NMR analysis of cardiac troponin C-troponin I complexes: effects of phosphorylation

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Abstract Phosphorylation of the cardiac specific amino-terminus of troponin I has been demonstrated to reduce the Ca^{2+} affinity of the cardiac troponin C regulatory site. Recombinant N-terminal cardiac troponin I proteins, cardiac troponin I(33–80), cardiac troponin I(1–80), cardiac troponin I(1–80)DD and cardiac troponin I(1–80)pp, phosphorylated by protein kinase A, were used to form stable binary complexes with recombinant cardiac troponin C. Cardiac troponin I(1–80)DD, having phosphorylated Ser residues mutated to Asp, provided a stable mimetic of the phosphorylated state. In all complexes, the N-terminal domain of cardiac troponin I primarily makes contact with the C-terminal domain of cardiac troponin C. The non-phosphorylated cardiac specific amino-terminus, cardiac troponin I(1–80), was found to make additional interactions with the N-terminal domain of cardiac troponin C.

Key words: Cardiac troponin C; Cardiac troponin I; Phosphorylation; Mutagenesis; NMR; Isotope labelling

1. Introduction

Troponin C is the Ca^{2+} binding subunit of the troponin complex that interacts with both troponin (Tn) I, the inhibitory subunit, and TnT, the Tm binding subunit. Upon binding Ca^{2+} to the regulatory domain of TnC, protein-protein interactions within the complex are altered relieving actomyosin ATPase inhibition and triggering muscle contraction. The cardiac isoform of TnC (cTnC) differs from the skeletal isoform (sTnC) in containing an insertion and several critical amino acid substitutions in Ca^{2+} binding site I rendering it inactive [1]. cTnI has a unique N-terminal extension of approximately 32 amino acids including two adjacent serines which are protein kinase A (PKA) phosphorylation sites. Phosphorylation at Ser-23 and Ser-24 in the cardiac specific amino-terminus of TnI, in response to β-adrenergic stimulation, has been demonstrated to modulate the myofilament sensitivity to Ca^{2+} by reducing the Ca^{2+} affinity for the regulatory site of cTnC [2].

cTnC has been shown to interact with cTnI in an anti-parallel fashion [3]. We have recently solved the structure of the C-terminal domain of cTnC bound to the N-terminal domain of cTnI comprising residues 33–80 [4]. The bound structure for the C-terminal domain of cTnC was found to be similar to the crystal structure of sTnC bound to sTnI(1–47) [5]. These studies suggest a common binding motif for the Ca^{2+}/Mg^{2+} dependent interaction site in the TnIC complex. However, structural effects of binding the cardiac specific amino-terminus comprising residues 1–32, both phosphorylated and non-phosphorylated, to cTnC have not been systematically investigated. The purpose of this study is to examine the structural consequences of binding of phosphorylated and non-phosphorylated cTnI proteins to cTnC and to determine if cTnI(1–80)DD is a suitable mimetic for examining the structural and dynamic consequences of phosphorylation.

2. Materials and methods

2.1. Sample preparation

Recombinant cTnC(C35S) was over-expressed in BL21(DE3) cells and purified as previously described [3,6,7]. Isotopic labelling was accomplished using 1 g of [^{13}C]glucose and/or [^{15}N]NH_4Cl per liter of minimal media. ^2H labelling was accomplished using the appropriate minimal media containing 98% ^2H_2O. cTnI N-terminal proteins were cloned into pET23d+ and expressed in Luria Broth (Abbott, unpublished). TnI proteins were extracted from inclusion bodies using 8 M urea followed by FPLC chromatography on CM-Sepharose and Superdex-75 columns. All proteins were judged to be homogeneous by SDS-PAGE and staining with Coomassie brillant blue.

