The Importance of the Carboxyl-terminal Domain of Cardiac Troponin C in Ca\(^{2+}\)-sensitive Muscle Regulation*

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The interactions between troponin I and troponin C are central to the Ca\(^{2+}\)-regulated control of striated muscle. Using isothermal titration microcalorimetry we have studied the binding of human cardiac troponin C (cTnC) and its isolated domains to human cardiac troponin I (cTnI). We provide the first binding data for these proteins while they are free in solution and unmodified by reporter groups. Our data reveal that the C-terminal domain of cTnC is responsible for most of the free energy change upon cTnC-cTnI binding. Importantly, the interaction between cTnI and the C-terminal domain of cTnC is 8-fold stronger in the presence of Ca\(^{2+}\) than in the presence of Mg\(^{2+}\), suggesting that the C-terminal domain of cTnC may play a modulatory role in cardiac muscle regulation. Changes in the affinity of cTnI for cTnC and its isolated C-terminal domain in response to ionic strength support this finding, with both following similar trends. At physiological ionic strength the affinity of cTnC for cTnI changed very little both following similar trends. At physiological ionic strength the affinity of cTnC for cTnI changed very little both following similar trends.

Cardiac and skeletal muscle contraction is regulated by the troponin complex and tropomyosin. The troponin complex consists of three proteins: the Ca\(^{2+}\) binding subunit, troponin C (TnC), the inhibitory subunit, troponin I (TnI), and tropomyosin T (TnT) which anchors the troponin complex to the thin filament. The binding of Ca\(^{2+}\) to TnC results in conformational changes and altered interactions within the thin filament which ultimately lead to muscle contraction (1). Central to the transmission of the Ca\(^{2+}\) signal are altered interactions between TnC and TnI.

Skeletal and cardiac TnC share ~70% sequence identity and consist of two globular domains (N- and C-terminal) connected by a central helix (2). Both proteins have four EF hand divalent binding sites, sites I and II in the N-terminal domain and sites III and IV in the C-terminal domain. All four sites bind Ca\(^{2+}\) in skeletal TnC (skTnC); but in cardiac TnC (cTnC) site I is unable to bind divalent cations (3).

Sites I and II of skTnC and site II of cTnC are low affinity Ca\(^{2+}\)-specific binding sites (K\(_{a}(Ca) = 5 \times 10^5 \text{M}^{-1}\) and 2 \times 10^6 \text{M}^{-1} for skTnC and cTnC, respectively) (4, 5). It is Ca\(^{2+}\) binding to these sites which is proposed to act as the trigger for the initiation of muscle contraction. In skTnC this involves an “opening” of the structure with increased exposure of an extensive hydrophobic patch to which skeletal troponin I (skTnI) binds, ultimately relieving inhibition on actin (6). There is, however, evidence that the details of interaction between TnC and TnI may differ in cardiac and skeletal muscle as the Ca\(^{2+}\)-saturated N-terminal domain of cTnC exists in a closed conformation (7), and a recent report suggests that the binding of cTnI\(_{147-163}\) is required for the “opening” of this domain (8).

Sites III and IV in cTnC are high affinity Ca\(^{2+}\)-binding sites that can also bind Mg\(^{2+}\) with a lower affinity (K\(_{a}(Ca) = 3 \times 10^8 \text{M}^{-1}\) and K\(_{a}(Mg) = 3 \times 10^9 \text{M}^{-1}\) (5). Under physiological conditions these sites are always occupied by Mg\(^{2+}\) or Ca\(^{2+}\). The C-terminal domain of TnC has been proposed as a Ca\(^{2+}\)-independent structural binding site for TnI and TnT, helping to anchor the troponin complex to the thin filament (9).

