Evidence for Two-Site Binding of Troponin I Inhibitory Peptides to the N and C Domains of Troponin C†

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ABSTRACT: The interactions of two troponin I peptides, Ip1 (residues 96−116) and Ip2 (residues 104−116), with spectral probe mutants F29W and F105W of intact troponin C (TnC) and of isolated N (residues 1−90) and C (residues 88−162) domains of TnC have been examined. Ip-induced fluorescence emission spectral changes were observed with all four proteins in the presence of Ca^{2+}. Different dependencies of these spectral changes on Ip concentration for intact F29W and F105W are interpreted in terms of two binding sites on TnC. The binding of Ip1 to the C domain (K_{D1} = 0.50 μM) is 20−40-fold stronger than to the N domain. The binding affinity of Ip1 to both the N and C domains is greater than that of Ip2. The binding strengths of Ip1 to the N domain of intact F29W and isolated F29W/ND are the same within experimental error; that to isolated F105W/CD is weakened by 5−6-fold relative to the C domain of intact F105W. Ip-induced fluorescence changes are dependent on the presence of Ca^{2+} and are not seen in the presence of Mg^{2+} alone nor in the absence of divalent cations. This is true even though Ip2 binds to TnC under all three conditions, as demonstrated by affinity chromatography. The accumulated evidence indicates that the F → W mutations have not significantly affected the binding of Ip peptides to TnC. The binding of Ip1 to the C domain of intact TnC increases its Ca^{2+} affinity without significantly affecting that of the N domain; only at high molar ratios of Ip1:TnC is the Ca^{2+} affinity of the N domain increased.

Troponin C (TnC)[1] together with troponin I (TnI) and troponin T (TnT) constitute the troponin complex, which with tropomyosin and F-actin form the regulated thin filament of skeletal and cardiac muscles. The binding of Ca^{2+} to TnC triggers a series of conformational changes that, when transmitted through the other troponin components to tropomyosin and actin, facilitate interaction with the myosin head and generation of ATPase activity and contraction [for reviews, see Potter and Johnson (1982), Leavis and Gergely (1984), Zot and Potter (1987), Parmacek and Leiden (1991), and Grabarek et al. (1992)].

X-ray crystallographic analyses of turkey (Herzberg & James, 1985a,b) and chicken (Sundaralingam et al., 1985; Satyshur et al., 1988) TnC have demonstrated the presence of two globular domains, N and C, separated by an extended α helix. In the crystals, grown at ~pH 5, the two high-affinity C domain Ca^{2+}/Mg^{2+} binding sites III/IV are occupied by Ca^{2+}; those of the low-affinity N domain sites I/II, specific for Ca^{2+}, are unfilled. Each of the four metal binding sites is arranged in the helix−loop−helix motif common to this class of proteins. In the N domain, helices A and B (site I) and helices C and D (site II) are complemented by an additional helix N (residues 3−13) whose side chains interact with helices A and D. This N helix has no counterpart in the C domain nor in other Ca^{2+} binding proteins such as calmodulin. The orientation of N domain helices B and C to those of the N, A, and D helices is different from that for the corresponding C domain helices E−H, whose sites III/IV are occupied by Ca^{2+}. Herzberg, Moult, and James (1986) have proposed a model for the Ca^{2+}-induced conformational transition of the N domain to a structure similar to that of the Ca^{2+}-filled C domain, with which it shows extensive sequence homology. As part of this structural change, a number of nonpolar residues, either partially or totally buried in the $2Ca^{2+}$ state, would become exposed on the surface of the protein to form a "hydrophobic patch" in the $4Ca^{2+}$ state. The fully Ca^{2+} loaded protein would now have two such nonpolar surfaces, one on each of the N and C domains, and each associated with a constellation of negatively charged residues. The Ca^{2+}-induced secondary structural changes proposed in this model have been confirmed by a recent NMR study (Gagné et al., 1994) of the apo (^1H and ^{15}N) and Ca^{2+}-saturated (^1H, ^{15}N, ^{13}C) N domain of TnC (residues 1−90). In the apo form, the spatial disposition of secondary structural elements was the same as that in the X-ray structure. In the Ca^{2+}-loaded state, the NMR secondary structural analysis was in general agreement with the model, although some differences were noted.

As part of a continuing study of the divalent cation-induced structural transitions of TnC and of its interactions with the other troponin components, several F → W mutants have been prepared and used successfully (Pearlstone et al., 1992; Trigo-Gonzales et al., 1992; Chandra et al. 1994) to monitor Ca^{2+}/Mg^{2+}-induced structural changes in the N and C domains of intact TnC. Since Trp is absent from wild-type protein (Golosinska et al., 1991), spectral properties could be unambiguously assigned to the single Trp. Recently, the

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[1] Abbreviations: TnC, troponin C; rTnC, wild-type recombinant TnC; F29W/ND, F29W N domain fragment (residues 1−90); F105W/CD, F105W C domain fragment (residues 88−162); TnI, troponin I; Ip1, troponin I inhibitory peptide (residues 96−116); Ip2, troponin I inhibitory peptide (residues 104−116); TnT, troponin T.

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F29W and F105W mutants were prepared in isolated N domain (residues 1−90) and in isolated C domain (residues 88−162) (Li et al., 1994), thus facilitating comparisons of the properties of the isolated domains with those in the intact F29W and F105W TnC molecules. Analyses of secondary structure and Ca^{2+} affinity by far-UV CD and fluorescence spectroscopy indicated that the N domain had virtually the same properties in both the isolated state and the intact protein. Those of the isolated C domain were similar, but not identical.

Recently, we have shown that the spectral features of F154W TnC can be applied to the measurement of binding affinities of TnI inhibitory peptides to the intact protein (Chandra et al., 1994). In the present report, we extend this approach to an investigation of the interaction of two TnI inhibitory peptides, Ip1 (residues 96−116) and Ip2 (residues 104−116), with intact F29W, intact F105W, and isolated N and C domains carrying these same F → W mutants. The observations are interpreted in terms of two binding sites on TnC, in which binding to the C domain is 20−40-fold stronger than that to the N domain.

EXPERIMENTAL PROCEDURES

TnC Mutants. The expression and preparation of intact F29W and F105W TnC proteins from cDNA clones of chicken skeletal muscle have been reported previously (Pearlstone et al., 1992; Trigo-Gonzalez et al., 1992). Isolated N (residues 1−90) and C (residues 88−162) domains, with and without the F29W and F105W mutations, were prepared as described (Li et al., 1994).

Preparation of Buffer and Sample. Detailed descriptions of stock buffers and protein sample preparation, with due care to eliminate Ca^{2+} contamination and oxidation during dialysis, have been reported previously (Golosinska et al., 1991). After dialysis against a 50 mM 3-morpholinopropanesulfonic acid, 100 mM KCl, 1 mM EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid), 1 mM dithiothreitol (pH 7.1) buffer, the 1 or 2 mg/mL stock protein solutions (for N and C domain fragments or for intact TnC proteins, respectively) were diluted with 0.22 μm filtered outer dialysate buffer. Final protein concentrations are given in the figure legends.

