A Cross-Linking Study of the N-Terminal Extension of Human Cardiac Troponin I[†]

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ABSTRACT: Phosphorylation of the unique N-terminal extension of cardiac troponin I (TnI) by PKA modulates Ca^{2+} release from the troponin complex. The mechanism by which phosphorylation affects Ca^{2+} binding, however, remains unresolved. To investigate this question, we have studied the interaction of a fragment of TnI consisting of residues 1−64 (I1−64) with troponin C (TnC) by isothermal titration microcalorimentry and cross-linking. I1−64 binds extremely tightly to the C-terminal domain of TnC and weakly to the N-terminal domain. Binding to the N-domain is weakened further by phosphorylation. Using the heterobifunctional cross-linker benzophenone-4-maleimide and four separate cysteine mutants of I1−64 (S5C, E10C, I18C, R26C), we have probed the protein−protein interactions of the N-terminal extension. All four I1−64 mutants cross-link to the N-terminal domain of TnC. The cross-linking is enhanced by Ca^{2+} and reduced by phosphorylation. By introducing the same monocysteine mutations into full-length TnI, we were able to probe the environment of the N-terminal extension in intact troponin. We find that the full length of the extension lies in close proximity to both TnC and troponin T (TnT). Ca^{2+} enhances the cross-linking to TnC. Cross-linking to both TnC and TnT is reduced by prior phosphorylation of the TnI. In binary complexes the mutant TnIs cross-link to both the isolated TnC N-domain and whole TnC. Cyanogen bromide digestion of the covalent TnI−TnC complex formed from intact troponin demonstrates that cross-linking is predominantly to the N-terminal domain of TnC.

Troponin acts as a Ca^{2+}-operated molecular switch regulating the contraction of vertebrate striated muscle. At resting Ca^{2+} concentrations, troponin together with tropomyosin prevents cross-bridge cycling and contraction. When the cytosolic Ca^{2+} increases sufficiently for troponin to bind Ca^{2+}, conformational changes in troponin and altered protein−protein interactions allow tropomyosin to move on the surface of actin, exposing additional myosin binding sites, and contraction ensues.

The muscle thin filament consists predominantly of a double helix of actin molecules (F-actin) (for review see ref *1*). Tropomyosin is found to be polymerized in a head to tail fashion, forming two continuous strands along the F-actin. Each tropomyosin monomer spans seven actin molecules. The troponin complex, sited at the overlap between adjacent tropomyosins, is composed of three subunits: troponin T (TnT),[1] troponin I (TnI), and troponin C (TnC). The N-terminal portion of TnT binds to tropomyosin, and the C-terminal portion binds to both TnC and TnI, anchoring troponin to the thin filament. Troponin C belongs to the EF-hand family of divalent cation binding proteins. The crystal stucture of TnC (*2*) reveals a dumbell-shaped molecule: an N-terminal domain connected by a short helix to a C-terminal domain. The two EF-hands in the C-terminal domain (sites III and IV) bind Ca^{2+} extremely tightly ($K_A \sim 1 \times 10^7\ M^{-1}$) but also bind Mg^{2+} ($K_A \sim 0.7 \times 10^3\ M^{-1}$) such that they would be expected to be permanently occupied by Mg^{2+}/Ca^{2+} in vivo (*3*). The two EF-hands in the N-terminal domain (sites I and II) bind Ca^{2+} specifically with an affinity ($K_A \sim 2 \times 10^5\ M^{-1}$) that responds to changes in Ca^{2+} concentration in the muscle and so act as the regulatory sites for contraction (*3*, *4*). TnI is the inhibitory subunit of troponin: binding to actin and inhibiting contraction. Ca^{2+} binding to troponin changes the interactions between TnC and TnI in such a way that the TnI can no longer bind to actin (reviewed in ref *5*).

While sharing high sequence identity with their skeletal counterparts, cardiac TnC and TnI also differ significantly. Site I in the N-terminal domain of cardiac TnC is unable to bind Ca^{2+} due to several amino acid changes (*6*). It is the binding of a single Ca^{2+} to site II that operates the troponin "switch" in cardiac muscle. Cardiac TnI has a 30-residue extension at its N-terminus. This extension shifts the sequence alignment of cardiac and skeletal TnI, and the numbering of homologous residues is therefore different. Unless specified otherwise, we refer to the numbering of the human cardiac sequence below.

The currently accepted model of TnI is that of a fairly extended molecule which adopts an antiparallel orientation to TnC (*7*, *8*). While we have only low-resolution stuctures of whole TnI in complex with TnC (*9*, *10*), there is a wealth of information on the regions of TnI that interact with TnC, TnT, and actin: residues 38−63 of TnI bind to the C-terminal domain of TnC (*11*), residues ∼90−130 form a coiled coil with TnT (*12*, *13*), and residues ∼129−138 form the "inhibitory region" (*14*−*16*) that binds alternately to actin or TnC. The C-terminal portion of TnI possesses additional binding sites for both actin and the N-terminal domain of TnC (*17*−*19*).

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[1] Abbreviations: TnC, troponin C; TnI, troponin I; TnT, troponin T; I1−64, TnI residues 1 to 64; $TnT_{166-288}$, TnT residues 166 to 288; TnC C-domain, residues 91−161 of TnC; TnC N-domain, residues 1−91 of TnC; BPM, benzophenone-4-maleimide; CNBr, cyanogen bromide; IAANS, 2-[4′-(iodoacetamido)anilino]naphthalene-6-sulfonic acid; PKA, protein kinase A.

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β-Adrenergic stimulation of the heart results in PKA-mediated bisphosphorylation of the N-terminal extension of TnI at serines 22 and 23 (*20*, *21*). Bisphosphorylation accelerates Ca^{2+} release from site II of TnC (*22*, *23*). This phosphorylation-induced acceleration of Ca^{2+} release results in a reduced affinity for Ca^{2+} observed as a 0.2−0.3 pCa unit shift to the right in plots of Ca^{2+} binding, actomyosin ATPase activity, or force versus pCa (*21*−*24*). It may be that TnI phosphorylation accelerates cardiac relaxation and hence maintains diastolic filling at increased heart rates; alternatively, TnI phosphorylation may act as a negative feedback mechanism limiting the inotropic effects of adrenaline (*5*). It remains unclear as to how phosphorylation of the N-terminal extension of TnI influences the Ca^{2+} binding properties of the N-terminal domain of TnC, especially in view of the proposed antiparallel orientation of the two proteins. Possible mechanisms can be separated into several models: long-range allosteric effects involving global conformational changes in TnI and/or TnC, direct effects due to the phosphorylation sites being close enough to the regulatory Ca^{2+} site to alter its properties directly, or perhaps phosphorylation altering the binding of the N-terminal extension to TnC. The last of these proposals is suggested by studies of peptides corresponding to residues 17−29 and 19−32 of TnI binding to TnC in the un- and bisphosphorylated state: binding was only observed in the absence of phosphorylation (*14*, *25*). This raises the question as to how the phosphorylated state can destabilize the Ca^{2+}-bound N-domain of TnC if it is no longer bound. The answer seems to be that it is the binding of the unphosphorylated extension to TnC that stabilizes Ca^{2+} binding (*23*). Interactions between the unphosphorylated N-terminal extension and the N-terminal domain of TnC have been demonstrated in various binary troponin complexes by the NMR experiments of Rosevear and co-workers (*26*−*29*). However, this study is the first to show that the N-terminal extension contacts the N-terminal domain of TnC in intact troponin comprised of full-length TnI, TnC, and TnT.

