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A ^{1}H NMR study of a ternary peptide complex that mimics the interaction between troponin C and troponin I

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Abstract

The troponin I peptide Nα-acetyl TnI (104–115) amide (TnIp) represents the minimum sequence necessary for inhibition of actomyosin ATPase activity of skeletal muscle (Talbot, J.A. & Hodges, R.S., 1981, *J. Biol. Chem. 256*, 2798–3802; Van Eyk, J.E. & Hodges, R.S., 1988, *J. Biol. Chem. 263*, 1726–1732; Van Eyk, J.E., Kay, C.M., & Hodges, R.S., 1991, *Biochemistry 30*, 9974–9981). In this study, we have used ^{1}H NMR spectroscopy to compare the binding of this inhibitory TnI peptide to a synthetic peptide heterodimer representing site III and site IV of the C-terminal domain of troponin C (TnC) and to calcium-saturated skeletal TnC. The residues whose ^{1}H NMR chemical shifts are perturbed upon TnIp binding are the same in both the site III/site IV heterodimer and TnC. These residues include F102, I104, F112, I113, I121, I149, D150, F151, and F154, which are all found in the C-terminal domain hydrophobic pocket and antiparallel β-sheet region of the synthetic site III/site IV heterodimer and of TnC. Further, the affinity of TnIp binding to the heterodimer (K_d = 192 ± 37 μM) was found to be similar to TnIp binding to TnC (48 ± 18 μM [Campbell, A.P., Cachia, P.J., & Sykes, B.D., 1991, *Biochem. Cell Biol. 69*, 674–681]). The results indicate that binding of the inhibitory region of TnI is primarily to the C-terminal domain of TnC. The results also indicate how well the synthetic peptide heterodimer mimics the C-terminal domain of TnC in structure and functional interactions.

Keywords: function; interaction of troponin C with troponin I; structure; synthetic ternary complexes

Muscle contraction involves multiple protein interactions. The thick filament protein myosin interacts with the thin filament proteins actin, tropomyosin, and troponin (which is composed of three subunits, TnC, TnI, and TnT). TnC binds calcium, TnI inhibits the magnesium-dependent ATPase activity of actomyosin (Hartshorne & Mueller, 1968; Schaub & Perry, 1969; Greaser & Gergely, 1971; Perry et al., 1972) through interactions with both actin and tropomyosin-actin (Potter & Gergely, 1974; Hitchcock, 1975), and TnT binds to tropomyosin. The inhibition of the actomyosin ATPase is neutralized when calcium-saturated TnC forms a complex with TnI (Perry et al., 1972; Weeks & Perry, 1978; Chong et al., 1983). This calcium-dependent interaction between TnC and TnI is one of the key processes in the regulation of contraction in skeletal muscle and thus is important to the understanding of the mechanism of muscle contraction on a molecular level (for recent reviews, see Leavis & Gergely, 1984; Zot & Potter, 1987).

Studies indicate that residues 1–21 and 96–116 of TnI interact with TnC (Moir et al., 1974; Cole & Perry, 1975; Syska et al., 1976). Residues 104–115 of TnI (Gly-Lys-Phe-Lys-Arg-Pro-Pro-Lys-Arg-Arg-Val-Arg) comprise the minimum sequence necessary for inhibition of actomyosin ATPase activity (Talbot & Hodges, 1979; Cachia et al., 1983, 1985; Van Eyk et al., 1991). This TnI pep-

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Abbreviations: s-TnC, skeletal troponin C; TnI, troponin I; TnT, troponin T; CaM, calmodulin; SCIII, Ac-(A101) (Y112) s-TnC (93–126) amide; SCIV, Ac-s-TnC (129–162) amide; TnIp, Nα-acetyl TnI (104–115) amide; TR_2C, C-terminal domain (residues 92–162) of s-TnC; NOE, nuclear Overhauser enhancement; TRNOE, transferred nuclear Overhauser enhancement; 2D NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; 2D DQF-COSY, two-dimensional double-quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; DSS, 2,2′-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

tide is extremely basic, with four arginines and two lysines within a 12-residue sequence. Alternating with these basic residues are hydrophobic residues. Van Eyk and Hodges (1988) and Van Eyk et al. (1991) have evaluated the contribution of each amino acid residue of the TnI inhibitory region (104–115) and have determined that the most important residues for binding and inhibitory activity were F106, R108, L111, R113, V114, and R115. The structure of the TnI peptide bound to calcium-saturated s-TnC, derived from 2D NOE ^{1}H NMR spectroscopy, reveals an amphiphilic helix-like structure, distorted in the center by two proline residues (Campbell & Sykes, 1989, 1991a,b). The central bend in the peptide functions to bring the residues on the hydrophobic face into closer proximity with each other to form a small hydrophobic pocket with the hydrophilic basic residues extending off the opposite face of the peptide.

