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# Calcium-Dependent Inhibitory Region of Troponin: A Proton Nuclear Magnetic Resonance Study on the Interaction between Troponin C and the Synthetic Peptide $N^{\alpha}$-Acetyl[FPhe$^{106}$]TnI-(104–115) Amide†

Paul J. Cachia, Brian D. Sykes, and Robert S. Hodges*

ABSTRACT: To investigate the calcium-dependent regulation of muscle contraction, a synthetic analogue of the inhibitory region of troponin I, $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide, has been made by solid-phase peptide synthesis. This region represents the minimum sequence necessary for inhibition of actomyosin ATPase activity in the presence of tropomyosin [Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798–2802]. Conformational changes induced by the formation of the synthetic peptide–troponin C complex are followed by proton nuclear magnetic resonance spectroscopy. Aliphatic (Leu and Val), aromatic (*p*-fluorophenylalanine), and charged (Arg) residues are perturbed by interaction with troponin C. In troponin C, peptide–protein interaction results in the redistribution of the Phe envelope of troponin C and perturbations in the aliphatic region. The observed effects on the protein resonances are in agreement with proposed interaction of the peptide with the N-terminal region of site III of troponin C. In the absence of calcium, this region of troponin I (104–115) is bound to actin–tropomyosin, inhibiting actomyosin ATPase activity [Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798–2802]. Our results suggest that the binding site for this region of troponin I is induced in troponin C in the presence of calcium and the formation of this complex releases actomyosin ATPase inhibition.

Full calcium-sensitive control of actomyosin ATPase activity requires both the troponin complex and tropomyosin. Troponin I can inhibit the ATPase activity of actomyosin by itself, but this inhibition, at ionic strengths where tropomyosin neither binds nor inhibits actomyosin, is greatly enhanced by the addition of tropomyosin (Eisenberg & Kielley, 1974; Eaton et al., 1975; Talbot & Hodges, 1979). Release of this inhibition is mediated by troponin C in the presence of calcium. Troponin I fragment studies conducted by Syska et al. (1976) demonstrated that two fragments are of special interest with respect to the function of this protein. One fragment, consisting of residues 1–21, includes the site of phosphorylation catalyzed by phosphorylase kinase (Thr-11), while the second site, which includes residues 96–116, is adjacent to a second site of phosphorylation (Ser-117) (Moir et al., 1974). Phosphorylation at both these sites is completely inhibited by an equimolar amount of troponin C (Cole & Perry, 1975). Furthermore, affinity chromatography using troponin C linked to Sepharose and gel electrophoresis in the presence of troponin C both indicate that these fragments form complexes with troponin C (Syska et al., 1976). The fragment, residues 96–116, also inhibits actomyosin ATPase activity. As with troponin I, the inhibitory effect was potentiated in the presence of tropomyosin but was released by troponin C in the presence or absence of calcium. Talbot & Hodges (1979, 1981) have reported on the biological activity of several analogues of the region 96–116 of troponin I. Their results indicate that the sequence 105–114 is the minimum inhibitory sequence, and analogues of this region are excellent mimics of troponin I, a protein 17 times larger. The tropomyosin specificity of these peptides is demonstrated by comparing the results obtained with salmine, a basic protein also known to inhibit actomyosin ATPase activity. In the presence of tropomyosin, the inhibition by salmine is suppressed, while that of troponin I and the inhibitory peptides is enhanced.

In the present study, we have undertaken the investigation of the interaction of the inhibitory region of troponin I with troponin C, employing the synthetic troponin I analogue $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide.[1] The interaction is monitored by $^1$H NMR spectroscopy to determine the nature of the residues in both species that are affected and thus the region of troponin C that binds the inhibitory region of troponin I during complex formation.

## Materials and Methods

*Materials.* All chemicals and solvents were reagent grade. Poly(styrene-*co*-1% divinylbenzene)benzhydrylamine hydrochloride resin (0.49 mmol of $NH_2$/g of resin) was purchased

† From the Department of Biochemistry and the Medical Research Council of Canada Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received December 17, 1982.* This investigation was supported by research grants from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research, a Postdoctoral Fellowship (P.J.C.), and a research allowance (P.J.C.).

[1] Abbreviations: FPhe, *p*-fluorophenylalanine; $N^{\alpha}$-acetyl[FPhe$^{106}$]-TnI-(104–115) amide, synthetic N-terminal acetylated rabbit skeletal troponin I fragment, residues 104–115 with a C-terminal amide and fluorophenylalanine substituted at position 106 for phenylalanine; CB-9, cyanogen bromide fragment 9, residues 84–135 of rabbit skeletal troponin C; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $^1$H NMR, proton nuclear magnetic resonance; TnI, troponin I; Tris, tris(hydroxymethyl)aminomethane; Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; Tos, tosyl; Z(Cl), 2-chlorobenzoxycarbonyl; TLC, thin-layer chromatography.

