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Order-Disorder Transitions in the Cardiac Troponin Complex

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Abstract

The troponin complex is a molecular switch that ties shifting intracellular calcium concentration to association and dissociation of actin and myosin, effectively allowing excitation-contraction coupling in striated muscle. Although there is a long history of muscle biophysics and structural biology, many of the mechanistic details that enable troponin's function remain incompletely understood. This review summarizes the current structural understanding of the troponin complex on the muscle thin filament, focusing on conformational changes in flexible regions of the troponin I subunit. In particular, we focus on order-disorder transitions in the C-terminal domain of troponin I, which have important implications in cardiac disease, and could also have potential as a model system for the study of coupled binding and folding.

Graphical Abstract

Keywords

troponin; coupled binding and folding; hypertrophic cardiomyopathy; disordered proteins; troponin I mobile domain

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Notes

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Introduction

Muscle contraction was one of the first areas of cellular biology to generate a biophysical sub-field, possibly due to its strong structural features enabling visualization with early electron microscopes. The sliding filament theory of striated muscle contraction was formulated in 1953 by Hugh Huxley,¹ which he continued to develop to describe how muscle fibers are shortened due to the inward sliding of actin filaments driven by myosin motors on thick filaments. Even in 1953, actin, myosin, and tropomyosin (which polymerizes along the actin filament) had already been identified and isolated. Troponin, the 'relaxing factor'² that acts as a molecular switch to link calcium (Ca^{2+}) concentration to filament sliding, was isolated a decade later by Setsuro Ebashi.^{3; 4} This molecular switch links Ca^{2+} signaling during muscle excitation and the ensuing contractile force generation, and since its discovery has been the subject of much dedicated research in multiple areas of biology. The troponin complex is an obligate heterotrimer composed of three subunits: troponin T (TnT), troponin I (TnI) and troponin C (TnC). Today, many mechanistic details of troponin remain to be fully characterized, despite persistent molecular and biophysical efforts, due to the complexity of the thin filament system and the multi-step conformational changes required for troponin's regulation of it. In particular, the reversible interactions between regions of the TnI subunit and their respective binding partners have become increasingly studied due to their associations with genetic cardiac diseases. This review details the recent structural and biophysical efforts to characterize conformational changes in TnI, placing them into their mechanistic context.

The thin filament and its activation states

The function of the troponin complex is to regulate the muscle thin filament in response to Ca^{2+} concentrations in the cell, thus linking muscle contraction to neurological signals. While the thin filaments of intact striated muscle fibers contain many additional proteins, *in vitro* studies of thin filament activation are frequently performed on reconstituted filaments containing actin, tropomyosin, and troponin. The actin of the cardiac muscle thin filament is a double-stranded helix; upon each strand is tropomyosin, an acetylated coiled-coil dimer that polymerizes to span the length of the filament.^{5; 6} The repeating unit of this complex is seven actin monomers, to which is bound one tropomyosin dimer. The final protein component, the troponin complex, binds in a 1:1 stoichiometry with the tropomyosin dimers. For contraction to proceed, myosin motor proteins must bind actin (basic mechanical details are reviewed in Szent-Gyorgi, 2004).⁷

The three-state model of thin filament activation proposes that during the cycle of contraction and relaxation, thin filaments pass through three structural states defined by azimuthal tropomyosin position: the blocked state, the closed state, and the open state.^{8; 9} The blocked state is the state of muscle relaxation, which occurs only at low intracellular Ca^{2+} concentrations. In this state, cryo-electron microscopy studies indicate that tropomyosin lies across the outer domain of the actin filament,⁹ where it is bounded on one strand by the stable binding site of the T1 domain of TnT (TnT1), and on the other strand by the TnI C-terminal domain (TnI_C) (Figure 1).^{10; 11; 12} This tropomyosin position is induced by troponin, and is unpopulated in its absence.¹³

During excitation-contraction coupling, intracellular Ca^{2+} concentrations rise and induce the closed state, in which inhibition of myosin is reduced but the motor protein has not yet bound. In this state, movement of the TnI_C domain allows azimuthal diffusion of tropomyosin to 25 degrees from its blocked state conformation, easing the steric inhibition of actomyosin contact.⁹ The TnI_C domain maintains limited contact with tropomyosin in this state, though whether this inhibits or enhances myosin binding is debated.^{10; 14; 15; 16} Despite the shift of tropomyosin away from the blocked state, the closed-state thin filament still has a lower myosin affinity than bare actin; however, the high local myosin concentrations on the thick filaments enable rapid transition into the open, active state.¹⁷

The final state of the thin filament, the open state, occurs when at least two myosin heads bind the Ca^{2+} -activated thin filament to prop open an area of the tropomyosin polymer.¹⁷ The binding of myosin wedges tropomyosin another 10 degrees farther from its low- Ca^{2+} position on the thin filament, which fully releases the tropomyosin steric inhibition and allows many more myosin heads access.^{9; 17} The TnI_C domain loses its contact with tropomyosin in the open state,^{18; 19} and tropomyosin's position on the thin filament becomes more fixed.²⁰ The open state persists while both Ca^{2+} and myosin are present to maintain it, with force generation continuing beyond the immediate loss of Ca^{2+} until myosin dissociates and relaxation can proceed.

