Switch Action of Troponin on Muscle Thin Filament as Revealed by Spin Labeling and Pulsed EPR*

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We have used pulsed electron-electron double resonance (PELDOR) spectroscopy to measure the distance between spin labels at Cys133 of the regulatory region of TnI (TnI133) and a native or genetically substituted cysteine of TnC (TnC44, TnC61, or TnC98). In the +Ca\(^2+\) state, the TnC44-TnI133 distance was 42 Å, with a narrow distribution (half-width of 9 Å), suggesting that the regulatory region binds the N-lobe of TnC. Distances for TnC61-TnI133 and TnC98-TnI133 were also determined to be 38 Å (width of 12 Å) and 22 Å (width of 3.4 Å), respectively. These values were all consistent with recently published crystal structure (Vinogradova, M. V., Stone, D. B., Malanina, G. G., Karatzaferi, C., Cooke, R., Mendelson, R. A., and Fletterick, R. J. (2005) Proc. Natl Acad. Sci. U.S.A. 102, 5038–5043). Similar distances were obtained with the same spin pairs on a reconstituted thin filament in the +Ca\(^2+\) state. In the −Ca\(^2+\) state, the distances displayed broad distributions, suggesting that the regulatory region of TnI was physically released from the N-lobe of TnC and consequently fluctuated over a variety of distances on a large scale (20–80 Å). The interspin distance appeared longer on the filament than on troponin alone, consistent with the ability of the region to bind actin. These results support a concept that the regulatory region of TnI, as a molecular switch, binds to the exposed hydrophobic patch of TnC and traps the inhibitory region of TnI away from actin in Ca\(^2+\) activation of muscle.

Troponin (Tn) is a calcium (Ca\(^2+\)) binding protein, and its sensitivity to Ca\(^2+\) concentration is important for regulation of the contraction of skeletal and cardiac muscles (1–6). TnC has two globular domains in its N- and C-terminal regions (the N-lobe and C-lobe). A number of events occur when the Ca\(^2+\) concentration is changed, as suggested mainly from biochemical studies. For one, Ca\(^2+\) binding to the N-lobe of TnC induces the exposure of a hydrophobic pocket (5, 7–11). When this hydrophobic pocket is open in the +Ca\(^2+\) state, the switch segment (amino acids 116–131) in the regulatory region (amino acids 116–182) of TnI binds to the N-lobe (12–15). Upon removal of Ca\(^2+\), the hydrophobic pocket of the N-lobe closes, and the switch segment moves out of the N-lobe of TnC. Finally, two regions (amino acids 104–115 and 140–182) of TnI bind actin, resulting in the inhibition of actomyosin ATPase (16–20). Crystal structures of the Tn core domain have been solved for human cardiac (21) and chicken skeletal troponin molecules (22). The Tn core domain has two characteristic subdomains: 1) the “regulatory head,” composed of the N-lobe of TnC and the C-terminal region of TnI, and 2) the “I–T arm,” composed of long coiled-coil regions from TnT2, TnI, and the C-lobe of TnC. The relative orientation of the regulatory head and the I–T arm may play a fundamental role in the regulatory mechanism of troponin. The optimal docking positions could be ascertained by fitting to a three-dimensional reconstruction from electron microscopic images of thin filaments (23, 24). Alternatively, the best orientation or the best spatial relationships could be identified by fitting to polarized fluorescence data from bifunctional rhodamine probes for TnC in muscle fibers (25, 26) or fluorescence resonance energy transfer (FRET) between probes attached to various Tn subunits (TnC, TnI, or TnT) on the thin filament (27).

