Interactions of Structural C and Regulatory N Domains of Troponin C with Repeated Sequence Motifs in Troponin I†

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ABSTRACT: The actomyosin ATPase inhibitory protein troponin I (TnI) plays a central regulatory role in skeletal and cardiac muscle contraction and relaxation through its calcium-dependent interactions with troponin C (TnC) and actin. Previously we have demonstrated the utility of F29W and F105W mutants of TnC for measurement of binding affinities of inhibitory peptide TnI_{96-116} to its regulatory N and structural C domains, both in isolation and in the intact TnC molecule [Pearlstone, J. R. & Smillie, L. B. (1995) *Biochemistry 34*, 6932−6940]. This approach is now extended to fragment TnI_{96-148}. Curve-fitting analyses of fluorescence changes induced in the intact TnC mutants and the isolated N and C domains by increasing [TnI_{96-148}] have permitted the assignments of K_D values (designated $K_{D,N}$ and $K_{D,C}$) to the interaction of TnI_{96-148} with the N and C domains, respectively, of intact TnC. Taken together with the previous data for TnI_{96-116} binding, it can be concluded that, within TnI_{96-148}, residues 96−116 are primarily responsible for binding to C domain of intact TnC and residues 117−148 to its N domain. Inspection of the available mammalian and avian skeletal muscle TnI amino acid sequences reveals a previously unrecognized conserved motif repeated 3-fold, once in the inhibitory peptide region (∼residues 101−114; designated α) and twice more in the region of residues ∼121−132 (β) and ∼135−146 (γ). The number and distribution of these motifs have important structural implications for the TnI·C complex. In the β motif of cardiac TnI, as compared with skeletal, several changes in charged amino acids are suggested as candidates responsible for the greater sensitivity of cardiac Ca^{2+}-regulated actomyosin to acidic pH as in ischemia.

The Ca^{2+} regulation of skeletal muscle contraction and relaxation is mediated by the troponin (Tn)[1] complex through its interactions with tropomyosin (TM) and the actin filament. Conformational changes induced by Ca^{2+} binding to TnC are transmitted through TnI and TnT (TM binding component) to TM-actin, facilitating productive interactions with myosin heads and subsequent contraction. These events have been extensively reviewed (Zot & Potter, 1987; Grabarek et al., 1992; Farah & Reinach, 1995; Tobacman, 1996). From X-ray diffraction analyses of TnC crystals grown at pH ∼5.0 (Herzberg & James, 1985; Sundaralingam et al., 1985), the molecule is seen as an extended dumbbell with N (regulatory) and C (structural) domains joined through a central helix, part of which is exposed to solvent. Each domain has two metal-binding EF-hand or helix−loop−helix motifs. The Ca^{2+}-specific sites I and II of N domain are of lower affinity with K_a values of $\sim 10^5-10^6$ M^{-1}; those of C domain, sites III and IV, are of higher affinity (K_a values $\sim 10^7$ M^{-1}) and also bind Mg ($K_a \sim 10^3$ M^{-1}). Helices are designated A−H, corresponding to the four pairs of helix−loop−helix motifs of sites I−IV. In addition, an extension of the NH_2 terminus forms the N helix; the central helix connecting site II to site III is designated D/E. In the pH 5.0 crystals, sites I and II are unoccupied; sites III/IV are liganded to Ca^{2+}. Substantial evidence exists that the regulatory role of TnC is exercised through Ca^{2+} binding to sites I/II. C domain sites III/IV are believed to be occupied by Ca^{2+}/Mg throughout the contraction/ relaxation cycle and to serve a structural role in anchoring TnC to the other Tn components.

A model for the Ca^{2+}-induced structural transition of N domain from a closed (apo) to open ($2Ca^{2+}$) conformation has been proposed (Herzberg et al., 1986) and largely supported by several biochemical studies (Grabarek et al., 1990; Fujimori et al., 1990; Pearlstone et al., 1992a,b; da Silva et al., 1993). The model's essential features have recently been confirmed by NMR structural determinations of the two states in solution at neutral pH (Gagné et al., 1995; Slupsky & Sykes, 1995). Ca^{2+} binding to sites I/II is accompanied by large changes in interhelical angles involving significant reorientation of helices B and C relative to N, A, and D. This closed to open conformational transition leads to the surface exposure of a number of previously fully and partially buried apolar residues to create a hydrophobic pocket. The intact TnC ($4Ca^{2+}$) would now possess two such nonpolar patches, one in each domain, with surrounding constellations of negatively charged residues. These have been proposed as putative interaction sites with TnI.