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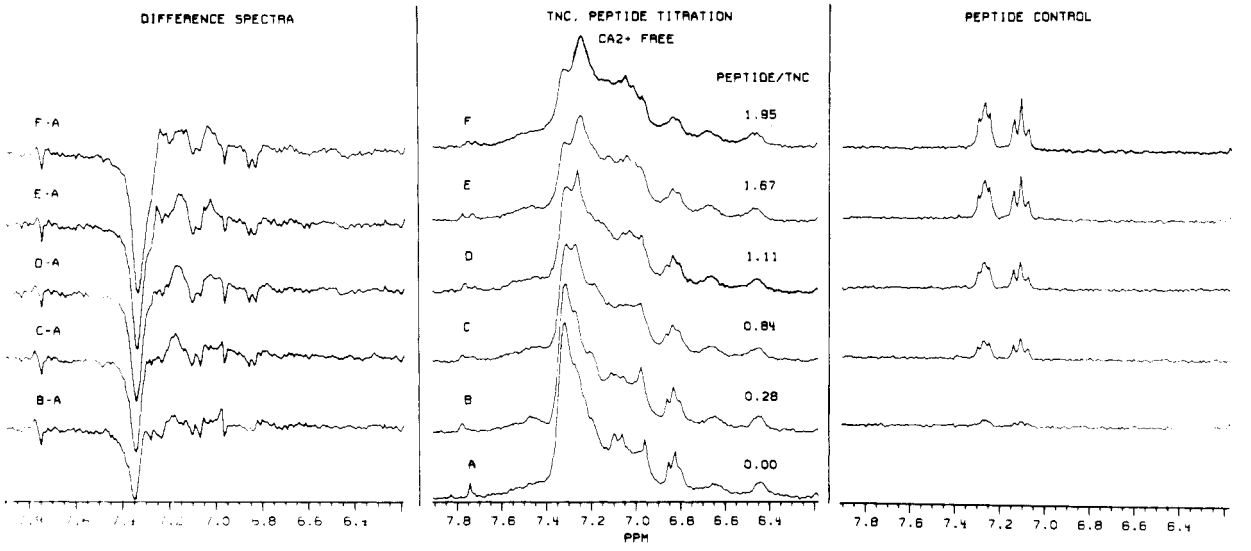


FIGURE 1: $^1H$ NMR spectra of the aromatic region (6.2–7.2 ppm) of $Ca^{2+}$-free troponin C at various stages of addition of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide (i.e., spectra B–F inclusive). The difference spectra are obtained by subtracting the troponin C spectrum (A) from each of the succeeding spectra B–F. [Troponin C] $\simeq 4.1 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.1). Negative peaks in the difference spectra are the result of conformational and/or environmental changes occurring in troponin C on successive additions of increasing amounts of peptide. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 3170.

from Bioproducts Peptide Department, Belgium. Rabbit skeletal muscle was purchased from Pel Freeze Biologicals Inc., Rogers, AR. The quantities of peptide and troponin C 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. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolysate was used to calculate the concentration of protein or peptide. Routine amino acid analyses were performed on a Durrum 500 amino acid analyzer. The pH values quoted are direct meter readings.

*Peptide Synthesis.* The troponin I inhibitory region analogue, $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide, was synthesized by using the general procedures for solid-phase peptide synthesis on a Beckman peptide synthesizer, Model 990. Amidation of the peptide COOH terminal was accomplished by coupling the C-terminal residue to the benzhydrylamine resin [0.31 mmol of Boc-Arg(Tos) and DCC/g of resin]. This resulted in a substitution level of approximately 0.29 mmol/g of amino acid resin as determined by the picrate monitoring method (Hodges & Merrifield, 1975). The remaining free amino groups were terminated by a 60-min treatment with acetic anhydride/benzene/pyridine (1:3:3, 50 mL/g of peptide resin) after prewashing the resin for 5 min with the same mixture. The α-amino groups of all amino acids used in the synthesis were protected with the Boc group, and the following side-chain blocking groups were used: Arg (Tos) and Lys (Z(Cl)). The amino acid Boc-L-*p*-fluorophenylalanine was synthesized from a racemic mixture (DL) of the methyl ester of *p*-fluorophenylalanine followed by resolution with α-chymotrypsin (vide infra). Boc-amino acids (3 equiv) in 5 mL of $CH_2Cl_2$ were added to 0.5 g of peptide resin followed by 3.5 mL of DCC (3 equiv). Boc-Arg(Tos) was dissolved in dimethylformamide/$CH_2Cl_2$ (1:5 v/v) due to the low solubility of this protected amino acid in $CH_2Cl_2$. Each coupling was performed twice for 60 min. Boc groups were removed at each cycle with 25 mL of 50% trifluoroacetic acid/$CH_2Cl_2$ (v/v) for 30 min. Neutralization was carried out with 25 mL of 5% diisopropylethylamine/$CH_2Cl_2$ (v/v). The programs used for the attachment of each amino acid have been previously described (Hodges et al., 1981). The N terminal of the peptide was acetylated as described above. The peptide was cleaved from the resin with hydrofluoric acid and purified by Bio-Rex 70 ion-exchange chromatography with a pyridine/acetate gradient as previously described (Talbot & Hodges, 1979, 1981). The amino acid composition of the purified peptide was as follows: Gly (0.90), Lys (1.86), FPhe (1.06), Arg (4.09), Pro (2.00), Leu (1.04), and Val (0.96). The elution position of FPhe was 53 min and 29 s as compared to Phe, which appears at 51 min and 6 s, and the color constant used was that of Phe.