Relaxation follows a different pathway, initiated by the dissociation of myosin under low- Ca^{2+} conditions.¹⁷ Tropomyosin diffuses azimuthally across the thin filament, passing through the closed-state position (likely the default position of tropomyosin).¹³ However, troponin transitions directly from the open state to the blocked state through a series of conformational changes, bringing the full thin filament to the blocked state.^{21; 22} (While a number of publications have proposed additional thin filament states or intermediates, this review uses the canonical three-state model characterized by cryo-electron microscopy.) The transition from open to blocked state is a vulnerable one for the cardiac thin filament, and mutations that dysregulate it are responsible for hypertrophic cardiomyopathies.

The troponin complex: subunits, regions and domains

Striated muscle troponin has two adult isoforms, skeletal and cardiac, with high sequence identity but subtle differences particularly in TnI . The cardiac troponin complex was crystallized in 2003 by Takeda *et al.*, providing high-resolution structural information on its stable core domains (Figure 2).²³ TnT is an elongated helical protein with two domains, T1 and T2. TnT1 , which was truncated in the crystallized construct, docks the complex onto the actin filament and assists in regulating tropomyosin position.¹² TnT2 forms a coiled-coil motif with TnI known as the IT arm, which binds to TnC to create the stable troponin core (Figure 2).²³

TnC is the only troponin subunit whose structure is stable outside of the heterotrimeric complex.²⁴ It is composed of two globular heads, both of which contain two EF-hand motifs. The C-terminal head contains two high-affinity Ca^{2+} sites (sites III and IV) that are constitutively occupied *in vivo*, maintaining the binding sites for the IT arm to bind the C-terminal TnC head.²³ The N-terminal head (TnC_N) also contains two EF-hand motifs to

form Ca^{2+} binding sites I and II; however, in cardiac troponin, site I is incapable of binding divalent cations.²³ Site II binds Ca^{2+} with a K_D of roughly 10 μM , and the reversible binding of Ca^{2+} in this site controls the accessibility of the hydrophobic cleft in this head and ultimately regulates muscle contraction. The TnC heads are connected by the DE linker (Figure 2), which is flexible in cardiac troponin.^{23; 24}

TnI is the third subunit, and undergoes the largest conformational changes during Ca^{2+} regulation of muscle contraction. In the troponin crystal structure,²³ only about half of TnI is visible: the coiled-coil IT arm and the switch peptide (residues 147–163). When Ca^{2+} occupies TnC site II, the switch peptide of TnI adopts a helical conformation and binds in the hydrophobic cleft of TnC_N. Between the IT arm region and the switch peptide lies the inhibitory region (IR, residues 128–147), a disordered region whose primary binding partner is the actin filament. Both termini of TnI are disordered, and both are critical for functioning of the complex. The N-terminus (TnI_N, residues 1–40) has been shown by NMR to interact with site I of TnC,²⁵ though the functional significance of this interaction is still debated. The C-terminal domain (TnI_C, residues 161–210), also called the mobile domain (residues 164–210), undergoes large conformational changes that are discussed further below.

Troponin conformation during muscle contraction

During muscle activation, the core of the troponin complex retains a stable conformation, while flexible regions of TnI undergo conformational changes that link the Ca^{2+} binding state of TnC site II to tropomyosin position.^{26; 27} While some conformations and their functions remain controversial, others have been established. In the blocked state, the IR and TnI_C are both bound to the thin filament.^{10; 28} TnI_C positions tropomyosin in a sterically hindering conformation, while the IR blocks myosin binding through some other mechanism.²⁸ The switch peptide and TnI_N are likely disordered and free.^{25; 29} In the closed state, the switch peptide adopts a helical conformation and binds the hydrophobic cleft of the TnC_N,²³ moving approximately 10 Å from its blocked state position.²⁹ TnI_N is also bound to TnC_N,²⁵ and TnI_C is bound to tropomyosin but likely in a different conformation.³⁰ The IR is likely not bound to the thin filament in the closed state.³¹ Finally, in the open state, TnI_C is dislodged from the thin filament.^{16; 22} (These conformations are boxed in Figure 5.)