Complex formation was carried out as previously described [3,4] and monitored using native and SDS polyacrylamide gels. Light scattering experiments were performed on Ca^{2+}-saturated cTnC/cTnI(33–80), cTnC/cTnI(1–80)DD and cTnC/cTnI(1–80) complexes under conditions of the NMR experiments. Samples of approximately 1.0 mM concentration were prepared in 10% ^2H_2O, 20 mM Tris-d^{11} buffer (pH = 6.8), 150 mM potassium chloride, 10 mM Ca^{2+}, 10 mM β-mercaptoethanol and 10 mM dithiothreitol. All samples contained 0.2 mM leupeptin and 0.4 mM pefabloc to inhibit protein degradation.

2.2. PKA phosphorylation of cTnI(1–80)

Purified cTnI(1–80) was loaded on a cTnC affinity column equilibrated in 50 mM KH_2PO_4 at pH = 7.0, 500 mM KCl, 10 mM $MgCl_2$ and 0.5 mM dithiothreitol and 125 U PKA/mg cTnI added directly to the column. ATP was added to the column to initiate the reaction. After 20 min at 30°C, the column was washed with buffer containing 50 mM MOPS at pH = 7.2, 500 mM KCl, 5 mM $CaCl_2$ and 0.5 mM dithiothreitol and cTnI(1–80)pp eluted with buffer containing 6 M

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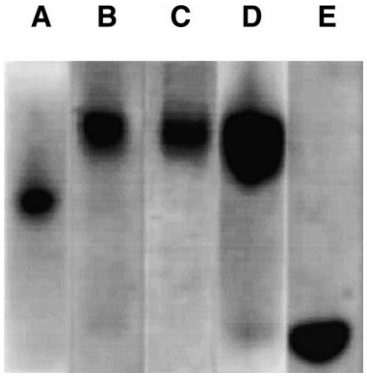

Fig. 1. Native 15% PAGE demonstrating complex formation of cTnC with the N-terminal domain of cTnI. (A) cTnC/cTnI(33–80) complex, (B) cTnC/cTnI(1–80)pp, (C) cTnC/cTnI(1–80), (D) cTnC/cTnI(1–80)DD and (E) free cTnC.

urea, 10 mM EDTA, 0.5 mM dithiothreitol and 50 mM MOPS at pH = 7.0. The extent of phosphorylation was quantitated by treatment with alkaline phosphatase and determination of inorganic phosphate using the EnzChek Phosphate assay.

2.3. NMR experiments

NMR experiments were performed at 40°C on 600 and 800 MHz Varian INOVA spectrometers. The following NMR experiments were used for backbone resonance assignment and secondary structure determination. Sensitivity-enhanced gradient ^{1}H-^{15}N HSQC [8], ^{1}H-^{15}N TROSY [9,10], ^{15}N-edited NOESY-HSQC with 70, 200 and 300 ms mixing times [11], $HNC_{\alpha}C_{\beta}$ [12], $(H_{\beta})C_{\beta}C_{\alpha}$(CO)NH [13], HNCO [14], HNHA experiment [15,16]. Acquisition parameters for the NMR experiments were previously described [4]. Data were processed and analyzed using Felix97.2.

3. Results

3.1. Complex formation, resonance assignment and secondary structure determination

Native gel electrophoresis was used to demonstrate tight complex formation between cTnC and N-terminal domain cTnI proteins (Fig. 1). Dynamic light scattering experiments on the complexes yielded similar radii of 3.6 nm with a polydispersity of 0.5 nm, indicating monodisperse solutions. In addition, gel filtration of the cTnC/cTnI(1–80)pp complex, using a Pharmacia Superdex 75 column, yielded a symmetrical peak at an elution volume consistent with the molecular mass for the complex (data not shown).

