Characterization of the Interaction between the N-Terminal Extension of Human Cardiac Troponin I and Troponin C†

Douglas G. Ward, Susan M. Brewer, Melanie J. Calvert, Clare E. Gallon, Yuan Gao, and Ian P. Trayer*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

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ABSTRACT: The N-terminal extension of cardiac troponin I (TnI) is bisphosphorylated by protein kinase A in response to β-adrenergic stimulation. How this signal is transmitted between TnI and troponin C (TnC), resulting in accelerated Ca^{2+} release, remains unclear. We recently proposed that the unphosphorylated extension interacts with the N-terminal domain of TnC stabilizing Ca^{2+} binding and that phosphorylation prevents this interaction. We now use ^1H NMR to study the interactions between several N-terminal fragments of TnI, residues 1−18 (I1−18), residues 1−29 (I1−29), and residues 1−64 (I1−64), and TnC. The shorter fragments provide unambiguous information on the N-terminal regions of TnI that interact with TnC: I1−18 does not bind to TnC whereas the C-terminal region of unphosphorylated I1−29 does bind. Bisphosphorylation greatly weakens this interaction. I1−64 contains the phosphorylatable N-terminal extension and a region that anchors I1−64 to the C-terminal domain of TnC. I1−64 binding to TnC influences NMR signals arising from both domains of TnC, providing evidence that the N-terminal extension of TnI interacts with the N-terminal domain of TnC. TnC binding to I1−64 broadens NMR signals from the side chains of residues immediately C-terminal to the phosphorylation sites. Binding of TnC to bisphosphorylated I1−64 does not broaden these NMR signals to the same extent. Circular dichroism spectra of I1−64 indicate that bisphosphorylation does not produce major secondary structure changes in I1−64. We conclude that bisphosphorylation of cardiac TnI elicits its effects by weakening the interaction between the region of TnI immediately C-terminal to the phosphorylation sites and TnC either directly, due to electrostatic repulsion, or via localized conformational changes.

The contractility of the myocardium is regulated by a Ca^{2+}-operated molecular switch, troponin. Troponin is a heterotrimeric protein complex, located on the thin filaments, which binds to both tropomyosin and actin. Like skeletal troponin, cardiac troponin consists of a tropomyosin binding subunit, TnT,[1] a Ca^{2+} binding subunit, TnC, and an inhibitory subunit, TnI. Ca^{2+} binding to TnC favors the binding of TnI to TnC over binding to actin, enabling strong actin−myosin binding, movement of tropomyosin, and cross-bridge cycling.

TnC is composed of four helix−loop−helix (EF-hand) cation binding sites organized pairwise into two domains linked by a short α-helix (*1*). Sites I and II in the N-terminal domain of skeletal TnC bind Ca^{2+} with a moderate affinity such that their occupancy varies under physiological conditions (*2*). Sites III and IV in the C-terminal domain of TnC bind Ca^{2+} extremely tightly and Mg^{2+} with a lower affinity. These sites would be permanently occupied by divalent cations in vivo and do not play a direct role in switching. TnI is an extended molecule, lying antiparallel to TnC (*3*) and making numerous contacts with TnC, TnT, and actin. TnT can be proteolytically cleaved into N-terminal (T1) and C-terminal (T2) fragments. T1 binds to tropomyosin, firmly anchoring troponin to the thin filament, whereas T2 interacts with TnC, TnI, and tropomyosin.

Our understanding of protein−protein interactions within the troponin complex improved with the recent crystal structure of the "core domain" of human cardiac troponin (*4*). TnI and T2 consist largely of several long α-helices and interact with each other predominantly via a parallel coiled coil. The interactions between cardiac TnI and TnC in the core domain structure are as predicted from binary complex work (*5*, *6*); however, several crucial regions of TnI (the cardiac-specific N-terminal extension and the inhibitory region) and the central linker of TnC are truncated or not resolved. Two well-defined interactions between TnI and TnC involve residues 42−64 of TnI binding to the C-terminal domain of TnC and residues 149−158 of TnI binding to the N-terminal domain of TnC. Additionally, the inhibitory region of TnI (residues 135−147) binds to TnC in the presence of Ca^{2+} (*7*), and TnC binding has been identified in the cardiac-specific N-terminal extension and C-terminal regions of TnI (*8*).

A fine degree of control of actomyosin interactions is required in the myocardium, and the cardiac isoforms of the troponin subunits have evolved to fulfill this role: although

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* Corresponding author. Tel: 44 (0)121 414 5401. Fax: 44 (0)121 414 5925. E-mail: i.p.trayer@bham.ac.uk.

[1] Abbreviations: TnC, troponin C; TnI, troponin I; TnT, troponin T; −18TnI, recombinant human troponin I with residues 1−18 deleted; T1, residues 1−167 of TnT; T2, residues 168−288 of TnT; I1−18, residues 1−18 of human cardiac TnI; I1−29, residues 1−29 of human cardiac TnI; I1−64, residues 1−64 of human cardiac TnI; N-domain of TnC, residues 1−91 of TnC; C-domain of TnC, residues 89−161 of TnC; PKA, cAMP-dependent protein kinase (EC 2.7.1.37); IAANS, 2-[4′-(iodoacetamido)anilino]naphthalene-6-sulfonic acid; TFE, trifluoroethanol.