An area of much recent interest has been the positioning of the core troponin complex on the thin filament, which has direct implications on understanding the interaction between the stable core (TnC and the IT arm) and the dynamic regions of TnI. The transition from the blocked to the closed state requires two disordered regions, TnI_N and the switch peptide, to both bind TnC_N.^{23; 25} While TnI_N likely has substantial mobility in the blocked state, the switch peptide is short and tethered to the thin filament by both the IR and TnI_C. Recent work supports a model in which TnC_N samples multiple positions close to the thin filament in the blocked state, which places it near to the switch peptide.^{32; 33} The interaction with TnI_N assists in stabilizing a TnC_N position in close proximity to the switch peptide, increasing the local concentration and thus encouraging binding.³² An alternative model is that TnC_N is fixed in space but the IT arm experiences a 20° tilt during activation,^{34; 35} moving TnC_N toward the actin outer domain and close to the switch peptide, and that the

binding of TnI_N compensates for the defective site I EF-hand motif in TnC_N to stabilize the hydrophobic cleft and increase Ca²⁺ affinity.^{25; 36}

Another unresolved area of research is that pertaining to the IR and its function. While early peptide studies suggested that the IR was responsible for the inhibition of myosin binding in the blocked state,²⁸ the stoichiometry was inconsistent with the physiological reality of the interaction. More recent work has highlighted the importance of TnI_C and TnT1 in positioning tropomyosin (with the switch peptide and TnI_N assisting the TnI_C transitions),^{10; 11} leaving the importance and function of the IR unclear. One difficulty in ascribing function to the IR is that the exact position of the switch peptide-TnC_N complex in relation to the thin filament is undetermined; without this knowledge, it is unclear how much conformational freedom the region could have in the closed and open states as a short region joining two folded domains.

Possible roles for the IR include inhibition of myosin binding, TnC_N positioning, interaction with TnI_N following PKA phosphorylation, and simply serving as a disordered linker giving necessary conformational freedom to the switch peptide; the function may be a combination of these roles. The argument for TnC_N positioning is based on the skeletal isoform, where the IR binds the helical conformation of the DE linker at high Ca²⁺ concentrations;³⁷ however, in cardiac troponin the DE linker between the TnC heads is disordered in all activation states.²³ The IR may still bind the disordered DE linker through electrostatic interactions,³⁸ but this has not been fully explored. The evidence supporting a role in blocked-state myosin inhibition is stronger, though still not conclusive. Cryo-EM studies suggest that the IR inhibits myosin binding through a steric mechanism,³⁹ which is consistent with results showing that inhibition by IR alone lack the cooperativity that characterizes thin filament interactions.⁴⁰ An alternative theory is that the IR induces a slight conformational shift in the thin filament that reduces its binding affinity for myosin.²⁸ Finally, it has been proposed that the IR interacts with TnI_N when the latter region is phosphorylated by PKA, which has the potential to affect many dynamic interactions within the troponin complex (further discussion of transient post-translational modifications is outside the scope of this review; the reader is referred to references 41–44).^{41; 42; 43; 44; 45; 46} Regardless of the mechanism the IR uses, its importance in the troponin complex is high: alanine scanning most residues in the IR, or replacing sections of the IR with an alanine-glycine linker, both have negative effects on the inhibition of myosin in the blocked state.^{39; 47}

TnI_C dominates transitions to and from the open state

While the transition from blocked to closed is dominated by movements of the switch peptide, TnI_N and IR, TnI_C conformational dynamics are critical for transitions involving the open state of the thin filament (both from closed to open, and from open to blocked). There are many questions surrounding the conformation of this 50-residue intrinsically disordered region in all three thin filament activation states. TnI_C is purported to have two binding sites for the thin filament, one near residues 186–193³⁰ and one that includes residue 166.⁴⁸ Of these two sites, the first is consistent with cryo-EM data showing that TnI 193–210 positions tropomyosin in the closed state.¹⁰ The second site has not been structurally confirmed, and

the original data only suggests that there is an unknown binding partner on the thin filament required for this interaction.⁴⁸

The conformation with which TnI_C binds the thin filament is also unknown. An NMR structure of TnI_C in the open state was published in 2005, and the group subsequently argued that this structure was consistent in all three activation states and could be docked onto the thin filament computationally.^{19; 49} However, this structure was immediately controversial,^{16; 18; 50} and even its own authors noted that they were surprised at the number of salt bridges between their structure and its putative actin binding site given the known low affinity between them.⁴⁹ More recent work has indicated that TnI_C is disordered in the open state,^{16; 18; 50; 51} which calls the NMR structure into question. While the structure could still prove to be relevant to understanding the blocked state conformation, the thin filament was not included in experimental measurements and thus its potential to be a blocked state conformation is unclear.