The 800 MHz NMR ^{1}H-^{15}N HSQC spectrum of [^{15}N, ^{2}H]cTnC/cTnI(1–80)DD and the ^{1}H-^{15}N TROSY spectrum of [^{15}N, ^{2}H]cTnC/cTnI(1–80) demonstrate that cTnC in the complexes is in slow exchange, on the NMR time scale, between the bound and free forms (Fig. 2). The ^{1}H, ^{13}C and ^{15}N backbone resonances for free [^{15}N, ^{13}C]cTnC and for [^{15}N, ^{13}C]cTnC bound to cTnI(33–80) and cTnI(1–80)DD were assigned from a series of NMR experiments ($HNC_{\alpha}C_{\beta}$, $(H_{\beta})C_{\beta}C_{\alpha}$(CO)NH, HNCO and ^{15}N-edited NOESY-HSQC). These experiments permitted the correlation of amide ^{1}H and ^{15}N resonances of each amino acid with the C_{α}, C_{β} and C′ signals of the *i*th and *i*th−1 residues. Amide ^{1}H and ^{15}N resonances for cTnC bound to cTnI(1–80) and cTnI(1–80)pp were initially assigned by comparison of ^{1}H-^{15}N TROSY and HSQC spectra with previously assigned ^{1}H-^{15}N HSQC spectra of cTnC bound to cTnI(1–80)DD. These assignments were then confirmed by the sequential assignment procedure using ^{15}N-edited NOESY-HSQC spectra.

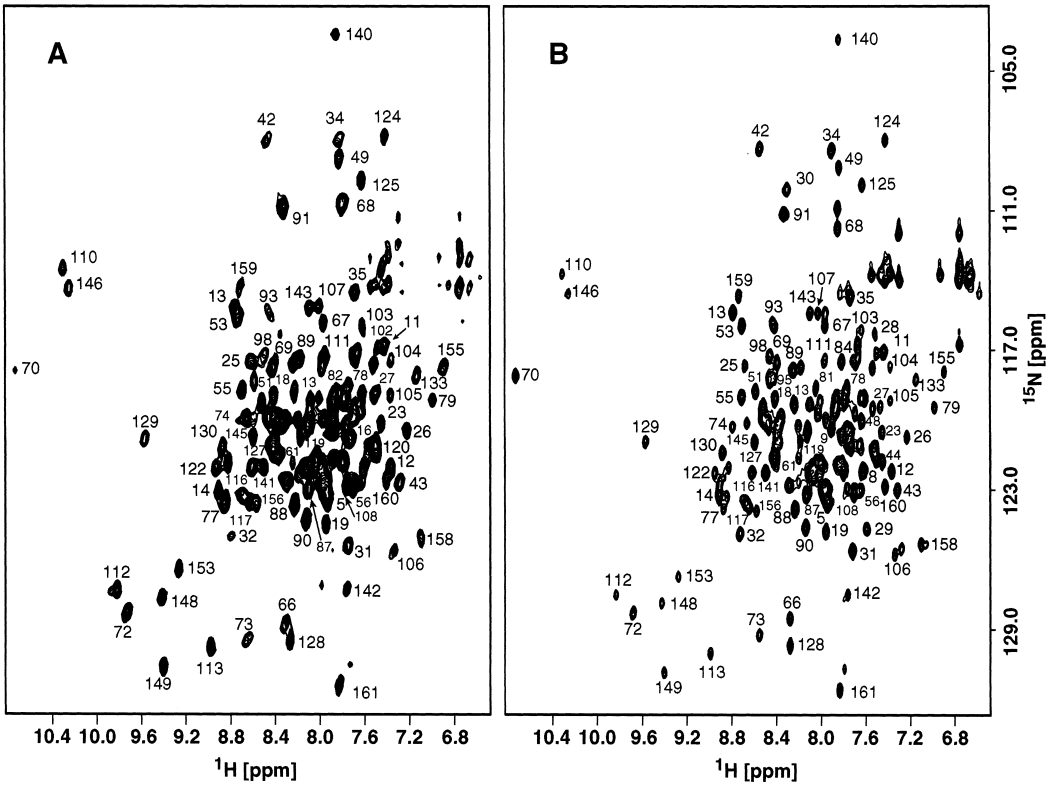

Fig. 2. Two dimensional ^{1}H-^{15}N HSQC and TROSY spectra of [^{15}N, ^{2}H]-labelled cTnC bound to cTnI(1–80)DD (A) and cTnI(1–80) (B) recorded at 800 MHz. Several of the correlations are labelled corresponding to the residue number.

