NMR spectroscopy. The two-dimensional 1H,15N HSQC NMR spectra of [13C,15N]CTnC in the beginning and the end of the titration are shown in Fig. 3a and b, respectively. Each cross-peak corresponds to 15N and 1H resonances of the amide of an individual amino acid. Assignments of some of the peaks are indicated in both spectra. The two spectra are clearly different. These differences were observed during the titration as a gradual shifting of the resonances of the residues affected by TnT-(160–193). The binding occurs with fast exchange kinetics on the NMR time scale. The peaks in each titration point correspond to the weighted average of peptide-free and peptide-bound peaks. This shifting can be seen in Fig. 4, which shows a superimposition of a region of the two-dimensional 1H,13C NMR spectra of [13C,15N]CTnC during the titration. While some cross-peaks almost do not change, like those for Ala-124 and Leu-137, others are greatly displaced in different directions, as exemplified by the cross-peaks for Glu-159 and Lys-156. The peaks stop moving when the [TnT-(160–193)]total/[CTnC]total ratio reaches 1, indicating the binding of only one TnT-(160–193) per CTnC.

Titration of whole TnC with TnT-(160–193) produced similar results for residues in the C-terminal domain. Residues in the N-terminal domain did not move significantly until the C-terminal domain was saturated, and then they began to move. Because the N domains of two TnC molecules can interact, forming a TnC dimer (18), chemical shift changes in the N domain can be caused by breaking of the dimer. Peak displacements (the total chemical shift changes) for each residue were calculated from the chemical shift changes of both nuclei using Equation 1 (9).

$$\Delta \delta_{\text{total}} = \sqrt{(\Delta \delta_{\text{N}})^2 + (\Delta \delta_{\text{H}})^2} \tag{1}$$

A comparison of the NMR chemical shift changes for residues in the C domain for both titrations can be seen in Fig. 5. The chemical shift changes for each residue were similar in both titrations, except for the first nine residues in CTnC. This difference is probably caused by the different environments for this region in the two proteins. While these residues are in the middle of the protein in TnC, in CTnC they are the N-terminal residues.

The residues in the C domain that had a chemical shift change greater than 100 Hz at the end of the titration were averaged and normalized in both titrations, and the results were plotted in the binding curves shown in Fig. 6. Five non-overlapping cross-peaks for residues in the N domain that moved more than 40 Hz were also averaged, normalized to the C domain values, and plotted in Fig. 6a. The curves were fitted to binding using Equation 2.

$$\text{N} + \text{C} \rightarrow \text{N} + \text{CTnC} \tag{2}$$

where TnC represents both [15N]TnC and [13C,15N]CTnC. The resulting dissociation constants were 30 ± 6 μM for the C domain of whole TnC and 38 ± 24 μM for CTnC. To calculate the N domain binding curve, TnT-(160–193) concentrations were adjusted at each point for the free TnT-(160–193) concentrations. The dissociation constant for the N domain cannot be accurately determined because the site did not saturate during the titration. It can be seen, however, that it is at least 10 times bigger than for the C domain and that the binding to the N domain is not significant until the C domain is 90% saturated. The curve for the N domain in Fig. 6 was plotted using $K_D = 400 \mu M$, the minimum value that fitted the data.

Peak displacements caused by the binding of TnT-(160–193) (shown in Fig. 5) were mapped on the protein surface both for
the C domain in the presence of \( \text{TnI}-(1-47) \) and for the whole \( \text{TnC} \) in the model of \( \text{TnC} / \text{H}18528 \). The surface was colored in a gradient according to the degree to which the residues were affected by \( \text{TnT}-(160-193) \) binding. Regions in white correspond to residues whose amide chemical shift, according to Equation 1, did not change by more than 20 Hz during the titration. Residues whose peaks shifted more than 120 Hz were colored in pure red. Through this mapping, the \( \text{TnT}-(160-193) \)-binding site on \( \text{TnC} \) can be visualized in the protein and in the binary complex model.

