Interaction of the Second Binding Region of Troponin I with the Regulatory Domain of Skeletal Muscle Troponin C as Determined by NMR Spectroscopy*

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Two dimensional $^1$H,$^15$N-heteronuclear single quantum correlation NMR was used to monitor the resonance frequency changes of the backbone amide groups belonging to the $^15$N-labeled regulatory domain of calcium saturated troponin C (N-TnC) upon addition of synthetic skeletal N-acetyl-troponin I 115–131-amide peptide (TnI$_{115-131}$). Utilizing the change in amide chemical shifts, the dissociation constant for 1:1 binding of TnI$_{115-131}$ to N-TnC in low salt and 100 mM KCl samples was determined to be 28 ± 4 and 24 ± 4 μM, respectively. The off rate of TnI$_{115-131}$ was determined to be 300 s$^{-1}$ from observed N-TnC backbone amide $^1$H,$^15$N-heteronuclear single quantum cross-peak line widths, which is on the order of the calcium off rates (Li, M. X., Gagné, S. M., Tsuda, S., Ray, C. M., Smillie, L. B., and Sykes, B. D. (1995) Biochemistry 34, 8330–8340), and agrees with kinetic expectations for biological regulation of muscle contraction. The TnI$_{115-131}$ binding site on N-TnC was determined by mapping of chemical shift changes onto the N-TnC NMR structure and was demonstrated to be in the “hydrophobic pocket” (Gagné, S. M., Tsuda, S., Li, M. X., Smillie, L. B., and Sykes, B. D. (1995) Nat. Struct. Biol. 2, 784–789).

One of the first intracellular steps required for skeletal muscle contraction is release of Ca$^{2+}$ ions in the muscle cell, leading to a protein-protein interaction cascade and sliding of the thin and thick filaments past one another in the contractile or power stroke (for reviews see Refs. 1–3). Cardiac and skeletal muscle cells have similar cascades, although the individual proteins involved and the molecular mechanism of regulation differ. The target for calcium is the troponin complex consisting of tropo- nin C (TnC),$^1$ troponin I (TnI), and troponin T. TnC is the calcium binding component and the best characterized member of the troponin complex. TnI inhibits the ATPase activity of myosin, whereas tropomin of Tp is thought to anchor the complex to actin/troponymosin. Calcium binding alters the interaction among the components of the troponin complex and with other proteins in the thin filament (4). Calcium-saturated TnC binds to TnI relieving the inhibition of muscle contraction (Refs. 5 and 6 and references therein).

The crystal structures of TnC revealed a dumbbell shaped molecule with two distinct domains joined by a helical linker (7, 8). TnC contains four EF-hand calcium binding motifs, two in each of the N- and C-terminal domains (9). The N-terminal or regulatory domain of TnC(N-TnC) was devoid of calcium under the crystallization conditions and showed a “closed” structure. This domain was postulated to open upon calcium binding in the Herzberg-Moul- James model for calcium-saturated conformation of TnC exposing nonpolar residues and creating a hydrophobic pocket (10). The opening of the regulatory domain in response to calcium binding was demonstrated by the NMR solution structures of the calcium-saturated N-terminal domain and whole TnC molecules (11, 12). Comparison of these structures demonstrated that isolation of the N-terminal domain does not significantly alter the effects of Ca$^{2+}$ binding. Interestingly, calcium-saturated cardiac N-TnC remains in the closed conformation (13).

Although there have been several studies investigating the interaction of TnC and TnI, no high resolution three-dimensional structures of TnI or the TnC/TnI complex are available (3, 14–26). We know that TnI binds TnC and actin and inhibits the actomyosin ATPase in the absence of calcium. However, it was not known exactly which residues of TnI bind to TnC nor where this complex occurs on TnC. Mutation studies (23), cross-linking experiments (17, 18, 22, 24, 27), and low angle x-ray diffraction structures (15) have suggested that the exposed TnC hydrophobic pockets are the site of TnC/TnI interaction. Interestingly the TnC/TnI complex formation was insensitive to calcium unless tropomysin and other members of the troponin complex were present, although calcium was found to stabilize the isolated TnC/TnI complex (6, 28). Farah et al. used deletion mutants to determine that TnC and TnI bind in an anti-parallel fashion (29).