We have produced a fragment of TnI consisting of residues 1 to 64 (I1−64). This fragment contains both the N-terminal extension of TnI (residues 1−31) and the major binding site for the C-domain of TnC (residues 38−63). We have studied the binding of this fragment to both whole TnC and the isolated domains of TnC. As expected, this fragment binds extremely tightly to TnC and to the TnC C-domain. We confirm our previous observation that the interaction between TnI and the TnC C-domain is strengthened by Ca^{2+} replacing Mg^{2+} in sites III and IV, and we show that phosphorylation reduces the affinity of I1−64 for the N-terminal domain of TnC. We have also used 2-[4′-(iodoacetamido)anilino]-naphthalene-6-sulfonic acid (IAANS) labeled TnC to monitor Ca^{2+} release from the TnC·I1−64 complex with and without phosphorylation. We find that phosphorylation of serines 22 and 23 accelerates Ca^{2+} release from the regulatory site even in this minimal binary complex.

Having investigated the behavior of "wt" I1−64, we produced a series of I1−64 mutants with cysteines introduced at positions 5, 10, 18, or 26. This allowed us to incorporate benzophenone-4-maleimide (BPM) at positions along the length of the N-terminal extension. The labeled I1−64s could then be reconstituted into binary complexes and cross-links formed to proteins within ~10 Å radius of the cysteine residue by exposure to UV light. We find that positions 5, 10, 18, and 26 all cross-link to the N-terminal domain of TnC. This cross-linking is Ca^{2+} sensitive and is substantially reduced if the I1−64 is phosphorylated. We then produced the corresponding monocysteine mutants in full-length TnI. This allowed us to probe the protein environment of the N-terminal extension of TnI in troponin and the changes that occur following Ca^{2+} binding and phosphorylation.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human cardiac TnC, the TnC C-domain, the TnC N-domain, TnI, and TnT were overexpressed and purified as described previously (*30*). All protein concentrations were determined by the bicinchoninic acid assay (Pierce) calibrated with bovine serum albumin. Oligonucleotide primers were produced by Alta Bioscience (University of Birmingham) and DNA sequenced at the Functional Genomics Facility (University of Birmingham). N-Terminal amino acid sequencing was by Alta Bioscience and Dr. A. Moir (University of Sheffield). Benzophenone-4-maleimide, maleic anhydride-*2,3*-^{14}*C*, PKA (catalytic subunit), and cyanogen bromide were from Sigma. Maleic anhydride and 4-aminobenzophenone were from Aldrich. Benzophenone-4-[2,3-^{14}C]maleimide was synthesized according to Tao et al. (*31*). Zip Tips were from Millipore. Sequencing grade trypsin was from Promega. Platinum Pfx DNA polymerase was from Invitrogen. All other DNA-modifying enzymes were from New England Biolabs. *Escherichia coli* strains were from Novagen.

Mutagenesis and I1−64 Preparation. Mutations were introduced into wt TnI DNA by oligonucleotide-directed mutagenesis by PCR as previously described (*30*). Monocysteine mutants of TnI were produced by sequentially introducing C78S and C96S mutations followed by the S5C, E10C, I18C, or R26C mutation. The mutant TnI DNAs were ligated into pET11c and sequenced prior to protein expression in *E. coli* Rosetta cells. All TnI mutants were purified exactly as per wt TnI (*30*). I1−64 was produced by CNBr digestion of TnI with a methionine introduced at position 64 (A64M). The purified A64M protein was dissolved at 10 mg/mL in 70% formic acid and an equal mass of CNBr added. Following an overnight incubation at room temperature in the dark, excess CNBr was removed by freeze-drying. I1−64 was separated from the other peptides in the CNBr digest by reverse-phase HPLC using a Vydac C5 semiprep column in 0.1% trifluoroacetic acid with a 25−45% acetonitrile gradient. The fractions containing I1−64 were identified by SDS−PAGE, pooled, and lyophilized.

Microcalorimetry. All experiments were carried out at 30 °C using a Microcal Inc. isothermal titration microcalorimeter. Experimental conditions for TnC and the TnC C-domain titrations were 300 mM KCl, 20 mM MOPS/KOH (pH 7.0), 3 mM $MgCl_2$, and 0.5 mM EGTA ± 1 mM $CaCl_2$. All proteins were dialyzed extensively against this buffer and centrifuged, and the protein concentration was determined prior to use. The 1.4 mL sample cell was filled with 3 μM ($+Ca^{2+}$) or 6 μM ($-Ca^{2+}$) I1−64 and titrated with 5 μL injections of 60 μM ($+Ca^{2+}$) or 160 μM ($-Ca^{2+}$) TnC or TnC C-domain. Titrations with the TnC N-domain were conducted under identical conditions except that the KCl concentration was reduced to 20 mM and the protein

concentrations were 20 μM I1−64 in the cell and 800 μM TnC N-domain in the syringe. Binding parameters were obtained using the Origin-ITC data analysis package in "single set of sites" mode. The phosphorylated I1−64 was prepared by dissolving at 5 mg/mL in the microcalorimetry buffer and incubating overnight with 2 mM ATP and 250 units/mL PKA at room temperature. The excess PKA and nucleotide were removed by HPLC, and the phosphorylation state of the I1−64 was assessed by MALDI-TOF mass spectrometry (Bruker Biflex IV with sinapinic acid as the matrix). The PKA-treated 1−64 was predominantly bisphosphorylated with no un- or monophosphorylated present, although at longer incubation times some (<10%) of the 1−64 appeared to be trisphosphorylated. Control experiments following the time course of phosphorylation of peptides corresponding to residues 1−32 and 30−78 demonstrated that the N-terminal extension is bisphosphorylated with very little, if any, spurious phosphorylation detectable on residues 30−64.