*Preparation of Boc-L-p-fluorophenylalanine.* DL-*p*-Fluorophenylalanine (5 g, 27.3 mmol) was suspended in anhydrous methanol (50 mL). Anhydrous HCl gas was passed through this suspension until the solid dissolved (ca. 10 min). The reaction was allowed to stand at room temperature for 8 h at which time it was evaporated to dryness and the remaining white solid dried over sodium hydroxide pellets for 8 h. This provided DL-*p*-fluorophenylalanine methyl ester hydrochloride salt (6 g, 94% yield). The product was checked by TLC (silica, 1-butanol/acetic acid/$H_2O$, 4:1:1 v/v/v) ($R_{f,acid}$ 0.31, $R_{f,ester}$ 0.40). Without further purification, the ester (4 g, 17 mmol) and triethylamine (4.0 mL) were dissolved in water (20 mL). 2-[(*tert*-Butoxycarbonyloxy)imino]-2-phenylacetonitrile (Boc-ON) (4.6 g, 19 mmol, 1 equiv) dissolved in *p*-dioxane (20 mL) was added to the aqueous mixture. The reaction was stirred for 18 h at room temperature, at which time it was diluted with diethyl ether (50 mL) and washed with saturated $NaHCO_3$ (3 × 10 mL) followed by water (3 × 20 mL) and saturated NaCl solution (1 × 20 mL). Drying over anhydrous $MgSO_4$ and evaporation of the solvent in vacuo provided $N^{\alpha}$-Boc-DL-*p*-fluorophenylalanine methyl ester as an amber oil. This product was taken immediately and suspended in 2 mM $NH_4HCO_3$/0.1 N KCl buffer (150 mL) at pH 7.9 and 38 °C. α-Chymotrypsin (15 mg) was added in the same buffer (2 mL), and the pH was kept constant (pH 7.9) with a pH-stat delivering 0.17 M NaOH. This reaction was stirred for 10 h at 38 °C, at which point it was allowed to separate into two distinct layers. The aqueous layer was extracted with diethyl ether (4 × 25 mL) and then acidified with 5 N HCl to pH 1.8. Extraction of the acidified solution with diethyl ether (5 × 50 mL), washing the pooled organic layers with saturated NaCl solution (1 × 50 mL), and

drying over anhydrous $MgSO_4$, followed by evaporation of the solvent in vacuo, provided a colorless oil. This oil contained a major impurity ($R_f$ 0.83), as well as the product Boc-L-*p*-fluorophenylalanine ($R_f$ 0.70), as judged by TLC on silica plates in isopropyl alcohol/$NH_4OH$/$H_2O$ (6:3:1 v/v/v) following treatment with HCl vapor before development. The impurity was separated from the product by dissolving the oil in saturated $NaHCO_3$ solution (100 mL) and extracting with $CH_2Cl_2$ (3 × 25 mL). Reacidification with 5 N HCl to pH 2.0 followed by extraction with $CH_2Cl_2$ (3 × 20 mL), drying over anhydrous $MgSO_4$, and evaporation of the solvent in vacuo provided pure Boc-L-*p*-fluorophenylalanine (883 mg), which was then used for peptide synthesis.

*Preparation of Troponin C.* Separation and isolation of troponin C was accomplished by using DEAE-Sephadex A-50 chromatography in the presence of 50 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, and 6 M urea buffer, pH 7.8, as described previously (Chong & Hodges, 1981). This method was a modification of the method of Greaser & Gergely (1973).

*$^1$H NMR Spectra.* Protein magnetic resonance spectra were obtained on a Bruker HXS 270-MHz instrument operating in the Fourier-transform mode with quadrature detection. Difference spectra were computed on a Nicolet 1180 interfaced with the spectrometer. A typical spectrum was obtained from 1000 acquisitions, a 0.5-s acquisition time, and 4096 data points, with an 8-$\mu$s pulse width (~80°), a ±2000-Hz sweep width, and a line broadening of 1 Hz.