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they maintain high overall sequence identity with their skeletal counterparts, their sequences do differ substantially in places. The predominant isoform of human cardiac TnT is 287 residues long compared with 267 for the skeletal isoform, the major difference being an 18-residue extension at the N-terminus of cardiac TnT. Isoform switching experiments indicate that the isoform of TnT present influences the cooperativity of thin filament regulation (*9*, *10*). The Ca^{2+} binding properties of cardiac and skeletal TnC are very different. Cardiac TnC is unable to bind Ca^{2+} at site I, and hence regulation of cardiac muscle contraction is by Ca^{2+} binding to site II only (the "regulatory site"). This alters the energetics of the Ca^{2+}-operated switching of TnI from actin to TnC. Binding of Ca^{2+} to site II of cardiac TnC does not produce the structural "opening" of the N-domain that is seen when Ca^{2+} binds to sites I and II of skeletal TnC (*11*). It is this opening that exposes a hydrophobic patch on the N-domain that acts as an additional TnI binding site in the presence of Ca^{2+}. Cardiac TnC requires the binding of both Ca^{2+} and TnI (or residues 147−163 thereof) to induce the opening (*5*). All mammalian cardiac TnIs have an N-terminal extension of approximately 30 residues compared with skeletal TnI (31 residues for human cardiac TnI). Conserved within this extension is an RRRSS sequence that acts as a protein kinase A (PKA) bisphosphorylation site (*12*). β-Adrenergic stimulation of the heart results in bisphosphorylation of S22 and S23 of TnI (*12*, *13*), and this reduces the affinity of the regulatory site on TnC for Ca^{2+}. This can be measured as a desensitization to Ca^{2+} in force or actomyosin ATPase versus pCa plots (*14*−*16*), as an increase in the rate of Ca^{2+} release from troponin (*16*, *17*), and as an increase in the rate of relaxation of cardiac myofibrils (*18*). It seems likely that the physiological role of TnI phosphorylation is to aid myocardial relaxation, allowing proper diastolic filling during periods of high heart rate.

Although we know where TnI is bisphosphorylated and some of the consequences of this phosphorylation, we do not understand the molecular events that link the two. For example, as the N-terminal extension is absent in even the most complete troponin structure available (*4*), we have no idea of how close the phosphorylation sites are to the Ca^{2+} binding site that they affect. Phosphorylation effects have been observed in the absence of TnT and in binary complexes as small as TnI + the N-terminal domain of TnC (*19*) and TnI residues 1−64 (I1−64) + TnC (*20*). It seems, therefore, that TnT is not required to transfer the phosphorylation signal and that the N-terminal extension of TnI and the N-terminal domain of TnC are the key players.

Previous studies have found that unphosphorylated synthetic peptides corresponding to residues 17−29 and 19−32 of TnI bind to TnC (*8*, *21*). Bisphosphorylation prevents these peptides from binding, hinting at how phosphorylation of the N-terminal extension may influence TnC. In this study we use several longer fragments of TnI, including I1−64 comprising the first 64 residues of TnI. I1−64 contains the N-terminal extension of TnI and a region (residues 42−64) that binds extremely tightly to the C-terminal domain of TnC (*4*, *6*, *20*, *22*). I1−64 forms a 1:1 complex with TnC in which the N-terminal extension should be anchored in the same orientation relative to TnC as one would expect in the troponin complex. We have previously demonstrated that I1−64 bound to TnC is able to influence the Ca^{2+} binding properties of the regulatory site (*20*). Hence, I1−64 retains a functional N-terminal extension and phosphorylation mechanism in a small, soluble complex that is amenable to physical studies.

Here, we use 1D ^{1}H NMR spectroscopy to study how the first 64 residues of TnI interact with TnC, how phosphorylation affects the binding, and how this influences the N- and C-terminal domains of TnC. The interplay between the N-terminal extension and Ca^{2+} binding to TnC is explored using an E76A mutant of TnC that is unable to bind Ca^{2+} at the regulatory site. We also use circular dichroism (CD) spectroscopy to determine whether bisphosphorylation produces gross structural changes in I1−64. Our major finding is that the region of TnI immediately C-terminal to S22 and S23 unphosphorylated TnI binds to TnC and that this binding influences the structure of TnC. Phosphorylation weakens this binding and hence reduces the influence of the N-terminal extension over the regulatory Ca^{2+} site.

EXPERIMENAL PROCEDURES

Protein Preparation. Recombinant human cardiac TnI, TnC, and TnC N- and C-terminal domains were expressed in *Escherichia coli* and purified as described previously (*23*, *24*). Mutations were introduced using the megaprimer PCR method. N-Terminally acetylated I1−18 and I1−29 were synthesized by Alta Bioscience, University of Birmingham. I1−64 was produced by CNBr digestion of a mutant TnI with a methionine residue introduced at position 64 and with the naturally occurring methionines (positions 153, 154, and 200) converted to leucine (*20*). The I1−64 was purified from the CNBr digest of the mutant TnI by reverse-phase HPLC using a Phenomenex Jupiter C5 semiprep column (0.1% trifluoroacetic acid with a 25−45% acetonitrile gradient). Purity and identity were checked by SDS−PAGE and MALDI-TOF mass spectrometry (Bruker Biflex IV with sinapinic acid as the matrix). Protein solutions for NMR spectroscopy were prepared by dissolving the protein in the appropriate buffer, adjusting the pH (when necessary), and spinning at 45000 rpm for 20 min (Beckman TL-100) to remove any insoluble material. The protein concentrations of the supernatants were determined using the bicinchoninic acid protein assay (Pierce) calibrated with bovine serum albumin.