Although the crystal and solution structures of skTnC and cTnC are well characterized (2, 7, 10, 11) only low resolution data are available for the structure of TnI (12–14). Several lines of evidence indicate that TnI lies antiparallel to TnC and TnT (11, 15). This paper is available on line at http://www.jbc.org

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The abbreviations used are: TnC, troponin C; TnI, troponin I; TnT, troponin T; cTnC, human cardiac troponin C; cTnI, human cardiac troponin I; cTnC, human cardiac troponin C; cTnC N-domain, recombinant human cardiac troponin C N-terminal domain (residues 1–91); cTnC C-domain, recombinant human cardiac troponin C C-terminal domain (residues 91–161); skTnC, troponin C from skeletal muscle; skTnC, troponin C from skeletal muscle; ITC, isothermal titration microcalorimetry; MOPS, 3-(N-morpholino)propanesulfonic acid; Inf, logarithm of fringe displacement; Pipes, 1,4-piperazinediethanesulfonic acid.
effect of ionic strength and Ca\(^{2+}\) on the free energy, enthalpy, and entropy changes associated with the binding of cTnC and its isolated domains to cTnI. Our results suggest that the cardiac muscle domain of cTnC may play a more important role in the cardiac muscle regulation than previously thought.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, DNA-modifying enzymes, and deoxynucleotides were purchased from New England Biolabs, Taq polymerase was purchased from Roche Diagnostics Ltd., and oligonucleotide primers were designed by Alba Bioscience, University of Birmingham.

**Expression and Purification of cTnC, cTnC N-domain, and cTnC C-domain**—Polymerase chain reaction was used to generate DNA fragments encoding the isolated N-terminal domain of cTnC (cTnC N-domain, residues 1–91 inclusive of N-terminal Met), and cTnC D-domain (residues 91–161). The 5′-polymerase chain reaction primer (5′-GGGAATTCATATGGAAGAATCAGGAGGAGC-3′) was designed to amplify pET11c(cTnC) DNA from the first amino acid and also encoded an NdeI restriction site. The 3′-primer (5′-AAGGATCCATATGGAGAATCAGGAGGAGC-3′) was complementary to pET11c(cTnC) DNA encoding the C terminus of cTnC; this primer also encoded a stop codon and BamHI site.

The resulting polymerase chain reaction products were purified to a single band by gel electrophoresis on a 7.5% polyacrylamide gel and electroeluted. The clean products were digested with NdeI and BamHI, ligated into NdeI/BamHI-digested pET11c vector, and used to transform competent Escherichia coli JM101 cells. Successful clones were screened by restriction analysis and verified by DNA sequencing and by amino acid sequencing of the first 10 residues of the purified protein.

The cTnC, cTnC N-domain, and cTnC C-domain pET11c constructs were transformed into and expressed in BL21(DE3) cells. The cells were grown at 37 °C in NZCYM medium with 0.3 mM ampicillin. The expression plasmid was contained on a 12-plasmid copy number vector. The cTnC-D domain was expressed at a yield of 9 × 10\(^{14}\) copies per cell at level 6 using a HeatSystems Inc. sonicator. Inosol cell debris was pelleted by centrifugation at 75,000 × g for 40 min at 4 °C. The cell extract was loaded onto a DEAE-Sepharose Fast Flow column (2.5 × 20 cm), equilibrated with 25 mM triethanolamine hydrochloride, pH 7.5, 8 mM urea, 2 mM EDTA, 1 mM dithiothreitol. The fractions containing cTnC or its domains were identified by absorption at 280 nm and SDS-polyacrylamide gel electrophoresis, pooled, dialyzed against 1 mM HCl, freeze dried, and stored at −20 °C.

**Analytical Methods**—12% SDS-polyacrylamide gel electrophoresis gels were run at 35 mA and stained with Coomassie Blue (19). DNA and N-termine protein sequencing were carried out on Applied Biosystems 373A and 487A automated sequencers respectively by Alta Bioscience, School of Biosciences, University of Birmingham. Protein concentration were determined by the biuretichinic acid method calibrated with bovine serum albumin (Pierce Chemical Company).