Moving from the blocked to the closed state, TnI_C appears to shift from an orientation positioned toward the outer domain of actin, to an orientation closer to the inner domain and the phalloidin binding site on actin.⁵² While the region including residues 193–210 likely retains contact with tropomyosin in this state,¹⁰ TnI_C in the closed state also exhibits greater dynamics throughout the chain than in the blocked state.¹⁶ These findings can be reconciled if there is a conformational change that accompanies the shift from blocked to closed conformation, such as a dissociation of the proposed binding site containing residue 166.

When myosin binds the thin filament, TnI_C is dislodged and is believed to be disordered. The conformation of TnI_C in the open state has been the subject of much contradictory structural work; in the past 15 years, publications have included two crystal structures, an NMR structure, NMR dynamics measurements, and hydrogen-deuterium exchange mass spectrometry protection factors showing folded regions.^{18; 19; 22; 23; 37; 50; 53} The separate structural models were recently established as one conformational ensemble using an integrated single-molecule fluorescence and simulation approach, which showed that the published models represent clusters of conformers favored in the disordered state (Figure 3, bottom panel).⁵¹ The conformational ensemble was clustered by mapping 3.5 Å contacts, which highlights helices as strong contacts along the i,i+3 diagonal. The helical propensities clustered by the contact maps match the helical propensities in published structures: a single N-terminal helix in Cluster 1 matching the 1J1E structure,²³ two short helices in areas highlighted in the 1VDJ structure,¹⁹ and a single C-terminal helix similar to that proposed by NMR and crystallography.^{37; 50} The shorter helical length *in silico* is likely due to a combination of stabilized secondary structure in published models and increased *in silico* flexibility from simulating the peptide in isolation.⁵¹

The conformational ensemble as a whole favors an extended conformation, as evidenced by a strong correlation between inter-residue distance and inter-residue sequence separation (Figure 3, top panel). There is a dearth of sequence-separated 3.5 Å contacts, which also supports an extended conformation (Figure 3, bottom panels). The heterogeneity in shorter-range contacts at the C-terminus indicates increased disorder in this area, which has the

potential to assist a fast structural transition to the bound, blocked state through coupled binding and folding (discussed further below).⁵¹

Moving from the open state to the blocked state does not proceed through the closed state, but rather proceeds through a different pathway. Kinetic studies of the troponin complex indicate that the movement of TnI_C back into its blocked-state position likely precedes the movements of the switch peptide and IR.^{21; 54} To confirm that a loss of Ca²⁺ does not lead to dissociation of the switch peptide and IR from TnC_N, we performed single-molecule Förster Resonant Energy Transfer (FRET) measurements on a solution-state troponin complex under high- and low- Ca²⁺ conditions (methods and controls may be found in Metskas and Rhoades, 2015).⁵¹ Donor and acceptor fluorophores were conjugated to TnI 152 (in the switch peptide) and TnC 35 (in TnC_N), and monitored for changes in FRET that would indicate dissociation of the interaction. The use of a stable solution-state troponin complex (without the presence of actin or tropomyosin) ensured that the switch peptide, IR, and TnI_C were unbound from the thin filament, which is otherwise difficult to achieve as an isolated state. Our results indicate that the switch peptide remains bound to TnC_N following loss of Ca²⁺ from site II of TnC_N: the mean transfer efficiency (ET_{eff}) between TnC and the switch peptide (TnI 152/TnC 35 pair) is unchanged when Ca²⁺ is removed (Figure 4). This finding supports the hypothesis that the interaction of TnI_C with the thin filament is necessary to pull the switch peptide and IR away from their interactions with TnC_N. We repositioned the fluorophores to monitor different areas of TnI_C, and repeated the single molecule FRET measurements (Figure 4, Table). Although small changes in the mean ET_{eff} were noted for some of the labeling positions as a function of Ca²⁺ concentration (Figure 4, Table) the changes correspond to conformational changes less than ~3 Å. This confirms that Ca²⁺ does not strongly influence the extended conformation of TnI_C, supporting the hypothesis that the transition to the blocked state involves coupled binding and folding.

A plausible mechanism for troponin function

With so many ongoing debates and key pieces of information unknown, any proposed mechanism of troponin function must accept some hypotheses and reject others. Given that caveat, one reasonable functional progression for the troponin complex is outlined here (Figure 5).

In the blocked state, troponin positions tropomyosin on the thin filament to sterically block myosin binding. The TnT1 domain of one troponin complex defines one boundary for tropomyosin, and the TnI_C domain of another troponin complex binds tropomyosin and serves as a second boundary for tropomyosin's azimuthal diffusion. The IR is bound to actin, and TnI_N floats freely. The TnC_N head diffuses in space on its disordered DE linker, but favors conformations close to the thin filament where it is in close proximity to the free, disordered switch peptide.