Phosphorylation of I1−64. I1−64 was dissolved at 5 mg/mL in 300 mM KCl, 20 mM MOPS/KOH (pH 7.0), 3 mM $MgCl_2$, 2.5 mM $ATPNa_2$, and 1 mM DTT. The PKA catalytic subunit (Sigma) was added to a final concentration of 250 units/mL and the phosphorylation allowed to proceed overnight at room temperature. The I1−64 was purified by HPLC (as above) and the extent of phosphorylation determined by MALDI-TOF mass spectrometry (>95% bisphosphorylation) as described previously (*20*).

NMR Spectroscopy. All spectra were collected on a Bruker DRX500 spectrometer equipped with a cryoprobe. One-dimensional spectra were collected using a 5 kHz (10 ppm) sweep width with an interpulse delay of 3 s to allow full relaxation. Typically, 128 scans were acquired at 20 °C using 300 μM protein dissolved in 20 mM KCl, 20 mM Tris-d_{11}, and 2.5 mM $CaCl_2$ in D_2O at pH 7.4. Each experiment was repeated independently at least three times and using different

protein preparations whenever possible. I1−29 and I1−64 resonances were assigned using NOESY and TOCSY spectra (3 mM I1−64 in H_2O at pH 5.3). TnC peaks in the 1D spectra have been identified using the spectra of isolated N- and C-terminal domains of TnC, Ca^{2+} titrations, and previously published assignments (*25*, *26*).

CD Spectroscopy. Spectra were collected using a Jasco J-810 spectropolarimeter with 0.1 mg of protein/mL in 5 mM K_2HPO_4/H_3PO_4 (pH 7.0) at 20 °C. The isolated domains of TnC and I1−64 were extensively dialyzed and centrifuged at 45000 rpm for 20 min prior to protein concentration determination. All solutions had an absorbance of less than 0.7 across the CD measurement range (1 mm path length).

Ca^{2+} Binding to TnC. Ca^{2+} binding to wt and E76A TnC was measured using the environmentally sensitive extrinsic fluorophore IAANS. The TnCs were labeled with IAANS by incubation overnight at 4 °C with a 5-fold molar ratio of IAANS to protein in 6 M urea, 25 mM triethanolamine/HCl (pH 7.0), and 1 mM EDTA. Unincorporated label was removed by dialysis against 20 mM MOPS/KOH (pH 7.0) and centrifuged at 45000 rpm for 20 min prior to protein concentration determination. The amount of label covalently attached to the TnCs was determined from the absorbance at 325 nm, using a molar extinction coefficient of 24900 M^{-1} cm^{-1} for IAANS (*27*). The labeling ratio was 1.8 mol of IAANS/mol of wt TnC and 1.5 mol of IAANS/mol of E76A TnC. Fluorescence studies were performed on a Perkin-Elmer LS 50B luminescence spectrometer at 30 °C ($\lambda_{excitation}$ = 325 nm, $\lambda_{emission}$ = 443 nm). The labeled TnC was diluted to 0.5 μM in 20 mM MOPS (pH 7.0), 0.3 M KCl, 3 mM $MgCl_2$, 0.5 mM EGTA, and sufficient $CaCl_2$ to give pCa values between 9 and 3.5.

RESULTS

Interaction of I1−29 with TnC. Parts a and c of Figure 1 show the NMR spectra of 300 μM I1−29 in the un- and bisphosphorylated states. Bisphosphorylation produces limited changes in the 1D spectrum of I1−29: most of the spectrum is identical, but we observe chemical shift differences of signals from I18, N24, Y25, and Y28 (other shifts may be occurring in regions of the spectrum where there is signal overlap and individual peaks cannot be resolved). Examination of the spectrum of a 2:1 molar ratio mix of I1−29 to TnC (Figure 1b) indicates that binding is occurring between some regions of I1−29 and TnC. Signals from the side chain protons of I18, the α- and β-protons of N24, and the side chain protons of Y25 and Y28 of I1−29 are substantially broadened by interaction with TnC. In contrast, the corresponding signals from bisphosphorylated I1−29 are not influenced by TnC (Figure 1d): bisphosphorylation prevents, or greatly reduces the affinity of, the interaction between the C-terminal region of I1−29 and TnC. Signals from the methyl protons of the N-terminal acetyl group and the β-protons of D2 and D6 of un- or bisphosphorylated I1−29 are not affected by TnC. This indicates that, unlike the C-terminal region of this peptide, the N-terminal region does not bind to TnC.

Binding Studies with I1−18. This peptide, corresponding to the region of the N-terminal extension N-terminal to the RRRSS bisphosphorylation motif, was synthesized with either α-N-terminal acetylation (as in native TnI) or α-N-terminal dansylation (to introduce a fluorophore). One-dimensional ^1H NMR was used to determine whether the N-terminally acetylated peptide binds to TnC. The peptide was diluted to 150 μM in 20 mM Tris-d_{11} in D_2O at pH 7.4 and titrated with up to 50 μM TnC. I1−18 did not appear to bind to TnC (the I1−28 spectrum remained sharp; data not shown). We decided to test several other sarcomeric proteins as potential binding partners for the extreme N-terminal region of TnI by repeating the I1−18 experiment with −18TnI, T1, T2, actin, myosin S1, and myosin. With intact myosin it was necessary to add 0.4 M KCl, and the maximum myosin concentration was 10 μM. None of these proteins altered the NMR spectrum of I1−18. In a second series of experiments the dansylated peptide was used to detect binding by fluorescence. The emission at 530 nm (excitation at 335 nm) of 2 μM peptide in 50 mM KCl, 25 triethanolamine/HCl (pH 7.4), 1 mM DTT, 3 mM $MgCl_2$, and 0.5 mM EGTA ± 1 mM $CaCl_2$ was measured before and after addition of 2 μM TnC, −18TnI, T1, T2, actin, myosin S1, or myosin. We did not observe any changes in fluorescence emission intensity or polarization anisotropy (data not shown). The dansylated I1−18 was also used in spinning down assays where 1 μM peptide was mixed with 2 μM reconstituted thin filaments and centrifuged at 100000 rpm for 30 min. SDS−PAGE verified that the thin filament proteins were pelleted by this procedure. Fluorescence measurements demonstrated that all of the I1−18 fluorescence remained in the supernatant. None of our experiments with I1−18 provided any evidence that the extreme N-terminal region of cardiac TnI interacts with any of the sarcomeric proteins tested.