**DISCUSSION**

Among the five peptides covering the whole T2 region in rabbit fast skeletal muscle \( \text{TnT} \), two peptides, containing residues 160–193 and 228–260, bind \( \text{TnC} \) according to non-denaturing gel electrophoresis data. These regions must therefore contain the physiologically important \( \text{TnC} \)-binding sites in \( \text{TnT} \). Any binding too weak to be seen as a band in the gel under the conditions used is not likely to be physiological. These results are in good agreement with previous results from the literature. Most studies showed Ca\(^{2+}\)-dependent binding to \( \text{TnC} \) of larger peptides from the T2 region or the whole T2 containing residues 160–193 (31–34). The smallest of them contained residues 160–210. There is also evidence of binding for peptides containing residues 207–259, 177–231, and 240–260 (31), although the last two peptides did not form complexes that could be detected by electrophoresis (35). The peptide containing residues 177–231 contains half of \( \text{TnT}-(160-193) \), whereas 207–259 and 240–260 overlap with \( \text{TnT}-(228-260) \). As in our experiments, these studies showed weaker binding in the absence of Ca\(^{2+}\), even in the presence of Mg\(^{2+}\) (31–33). Different studies have suggested that the C domain of \( \text{TnC} \) adopts different conformations when bound to Ca\(^{2+}\) or Mg\(^{2+}\) (36, 37).

\( \text{TnT}-(228-260) \) seems to have a primary binding site on \( \text{TnC} \) but also binds \( \text{TnC} \) nonspecifically in increased concentrations. This nonspecific binding can affect affinity measurements and binding site mapping. Preliminary NMR studies showed that the peptide aggregates when in solution, and the complex \( \text{TnC} / \text{TnT}-(228-260) \) precipitates in the NMR tube (data not shown). For these reasons, we centered this work on the characteristics of \( \text{TnT}-(160-193) \) binding to \( \text{TnC} \).

The binding site for \( \text{TnT}-(160-193) \) is located on the C-terminal domain of \( \text{TnC} \). Not only is \( \text{TnT}-(160-193) \) capable of binding to \( \text{CTnC} \), but the same residues are involved in the binding of \( \text{TnT}-(160-193) \) to the whole protein and to \( \text{CTnC} \), showing that the peptide is bound to the same site in both situations. When the C domain is saturated, \( \text{TnT}-(160-193) \) binds to the N domain of \( \text{TnC} \). That binding, however, occurs with much lower affinity (more than 10 times lower) and is likely non-physiological.

The dissociation constant for \( \text{TnC} / \text{TnT}-(160-193) \) binding is \( 30 \pm 6 \mu\text{M} \). The affinity between \( \text{TnC} \) and \( \text{TnT} \) was previously reported as almost 1000 times stronger (38). This difference can be caused by different conditions or different measuring methods, but most probably because we used a small peptide.
The region containing residues 160–193 in TnT involved in TnC binding is immediately N-terminal from the TnI-binding region. Residues 198–251 of TnT contain a hydrophobic heptad repeat, typical of a coiled-coil structure, that has been proposed to interact with residues 57–106 of TnI, which also contain a hydrophobic heptad repeat (39). A TnT fragment containing residues 177–231 of TnT bind to TnI (39), while a chicken TnT mutant missing residues 216–263 (40) and a bovine TnT mutant missing residues 203–214 (41) cannot bind TnI. These regions correspond to residues 211–260 and 205–216 in rabbit TnT2f, respectively. Residues 224–228 were also shown to be critical for TnI binding (33).

Since regions of TnT responsible for TnC and TnI binding are located side by side, it is expected that the corresponding regions of TnC and TnI that interact with TnT would be closely located in the troponin complex. In the model in Fig. 7, the region containing the hydrophobic heptad repeat in TnI (residues 57–106) corresponds mostly to the α-helix (seen in blue to the left of the C domain of TnC), which does not interact with TnC in the complex. It is, however, close enough to the mapped TnT-binding site on TnC (colored in red) to suggest that these regions could interact with consecutive regions in TnT.