Ca^{2+} Release Measurements. Wild-type human cardiac TnC was labeled with IAANS by dissolving in 6 M urea, 25 mM triethanolamine/HCl (pH 7.5), and 1 mM EDTA and incubating at 4 °C overnight with a 10-fold molar excess of IAANS. TnC-IAANS, I1−64, and phosphorylated I1−64 (prepared as for microcalorimetry) were dialyzed against "stopped-flow buffer" (100 mM KCl, 20 mM MOPS/KOH (pH 7.0), 3 mM $MgCl_2$, 100 μM $CaCl_2$). The rate of Ca^{2+} release was determined from the decrease in IAANS fluorescence following rapid chelation of Ca^{2+}. This was achieved by mixing 90 μL of 1 μM TnC-IAANS ± 2 μM I1−64 in the stopped-flow buffer with 10 μL of 100 mM EGTA/KOH (pH 7.0) at 15 °C using an Applied Photophysics SX-18MV stopped-flow fluorometer equipped with a 400 nm filter (excitation at 325 nm). Rate constants were obtained by least-squares fitting of the data between 2 and 20 ms to a single-exponential function.

Cross-Linking. The I1−64 (or TnI) was labeled with BPM by dissolving in 8 M urea, 25 mM triethanloamine/HCl (pH 7.4), and 1 mM EDTA and incubating at 4 °C overnight in the dark with a 5-fold molar excess of BPM. Excess BPM was removed by extensive dialysis. The proteins used in binary complex experiments were dialyzed against "cross-linking buffer" [50 mM KCl, 20 mM triethanolamine/HCl (pH 7.4), 1 mM dithiothreitol, 0.5 mM EGTA] prior to mixing in a 1:1 molar ratio. The proteins used in ternary troponin complex experiments were mixed in a 1:1:1 TnI:TnC:TnT ratio in 8 M urea and then dialyzed against the cross-linking buffer. When the effect of phosphorylation was to be studied, 1 mM ATP was added to all samples. PKA (previously dialyzed against the cross-linking buffer) was added at 250 units/mL to the samples containing 20 μM I1−64 or TnI and the reaction allowed to proceed for 30 min at room temperature in the dark before commencing cross-linking. According to previous determinations of the rate of TnI phosphorylation this protocol would have resulted in full bisphosphorylation (*32*). Cross-linking was achieved using a 366 nm UV lamp at room temperature. The samples (50 or 100 μL) contained 20 μM protein and were irradiated in 96-well plates. The standard UV exposure time was 30 min. Samples were then subjected to SDS−PAGE on 12% slab gels, followed by staining with Coomassie blue.

Table 1: Binding Parameters Obtained by Isothermal Titration Microcalorimetry for Complex Formation between I1−64 and TnC (a), TnC C-domain (b), and TnC N-domain (c) at 30 °C[a]

(a) I1−64 Binding to TnC

	binding constant × 10^{-7} (M^{-1})		binding enthalpy (kcal/mol)	
	1−64	1−64 Phos	1−64	1−64 Phos
−Ca	2.97 ± 0.37	1.98 ± 0.63	−6.66 ± 0.67	−4.84 ± 0.60
+Ca	10.9 ± 4.5	6.01 ± 2.1	−11.5 ± 1.0	−8.57 ± 0.48

(b) I1−64 Binding to TnC C-Domain

	binding constant × 10^{-7} (M^{-1})		binding enthalpy (kcal/mol)	
	1−64	1−64 Phos	1−64	1−64 Phos
−Ca	1.67 ± 0.38	1.24 ± 0.36	−5.04 ± 0.43	−4.75 ± 0.66
+Ca	15.2 ± 3.4	12.6 ± 1.8	−11.5 ± 0.3	−10.8 ± 0.2

(c) I1−64 Binding to TnC N-Domain

	binding constant × 10^{-5} (M^{-1})		binding enthalpy (kcal/mol)	
	1−64	1−64 Phos	1−64	1−64 Phos
+Ca	3.67 ± 0.88	0.87 ± 0.21	−2.84 ± 0.38	−2.96 ± 0.30

[a] TnC and TnC C-domain results were obtained at 300 mM KCl, and TnC N-domain results were obtained at 20 mM KCl. All other assay conditions can be found in Experimental Procedures.

Analysis of Cross-Linking Sites on TnC. Following cross-linking using benzophenone-4-[2,3-^{14}C]maleimide in the presence of Ca^{2+} for 30 min, the cross-linked I1−64−TnC was isolated by C5 RP-HPLC (as above). Similarly, cross-linked TnI−TnC was purified following 30 min of cross-linking whole troponin in the presence of Ca^{2+}. The cross-linked TnI−TnC was isolated by successive CM- and DEAE-Sepharose chromatography. The cross-linked I1−64−TnC and TnI−TnC were freeze-dried and either dissolved in 70% formic acid and digested overnight with CNBr (as above) or dissolved at approximately 1 mg/mL in 100 mM ammonium bicarbonate and digested overnight at room temperature with 40 μg/mL trypsin. The digests were lyophilized and either dissolved in 0.1% trifluoroacetic acid, desalted using C18 Zip Tips, and analyzed by MALDI or subjected to SDS−PAGE on 10−20% gradient gels or 16.5% gels containing 8 M urea (*33*) followed by autoradiography. The bands identified as containing radiolabel were cut out from duplicate gels, blotted, and sequenced.

RESULTS

Microcalorimetry Experiments. This series of experiments allowed us to examine the effect of phosphorylation of I1−64 on its binding to TnC and the isolated domains of TnC. I1−64 contains the phosphorylatable N-terminal extension of TnI and the primary binding site for the C-domain of TnC, which forms the strongest site of interaction between the two proteins. In this system the influence of the TnC binding sites in the C-terminal region of intact TnI is absent, simplifying the interpretation of the results. Parts a, b, and c of Table 1 show the binding constants and enthalpies obtained from titrations of I1−64 and bisphosphorylated I1−64 with TnC, the TnC C-domain, and the TnC N-domain, respectively. The stoichiometry of binding was approximately 0.7 TnC (or N/C-domain) per I1−64 in all cases. Both unphosphorylated and bisphosphorylated I1−64 bind to TnC and the TnC C-domain with a K_A of ~2 × 10^7 M^{-1} in the absence of Ca^{2+} (but in the presence of Mg^{2+}) which increases to ~1 × 10^8 M^{-1} in the presence of Ca^{2+}. The