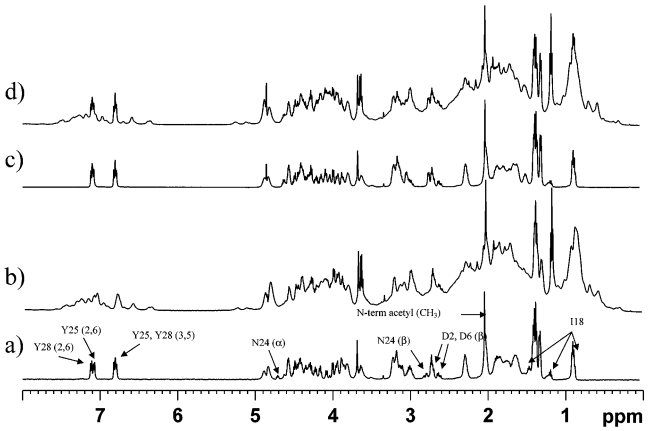

FIGURE 1: Binding of un- and bisphosphorylated I1−29 to TnC. 1D ^1H NMR spectra of (a) 300 μM I1−29, (b) 300 μM I1−29 + 150 μM TnC, (c) 300 μM bisphosphorylated I1−29, and (d) 300 μM bisphosphorylated I1−29 + 150 μM TnC. Spectra were collected as described in Experimental Procedures. Signals in the 1D spectra have been assigned from the NOESY spectrum of I1−29.

One-Dimensional ^1H NMR Spectra of I1−64, TnC, and the I1−64·TnC Complex. Figure 2 shows the spectra of 400 μM solutions of the two proteins under investigation, both separately and when mixed in a 1:1 ratio. In addition, we have plotted the sum of the spectrum of I1−64 and the spectrum of TnC for comparison with the spectrum of the 1:1 mix. The spectrum of I1−64 consists of sharp peaks as expected for a flexible peptide. Signals that are particularly useful to this study are those of the side chain protons from Y25, Y28, and H33. These signals are easily distinguished

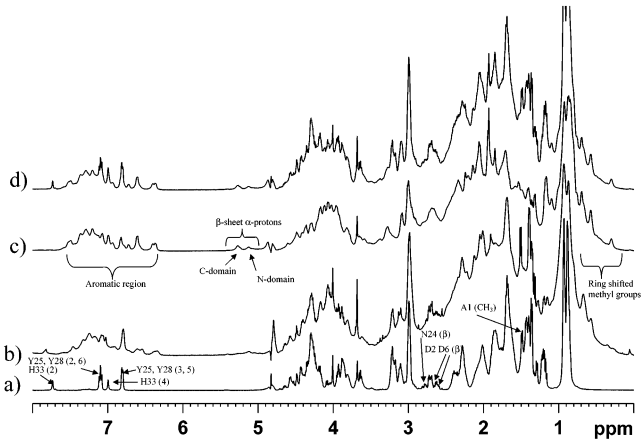

FIGURE 2: Binding of I1−64 to TnC. 1D ^{1}H NMR spectra of (a) 400 μM I1−64, (b) 400 μM I1−64 + 400 μM TnC, (c) 400 μM TnC, and (d) the sum of the spectrum of 400 μM I1−64 and the spectrum of 400 μM TnC. Several informative regions of the TnC spectrum and peaks in the I1−64 spectrum have been labeled.

in an uncluttered region of the spectrum and are close enough to serines 22 and 23 to report on binding events immediately C-terminal to the bisphosphorylation site.

The spectrum of TnC is more complex than that of I1−64 and largely consists of broader peaks as expected for a structured protein. Few of the signals can be assigned to a unique residue in TnC; however, careful analysis of the spectra of the isolated domains of TnC and Ca^{2+} titration of intact TnC allow assignment of some of the signals in the 1D spectrum to one domain or the other. This is particularly true for the aromatic signals (6.3−7.6 ppm), ring current shifted methyl group signals (0.2−0.8 ppm), and the signals from the α-protons of the β-sheets formed by the back-to-back interaction of the EF-hands in each domain (4.9−5.5 ppm).

Due to the very high affinity of I1−64 for TnC ($K_a = 10^8$ M^{-1}) (*20*) the 1:1 mixture would consist almost exclusively of binary complex with little free I1−64 or TnC. Hence, the spectrum obtained for a mixture of 400 μM I1−64 and 400 μM TnC (Figure 2) can be assumed to be that of the binary complex. Comparison of the spectrum of the complex with the sum of the spectra of I1−64 and TnC reveals that binding of I1−64 to TnC results in broadening of the N24, Y25, Y28, and H33 signals arising from I1−64, indicating that this region binds to TnC. Signals arising from A1, D2, and D6 of I1−64 are not broadened by TnC in the complex, indicating that the extreme N-terminal region of I1−64 remains highly mobile, i.e., does not bind directly to TnC. Binding of I1−64 to TnC also produces substantial changes in the NMR spectrum of TnC. I1−64 binding produces shifts and broadening in signals arising from aromatic residues in TnC, ring-shifted methyl groups, and the α-protons of the β-sheets formed between the Ca^{2+} binding loops. Crucially, these changes occur not only in the C-terminal domain of TnC but also in the N-terminal domain. This demonstrates directly that I1−64 binding to TnC affects the structure of the N-terminal domain. The effect of I1−64 on the N-terminal domain could result from a communication between the N- and C-terminal domains, although the linking E/F helix is partially unwound and flexible in solution (*11*), and it seems more likely that I1−64 directly contacts the N-terminal domain, probably via the region around residues N24 to H33.