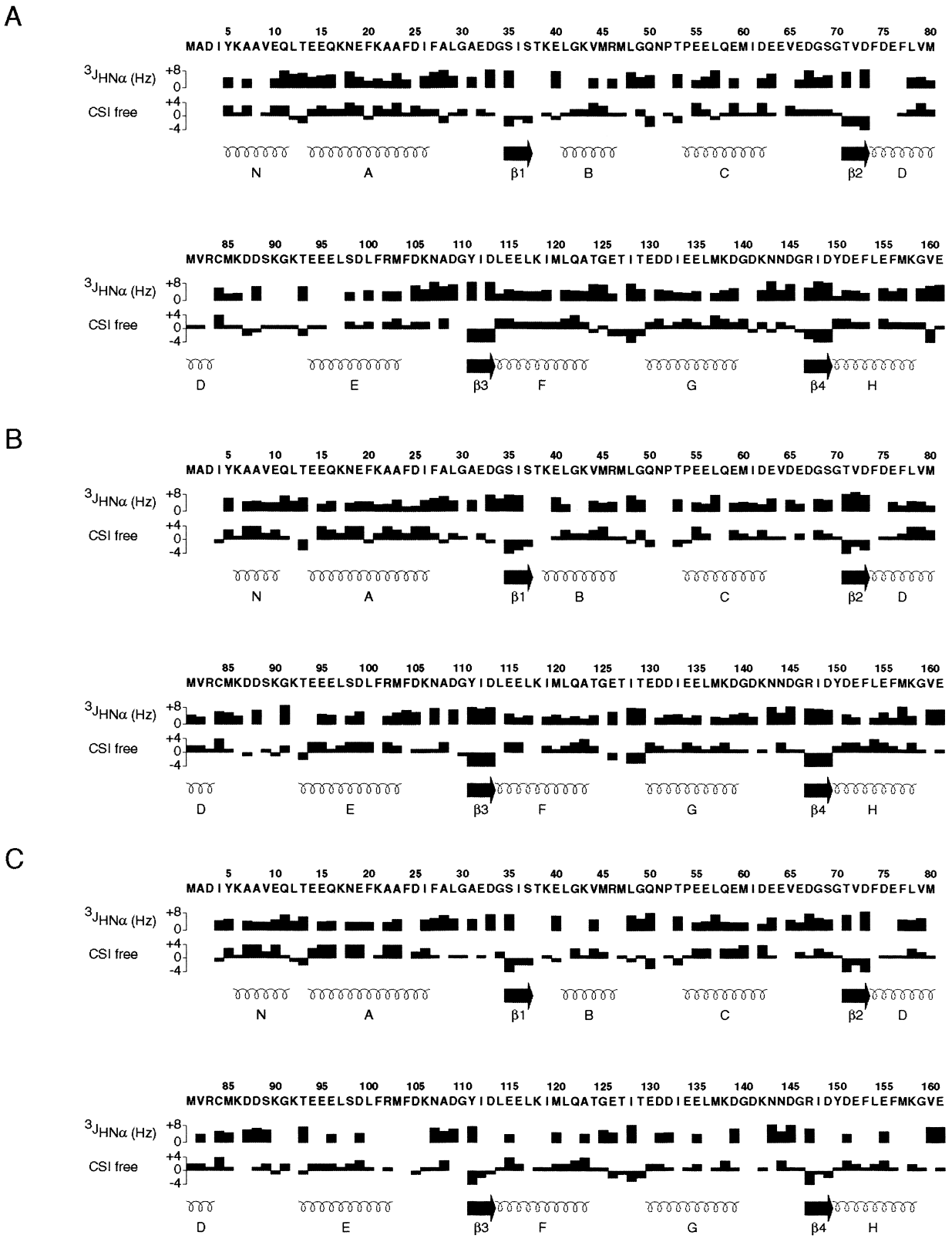

Fig. 3. Summary of the consensus CSI and $^3J_{HNH\alpha}$ coupling constants for cTnC, (A) free, (B) bound to cTnI(33–80) and (C) bound to cTnI(1–80)DD. Helices and β-strands determined form the data are shown aligned with the amino acid sequence.