**Isothermal Titration Microcalorimetry**—Experiments were carried out using a Microcal Inc. isothermal titration microcalorimeter. Proteins were dialyzed extensively against 20 mM MOPS, pH 7.0, 7 mM MgCl\(_2\), 0.5 mM EGTA, +1 mM dithiothreitol, loaded on a CM-Sepharose Fast Flow column (2.5 cm), and eluted in the same buffer. The fractions containing cTnC, cTnC N-domain, or cTnC C-domain. Protein concentrations were 3 µM (+Ca\(^{2+}\)) or 6 µM (−Ca\(^{2+}\)) cTnC solution in the cell titrated with 60 µM (+Ca\(^{2+}\)) or 160 µM (−Ca\(^{2+}\)) cTnI or cTnC D-domain. When cTnI was titrated with cTnC N-domain in the presence of Ca\(^{2+}\) concentrations were 6 and 160 µM, respectively. The injection size was 5 µl, with duration of 10 s, at 210-s intervals with stirring at 350 rpm. Control titrations of buffer with cTnI or its isolated domains and titrations of cTnI with buffer indicated that heats of dilution were small and constant. In each experiment the heat of dilution was obtained from additional injections following complete saturation and was subtracted from the binding isotherm. Origin™ ITC data analysis software (Microcal Inc.) was used in the “one set of sites” mode to analyze all binding isotherms.

**RESULTS**

**The Effect of Temperature on the ΔH of cTnC/cTnI Binding**—To determine the optimum temperature at which to monitor cTnC/cTnI binding, the effect of temperature on the binding enthalpy was studied between 16 and 37 °C (Fig. 1). In the absence of Ca\(^{2+}\) (but in the presence of Mg\(^{2+}\)) the ΔH was +4 kJ mol\(^{-1}\) at 16 °C. As the temperature was increased, ΔH decreased linearly to −46 kJ mol\(^{-1}\) in the absence of Ca\(^{2+}\) (Fig. 2). These results also indicate that the minimum temperature at which ΔH was large enough to obtain good data in the absence of Ca\(^{2+}\) was 30 °C.

This temperature was therefore used for all further experiments.
ments. In the presence of Ca\(^{2+}\) the binding process was considerably more exothermic across the entire temperature range, although \(\Delta C_p\) was similar \((-2.2 \pm 0.1 \text{ kJ mol}^{-1} \text{ K}^{-1})\). The large negative value of \(\Delta C_p\) upon cTnC-cTnI binding is probably accounted for by a decrease in the solvent-exposed nonpolar surface area \(23\). This could be the result of movement of apolar residues within cTnC and/or cTnI on interaction or, more probably, the burial of hydrophobic residues at the binding interface. The fact that \(\Delta C_p\) is very similar in the presence and absence of Ca\(^{2+}\) is consistent with the binding of the cTnC N-domain to cTnI being thermally neutral under these conditions \(23\) (see Fig. 5a). This suggests that the area of nonpolar residues buried at the cTnC C-terminal domain-cTnI interface is similar in the presence of Mg\(^{2+}\) or Ca\(^{2+}\).

Proton Release during cTnC-cTnI Binding—If the cTnC-cTnI binding process results in a net release or absorption of protons then an equivalent number of protons must be absorbed or released by the buffer. This process will contribute to the observed \(\Delta H\) for cTnC-cTnI binding and therefore has to be accounted for. To assess whether there is a significant absorption or release of protons upon cTnC-cTnI complex formation \(\Delta H\) was measured in experiments using buffers with enthalpies of ionization ranging from \(-2.35\) to \(-36.51 \text{ kJ mol}^{-1}\) (cacyclate, Pipes, MOPS, and imidazole) \(24\). The results in Fig. 2 show that the observed \(\Delta H\) changed very little, either in the presence or absence of Ca\(^{2+}\), and was not dependent on the buffer, demonstrating that there was little or no net release or absorption of protons during binary complex formation.

Binding of cTnC to cTnI—Initially the experiments were performed in the presence of 0.3 M KCl to allow comparison with previous work \(25,26\). The result of a typical ITC experiment is illustrated in Fig. 3. A trace of the calorimetric titration of cTnI with cTnC in the absence of Ca\(^{2+}\) is shown \(25\). The negative peaks show that the interaction is exothermic. Each deflection represents the heat released by cTnC binding to cTnI with each injection. The binding isotherm derived from these data is plotted in Fig. 3b. This graph shows the integrated heats for each cTnC injection versus the molar ratio of cTnC to cTnI. From these data the stoichiometry \(n\), binding constant \(K_n\) and enthalpy \(\Delta H\) of binding were obtained directly, and the changes in Gibbs free energy \(\Delta G\) and entropy \(\Delta S\) of binding were calculated using Equation 2.

\[
\Delta G = -RT \ln K_n = \Delta H - T\Delta S
\]

(Eq. 2)

The average \(K_n\), \(\Delta H\), \(\Delta G\), and \(\Delta S\) values from a minimum of five independent ITC binding experiments are given in Table I. As expected, the \(K_n\) was ~6-fold higher \((17.4 \times 10^7 \text{ M}^{-1})\) in the presence of Ca\(^{2+}\) than in the absence of Ca\(^{2+}\). The binding stoichiometry was essentially 1:1 under both sets of conditions.