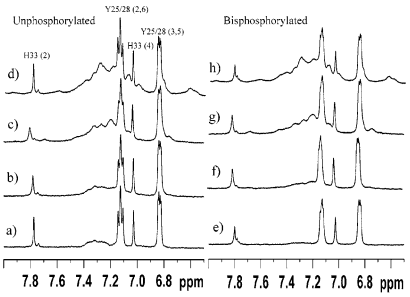

FIGURE 3: Titration of I1−64 with increasing concentrations of the N-terminal domain of TnC. The spectra in the left-hand panel show the aromatic region of the spectrum of 200 μM unphosphorylated I1−64 plus 0 μM (a), 40 μM (b), and 120 μM (c) N-terminal domain of TnC. The right-hand panel shows the spectra of 200 μM bisphosphorylated I1−64 plus 0 μM (e), 40 μM (f), and 120 μM (g) N-terminal domain of TnC. Traces d and h show the sums of the spectrum of 120 μM N-terminal domain of TnC and the spectrum of 200 μM un- or bisphosphorylated I1−64, respectively.

Titration of I1−64 with the Isolated N-Terminal Domain of TnC. Figure 3 shows the Y25, Y28, and H33 peaks of I1−64 (200 μM) following addition of 0, 40, and 120 μM N-terminal domain of TnC and, for comparison, the sum of the spectrum of 200 μM I1−64 and the spectrum of 120 μM N-terminal domain of TnC. At the lower concentrations used in this experiment the N-terminal domain makes only a small direct contribution to the spectrum, and the effects of binding on the spectrum of I1−64 can be clearly observed. The N-terminal domain of TnC binds to I1−64 but has a relatively low affinity (*20*) and dissociates rapidly. Hence each N-domain molecule can bind to, and broaden the peaks of, many I1−64 molecules on the time scale of the NMR experiment (the high affinity of the C-terminal domain of TnC for I1−64 precludes the observation of fast exchange with the isolated C-terminal domain of TnC). Low concentrations of the N-terminal domain of TnC broaden the peaks from Y25, Y28, and H33 of unphosphorylated I1−64. Bisphosphorylation of I1−64 broadens the signals from Y25 and Y28 of the free peptide. However, the extent of broadening of the Y25, Y28, and H33 signals induced by the N-terminal domain of TnC is much reduced in the case of bisphosphorylated I1−64 compared with unphosphorylated I1−64. These results suggest that, as with I1−29, the region of TnI immediately C-terminal to the bisphosphorylation site can bind to the N-terminal domain of TnC and that bisphosphorylation weakens this interaction.

Effects of Bisphosphorylation on the I1−64·TnC Complex. Figure 4a shows the aromatic region of the spectra of the unphosphorylated and bisphosphorylated I1−64·TnC complex. The peaks arising from the side chain protons of Y25 and Y28 of I1−64 are substantially sharper in the bisphosphorylated complex compared with the unphosphorylated complex. The phosphorylation-induced sharpening of the peaks results from increased environmental exchange rates demonstrating a weakening of the binding of residues Y25 and Y28 of I1−64 to TnC (an increase in the dissociation rate). The bisphosphorylation of I1−64 is "sensed" by TnC, producing small spectral changes in the aromatic (Figure 4a), β-sheet (Figure 4b), and ring-shifted methyl (data not shown) signals from TnC. These changes occur in both domains of TnC. The phosphorylation-induced changes observed in the TnC spectrum are small compared with those produced by

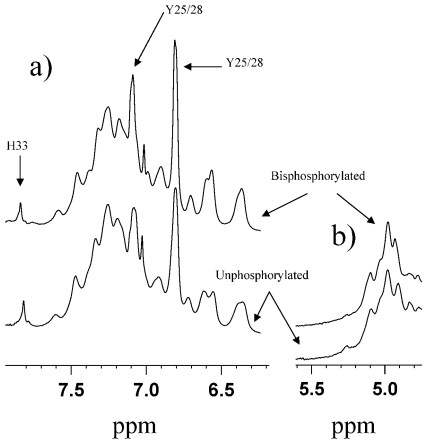

FIGURE 4: Effect of bisphosphorylation of the I1−64·TnC·3Ca^{2+} complex. The lower traces in both panels are the spectrum of the unphosphorylated complex (400 μM I1−64 + 400 μM TnC). The upper traces are the spectrum of the bisphosphorylated complex. Panel a shows the aromatic regions of the spectra, and panel b shows the α-proton signals from the β-sheets of TnC. The vertical gain is doubled in panel b over that in panel a.