In regards to TnI, Syska et al. showed that TnI bound actin, and they were the first to show that only a portion of TnI (specifically region 96–117) was needed for full inhibition of ATPase activity (5). Subsequently, synthetic TnI peptides provided an attractive alternative to the use of highly insoluble whole TnI in studies of muscle protein interactions. Talbot and Hodges showed that the synthetic TnI peptide corresponding to region 96–116 behaved identically to the cyanogen bromide-cleaved fragment (26). Talbot and Hodges also identified the TnI peptide (TnI residues 104–115), which was the minimum length inhibitory peptide of TnI still regulated by TnC, and able to inhibit ATPase activity (30). Campbell et al. solved the structures of the synthetic TnI peptide while bound to intact skeletal and cardiac TnC using the transferred nuclear Overhauser effect technique (16, 31). TnI had an amphiphilic α-
helical structure distorted around the central proline residues in both cases. Ngai et al. determined that the TnIp fragment cross-linked to the C-terminal domain of TnC and modeled the NMR-derived TnIp structure into the TnC crystal structure (17).

Recently Tripet et al. have mapped a second TnC binding site on TnI and postulated that the region corresponding to residues 115–131 (TnI_{115–131}) interacts with the N-terminal domain of TnC in the Ca^{2+}-regulated hydrophobic pocket (19). In this paper the interaction of calcium-saturated N-TnC with TnI_{115–131} was explored with multinuclear, multi-dimensional NMR spectroscopy. We monitored 15N-labeled N-TnC upon the addition of TnI_{115–131} to determine the stoichiometry of binding, dissociation constant of the complex, and location of chemical shifts induced in the N-TnC molecule. We have mapped those chemical shift changes onto the structure of Ca^{2+}-bound N-TnC. Further we monitored the change in cross-peak line width to determine the reaction rate constants. These results provide direct evidence for TnI binding in the hydrophobic pocket of the regulatory domain and have implications for the kinetic competence of the complex with respect to muscle contraction.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides**—The cloning, expression, and purification of 50% deuterated, uniformly 15N-labeled N-TnC was done following the protocols described in Gagné et al. for nondeuterated N-TnC (32). The synthetic N^a-acetylated N-TnC (115–131)-amide rabbit skeletal peptide was prepared as described previously (19) and lyophilized repeatedly to remove residual organic solvents. The sequence was confirmed by amino acid analysis, and the mass was verified by electrospray mass spectrometry.

**NMR Sample Preparation**—Two NMR samples (500 μl) of [U-^{15}N; 50% ^2H]-N-TnC were prepared for titration with the TnI_{115–131} peptide differing only in the concentration of added KCl. The first sample (designated high salt) contained 100 mM KCl, whereas the second had no added KCl (low salt). The N-TnC was dissolved in a buffer containing 90% H_2O, 10% D_2O, and 10 mM deuterated imidazole, with 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate as an internal reference. The only other salts were the slight amounts of HCl or NaOH necessary to put the pH level of the samples to 6.85.

The concentrations of N-TnC and TnI_{115–131} were determined by amino acid analysis performed in triplicate. The initial concentration of the high salt sample was 1.54 ± 0.1 mM N-TnC, whereas the low salt sample was 1.36 ± 0.03 mM N-TnC. The concentrations of CaCl_2 for the high and low salt samples were 6 and 5.4 mM, respectively.

**NMR Spectroscopy**—All experiments were conducted at 31 °C on a Varian Unity-600 spectrometer. The HSQC spectra of the high salt sample were acquired with a sweep width of 8000 Hz for ^1H (512 complex points) and 1650.2 Hz for ^15N (128 complex points) with 64 transients/increment, whereas the low salt HSQC spectra were performed using a sweep width of 8000 Hz for ^1H (512 complex points) and 1800 Hz for ^15N (128 complex t_1 points) with 28 transients/increment. Acquisition time was approximately 4.5 h/spectra. All experiments were processed using the software package NMRPipe (33) and analyzed using the program PIPP (34). Data were zero filled in t_2 to 1024 complex points. The t_1 points were increased by linear prediction to 256 complex points and then zero filled to 512 complex points. Spectra were apodized using a shifted sine bell before Fourier transformation.