When Ca²⁺ binds TnC_N, TnI_N also binds nearby, which both fixes the position of TnC_N and stabilizes its hydrophobic binding cleft. The switch peptide binds TnC_N, pulling the IR off the thin filament. At this point the IR may bind the DE linker to assist in bringing the TnC_N head fully up to its closed position.³⁸ These interactions bring TnI_C into the closed

conformation, which likely retains a conformation similar to the blocked conformation around residues 193–210 but is unbound and dynamic between residues 161–192. This concludes the pre-activation of the thin filament, and is consistent with findings that the principal components of thin filament activation are independent of myosin binding.⁵⁴ Activation then simply requires myosin binding, which dislodges TnI_C. TnI_C floats freely in solution, where its disordered conformation may allow low-affinity interaction with the thin filament to sample the binding state of myosin and determine whether contraction is still progressing.

Relaxation is less well studied than activation. It is known that myosin lifetimes are not affected by regulatory proteins, so myosin must finish its cross-bridge cycle and dissociate normally.¹⁷ After this occurs, TnI_C makes contact with the thin filament, coupling binding and folding to return to its blocked state conformation. The absence of Ca²⁺ from site II on TnC_N will have destabilized the TnC_N interactions such that the TnI_C interaction with the thin filament is enough to dislodge the switch peptide from TnC_N. Dissociation of the switch peptide gives greater conformational freedom to the inhibitory region, which dissociates from the DE linker and rebinds the thin filament (this assumes low affinity of the inhibitory region for the DE linker such that it only interacts when held nearby, but this assumption is consistent with the fact that the association has been so elusive).

The mechanism above is supported by recent kinetics studies of thin filament activation and relaxation. Binding and dissociation of Ca²⁺ from site II is rapid, making it the first step in activation or relaxation.⁵⁴ Large conformational changes in TnI occur on the order of 7–8 ms during both activation and relaxation, and this timescale is similar to tropomyosin movements; however, the IR binds the actin filament with a slightly longer timescale, supporting its place as the last step in deactivation.^{21; 54} The rates of myosin binding and force generation are at least an order of magnitude slower than any of these conformational changes,⁵⁴ which is consistent with thin filament activation processes occurring before the actual myosin binding event.¹⁷

Coupled binding and folding of TnI_C: Evidence and potential implications

In the mechanism outlined above, the conformational changes leading to relaxation are initiated by TnI_C binding the thin filament and dragging the other TnI regions from their open state positions (preceded by the dissociation of myosin and Ca²⁺). This is likely a coupled binding and folding event, where TnI_C transitions from a disordered, unbound conformation to a folded (or partially-folded) bound one.^{18; 21; 22} Coupling binding and folding can have important implications for binding kinetics and their modulation, and insight into this aspect of troponin's mechanism could be relevant to understanding the role of TnI_C point mutations in genetic cardiac disease.

Coupled binding and folding can occur through conformational selection, a two-state process where the folded conformation must be sampled prior to binding; induced fit, where the folded conformation is adopted only after initial contact with the binding site; or a mixed mechanism falling on a spectrum between these two extremes.^{55; 56} There has been much recent interest in classifying binding mechanisms of intrinsically disordered proteins and

domains (for recent reviews on the subject, see references 57 and 58).^{57; 58} Conformational selection would require adoption of the bound-state conformation in the disordered ensemble, and examples of this binding mechanism typically have a significant population of the disordered state in a binding-ready conformation.⁵⁹ However, current experimental evidence suggests that TnI_C 186–210 (the region containing putative thin filament binding sites) is dynamic and lacking secondary structure in a large population of the conformational ensemble.^{16; 18; 23; 50; 51} Though a conformational selection mechanism for TnI_C cannot be conclusively dismissed without folding kinetics studies or a known actin-bound conformation, we conclude that there is currently little experimental evidence to support such a binding mechanism. Therefore, this section concentrates on the induced fit and mixed mechanisms, which are likely the favored interaction mechanisms for association of disordered regions with their binding targets.⁵⁸

One important feature of an induced fit or mixed mechanism is the concept of fly-casting, which relates the disorder-order transition and the kinetics of the system. The fly-casting mechanism, first proposed by the Wolynes group, postulates that a disordered protein has a larger contact radius than a folded protein at the same position, which allows the distance between the protein and its binding site to be crossed by fast intra-chain diffusion rather than slower translational diffusion.⁶⁰ This characteristic is beneficial in cases involving binding partners held in close proximity by other protein contacts, such as an intrinsically disordered region binding to another area of the same protein complex, where the slower translational diffusion of disordered proteins would be irrelevant. Another feature of fly-casting is that the initial collision between the protein and its binding site is more likely to result in successful binding because there are more allowable orientations for the contact; the increased probability for successful binding decreases the average number of encounters necessary for binding, thus increasing binding kinetics.⁶¹ The fly-casting mechanism is hypothesized to result in faster binding kinetics without significant changes in the thermodynamics of the system, a theory which continues to be debated with insufficient experimental evidence due to substantial experimental difficulties.^{58; 60; 61; 62} Whether or not the average association rates of disordered domains proves to be faster than those of folded domains, a subset of disordered domains do use the induced fit mechanism with very fast association rates.^{58; 63; 64}