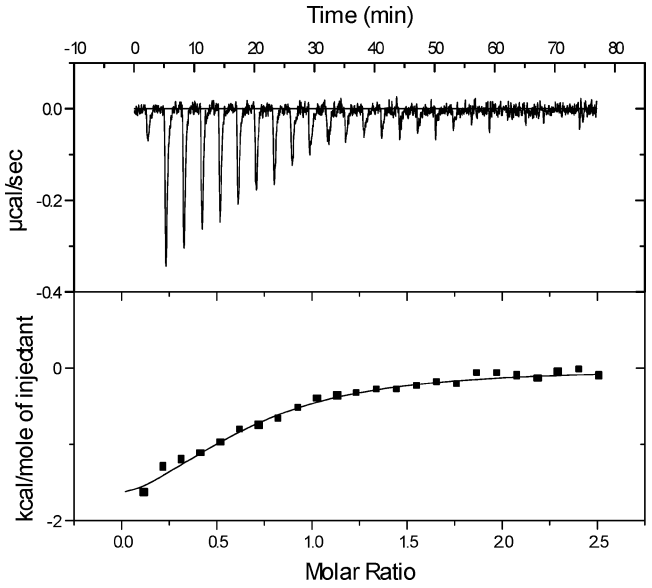

FIGURE 1: Microcalorimetric titration of I1−64 with the TnC N-terminal domain. The top panel shows the raw data from a titration of 20 μM I1−64 with 5 μL injections of 800 μM TnC N-domain at 30 °C, 20 mM MOPS/KOH (pH 7.0), 20 mM KCl, 3 mM $MgCl_2$, and 1 mM $CaCl_2$. The bottom panel shows the binding isotherm (integrated data and curve fit).

effect of Ca^{2+} on the binding of I1−64 to TnC and the TnC C-domain is also reflected in the binding enthalpies. Phosphorylation does not produce any dramatic changes in the binding constant obtained for the interaction between I1−64 and TnC or the TnC C-domain. However, in the case of TnC, the extremely tight binding of I1−64 to the C-terminal domain would mask any effects of phosphorylation on I1−64 binding to the N-terminal domain. The TnC and TnC C-domain titrations were carried out at 300 mM KCl to allow comparison with previous experiments with full-length TnI (*30*, *32*). We find that the binding of I1−64 to TnC is not measurably weaker than that of full-length TnI, emphasizing the importance of TnI residues 38−63 binding to the C-domain of TnC for maintaining the structural integrity of the complex. We previously found that the binding of TnI to the TnC N-domain could only be measured by microcalorimetry at low salt concentrations and in the presence of Ca^{2+} (*30*). We now find that the binding of I1−64 to the TnC N-domain also cannot be measured at 300 mM KCl (data not shown), and we therefore reduced the salt to 20 mM in the TnC N-domain experiments. Figure 1 shows a titration of I1−64 with the N-domain under these conditions. At 20 mM KCl the binding of I1−64 to the N-terminal domain of TnC is approximately 100-fold weaker than to the C-domain or TnC at 300 mM KCl. It is now possible to see a 4−5-fold decrease in affinity of I1−64 for the N-terminal domain produced by phosphorylation.

Effect of I1−64 Phosphorylation on Ca^{2+} Release from TnC. We rapidly mixed the Ca^{2+}-saturated TnC-IAANS·I1−64 complex with excess EGTA at 15 °C and followed Ca^{2+} release by monitoring the decrease in IAANS fluorescence. The release of Ca^{2+} from TnC-IAANS resulted in a 30% decrease in fluorescence; the corresponding decreases in the un- and bisphosphorylated TnC-IAANS·I1−64 complexes were 11% and 19%, respectively. Figure 2 shows typical experiments with the TnC-IAANS·I1−64 complex in the un- and bisphosphorylated states. The mean (±SEM, $n = 10$)

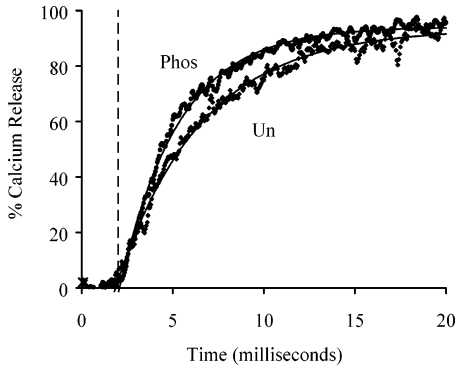

FIGURE 2: Effect of phosphorylation of I1−64 on Ca^{2+} release from the TnC-IAANS·I1−64 binary complex. The rate of Ca^{2+} release was monitored from the decrease in IAANS emission as described under Experimental Procedures. The graph shows averaged data from eight repetitions and the least-squares fittings to single-exponential functions for the unphosphorylated (Un) and phosphorylated complex (Phos). Data to the left of the dashed line (2 ms) were not included in the curve fitting.

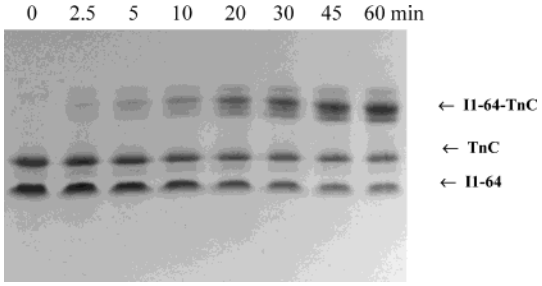

FIGURE 3: Time course of BPM-labeled I1−64 (I18C) cross-linking to TnC. The samples containing 20 μM I1−64 and 20 μM TnC were irradiated for varying times as indicated in the presence of $CaCl_2$. The samples were then subjected to SDS−PAGE on 12% slab gels. The gel has been stained with Coomassie blue.

rate constants that we obtained were 279 ± 5 s^{-1} for TnC, 238 ± 6 s^{-1} for TnC·I1−64, and 316 ± 5 s^{-1} for phosphorylated TnC·I1−64. It appears that the Ca^{2+} off-rate from TnC·I1−64 is very similar to that of TnC and not that of whole troponin [25 s^{-1} at 25 °C (*23*)], suggesting that the major influences stabilizing Ca^{2+} binding in whole troponin come from the C-terminal regions of TnI and perhaps TnT. Nevertheless, the unphosphorylated I1−64 does exert a small stabilization of Ca^{2+} binding to TnC whereas phosphorylated I1−64 has the reverse effect, producing an acceleration of Ca^{2+} release over that of TnC alone. It is interesting to note that the percentage increase in off-rate produced by phosphorylation of TnC·I1−64 (∼33%) is similar to the ∼29% that we recently reported for phosphorylation of whole troponin (*23*); albeit the rates were much slower in the latter. These results suggest that the N-terminal extension of TnI interacts with TnC in a similar manner in both TnC·I1−64 and whole troponin.