Continuous wave electron paramagnetic resonance (CW-EPR) was used for pioneering studies on spin-labeled troponin (28–30). Recently, we measured CW-EPR spectra from a spin label located on the Cys133 residue next to the switch segment in the regulatory region of TnI to identify Ca\(^2+\)-induced structural changes (31) based on the sensitivity of spin-label mobility to flexibility and tertiary contact with a polypeptide (32). The slow spin-label mobility observed for the thin filament in the −Ca\(^2+\) state indicated tertiary contact of Cys133 with actin because similar slow mobility was found for TnI-actin and TnI-tropomyosin-actin filaments lacking TnC, TnT, or tropomyosin. Furthermore, using nuclear magnetic resonance (NMR) and electron microscopic reconstruction, Murakami et al. (23) determined the structure of the C-terminal portion (131–182) of the regulatory region, including this cysteine, which binds to actin in the −Ca\(^2+\) state.
Global movement of Cys^{133} of TnI in the complex and on the actin thin filament has been investigated by FRET and chemical cross-linking. Tao et al. (34, 35) measured the average distance between TnI and TnC by FRET and found that the segment near the Cys^{133} region of TnI was located near TnC in the +Ca^{2+} state but moved away in the -Ca^{2+} state. This result was supported by Ca^{2+}-dependent cross-linking between Cys^{133} and TnC (36, 37). Using FRET, Miki et al. (38, 39) also found that Cys^{133} of TnI moved relative to Gln41, Lys61, and Cys^{374} of actin on the thin filament. However, the observed changes in the average distance were relatively small (~5 Å), suggesting that Cys^{133} of TnI is released from the N-lobe of TnC and moves toward the actin surface. However, the N-lobe of TnC is close to the actin surface and remains near Cys^{133} of TnI in the -Ca^{2+} state. Recently, CW-EPR and PELDOR (or DEER) spectroscopy have been used to investigate the distance between intra- and intersubunit sites in human cardiac binary TnC-I complexes and in reconstituted muscle fibers (10, 11, 40). These studies have verified the structures of the TnC monomer (10, 11) and binary TnC-I complex (40) derived from x-ray crystallography and NMR studies. Additionally, the Ca^{2+}-induced conformational transition of TnC in the binary complex and in fibers was determined (10, 11). Here, we measured the distance distribution between Cys^{133} in TnI and several residues in TnC using PELDOR to investigate the Ca^{2+}-dependent structural changes in reconstituted thin filaments, which may be physiologically relevant. Our results suggest that the switch segment near Cys^{133} of TnI is physically relevant and probably interacts with an actin surface in the thin filament.

**EXPERIMENTAL PROCEDURES**

**Reagents**—4-Maleimido-2,2,6,6-tetramethyl-1-piperidinoxy (MSL) was purchased from Sigma. The BCA protein assay reagents were of analytical grade. Other reagents were from Pierce. Other reagents were of analytical grade.

**Protein Preparation**—Rabbit muscle protein was prepared from back and leg muscles, and chicken muscle protein was prepared from breast muscles. Actin was prepared from acetone powder of rabbit skeletal muscles, according to the method described by Spudich and Watt (41). Native troponin complexes (TnC-I-T complexes) and tropomyosin were prepared from rabbit and chicken skeletal muscle residues after extraction of myosin by methods described by Ebashi et al. (42). After dialysis with 20 mM Tris-HCl (pH 7.5), 30 mM KCl, and 0.1 mM CaCl_2 (buffer A) with 1 mM dithiothreitol (DTT), troponin was purified using a Q-Sepharose Fast Flow column (2.5 × 10 cm) and eluted with a linear 30–400 mM KCl gradient. Fractions containing TnC-I-T complexes were identified by measuring absorbance at 280 nm and by SDS-PAGE, collected, and finally dialyzed against buffer A containing 1 mM DTT. Single cysteine mutant proteins of chicken skeletal TnC (G44C and A61C) were expressed and purified according to the method described by Nakamura (11). G44C and A61C were mutated using a Mutant-Super Express Km kit (Takara Bio Co.). The TnC gene fragments were ligated into pET15b vectors and used to transform competent *Escherichia coli* cells. Cells were grown at 37 °C in LB medium with 0.5 mg/ml ampicillin and chloramphenicol. Cells were then induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside and incubated for 4–5 h. Cells collected by centrifugation were sonicated in 50 mM Tris-HCl (pH 7.5) with 1 mM EDTA and 1 mM DTT. Next, ammonium sulfate (60% w/v) was added to the supernatant in 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT and centrifuged at 15,000 × g for 10 min. After the supernatant was dialyzed against 10 mM Tris-HCl (pH 7.5) with 2 mM EDTA and 1 mM DTT, mutant TnC was purified using a Q-Sepharose Fast Flow column (2.5 × 10 cm) and eluted with a linear 30–900 mM KCl gradient. Fractions containing TnC were identified by measuring absorbance at 280 nm and by SDS-PAGE, collected, and finally dialyzed against buffer A containing 1 mM DTT.