**TnI_{115–131} Titrations**—TnI_{115–131} was added in 5-μl aliquots to 500-μl N-TnC samples to final TnI_{115–131} to N-TnC ratios of 1.7:1 and 1.4:1 for the high and low salt samples, respectively. Two-dimensional HSQC spectra were acquired at TnI_{115–131} to N-TnC ratios of 0, 0.3, 0.7, 1.4, and 1.7 for the high salt sample and ratios of 0, 0.1, 0.3, 0.5, 0.6, 0.8, 1, 1.1, 1.3, and 1.4 for the low salt sample. TnI_{115–131} was dissolved in the same stock buffer as N-TnC, and the pH of the NMR sample was checked after each addition. N-TnC backbone amide HSQC cross-peaks were followed during each point of the titration, and the total chemical shift change (Δδ) was determined as in Equation 1.

$$
Δδ_j = ((Δδ_{1H})^2 + (Δδ_{15N})^2)^{1/2}
$$

where Δδ_{15N} and Δδ_{1H} are the ^15N and ^1H chemical shift changes for a particular backbone amide residue j. The total chemical shift change for the N-TnC molecule (Δδ_{total}) is shown in Equation 2.
...the best fit (line shows the best fit for the experimental data. \(k\) was used to simulate the spectral line shapes for various values of \(K_{\text{off}}\); it showed a smaller change of \(\Delta \delta_{\text{total}}\). In Equation 3, analysis (25, 36) using the program Xcrvfit (available at www.pence.ualberta.ca). In Equation 3, \(P\) is the free N-TnC protein, \(L\) is the free TnI\(_{115-131}\) peptide, and \(PL\) is the complex. The fit of the results also establishes the stoichiometry of the reaction.

Calculation of Dissociation Constants—The dissociation constant \((K_{\text{off}})\) for the reaction,

\[
P + L \rightleftharpoons PL
\]

was determined from the \(\Delta \delta_{\text{total}}\) by an iterative nonlinear least squares analysis (25, 36) using the program Xcrvfit (available at www.pence.ualberta.ca). In Equation 3, \(P\) is the free N-TnC protein, \(L\) is the free TnI\(_{115-131}\) peptide, and \(PL\) is the complex. The fit of the results also establishes the stoichiometry of the reaction.

**Off Rate Constant (\(k_{\text{off}}\)) Determination—**Two backbone amide cross-peaks were selected from the low salt titration spectra. They were selected because their shifts were parallel to the \(^1H\) NMR axis, and therefore traces through the cross-peaks demonstrated line shape changes and were not complicated by unresolved splittings in the 50% \(^2H\) N-TnC sample. Methionine 46 was chosen because it showed one of the largest \(\Delta \delta\) of \(~130\) Hz, whereas asparagine 52 was selected because it showed a smaller change of \(~30\) Hz. The program Mathematica (37) was used to simulate the spectral line shapes for various values of \(k_{\text{off}}\). Input parameters included the determined \(K_{\text{off}}\), protein and ligand concentrations, free and bound chemical shifts, and free and bound line widths for each set of peaks, using the equations for the effect of site exchange on NMR spectra (38). From these simulations the \(k_{\text{off}}\) rate for the TnI\(_{115-131}\) peptide in the TnI\(_{115-131}\)-N-TnC complex was determined.

**RESULTS**

The interaction of TnI\(_{115-131}\) with calcium-saturated N-TnC was studied using two-dimensional \(^1H,\(^{15}N\)-HSQC NMR spectroscopy. The TnI\(_{115-131}\) was unlabeled, whereas the N-TnC was uniformly \(^{15}N\)-labeled, allowing for specific monitoring of the N-TnC component of the complex without interference from TnI signals. N-TnC was partially deuterated (50%) to reduce the line width of the observed cross-peaks. HSQC spectra show approximately 90 backbone amide and 6 Asn, Gln NH\(_2\) side chain cross-peaks that can be used to follow the titration in great detail. Each cross-peak responds to changes in the local environment of the atom, and this allows sensitive monitoring of each amino acid.