The non-denaturing gel electrophoresis data (Fig. 1c) showed that TnT-(160–193) and TnI-(1–40) can bind to TnC at the same time. The region of TnI corresponding to TnI-(1–40) is shown in green in Fig. 7. Even though both peptides bind to the same region of TnC, they do not interact with the same residues and would not compete for TnC binding. NMR chemical shift changes mapping the TnI-(1–40)-binding site on CTnC
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Fig. 7. Molecular surface mapping of the TnT-(160–193) binding site in the C domain of TnC. The TnT-(160–193) binding site is shown in TnC-TnI complexes. TnC is shown as a surface, while TnI is shown in ribbon representation. a, mapping of TnT-(160–193) binding to TnC, shown on TnC crystal structure complexed to TnI-(1–47) (11); b, mapping of TnT-(160–193) binding to whole TnC, shown on the model L of the binary complex TnC-TnI (15). The surfaces were colored according to the gradient on the figure, going from white, for the residues whose cross-peaks moved less than 20 Hz, to red, for the residues whose cross-peaks moved more than 120 Hz. In b, the region of TnI corresponding to the peptide TnI-(1–40) is colored in green, while the region corresponding to TnI-(96–139) is colored in orange.

showed that the binding affects the residues in the hydrophobic pocket of CTnC (14), while TnT-(160–193) affects mostly the external surface of helices F and G in the region exposed to the solvent.

TnI-(96–139) (Fig. 7, orange region) and TnI-(56–115) compete with TnT-(160–193) binding to TnC, indicating that they

share at least part of the binding site on TnC. TnI-(96–139) contains the inhibitory peptide (residues 96–115), shown as the orange β-hairpin to the right of TnC in Fig. 7, and an α-helical region (residues 116–139) that binds to the N domain of TnC (9). The inhibitory peptide is the only common region between the two peptides, and must, therefore, be the region that competes with TnT-(160–193). The model for the TnC-TnI binary complex shown in Fig. 7 does not account for that competition.

We suggest that the binding site for the inhibitory peptide on TnC overlaps the TnT-binding site, at least partially. The inhibitory peptide is located close to TnT in the troponin complex, and the presence of TnT can protect the inhibitory region from chymotryptic digestion (42). As for TnT-(160–193), TnI-(96–115) has both electrostatic and hydrophobic interactions with CTnC, while TnI-(1–40) has mostly hydrophobic interactions (14). TnT-(160–193) and TnI-(96–115) may share some of the electrostatic contacts and therefore compete for TnC.

It is interesting to observe that while TnI-(96–115) competes with TnI-(1–40) and presumably with TnT-(160–193), TnI-(1–40) and TnT-(160–193) do not compete. The binding site for the inhibitory peptide probably shares different residues with TnI-(1–40)- and TnT-(160–193)-binding sites, so that the last two sites either do not overlap or the overlapping region is so small that it does not significantly affect the binding.

Thus our results support much of model L from Tung et al. (15), except for the exact position of the inhibitory peptide. Model R is not compatible with our results. The position of TnI residues 3–33 in model R, overlapping the mapped TnT-(160–193)-binding site, would cause competition between TnI-(1–40) and TnT-(160–193). We suggest that model L is probably closer to the actual structure of the binary TnC-TnI complex. This is the first time that a TnT-binding site has been mapped on TnC. When combined with the information available on TnI binding to TnC, the mapping offers insights into how the three components come together in the structure of troponin tertiary complex.

Acknowledgments—We thank Robert Boyko for developing xcrvf software, David Corson for expressing and purifying TnC and its domains, and Gerry McQuaid for maintenance of the NMR spectrometers. We thank Pascal Mercier and Dr. Leo Spyracopoulos for advice and help during this work.

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