Although the inhibitory region of TnI that interacts with s-TnC has been defined, the region on s-TnC, which interacts with the inhibitory region of TnI, is not clearly defined. The crystal structure of s-TnC (Herzberg & James, 1985, 1988; Sundaralingam et al., 1985; Satyshur et al., 1988) reveals a dumbbell-shaped molecule with two globular domains connected by a nine-turn α-helix. Each domain contains two calcium-binding sites of the helix-loop-helix structural motif. NMR studies of tryptic fragments of TnC indicate that both halves of the molecule retain a structure in the apo and calcium-saturated forms, which resemble the structure of the intact protein (Drakenberg et al., 1987). Recently, it has been shown that synthetic peptides representing the calcium-binding site III (SCIII) or site IV (SCIV) of TnC, in the presence of calcium, assemble to form symmetric dimers that are structurally very similar to the C-terminal domain of TnC (Shaw et al., 1990, 1991a–d; Kay et al., 1991). Further, when equimolar amounts of SCIII and SCIV are mixed in the presence of calcium, SCIII/SCIV heterodimers are stoichiometrically and preferentially formed that even more closely mimic the C-terminal domain of s-TnC in structure (Shaw et al., 1991b,c).

Several studies indicate that the TnI peptide interacts with the C-terminal domain of TnC (Weeks & Perry, 1978; Grabarek et al., 1981; Cachia et al., 1983; Drabikowski et al., 1985; Leszyk et al., 1987, 1988; Lan et al., 1989). In the present study we use ^{1}H NMR spectroscopy to compare the binding of TnIp to the synthetic noncovalent heterodimer comprised of site III and site IV peptides with the binding of TnIp to calcium-saturated s-TnC.

Results and discussion

TnIp has been shown to interact with the C-terminal domain of TnC (Leavis et al., 1978; Weeks & Perry, 1978; Chong & Hodges, 1981; Grabarek et al., 1981; Wang & Cheung, 1984; Tao et al., 1986, 1989; Leszyk et al., 1987; Lan et al., 1989). Here, we present a ^{1}H NMR comparison of TnIp binding to s-TnC and TnIp binding to an SCIII/SCIV heterodimer. Identification of specific residues in s-TnC and the SCIII/SCIV heterodimer that are perturbed upon binding of TnIp requires spectral assignment of TnIp, s-TnC, and the SCIII/SCIV heterodimer. A complete proton assignment of TnIp has been published (Campbell & Sykes, 1991b). As well, a complete assignment of the calcium-saturated SCIII/SCIV heterodimer has been accomplished (Shaw, G.S. & Sykes, B.D., in prep.), the partial assignment of which has been published (Shaw et al., 1991b, 1992). The partial assignment of whole turkey skeletal s-TnC was accomplished using standard methods employing one-dimensional ^{1}H NMR calcium titration data and 2D DQF-COSY and NOESY data and will not be presented. Partial proton assignments of the C-terminal domain of rabbit s-TnC and whole rabbit s-TnC have been published (Drabikowski et al., 1985; Tsuda et al., 1988, 1990) and are in agreement with those assignments made of turkey s-TnC. These proteins are highly homologous in sequence and share a similarity in sequence and structure to CaM. The entire ^{1}H spectral assignment of *Drosophila* recombinant CaM has been accomplished (Ikura et al., 1990). Similarities in the crystal structures of CaM and s-TnC (Sundaralingam et al., 1985; Herzberg & James, 1985, 1988; Babu et al., 1988; Satyshur et al., 1988) suggest that there may be similarities in the ^{1}H NMR spectra of these proteins. The chemical shifts found for some of the resonances in s-TnC are very similar to some of the chemical shifts found in CaM (Ikura et al., 1990). Figure 1 illustrates the partial sequences of turkey s-TnC, SCIII/SCIV, rabbit s-TnC, and *Drosophila* recombinant CaM.

Comparison of heterodimer and s-TnC spectra

Figure 2 illustrates a comparison of the aromatic region of the 600-MHz ^{1}H NOESY spectra of s-TnC and the SCIII/SCIV heterodimer. There are a total of five aromatic residues in the C-terminal domain of turkey s-TnC: F102, F105, F112, F151, and F154. The F105 protons do not exhibit much spectral dispersion and therefore are not indicated in either spectrum. The striking similarity of the NOESY spectra in Figure 2 indicates that the C-terminal domain of s-TnC and the SCIII/SCIV heterodimer form similar structures in solution. Three of the residues (F102, F151, and F154) form an aromatic cluster in the X-ray structures (Herzberg & James, 1985, 1988; Sundaralingam et al., 1985; Satyshur et al., 1988) and Figure 2 illustrates NOESY connectivities between these residues. F102 makes strong contacts to F151 and F154. As well, contacts may be seen between F151 and F154. Figure 3 shows a comparison of the amide NH and αCH to aliphatic regions of the NOESY spectra of s-TnC (A) (the amide and α regions in D_2O) and the SCIII/SCIV heterodimer (B) (the amide region in H_2O and the α region in D_2O). The amide hydrogen of I113 of s-TnC is involved in the anti-