In contrast to the extensive tertiary structural information now available for TnC, that for TnI both in isolation and in complex with TnC/TnT is minimal; see, however, Campbell and Sykes (1991). On the basis of many biochemical,

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[1] Abbreviations: Tn, troponin; TM, tropomyosin; TnC, Ca^{2+}-binding component of troponin; TnI, inhibitory component of troponin; F29W/ND, F29W N domain of troponin Ca^{2+}-binding component (residues 1−90); F105W/CD, F105W C domain of troponin Ca^{2+}-binding component (residues 88−162); TnI_{96-116} and TnI_{96-148}, troponin inhibitory component fragments encompassing residues 96−116 and 96−148, respectively; RLC, scallop myosin regulatory light chain.

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chemical, and biophysical investigations, however, the interface between TnI and the N and C domains of TnC has been shown to be antiparallel in arrangement and to involve multiple side-chain interactions between the two proteins [reviewed by Farah and Reinach (1995); see also Krudy et al. (1994), Sheng et al. (1992), and Kobayashi et al. (1995)]. Functionally, the TnI polypeptide chain may be divided into three regions. TnI N domain (∼residues 1−95) has been shown to interact strongly with the structural C domain of TnC when the latter's sites III/IV are occupied by Ca^{2+}. When the sites are filled by Mg^{2+}, this binding strength is weakened but still significant. This interaction, responsible in part for the anchoring of TnC to the other members of the troponin complex and to the regulated thin filament, thus possesses both a Ca^{2+}-dependent and a Ca^{2+}-independent component. The second region (∼residues 96−116) is largely responsible in the absence of TnC and TnT for the actomyosin ATPase inhibitory activity of TnI. The minimal length for this inhibition has been shown to reside within residues 105−115 (Talbot & Hodges, 1981). Larger molar equivalents of inhibitory peptide are required for maximum inhibition than with intact TnI. Inhibition is potentiated by tropomyosin, partially relieved by TnC ($-Ca^{2+}$), and fully released by TnC in the presence of Ca^{2+}. In the absence of Ca^{2+}, this inhibitory region is postulated to bind to actin, preventing its productive interaction with the myosin head. With Ca^{2+} present, inhibition would be released by switching of the inhibitory region to complex formation with TnC.

The importance of the third major domain [∼residues 117−181 (or 182)] in the Ca^{2+} regulation of tropomyosin actomyosin ATPase has recently been demonstrated by Farah et al. (1994) using troponin complexes reconstituted with intact TnC, TnT, and recombinant fragments of TnI. While troponin reconstituted with $TnI_{103-182}$ or TnI_{1-156} retained actomyosin regulatory activity in a Ca^{2+} -dependent manner similar to that of the intact protein, a deletion mutant, TnI_{1-116}; lacking the COOH-terminal domain was ineffective. Qualitative binding studies in benign and denaturing electrophoretic gels as well as cross-linking studies of intact and deletion mutants of TnC and TnI indicate Ca^{2+}-dependent interactions between the N domain of TnC and the COOH-terminal domain (residues 117−182) of TnI [see Farah and Reinach (1995) and Kobayashi et al. (1995) and references therein]. Taken together, these studies suggest a critical regulatory role for the region of TnI residues 117−156 in its Ca^{2+}-dependent interaction with the N domain of TnC.

Previously we have taken advantage of the fluorescence properties of TnC mutants F29W and F105W to measure the binding properties of the TnI inhibitory peptide TnI_{96-116} to the intact molecule and its isolated N and C domains (Pearlstone & Smillie, 1995). The data were interpreted in terms of two relatively independent binding sites on TnC, that on C domain being approximately 20−40-fold stronger than that on N domain. In the present report we extend these binding analyses using a highly soluble recombinant TnI fragment, TnI_{96-148}, encompassing the inhibitory region and a portion of the TnI COOH-terminal regulatory domain. The data demonstrate that within TnI_{96-148}, residues 96−116 are primarily responsible for binding to the structural C domain of TnC and 117−148 to the regulatory N domain. We also report the presence in TnI of a previously unrecognized and conserved sequence motif, repeated 3-fold, once in the inhibitory region (residues 101−114) and twice more in the region of residues 121−146. The number and distribution of these have certain structural implications for the TnI·C complex. Upon comparison with the skeletal TnI sequence, several conserved changes in the consensus sequence of the cardiac β motif are suggested as candidates responsible for the greater sensitivity of cardiac Ca^{2+}-regulated actomyosin to acidic pH as in ischemia.