Secondary structures for cTnC, free and bound to cTnI-(33–80) and cTnI(1–80)DD, were determined from the chemical shift index (CSI) and analysis of $^3J_{HNH\alpha}$ coupling constants. The CSI was compiled utilizing C_α, C', C_β and H_α chemical shift values [17,18]. These data are summarized in Fig. 3. The secondary structure of Ca^{2+}-saturated cTnC bound to cTnI(33–80) and cTnI(1–80)DD was qualitatively

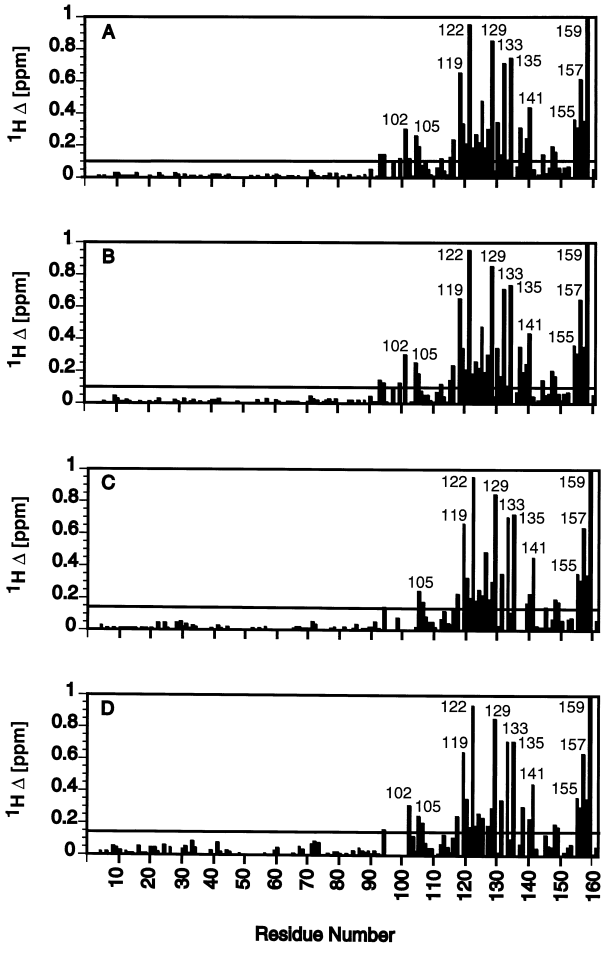

Fig. 4. Absolute values of the amide proton chemical shift differences between Ca^{2+}-saturated cTnC and the various N-terminal cTnI proteins. (A) cTnC/cTnI(33–80) minus cTnC, (B) cTnC/cTn-(1–80)DD minus cTnC, (C) cTnC/cTnI(1–80)pp minus cTnC and (D) cTnC/cTnI(1–80) minus cTnC. Horizontal lines represent average chemical shift differences plus one S.D.

the same as the free protein (Fig. 3) and consistent with that previously reported for cTnC [19].