**Spin Labeling of the Troponin Complex and Isolation of Subunits**—To remove DTT, the protein solution was dialyzed against buffer A. Troponin complexes were reacted with an equimolar amount of MSL overnight in the dark at 4 °C. To remove unreacted MSL, the sample was dialyzed exhaustively against buffer A. To confirm the reaction and the removal of unreacted MSL, the dialyzed solution was measured by EPR.

MSL-labeled TnI (MSL-TnI), TnC, and TnT were isolated from MSL-Tn complexes as per Ojima and Nishita (43). MSL-Tn was dialyzed against urea buffer (6 mM urea, 30 mM KCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.6)) and applied to a CM-Toyopearl 650 M column (2.5 × 10 cm). Following pre-equilibration with the same buffer to elute isolated TnC, MSL-Tn and TnT were eluted with a linear gradient of 30–450 mM KCl in succession. Fractions containing TnC, MSL-TnI, and TnT were identified by measuring absorbance at 280 nm and by SDS-PAGE. TnC could be isolated almost completely from labeled TnI and TnT. Because TnI does not have a cysteine, the spin label cannot attach to TnI. Actually, a TnT-rich fraction showed a very weak EPR signal. Furthermore, a weak or no EPR signal was observed with TnI and TnT fractions isolated from the chicken MSL-Tn complex where TnI has Asn^{133} (22). These results confirmed that Cys^{133} of TnI was exclusively spin-labeled in the Tn complex from the rabbit (31, 44). Therefore, we did not try further to separate TnT from labeled TnI fractions. Samples were stored in urea isolation buffer. Isolated TnC was used for spin labeling of Cys^{99}.

**Spin Labeling of Single Cysteines on TnCs**—The mutated TnCs and TnCs isolated from native troponin complexes were dialyzed against buffer A to remove DTT. TnCs were reacted with a 10-fold molar excess of MSL overnight in the dark at 4 °C. To remove unreacted MSL, the solution was dialyzed against buffer A twice. Other reagents were of analytical grade.

**Reconstitution of the Doubly Labeled Troponin Complex**—MSL-TnI and TnT isolated from rabbit MSL-troponin complexes were mixed with MSL-labeled mutant TnC and incubated at 20 °C for 1 h in urea buffer (6 mM urea, 1 mM KCl, 0.1 mM CaCl_2, and 20 mM Tris HCl (pH 7.6)). For reconstitution of doubly labeled troponin, this solution was gradually dialyzed to remove urea and KCl and finally dialyzed against buffer A. The reconstituted doubly labeled troponin was purified using a
Q-Sepharose Fast Flow column (2.5 × 10 cm) and eluted with a linear 30–400 mM KCl gradient. Fractions containing TnC-I-T complexes were identified by measuring absorbance at 280 nm and by SDS-PAGE and subsequently pooled. The collected solution was dialyzed against buffer A. For EPR measurement, the doubly labeled troponin solution was concentrated to ~100 μM. This solution represented the +Ca²⁺ state without any further treatment or the −Ca²⁺ state following the addition of 5 mM EGTA.

Reconstitution of Thin Filaments—G-actin was polymerized in 20 mM imidazole-HCl (pH 7.0) with 100 mM KCl and 4 mM MgCl₂ for 30 min. Tropomyosin and MSL-Tn were added in excess to the F-actin solution (~100 μM, 0.3–0.5 ml) in the presence of 1 mM CaCl₂ (+Ca²⁺ state) or 5 mM EGTA (−Ca²⁺ state) and incubated for an additional hour. The molar ratio was set at 1:1:3 for tropomyosin, MSL-Tn complexes, and actin. Then, the mixture was centrifuged at 100,000 g for 30 min, and the pellet cosedimented with MSL-Tn was suspended in a small volume (20–100 μl) of 20 mM imidazole–HCl (pH 7.0) with 100 mM KCl, 4 mM MgCl₂, and 1 mM CaCl₂ or 5 mM EGTA. The amounts of TnC, TnI, TnT, tropomyosin, and actin were assessed by SDS-PAGE. The full binding was also confirmed by proper amounts of tropomyosin and MSL-Tn left in the supernatant. The suspension, containing 20–40 μM MSL-troponin, was used for EPR measurements.