MATERIALS AND METHODS

Proteins and Fragments. The preparation of F105W, F29W, F105W/CD, and F29W/ND has been described (Pearlstone et al., 1992a; Pearlstone & Smillie, 1995; Trigo-Gonzalez et al., 1992; Li et al., 1994).

For construction of an expression vector containing TnI_{96-148}, PCR technology was applied to a chicken TnI construct in pET3a as template kindly provided by F. C. Reinach, Sao Paulo, Brazil (Farah et al., 1994). Briefly, this involved the preparation of a PCR product containing codons corresponding to residues 96−148, an initiation Met at 95 as part of an *Nde*I site, a stop codon at 149, and a *Sac*I site 9−14 nucleotides in the 3′ direction from the stop codon. Ser-96 in chicken was also mutated to Asn as in rabbit TnI_{96-116}. After cleavage with *Nde*I and *Sac*I, ligation was into *Nde*I/*Sac*I-cleaved pAED4 (kindly donated by Dr. D. Doering, Whitehead Institute). Validity of the construct was confirmed by plasmid sequencing. The pAED4·TnI_{96-148} was transformed into *E. coli* strain BL21·DE3·pLysS (Novagen) for inducible protein expression with IPTG (Studier et al., 1990). A 50 mM Tris, 6 M urea, 0.1 mM EDTA, and 0.1% NaN_3, pH 7.5, extract of a dried acetone powder of cell pellet from an 8 L culture was fractionated on a CM-cellulose column at pH 7.5. Further purification was on a C_8 HPLC Synchropac column (0.05% TFA/acetonitrile gradient) and by gel filtration on Sephadex G-75 in 1% formic acid. Final purity was established by SDS gel electrophoresis and amino acid analyses. During expression the initiation Met was partially removed as assessed by sequencing [(M)NQKLFDLRGKF-].

Fluorescence Measurements. Stock solutions of the intact F29W and F105W proteins (2 mg/mL) as well as the F29W/ND and F105W/CD preparations (1 mg/mL) were prepared as described (Golosinska et al., 1991), using precautions to eliminate Ca^{2+} contamination and oxidation during dialysis against 50 mM 3-(4-morpholino)propanesulfonic acid, 100 mM KCl, 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, and 1 mM dithiothreitol buffer, pH 7.1. The proteins were centrifuged using 0.22 μm-filter Spin-X tubes (Costar), their concentrations were determined, and then the samples were diluted with similarly filtered outer dialysate buffer prior to the fluorescence measurements. Stock solutions of TnI_{96-148} (1.1 mM) were made using the same final filtered buffer as that used for dilution of the TnC proteins. Concentrations were determined by amino acid analyses in duplicate and final values for proteins and peptides are given in the figure legends. As described previously (Pearlstone et al., 1992a; Pearlstone & Smillie, 1995), the Trp fluorescence measurements were carried out using a Perkin-Elmer MPF-44B fluorescence spectrophotometer operating in the ratio mode with the temperature maintained at 20 °C with a water bath. To correct for the solvent blank, a DSCU-2 differential corrected spectra unit was used with the difference setting in the blank mode. Excitation wavelength was