Cross-Linking of I1−64 Mutants. The results shown in Figures 3−5 were obtained using the I18C mutant. They are typical of the results obtained with the four mutants (S5C, E10C, I18C, R26C) of I1−64 which all produced similar patterns of cross-linking, although the extent of cross-linking increased with the distance of BPM attachment from the N-terminus. Figure 3 shows how cross-link formation between I1−64 and TnC mixed in a 1:1 ratio in the presence of Ca^{2+} increases with increased time of exposure to UV light. Substantial quantities of cross-linked I1−64−TnC are

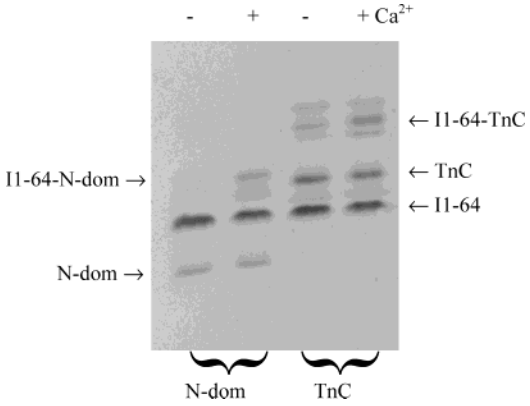

FIGURE 4: Cross-linking of BPM-labeled I1−64 (I18C) to TnC and the TnC N-domain in the presence and absence of $CaCl_2$ (3 mM $MgCl_2$ present throughout). Conditions are as described in Experimental Procedures.

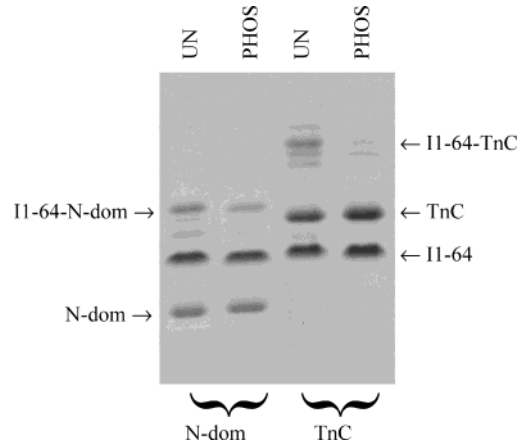

FIGURE 5: Effect of phosphorylation on the cross-linking of BPM-labeled I1−64 (I18C) to TnC and the TnC N-domain (+$CaCl_2$).

formed after 30−60 min. The high level of cross-linking suggests that the N-terminal extension lies in close proximity to TnC in this binary complex. The fact that the cross-linked product appears as two or three bands on the gel suggests that the N-terminal extension may be sufficiently flexible to cross-link at several different locations on TnC. We found that I1−64 also cross-linked to the TnC N-domain (Figure 4) but not to the TnC C-domain nor to a soluble fragment of TnT ($TnT_{166-288}$) (data not shown). Control experiments demonstrated that the cross-links are forming between the binding partners of tight noncovalent protein complexes and do not result from random encounters: the cross-linking to TnC was just as effective at 10-fold lower protein concentrations but was abolished by addition of 1% SDS (w/v). No cross-linking was observed when BPM-labeled I1−64 alone, or in the presence of a large excess of bovine serum albumin, was exposed to UV light.

Figure 4 shows that the extent of cross-linking to both TnC and the TnC N-domain is much reduced in the absence of Ca^{2+}. From the data in Table 1 one can calculate that at concentrations of 20 μM I1−64 and 20 μM TnC essentially all of the protein would be in the binary complex regardless of the presence of Ca^{2+}. Therefore, the reduced cross-linking to TnC in the absence of Ca^{2+} resulted not from dissociation of the I1−64 from TnC but from a reduced affinity of the N-terminal domain of intact TnC for I1−64 in the absence of Ca^{2+} (while the TnC and I1−64 remain tightly bound via the C-terminal domain of TnC). This implies that cross-linking in the I1−64−TnC complex occurs via the N-terminal domain of TnC although we cannot exclude possible effects of Ca^{2+}/Mg^{2+} exchange at the C-terminal domain of TnC altering the relative orientation of the two proteins and producing a conformation that does not allow cross-linking to take place, although this seems unlikely. The reduced cross-linking to the isolated TnC N-domain in the absence of Ca^{2+} may well arise directly from a lower concentration of binary complex in the absence of Ca^{2+}.

In the experiment shown in Figure 5 we test the effect of phosphorylating the I1−64 prior to cross-linking to TnC and the TnC N-domain. In both cases phosphorylation substantially reduces cross-linking. As argued for the effects of Ca^{2+}, the binding constants obtained by microcalorimetry show that the I1−64 would be tightly bound to TnC under these conditions regardless of its phosphorylation state. Hence, the reduction in cross-linking to TnC caused by phosphorylation must arise from the reduced affinity of phosphorylated I1−64 for the N-terminal domain of TnC. The effect of phosphorylation on cross-linking to the TnC N-domain can be related directly to the reduced affinity of phosphorylated I1−64 for the TnC N-domain: the affinity of I1−64 for the TnC N-domain is such that phosphorylation would significantly reduce the amount of noncovalent complex present during the cross-linking experiments (Table 1c).

Although the I1−64 mutants cross-linked to the isolated TnC N-domain and not to the isolated TnC C-domain, we could not be sure that the cross-links were also being formed to the N-terminal domain in the complex with intact TnC. To answer this question, we cross-linked I1−64 to TnC using benzophenone-4-[2,3-^{14}C]maleimide. The cross-linked product was purified and digested with trypsin, and the digests were analyzed by MALDI, SDS−PAGE, and autoradiography. We were able to identify a unique peak in the mass spectrum of the digest of each of the cross-linked products (S5C, E10C, I18C, R26C) corresponding to the sum of the mass of the tryptic fragment of TnI containing the BPM attachment site, the mass of BPM, and the mass of a tryptic fragment of TnC. The TnC fragment involved in each case was residues 47−83. The digests were subjected to SDS−PAGE and autoradiography. The radiolabeled bands were blotted and five N-terminal residues identified by amino acid sequencing. In each case we obtained two sequences, one starting at the relevant tryptic cleavage in TnI and one starting at TnC residue 47. To further delineate the region(s) of TnC involved in cross-linking, we digested the purified cross-linked products with CNBr. These digests were analyzed by SDS−PAGE and autoradiography and the labeled bands sequenced. We found that all four I1−64s cross-linked not only to CNBr fragments 48−60 and 61−80 of TnC, which are consistent with the tryptic fragments mentioned above, but also to TnC residues 2−45. Together, these fragments cover the whole of the N-domain and do not identify a specific region involved in binding to the N-terminal extension of TnI. Previous reports have shown that the N-terminal extension of TnI induces amide proton NMR shifts throughout N-domain but concentrated in the Ca^{2+} binding loops (*28*, *29*). Our data are consistent with the N-terminal extension of TnI contacting both EF-hands of the N-domain. There was no evidence for cross-linking to the C-terminal domain of TnC.