Binding of cTnI to the cTnC N- and C-domains—Initial studies of the binding of the cTnC N- and C-domains were carried out under conditions identical to those employed for intact cTnC. The results summarized in Table I show striking similarities for the thermodynamic properties of whole cTnC and cTnC C-domain binding to cTnI. The large negative enthalpy change drives complex formation in both cases although there is a small entropic contribution in the absence of Ca\(^{2+}\). The affinities of cTnC and cTnC C-domain for cTnI were very similar in the presence of Ca\(^{2+}\) but higher than those in the absence of Ca\(^{2+}\). This indicates that the C-terminal domain of cTnC is the major contributor to the overall binding affinity of cTnC to cTnI. We find that Ca\(^{2+}\)-saturated cTnC C-domain binds to cTnI with an 8-fold higher affinity than the Mg\(^{2+}\)-saturated cTnC C-domain. Control binding assays with increased concentrations of Mg\(^{2+}\) \((\text{up to 10 mM})\) gave binding parameters identical to assays performed under standard conditions, indicating that the cTnC C-domain was fully saturated with Mg\(^{2+}\) in the presence of 3 mM Mg\(^{2+}\).

Attempts to monitor the binding of the isolated cTnC N-domain to cTnI in the presence of Ca\(^{2+}\) proved unsuccessful in the presence of 0.3 M KCl, even at different temperatures (data not shown). Consequently studies were undertaken to investigate the effect of ionic strength on the binding of whole cTnC, cTnC N- and C-domains to cTnI.

Effect of Ionic Strength on cTnC and Its Isolated Domains Binding to cTnI—The effect of increasing ionic strength on the binding of cTnC to cTnI is shown in Fig. 4a. In the absence of Ca\(^{2+}\) \((\text{Mg}^{2+}\) only) the affinity of cTnC for cTnI decreased as the ionic strength was increased, whereas in the presence of Ca\(^{2+}\) the affinity increased as ionic strength was increased. Similar trends were observed when cTnI was titrated with cTnC C-domain \(25\). In the absence of Ca\(^{2+}\) the affinity of the cTnC C-domain for cTnI decreased dramatically as the ionic strength was increased, and in the presence of Ca\(^{2+}\) the affinity of cTnC C-domain for cTnI increased slightly as ionic strength was increased.

cTnC N-domain bound to cTnI in both the presence and absence of Ca\(^{2+}\) in the 0–0.1 M KCl range. In the presence of Ca\(^{2+}\), binding of the cTnC N-domain to cTnI was exothermic, equimolar, decreased slightly as ionic strength was increased, and was ~8-fold weaker than that of the cTnC C-domain under

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**Fig. 1.** The temperature dependence of \(\Delta H\) associated with cTnC binding to cTnI. The \(\Delta H\) for cTnC binding to cTnI was measured at eight temperatures. Assay conditions were 20 mM MOPS, pH 7.0, 0.3 M KCl, 3 mM MgCl\(_2\), 0.5 mM EGTA, in the absence or presence of 1 mM CaCl\(_2\). Protein concentrations were: 6 \(\mu\text{M}\) cTnI and 160 \(\mu\text{M}\) cTnC \((-\text{Ca}^{2+}\) + \(\bullet\)) or 3 \(\mu\text{M}\) cTnI and 60 \(\mu\text{M}\) cTnC \((+\text{Ca}^{2+})\) \((\square)\).
similar conditions (Fig. 4b). In the absence of Ca\(^{2+}\), binding was endothermic and therefore must be entropically driven. However, conditions could not be found to increase the cTnC N-domain/cTnI stoichiometry much beyond ~0.5, making the data difficult to interpret. The endothermic binding of cTnC N-domain to cTnI in the absence of Ca\(^{2+}\) also appeared to contribute to the binding of whole cTnC to cTnI, at concentrations of KCl less than 0.1 M, giving rise to a two-site binding curve that was difficult to interpret, hence these data are not shown.

