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Cardiac troponin I inhibitory peptide: location of interaction sites on troponin C

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Abstract Cardiac troponin I(129–149) binds to the calcium saturated cardiac troponin C/troponin I(1–80) complex at two distinct sites. Binding of the first equivalent of troponin I(129–149) was found to primarily affect amide proton chemical shifts in the regulatory domain, while the second equivalent perturbed amide proton chemical shifts within the D/E linker region. Nitrogen-15 transverse relaxation rates showed that binding the first equivalent of inhibitory peptide to the regulatory domain decreased conformational exchange in defunct calcium binding site I and that addition of the second equivalent of inhibitory peptide decreased flexibility in the D/E linker region. No interactions between the inhibitory peptide and the C-domain of cardiac troponin C were detected by these methods demonstrating that the inhibitory peptide cannot displace cTnI(1–80) from the C-domain.

Key words: Cardiac troponin C; Cardiac troponin I; Troponin I inhibitory peptide; Nuclear magnetic resonance; Dynamics; Peptide binding

1. Introduction

Cardiac muscle contraction is regulated by calcium dependent interactions between members of the troponin complex and other thin filament proteins including actin and tropomyosin. The troponin complex consists of three proteins: troponin I (TnI) which inhibits the actomyosin Mg^{2+}-ATPase, TnC which regulates TnI inhibition of the Mg^{2+}-ATPase, and TnT which makes primary protein–protein contacts with tropomyosin. TnC is the Ca^{2+} binding component of the troponin complex that is required to confer Ca^{2+} sensitivity on the actin–myosin interaction. The cardiac isoforms of TnC and TnI differ significantly from skeletal isoforms. In the cardiac isoform of TnC, Ca^{2+} binding site I is naturally inactive and was found to undergo chemical exchange consistent with an equilibrium between 'closed' and 'open' forms [1,2]. Calcium binding to the isolated cTnC regulatory domain was shown not to induce a structural opening similar to that seen in the skeletal isoform [1,3]. The cardiac isoform of TnI is also unique in that it contains an additional N-extension of approximately 32 residues. This extension contains two adjacent serine residues that can be phosphorylated by cAMP dependent protein kinase A [4].

Interactions between TnI and TnC have been recently reviewed [5–12]. Cardiac TnC and TnI are known to interact in an antiparallel manner such that the C-domain of TnC interacts with the N-domain of TnI [13]. The inhibitory region of TnI plays a key regulatory role by inhibiting contraction at low Ca^{2+} concentrations [14]. Available biochemical data support models for interactions of TnIp, corresponding to cTnI residues 129–149, with either both globular domains, the C-domain, and/or the D/E linker region of TnC [7–9,15–19]. These models suggest exposure of hydrophobic patches on each globular domain of cTnC upon the binding of Ca^{2+} and interaction of an amphiphilic α-helical TnIp with the exposed hydrophobic surface [20]. However, other regions of cTnI have been shown to interact with the globular domains of cTnC. In the intact TnIC complex, the C-terminal hydrophobic pocket of TnC is occupied with the N-domain of TnI and may not be available for interaction with cTnIp [13,21–23]. Cardiac TnI(147–163), a region of cTnI C-terminal to the inhibitory region (cTnIp), has been shown to bind the isolated cTnC regulatory domain and induce a structural opening similar to that observed in sTnC upon Ca^{2+} binding [8,9,24]. Further, in the cardiac isoform of TnC, exposure of the N-domain hydrophobic pocket does not occur simultaneously with Ca^{2+} binding [3,25]. Binding of full length cTnI(1–211) to Ca^{2+} saturated cTnC has been shown to decrease flexibility in the linker region between the N- and C-domains (Abbott, unpublished), while the D/E linker remained flexible in the presence of cTnI(1–80) [2]. Thus, the role of the inhibitory region in Ca^{2+} dependent regulation of muscle contraction remains controversial and requires further investigation.