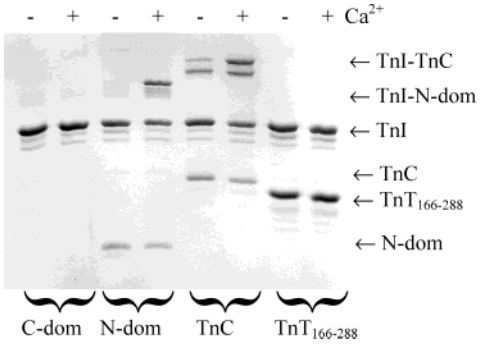

FIGURE 6: Cross-linking of BPM-labeled TnI (I18C) in binary complexes. In this experiment the cross-linking of 20 μM TnI to 20 μM TnC C-domain, TnC N-domain, TnC, and $TnT_{166-288}$ has been measured ($\pm CaCl_2$). The gel has been run a sufficient distance to satisfactorily resolve the cross-linked products resulting in the TnC C-domain (lanes 1 and 2) migrating off the gel.

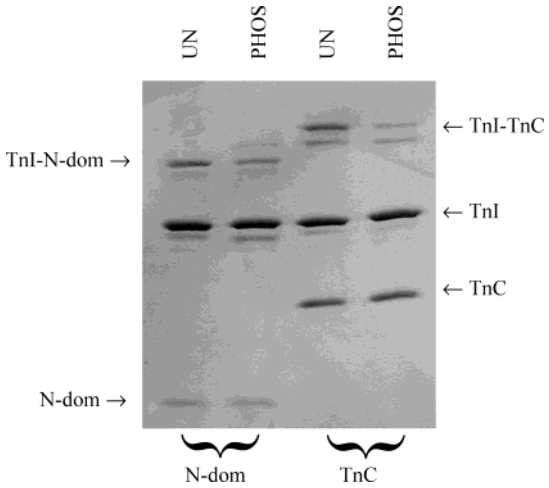

FIGURE 7: Effect of phosphorylation on the cross-linking of BPM-labeled TnI (I18C) in binary complexes ($+CaCl_2$).

Cross-Linking with Full-Length TnIs. Initially, the full-length monocysteine TnIs were cross-linked in binary complexes (Figure 6). In the binary complexes the four mutants behaved qualitatively identically: no cross-linking to the TnC C-domain or $TnT_{166-288}$ but high-yield cross-linking to TnC and the TnC N-domain. As with the I1−64 the amount of cross-linking was increased by Ca^{2+}, indicating a higher affinity of the N-terminal extension of TnI for the Ca^{2+}-bound rather than the Ca^{2+}-free conformation of the N-terminal domain of TnC. Phosphorylation of TnI again reduces the extent of cross-linking to both TnC and the TnC N-domain (Figure 7). This result shows that phosphorylation reduces the affinity of the N-terminal extension of TnI for the N-terminal domain of TnC not only in I1−64 but also when the C-terminal part of TnI (containing the inhibitory and regulatory regions) is present.

We reconstituted the ternary troponin complex in the presence of 8 M urea and dialyzed extensively against cross-linking buffer. This method has previously been shown to produce a functional complex that is able to regulate actomyosin ATPase activity in a Ca^{2+}-sensitive manner (*23*). Figure 8 shows the time course of cross-linking in troponin containing BPM-labeled I18C TnI. As the period of exposure to UV light was increased, we see that two populations of cross-linked products are formed. The identities of the gel bands were verified by several methods. Blotting and staining

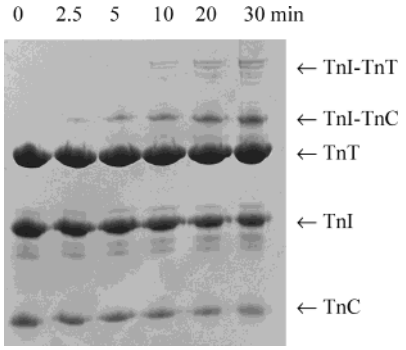

FIGURE 8: Time course of cross-linking of BPM-labeled TnI (I18C) in the troponin complex ($+CaCl_2$). The samples were irradiated for the times indicated prior to analysis by SDS−PAGE.

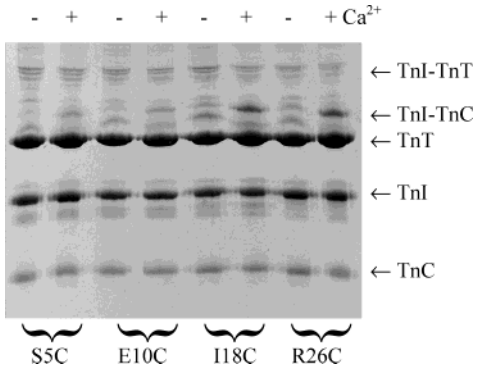

FIGURE 9: Comparison of cross-linking results with C5S, E10C, I18C, and R36C TnI labeled with BPM and reconstituted into troponin ($\pm CaCl_2$).

with an anti-TnI antibody (*34*) demonstrated the presence of TnI in all the higher molecular weight species, and the use of fluorescently labeled TnC or TnT demonstrated their presence in the lower and upper bands, respectively. N-Terminal sequencing yielded sequences corresponding to the N-termini of TnI and either TnC or TnT, confirming that the lower product bands correspond to cross-linked TnI−TnC and the upper bands to TnI−TnT as labeled in Figure 8. Cross-linked TnI−TnC appeared as two or three bands whereas there are numerous bands of TnI−TnT presumably caused by cross-link formation to positions separated in the TnT primary structure. Although we cannot exclude that the multiple bands arise from conformational heterogeneity, it seems most likely that they arise from local flexibility and the reactivity of the triplet biradical state of the activated benzophenone group toward any neighboring C−H bond. Figure 9 shows the patterns of cross-linking produced by BPM anchored at positions 5, 10, 18, or 26 of TnI. In whole troponin all four mutants produce a similar quantity of TnI−TnT, suggesting that the whole of the N-terminal extension lies in proximity to TnT. Cross-linking to TnT does not appear to be strongly influenced by the presence of Ca^{2+}, suggesting that the N-terminal extension of TnI lies close to TnT regardless of whether it is directly bound to TnC. All four mutants also cross-link to TnC. However, positions 5 and 10 do not cross-link as well as positions 18 and 26; i.e., the extreme N-terminus of TnI may not bind as tightly to TnC as the region around the bisphosphorylation site. As with the binary complexes, Ca^{2+} enhances the cross-linking to TnC at all positions of cross-linker but especially at positions 18 and 26, possibly at the expense of cross-linking to TnT. The quantity of cross-linked TnI−TnC in the presence