ATPase Assay—The inhibitory activity of troponin was measured using an actomyosin ATPase assay, as described previously (31). The ATPase reaction was monitored by NADH absorbance using a pyruvate-lactate dehydrogenase-coupled system (45). Inhibitory activity was defined as [ATPase rate (−Ca²⁺ state)]/[ATPase rate (+Ca²⁺ state)] of 20 mM imidazole–HCl (pH 7.0) with 100 mM KCl, 4 mM MgCl₂, and 1 mM CaCl₂ or 5 mM EGTA. The ATPase reaction was monitored at an actomyosin ATPase assay, as described previously (31). The ATPase reaction was monitored by NADH absorbance (31). These results indicate that the distance is long and the distribution is relatively small volume (20–100 μl) of 20 mM imidazole–HCl (pH 7.0) with 100 mM KCl and 4 mM MgCl₂, and 1 mM CaCl₂ or 5 mM EGTA. The amounts of TnC, TnI, TnT, tropomyosin, and actin were assessed by SDS-PAGE. The full binding was also confirmed by proper amounts of tropomyosin and MSL-Tn left in the supernatant. The suspension, containing 20–40 μM MSL-troponin, was used for EPR measurements.

RESULTS AND DISCUSSION

Spin Labeling of the Cys¹³³ Residue of TnI and TnCs—We measured dipolar interactions between spin labels located at Cys¹³³ in the regulatory segment of TnI and a few sites (Cys⁴⁴, Cys⁶¹, and Cys⁹⁸) in TnC. Rabbit skeletal TnI was labeled exclusively with MSL because Cys¹³³, near the switch segment of TnI, is the most reactive cysteine in the complex state (44). The labeling efficiency for rabbit troponin, estimated from double integration of the spectrum, was >0.8 mol/mol troponin complex. On the other hand, single cysteine-labeled TnCs (designated as TnC⁴⁴, -61, and -98) yielded labeling efficiencies and actomyosin ATPase inhibitory activities of >90%. Labeled TnCs were reconstituted with labeled TnI (designated as TnI¹³³) and TnT. Doubly labeled troponin C-I-T complexes also showed Ca²⁺-dependent actomyosin ATPase activity (>80%). CW-EPR spectroscopy from doubly labeled troponin complexes showed Ca²⁺-dependent mobility changes similar to that of singly labeled troponin complexes labeled at Cys¹³³ of TnI, as reported previously (31). These results indicate that doubly labeled troponin complexes function similarly to the native complex. These samples are designated as TnC⁴⁴-TnI¹³³-TnT, TnC⁶¹-TnI¹³³-TnT, and TnC⁹⁸-TnI¹³³-TnT.

Distances between Pairs of Spin Labels Attached to TnI and TnC in the +Ca²⁺ State—To investigate how the N-lobe of TnC changes or modulates its interaction with the regulatory region of TnI upon Ca²⁺ binding, we measured the distances between Cys¹³³ in regulatory domain of TnI and three positions, Cys⁴⁴, Cys⁶¹, and Cys⁹⁸, of the N-lobe and central helix of TnC using PELDOR. The modulation of ESE from the TnC⁴⁴-TnI¹³³-TnT (Fig. 1a) or TnC⁶¹-TnI¹³³-TnT (Fig. 1b) complex decayed monotonically to baseline within 0.8 μs. This suggests that the distance is long and the distribution is relatively

FIGURE 1. Analysis of the PELDOR spectra of doubly labeled TnC and TnI in the reconstituted heterotrimer complex. Spectra were taken with a four-pulse sequence, both in the presence (red line) and absence (blue line) of Ca²⁺ ions at 80 K. Spin pairs were analyzed between Cys¹³³ in TnI and three different residues in TnC: Cys⁴⁴ (a), Cys⁶¹ (b), and Cys⁹⁸ (c). For each pair, the left panel compares the experimental spectrum modulation of the doubly labeled complex (dotted line) with the fitted spectrum modulation (continuous line). The single or double Gaussian distribution shown in the right panel fits the experimental spectra satisfactorily. In the right panel of a, a second population (56%) of widely separated noninteracting spins (>80 Å) in addition to the single Gaussian distribution is needed (not shown) to obtain the best fit for the complex in the −Ca²⁺ state (blue line).
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TABLE 1
Distance distributions between MSLs bound to Cys<sup>133</sup> of troponin I and Cys<sup>44</sup>, Cys<sup>61</sup>, or Cys<sup>98</sup> of troponin C in the troponin C-I-T complex in solution and on the thin (actin) filament in the +Ca<sup>2+</sup> state