We have used chemical shift mapping, ^{15}N relaxation experiments, and $^{15}N\{^1H\}$NOEs to further define the interaction between cTnIp and cTnC in the troponin complex. As a consequence of the size and complexity of the system, there are presently no high resolution data on the intact system. Instead, structural studies on isolated domains and peptides derived from these domains are being used to study the molecular details of this system. To complement our structural and dynamic studies on the intact cTnIC complex and to develop a more complete model system, we have studied the

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Abbreviations: Tn, troponin; cTnC, recombinant cardiac troponin C (desMet[1]-Ala[2], Cys35Ser); sTnC, skeletal TnC; cTnI, cardiac troponin I; sTnI, skeletal troponin I; TnT, troponin T; cTnI(33–211), recombinant cardiac troponin I corresponding to residues 33 through 211; cTnI(33–80), recombinant cardiac troponin I corresponding to residues 33 through 80; cTnI(1–80), recombinant troponin I corresponding to the first 80 amino acid residues of cTnI; NOE, nuclear Overhauser effect; cTnIp, synthetic *N*-acetyl-TQKIYDLRGKFKRPTLRRVRI-amide, corresponding to mouse cTnI(129–149); cTnIC, cardiac TnC/TnI binary complex; R_2, ^{15}N transverse relaxation rates

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binding of cTnIp to Ca^{2+} saturated cTnC/cTnI(1–80). Chemical shift perturbation mapping and ^{15}N transverse relaxation values identify two binding sites for the inhibitory peptide, cTnIp, on the cTnC/cTnI(1–80) complex. Binding of one equivalent of cTnIp to form a cTnC/cTnI(1–80)/cTnIp complex reduced mobility in defunct Ca^{2+} binding site I. Binding of the second equivalent of cTnIp was found to result in decreased flexibility within the D/E linker region. However, $^{15}N\{^1H\}$ heteronuclear NOE values indicate that some fast picosecond motion persists in the linker region in the presence of two equivalents of cTnIp.

2. Materials and methods

2.1. Proteins and peptides

The synthetic peptide *N*-acetyl-TQKIYDLRGKFKRPTLRRVRI-amide, corresponding to mouse cTnIp, was synthesized by Protein Express (Cincinnati, OH) and the sequence confirmed by mass spectroscopy. The purity of the peptide was confirmed by high performance liquid chromatography. ^{15}N and ^{2}H labeled cTnC and cTnI(1–80) were purified and complex formation carried out as previously described [2]. For NMR experiments, cTnC/cTnI(1–80) complex was prepared at 1.0 mM concentration in 10% $^{2}H_2O$, 20 mM Tris-d[11] buffer (pH 6.8), 150 mM potassium chloride, 10 mM Ca^{2+}, 5 mM dithiothreitol [2]. Protein concentrations for cTnC were determined by UV and Bradford analysis. Cardiac TnI concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Amino acid analysis was used to calibrate the colorimetric methods and confirm concentrations. Synthetic peptide concentrations were determined by amino acid analysis. Complete EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Germany) was added to the NMR samples to prevent proteolysis during long NMR experiments.

2.2. NMR spectroscopy

All experiments were carried out on Varian Inova 600 or 800 MHz spectrometers. Amide ^1H and ^{15}N resonances for cTnC in the Ca^{2+} saturated cTnC/cTnI(1–80) complex have previously been assigned by means of heteronuclear triple resonance NMR experiments [26]. Nitrogen-15 relaxation experiments were performed and processed as described by Gaponenko et al. [2] using the CURVEFIT software [27]. Spectra were referenced to an external 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt standard.

3. Results

3.1. Mapping of interactions between cTnC/cTnI(1–80) and cTnIp

Previously, we have shown that the N-domain of cTnI interacts with the C-domain of cTnC [13,21]. In addition, the cardiac specific amino-terminus, consisting of residues 1–32, was found to interact with the regulatory domain of cTnC and decrease chemical exchange, presumably changing the dynamic equilibrium between 'open' and 'closed' conformations [2]. In view of these recent structural data, the proposed binding site for cTnIp on cTnC warrants further investigation. We have studied the binding of the well known cardiac inhibitory peptide cTnIp, or cTnI(129–149), to the Ca^{2+} saturated [^{15}N, ^{2}H]cTnC/cTnI(1–80) complex. Assignments in the ternary complex, [^{15}N, ^{2}H]cTnC/cTnI(1–80)/cTnIp, were made by comparison with those obtained in the [^{15}N, ^{2}H]cTnC/cTnI(1–80) complex [26] and confirmed by analysis of sequential NOEs in NOESY-HSQC spectra (data not shown). The majority of cTnC amide resonances, upon titration of the cTnC/cTnI(1–80) complex with cTnIp, were in fast exchange with a few resonances in the slow to intermediate exchange regime. Amide resonances found to be in slow to intermediate exchange were Val29, Ala31, Gly34, and Asp73. These residues have previously been observed to be in slow exchange in the intact cTnC/cTnI complex (Abbott, unpublished).