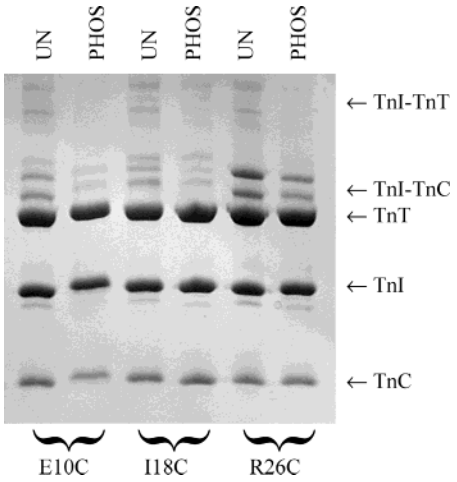

FIGURE 10: Effect of TnI phosphorylation on cross-linking in the ternary troponin complex (+$CaCl_2$).

of Ca^{2+} is especially high at positions 18 and 26, providing evidence that the phosphorylation region of the N-terminal extension binds to TnC in a Ca^{2+}-sensitive manner. At positions 18 and 26 cross-linking to TnC in the presence of Ca^{2+} far outweighs cross-linking to TnT. Ca^{2+} appears to slightly decrease cross-linking to TnT at positions 18 and 26, suggesting a movement of the extension from TnT to TnC.

Figure 10 shows the effect of phosphorylation on cross-linking in troponin. Phosphorylation reduces cross-linking to both TnC and TnT. These experiments confirm our conclusions from the binary complexes that this cannot be due to dissociation of TnI from the other troponin subunits but rather reflects the changes in the protein−protein interactions made by the N-terminal extension of TnI upon phosphorylation. The effect of phosphorylation is to reduce the contact between the entire length of the extension and both TnC and TnT. This result could be explained by the extension either moving out of the troponin complex or folding back on itself when it is bisphosphorylated. The effects of phosphorylation observed in the experiment of Figure 10 must be due to bisphosphorylation of serines 22 and 23 of TnI as these have been shown to be the only residues phosphorylated by PKA in the troponin complex (*32*).

To further characterize the interaction between the unphosphorylated extension and TnC, we purified benzophenone-4-[2,3-^{14}C]maleimide cross-linked TnI−TnC from irradiated troponin and digested with CNBr. The resulting peptide mixture was separated by SDS−PAGE, and the radiolabeled bands were blotted and N-terminally sequenced. For each cross-linked product (S5C, E10C, I18C, R26C) we were able to identify the TnI CNBr fragment 1−153 cross-linked to CNBr fragments 2−45, 48−60, and 61−80 of TnC. These results confirm that the extension cross-links to the N-terminal domain of TnC not just in binary complexes but also in whole troponin.

DISCUSSION

The PKA-mediated phosphorylation of TnI, first reported over 20 years ago (*21*, *35*), remains an enigmatic phenomenon. It is well-known that phosphorylation of TnI reduces Ca^{2+} sensitivity in several steady-state assays: actomyosin ATPase activity, force production, and fluorescent changes induced by Ca^{2+} binding to TnC (*21*−*24*). It has also been shown that TnI phosphorylation accelerates Ca^{2+} release from troponin reconstituted with TnC labeled with IAANS (*22*, *23*). Zhang et al. (*36*) demonstrated that TnI phosphorylation accelerates the relaxation of skinned fibers upon rapid chelation of Ca^{2+}, although another group reported that phosphorylation did not affect the rate of relaxation (*37*). It is known that adrenergic stimulation of the heart produces a lusitropic effect, and although the relative contributions of TnI, C-protein, and phospholamban phosphorylation remain a topic of discussion (*38*, *39*), it seems likely that TnI is involved. If phosphorylation of TnI does accelerate relaxation in intact cardiac muscle, it would contribute to the lusitropic effect maintaining diastolic function at high heart rates. It has also recently been reported that TnI phosphorylation accelerates cross-bridge cycle kinetics (*40*, *41*).

The effects of TnI phosphorylation on the structure and properties of TnI and TnC have been studied in some detail. FRET experiments have shown that upon phosphorylation the N-terminal extension of TnI moves 10−12 Å toward the C-terminal regions of TnI (*42*). Consistent with this, fluorescence anisotropy measurements have been interpreted as showing TnI becoming more compact upon phosphorylation (*43*, *44*). The changes in cross-linking produced by TnI phosphorylation seen in this study are consistent with movement of the N-terminal extension of TnI away from TnC and TnT out of the complex or possibly toward TnI (we would probably not have observed intramolecular cross-linking in our experiments). It has previously been reported that phosphorylation reduces the affinity of TnI for TnC and the TnC N-domain (*42*, *45*) although it is not clear whether this arises from global changes in the conformation of TnI or directly from changes in the TnC binding properties of the cardiac TnI N-terminal extension. Peptides corresponding to parts of this N-terminal extension have been shown to bind to TnC only when unphosphorylated (*14*, *25*). More recently, the NMR experiments of Rosevear and co-workers (*26*−*29*) have demonstrated that phosphorylation or deletion of residues 1−32 from TnI or TnI 1−80 (a fragment of TnI containing residues 1−80) produces amide ^{1}H shifts and influences conformational exchange of the N-terminal domain of TnC in binary complexes. These experiments are most easily explained if the unphosphorylated N-terminal extension makes contacts with the TnC N-domain that are disrupted by phosphorylation. The influence of residues 1−32 of TnI on the structure of the N-terminal domain of TnC is small compared with that of residues 33−80 of TnI on the C-terminal domain of TnC (*28*).

The ability of TnI phosphorylation to accelerate Ca^{2+} release from the regulatory site has not previously been demonstrated in a binary complex of TnC and a fragment of TnI such as I1−64. It is, however, debatable as to whether the N-terminal extension influences Ca^{2+} release in the same way in the TnC·I1−64 complex as in intact troponin: the relative increase in the Ca^{2+} off-rate is similar in both cases, but the absolute rates are at least 10-fold slower in troponin. We previously reported evidence that in troponin it is the unphosphorylated N-terminal extension of TnI that stabilizes Ca^{2+}-bound TnC and that phosphorylation prevents this stabilization. In the TnC·I1−64 complex it appears that unphosphorylated I1−64 has a small stabilizing effect on Ca^{2+}-saturated TnC and reduces the Ca^{2+} off-rate with respect to uncomplexed TnC. However, phosphorylated I1−

64 exerts a destabilizing influence and increases the Ca^{2+} off-rate with respect to TnC. What is clear is that if the N-terminal extension of TnI is anchored at its C-terminus to the C-terminal domain of TnC, then it is able to interact with the N-terminal domain of TnC and influence its properties.