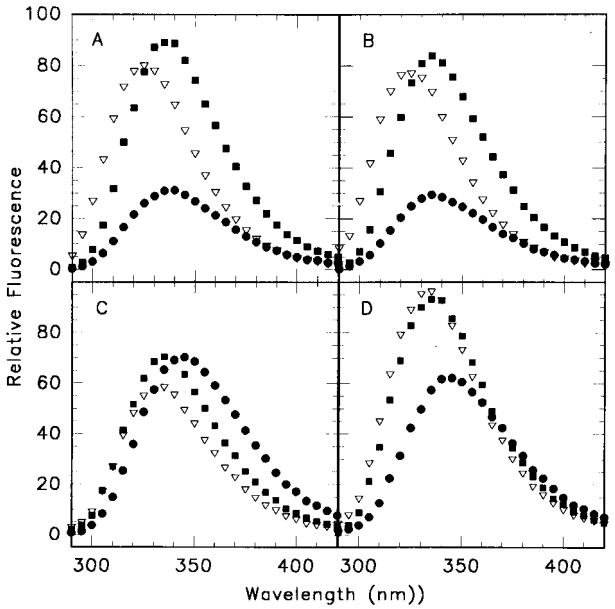

FIGURE 1: Fluorescence emission spectra of (A) F29W, (B) F29W/ND, (C) F105W, and (D) F105W/CD under apo conditions (●), in the presence of 2 mM $CaCl_2$ (■), and in the presence of 2 mM $CaCl_2$ and TnI_{96-148} (▽). The apo buffer and instrumentation are described in Materials and Methods. Protein concentrations were 0.75 μM. For F29W, F29W/ND, F105W, and F105W/CD, the fragment TnI_{96-148} was added to a molar ratio to the TnC mutants of 4, 5, 3, and 6, respectively. Under apo conditions or in 44 mM Mg^{2+}, addition of TnI_{96-148} showed little or no change in the fluorescence spectra of any of the four F → W mutants (not shown).

at 282 nm. Slit band widths were 7 nm for excitation and 10 nm for emission. A 1-cm semimicrocell (Hellma) was used, and detection was at right angles. Prior to titration with TnI_{96-148}, Ca^{2+} to 2 mM was added to saturate the TnC proteins. Sequential additions of TnI fragment were made using a 10 μL Hamilton syringe, and the increase in fluorescence emission intensity monitored at one wavelength (~350 nm) as described previously (Pearlstone & Smillie, 1995). For curve fitting and derivation of dissociation constants, nonlinear least-squares computer in-house programs were used, assuming 2:1, 1:1, or 1:2 binding of TnI_{96-148} to protein. The change in fluorescence intensity was analyzed as a function of total fragment concentration and the concentration of fragment bound forms obtained as described (Williams et al., 1985; B. D. Sykes, unpublished results).

RESULTS AND DISCUSSION

Effects of TnI_{96-148} on the Fluorescence Emission Spectra of F29W, F29W/ND, F105W, and F105W/CD. Changes in the fluorescence spectra of each of the four TnC mutants upon addition of a molar excess of the TnI fragment were examined in the absence of divalent cations and in the presence of 44 mM Mg^{2+} or 2 mM Ca^{2+}. Little or no change was observed in the apo state nor in the presence of Mg^{2+} (not shown), whereas under conditions in which all four binding sites of TnC would be Ca^{2+} saturated, significant effects upon the addition of TnI_{96-148} (3−6-fold molar excess) were seen (Figure 1). The results for intact F29W (panel A) and F29W/ND (panel B) were the same. Both apo spectra had an emission maximum at 336 nm and addition of 2 mM Ca^{2+} caused a 3-fold increase in fluorescence intensity as observed previously (Pearlstone et al., 1992a; Pearlstone & Smillie, 1995). Subsequent addition of 4−5-fold molar excess of TnI_{96-148} to either Ca^{2+}-saturated protein resulted in a decrease in fluorescence intensity and a blue shift in emission maximum from 336 to 324 nm.

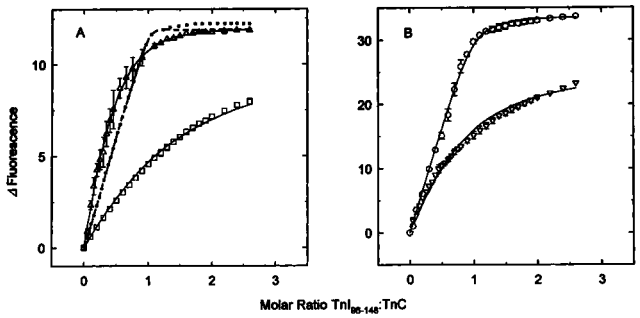

FIGURE 2: Fragment TnI_{96-148} fluorescence titration of Ca^{2+}-saturated (A) F105W (△) and F105W/CD (□) and (B) F29W (▽) and F29W/ND (○). For F105W in panel A, curve-fitting analyses are shown based on assumption of 1:1 (•••), 2:1 (- - -), and 1:2 (—) binding of TnI_{96-148} to TnC mutant. Curve-fitting analyses (—) for F29W in panel B assumed 1:2 binding; curve fitting (—) for F105W/CD in panel A and F29W/ND in panel B was for 1:1 binding. For each mutant the averaged values for two titrations are shown by the symbols with standard deviations as vertical bars. Protein concentrations were 0.75 μM for the two intact proteins and 1.5 μM for the two domains.