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                      93                 100                 110
Turkey s-TnC          K S E E E L A N C (F) R (I) F D K N A D G [(F)(I) D] I E
                      93
SCIII/SCIV            K S E E E L A N A (F) R (I) F D K N A D G [(Y)(I) D] I E
                      90                    100                   110
Rabbit s-TnC          K S E E E L A E C F R I F D R N A D G [Y I D] A E
                      80                    90                    100
Drosophila CaM        D S E E E I R E A F R V F D K D G N G [F I S] A A

                          120                 130                    140
Turkey s-TnC          E L G E (I) L R A T G E H V T E E E I E D L M K D

SCIII/SCIV            E L G E (I) L R A T G     V T E E D I E D L M K D
                                  120                 130
Rabbit s-TnC          E L A E I F R A S G E H V T D E E I E S L M K D
                                  110                 120
Drosophila CaM        E L R H V M T N L G E K L T D E E V D E M I R E

                                        150                  160
Turkey s-TnC          S D K N N D G [R (I)(D)] (F) D E (F) L K M M E G V Q

SCIII/SCIV            S D K N N D G [R (I)(D)] (F) D E (F) L K M M E G V Q
                          140                 150
Rabbit s-TnC          G D K N N D G [R I D] F D E F L K M M E G V Q
                          130                 140
Drosophila CaM        A N I D G D G [Q V N] Y E E F V T M M T S K
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Fig. 1. Partial amino acid sequence alignment of turkey s-TnC (Wilkinson, 1976; Golosinska et al., 1991), SCIII/SCIV (Shaw et al., 1991b) (Ac-(A101)(Y112) TnC (93–126) amide/Ac-TnC (129–162) amide), rabbit s-TnC (Collins et al., 1973), and *Drosophila* recombinant CaM (Ikura et al., 1990). The amino acid numbering is indicated at the top of each sequence. Circled residues are those that are perturbed upon binding of the inhibitory TnI peptide. Boxed residues are those involved in the antiparallel β-sheet formation between calcium-binding loops III and IV. For the SCIII/SCIV heterodimer, C101 was changed to A101 to prevent intermolecular cross-links from occurring. F112 was changed to Y112 to aid in making ^{1}H NMR assignments. Y112 in the heterodimer is equivalent to Y109 in rabbit s-TnC and therefore is a conservative change.

parallel β-sheet hydrogen bonding to I149 carbonyl oxygen and therefore exhibits slow exchange with the solvent (allowing us to observe the amide resonance on a 2D NOESY spectrum in D_2O) as well as an extreme downfield shift. The amide hydrogen of I113 of the heterodimer exhibits faster exchange with the solvent (and is therefore not observable on a 2D NOESY spectrum in D_2O) but still exhibits the extreme downfield shift. This residue is analogous to I100 of CaM, which also exhibits slow exchange behavior in D_2O (Ikura et al., 1991). Similar NOEs are observed for s-TnC and the SCIII/SCIV heterodimer in this region. A strong $d_{\alpha N}$ crosspeak between F112α/Y112α and I113NH, and between I149α and D150NH (data not shown) may be observed as well as a cross-sheet $d_{\alpha\alpha}$ between F112α and D150α in s-TnC (Y112α and D150α in SCIII/SCIV). A d_{NN} between I113NH and I149NH and a weak $d_{\alpha N}$ between I113NH and D150α were also observed. COSY and NOESY connectivities between the NH, αCH, βCH, γCH_2 and δCH_3 protons were observed for I113 for s-TnC and the SCIII/SCIV heterodimer.

Interactions between s-TnC and the TnI peptide

We have used ^{1}H NMR at 600 MHz to follow several residues on turkey s-TnC and the heterodimer that are perturbed upon binding of the inhibitory TnIp. A calcium-