TnI Inhibitory Peptide Titrations of Ca^{2+}-Saturated F105W and F29W Mutants of TnC. Two rabbit skeletal TnI inhibitory peptides Ip1 (residues 96−116 with free NH_2 and COOH termini) and Ip2 (residues 104−116 with acetylated NH_2 and free COOH termini) were synthesized by the Alberta Peptide Institute, University of Alberta. Stock solutions of these peptides were made with the same final filtered buffer that was used for dilution of the TnC proteins. TnI peptide concentrations were determined by duplicate amino acid analyses as described previously (Golosinska et al., 1991). Ca^{2+} to 2 mM was added to the protein solution prior to titration.

Fluorescence Measurements. Trp fluorescence measurements were conducted on a Perkin-Elmer MPF-44B fluorescence spectrophotometer operating in the ratio mode and equipped with a water bath at 20 °C, as described previously (Pearlstone et al., 1992). A DSCU-2 differential corrected spectra unit was used with the difference setting in the blank mode in order to correct for the solvent blank. The slit bandwidth was 5 nm for both excitation and emission. The A_{282} did not exceed 0.05, eliminating the need to make inner filter corrections. Measurements were with a 1 cm semimicrocell from Hellma, and detection was at right angles. The excitation wavelength was 282 nm. Titrations were monitored by following the increase in fluorescence emission intensity at 350 nm. For Ca^{2+} titrations, the calculations of free [Ca^{2+}] and the fitted curve were obtained as described previously using a nonlinear iterative least-squares procedure for one or two binding components (Golosinska et al., 1991). For TnI peptide titrations of Ca^{2+}-saturated TnC F → W mutants, the calculated fitted curves and dissociation constants from these curves were derived using a nonlinear least-squares computer program for 1:1 or 2:1 binding of ligand to protein, in which the change in fluorescence intensity is analyzed as a function of total ligand concentration, and the concentration of the ligand-bound form is obtained as described by Williams et al. (1985).

Affinity Chromatography. Inhibitory peptide Ip2 (residues 104−116) was coupled to cyanogen bromide-activated Sepharose 4B according to instructions provided by Pharmacia. As a control, activated Sepharose 4B was subjected to the coupling procedure without inclusion of the inhibitory peptide. By taking precautions for eliminating inherent Ca^{2+} during the preparation and storage of stock buffers as reported earlier (Golosinska et al., 1991), the following three buffers were used: apo (50 mM MOPS, 0.1 M NaCl, and 0.1 mM EGTA, pH 7.1); $+Ca^{2+}$ (same as apo buffer plus 0.3 mM $CaCl_2$); $+Mg^{2+}$ (same as apo buffer plus 5 mM $MgCl_2$). The column (a 1 mL plastic syringe, 5 × 40 mm) was equilibrated with starting buffer using a flow rate of 10 mL/h. Stock sample solutions were made in each of the three buffers by dissolving 1.0 (N or C domain fragment) or 2 mg/mL (intact TnC) in the appropriate buffers, to which solid DTT (1 mM final concentration) had been added. Following centrifugation in prerinsed Spin-X tubes (Costar) equipped with 0.22 μm nylon filters and determination of protein concentrations by duplicate amino acid analyses, samples were stored frozen prior to the application of 14 nmol to the column. After the column was washed with several column volumes of starting buffer, a linear gradient of 0.1−1.0 M NaCl was applied, and the column profile was monitored at 280 nm for W mutants and at 230 nm for wild-type proteins. Samples from the eluted peaks were analyzed by SDS−PAGE to confirm their identities (results not included).

RESULTS

Ca^{2+} Titrations of F29W and F105W in the Presence of Increasing Molar Ratios of Ip1 to Protein. Previous reports (Pearlstone et al., 1992; Trigo-Gonzalez et al., 1992) have demonstrated that F29W and F105W serve as useful spectral probes for monitoring the Ca^{2+}-induced structural transitions of the N and C domain, respectively. As a preliminary qualitative assessment of the strength of binding of Ip1 to these intact mutant proteins and its effects on the Ca^{2+} affinities of the two domains, Ca^{2+} titrations of the fluorescence change with increasing molar ratios of Ip1 to protein were carried out. Selected representative data for both mutant proteins are shown in Figure 1. For F29W, the addition of Ip1 to protein at molar ratios of 1:1 and 2:1 has only a small effect in shifting the curve to higher pCa values. When the ratio was increased to 50:1, the pCa_{50} value was increased from 5.7 for F29W alone (Pearlstone et al., 1992) to 6.2 for F29W plus Ip1. These observations may be contrasted with the effects of the addition of Ip1 to F105W.

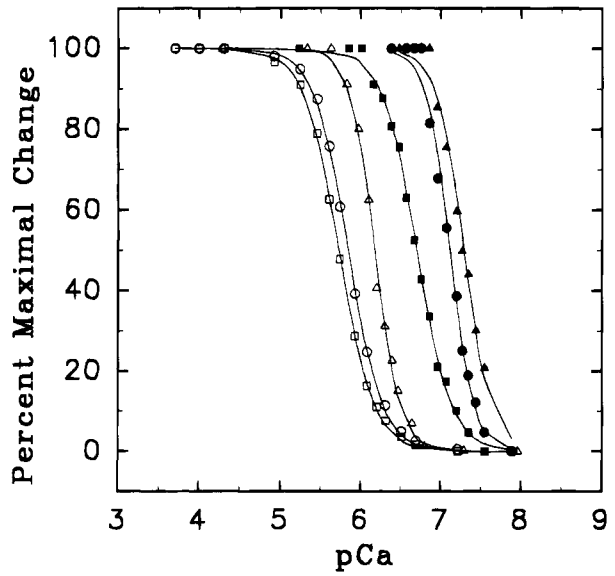

FIGURE 1: Ca^{2+} titrations of F29W and F105W TnC's in the presence of various molar ratios of Ip1 monitored by W fluorescence emission measurements. The apo starting buffer conditions are described in Experimental Procedures (temperature 20 °C); protein concentrations were in the range of 2.6−3.5 μM. For each titration, the data points are indicated by symbols and the calculated fitted curves by solid lines: Ip1:F29W = 0:1 (□), 2:1 (○), 50:1 (△), Ip1:F105W = 0:1 (■), 3:1 (●), 27:1 (▲).

At a molar ratio of Ip1:F105W of 3:1, the pCa_{50} value is shifted to 7.2 from 6.7 for F105W alone. Increasing the ratio to 27:1 led to an additional but smaller increase in pCa_{50} to ~7.3. Similar results were obtained with isolated F29W/ND and isolated F105W/CD (data not shown). On the basis of the assumption that the two spectral probe mutations have not affected the strength of Ip1 binding to TnC significantly, the data are most simply interpreted in terms of two-site binding to intact TnC. Higher affinity binding to the C domain would lead to significantly increased Ca^{2+} affinity at relatively low ratios of Ip1:F105W; weaker affinity binding to the N domain would require much higher ratios. A corollary to this interpretation is that the binding of Ip1 to the higher affinity site of the C domain has little effect on the Ca^{2+} affinity of the N domain.