Unlike the similarities observed with intact F29W and F29W/ND, quantitative differences were observed in the fluorescence emission spectra of F105W (panel C) and F105W/CD (panel D), as reported earlier (Li et al., 1994; Pearlstone & Smillie, 1995). In intact F105W (panel C), addition of 2 mM Ca^{2+} merely shifted the emission maximum from 344 to 336 nm, while subsequent additions of 3-fold excess TnI_{96-148} caused a decrease in fluorescence intensity and a further blue shift in emission maximum from 336 to 332 nm. In contrast, F105W/CD (panel D) exhibited ~1.5-fold increase in fluorescence intensity in addition to a shift in emission maximum from 344 to 336 nm with 2 mM Ca^{2+}. Addition of TnI_{96-148} effected a 2 nm shift in emission maximum from 336 to 334 nm with the fluorescence intensity remaining essentially unchanged. On the basis of these TnI peptide-induced changes in the fluorescence properties of the TnC mutants, it has been possible to derive quantitative binding data for their interactions as described below.

Titration with TnI_{96-148} of the F → W Mutants within the Intact TnC Molecule and in Their Isolated Domains. The titrations of intact F105W and F29W with TnI_{96-148} are shown in panels A and B of Figure 2. In the case of F105W, the fluorescence data approached maximal values at less than 1 equiv of fragment/equiv of protein. Analyses of these data, assuming either 1:1 or 2:1 binding of fragment to protein, provided poorly fitted curves that lay significantly to the right of the experimental data. Similar curve fitting assuming 1:2 binding of fragment to protein, however, fitted the data closely, suggesting two binding sites on TnI_{96-148} for F105W. Two dissociation constants were derived with values of 0.12 μM and 1.6 μM. The titration of intact F29W with TnI_{96-148} under identical conditions is shown in Figure 2B. In this case, curve-fitting analyses assuming 1:2 binding of peptide to protein were only marginally better than for 1:1 and 2:1 (not shown). However, since intact F29W and F105W are identical proteins except for the position of the Phe → Trp mutant and thus are expected to bind TnI_{96-148} in a similar manner, the data for intact F29W were also analyzed

Table 1: Dissociation Constants of TnI Fragment Interaction with Intact TnC and Its Isolated C and N Domains in the Presence of Ca^{2+} [a]

	TnI_{96-116}			TnI_{96-148}		
	binding analysis I:C	C domain binding $K_{D,C}$ (μM)	N domain binding $K_{D,N}$ (μM)	binding analysis I:C	C domain binding $K_{D,C}$ (μM)	N domain binding $K_{D,N}$ (μM)
intact F105W	2:1	0.5 ± 0.2 (2)	10 ± 5 (2)	1:2	1.6 ± 0.2 (2)	0.12 ± 0.01 (2)
isolated F105W/CD	1:1	2.8 ± 0.2 (3)		1:1	1.2 ± 0.2 (2)	
intact F29W	2:1	0.5 ± 0.1 (5)	24 ± 9 (5)	1:2	1.7 ± 0.7 (2)	0.26 ± 0.04 (2)
isolated F29W/ND	1:1		20 ± 2 (3)	1:1		0.03 ± 0.02 (2)

[a] The number of titrations that were averaged is shown in parentheses. The standard deviations are obtained from the average of the K_D values from 2−5 titrations, as indicated. Dissociation constants were derived from the calculated fitted curves as described in Materials and Methods.