Contour plots of an expanded region of the HSQC spectra taken during the titration are shown in Fig. 1 (A and B) for the high salt and low salt samples, respectively. Some N-TnC amide resonances are not affected by the addition of TnI\(_{115-131}\) (e.g. Gly\(_{33}\) and Asp\(_{36}\)), whereas other resonances are significantly altered in either the \(^1H\), \(^{15}N\), or both dimensions (e.g. Met\(_{46}\), Gly\(_{50}\), and Met\(_{82}\)). Virtually all amide resonances shift very similarly in both samples. To a first approximation (see detailed line shape analysis below) these spectra are in the NMR intermediate-fast exchange limit. Only a single resonance peak is observed for each amide whose position is the weighted average of the free and bound chemical shifts (Equation 4).

\[
\delta_{\text{obs}} = P_f \delta_f + P_b \delta_b = (1 - P_f) \delta_f + P_b \delta_b \tag{4}
\]

where \(\delta_{\text{obs}}\) is the observed chemical shift of the backbone amide cross-peak, \(P_f\) and \(P_b\) are the fraction free and fraction bound of N-TnC, respectively, and \(\delta_f\) and \(\delta_b\) are the chemical shifts for the free and bound species, respectively.

**Chemical Shift Analysis and Determination of Dissociation Constants—**A total of 77 N-TnC backbone amide nitrogen and hydrogen pairs were followed throughout the TnI\(_{115-131}\) titration of the high salt sample. The NMR chemical shift change was calculated for each amide for each point in the titration, and individual \(\Delta \delta\) values were summed yielding the \(\Delta \delta_{\text{Total}}\).

Initial assignments of N-TnC backbone amide nitrogen and...
hydrogen atoms to be followed through the titration were taken from Gagné et al. (32). Residues Ala1, Ser2, Met3, Ala25, Met28, Glu57, Phe78, and Leu79 were not followed throughout the titration due to rapid amide exchange or resonance overlap. Pro53 lacks a backbone amide hydrogen atom and thus does not show a cross-peak in the HSQC spectra. Thr4 resonated in a crowded region in the high salt sample and was therefore not included in the titration. Asp66 and Leu49 were not assigned in the original N-TnC NMR spectra. An attempt was made to assign these two residues in the TnI115–131 N-TnC complex and interpolate back to where the cross-peak originated from in the free N-TnC spectra. From $\Delta \tau_{\text{total}}$ values acquired during the titration, a $K_d$ of 24 $\pm$ 4 mM was determined for a 1:1 binding complex (Fig. 2A).

The low salt N-TnC titration was analyzed as described above, but a total of 78 peaks were followed. In the low salt sample Thr4 was isolated enough to follow through half the titration and did not shift upon TnI115–131 addition. A $K_d$ of 40 $\pm$ 23 for a 1:1 complex was determined for a 1:1 binding complex (Fig. 2A). The assumption was considered reasonable because both samples had almost identical responses to binding of the peptide and the same backbone amide groups were followed throughout. The only exception is threonine 4, which was followed for the low salt sample; however, Thr4 did not alter the binding data because it does not shift during the titration. The completeness of the titration is evident because a majority of the peaks ceased chemical shift changes before the end of the titration.

**Line Shape Analysis and Determination of Exchange Rates**

Closer inspection of individual HSQC NMR cross-peaks during the titration reveals differential broadening of resonances (for examples see Fig. 1, A and B, residue Gly50 (G50)), indicating exchange broadening. One-dimensional traces through the cross-peaks of N-TnC residues Met46 and Asn52 are shown in Fig. 3 (A and E, respectively) to display the effect of exchange at various points during the addition of peptide. For Asn52, where the $\Delta \delta_H$ was 30 Hz, the line shape only slightly broadens during the titration. On the other hand Met46, which has a $\Delta \delta_H$ of 130 Hz, broadens substantially during the titration and then sharpens dramatically at the end. Different broadening is expected because the effect of chemical exchange on line width in the intermediate exchange limit has a dependence on the chemical shift difference between the free and bound species:

$$\Delta V_{\text{ex}} = P_f P_b \tau_{\text{ex}} (\Delta \delta)^2$$

(Eq. 5)

where $\Delta V_{\text{ex}}$ is the observed line width, $P_f$ and $P_b$ are the populations of free and bound N-TnC, and $\tau_{\text{ex}}$ is the exchange lifetime defined as $(\tau_f + \tau_b)^{-1}$.