Effects of Ip1 on the Fluorescence Emission Spectra of F29W, F29W/ND, F105W, and F105W/CD. To assess the feasibility of measuring the binding affinities of Ip1 and Ip2 to these Trp-labeled proteins, their fluorescence emission spectra were examined in the absence and presence of high ratios of Ip peptide to protein in the absence of divalent metal, in the presence of 44 mM Mg^{2+}, and in the presence of 1 mM excess Ca^{2+}. The fluorescence emission spectra of the two TnC F29W mutants in the absence and presence of Ca^{2+} versus Mg^{2+} ions and the effect of a 100-fold molar excess of TnI inhibitory peptide Ip1 (residues 96−116) are shown in Figure 2A (intact F29W) and Figure 2B (F29W/ND). The results for intact F29W and F29W/ND were the same. The apo and 44 mM Mg^{2+} spectra were superimposable, with an emission maximum at 336 nm as reported previously (Pearlstone et al., 1992). The addition of 100-fold molar excess Ip1 to either the apo (not shown) or the 44 mM Mg^{2+} state had no effect on the fluorescence spectra. In contrast, subsequent addition of 1 mM excess Ca^{2+} to the 44 mM Mg^{2+}/Ip1 form resulted in a 2.6-fold increase in fluorescence intensity and a blue shift in the emission maximum from 336 to 332 nm (results not included). The addition of 1 mM excess Ca^{2+} directly to the apo state resulted in a 3-fold increase in fluorescence intensity with the emission maximum unchanged at 336 nm, as seen previously (Pearlstone et al., 1992). The subsequent addition of 100-fold excess Ip1 caused a decrease in fluorescence intensity and a blue shift in the emission maximum from 336 to 331 nm, equivalent to the results with 44 mM Mg^{2+}/Ip1/Ca^{2+}. Ip1-induced perturbations in the fluorescence spectra were observed only in $2Ca^{2+}$ F29W/ND and $4Ca^{2+}$ intact F29W, indicating the Ca^{2+}-specific nature of these structural transitions in the N domain of TnC.

The fluorescence emission spectra of the two F105W mutants in Ca^{2+} versus Mg^{2+} and in the presence of 10-fold molar excess Ip1 are shown in Figure 2C (intact F105W) and Figure 2D (F105W/CD). Unlike the equivalence in spectra observed between the N domains of intact F29W (panel A) and F29W/ND (panel B), there were quantitative differences in the emission spectra of the two F105W mutants, as observed previously by Li et al. (1994). Nevertheless, the qualitative results were similar. Both apo spectra had an emission maximum at 344 nm. The addition of 44 mM Mg^{2+} caused a ~1.5-fold increase in fluorescence intensity and a blue shift from 344 to 338 nm in panels C and D, as reported by Li et al. (1994). As with the N domain, no Ip1-induced changes were observed with either the apo (results not shown) or Mg^{2+}-saturated states. Direct addition of 1 mM excess Ca^{2+} to the apo state resulted in a shift in the emission maximum for intact F105W (panel C) and a similar shift in the emission maximum plus a ~1.5 fold increase in fluorescence intensity for F105W/CD (panel D). The subsequent addition of 10-fold excess Ip1 caused a slight decrease in fluorescence intensity and a further shift in the emission maximum from 335 to 333 nm for both F105W mutants (panels C and D). These Ip1-induced structural transitions in the C domain were observed only with $2Ca^{2+}$ F105W/CD and $4Ca^{2+}$ intact F105W, indicating the Ca^{2+}-specific nature of these transitions, analogous to the situation seen with the N domain.

Fluorescence Difference Spectra of Ca^{2+}-Saturated TnC W Mutants upon the Addition of Ip1. The changes observed in the fluorescence spectra of Figure 2 upon the addition of Ip1 to the Ca^{2+}-saturated form suggested that binding constants could be obtained by titrating the Ca^{2+}-saturated TnC W mutants with Ip1. In Figure 3, the spectra of the four Ca^{2+}-saturated TnC W mutants were put, in turn, into the memory of the spectrofluorimeter and indicated as zero baseline. The resultant difference spectra are due to the blue shift in the wavelengths of the emission maxima. Figure 3 shows examples of the fluorescence difference spectra of F105W and F105W/CD (panel A) and of F29W and F29W/ND (panel B) upon the addition of 9-fold molar excess Ip1 over TnC. In each case, the fluorescence difference was quite measurable, and titration with Ip1 was monitored at ~350 nm.

Inhibitory Peptide Titrations of Ca^{2+}-Saturated F105W and F29W Mutants of TnC. The data for titration of F105W and F105W/CD are shown in Figure 4A; those for intact F29W and for F29W/ND are shown in Figure 4B. The interaction of Ip1 with the two F105W proteins is seen to approach saturation levels at molar ratios of Ip1 to protein of 10:1 and greater. Those with the F29W proteins require higher ratios since saturation is not reached even at ratios

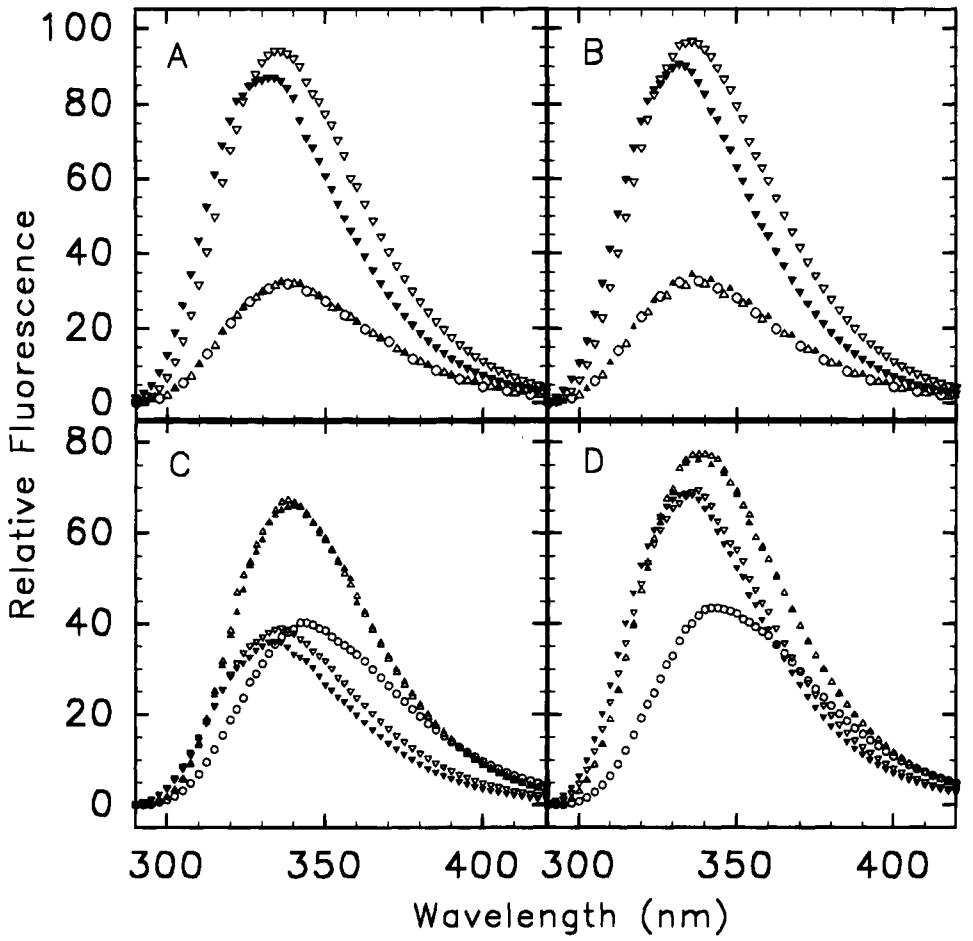

FIGURE 2: Fluorescence emission spectra of F29W (A), F29W/ND (B), F105W (C), and F105W/CD (D) TnC W mutants in apo, $+Mg^{2+}$, $+Ca^{2+}$, and in the presence of TnI inhibitory peptide Ip1. Excitation was at 282 nm. Buffer conditions were as for Figure 1. Protein concentrations were all 3.0 μM. Apo (○); Mg^{2+} added to 44 mM (△); 44 mM Mg^{2+} plus Ip1 (▲); Ca^{2+} added to 2 mM (▽); 2 mM Ca^{2+} plus Ip1 (▼). For F29W and F105W mutants, 100-fold and 10-fold excess Ip1, respectively, over TnC was added. Since the addition of Ip1 to the apo form showed no change in the spectrum for all four TnC W mutants, these results were omitted from the figure for the sake of clarity.