assuming 1:2 binding. The good agreement of the derived K_D values (see Table 1) with those obtained with F105W provides confidence in the reliability of these curve-fitting analyses. To establish a relationship between the two K_D values and the sites of binding of TnI_{96-148} on the N or C domains of the TnC molecule, similar TnI fragment titrations were carried out with the isolated F → W mutated C and N domains. The fitted curves assuming 1:1 binding of TnI fragment to the two isolated domains were in good agreement with the experimental data (see Figure 2). The derived K_D value of 1.2 ± 0.2 μM (designated $K_{D,C}$ in Table 1) for the interaction of TnI_{96-148} and F105W/CD is seen to be in reasonable agreement with two of the K_D values derived from the binding data for intact F105W and F29W (1.6 ± 0.2 μM and 1.7 ± 0.7 μM, respectively; see Table 1). It is therefore possible to assign these K_D values for the two intact proteins to the binding of TnI_{96-148} to their C domains. These are designated $K_{D,C}$ in Table 1. In the case of the data for titration of F29W/ND with TnI_{96-148}, comparison of the derived K_D value = 0.03 μM (designated $K_{D,N}$ in Table 1) with the K_D values for the two intact proteins (0.12 μM and 0.26 μM) indicates that TnI_{96-148} binding to the isolated N domain is 4−8-fold stronger than to the N domain of the intact protein. However, all of these values show that binding of TnI_{96-148} to N domain both in isolation and in the intact proteins is appreciably stronger than with C domain. These values (0.12 μM and 0.26 μM) have therefore been designated $K_{D,N}$ in Table 1.

Comparison of Binding Data for Interaction of TnI Fragments with TnC and Its Domains. We have previously published an investigation (Pearlstone & Smillie, 1995) in which K_D values were reported for the interaction of TnI_{96-116} with intact F105W and F29W as well as the corresponding F → W mutants of the isolated domains. As in the present work, it was possible to assign K_{D1} and K_{D2} values to the interactions of TnI_{96-116} with the C and N domains (redesignated herein as $K_{D,C}$ and $K_{D,N}$, respectively). Since these data were collected under identical experimental conditions as presently, we have, for comparative purposes, reproduced these data in Table 1 together with those for TnI_{96-148}. Inspection of the assembled data permits several observations and correlations.

First of all, titration of the two intact proteins provides values of $K_{D,C}$ and $K_{D,N}$ for each TnI fragment in reasonable agreement irrespective of whether they are derived from the fluorescence properties of F105W or F29W. As concluded before (Pearlstone & Smillie, 1995), this indicates that the F → W mutations have not grossly altered the inhibitory protein fragments' binding properties to wild-type TnC. Second, taking $K_{D,C}$ and $K_{D,N}$ as measures of affinity to TnC C and N domains, respectively, it may be concluded that binding of TnI_{96-148} has a very significantly increased affinity for N domain as compared with TnI_{96-116}. Thus the inclusion of residues 117−148, in addition to 96−116, increases affinity of binding to N domain dramatically (~90-fold). On the other hand, comparison of $K_{D,C}$ values for TnI_{96-116} and TnI_{96-148} indicates at most only a modest negative effect of residues 117−148 on affinity of binding to C domain, which may or may not be significant. Thus the $K_{D,C}$ values for TnI_{96-148} and the intact TnCs are increased about 3−4-fold when compared with those of TnI_{96-116}. Comparison of the $K_{D,N}$:$K_{D,C}$ ratios for the interaction of the two TnI fragments with intact TnC is also instructive in assessing the relative importance of regions 96−116 and 117−148 to the binding to C and N domains. These are in the range of 20−46 for TnI_{96-116} and 0.08−0.15 for TnI_{96-148}. That the interactions involving TnI residues 117−148 and N domain of TnC are highly Ca^{2+}-sensitive is indicated by our inability to demonstrate fragment-induced fluorescence changes in F29W or F105W in the absence of Ca^{2+}, an observation consistent with the absence of detectable complexes of $TnI_{103-182}$ and $TnI_{120-182}$ with TnC ($-Ca^{2+}$) as assessed by gel electrophoretic studies (Farah et al., 1994).

Overall, the observations are consistent with an antiparallel molecular arrangement of the TnC·I complex as proposed in several reports based on more qualitative cross-linking studies [summarized in Kobayashi et al. (1995)], NMR analyses (Krudy et al., 1994), and other observations (Farah & Reinach, 1995).