The line widths of the free ($\Delta \delta_H$) and bound ($\Delta \delta_H$) N-TnC and the starting ($\delta_b$) and final ($\delta_f$) resonance positions were determined from the NMR spectra without TnI115–131 and at the highest TnI115–131 to N-TnC ratio used. The starting line width of the free N-TnC of 24 Hz (Fig. 3A, right peak) sharpens to 20 Hz (Fig. 3A, left peak) for the bound complex. This results...
because N-TnC transfers from a weak dimer to a tighter TnI complex (39). Line shapes were simulated using the experimentally derived values for \( D_{nf}, D_{nb}, d_{nf}, d_{nb}, K_d \), and adjusting \( k_{off} \). The results of using \( k_{off} \) rates of 350, 35, and 3500 \( s^{-1} \) were shown in Fig. 3 (B–D and F–H) for Met^{46} and Asn^{52}, respectively. A \( k_{off} \) of \( -3 \times 10^2 \) \( s^{-1} \) (Fig. 3, B and F) provides

**FIG. 4.** The \( \delta\Delta \) for each individual backbone amide of N-TnC upon addition of TnI_{115–131}. The 100 mM KCl sample is shown in A, whereas the 0 mM KCl sample is shown in B. The high salt residues marked with an asterisk were not followed over the entire titration, and the values shown are either extrapolated based on the data acquired (e.g., Asp^{66}, Leu^{68}, and Ala^{25}) or shown for as far as they were followed in the titration. For B no residues were extrapolated. Residues in B with an asterisk were followed until lost in the titration, and the chemical shift magnitude indicates the last known chemical shift position. The dashed lines in A and B indicate the mean chemical shift change plus one standard deviation. One-letter codes are used for the residues in the figure.
residues Ile19, Ala25, Phe26, Leu42, Val45, Met46, Met48, Leu49, Gly50, Asp59, Ile61, Val65, Met81, Met82, and Gln85 changed
D
TnI115–131 under both salt conditions and is presented in Fig. 4
cal shift upon addition of TnI 115–131 are shown in Fig. 5. The
peptide TnI115–131 binds N-TnC with 1:1 stoichiometry under
occurs in the hydrophobic pocket of N-TnC.
residues. The structure represents N-TnC by itself in solution not the structure of N-TnC while bound, nor is the
TnI115–131 shown. These marked residues clearly indicate that binding
occurs in the hydrophobic pocket of N-TnC.

Identification of TnI115–131 Binding Site on N-TnC—The
binding site of TnI115–131 on N-TnC was mapped by following
backbone amide chemical shift changes during the titration.
The maximum Δδ was obtained for each residue at saturating
TnI115–131 under both salt conditions and is presented in Fig. 4
(A and B). The average Δδ was 35 and 29 Hz for the high and
low salt samples, respectively. A standard deviation of 38 and
27 Hz was calculated for the Δδ of the high salt and low salt
samples, respectively. Values of Δδ greater than or approximately
equal (within the digital resolution) to one standard
deviation above the mean Δδ were considered to be statistically
significant (Fig. 4, A and B, dotted line). In the high salt sample
residues Ile19, Ala25, Phe26, Leu22, Val145, Met46, Leu49,
Gly50, Gly51, Ile61, Val65, Met82, and Gln85 showed significant
Δδ, whereas residues Ile19, Phe26, Phe29, Leu42, Val145, Met46,
Gly50, Asp59, Ile61, Val65, Met81, Met82, and Gln85 changed
significantly in the low salt sample.
N-TnC resonances that show a significant change in chemical
shift upon addition of TnI115–131 are shown in Fig. 5. The
structure used is that of Ca
2+
saturated N-TnC (11). N-TnC
residues that have a significant Δδ are shown in red and line
the hydrophobic pocket.

DISCUSSION
We have used HSQC NMR spectroscopy to show that the
peptide TnI115–131 binds N-TnC with 1:1 stoichiometry under
both high salt and low salt conditions, to measure the
dissociation and off rate constants for the complex, and to
demonstrate that binding occurs in the hydrophobic pocket of N-TnC
(10, 11). The high resolution HSQC spectra provides a unique
tool for monitoring each individual residue in the protein. This
allows one to observe protein complex formation in solution
without the potential hazards of introducing a chemical modi-

ification or sequence mutation in the protein, which are used to
introduce a localized, single spectrographic probe.