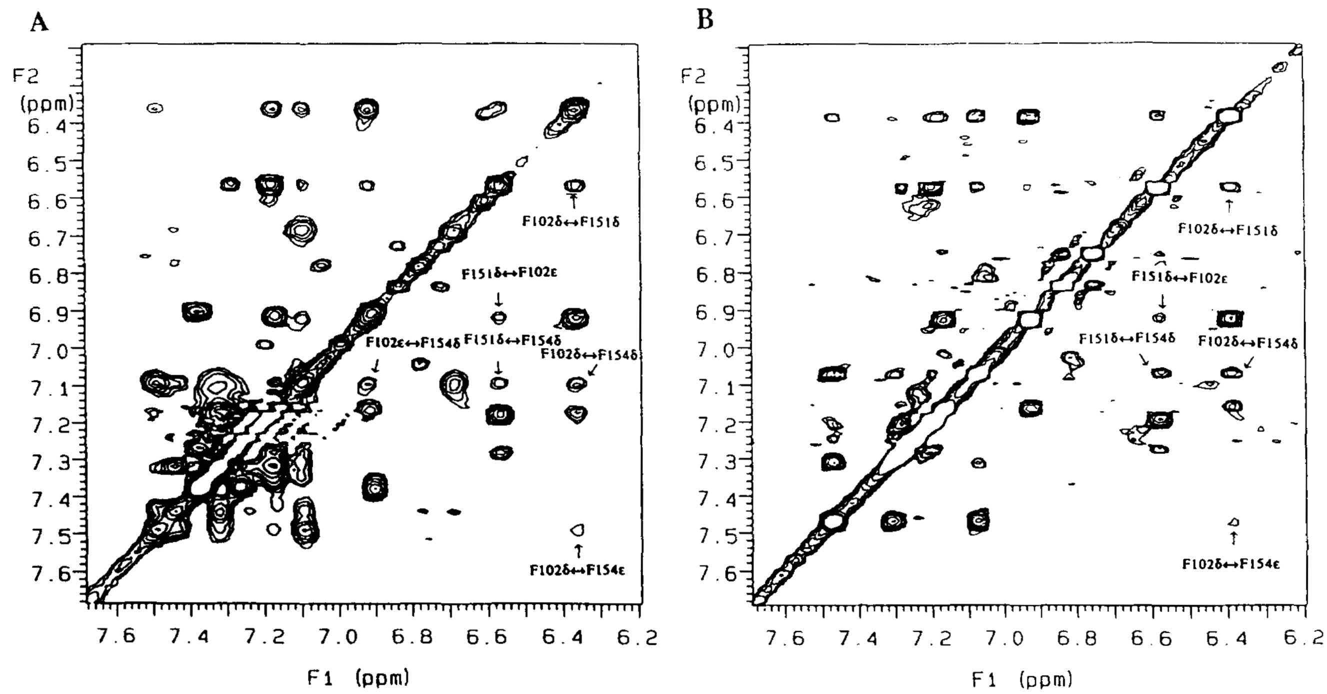

Fig. 2. 600-MHz ^{1}H NOESY spectra in D_2O showing NOE connectivities between the phenylalanine rings of residues F102, F151, and F154 for (**A**) calcium-saturated TnC (2 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl, pD 6.2) and (**B**) calcium-saturated SCIII/SCIV heterodimer (4 mM SCIII/SCIV, 10 mM $CaCl_2$, 50 mM KCl, pD 7.2). τ_m = 150 ms for both spectra.

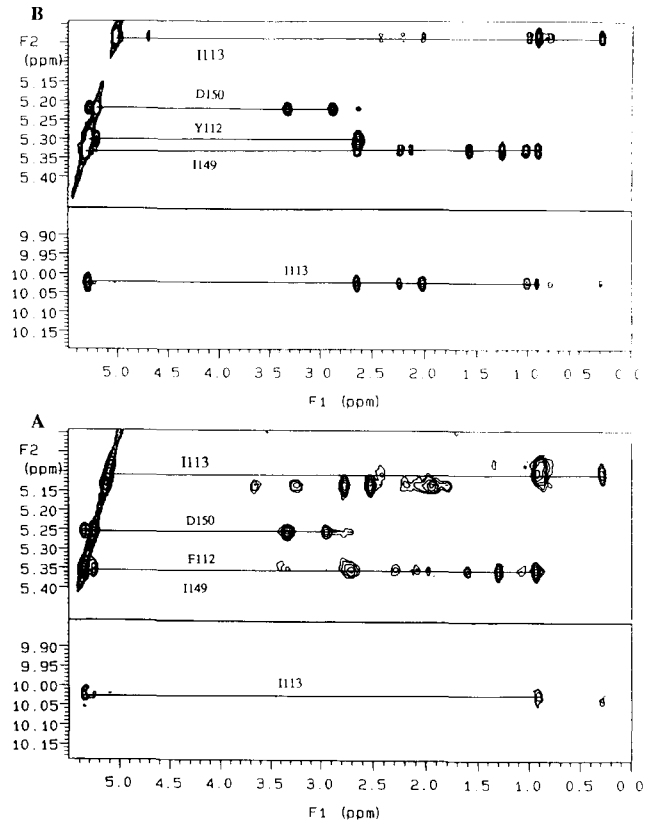

Fig. 3. 600-MHz ^{1}H NOESY spectra of (**A**) calcium-saturated TnC (2 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl, pD 6.2) and (**B**) calcium-saturated SCIII/SCIV heterodimer (4 mM SCIII/SCIV, 10 mM $CaCl_2$, 50 mM KCl [amide region in H_2O, pH 7.2, and αCH region in D_2O, pD 7.2]) illustrating the amide and αCH regions. τ_m = 150 ms for both spectra.