Presence of Repeated Sequence Motifs in TnI. Our demonstration that, in the TnI_{96-148} fragment, the major contribution to TnC C domain binding is via residues 96−116 and to N domain through residues 117−148 suggests a common structural mode of interaction between these two segments of TnI and the corresponding hydrophobic patch regions of N and C domains of $4Ca^{2+}$ TnC. To explore this possibility we examined the available mammalian and avian TnI primary structures and detected a previously unrecognized common sequence motif (see Figure 3) repeated 3-fold. In the complete TnI sequences these are observed only in the region of residues 101−146. They have been designated α (inhibitory peptide residues ~101−114), β and γ (residues ~121−132 and ~135−146, respectively). For maximal alignment, two-residue gaps were introduced into the β and γ motifs corresponding to Pro residues 109−110 of the α motif. Motif positions have been designated I−XII. The most characteristic features of these motifs, present in all three, are a common LRG, LKA, or LRA sequence at positions II−IV, a nonpolar residue, F or L, at position VI, and two basic residues, R, K, or H, at positions X and XI. Features common to only two of the motifs include D at position I (in α and γ), K at position VII (in α and γ),

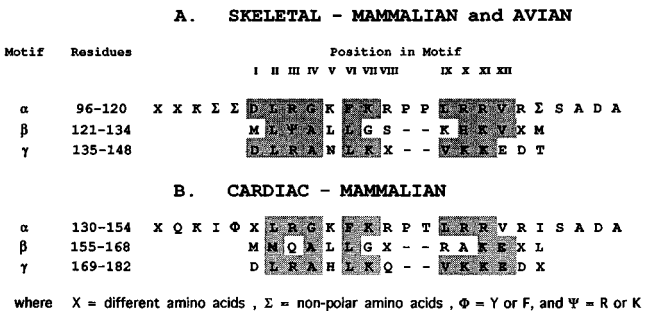

FIGURE 3: Repeated TnC domain binding motifs in TnI consensus sequences from skeletal (mammalian and avian) fast and slow muscles (A) and from mammalian cardiac muscles (B). In each of the skeletal and cardiac sequences, three repeated motifs are evident and are aligned vertically to demonstrate identical and conserved residues (shaded). Gaps (- -) have been introduced to maximize alignment. Residues designated by the one-letter amino acid symbols are identical in all TnI sequences included. In panel A, residue numbers are for rabbit and chicken fast skeletal TnI. The following skeletal muscle TnIs are included: rabbit fast (Wilkinson & Grand, 1978; Sheng et al., 1992); rabbit slow (Wilkinson & Grand, 1978); mouse fast (Koppe et al., 1989); human fast (Zhu et al., 1994); human slow (Wade et al., 1990; Corin et al., 1994); chicken fast (Wilkinson & Grand, 1978; Nikovits et al., 1986; Quaggio et al., 1993); quail fast (Baldwin et al., 1985), and quail slow (Hastings, 1996). In panel B, residue numbers are for bovine cardiac TnI. The following cardiac TnIs are included in the comparison: bovine (Lesyk et al., 1988), rabbit (Grand et al., 1976; Lesyk et al., 1988), rat (Murphy et al., 1991), mouse (Guo et al., 1994; Ausoni et al., 1994), and human (Vallins et al., 1990; Hunkeler et al., 1991). Two avian cardiac sequences (quail and chicken) have been reported (Hastings et al., 1991) but not included in this consensus. They show significant differences in the region of residues 135−148, indicating considerable divergence in the α motif of the avian cardiac protein. Their β and γ motifs, however, retain the major features of the mammalian cardiac consensus sequence.

nonpolar residues (L or V) at position IX (α and γ) and V at position XII (α and β). Variations among the motifs are to be expected since the exact mode of interaction of any one of these with TnC will be dependent on the precise structure of that TnC binding site. Such variation is particularly evident at positions V and VIII, where the chemical nature of the side chain appears to be of little significance, and of course in the α motif with the insertion of PP.

The number and distribution of these motifs along the TnI polypeptide chain has certain structural implications for the TnC·I complex. First, the length of polypeptide chain encompassed by all three is 43−45 residues, greatly in excess of the target polypeptide chain lengths of about 18−19 residues observed in calmodulin binding (Grivici & Ikura, 1995). Thus a compact structure of TnC analogous to that of calmodulin in its complexes with target α-helical peptides is unlikely. On the other hand, the short segment of polypeptide chain (six residues; <2 turns of α-helix) separating the α and β motifs would appear to be inconsistent with a proposed fully extended TnC structure in the C·I complex based on modeling of low-angle X-ray and neutron scattering data of the complex in solution (Olah & Trewhella, 1994). The presence of the PP in the α motif precludes the existence of this region (residues 101−146) as fully helical. It may also be pertinent that secondary structure predictions (Wishart et al., 1994) suggest the possibility of helical breaks (see Figure 4) in the region of residues 128−131 and 145−148 (β and γ motifs, respectively). A more likely scenario