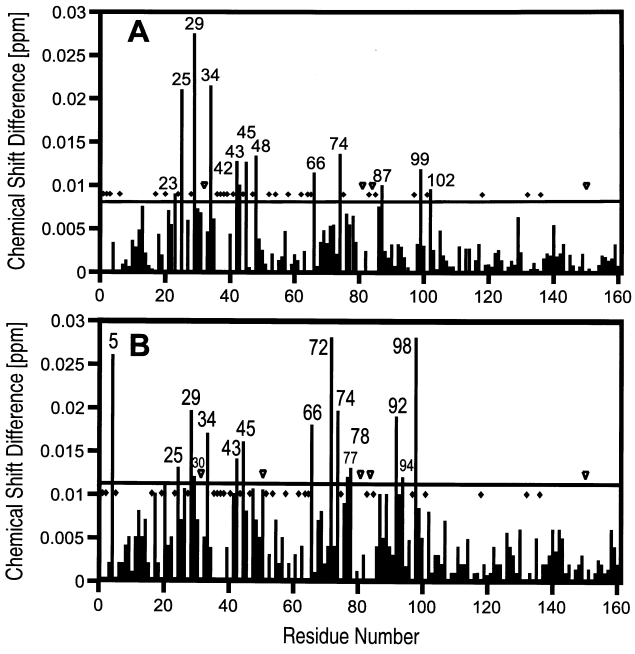

Fig. 1. Amide proton chemical shift differences between Ca^{2+} saturated [^{15}N, ^{2}H]cTnC/cTnI(1–80) and cTnC/cTn(1–80)/cTnIp at stoichiometric ratios of 1:1:1.4 (A) and 1:1:2.6 (B). The horizontal line represents the average chemical shift difference plus one S.D., 0.004 ± 0.004 ppm in A, and 0.006 ± 0.006 ppm in B. Filled diamonds mark residues for which resonance assignments in ^1H-^{15}N correlation spectrum could be not confirmed due to a lack of sequential NOEs in NOESY-HSQC spectra. These residues are 1, 2, 3, 6, 17, 20, 24, 28, 36, 37, 38, 39, 41, 44, 47, 58, 62, 64, 65, 75, 83, 85, 97, 101, 118, 132, and 136. Residues 52 and 54, also marked with black diamonds, are prolines. Cross-peaks for 32, 81, 84, and 150, marked by open triangles, are broadened beyond detection in ^1H-^{15}N correlation spectra of cTnC/cTnI(1–80) or cTnC/cTnI(1–80)/cTnIp at both concentrations of cTnIp. The resonance for residue 51, also indicated by an open triangle, is broadened only at high concentrations of cTnIp.

Chemical shift perturbations were used to monitor cTnIp interactions in the [^{15}N, ^{2}H]cTnC/cTnI(1–80) complex. Fig. 1 shows chemical shift changes for amide ^1H resonances of [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) observed in the presence of 1.4 equivalents of cTnIp. The largest amide ^1H chemical shifts were observed in defunct site I and active Ca^{2+} binding site II suggesting cTnIp binds to the regulatory domain in the cTnC/cTnI(1–80) complex. Small amide ^1H chemical shifts were also observed for residues 66 and 74 in helix D as well as residues 99 and 102 in helix E, surrounding the flexible linker region. No significant amide ^1H chemical shift changes were found in the C-domain (Fig. 1A). Addition of another 1.3 equivalents of cTnIp to form a 1:1:2.7 cTnC/cTnI(1–80)/cTnIp solution resulted in additional amide ^1H chemical shift changes in helices D and E (Fig. 1B). Specifically, chemical shift changes were observed in the C-terminus of helix D, the N-terminus of helix E, as well as in residue 92 within the linker region (Fig. 1B). The linker region corresponds to residues 83–93 [26]. Again, no significant chemical shift changes were observed in the C-domain. Thus, chemical shift changes could be used to map a second cTnIp binding site to the D/E linker region of cTnC.