| Paired residues       | Center | Half-width of half-maximum | Model<sup>a</sup> | α–α | β–β |
|-----------------------|--------|-----------------------------|-------------------|-----|-----|
| Ternary complex       |        |                             |                   |     |     |
| TnC44/TnI133          | 41.7   | 9.1                         | 27.33             | 30.28<sup>b</sup> |
| TnC61/TnI133          | 37.8   | 11.7                        | 28.98             | 30.56 |
| TnC98/TnI133          | 21.8   | 3.4                         | 23.05             | 21.85 |
| Thin filament         |        |                             |                   |     |     |
| TnC44/TnI133          | 41.4   | 7.7                         | 27.33             | 30.28 |
| TnC61/TnI133          | 33.9   | 17.5                        | 23.05             | 21.85 |

<sup>a</sup> α–α and β–β distances were calculated from the crystal structure model (Protein Data Bank code 1YTZ).

<sup>b</sup> Residue 44 corresponds to Gly<sup>42</sup> in the crystal structure.

The large observed half-widths (~10 Å) seem inconsistent with published crystal structure data (Protein Data Bank code 1YTZ). TnC<sup>44</sup> and TnC<sup>61</sup> are not thought to be flexible because the intramolecular distances in TnC have very narrow distributions (10, 11). TnI133 is exposed and moves out from the switch segment, which is clutched by the N-lobe in the crystal structure. Therefore, the side chain of Cys<sup>133</sup> fluctuates thermally. Additionally, the backbone of the segment including Cys<sup>133</sup> is thought to swing toward Cys<sup>44</sup> of the N-lobe. The interspin distance in TnC98-TnI133-TnT was 21.8 Å. Because the direction of the side chain of Cys<sup>98</sup> is nearly parallel with that of TnI133, it is reasonable to assume that the distance between these residues is nearly identical to that observed in the crystal structure (23.1 Å). A narrow distance distribution (~3.4 Å) suggested that the swing direction of the region including Cys<sup>133</sup> was vertical with respect to TnC98. In summary, the segment adjacent to Cys<sup>133</sup> of Tnl binds to the N-lobe of TnC in the +Ca<sup>2+</sup> state as in the crystal structure, but the Cys<sup>133</sup> region protrudes and swings thermally in solution (see Fig. 3a, left panel). This conclusion is supported by our previous data (31). Spin label experiments indicated that TnI133 exhibited decreased mobility but remained mobile on a nanosecond time scale in the +Ca<sup>2+</sup> state.

Next, we measured PELDOR from reconstituted thin filaments in the +Ca<sup>2+</sup> state. The filaments were prepared by mixing a spin-labeled troponin complex (TnC44-TnI133-TnT or TnC61-TnI133-TnT) with actin and tropomyosin (Tm). For TnC44-TnI133-TnT-Tm-actin<sub>v</sub> (Fig. 2a) and TnC61-TnI133-TnT-Tm-actin<sub>v</sub> (Fig. 2b), the modulation of ESE exhibited a monotonic decay within 0.8 µs. The distance distribution found to fit the decay curves was centered at 41.4 Å with a Gaussian half-width of 7.7 Å for TnC44-TnI133-TnT-Tm-actin<sub>v</sub> and at 33.9 Å with a width of 18.0 Å for TnC61-TnI133-TnT-Tm-actin<sub>v</sub>.

These values of the reconstituted filaments are similar to or only slightly different from those of troponin alone. Therefore, our results indicate that the bond between the N-lobe of TnC and the regulatory region of Tnl is not much altered by reconstitution of the troponin complex on the thin filament in the +Ca<sup>2+</sup> state. This conclusion was supported by a previous report, which showed that the moderate mobility of a spin label...
at TnI133 was identical in the TnC-I-T complex alone and in reconstituted filaments in the Ca<sup>2+</sup> state (31). Therefore, in a reconstituted thin filament, the regulatory region of TnI binds the N-lobe of TnC in a similar way to the Tn complex alone in the +Ca<sup>2+</sup> state (Fig. 3b, left panel). However, a small difference in the distances may explain why the Ca<sup>2+</sup> affinity is several-fold weaker for the filament than for the complex alone (46, 47). Our previous spin-labeling study showed that the geometry of TnC differed slightly (by <1.5 Å) in the monomer and in the TnC-I complex, to explain their nearly 10-fold affinity difference (10).