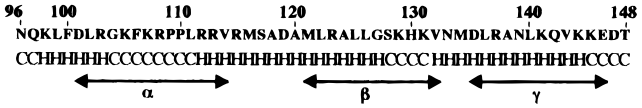

FIGURE 4: Secondary structural predictions (Wishart et al., 1994) of TnI residues 96−148. H, helical; C, coil. The α, β, and γ motifs are as in Figure 3A.

is one analogous perhaps to that observed for the interaction of scallop myosin regulatory light chain (RLC) and its heavy chain (Xie et al., 1994). By virtue of a sharp bend or "hook" in the otherwise continuous heavy chain α-helix, both N and C domains of RLC interact with adjacent but nonoverlapping segments of heavy chain encompassing a region of ~40 residues. This occurs with only a slight unwinding of the linker helix between domains in the region of its Gly-82. Other arrangements are, of course, possible. Clearly answers to these questions must await further detailed structural analyses of the TnC·I complex. Hopefully the results of such investigations will be reconcilable with the extensive cross-linking evidence [summarized in Kobayashi et al. (1995)] indicating interaction of the extended inhibitory region of TnI with both the N and C domains of TnC as well as their connecting central helix.

A surprising aspect of these observations with TnI is the presence of two motifs, β and γ, encompassing 26 residues in addition to the single α motif (14 residues). This implies a larger region of TnI_{96-148} contact with TnC N domain than with C domain. Conceivably this may be related to the larger apolar surface area exposed in the former in its open form as reported for the NMR solution structure (Slupsky & Sykes, 1995). Alternatively and as indicated by cross-linking studies, the γ motif may interact with a region distinct from the apolar patch. Employing mutants to position Cys residues at strategic locations in TnC, Kobayashi et al. (1994) observed that one of these (Cys-12 in the rabbit sequence; Cys-15 in chicken TnC) was extensively cross-linked by 4-maleimidobenzophenone to residues 132−141 of intact TnI. These residues are immediately adjacent to and encompass part of the γ motif (residues 135−146) of the amino acid sequence (see Figure 3). As pointed out by these workers, this would place this portion of TnI on the opposite side of the N-terminal domain from the exposed nonpolar surface of the 2 Ca^{2+} open conformation. The exposed surface of this "back side" is contributed largely by the side chains of amino acid residues on helices N, A, and D, whose main-chain relative orientations one to the other are little altered in the Ca^{2+}-induced closed to open transition. However, inspection of this region in the recently determined 1.75 Å resolution structure of the Ca^{2+}-saturated N domain structure (Strynadka et al., submitted for publication) shows significant changes in sidechain orientations and interactions when compared with the apo form (N. Strynadka, personal communication). These alterations, presumably arising from the structural reorganization associated with the large movements of helices B and C as a unit relative to N, A, and D, clearly deserve special consideration and investigation as a putative Ca^{2+}-sensitive interaction site for the γ motif region of TnI and its immediate environs.

Detrimental effects of increased acidity on the contractile activity of heart tissue as compared with skeletal muscle have been long recognized as one of the expected manifestations of cardiac ischemia. Recent evidence from at least three laboratories (Metzger et al., 1993; Ball et al., 1994; Ding et

al., 1995; Metzger, 1996) has implicated differences in Ca^{2+} sensitivity to acidic pH of the cardiac and skeletal TnC·I complexes as being responsible, at least in part, for this phenomenon. Comparison of the consensus sequences for the repeating motifs in skeletal and cardiac TnIs (Figure 3) indicates highly conserved differences, especially in the β motif. Skeletal R or K at residue 123 is replaced by Q157, H130 by A164, and V132 by E166. Since this region is undoubtedly intimately involved in the Ca^{2+}-dependent regulatory process, we propose these substitutions, all involving charged residues, as prime candidates responsible for this differential pH effect on the Ca^{2+} sensitivities of the skeletal and cardiac systems, a hypothesis currently under investigation in our laboratory.

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