Effect of Ionic Strength on Enthalpy and Entropy of Binding.—The effect of varying the ionic strength on \(\Delta H\) and \(T\Delta S\) of cTnC and its isolated domains binding to cTnI resulted in the linear plots shown in Fig. 5, a and b, respectively. The thermodynamic parameters of cTnC C-domain binding to cTnI closely paralleled those obtained with whole cTnC both in the presence and absence of Ca\(^{2+}\) and were quite dissimilar to those observed for the cTnC N-domain. The \(\Delta H\) observed for cTnC N-domain binding to cTnI approached zero as the ionic strength was increased. Because ITC monitors the heat change

as a result of complex formation a very small \(\Delta H\) made it difficult to measure cTnC N-domain/cTnI binding affinities at ionic strengths greater than 0.1 M. The \(\Delta H\) and \(T\Delta S\) results show that changes in enthalpy were the driving force for the interaction between cTnC and cTnC C-domain with cTnI both in the presence and absence of Ca\(^{2+}\), as both \(\Delta H\) and \(T\Delta S\) values were negative over most of the range of salt concentrations studied. The interaction between cTnC N-domain and cTnI is entropically favorable at low salt concentrations and entropy would be the only driving force at physiological ionic strength (as determined from extrapolation of Fig. 5, a and b).

Sedimentation Equilibrium Studies on cTnI—Because of the potential of cTnI to aggregate it was important to show that aggregation was not the cause of differences in cTnC-cTnI binding observed at different ionic strengths. Sedimentation equilibrium experiments were therefore performed on cTnI under the conditions used for ITC. A typical plot illustrating the relationship between the lnF and the square of the distance from the axis of rotation (\(\rho^2\)) is shown in Fig. 6. The linear relationship of this analysis over the entire length of the solution column demonstrates that the cTnI is monodisperse in solution. Furthermore, this linearity was observed over all of the KCl and protein concentrations used and in the presence and absence of Ca\(^{2+}\). The estimated molecular mass obtained for cTnI by sedimentation equilibrium, by averaging the molecular mass at all KCl conditions, was 22.6 ± 1.1 kDa (S.E.). This agrees with the known molecular mass of cTnI (23,906 Da) calculated from the amino acid composition and observed in this laboratory by mass spectrometry.2

DISCUSSION

Fluorescence and surface plasmon resonance studies have been used previously to establish the affinity of cTnC for cTnI, e.g. (25–28). Although these methods have provided much valuable information, fluorescence techniques require modification of the proteins with reporter groups, and surface plasmon resonance produces orientation constraints because of protein immobilization. We have used ITC, a technique for directly measuring \(K_a\) and \(\Delta H\) values in biological systems, to examine the binding of cTnC to cTnI under a variety of ionic conditions. No protein modification was required, and the experiments were performed with the proteins free in solution. Furthermore, we have studied the binding of the recombinant N- and C-terminal domains of cTnC to cTnI. Previous studies, using a

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2 M. J. Calvert, D. G. Ward, P. Ashton, and I. P. Trayer., unpublished results.
A variety of techniques, have shown that the isolated domains of both skeletal and cardiac TnC have structures and properties very similar to those in the intact proteins (16, 29, 30).

Because it is known that TnI has a tendency toward self-association, it was important to ascertain whether it was monomeric or forming aggregates in solution, under the conditions used in the ITC experiments. From the sedimentation equilibrium studies the linearity of the graphic analysis was consistent with sample homogeneity. No indication of dimerization or higher order aggregation was ever observed, even in the absence of KCl, in the presence or absence of Ca$^{2+}$, with protein concentrations higher than those used for ITC. Furthermore, previous studies from our laboratory (31) have demonstrated that both cTnC and the cTnC-cTnI complex show no tendency toward aggregation at and above the protein concentrations used in these ITC experiments. These data indicate that the protein solutions used for the ITC experiments were all monodisperse and that effects of ionic strength on binding were not caused by protein aggregation. The fact that all observed binding ratios of cTnC (or its isolated domains) with cTnI were essentially 1:1 under all conditions studied is consistent with this finding.