Heterobifunctional cross-linking is a well-established technique which has been used successfully with troponin in the past (*46*−*49*). An advantage of using BPM is that the benzophenone moeity can be excited many times over until it finds another molecule to react with. Hence, the cross-linking will report on contacts made during the excitation period; i.e., the cross-linked product(s) represent(s) a time-averaged picture with the extent of cross-linking to a certain protein reporting on the amount of time in contact with that protein. It is difficult for cross-linking to distinguish between a tight-binding and close proximity. Cross-linking can, however, give reliable information about the neighboring proteins that the labeled protein can contact in multiprotein complexes. Here, we provide for the first time evidence that the N-terminal extension lies close to the N-terminal domain of TnC in *intact* troponin. We demonstrate a proximity or interaction between the N-terminal extension of TnI and the N-terminal domain of TnC that is modulated both by Ca^{2+} and by phosphorylation. We observed cross-linked TnI−TnC and TnI−TnT formation in troponin and also in reconstituted thin filaments (data not shown) although we did not examine the effects of Ca^{2+} and phosphorylation in the filaments.

We produced the monocysteine mutations at positions 5, 10, 18, and 26 in the N-terminal extension for several reasons. First, one criticism of cross-linking as a method is that the mutation of an amino acid and attachment of the cross-linker may alter the very interaction that one is trying to measure. However, it seems very unlikely that this would be true for all of the mutants that we have tested. Second, it has allowed us to investigate how the protein−protein interactions change along the length of the extension: we find that the interaction with TnC is strongest in the vicinity of the phosphorylation sites (positions 18 and 26). Third, our a priori expectation was that the extension would contact the N-terminal domain of TnC. By using the four mutants, we hoped to trace the binding "footprint" of the extension across TnC. That we have not been able to achieve this goal may have been due to the lack of specificity of the cross-linking reagent (the activated benzophenone reacts primarily with C−H bonds). This could allow cross-links to form to several TnC residues that are spatially close in the folded protein but separated in the amino acid sequence. This effect may well have been compounded by flexibility of the N-terminal extension and a degree of promiscuity in its interaction with TnC.

Our cross-linking experiments show that the N-terminal extension of TnI interacts with the N-terminal domain of TnC in the presence of Ca^{2+} and to a lesser extent in the absence of Ca^{2+}. We have recently shown that deletion of the N-terminal extension of TnI mimics phosphorylation (*23*). This led us to propose that it is the unphosphorylated extension that binds to TnC and stabilizes the "open" Ca^{2+}-bound state of the N-terminal domain. Phosphorylation (or deletion) would then reduce (or prevent) binding of the extension to the N-terminal domain of TnC, removing the stabilizing influence and accelerating Ca^{2+} release. The extent of cross-linking of all four TnI mutants to TnC is reduced by phosphorylation, lending further support to this hypothesis. Cross-linking to TnC is most extensive when the cross-linker is attached to TnI near the bisphosphorylation site (I18C and R26C), supporting previous peptide binding studies showing that residues ∼17−32 of TnI can bind to TnC (*14*, *25*).

The cross-linking results also show that the N-terminal extension is positioned close to TnT but moves further away upon phosphorylation. Clearly, TnT is positioned such that it could play a role in transmission of the phosphorylation signal from TnI to TnC. However, TnI phosphorylation has been shown to reduce the Ca^{2+} affinity of the N-terminal domain of TnC in the absence of TnT (*45*), so TnT is clearly not a key player. The proximity between the N-terminal extension and TnT that we observe is not surprising in view of the ^{31}P NMR experiments of Jaquet and co-workers (*50*, *51*) in which both TnC and TnT contribute to the environment of phosphoserines 22 and 23 of TnI.

The N-terminal domain of skeletal TnC adopts an open conformation when saturated with Ca^{2+}: the A and D helices move apart from the B and C helices, exposing a hydrophobic patch (*52*, *53*). This hydrophobic patch is then able to interact with TnI. In cardiac TnC it is only site II that is able to bind Ca^{2+}, and Ca^{2+} binding alone does not induce an opening of the cardiac N-domain (*54*−*59*). However, binding of a peptide consisting of residues 147−163 of TnI (or whole TnI) and Ca^{2+} stabilizes an open conformation (*54*−*58*). The interaction of TnI with TnC increases the affinity of the N-domain for Ca^{2+} approximately 10-fold (*3*), presumably due to the interaction of the C-terminal region of TnI with the N-terminal domain of TnC. Recently, Abbott et al. (*27*) titrated TnI 129−166 into a complex of TnC with TnI 1−80. TnI 1−80 increased the affinity of TnC for TnI 129−166, and phosphorylation reduced the affinity back toward that of TnC alone. It seems that the binding of the N-terminal extension to the N-terminal domain of TnC stabilizes binding of both Ca^{2+} and the C-terminal regions of TnI. This raises the question of how the extension influences the linked equilibria in this complex with at least four components (Ca^{2+}, TnC, and the N- and C-terminal regions of TnI and possibly TnT). Is it via stabilizing TnI 147−163 binding directly or a short-range effect on the conformation or flexibility of the Ca^{2+} binding loop or on the ionization state of a single group? The Ca^{2+} affinity of the regulatory site is sensitive to many influences including mutations in TnC (*60*), TnI (*61*), and TnT (*62*, *63*) and the binding of hydrophobic compounds such as EMD 57033, bepridil, and trifluoperazine (*64*). While structural determinations of TnC in complex with these Ca^{2+}-sensitizing drugs show ways in which the Ca^{2+} affinity of the regulatory site may be increased, their mode of action may be quite distinct from the mode of action of the N-terminal extension (*65*, *66*). To fully understand how the N-terminal extension of TnI alters the Ca^{2+} binding properties of TnC, we are going to need information about the protein−protein interaction at the atomic level.

In conclusion: the cross-linking experiments reported in this paper demonstrate a proximity between the phosphorylatable N-terminal extension of TnI and the N-terminal domain of TnC. These results reinforce our earlier suggestion (*23*) of a direct, rather than allosteric, model to explain the consequences of TnI phosphorylation: the unphosphorylated

N-terminal extension binds to and stabilizes the Ca^{2+}-bound N-terminal domain of TnC. Phosphorylation reduces the affinity of the extension for the TnC N-domain and hence reduces the stabilizing influence, accelerating Ca^{2+} release.

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