3.2. *Effects of binding cTnI proteins to Ca^{2+}-saturated cTnC*

Absolute values of amide ^{1}H chemical shift perturbations in Ca^{2+}-saturated cTnC observed upon the binding of cTnI-(33–80), cTnI(1–80), cTnI(1–80)pp and cTnI(1–80)DD are summarized in Fig. 4. Localization of large chemical shift perturbations to the C-terminal domain of cTnC could be used to map the primary cTnI binding site to the Ca^{2+}/Mg^{2+} dependent interaction site in the C-terminal domain of cTnC. Addition of the 32 residue cardiac specific amino-terminus does not appear to alter the Ca^{2+}/Mg^{2+} dependent interaction site. No significant chemical shift changes were observed in the N-terminal domain of cTnC upon binding cTnI(33–80), cTnI(1–80)pp or cTnI(1–80)DD (Fig. 4). In contrast, cTnI(1–80) binding to cTnC produces small, but significant, chemical shift perturbations in N-terminal domain resonances. This can be most easily seen by comparing the absolute value of the amide ^{1}H chemical shift differences between cTnC/cTnI(1–80) and cTnC/cTnI(1–80)pp (Fig. 5). Residues in the N-terminus of cTnC whose amide ^{1}H chemical shifts are affected include Lys-21, Ala-22, Leu-29, Gly-34, Gly-42, Leu-57, Gly-68, Thr-71 and Asp-73. Both Leu-29 and Gly-34 are located in the inactive Ca^{2+} binding site I with Gly-34 located at the beginning of the β-sheet in site I. Gly-68, Thr-71 and Asp-73 are located in Ca^{2+} binding site II. In addition, Thr-71 and Asp-73 form a part of the short β-sheet between Ca^{2+} binding sites I and II. Met-81, Cys-84 and Lys-90 form a part of the linker region between the N- and C-terminal domains of cTnC. Comparison of amide ^{1}H chemical shift differences for cTnC bound to cTnI(1–80)pp and cTnI(1–80)DD showed that both cTnI proteins induce similar spectral perturbations. This similarity provides structural evidence in support of biochemical data that cTnI-(1–80)DD provides a suitable paradigm for examining the structural and dynamic consequences of PKA phosphorylation of the cardiac specific amino-terminus.

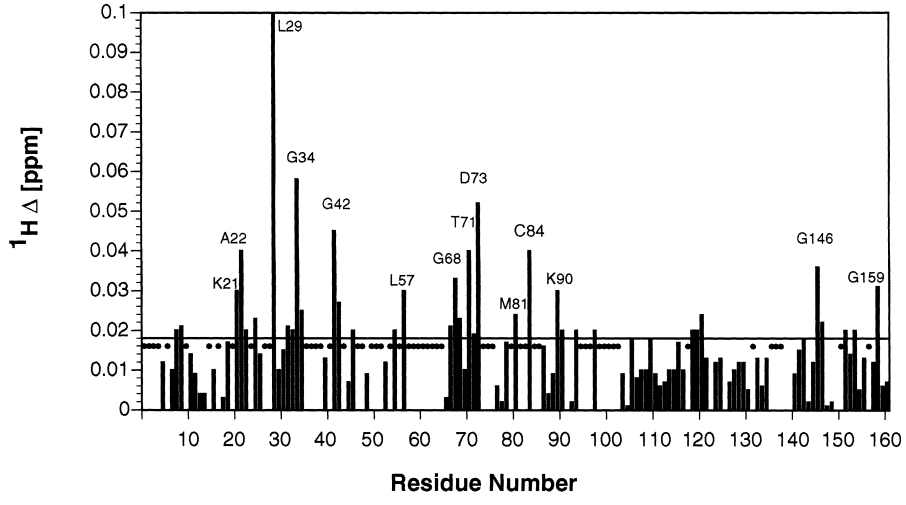

Fig. 5. Absolute value of the amide proton chemical shift differences between Ca^{2+}-saturated cTnC/cTnI(1–80) and cTnC/cTn(1–80)pp. Horizontal lines represent average chemical shift differences plus one S.D. Filled circles mark residues for which resonance assignments in one of the ^{1}H-^{15}N correlation spectra could not be confirmed due to a lack of sequential NOEs or as a consequence of chemical exchange [20].

4. Discussion

Native PAGE was used to demonstrate high affinity complex formation between cTnC and several N-terminal domain cTnI proteins (Fig. 1). We have assigned amide ^{1}H and ^{15}N resonances in cTnC, free and bound to cTnI(33–80), cTnI(1–80), cTnI(1–80)pp and cTnI(1–80)DD. No significant secondary structural changes were observed in cTnC bound to cTnI(33–80) or cTnI(1–80)DD (Fig. 3). In each of the complexes, amide resonances for residues Val-28, Ile-36, Ser-37, Thr-38, Lys-39 and Glu-40, located at or near inactive Ca^{2+} binding site I, show significant broadening due to chemical exchange [20]. One consequence of this exchange is that the secondary structure within this region is less well-defined.