saturated s-TnC sample as well as a calcium-saturated heterodimer sample (representing the C-terminal domain of s-TnC) were titrated with TnI peptide. Figure 4a (A and B) shows the aromatic region of the ^{1}H NMR spectra. In both cases, a slight downfield shift is observed for the F102δCH2 and F154δCH2 resonances, and an upfield shift is observed for the F154ϵCH resonance. The resonances at 6.7 ppm, which arise from the N-terminal domain F75δCH2, F26δCH2 protons (W.A. Findlay and B.D. Sykes, unpubl.), are also observed to shift slightly upon binding of TnIp. As the titration progresses, TnI peptide F106δCH2 and F106ϵCH2, F106ζCH resonances are observed to emerge at 7.25 and 7.30 ppm, respectively, with increasing peptide concentration. Figure 4b (A and B) shows the α-proton region of the spectra upon binding of TnIp. In both cases, an upfield shift for the I113αCH resonance, a downfield shift for the D150αCH proton, and upfield and downfield shifts for I149αCH and F112(Y112)αCH, respectively, were observed. These residues are all situated within the β-sheet region between the two calcium-binding sites. The resonances from D114αCH and R148αCH, which are also present in the β-sheet, are obscured because their resonances are almost coincident with the water resonance. Figure 4c (A and B) shows the upfield-shifted methyl region of the ^{1}H NMR spectra during the titration of s-TnC and the heterodimer with the TnI peptide. Marked downfield shifts for the I113δCH_3 and I121γCH_3 resonances and a marked upfield shift for the I104γCH_3 resonance are observed.

The spectral changes presented in Figure 4 indicate that TnIp perturbs the same residues in s-TnC as it does in the SCIII/SCIV heterodimer and that they are perturbed in the same manner. These residues are from the hydrophobic region formed by the amphipathic helices from calcium-binding sites III and IV and the β-sheet region between the two calcium-binding loops. The perturbed resonances include the aromatic side chains of F102 and F154, the methyl resonances of I104, I113, and I121, and the backbone αCH resonances of F112(Y112), I113, I149, and D150. This suggests that TnIp binds to the SCIII/SCIV heterodimer and s-TnC in the same way implying, therefore, that the interaction of the TnIp with s-TnC is primarily with the C-terminal domain.

Further evidence for the similarity of the interaction of the TnIp with the heterodimer and s-TnC is obtained from the determination of the stoichiometry and dissociation constant of the TnIp from the NMR data. The change in chemical shift of residues Y112α, I149α, D150α, I113α, I104γCH_3, I121γCH_3, and I113δCH_3 for the heterodimer were plotted as a function of added [TnIp], and a dissociation constant (K_d) calculated (Fig. 5). The dissociation constant obtained for the heterodimer (for a 1:1 complex of TnIp with the SCIII/SCIV heterodimer) was 192 $\pm$ 37 μM which is similar to that published for s-TnC using ^{19}F NMR spectroscopy (K_d = 48 $\pm$ 18 μM [Campbell et al., 1991]). The slightly larger dissociation constant obtained for the heterodimer indicates that the peptide is bound more tightly to s-TnC perhaps because of additional interactions with the N-terminal domain (see below).

The residues that are perturbed in the C-terminal domain of s-TnC upon binding of TnIp based upon the TnIp titration studies with s-TnC and the SCIII/SCIV heterodimer are all located in a hydrophobic pocket (a view of the C-terminal domain of TnC showing the locations of the perturbed resonances is presented in Kinemage 1). These residues are surrounded by acidic residues (for color diagram see Fig. 2a of Strynadka & James, 1990). In the bound structure of TnIp (Campbell & Sykes, 1991b), the hydrophobic residues of TnIp form a surface that is surrounded by the basic residues of the TnIp. This result certainly suggests that a prime spot for TnIp binding is the C-terminal domain hydrophobic pocket of s-TnC. The F106NH resonance of TnIp is the most shifted amide resonance of TnIp upon binding to s-TnC (Campbell & Sykes, 1991b). This supports the proposed interaction of the hydrophobic surface of the TnIp with the hydro-