The results show that the affinity of cTnC for cTnI is strongly dependent on ionic strength. All previously reported measurements of the affinity of cTnC for cTnI were obtained at concentrations of 0.3 M KCl or greater (25–28). At these high ionic strengths the affinity of cTnC for cTnI was significantly higher in the presence of Ca$^{2+}$ than in the presence of Mg$^{2+}$, as we have found here. At physiological ionic strength, however, we found no increase in the affinity of cTnC for cTnI upon addition of Ca$^{2+}$. These data suggest that around physiological ionic strength the cTnC/cTnI interaction in the presence and ab-

### Table I

| Species bound to cTnI | $K_a \times 10^{-7}$ | $n$ | $\Delta H$ | $\Delta G$ | $T\Delta S$ |
|----------------------|---------------------|----|------------|------------|-------------|
| cTnC ($-\text{Ca}^{2+}$) | 2.7 (±0.3) | 0.91 (±0.02) | -34.6 (±1.4) | -43.1 | 8.5 |
| cTnC ($+\text{Ca}^{2+}$) | 17.4 (±1.6) | 0.79 (±0.02) | -92.5 (±2.0) | -47.8 | -44.6 |
| cTnC C-domain ($-\text{Ca}^{2+}$) | 2.2 (±0.2) | 0.89 (±0.05) | -39.5 (±2.4) | -41.1 | 1.6 |
| cTnC C-domain ($+\text{Ca}^{2+}$) | 10.0 (±2.5) | 0.79 (±0.05) | -76.6 (±5.2) | -46.4 | -30.2 |

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Fig. 4. Effect of ionic strength on the binding of cTnC and its isolated domains to cTnI. Assay conditions were: 20 mM MOPS, pH 7.0, 3 mM MgCl$_2$, 0.5 mM EGTA, 30 °C, with increasing KCl concentrations, in the absence or presence of 1 mM CaCl$_2$. Panel a, effect of increasing ionic strength on the binding of cTnC to cTnI. Protein concentrations were 6 μM cTnI and 160 μM cTnC ($-\text{Ca}^{2+}$) (○) or 3 μM cTnI and 60 μM cTnC ($+\text{Ca}^{2+}$) (●). Panel b, effect of increasing ionic strength on the binding of cTnC C-domain and cTnC N-domain to cTnI. Protein concentrations were: 6 μM cTnI and 160 μM cTnC C-domain ($-\text{Ca}^{2+}$) (●), 3 μM cTnI and 60 μM cTnC C-domain ($+\text{Ca}^{2+}$) (×) or 6 μM cTnI and 160 μM cTnC N-domain ($+\text{Ca}^{2+}$) (●). Each data point shown is the mean of at least three independent determinations ± S.E.
sence of Ca\(^{2+}\) is finely balanced, although Ca\(^{2+}\) clearly changes the mode of interaction as demonstrated by changes in \(\Delta H\) and \(T\Delta S\). It should be noted, however, that we have of necessity investigated only the isolated binary interaction between cTnC and cTnI in these studies. It is possible that the other thin filament protein components could influence the effect of ionic strength on the Ca\(^{2+}\) sensitivity of the cTnC/cTnI interaction.

We find that the effect of Ca\(^{2+}\) and ionic strength on the affinity and thermodynamic parameters of the cTnC C-domain binding to cTnI closely parallel those found with whole cTnC.

**Fig. 5.** Effect of increasing ionic strength on the enthalpy and entropy associated with the binding of cTnC and its isolated domains to cTnI. Panel a, \(\Delta H\), and panel b, \(T\Delta S\). Data were obtained from the binding of cTnI to cTnC in the presence (○) and absence (●) of Ca\(^{2+}\), cTnC C-domain in the presence (×) and absence (●) of Ca\(^{2+}\), and cTnC N-domain the presence of Ca\(^{2+}\) (△). Each data point shown is the mean of at least three independent determinations (± S.E. shown for \(\Delta H\)). For conditions, see the legend to Fig. 4.