The molecular mechanism responsible for transmitting the PKA phosphorylation signal remains an enigma. It is known that phosphorylation of Ser-23 and Ser-24 decreases the apparent affinity of the TnC regulatory site for Ca^{2+} [2]. The resulting reduction in sensitivity of myofilament force development to Ca^{2+} induced by phosphorylation of cTnI can be mimicked by the exchange of cTnI with cTnI-DD, in which Ser-23 and Ser-24 have been mutated to Asp [21]. Cardiac TnI-DD is also able to mimic effects of phosphorylation on steady state and pre-steady state cTnI binding to cTnC [22]. However, the structural consequences of substituting Asp for phosphoserine residues at position 23 and 24 have not previously been explored. Amide chemical shift differences provide a powerful tool for mapping protein-protein interaction sites and monitoring conformational changes induced by protein binding [23–26]. As can be seen in Fig. 3, binding of N-terminal cTnI proteins induces large chemical shift perturbations in the C-terminal domain of cTnC. These perturbations map out the Ca^{2+}/Mg^{2+} dependent interaction site between cTnC and cTnI.

Several cTnC N-terminal amide residues undergo chemical shift changes upon binding cTnI(1–80), suggesting that the non-phosphorylated cardiac specific amino-terminus contacts both cTnC domains (Fig. 5). In support of cTnI(1–80) contacting both cTnC domains, relaxation data have shown that the N- and C-terminal domains of cTnC do not tumble independently when they are bound to cTnI(1–80) [20]. In contrast, the N- and C-terminal domains of cTnC bound to cTnI(1–80)DD do tumble independently. However, interaction of both cTnC domains with cTnI(1–80) does not preclude flexibility in the linker region of cTnC. Within the N-terminus of cTnC, interaction sites for the cardiac specific amino-terminus of cTnI(1–80) are mapped to the short β-strands between inactive Ca^{2+} binding site I and active site II. This region in cTnC has previously been implicated in modulating a chemical exchange in the Ca^{2+}-saturated regulatory domain [20]. Data presented here allow us to propose a model in which the cardiac specific amino-terminus interacts with the regulatory domain of cTnC, modulating the chemical exchange between 'open and closed' conformations. In cTnC, the short β-sheet located between inactive and active Ca^{2+} binding sites I and II, respectively, is solvent accessible in both the 'closed' [19] and proposed 'open' forms [27]. Thus, the non-phosphorylated cardiac specific amino-terminus of cTnI could interact at or near sites I and II. PKA phosphorylation of the cardiac specific amino-terminus at Ser-23 and Ser-24 would disrupt the interaction with the N-terminal domain of cTnC. The phosphorylated cardiac specific amino-terminus could then fold back onto cTnI as previously suggested [2] or simply assume a more random structure. The loss of interactions with the regulatory domain of cTnC could partially account for the observed decrease in affinity of phosphorylated cTnI for cTnC [28]. Our proposed mechanism by which phosphorylation modulates the Ca^{2+} affinity at the regulatory site utilizes the unique isoform differences of both TnI and TnC. Of course, it is always possible that the observed N-terminal cTnC perturbations may be modified in the intact cTnIC or the ternary troponin complex.

In summary, we have assigned backbone resonances for cTnC, both free and bound to cTnI(33–80), cTnI(1–80), cTnI(1–80)DD and cTnI(1–80)pp. NMR studies presented here provide the first direct evidence for the location of interaction sites between intact cTnC and the N-terminal domain of cTnI, in the presence and absence of phosphorylation. These studies provide a model of the molecular mechanism by which phosphorylation of Ser-23 and Ser-24 in cTnI modulates the Ca^{2+} affinity in the regulatory domain of cTnC. In addition, chemical shift mapping has shown that cTnI-(1–80)DD provides a good structural mimetic for cTnI-(1–80)pp, which will facilitate future biophysical studies.

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