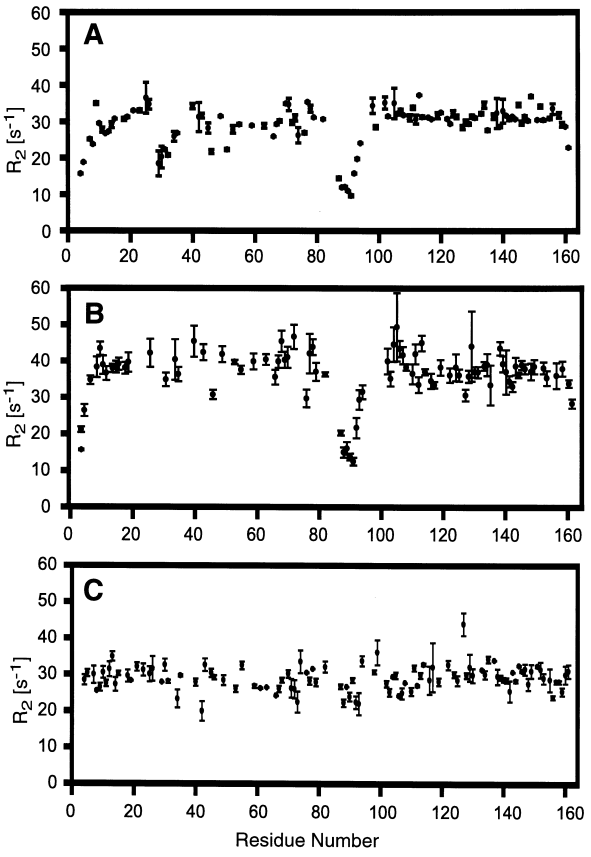

Fig. 2. Plots of ^{15}N transverse relaxation rates, R_2 (●) for Ca^{2+} saturated [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) (A) and to cTnI(1–80)/cTnIp with a ratio of 1:1.4 (B) and to cTnI(1–80)/cTnIp with a ratio of 1:2.6 (C). Residues 21, 22, 27, 33, 48, 50, 56, 57, 86, 60, 95, 96, 100, 109, 115, 120, 121, 123, 126, 131, 144, 147, and 154 were excluded from the analysis due to peak overlap in the ^{1}H-^{15}N correlation spectra of cTnC in the presence of cTnI(1–80) and cTnIp(129–149). Cross-peaks for 29, 30, 71, 74, and 80 were too broad for reliable R_2 determination. Values for Ca^{2+} saturated [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) were taken from Gaponenko et al. [2].

3.2. Dynamic consequences of cTnIp binding to cTnC/cTnI(1–80)

Transverse relaxation rates (R_2) and $^{15}N\{^{1}H\}$ heteronuclear NOE values were used to study the interaction of cTnIp with the binary cTnC/TnI(1–80) complex. ^{15}N R_2 values were measured for [^{15}N, ^{2}H]cTnC/cTnI(1–80) and for [^{15}N, ^{2}H]cTnC/cTnI(1–80)/cTnIp samples having either approximately one or two equivalents of cTnIp bound (Fig. 2). ^{15}N R_2 values for the cTnC/cTnI(1–80) complex, taken from Gaponenko et al. [2], are included for comparison. ^{15}N R_2 values depend on the rotational correlation time of the complex in solution, as well as chemical exchange and cross-relaxation. The presence of one equivalent of cTnIp dramatically increases ^{15}N R_2 values in inactive calcium binding site I, consistent with reduced millisecond motion of this previously flexible loop. Low ^{15}N R_2 values persist for residues within the D/E linker region, suggesting flexibility in this region is unaffected by cTnIp. The average ^{15}N R_2 values for the N- and C-domains in [^{15}N, ^{2}H]cTnC/cTnI(1–80) and [^{15}N, ^{2}H]cTnC/cTnI(1–80)/cTnIp are similar indicating uniform tumbling across the molecule in both complexes (Fig. 2B,C). As expected, ^{15}N R_2 values decrease at the N- and C-termini of cTnC for the two complexes (Fig. 2A,B). Higher average ^{15}N R_2 values in the [^{15}N, ^{2}H]cTnC/cTnI(1–80)/cTnIp complex may be due to the increased molecular mass or a more extended conformation resulting in a larger rotational correlation time for this complex.