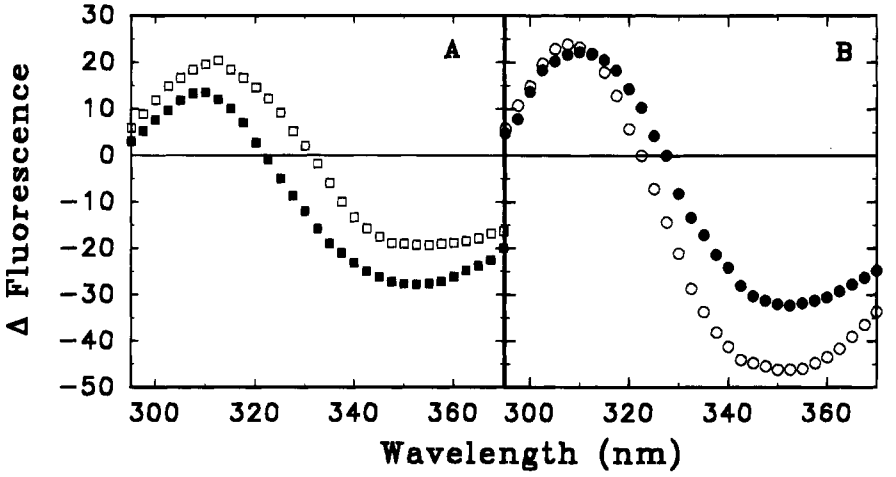

FIGURE 3: Fluorescence difference spectra of Ca^{2+}-saturated TnC W mutants upon the addition of Ip1. Panel A: F105W (■); F105W/CD (□). Panel B: F29W (●); F29W/ND (○). The zero baseline indicates the fluorescence spectrum of Ca^{2+}-saturated TnC W mutant. A 9-fold molar excess of Ip1 was added, and the spectrum was scanned from 290 to 370 nm. Excitation was at 282 nm.

greater than 15:1. These different concentration dependencies are inconsistent with a single binding site on the intact TnC molecule and suggest the likelihood of two binding sites: a stronger site on the C domain reported by the F105W probe and a weaker N domain site perturbing the fluorescence properties of F29W. For this reason, the Ip1 titration data for the two intact F105W and F29W proteins have been analyzed using a curve-fitting program for 2:1 binding of Ip1 to intact protein (Williams et al., 1986). Those for the isolated N and C domains have been fitted by assuming 1:1 binding. In these analyses, the fraction of the total fluorescence change (ΔF_t) contributed by the filling of the first site by Ip1 is designated ΔF_1; that attributable to the second site is ΔF_2. The corresponding macroscopic dissociation con-

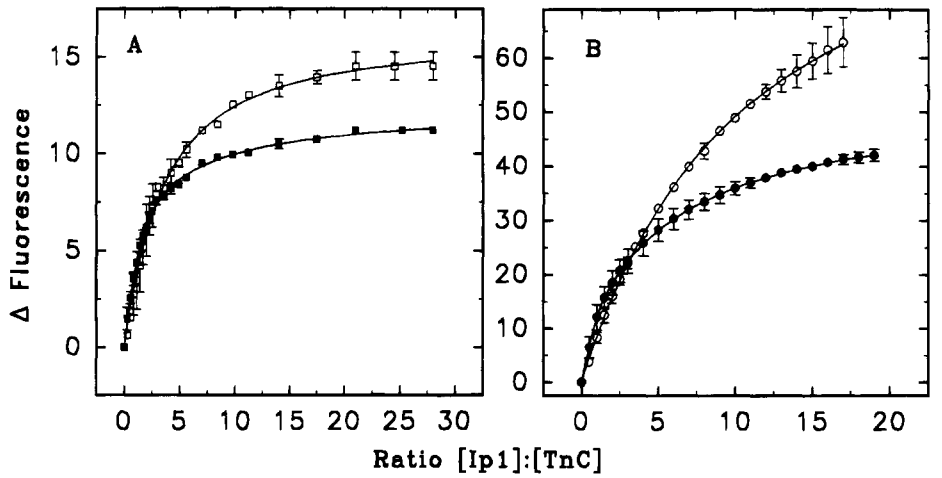

FIGURE 4: Ip1 titrations of Ca^{2+}-saturated TnC W mutants monitored by fluorescence emission measurements. Buffer conditions were as in the Experimental Procedures (temperature 20 °C); protein concentrations were 3.39 μM. Panel A: F105W (■); F105W/CD (□). Panel B: F29W (●); F29W/ND (○). For each mutant, the averaged values for 2−5 titrations are shown by symbols and the calculated fitted curves by solid lines. The calculated fitted curves were derived as described in the Experimental Procedures, assuming 2:1 binding of ligand to protein for the intact TnC's F105W and F29W and 1:1 binding for the isolated domains F105W/CD and F29W/ND. The standard deviations of the data points are indicated by vertical bars.

Table 1: Dissociation Constants for the Binding of Inhibitory Peptides Ip1 and Ip2 to the N and C Domains of Intact TnC and to the Isolated Domains[a]

		Ip1		Ip2	
proteins	binding analysis	K_{D1} (μM)	K_{D2} (μM)	K_{D1} (μM)	K_{D2} (μM)
intact F105W	two-site	0.47 ± 0.2 (2)	9.6 ± 5.0 (2)	8.0 ± 1.9 (3)	68 ± 24 (3)
isolated F105W/CD	one-site	2.8 ± 0.2 (3)		75 ± 5.3 (3)	
intact F29W	two-site	0.52 ± 0.1 (5)	24 ± 9 (5)	5.4 ± 1.8 (3)	75 ± 11 (3)
isolated F29W/ND	one-site		20 ± 2 (3)		72 ± 20 (5)

[a] The number of runs that were averaged are indicated in parentheses. The standard deviation was obtained from the average of the K_D values from 2−5 separate runs, as indicated. Dissociation constants were derived from the calculated fitted curves as described in the Experimental Procedures.