**Fig. 6.** Sedimentation equilibrium of cTnI. Analytical ultracentrifugation conditions were: 20 mM MOPS, pH 7.0, 0.1 mM KCl, 3 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.5 mM EGTA, 30 °C. An AN-D rotor was used to centrifuge cTnI (20 μM) at 35,000 rpm for 7 h. The plot shows the radial distance squared \((r^2)\) versus the lnf. The molecular mass calculated from the linear regression of \(r^2\) versus lnf was 23,670 Da. The bottom of the cell is indicated by a black arrow.
These data suggest that the C-terminal domain of cTnC dominates the steady-state interaction with cTnI in both the presence and absence of Ca\(^{2+}\). These results contrast sharply with the binding of cTnC N-domain to cTnI over the limited ionic strength range where we were able to obtain data. Even in the presence of Ca\(^{2+}\), the affinity of cTnI for the cTnC N-domain was significantly lower than that measured for the cTnC C-domain. It is interesting to note that where data for the binding of cTnI to the isolated domains of cTnC can be compared with whole cTnC, considerable constraints occur in the latter interaction. For example, at 0.1 M KCl in the presence of Ca\(^{2+}\), if we consider that cTnI binds simultaneously to both domains of cTnC, the overall affinity is the product of the individual affinities for each domain, then one would expect a $K_a$ of $10^{12}$ M\(^{-1}\) for cTnC-C-TnI in the $10^{14}$ M\(^{-1}\) range (Fig. 5b). Clearly the interactions of the two domains of whole cTnC with cTnI are not independent and influence one another.

We observed that the binding of cTnC and cTnC C-domain to cTnI were dramatically affected by the addition of Ca\(^{2+}\) over a range of ionic strengths. In the absence of Ca\(^{2+}\), the reduction in $K_a$ as the ionic strength was increased, both for the C-terminal domain of cTnC and whole cTnC, may suggest that electrostatic interactions dominate the binding when Mg\(^{2+}\) occupies sites III and IV. On the other hand, when Ca\(^{2+}\) is bound to these sites, increasing the ionic strength increased the affinity, especially between the intact proteins, a characteristic of interactions in which hydrophobic forces dominate. This agrees with NMR (16) and crystallographic data (15) that show extensive Van der Waals interactions between TnC and TnI in the presence of Ca\(^{2+}\). It also suggests a difference in the structure of the C-terminal domain of cTnC when it is occupied by Mg\(^{2+}\) or Ca\(^{2+}\). It may be that Ca\(^{2+}\) occupancy of sites III and IV “opens” up the paired EF hands exposing hydrophobic sites as seen in the crystal structures of skTnC (2) and skTnC bound to the N-terminal region of skTnI (15), whereas Mg\(^{2+}\) is not able to do this fully. There is presently no high resolution structure for TnC with Mg\(^{2+}\) bound in sites III and IV, which would be particularly valuable to compare with the Ca\(^{2+}\)-bound state. Several previous studies are consistent with our findings. Different fluorescence changes were found within the C-terminal domain of skTnC depending upon whether Mg\(^{2+}\) or Ca\(^{2+}\) were bound (32, 33). Analysis of the binding of Mg\(^{2+}\) and Ca\(^{2+}\) to cTnC by microcalorimetry suggests that Mg\(^{2+}\) and Ca\(^{2+}\) induce different conformations of the C-terminal domain of cTnC (34). Microcalorimetry has also been used to show that conformational changes occur in the C-terminal domain of skTnC when Ca\(^{2+}\) replaces Mg\(^{2+}\) in sites III and IV, although this differs somewhat from those observed in the cardiac isoform (35).