Although there is no direct experimental support of an induced fit mechanism for TnI_C binding to the thin filament, several studies of conformational dynamics are consistent with this mechanism. NMR measurements of the skeletal isoform (75% sequence identity) show dynamic behavior throughout TnI_C with higher T₂ relaxation parameters near the C-terminal end,⁵⁰ and fluorescence anisotropy of the cardiac isoform has values consistent with intrinsic disorder.¹⁶ However, neither can support an induced fit over conformational selection, particularly because there remained disagreement over secondary structure propensities throughout the domain at the time of the publications. More recently, the question of helical propensities was revisited *in silico*, where it was found that both helical and non-helical conformations are sampled, but that global conformation is independent of local helicity.⁵¹ This study found that global conformational sampling was limited, particularly in the central portion of TnI_C, and that the greatest variability in conformation was at the C-terminal end; this supports a mechanism in which the C-terminal end of TnI_C contacts the thin filament

through an induced fit mechanism.⁵¹ While it is possible that the C-terminal end is not in fact the first domain contact with the thin filament despite being a known binding site, the homogeneously extended nature of TnI_C and the stable switch peptide interaction at its N-terminal end would suggest that the TnI_C C-terminal end is in the most favorable position for the initial contact.

The coupled binding and folding of TnI_C during the transition from open to blocked state has several implications. In the case of a disordered region such as TnI_C, positioned near its binding site through protein-protein interactions, both the disorder in the proximal site and the positioning of the site by distal regions should be important. A loss of disorder would decrease intrachain diffusion, while positioning the region farther from its binding site would increase the distance traveled by translational diffusion (slower in disordered proteins). Importantly, both of these effects are independent of much of fly-casting theory: whether the association rate is faster than a folded protein or not, the association rate of TnI_C will likely be affected by alterations to intrachain diffusion or average distance from a binding site. Moreover, a model in which a small number of residues samples the binding site would allow TnI_C to monitor the activation state of the thin filament, permitting transient low-affinity contacts without requiring full binding that could disrupt other protein-protein interactions occurring during muscle contraction. Therefore, any alteration to TnI_C (by mutation or post-translational modification) that affects global conformation, conformational dynamics, or affinity in the 186–210 region could have effects on binding kinetics and downstream effects on physiological function and cardiac contraction.

The role of coupled binding and folding in cardiomyopathy

The study of TnI_C has become the focus of many recent publications due to its link with hypertrophic cardiomyopathy (HCM), a genetic cardiac disease affecting approximately 0.2% of the global population and the leading cause of sudden cardiac death in children.^{65; 66} Typical HCM disease is asymptomatic at onset, and clinical presentation of symptoms is frequently severe (gross hypertrophy of the left ventricle, arrhythmia, impaired blood movement and/or death).⁶⁷ Several mechanisms have been proposed to explain the events leading to the onset of hypertrophy in HCM patients, with many directly or indirectly related to impaired handling of Ca²⁺ signaling or sensitivity.^{65; 68; 69; 70}

While only a small percentage of HCM mutants are within TnI_C, troponin HCM mutations typically generate less classical hypertrophy and less obstruction in the left ventricle, but have higher rates of severe symptoms.⁷¹ However, efforts to understand or classify the effects of specific disease-causing point mutations in TnI_C have faltered because many experiments are contradictory. One particularly good example of this is the R145G mutation, which was characterized as an HCM mutation relatively early and thus has been the subject of more extensive exploration than other mutation sites. The effect of the mutation on Ca²⁺ sensitivity has been studied by multiple labs using simulations, *in vitro* ATPase and sliding filament assays, skinned trabeculae force generation, and transgenic mouse models. However, different laboratories and techniques give conflicting results, with determinations of increased calcium sensitivity compared with wild-type, decreased calcium sensitivity, and no or statistically insignificant change.^{72; 73; 74; 75; 76} Even in cases without conflicting

results, the effects of HCM mutations within TnI_C *in vitro* are highly dependent on method and system complexity.