Addition of the second equivalent of cTnIp, to form a 1:1:2 cTnC/cTnI(1–80)/cTnIp complex, increased ^{15}N R_2 values for the D/E linker region of cTnC suggesting that conformational flexibility in this region is considerably reduced by binding the second equivalent of cTnIp (Fig. 2C). In addition to the loss of flexibility in the linker region, a significant loss in flexibility was observed in both the N- and C-termini of cTnC (Fig. 2C). Some conformational flexibility was still observed in defunct Ca^{2+} binding site I as judged by a decrease in ^{15}N R_2 values for residues G34 and G42 compared with the average ^{15}N R_2 value, 29.2 ± 3.4 s^{-1} (Fig. 2C). The average ^{15}N R_2 value for this complex was smaller than the average ^{15}N R_2 value for the 1:1:1 cTnC/cTnI(1–80)/cTnIp complex, suggesting a shorter rotational correlation time, consistent with a more compact structure for the complex and loss of mobility at

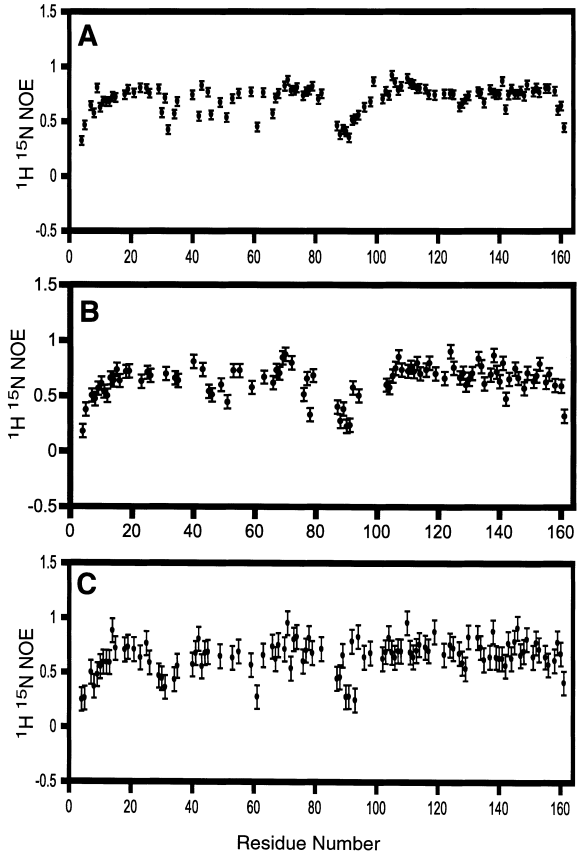

Fig. 3. $^{15}N\{^{1}H\}$ heteronuclear NOE values for Ca^{2+} saturated [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) (A) and Ca^{2+} saturated [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) and cTnIp at a ratio of 1:1:1.4 (B) and Ca^{2+} saturated [^{15}N, ^{2}H]cTnC bound to cTnI(1–80) and cTnIp at a stoichiometry of 1:1:2.6 (C). Accurate measurement of $^{15}N\{^{1}H\}$ NOE for residues 29, 30, 32, 74, 77, 93, 102, and 159 was not possible in the cTnC/cTnI(1–80) at one equivalent of cTnIp (B). At higher concentrations of cTnIp, accurate measurement of $^{15}N\{^{1}H\}$ NOE for residues 51, 80, 81, 84, and 159 also became impossible (C).