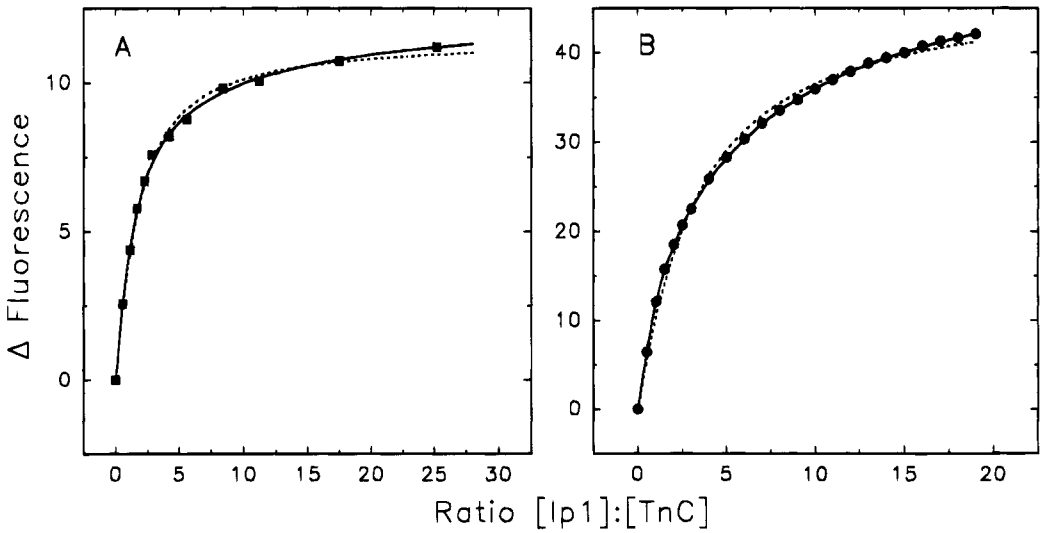

FIGURE 5: Theoretical calculated fitted curves for 2:1 versus 1:1 binding of Ip1 to F105W (A) and F29W (B). The averaged data points (same as in Figure 4) from two titrations of $4Ca^{2+}$·F105W and five titrations of $4Ca^{2+}$·F29W with Ip1 are shown by solid squares (■) and circles (●), respectively. The data points were analyzed by a nonlinear least-squares curve-fitting program (Williams et al., 1985), assuming 1:1 (•••) versus 2:1 (—) binding of ligand to protein.

stants are K_{D1} and K_{D2}, respectively. The derived best fit to the experimental data for each of the four proteins based on this approach is shown as the solid line in Figure 4A,B. The derived K_{D1} and K_{D2} values for each analysis are presented in Table 1. For comparative purposes with the 2:1 binding analyses, the data for intact F105W and F29W have also been fitted by assuming 1:1 binding and are presented in Figure 5A,B. The 2:1 binding analyses are seen to give marginally better fits than the assumption of 1:1 binding. Interestingly, the best fits for the 2:1 binding analyses were obtained when both ΔF_1 and ΔF_2 were allowed to vary. Thus, for example in the case of F29W, the best fit was obtained with ΔF_1 and ΔF_2 contributing 31% and 69% of ΔF_t, respectively. This indicates that the binding of Ip1 to

site 1 of the C domain leads to a change in the fluorescence of W29 of the N domain, indicating some degree of interaction between domains.

Inspection of the data of Table 1 permits certain observations and correlations. First of all, the values of K_{D1} (taken as a measure of binding affinity of Ip1 and Ip2 to the C domain) and K_{D2} (a measure of N domain affinity) are in good agreement, irrespective of whether they are derived from the titration of intact F105W or intact F29W. This indicates that the F → W mutants have not significantly affected the inhibitory peptide binding properties of the wild-type protein.[2] Second, binding of both Ip1 and Ip2 to the C domain is stronger than that to the N domain. For Ip1 the ratio K_{D2}:K_{D1} is 20−46; for Ip2 the corresponding ratio is 8−14. Third, the binding affinity of either Ip1 or Ip2 to the N domain appears to be unaffected by the presence (as in intact F29W or F105W) or absence of the C domain (as in F29W/ND). Thus, the K_{D2} values with intact F105W, F29W, and F29W/ND are all, within experimental error, the same. The binding affinities of Ip1 and Ip2 to the C domain, on the other hand, are affected by the presence or absence of the N domain. Thus, K_{D1} values for Ip1 and Ip2 binding to isolated F105W/CD are significantly higher than those for intact F29W and F105W. Finally, binding of Ip1 to both the C and N domains is significantly stronger than that of Ip2.

Interactions of TnC Mutants with Ip2 As Assessed by Affinity Chromatography. The strength of interactions of intact F29W and F105W, as well as the isolated domains carrying the same F → W mutants, was assessed with an affinity column of Ip2 coupled to cyanogen bromide-activated Sepharose 4B. The column was operated under three conditions (see Experimental Procedures) for each protein: apo (absence of divalent ions); apo plus 0.3 mM Ca^{2+}; apo plus 5 mM $MgCl_2$. The elution position of the mutant protein upon the application of a linear gradient from 0.1 to 1 M NaCl was taken as a measure of its strength of binding to the Ip2 ligand. As a control, an identical column was prepared with activated Sepharose 4B that had been subjected to the coupling procedure in the absence of Ip2. TnC applied to this column failed to bind and was eluted in the flow through. The results of these analyses are summarized in Table 2. All three intact proteins (rTnC, F29W, and F105W) behaved identically in this system. In the plus Ca^{2+} buffer, the peak position of elution was at 0.60 M NaCl; in plus Mg^{2+} it was at 0.37−0.38 M, and in apo buffer it was at 0.43 M. Thus, their strength of binding to Ip2 was in the order plus Mg^{2+} < apo < plus Ca^{2+}. The isolated wild-type domain, F29W/ND, and F105W/CD also behaved similarly on this Ip2 column, in agreement with the very similar K_{D2} and K_{D1} values for the interactions of the latter two with Ip2 in their Ca^{2+}-saturated states (see Table 1). As with intact F → W mutants, the strength of interaction of these mutant domains with Ip2 was in the order plus Mg^{2+} < apo ≤ plus Ca^{2+}. The data thus demonstrate, at least as assessed by this criterion, that interaction with inhibitory peptide occurs irrespective of the metal-bound state of the protein. While the strength of interaction of the Ca^{2+}-loaded state is strongest, the differences with the apo and Mg^{2+} states are not dramatic. The data also indicate that the introduction of the F → W mutants has not significantly affected the affinity of the protein for Ip2.

[2] Comparable values for K_{D1} and K_{D2} have been obtained using two other Trp mutants: F78W and I61W (M. Chandra et al., unpublished results).

Table 2: Binding of Wild-Type and F → W Mutant TnC's to an Ip2 Affinity Column

protein sample	buffer[a]	elution range[b] [NaCl]	peak position[c] [NaCl]
intact F105W	Ca^{2+}	0.49−0.75	0.60
intact F29W	Ca^{2+}	0.50−0.72	0.60
intact rTnC	Ca^{2+}	0.50−0.69	0.60
F105W/CD	Ca^{2+}	0.24−0.39	0.32
rTnC/CD	Ca^{2+}	0.26−0.38	0.32
F29W/ND	Ca^{2+}	0.28−0.41	0.34
rTnC/ND	Ca^{2+}	0.28−0.42	0.34
F105W/CD + F29W/ND	Ca^{2+}	0.19−0.42	0.30
intact F105W	apo	0.38−0.54	0.43
intact F29W	apo	0.36−0.54	0.43
F105W/CD	apo	0.25−0.39	0.30
F29W/ND	apo	0.23−0.37	0.27
F105W/CD + F29W/ND	apo	0.23−0.40	0.28
intact F105W	Mg^{2+}	0.30−0.52	0.38
intact F29W	Mg^{2+}	0.30−0.51	0.37
F105W/CD	Mg^{2+}	0.17−0.28	0.21
F29W/ND	Mg^{2+}	0.15−0.33	0.23

[a] Buffer compositions were as follows: apo (50 mM MOPS, 0.1 M NaCl, 0.1 mM EGTA, pH 7.1); Ca^{2+} (same as apo buffer plus 0.3 mM $CaCl_2$); Mg^{2+} (same as apo buffer plus 5 mM $MgCl_2$). A gradient of 0.1−1.0 M NaCl was applied after the column was washed with several column volumes of the starting buffer. Samples under the peak were analyzed via SDS−PAGE. [b] The range of [NaCl] is given for the initial and final positions of elution of the sample. [c] The [NaCl] at the peak position of the eluted sample is given.