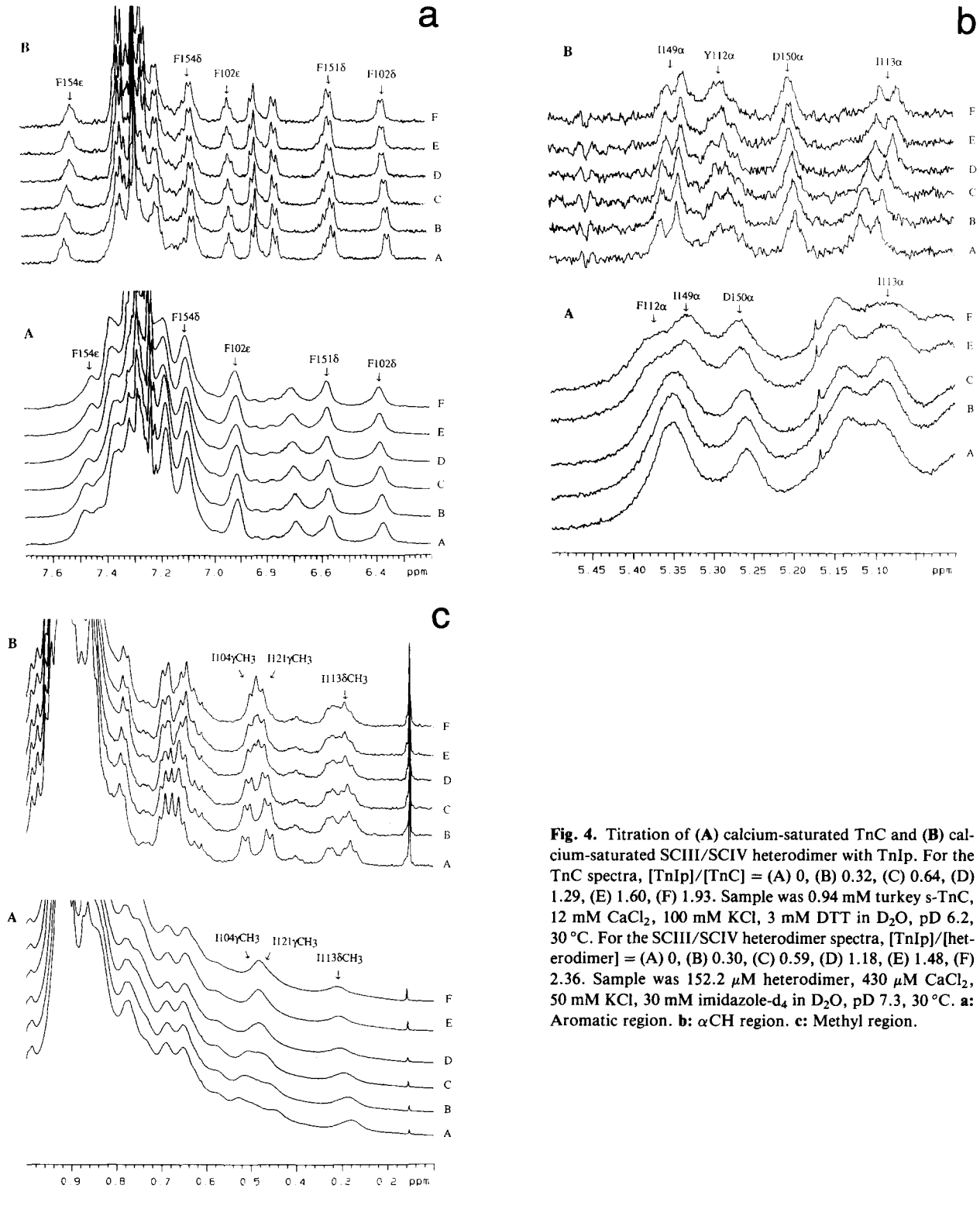

Fig. 4. Titration of (**A**) calcium-saturated TnC and (**B**) calcium-saturated SCIII/SCIV heterodimer with TnIp. For the TnC spectra, [TnIp]/[TnC] = (A) 0, (B) 0.32, (C) 0.64, (D) 1.29, (E) 1.60, (F) 1.93. Sample was 0.94 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl, 3 mM DTT in D_2O, pD 6.2, 30 °C. For the SCIII/SCIV heterodimer spectra, [TnIp]/[heterodimer] = (A) 0, (B) 0.30, (C) 0.59, (D) 1.18, (E) 1.48, (F) 2.36. Sample was 152.2 μM heterodimer, 430 μM $CaCl_2$, 50 mM KCl, 30 mM imidazole-d_4 in D_2O, pD 7.3, 30 °C. **a:** Aromatic region. **b:** αCH region. **c:** Methyl region.

phobic region in C-terminal domain of s-TnC. The fact that the s-TnC residues are perturbed upon TnIp binding demonstrates that the protein must change in some manner to accommodate the TnIp, possibly by opening slightly to expose the hydrophobic residues more. At this point, however, it is unproven whether TnIp actually interacts directly with these residues in the hydrophobic pocket or if TnIp merely binds somewhere else and perturbs these residues. A major influence on chemical shift in this region of s-TnC is the ring current effects of F102,