**Distances between Spin-labeled TnC and -I in the −Ca<sup>2+</sup> State**—We also tried to measure the distance between the regulatory region of TnI and the N-lobe of TnC in the −Ca<sup>2+</sup> state using PELDOR, to examine how they move in response to Ca<sup>2+</sup> removal. PELDOR spectra of doubly labeled troponin in the −Ca<sup>2+</sup> state indicated a gradual decay in the modulation of ESE (Fig. 1). These results suggest that a majority of spins have either weak spin-spin interactions or none at all (distance >50 Å) and broad distance distributions. However, these parameters could not be determined solely by spectral fit. We can assume that the number of spin pairs formed in the complex in the +Ca<sup>2+</sup> state is the same as the number in the −Ca<sup>2+</sup> state because we used the same preparations. We also used the same depth factor for the −Ca<sup>2+</sup> state as that obtained from the fit in the +Ca<sup>2+</sup> state. To obtain the best fit, single Gaussian distance distribution fitting was applied to the data from TnC44-TnI133-TnT (Fig. 1a) or TnC98-TnI133-TnT (Fig. 1c) interactions in the complex. The best fit showed similar broad distributions. The distances and half-widths were 29.1 and 25.2 Å for TnC44-TnI133-TnT and 41.7 and 22.7 Å for TnC98-TnI133-TnT, respectively. For TnC44-TnI133-TnT, partitioning of a second population of widely separated (>80 Å, noninteracting) spins (56%) was necessary to obtain the best fit. Inclusion of a second Gaussian function also improved the fit of TnC61-TnI133-TnT (Fig. 1b). The first population (29%) had a distance and half-width of 25.9 and 8.6 Å, whereas the second (71%) had values of 68.2 and 31.0 Å. The result obtained by two-component fit did not alter the conclusion that the distance distribution between the N-lobe of TnC and the regulatory region of TnI is very broad in the −Ca<sup>2+</sup> state. It is therefore likely that the TnI133 region dissociates from the N-lobe of TnC and swings around it upon Ca<sup>2+</sup> removal (Fig. 3a, right panel). This agrees well with previous data indicating that spin labels of TnI133 in the Tn complex is highly mobile in the −Ca<sup>2+</sup> state (31).

A gradual decay in ESE modulation in PELDOR spectra was also observed for reconstituted thin filaments prepared by mixing spin-labeled TnC-I-T with tropomyosin and actin (TnC44-TnI133-TnT-Tm-actin<sub>2</sub> and TnC61-TnI133-TnT-Tm-actin<sub>7</sub>). To obtain the best fit, single or double Gaussian component fitting was applied to the data obtained from TnC44-TnI133-TnT-Tm-actin<sub>2</sub> (Fig. 2a) and TnC61-TnI133-TnT-Tm-actin<sub>7</sub> (Fig. 2b). Broad distance distributions were obtained in both cases. However, the distributions were less uniform than that of the troponin complex alone in solution. Indeed, the population with distances of 60−80 Å or larger was dominant in the reconstituted thin filaments. This suggests that the regulatory region around TnI133 is released from TnC, fluctuates around TnC, and stays for some time at a site far away on the thin filament (Fig. 3b, right panel). However, using NMR and electron microscopic image reconstruction, Murakami et al. (23) recently showed that the C-terminal region near TnI133 binds to the surface of actin in the −Ca<sup>2+</sup> state. It is reasonable to resolve this discrepancy by assuming that the N-lobe of TnC is mobile and tumbling relative to the C-lobe of TnC and the actin surface (26, 48), whereas the regulatory region of TnI is released from TnC and fixed on the actin surface. The flexibility of the N-lobe of TnC in the complex was reported by Blumenschein et al. (49) using NMR. In the crystal structure of chicken skeletal troponin, the central helix is melted in the −Ca<sup>2+</sup> state, whereas it is folded in the +Ca<sup>2+</sup> state (22). On the other hand, we previously found that a part of the spin labels of TnI133 in the −Ca<sup>2+</sup> state were immobilized completely on actin alone, as well as on the thin filament, but the remaining population were still highly mobile or moderately immobilized as in the +Ca<sup>2+</sup> state (31), suggesting that the regulatory region of TnI is in equilibrium between being fixed on the actin surface and freely approaching the N-lobe of TnC. It is therefore likely that in a response to Ca<sup>2+</sup> the N-lobe traps a favored conformation of the regulatory region that fluctuates between the N-lobe and a site >50 Å away on the actin surface.