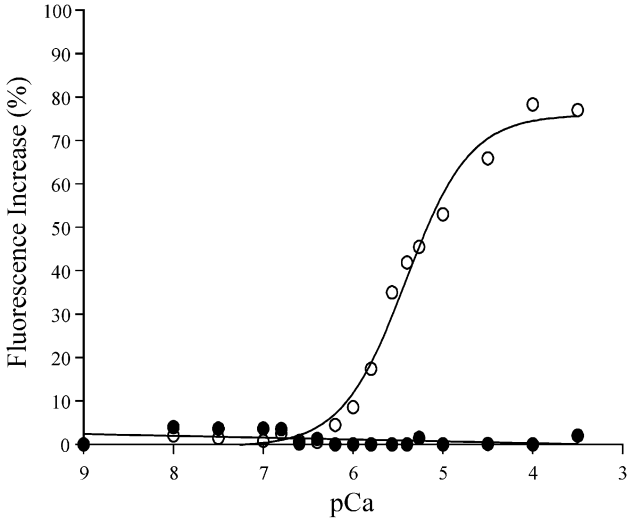

FIGURE 5: Ca^{2+} titration of wt and E76A TnC. The percentage increase in fluorescence emission at 443 nm of wt (○) and E76A (●) TnC−IAANS is shown as a function of pCa (the labeling ratio was 1.8 mol of IAANS/mol of wt TnC and 1.5 mol of IAANS/mol of E76A TnC). Each data point represents the mean ± SEM of triplicate determinations (error bars are within the symbols). The experiment was conducted as described in Experimental Procedures.

the original I1−64 binding, and it is not possible to say whether the TnC in the un- or bisphosphorylated complex more resembles free TnC.

Effect of Ca^{2+} Binding on the I1−64·TnC Complex. We have produced a mutant TnC, E76A TnC, with the glutamic acid in the −Z position of site II converted to alanine. E76A TnC did not bind Ca^{2+} at site II in fluorescence titration assays (Figure 5) and was unable to restore Ca^{2+} sensitivity to reconstituted thin filament myosin ATPase assays (data not shown). The 1D ^{1}H NMR spectrum of E76A TnC in the presence of varying Ca^{2+} concentrations reaffirms that site II of E76A TnC does not bind Ca^{2+} (data not shown). E76A TnC does, however, retain a similar affinity to wt TnC for intact TnI with binding constants of (4.0 ± 1.0) × 10^7 M^{-1} (Mg^{2+}/EGTA) and (19 ± 3) × 10^7 M^{-1} (Ca^{2+}) determined by microcalorimetry as described in ref *24*. The effect of Ca^{2+} on E76A TnC binding to TnI is due to Ca^{2+}/Mg^{2+}

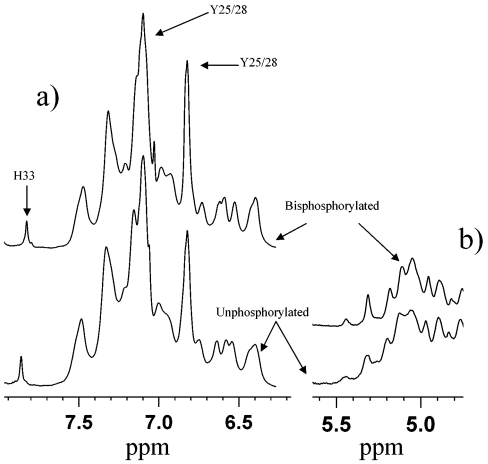

FIGURE 6: Effect of bisphosphorylation of the I1−64·E76A TnC·2Ca^{2+} complex. The lower traces in each panel are the spectrum of the unphosphorylated complex, and the upper traces that of the bisphosphorylated complex. Panel a shows the aromatic region of the spectra and panel b the α-proton signals from the β-sheets of E76A TnC. The vertical gain is doubled in panel b over that in panel a.

exchange at the C-terminal domain of E76A TnC (*24*). The advantage of using E76A TnC in the presence of Ca^{2+} over using wt TnC in the presence of Mg^{2+} and EGTA is that sites III and IV in the C-domain are saturated with Ca^{2+}. Therefore, any changes in the interaction between I1−64 and wt TnC or E76A TnC must be due to the Ca^{2+} occupancy of the N-terminal domain of TnC. The results that we obtained with E76A TnC (Figure 6) in complex with unphosphorylated and bisphosphorylated I1−64 are similar to those obtained with wt TnC (Figure 4). Although the E76A TnC component of the spectrum is different because the regulatory Ca^{2+} site is empty, we can still observe that I1−64 binding to E76A TnC broadens the signals from Y25, Y28, and H33 of I1−64, indicating that this region of the N-terminal extension can bind to E76A TnC. However, the extent of broadening of the Y25, Y28, and H33 signals is not as great with E76A TnC as with wt TnC. This suggests that the N-terminal extension binds with a lower affinity to the Ca^{2+}-free than to the Ca^{2+}-saturated N-terminal domain of TnC. The same residues of unphosphorylated I1−64 are involved in binding to E76A and wt TnC. Bisphosphorylation of the I1−64·E76A TnC complex sharpened the signals from Y25 and Y28 of I1−64, indicating that, as in the wt complex, phosphorylation weakens the interaction between the N-terminal extension and TnC. Bisphosphorylation of I1−64 also influenced the structures of both domains of E76A (Figure 6).

CD Spectroscopy of Un- and Bisphosphorylated I1−64. Figure 7a shows the CD spectra of un- and bisphosphorylated I1−64 in 0% and 15% (v/v) trifluoroethanol (TFE). The un- and bisphosphorylated spectra are very similar. In the absence of TFE I1−64 produces a spectrum characteristic of an unstructured peptide: there are no peaks at 208 and 222 nm to indicate the presence of an α-helix. The spectral changes upon addition of TFE are indicative of helix formation, presumably in the C-terminal region of I1−64 (I1−29 gave a random coil type spectrum regardless of the addition of TFE; data not shown). There was no significant difference in the extent of helicity of the un- and bisphosphorylated I1−64 at 0% or 15% TFE (or at intermediate concentrations).