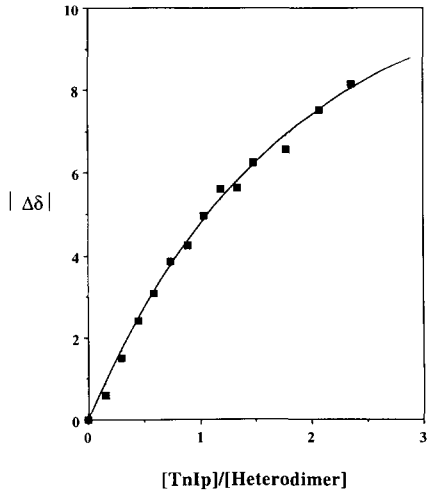

Fig. 5. TnIp titration plot for 152.2 μM heterodimer derived from the heterodimer titration data shown in Figure 4. The data are the average absolute values of the chemical shift changes of I113αCH, Y112αCH, D150αCH, I149αCH, I104γCH$_3$, I121γCH$_3$, and I113δCH$_3$ as TnIp is added to the SCIII/SCIV heterodimer. The curve for the binding was calculated using an iterative nonlinear least-squares analysis allowing the K_d and the total shift parameters to be simultaneously changed to achieve a best fit, giving a value for $K_d = 192 \pm 37$ μM.

F151, and F154, and it is possible that the alteration of the disposition of one of these aromatic rings could be enough to affect all of the residues discussed above. Detailed NOE experiments should reveal where the site of TnIp binding is if peptide–protein NOEs can be observed. Several reports suggest that the binding of the TnI peptide is to the N-terminal portion of site III (Leavis et al., 1978; Weeks & Perry, 1978; Chong & Hodges, 1981; Grabarek et al., 1981; Wang & Cheung, 1984; Tao et al., 1986, 1989; Leszyk et al., 1987; Lan et al., 1989). Certainly, this region is highly conserved in s-TnC and CaM and is fairly acidic (Fig. 1).

These results do not preclude additional possible interactions of TnIp with the N-terminal region of s-TnC. A shift of the δCH2 protons of F26/75 (in the N-terminal domain) was seen, but no other assignable shifts were observed. Cross-linking studies suggest that there is an interaction (Leszyk et al., 1990; Kobayashi et al., 1991) between the N-terminal domain of s-TnC and TnIp. Because the N-terminal region of s-TnC contains the regulatory sites, it seems probable that there must be some interaction. Perhaps the C-terminal domain serves to anchor the TnIp portion of TnI to s-TnC and regulation comes by interaction of an extended portion of TnIp or another portion of TnI with the regulatory sites. Extensive NMR studies of CaM complexed to the myosin light chain kinase (MLCK) M13 peptide have shown an interaction of the peptide with both domains of CaM (Ikura et al., 1992). The residues in the C-terminal domain of CaM with which the M13 peptide interacts are analogous to some of the residues observed here to shift upon binding of TnIp (namely F102, I104, I113, and I121 of s-TnC). The M13 peptide also interacts with the N-terminal domain of CaM changing the overall shape of CaM in complex versus CaM alone. This interaction, however, relies on the M13 peptide being entirely helical with a 12-residue span between the first residue that interacts with the C-terminal domain of CaM and the residue that interacts with the N-terminal domain of CaM. Shortened peptides that do not contain this 12-residue amino acid span only bind to the C-terminal domain of CaM (Kataoka et al., 1991). These shortened peptides could interact with CaM in an analogous manner to the TnIp interaction with TnC.

The results here suggest that the SCIII/SCIV heterodimer is a good model to study the C-terminal domain of s-TnC and its interaction with other muscle proteins. Synthetic peptides representing specific portions of a protein not only aid in our understanding of the folding properties of proteins but allow us to observe the interactions of specific portions of proteins with other proteins. As well, this type of research lays the groundwork for the design of novel proteins with desired activities.

In conclusion, we have compared the residues in the SCIII/SCIV heterodimer and in turkey s-TnC involved in binding the inhibitory TnI peptide and have found the interactions to be similar. We have previously determined the structure of the TnI peptide when bound to calcium-saturated s-TnC (Campbell & Sykes, 1991b), and here we attempt to bring together the structure and the location of binding of the TnI peptide in a model of the interaction of the inhibitory region of TnI with calcium-saturated s-TnC using synthetic peptide models and whole s-TnC. The relief of inhibition of actomyosin ATPase when calcium-saturated s-TnC forms a 1:1 complex with TnI is a key step in the initiation of muscle contraction. An understanding of the interaction between s-TnC and TnI at the molecular level is the key to understanding muscle contraction.

Materials and methods

Sample preparation

Nα-acetyl-TnI (104–115) amide was synthesized using the standard procedures for solid-phase peptide synthesis (Erickson & Merrifield, 1976; Talbot & Hodges, 1981; Hodges et al., 1988) on an Applied Biosystems 430A peptide synthesizer (Foster City, California). The synthesis of the site III and site IV peptides, Ac-(A101)(Y112)TnC (93–126) amide (SCIII) and Ac-TnC (129–162) amide (SCIV), respectively, of TnC, have been previously described (Shaw et al., 1991a,d). Troponin was isolated as described previously (Campbell & Sykes, 1991b). TnC was found to be homogeneous when examined by SDS-PAGE and UV spectrophotometry ($\epsilon_{\lambda 259nm} = 0.109$/mg/mL for turkey s-TnC). The concentrations of peptide and turkey

s-TnC used were determined from amino acid analysis after hydrolysis in 100–200 μL of 6 N HCl in evacuated sealed tubes for 24 h at 110 °C or 1 h at 160 °C. The mean of the molar ratios of all accurately measurable amino acids (alanine and leucine) in the acid hydrolysate were used to calculate the concentration of peptide or protein upon comparison to a standard amino acid preparation.