DISCUSSION

Previously, we demonstrated the usefulness of two Phe → Trp mutants, F29W and F105W, as fluorescence probes for monitoring the Ca^{2+}- and/or Mg^{2+}-induced structural transitions in intact TnC and its isolated N and C domains (Pearlstone et al., 1992; Trigo-Gonzalez et al., 1992; Li et al., 1994). In the present study, we employed these same mutants in an investigation of the interaction of the inhibitory peptides of TnI (Ip1 and Ip2) with intact TnC and its domains. The binding of Ip peptide to intact TnC can be expected to perturb the fluorescence properties of the Trp residues either by affecting their environment directly or through the induction of structural changes. Such binding to or structural alterations in the N domain would be reflected in the spectral features of F29W; similarly, the fluorescence properties of F105W would report binding to and/or structural changes in the C domain. By assuming one-site binding and no significant effects of the F → W mutations of the affinity of Ip binding, the dependencies of the fluorescence changes on Ip concentration should be the same irrespective of the F → W mutant employed to monitor binding.

The present data (Figures 1 and 4) show that this dependency on Ip1 concentration is very different for the F29W and F105W mutants, which is inconsistent with simple 1:1 binding of Ip1 to intact TnC. The binding data therefore have been analyzed in terms of two-site binding of Ip to intact TnC, yielding two dissociation constants, K_{D1} and K_{D2}, corresponding to sites 1 and 2 (Table 1). These values are seen to be in excellent agreement irrespective of whether intact F29W or intact F105W is used in the binding analysis. This is true for both Ip peptides and indicates that the F →

W mutants have not significantly affected the binding affinities of the peptides to TnC. This conclusion is also supported by the affinity chromatography experiments (see Table 2) in which no differences were observed in the salt concentrations required for the elution of rTnC, F29W TnC, and F105W TnC from a column of Ip2 coupled to Sepharose 4B.

Since the fluorescence change observed with F105W is more highly dependent on Ip concentration than that with F29W, it can be assumed that the binding of Ip peptide to site 1 is to the C domain of TnC, while that to site 2 is to the N domain. This is supported by single binding site analyses of the data for isolated F29W/ND and isolated F105W/CD. The derived K_{D2} values for Ip1 and Ip2 binding to isolated F29W/ND are seen to be in good agreement with those obtained with intact F29W. This good agreement is consistent with our previous observations (Li et al., 1994) in which we could demonstrate no differences between the structural features and Ca^{2+} binding properties of isolated F29W/ND and those of the N domain in intact F29W.

The data for Ip1 and Ip2 interactions with F105W/CD, however, give K_{D1} values significantly higher than those seen with intact F105W, indicating that Ip binding to isolated F105W/CD is ~5−10-fold weaker than that to the C domain in intact F105W. This observation is also consistent with our earlier demonstration that the properties of isolated F105W/CD are similar, but not identical, to those of intact F105W (Li et al., 1994).

Several previous studies have examined the binding affinities of inhibitory peptides with intact TnC, either with Ip1 (Tsuda et al., 1992; Chandra et al., 1994), Ip2 (Chandra et al., 1994), or moderately altered versions of Ip2 (Cachia et al., 1983; Melencik & Anderson, 1984; Lan et al., 1989; Campbell et al., 1991; Swenson & Fredricksen, 1992; Chandra et al., 1994). The reported binding affinities, with some variation, are in reasonable agreement with those reported herein for Ip1 and Ip2. The consensus of these reports is that the major binding site for inhibitory peptide binding is associated with the C domain, and in most cases the data were analyzed by assuming one-site binding with the formation of a binary complex TnC·Ip. In one case (Campbell et al., 1991) the authors, on the basis of curve-fitting analyses, were unable to distinguish between one-site and two-site binding. In agreement with the present study, their analyses allowed for the possibility of a second peptide binding site on calcium-saturated TnC with an affinity 10−20-fold weaker than that of the first site.

Two of the reports, however, provided evidence that, while inhibitory peptide binding to TnC involves both the N and C domains, the stoichiometry of such binding to intact TnC is close to 1:1. Tsuda et al. (1992) followed the ^1H NMR spectral shifts of several N and C domain residues of intact rabbit Ca^{2+}-saturated TnC upon successive additions of Ip1. Spectral changes involving Tyr10, Phe23, and Phe72 were observed, as well as a number of C domain residues. These changes apparently approached maxima at a molar ratio of Ip1:TnC of ~1.3:1. By using the K_{D1} and K_{D2} values derived in the present study, it can be calculated that, at a concentration of 1 mM TnC used by Tsuda et al. (1992) and at a molar ratio of Ip1:TnC of 2:1, both sites 1 and 2 would be largely occupied with Ip1. Thus, during titration with Ip1, perturbation in the spectral shifts of both the C and N domains would be expected under these conditions, as observed by the authors. The reliability of the estimation of binding stoichiometry is, of course, dependent on the accuracy of the concentration determinations for Ip1 and TnC. These were by weight for Ip1 and spectrophotometrically for TnC. For various reasons, these may be considered as inadequate for this purpose. In the present study such concentration determinations were by amino acid analyses in duplicate or triplicate. Swenson and Fredricksen (1992) reported the interaction of a modified inhibitory peptide (N^{α}-acetyl-Trp^{103}-TnI-(104−115)-amide) with intact TnC, as well as isolated N and C domain TnC fragments (residues 1−98 and 99−159; rabbit TnC numbering). While interaction was observed with both fragments, they reported close to 1:1 stoichiometry of peptide binding to intact TnC on the basis of their curve-fitting analyses. Unfortunately, this conclusion is difficult to critically assess since comparative curve-fitting analyses or other supporting evidence was not provided. In our experience, such procedures are inadequate by themselves in distinguishing between two-site and one-site binding in situations where the binding affinity of one site is much weaker than that of the other [see, for example, the fitted curves for one- and two-site binding in Figure 5 of this report and in Figure 3B of Campbell et al. (1991)]. Rather, in the present report, our conclusions are based on the very different Ip concentration dependencies observed with the two F → W fluorescence probes in intact TnC, one in the N domain and one in the C domain, and the good agreement between the derived K_D values for both intact mutants and the isolated F29W/ND.