Interestingly, the high and low salt samples showed similar
d deviations for K
D
. A previous study had shown that salt concentrations
played a part in TnI-TnC affinity chromatography (19). Further, Van Eyk et al. showed the importance of basic resi-
dues in their TnIα-cardiac TnC affinity studies (40), and therefore
high salt was expected to screen electrostatic interactions and
decrease complex affinity (19, 41). However, this was not
observed. A possible explanation for the difference in results
may be that high salt concentrations disrupt nonspecific inter-
actions occurring in the affinity chromatography.

Dimerization of N-TnC was a concern in affinity calculations
because Slupsky et al. had shown previously that the
dimerization interface occurred in the hydrophobic pocket of N-TnC
(39). To test this hypothesis we simulated binding curves with an
increasing dimerization strength (data not shown). The first
two simulations with dimerization dissociation constants
(K
dimers
) of 100 and 10 mM indicated that K
dimers
in this range
would be insufficient to affect calculated protein-peptide com-
plex dissociation constants. For a K
dimers
of 1 mM, there was
a slight reduction in the observed K
D
. For example an apparent
K
D
of 22 μM would actually correlate to a K
D
of approximately
16 μM. Slupsky et al. showed a K
dimers
of approximately 1 mM at
30 °C for intact TnC, and our line shape analysis indicated an
N-TnC dimerization dissociation for N-TnC of ~3 mM.9 Therefore
the reported K
D
values for the TnI115–131-N-TnC complex
are probably closer to the lower experimental value reported.

We used the chemical shift changes of the backbone amide
resonances to demonstrate the position of the TnI peptide bind-
ing. This technique has been used to identify extremely specific
and tight binding compounds for enzyme active sites (42).

Cross-peaks that shifted more than one standard deviation
from the mean chemical shift change were marked in Fig. 4 (A
and B) and shown on the N-TnC structure in Fig. 5. These
residues are predominantly hydrophobic and line the N-TnC
hydrophobic pocket. These results agree with mutational stud-
ies that demonstrated that residues such as Met48 and Met46
were critical for proper regulation of muscle contraction (43,
44). In addition our results agree with cross-linking studies
showing that the N-TnC interface consisted of the interac-
tions between TnI residues 96–145 to whole TnC (22, 24, 45).
More specifically TnIβ was shown to cross-link to TnC residues
84–94 and either 60 or 61.

The interaction of TnI with the C-terminal domain of TnC
(17) and the interaction of TnI115–131 with N-TnC (19) support
the anti-parallel model of Farah et al. (3). The lack of an
extended spacer between the two TnI binding regions suggests
a more compact bound TnC structure than indicated by low
resolution neutron scattering experiments (14, 15). A compact
bound TnC structure would also agree with the homologous
calmodulin (46, 47) and myosin (e.g. regulatory light chain and
essential light chain) crystal structures (48, 49).

We determined off-rate constants from detailed line shape
analysis of HSQC cross-peaks. The simulations of cross-peak
traces (Fig. 3, B–D and F–G) show that one can easily distin-
guish between k
off
rates differing by a factor of ten. However,
the precision is limited to approximately ± 100 s
–1. Line shape
was sensitive to both k
off
and K
D
. A limitation of the line shape
fitting procedure was the quality of the spectra obtained as
traces through HSQC spectra. There are issues such as limit-
ing spectral resolution and also the varying T2 during the
intermediate sections of the titration, which leads to small
differential intensities in the cross-peaks.

The measured k
off
is fast enough to be kinetically competent

The K
dimers
was determined using the observed line width for both
the TnI115–131-N-TnC complex and partially dimerized N-TnC molecule
and assuming that the fully dimerized N-TnC would have a line width
double that of the monomer.
for muscle contraction. For example, the contraction frequency of insect flight muscle can be as fast as 600–1000 Hz (50).

Interestingly, the \( k_{\text{off}} \) for our complex is of the same order as the \( k_{\text{off}} \) for calcium (Ref. 51 and references therein), implying that the steps of calcium binding, opening of the hydrophobic pocket, and TnI binding all occur on similar time scales.

This study offers a unique starting point for kinetic analysis of other troponin interactions. These values will be compared with future kinetic work on even larger TnC and/or TnI protein fragments and is only the beginning of fully dissecting the molecular kinetics of muscle contraction.

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