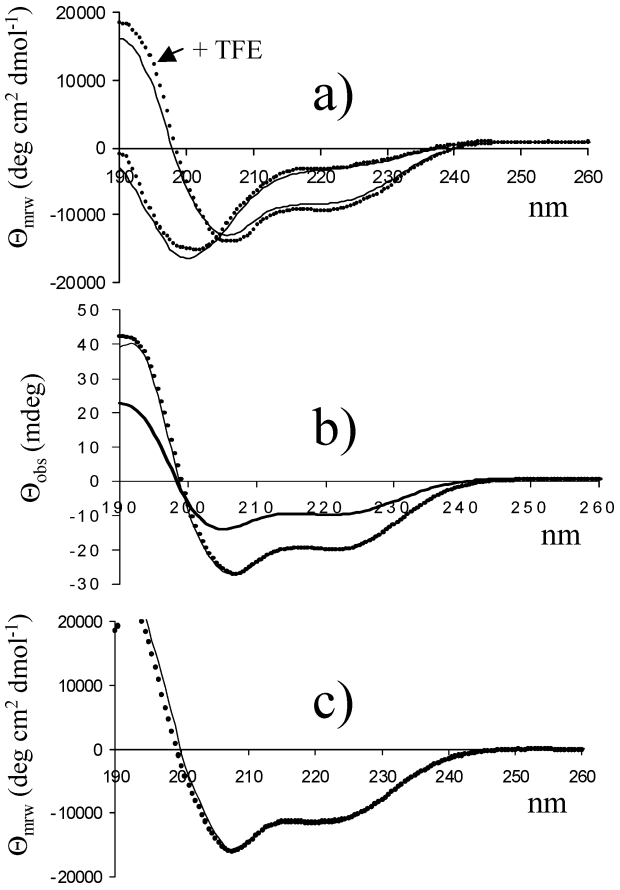

FIGURE 7: Circular dichroism spectra of I1−64. Panel a shows the CD spectra of un- (solid line) and bisphosphorylated (dotted line) I1−64, with or without 15% TFE as indicated. Panel b shows the spectra of 14 μM C-terminal domain of TnC (heavy solid line), 14 μM C-terminal domain of TnC + 14 μM I1−64 (solid line), and 14 μM C-terminal domain of TnC + 14 μM bisphosphorylated I1−64 (dotted line). Panel c shows the spectra of un- (solid line) and bisphosphorylated (dotted line) I1−64 while bound to the C-terminal domain of TnC, obtained by subtracting the C-terminal domain spectrum in panel b from those of the complexes in panel b. In panels a and c the data are plotted as molar ellipticity against wavelength, whereas the raw data are plotted in millidegrees in panel b to provide a better visual display of the I1−64 contribution in the complexes. All spectra represent the mean of at least 30 scans collected from two to three experiments.

We conclude that bisphosphorylation does not alter the propensity of free I1−64 to form secondary structures. We also investigated the secondary structure of I1−64 when bound to the C-terminal domain of TnC (Figure 7b). By subtracting the spectrum of the free C-terminal domain of TnC from the spectrum of an equimolar mixture of I1−64 and the C-terminal domain of TnC, we were able to obtain an estimate of the CD spectrum of bound I1−64 (Figure 7c). This approach requires that the CD spectrum of the C-terminal domain of TnC does not change upon binding of I1−64. Although this is difficult to test experimentally, it has been reported that binding of TnI or I1-64-like fragments of TnI does not alter the secondary structure of the C-terminal domain of TnC (*22*). Additionally, the CD spectra of the C-terminal domain of TnC were identical in 0% and 15% trifluoroethanol, indicating that the helical content of this globular folded domain is stable under the experimental conditions. The CD spectra of un- and bisphosphorylated I1−64 bound to the C-terminal domain of TnC indicate a similar level of helicity to that induced by 15% trifluoroethanol, suggesting that the helical conformation of the I1−64 binding site for the C-terminal domain of TnC is stabilized by the interaction between the two proteins. The CD data could be explained by the C-terminal region of I1−64 binding to the C-terminal domain of TnC as an α-helix as is seen in a comparable skeletal isoform binary complex (*6*) and as in the core domain structure of cardiac troponin (*4*). As with the spectra of the free I1−64, the phosphorylation state of I1−64 when bound to the C-terminal domain of TnC does not appear to substantially affect secondary structure.

DISCUSSION

In this paper we have presented NMR data that provide evidence for the region immediately C-terminal to S22 and S23 of TnI binding to TnC in a phosphorylation-sensitive manner. This is clearly demonstrated by TnC broadening the signals from I1−64 residues Y25 and Y28 and the reversal of this broadening upon bisphosphorylation in the 1D NMR experiments presented here. Experiments with I1−29 indicate that this TnC binding site may reach as far as I18, and experiments with I1−64 indicate that it extends as far as H33 of TnI. We propose that this region of the N-terminal extension acts as a "phosphorylation switch" by binding to the N-terminal domain of TnC and stabilizing Ca^{2+} binding to the regulatory site. Bisphosphorylation disrupts binding and hence alleviates the stabilizing influence causing an apparent acceleration of Ca^{2+} release. The disruption of binding may be due to the stabilization of a looped conformation immediately C-terminal to S22 and S23 as a result of their phosphorylation (*21*) or result directly from the presence of the two large, negatively charged, phosphate groups. Similar disruption of protein−protein interactions has previously been shown to underlie the effects of phosphorylation in other systems (*28*, *29*). The experimental system that we have used, TnC in complex with residues 1−64 of TnI, more closely resembles the in vivo situation than previous studies with short synthetic peptides (*8*, *21*). It appears that the phosphorylation mechanism is functional in the I1−64·TnC system in that phosphorylation accelerates Ca^{2+} release (*20*) although additional contributions from the C-terminal regions of TnI or other thin filament proteins in vivo cannot be excluded.