Salt-free apo TnC was prepared by dissolving approximately 30 mg of TnC in 1 mL of 25 mM NH_4HCO_3, 1 M EDTA (pH 8), and 10 mM DTT and applied to a 1.5 × 90-cm Sephadex G-25 medium gel size exclusion column (which had been previously decalcified by eluting 5 mL of 1 M EDTA, pH 8) equilibrated with 25 mM NH_4HCO_3 at room temperature. The EDTA-free apo TnC was collected in an acid-washed plastic vial and lyophilized.

The TnC sample for ^{1}H NMR assignments was 2.0 mM turkey s-TnC, 12 mM $CaCl_2$, and 100 mM KCl dissolved in 650 μL of D_2O at a pD of 6.2. DSS (0.1 mM) was added to the sample as a chemical shift standard; 3 mM DTT was also added to the sample to prevent oxidation of the sulfhydryl of s-TnC (C101) and thus prevent formation of intermolecular cross-links. The SCIII/SCIV sample for the 2D NOESY spectrum was 4 mM SCIII/SCIV, 10 mM $CaCl_2$, 50 mM KCl in D_2O, pD 7.2. The TnC sample for the ^{1}H NMR-monitored TnI peptide titration was desalted and made apo as described above. Lyophilized TnC was dissolved in 100 mM KCl, 5 mM $CaCl_2$, 0.1 mM DSS, and 3 mM DTT in D_2O, pD 6.2, to make a final [TnC] = 0.94 mM. The TnI peptide was dissolved to a final concentration of 30.3 mM in 100 mM KCl, 5 mM $CaCl_2$ in D_2O. Small aliquots of the TnI peptide sample were added to the TnC sample using a carefully calibrated Hamilton syringe. The SCIII/SCIV heterodimer sample for the ^{1}H NMR TnI peptide titration was composed of 152.2 μM SCIV, 171.4 μM SCIII, 430 μM $CaCl_2$, 50 mM KCl, and 30 mM imidazole-d_4, pH 7.30. Small aliquots of TnI peptide (11.81 mM in 430 μM $CaCl_2$, 50 mM KCl, and 30 mM imidazole-d_4, pH 7.30) were added to the 152.2 μM heterodimer sample using a carefully calibrated Hamilton syringe.

Calculation of interaction affinity

Dissociation constants for the reaction $P + L \leftrightarrow PL$ were calculated as described previously (Shaw et al., 1991a) using an iterative nonlinear least-squares analysis. The equation used was

$$K_d = \frac{[P][L]}{[PL]},$$

where $[P]$ is the heterodimer concentration, $[L]$ is the free TnIp concentration, and $[PL]$ is the heterodimer – TnIp complex concentration. $[PL]$ was calculated from the total concentrations of P and L according to

$$[PL] = \frac{(P_0 + L_0 + K_d) - \sqrt{(P_0 + L_0 + K_d)^2 - 4P_0L_0}}{2}.$$

NMR spectroscopy

^{1}H NMR spectra of s-TnC and the heterodimer were obtained at 600 MHz on a Varian Unity 600 NMR spectrometer. All spectra were obtained at 30 °C. Two-dimensional experiments were taken with 2,048 data points along t_2 for 256 or 312 t_1 increments and spectral widths of 7,500 Hz. NOESY data sets were acquired using the phase-sensitive method of States et al. (1982). The appropriate phase cycling was used to achieve quadrature detection, to eliminate axial peaks, and, in the case of NOESY spectra, to eliminate multiple quantum coherences. For NOESY spectra, a random delay of between ±10 ms was incorporated to suppress zero quantum coherences. For all data sets, the water resonance was suppressed by presaturation (1.6–2.3 s). The decoupler offset was set equal to the transmitter offset to prevent phase distortions around the water peak due to imperfect cancellation of the dispersive component of residual water magnetization (Hoult, 1976). For NOESY spectra, mixing times (τ_m) of 150 ms and 75 ms were used to assign resonances in the calcium-saturated s-TnC protein. For processing of one-dimensional spectra, a line broadening of 0.5 Hz was used. Two-dimensional NOESY data sets were processed by applying a $\pi/8$ shifted sine bell function to the data in t_2 and a $\pi/6$ shifted sine bell function to the data in t_1 and zero-filling in t_1 to 2K data points.

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