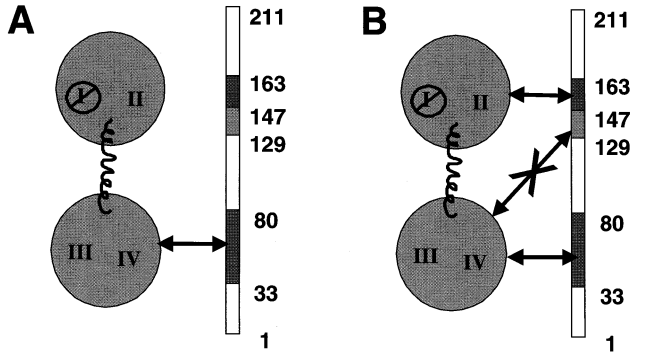

Fig. 4. Summary of proposed cTnC and cTnI interactions. Roman numerals indicate the Ca^{2+} binding sites in cTnC. Site I (naturally inactive) and site II are located in the N-domain, while sites III and IV lie in the C-domain. The two globular domains are connected by the D/E linker (residues 83–93). Functionally significant regions of cTnI are shaded. The cardiac specific N-terminus of cTnI consists of residues 1–32. Residues 33–80 comprise the first cTnC binding site (dark gray), while residues 147–163 constitute the second cTnC binding site (dark gray). The inhibitory region (light gray) includes residues 129–147. Arrows represent proposed interactions. In the absence of bound Ca^{2+} in the regulatory domain (A), residues 33–80 of cTnI interact with the C-domain of cTnC [21]. Upon Ca^{2+} binding to the regulatory domain (B), cTnI residues 147–163 interact with the N-domain of cTnC [24]. The inhibitory region of cTnI, corresponding to residues 129–147, may interact with the D/E linker. No evidence for interactions between cTnIp and the C-domain of cTnC was found in the presence of cTnI(1–80), suggesting that the inhibitory region does not compete with the cTnI N-domain (residues 33–80) for binding the cTnC C-domain, as proposed by the TnI switching model [9,12,28,29].

the N- and C-termini of cTnC in the presence of the second equivalent of cTnIp.

To further investigate dynamic consequences of binding cTnIp to the binary complex, on the picosecond time scale, $^{15}N\{^1H\}$ heteronuclear NOEs for ^{15}N perdeuterated cTnC/cTnI(1–80) and cTnC/cTnI(1–80)/cTnIp with approximately one and two equivalents of cTnIp bound were compared (Fig. 3A–C). Lower heteronuclear NOE values are observed in inactive calcium binding site I and calcium binding site II as well as the linker region of cTnC bound to cTnI(1–80). Thus, these regions are expected to have rapid internal motions. Addition of cTnIp to the binary complex does not appear to greatly alter the magnitude of $^{15}N\{^1H\}$ heteronuclear NOE values in the two globular domains or in the D/E linker region at either cTnIp concentration (Fig. 3B,C). These results suggest relatively uniform motion on a nanosecond time scale across both domains of cTnC in the ternary complex.

Both chemical shift perturbations and ^{15}N R_2 changes are consistent with two binding sites for cTnIp in the cTnC/cTnI(1–80) complex. The first equivalent of cTnIp results in chemical shift changes and increased ^{15}N R_2 values in the regulatory domain of cTnC. The second equivalent of cTnIp results in chemical shift perturbations and decreased flexibility in both the linker region and the N- and C-termini of cTnC. Lower average ^{15}N R_2 values suggest a more compact shape for the complex with two equivalents of cTnIp bound. No chemical shift changes or perturbation of dynamic parameters were observed in the C-domain of cTnC. Taken together, the data clarify existing biochemical data and demonstrate possible pitfalls of using peptides to model interactions of intact proteins.