It has previously been reported that the N-terminal extension of TnI makes contacts with the N-terminal domain of TnC that are absent if the extension is phosphorylated or deleted (*16*, *20*, *30*−*32*). Rosevear and co-workers have used NMR to demonstrate small but significant structural changes in the N-domain of TnC induced by the unphosphorylated extension (*30*−*32*). We now provide evidence that this is also the case with the human cardiac isoforms of TnI and TnC: the unphosphorylated extension does induce changes in ^{1}H NMR signals arising from the N-terminal domain of TnC. Previous NMR studies (*30*−*33*) have focused on the effects of the N-terminal extension of TnI on TnC. In our experiments we have been able to study the effects of TnC on the N-terminal extension of TnI. We are able to identify some TnI residues that play a role in the phosphorylation switch and those that do not. N-Terminal to S22 and S23 is a $(Xaa\text{-}Pro)_4$ sequence. It seemed possible that this region could act as a rigid spacer arm (*34*) connecting the bisphosphorylation site to a protein−protein interaction site at the

extreme N-terminus of TnI, as seen with the myosin A1 light chain (*35*, *36*). Recent experiments with N-terminal deletion mutants of TnI showed that the N-terminal 16 residues of TnI are not required to transfer the phosphorylation signal (*16*), arguing against such a mechanism. Now, we provide further evidence that the N-terminal 18 residues of un- or bisphosphorylated I1−64 do not bind to TnC. What is more, we were unable to detect any binding between a synthetic peptide corresponding to residues 1−18 of TnI and TnC, TnT, −18TnI, tropomyosin, actin, or myosin. This further argues against the spacer arm/binding site at the N-terminus of the TnI mechanism of phosphorylation signal transfer.

In the crystal structure of the core domain of troponin (*4*), helix "H1" of TnI, which binds to the C-terminal domain of TnC, consists of residues 43−79. Because the TnI used in this structure was truncated at residue 31 (in addition, residues 31−34 were disordered), it is unclear as to whether the helix might continue further toward S22 and S23 in intact troponin. Another possible mechanism of phosphorylation signal transfer was that bisphosphorylation of S22 and S23 might extend or unwind the N-terminal region of helix H1. This possibility seems very unlikely in view of the CD data presented here indicating very similar secondary structures for un- and bisphosphorylated I1−64 in complex with the C-terminal domain of TnC.

Deletion of residues 16−29 of TnI mimics the effects of phosphorylation (*16*), indicating that residues between 16 and 29 are required to maintain the "unphosphorylated" or high Ca^{2+}-affinity state of the troponin complex. The binding of this region of the N-terminal extension of TnI to the N-terminal domain of TnC is inferred in this study by the changes induced in the spectrum of I1−64 by the isolated N-terminal domain of I1−64, by the effects of the I1−64 on both domains of TnC, and by the effect of the E76A mutation in TnC on the I1−64·TnC complex. This interaction has also been demonstrated in a previous cross-linking study (*20*): benzophenone-4-maleimide attached to cysteines introduced at positions 5, 10, 18, or 26 of TnI forms cross-links to the N-terminal domain of TnC in the intact reconstituted troponin complex. What is more, the cross-linking was favored by Ca^{2+} but reduced by bisphosphorylation, confirming the NMR data that demonstrate weaker binding of the bisphosphorylated extension to TnC.

FRET and fluorescence polarization anisotropy measurements suggest that isolated TnI becomes more compact upon phosphorylation (*37*−*39*). This has been taken as evidence that phosphorylation produces global conformational changes in TnI. TnI seems to be a fairly flexible protein that gains its three-dimensional structure from its interactions with TnC and TnT (*4*). Therefore, caution must be exercised when interpreting observations of isolated TnI in solution. These studies also found that phosphorylation caused TnI to become more compact in a binary complex with TnC and the N-terminal extension to move toward the bulk of the TnI molecule. This could reflect the release of the N-terminal extension from a binding site on TnC and possibly movement toward a binding site on the C-terminal region of TnI for the phosphorylated N-terminal extension. Bisphosphorylation of TnI produces a slight decrease in the affinity of TnI for TnC (*40*, *41*). This reduction in affinity could arise directly from the loss of the binding contribution from the phosphorylation switch although there is evidence that phosphorylation of the extension alters the affinity of the C-terminal region of TnI for TnC (*30*).

The NMR data presented in this paper demonstrate that the unphosphorylated N-terminal extension of TnI binds to the N-terminal domain of TnC via residues immediately C-terminal to S22 and S23, consistent with our previous deletion mutant (*16*) and cross-linking (*20*) studies. Several questions still remain. Is there an alternative binding site for the phosphorylated extension on the thin filament (cross-linking experiments do not provide any evidence for this) and what role do the N-terminal 18 residues of TnI play? What is clear is that we need to determine exactly the region of TnI involved in phosphorylation-sensitive binding to TnC and how it binds to TnC if we are to fully understand how phosphorylation of TnI influences the Ca^{2+} sensitivity of the heart.

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