The evidence for two-site binding is further supported by the effect of increasing Ip1 concentration on the Ca^{2+} affinity of C domain sites III/IV and N domain sites I/II, as assessed by the fluorescence changes of F105W and F29W. Previous studies (Trigo-Gonzalez et al., 1992; Pearlstone et al., 1992; Li et al., 1994) have demonstrated the utility of these probes for monitoring the structural transitions associated with the filling of N and C domain sites by Ca^{2+} and Ca^{2+}/Mg^{2+}, respectively. These have been shown to be specific for their respective domains and not to be significantly influenced by metal-induced conformational events in the other domain. Ca^{2+} titrations of these two mutants as monitored by fluorescence changes in the presence of increasing molar ratios of Ip1:TnC (see Figure 1) again show very different dependencies on inhibitor concentration. At relatively low molar ratios of Ip1:F105W, the Ca^{2+} affinity of sites III/IV is sharply increased as monitored by the shift in pCa_{50} to higher values. Higher ratios of Ip1:F29W are required to produce a corresponding shift in pCa_{50} of the N domain sites I/II. These observations are consistent with the presence of two Ip1 binding sites on intact TnC, with a higher affinity site on the C domain and a lower affinity site on the N domain. At relatively low ratios of Ip1:protein, the higher affinity site would be occupied, leading to significant increases in the Ca^{2+} affinity of C domain sites III/IV. At higher ratios, the filling of the lower affinity sites by Ip1 would lead to the observed shift of the pCa curve to lower $[Ca^{2+}]$, i.e., higher pCa. These fluorescence results are in agreement with far-UV CD Ca^{2+} titration curves of rTnC and F154W TnC in the presence of Ip1 (Chandra et al., 1994), in which the Ca^{2+} binding affinity of the C domain sites III/IV was increased by 3−4.5-fold, but with minimal effect on that of the N domain sites I/II. These data are

also consistent with the effects of intact TnI which, when complexed with TnC, has been shown to increase the Ca^{2+} affinity of both N and C domain sites by an order of magnitude (Potter & Gergely, 1975; Potter et al., 1976; Wang & Cheung, 1985). Our data also indicate that the binding of Ip1 to the higher affinity C domain site has little effect on the Ca^{2+} affinity of N domain sites I/II. These observations may be contrasted with the data reported by Van Eyk et al. (1991). Using a single Ip2:rabbit TnC ratio of 1:1 and monitoring the intrinsic fluorescence due to Tyr and mean residue molar ellipticity at 222 nm, the authors reported no apparent change in the Ca^{2+} affinity of C domain sites III/IV but a sharp increase in Ca^{2+} affinity attributable to N domain sites I/II. The reliability of these observations is, in our view, suspect since at the protein concentrations employed and at a molar ratio of 1:1, the occupancy of the Ip binding sites would be only partial. The authors also have given no indication of the reproducibility of their data; the reported pCa curves are apparently observations from single Ca^{2+} titrations. In light of these considerations and the observations reported in the present and previous communications, (Chandra et al., 1994), we believe the interpretations and conclusions of their study as they relate to the effects of the binding of skeletal and cardiac Ip peptides on the Ca^{2+} affinities of the N and C domains of skeletal and cardiac TnC's to be invalid.

The affinity chromatography experiment described in the present report was designed to give information on two areas of some uncertainty. The first was to provide further evidence that the two F → W mutants F29W and F105W did not affect the strength of the interaction with Ip peptide as compared to wild-type rTnC. Examination of the NaCl concentrations required for elution of the three proteins, F29W, F105W, and rTnC, from the immobilized Ip2 column (Table 2) shows that no differences among the proteins were observed under $+Ca^{2+}$ conditions. This was also true for the isolated domains. That is to say, F29W/ND was eluted at the same [NaCl] as rTnC/ND; F105W/CD behaved the same as rTnC/CD. These data thus support our earlier deduction that the substitution of F29 and F105 by W has no significant effect on the strength of interaction of TnC with the inhibitory peptide(s) of TnI.

The second purpose of these affinity chromatography experiments was to reexamine the question of the divalent cation dependencies of the interaction of TnI inhibitory peptides and TnC. This was prompted by our observation that fluorescence changes elicited upon the addition of Ip peptides to F29W and F105W were not observed in either plus Mg^{2+} or apo conditions and by the report of Van Eyk and Hodges (1987) that although TnC bound to an Ip2 affinity column in the presence of 5 mM $MgCl_2$ or 2 mM $CaCl_2$ such binding did not occur under apo conditions (0.1 mM EDTA). The present affinity chromatography data (Table 2) collected under conditions similar to those of Van Eyk and Hodges (1987), however, indicate that binding of TnC to Ip2 does occur in the absence of divalent cations and with similar affinity as in the Mg-saturated state. We have no explanation for the failure to detect such an interaction in the previous report of Van Eyk and Hodges (1987) since an earlier study from the same laboratory (Cachia et al., 1986) did, in fact, demonstrate that in 1 mM EGTA [i.e., 10-fold higher than the concentration used by Van Eyk and Hodges (1987)] the same shorter Ip peptide (residues 104−115) did indeed bind to TnC, resulting in an increase in negative ellipticity due to complex formation. Since the addition of Ip1 or Ip2 to F105W or F29W in the apo and plus Mg^{2+} states produced no fluorescence changes, we have been unable to obtain binding affinities for the interaction of TnC and Ip. However, the present affinity chromatography data are in agreement with the observations of others, using alternative methodological approaches, that TnC in both its apo and Mg^{2+} states does interact measurably with the inhibitory peptides (Cachia et al., 1983; Campbell et al., 1991; Tsuda et al., 1992; Swenson & Fredericksen, 1992). Typical association constants as reported by Swenson and Fredericksen (1992), for example, are $(9.5 \pm 4.2) \times 10^4$ M^{-1} in the apo state, $(6.4 \pm 2.2) \times 10^4$ M^{-1} in the presence of 3 mM $MgCl_2$, and $(5.3 \pm 1.1) \times 10^5$ M^{-1} in the Ca^{2+}-saturated state. On the basis of these binding affinities and the concentrations of proteins and peptides used in the present studies, a significant fraction of the TnC would be present in the protein−peptide complex not only in plus Ca^{2+} conditions but also in the presence of Mg^{2+} or in the apo state. Our inability to demonstrate Ip1-induced fluorescence changes under these latter two conditions indicates that the structural interface between TnC and the Ip peptides is different for the Mg^{2+} and apo states as compared with the Ca^{2+}-saturated protein. We came to a similar conclusion on the basis of our studies of the interaction of Ip peptides with the fluorescence probe mutant F154W TnC (Chandra et al., 1994).

The cumulative data of Swenson and Fredericksen (1992) and other reports cited earlier, as well as the present results, indicate that the effect of the presence of Ca^{2+} ions on the TnC/Ip interaction is rather modest, leading to an increase in affinity of only 6−10-fold over that seen under apo or plus Mg^{2+} conditions. This may be compared with the interaction of TnC and intact TnI, where the presence of Ca^{2+} increases affinity 40−120-fold over that seen under plus Mg^{2+} conditions and 1000−8000-fold compared with the apo state (Ingraham & Swenson, 1984; Wang & Cheung, 1985). From this perspective, the TnI peptides Ip1 and Ip2 are rather poor surrogates for the intact protein. Clearly, other regions of the TnI molecule are of vital importance in conferring such a high degree of Ca^{2+} sensitivity to the interaction of these two proteins.