The most striking aspect of TnI_C mutations causing HCM is that they are subtle, as evidenced by the difficulty in isolating their effects *in vitro*. An HCM mutation will cause a shift in probability of correct troponin function, but a portion of the population will still function correctly during a contraction cycle. The thin filament is highly cooperative and involves many proteins, which allows functional units on the thin filament to compensate for each other's defects.⁶⁹ This is well established in the literature for multiple scales of complexity. A study of TnC mutations with Ca²⁺ affinity changes in isolated troponin complexes demonstrated that docking these mutants onto the thin filament rescued the defect, with Ca²⁺ affinity comparable to wild-type protein function.⁷⁷ A similar effect was seen for TnI mutations that showed altered Ca²⁺ sensitivity and maximal velocity in sliding filament assays but no changes in permeabilized cardiac trabeculae.⁷⁸ Because the organization of striated muscle allows a certain amount of troponin failure without functional effects, a functional defect in the thin filament requires multiple adjacent troponin molecules to fail simultaneously, and dysfunction in a myofibril is only apparent when multiple thin filaments fail during the same contraction cycle. The failure of the myofibril thus becomes a rare, sporadic event, consistent with the late onsets and low penetrance observed *in vivo*.^{65; 71}

While doubtlessly some HCM mutations are simple binding affinity changes that can be understood in the traditional context of protein-protein interactions, some TnI_C mutations suggest a defect in the open state of TnI_C that then impacts the regulation process. The fly-casting mechanism proposed for TnI_C makes conformational dynamics the driving factor in rapid binding of TnI_C to the thin filament, which starts the cascade of conformational changes needed for relaxation. Specifically, a mutation that alters global conformation anywhere in the domain to increase the distance between binding partners could affect the association rate of TnI_C with the thin filament, while a change in local conformational dynamics in the 186–210 region could either affect exploration of conformational space (leading to fewer contacts) or cause an increased rigidity in binding site contact such that a larger number of collisions is required to achieve binding.

Only a small number of studies have examined conformational dynamics of HCM mutants in the open conformation of troponin or its association rate with the thin filament. A kinetics study using FRET observed a slowing of all conformational transitions in the R146G, R146Q, and R163W mutants,⁷⁹ which is consistent with a defect in conformational dynamics but unfortunately probes the area of the domain least likely to participate directly in fly-casting. Two NMR studies have examined local conformational dynamics in the distal area of TnI_C, but did not relate them to binding kinetics. One study showed altered conformational dynamics in the K183 and G203S mutants, though experimental limitations prevented the author from drawing strong conclusions.⁸⁰ The other study observed small changes in chemical shifts with the K178E mutation that affected a small number of adjacent residues in the open state,⁸¹ though it is unclear whether this could affect sampling of the thin filament binding site. These studies support the theory that a shift in the coupled binding and folding of TnI_C may be involved in HCM, but more targeted efforts are needed

to fully establish the relationship between conformational dynamics of TnI_C, binding kinetics between TnI_C and the thin filament, and functional defects of cardiac myofibrils.

CONCLUSION

The troponin complex undergoes multiple conformational changes to perform its role in regulating tropomyosin position on the thin filament in response to intracellular Ca²⁺ concentrations. These conformational shifts originate with and are carried out by order-disorder transitions within regions of TnI: the TnI_N, IR, switch peptide and TnI_C/mobile domain. Transitioning from the blocked to the closed state of TnI involves the TnI_N, the IR, and the switch peptide, while TnI_C plays the largest role in transitions involving the open state. The transition from the open state to the blocked state, which occurs during muscle relaxation, involves a coupled binding and folding event within TnI_C that may be dysregulated in hypertrophic cardiomyopathy.

The coupled binding and folding performed by TnI_C has been the focus of multiple recent papers that study both the conformational changes and their kinetics. The transition from the open state to the blocked state of TnI is of great importance in disease, but also has potential to serve as a useful model for the study of coupled binding and folding. The 50-residue length of TnI_C is a convenient size for many different experimental techniques, there are well-established functional assays and structural controls, and its participation in a complex that holds it close to its binding site allows study under single-molecule conditions without the necessity of diffusion-based random contacts. The fields of muscle biology and disordered proteins have both advanced considerably in recent years; the troponin complex provides an opportunity for techniques from both areas to provide complementary information to study the many remaining questions in the function of this dynamic protein.

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Abbreviations

TnC_N	Troponin C N-terminal head
TnI_N	troponin I N-terminal extension
TnI_C	troponin I C-terminal domain (residues 161–210)
TnT1	Troponin T T1 domain
Ca²⁺	Calcium

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HIGHLIGHTS

- Several regions of the troponin complex undergo order-disorder transitions during muscle contraction
- We review conformations and binding partners of disordered troponin regions
- We summarize current knowledge into a plausible mechanism for transitions between troponin activation states
- The potential of troponin to explain aspects of human cardiomyopathy is discussed
- This review discusses current knowledge of conformational changes in troponin leading to muscle contraction and relaxation