4. Discussion

Localization of interactions between cTnIp and cTnC is crucial to understanding the mechanism of the Ca^{2+} triggered troponin molecular switch. In our efforts to understand the interactions between intact cTnC and cTnI, we chose to map the structural and dynamic consequences of cTnIp binding to cTnC/cTnI(1–80). The binary cTnC/cTnI(1–80) complex was used since it is known that cTnI residues 33–80 interact in the C-domain hydrophobic pocket of cTnC [13,21]. In the absence of the N-domain of cTnI, cTnIp has been shown to bind to the C-domain of cTnC [19]. To determine if cTnIp could still bind the C-domain of cTnC in the presence of the N-domain of cTnI, cTnIp was titrated into the binary cTnC/cTnI(1–80) complex. Two cTnIp binding sites were identified by chemical shift perturbations and altered dynamics in discrete regions of cTnC. Interactions of cTnIp that affect defunct Ca^{2+} binding site I did not preclude the observation of multiple NMR detectable conformers for the regulatory domain. Addition of a second equivalent of cTnIp induced chemical shift changes in residues at the end and at the beginning of helices D and E, respectively (Fig. 1). Interactions of cTnIp with the D/E linker region resulted in a decrease in flexibility within this region as judged by measured ^{15}N transverse relaxation rates (Fig. 2). Weaker binding of cTnIp to the D/E linker region may reflect the entropic cost of restricting flexibility within this region. No evidence for cTnIp interacting with the C-domain of cTnC in the complex was observed.

Under our conditions, cTnIp cannot displace cTnI(1–80) from the C-domain of cTnC as would be predicted by the 'TnI switching' model [28,29]. The TnI switching model postulates that residues 33–80 of cTnI (corresponding to skeletal TnI residues 1–40) and the inhibitory region of cTnI compete for the C-domain of cTnC, with Ca^{2+} saturation favoring the binding of the inhibitory region [9,12,28,29]. A combination of chemical shift mapping and relaxation studies has now demonstrated that the inhibitory peptide can bind to both the regulatory domain and the D/E linker region but not the C-domain of cTnC in the presence of cTnI(1–80) (Fig. 4). Cross-linking data for sTnIp binding to sTnC also support interactions for TnIp with both the regulatory domain and the linker region of TnC [16,30,31]. The recent NMR structure of cTnI(147–163) bound to the isolated regulatory domain of cTnC [24] suggests that in the intact cTnC/cTnI complex, cTnIp may interact with the D/E linker region of cTnC. In the X-ray crystallographic structure of sTnC bound to sTnI(1–47), the linker region of TnC is unwound and bent by 90° between the D and E helices [22]. This region corresponds to residues Ser89-Lys90-Gly91-Lys92-Thr93 in the cardiac isoform and is the region, as identified by chemical shift mapping and relaxation data, that binds the second equivalent of cTnIp in the Ca^{2+} saturated cTnC/cTnI(1–80)/cTnIp complex. Spin labeling studies on cTnC/cTnI(33–211) complex are consistent with a decrease in flexibility of the linker region in cTnC upon complex formation with cTnI [23]. Recent detailed dynamic studies of the cTnC/cTnI(1–211) complex also indicate reduced flexibility in the linker region of cTnC compared to the cTnC/cTnI(1–80) complex (Abbott, unpublished). These data suggest that a region of cTnI between residues 80 and 211, possibly corresponding to cTnIp, restricts the mobility of the linker region in the intact cTnC/cTnI complex.

Thus, the physiologically relevant cTnIp binding site on cTnC may be the D/E linker region.

Taken together, the available data for the cardiac TnIC interaction are more consistent with the model proposed by Maeda's group for the skeletal isoforms [22]. In this model, the N-domain for cTnI comprising residues 33–80 (corresponding to residues 1–47 in the skeletal isoform) are bound to the C-domain of TnC in both the presence and absence of Ca^{2+} at the regulatory site (Fig. 4). The inhibitory region of TnI, residues 129–149 in the cardiac isoform (corresponding to residues 96–115 in the skeletal isoform), would interact with the D/E linker region in TnC. The 'second Ca^{2+} dependent interaction site' [9] of TnI corresponding to residues 147–163 for the cardiac isoform (residues 115–131 in the skeletal isoform) would bind to the N-domain of TnC in a Ca^{2+} dependent manner. Regions of TnI C-terminal to this region are believed to make interactions with other thin filament proteins. Modulation of the interaction between the 'second Ca^{2+} dependent interaction site' and the regulatory domain of cTnC by intracellular calcium flux may 'pull' the adjacent inhibitory region of cTnI away from actin, relieving the inhibition of muscle contraction.

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