The data presented in this paper provide, in our view, convincing evidence for the interaction of two Ip peptides with two sites on intact TnC, one site on the C domain, and one on the N domain. Binding to the stronger site on the C domain appears to have little effect on the weaker binding to the N domain. This interpretation is in contrast with the view that a single Ip peptide simultaneously binds to both the N and C domains through collapse of the central D/E helix, allowing both domains to interact with a single peptide, a situation now known to occur with calmodulin and its target peptide(s) (Ikura et al., 1992; Clore et al., 1993; Meador et al., 1992, 1993). The present evidence is, however, consistent with the small-angle X-ray scattering studies of Blechner et al. (1992), indicating that TnC remains in an extended structure upon binding the inhibitory peptide of TnI. In the present studies, with $4Ca^{2+}$·ThC in an extended conformation in solution and with each globular domain possessing a similar hydrophobic patch, it is not surprising to find the small Ip peptides interacting independently with each domain.

With intact TnI and TnC, on the other hand, in which the stoichiometry of complex formation is 1:1, substantial evidence exists for simultaneous interactions of the inhibitory region of TnI with both domains of TnC [reviewed by Grabarek et al. (1992); see also Ngai and Hodges (1994); Farah et al., 1994; Kobayashi et al., 1994]. On the basis of extensive cross-linking experiments [summarized in Kobayashi et al. (1994)] and interaction studies of deletion mutants of TnI and TnC and its isolated domains, Farah et al. (1994) have depicted the TnI and TnC polypeptide chains as interacting in an antiparallel fashion, with extensive contacts involving the NH_2-terminal region of TnI (residues 1−95) and the COOH-terminal domain of TnC and between the COOH-terminal TnI segment (residues 117−182) and the N domain of TnC. The TnI inhibitory region (residues 96−116) would interact with both domains in a divalent cation dependent manner. The question of whether this would require the TnC to adopt a more compact structure than that seen in the X-ray structure and similar to that in the complex of calmodulin and its target peptides presently is unclear. The recent low-angle X-ray and neutron scattering data of the TnC·TnI complex (Olah et al., 1994; Olah & Trewhella, 1994) have depicted a solution structure in which the TnC is fully extended as in the crystal. The central region of the TnI, presumably including the inhibitory peptide region, is represented as an extended helix spiraling through and interacting with the hydrophobic patch regions of both domains of TnC. Confirmation of this extended TnI·TnC structure and rationalization with the extensive chemical and interaction studies reported in the literature clearly will require more detailed structural analyses at higher resolution. Fortunately the application of multidimensional NMR techniques to the system promises to provide answers to such questions in the forseeable future.

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REFERENCES

Blechner, S. L., Olah, G. A., Strynadka, N. C. J., Hodges, R. S., & Trewhella, J. (1992) *Biochemistry 31*, 11326−11334.
Cachia, P. J., Sykes, B. D., & Hodges, R. S. (1983) *Biochemistry 22*, 4145−4152.
Cachia, P. J., Van Eyk, J., Ingraham, R. H., McCubbin, W. D., Kay, C. M., & Hodges, R. S. (1986) *Biochemistry 25*, 3553−3562.
Campbell, A. P., Cachia, P. J., & Sykes, B. D. (1991) *Biochem. Cell. Biol. 69*, 674−681.
Chandra, M., McCubbin, W. D., Oikawa, K., Kay, C. M., & Smillie, L. B. (1994) *Biochemistry 33*, 2961−2969.
Clore, G. M., Bax, A., Ikura, M., & Gronenborn, A. M. (1993) *Curr. Biol. 3*, 838−845.
Farah, C. S., Miyamoto, C. A., Ramos, C. H. I., da Silva, A. C. R., Quaggio, R. B., Fujimori, K., Smillie, L. B., & Reinach, F. C. (1994) *J. Biol. Chem. 269*, 5230−5240.
Gagné, S. M., Tsuda, S., Li, M. X., Chandra, M., Smillie, L. B., & Sykes, B. D. (1994) *Protein Sci. 3*, 1961−1974.
Golosinska, K., Pearlstone, J. R., Borgford, T., Oikawa, K., Kay, C. M., Carpenter, M. R., & Smillie, L. B. (1991) *J. Biol. Chem. 266*, 15797−15809.
Grabarek, Z., Tao, T., & Gergely, J. (1992) *J. Muscle Res. Cell Motil. 13*, 383−393.
Herzberg, O., & James, M. N. G. (1985a) *Nature 313*, 653−659.
Herzberg, O., & James, M. N. G. (1985b) *Biochemistry 24*, 5298−5302.
Herzberg, O., Moult, J., & James, M. N. G. (1986) *J. Biol. Chem. 261*, 2638−2644.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632−638.
Ingraham, R. H., & Swenson, C. A. (1984) *J. Biol. Chem. 259*, 9544−9548.
Kobayashi, T., Tao, T., Grabarek, Z., Gergely, J., & Collins, J. H. (1991) *J. Biol. Chem. 266*, 13746−13751.
Kobayashi, T., Tao, T., Gergely, J., & Collins, J. H. (1994) *J. Biol. Chem. 269*, 5725−5729.
Lan, J., Albaugh, S., & Steiner, R. F. (1989) *Biochemistry 28*, 7380−7385.
Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235−305.
Leszyk, J., Grabarek, Z., Gergely, J., & Collins, J. H. (1990) *Biochemistry 29*, 299−304.
Li, M. X., Chandra, M., Pearlstone, J. R., Racher, K. I., Trigo-Gonzales, G., Borgford, T., Kay, C. M., & Smillie, L. B. (1994) *Biochemistry 33*, 917−925.
Malencik, D. A., & Anderson, S. R. (1984) *Biochemistry 23*, 2420−2428.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251−1255.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1993) *Science 262*, 1718−1721.
Ngai, S.-M., & Hodges, R. S. (1994) *J. Biol. Chem. 269*, 2165−2172.
Olah, G. A., & Trewhella, J. (1994) *Biochemistry 33*, 12800−12806.
Olah, G. A., Rokop, S. E., Wang, C.-L. A., Blechner, S. L., & Trewhella, J. (1994) *Biochemistry 33*, 8233−8239.
Parmacek, M. S., & Leiden, J. M. (1991) *Circulation 84*, 991−1003.
Pearlstone, J. R., Borgford, T., Chandra, M., Oikawa, K., Kay, C. M., Herzberg, O., Moult, J., Herklotz, A., Reinach, F. C., & Smillie, L. B. (1992) *Biochemistry 31*, 6545−6553.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628−4633.
Potter, J. D., & Johnson, J. D. (1982) *Calcium Cell Funct. 2*, 145−173.
Potter, J. D., Seidel, J. C., Leavis, P. C., Lehrer, S. S., & Gergely, J. (1976) *J. Biol. Chem. 251*, 7551−7556.
Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628−1647.
Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science 227*, 945−948.
Swenson, C. A., & Fredricksen, R. S. (1992) *Biochemistry 31*, 3420−3429.
Trigo-Gonzalez, G., Racher, K., Burtnick, L., & Borgford, T. (1992) *Biochemistry 31*, 7009−7015.
Tsuda, S., Aimoto, S., & Hikichi, K. (1992) *J. Biochem. (Tokyo) 112*, 665−670.
Van Eyk, J. E., & Hodges, R. S. (1987) *Biochem. Cell. Biol. 65*, 982−988.
Wang, C.-K., & Cheung, H. C. (1983) *Biophys. J. 48*, 727−739.
Williams, T. C., Shelling, J. G., & Sykes, B. D. (1985) in *NMR in the Life Sciences* (Bradbury, E. M., & Nicolini, C., Eds.) pp 93−103, Plenum Press, New York.
Zot, A. S., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535−559.

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