Figure 1. Schematic of tropomyosin positioned by troponin in the blocked state

In the blocked state, troponin molecules on two separate strands of actin position tropomyosin. Tropomyosin (red) lies upon one strand of an actin filament (yellow) near the outer domain. Left: cryo-electron microscopy of decorated thin filaments in the blocked state shows TnI_C (cyan) positioned across the thin filament, blocking tropomyosin from accessing its closed-state conformation. One actin monomer is shaded in yellow; actin residues that are believed to bind tropomyosin in the closed state are colored according to charge. Reprinted from *J. Mol. Biol.* 379, Galinska-Rakoczy *et al.*, Structural Basis for the Regulation of Muscle Contraction by Troponin and Tropomyosin, 929–935, 2008, with permission from Elsevier. Right: The TnT1 domain of troponin (green) serves as a boundary for tropomyosin's diffusion, preventing it from shifting to a higher-affinity site on the opposite strand rather than the lower-affinity blocked state site. Troponin structures are from the 1J1E model;²³ data and concept for the schematic are from the Lehman group's electron microscopy studies.¹¹

Figure 2. The troponin complex in the open state

The troponin complex consists of three subunits: TnT (green), TnC (gray), and TnI (dark blue). Left: Folded domains of the troponin complex (PDB 1J1E).²³ TnT2 (green) forms a coiled-coil fold with TnI (dark blue) to form the IT arm. The IT arm binds the C-terminal head of TnC (gray). The N-terminal head of TnC binds the switch peptide (light blue) when Ca^{2+} (black spheres) is bound. Right: Regions missing from the troponin crystal structure. The TnT1 domain (green) binds to actin (yellow) and tropomyosin (red). TnI has several regions that are disordered in at least one thin filament state: the N-terminal domain (TnI_N) (magenta), the inhibitory region (purple), the switch peptide (light blue), and the C-terminal mobile domain or TnI_C (cyan). The TnC heads are connected by the disordered DE linker (orange). The DE linker and inhibitory region have been drawn based on connecting space limitations, TnI_C is shown in a conformation known to be sampled in the open state, and the TnI_N is positioned based on NMR studies from several groups.^{18; 36; 50; 51} Rectangles indicate helical structure.

Figure 3. TnI_C favors an extended conformation with specific conformational propensities in the open state

Monte Carlo simulations of TnI_C in solution show distinct conformational preferences. Top panel: An average of 10,000 conformers shows that all have residue-residue pairwise distances increasing over residue separation, indicating an extended conformation. Plot produced from published data.⁵¹ Bottom panel: The conformers can be clustered by 3.5 Å contact maps, which focus on secondary structure signatures. The three most populated clusters are similar to conflicting, published literature models of TnI_C. Contact map inserts show published structures (top left) and snapshots of simulated structures (bottom right). Adapted with permission from *J. Am. Chem. Soc.* 137(37), Metskas and Rhoades, Conformation and Dynamics of the Troponin I C-terminal Domain: Combining Single-Molecule and Computational Approaches for a Disordered Protein Region, 11962-9, 2015.

Figure 4. Single molecule FRET histograms of the switch peptide-TnC_N interaction at high and low calcium concentrations

At high Ca^{2+} (pCa 3, top plot), the histogram peak is fairly narrow; however, at low Ca^{2+} (bottom plot), the width increases. There is no observable peak shift, indicating that the switch peptide is not dissociating from its binding site on TnC, and there is also no observable TnI monomer population that would suggest a loss of signal for this state. A dashed line has been drawn through the plots at the mean energy transfer efficiency (ET_{eff}) for the pCa 3 state to guide the eye. The histograms shown here are representative of those measured for all labeling positions tested; absent or minor shifts in the mean ET_{eff} and slight broadening of the histograms were observed at low Ca^{2+} . The table summarizes these results. The mean ET_{eff} under high (pCa 3) and low (pCa 8) Ca^{2+} conditions and the standard error are given. Asterisks represent changes with $p < 0.01$ using a Student's T-Test.

Figure 5. One plausible model of troponin conformational changes

A model consistent with currently available data on troponin conformational changes is shown here; it is important to note that the data may be consistent with other mechanisms or models as well. Activation progresses from the blocked state to the closed state, and finally to the open state; relaxation proceeds from the open state directly to the blocked state through a different pathway. The IT arm, actin/tropomyosin filament, and TnC C-terminal head are sketched in black. TnC_N is in gray, with a black circle indicating Ca²⁺ bound at site II and a white circle indicating an unoccupied site. The DE linker is in orange. For the regions of TnI, the IR is in purple, the switch peptide is in teal, and TnI_C is in cyan. Transient helical structure is indicated by rectangles, and unknown but potentially stable folds are drawn as ovals. The TnI_N is in magenta; it is omitted in intermediates where available data do not support a specific hypothesis as to its conformation and binding state. Binding of myosin to the thin filament is indicated by thickening of the thin filament line. The stable blocked, closed and open states are boxed and indicated in the model.