Our data demonstrate, for the first time, that there are structural differences within the C-terminal domain of cardiac TnC, depending on whether Ca\(^{2+}\) or Mg\(^{2+}\) is bound, which affect the characteristics of the cTnC/cTnC interaction. Experiments with skeletal isoforms also suggest Ca\(^{2+}\) sensitivity in the binding of skTnC/C-terminal domain to skTnI. An increase in the affinity of a skTnC tryptic fragment, corresponding to the C-terminal domain of skTnC, for skTnI in the presence of Ca\(^{2+}\) has been noted previously (36). Additionally, Pearlstone and Smillie (37) found that a higher ionic strength was required to elute the C-terminal domain of skTnC from a skTnI peptide affinity column in buffer containing Ca\(^{2+}\) rather than Mg\(^{2+}\). The difference in cTnC-cTnI affinity would be important if Ca\(^{2+}\) can exchange for Mg\(^{2+}\) at sites III and IV in working cardiac muscle. It has not been determined if the C-terminal sites of TnC are bound with Mg\(^{2+}\) at all times or if the Ca\(^{2+}\) signal is of sufficient duration to displace Mg\(^{2+}\) in the contractile state. Although modeling studies (38) suggest that Mg\(^{2+}\) dissociation from sites III and IV is likely to be too slow to be completed in a single contraction-relaxation cycle, they also predict that Ca\(^{2+}\) occupancy of the C-terminal domain of cTnC is a measure of the intensity and frequency of muscle contraction and could therefore increase with increased heart rate. This would lead to a change in the structure of cTnC and enhanced affinity for cTnI, allowing the C-terminal domain of cTnC to play a modulatory role in the regulation of the contractile cycle. Our results give no indication as to whether the change in affinity of cTnC C-domain for cTnI, in the presence and absence of Ca\(^{2+}\), reflects a different mode of binding to residues 33–80 of cTnI or whether a different region of cTnI displaces the cTnI helix at positions 33–80 in response to Ca\(^{2+}\) as in the model proposed by Triplet (39).

The thermodynamic results obtained by ITC give some interesting insights into the cTnC/cTnI interaction. Previously the $\Delta G$ difference between Ca\(^{2+}\) and Mg\(^{2+}\)-bound states of cTnC binding to cTnI, at 0.4 M KCl, was attributed solely to Ca\(^{2+}\) binding to “regulatory” site II in the N-terminal domain of cTnC (40). This change in $\Delta G$ was thought to be responsible for Ca\(^{2+}\) activation in cardiac muscle. Our data suggest that at high ionic strengths most of the Ca\(^{2+}\)-dependent increase in $\Delta G$ of cTnC binding to cTnI arises from Ca\(^{2+}\) binding to sites III and IV. Our results demonstrate that any differences between the steady-state binding of the Mg\(^{2+}\) and Ca\(^{2+}\) forms of cTnC to cTnI cannot solely be attributed to Ca\(^{2+}\) binding to the N-terminal domain of cTnC.

Parallels can be drawn between our cTnC-cTnI work and published ITC data on calmodulin-target peptide recognition (41, 42). Quantitatively similar $\Delta H$, $\Delta T S$, and $\Delta C_p$ values and negligible proton release are seen in both cases. The negative $\Delta C_p$ values indicate internalization of hydrophobic surfaces upon complex formation, a process that is entropically favorable. Wintrode and Privalov (41) considered that burial of hydrophobic surfaces could not be the major driving force in the enthalpically driven binding of calmodulin to target peptide. However, it cannot be excluded that the entropy associated with the burial of hydrophobic surfaces is important in offsetting unfavorable entropy changes arising from noncovalent bond formation and increased order in the cTnC-cTnI complex. Both domains of calmodulin appear to contribute similarly to the overall $\Delta H$ of target peptide binding (41), whereas tissue-specific adaptations of the cardiac homolog, cTnC, to muscle regulation result in very different contributions of the cTnC N-terminal and C-terminal domains to the overall $\Delta H$.

This study has revealed the importance of ionic strength on the cTnC-cTnI interaction, which must be considered when interpreting data. Large changes in the $\Delta H$ of cTnC-cTnI binding, but little change in affinity at physiological ionic strength, could imply that the mode but not the strength of the cTnC/cTnI interaction changes in response to Ca\(^{2+}\). We have also demonstrated that the interaction between cTnC C-domain and cTnI is Ca\(^{2+}\)-sensitive and that cTnC C-domain is responsible for most of the energy difference observed between cTnC-cTnI binding in the presence and absence of Ca\(^{2+}\). Further studies are required to clarify the importance of the cTnC C-domain in regulation. It will be interesting to compare the results obtained for cTnC and its isolated domains binding to cTnI with data obtained using the skeletal isoforms, where significant differences in sequence and binding occur.

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3. D. G. Ward and M. J. Calvert, unpublished data.
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