**The Relationship to Previous Distance Measurements**—From FRET data derived from the ternary complex in the Ca<sup>2+</sup> state,
Luo et al. (35) showed that the distance between TnC41 or 49 and TnI133 was 40 Å, which is very close to the distance we measured between TnC44 and TnI133 (42 Å). Tao et al. (34) also showed that the distance between TnC98 and TnI133 was at least 27 Å, which was a little larger than the value our experiments yielded (22 Å). Because an attached fluorescence label is larger in size than a nitroxide spin label, it might interfere sterically with a closer approach. This close proximity was supported by disulfide bond formation between TnC98 and TnI133 (36, 37). The FRET studies described above have all shown that the average distances between TnI133 and the residues of TnC in the ternary complex were only ~5 Å larger in the −Ca²⁺ state than in the +Ca²⁺ state. The average distance could not be directly compared because PELDOR yielded a much broader and more complex distance distribution within the Tn complex in the −Ca²⁺ state than in the +Ca²⁺ state. In agreement with FRET data, our PELDOR results indicate that the center of the Gaussian distance distribution between TnC98 and TnI133 increased by 7 Å (from 22 to 29 Å).

Miki and coworkers (38, 39) measured FRET between TnI133 and residue 41 or 374 of the nucleotide binding site of actin and showed that the average distances between them decreased by 5–15 Å upon Ca²⁺ removal. Xing et al. (50) recently showed a shift in the distance distribution by 10–15 Å between sites in TnI and residue 374 of actin in cardiac thin filaments using time-resolved FRET. Therefore, TnI133 moves inwards on the actin filament upon Ca²⁺ removal. The present study showed that the distances between TnI133 and various TnC sites increased, and their distributions shifted by >20 Å upon removal of Ca²⁺ from the thin filament. Taken together, it appears that TnI133 dissociates from TnC and then moves inward on the actin filament. The differences in the amplitude of shift and the width of the distribution could be explained by increased flexibility and outward movement of the free N-lobe of TnC in the −Ca²⁺ state.

Conformational Changes of TnI Regulatory Region (Switch Action)—Based on the data described above, we postulated a model of the TnI regulatory region (Fig. 3b). In the −Ca²⁺ state, the N-lobe of TnC releases the regulatory region and begins swinging thermally in Brownian motion. The regulatory region of TnI dissociates from the N-lobe of TnC, weakly binds to a site of actin surface, and may fluctuate between TnC and actin sites. Therefore, the interspin distance distributions were broad but dominated by long distances (>50 Å). This is a stand-by state for the binding of the regulatory region to TnC (Fig. 3b, right panel). Next, when the N-lobe binds Ca²⁺, it opens a hydrophobic pocket, which allows trapping a switch segment (amino acids 116–131) of the regulatory region of TnI. The distance between TnC and TnI133 showed a relatively narrow distribution in the +Ca²⁺ state, suggesting that the switch segment immediately adjacent to TnI133 was fixed to the N-lobe of TnC (Fig. 3b, left panel). The release of the C-terminal actin-binding portion (amino acids 140–182) of the regulatory region from the actin surface induces activation of muscle contraction. It is a speculative but attractive possibility that the other actin-binding site (amino acids 104–115) as well as the C-terminal binding portion of TnI are pulled up from the actin surface by the refolding and extension of the central helix of TnC (Fig. 3b).

When the N-lobe of TnC releases Ca²⁺+, the hydrophobic pocket closes, and the affinity of TnI for the switch segment decreases. Then, the regulatory region begins tumbling and binds again to the actin surface. Therefore, the switch segment moves outward on the filaments upon Ca²⁺ binding as postulated earlier by Miki et al. (39).

Although the Ca²⁺+-induced shift of tropomyosin appears to be well established by electron microscopic studies (51–54), it has not yet been satisfactorily demonstrated by distance measurements using FRET (55–57). The present PELDOR measurements clearly demonstrate the existence of large spatial rearrangements within the troponin complex. This method would be useful to test tropomyosin movement along the thin filament. The shutting of TnI between TnC and actin sites observed here may induce changes in the flexibility of tropomyosin, which may, in turn, induce changes in the flexibility of the actin filament (33, 55, 57). Alternatively, the C-terminal region of TnI may anchor the protein onto actin to pull tropomyosin laterally to the outer domain of the actin filament and retain the blocked state of tropomyosin (Fig. 3b) (53).

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