*Preparation of Troponin C NMR Samples.* Calcium-free troponin C was prepared in the following manner. Troponin C was dissolved in 1.0 mL of 10 mM DTT, 1 mM EGTA, 50 mM KCl, and 20 mM $NH_4HCO_3$ at pH 8.39. This sample was then dialyzed against this same buffer system for 22 h at 4 °C. Freeze-drying provided protein that was then redissolved in water (Millipore Q grade) and dialyzed against water containing Chelex-100 at 4 °C for 18 h. Both dialyses were conducted in degassed solutions under an atmosphere of nitrogen gas. Freeze-drying then provided calcium-free troponin C. The calcium and calcium-free NMR samples were prepared by dissolving the protein in 50 mM KCl/20 mM $NH_4HCO_3$ in $D_2O$ at pH 8.10 to a concentration of 7.37 mg/mL. In the calcium-saturated sample, the buffer system contained 3 mM $CaCl_2$. Finally, 350 $\mu$L of the troponin C stock solutions was transferred to 5-mm round-bottom NMR tubes, and 2 $\mu$L of a 200 mM DSS solution was added as a reference compound.

*Preparation of Peptide Titrants.* Peptide samples were dissolved in 80 $\mu$L of the calcium or calcium-free buffer systems to a concentration of 1.5 mg/mL. Small aliquots (1–4 $\mu$L) of these peptide solutions were taken to titrate troponin C samples. The pH during these titrations was adjusted when necessary with 5 M HCl or 5 M KOH solutions in $H_2O$. Standard peptide spectra were recorded in buffer systems in the absence of protein at pH 8.0.

*Preparation of Peptide NMR Samples.* The peptide was dissolved to a final concentration of 9.6 mg/mL in the calcium-free buffer system at pH 8.0. Subsequently, a 20-$\mu$L aliquot of the solution (0.19 mg) was taken and placed in a 5-mm round-bottom NMR tube, and the volume was made up to 350 $\mu$L with either the calcium-free or calcium buffer system. In peptide samples containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) (Figures 2 and 4), two weak resonances appear at $\delta$ 0.65 and 2.91. These signals are independent of peptide concentration and are associated with the spectrum of DSS.

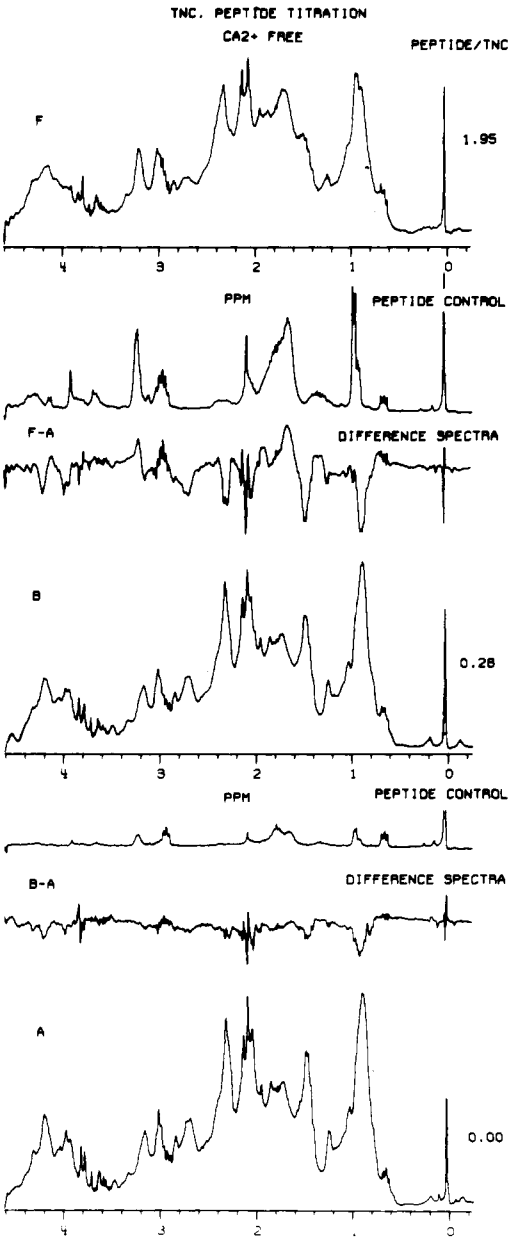


FIGURE 2: $^1$H NMR spectra of the aliphatic region (0.0–4.6 ppm) of $Ca^{2+}$-free troponin C at various stages of addition of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide (i.e., spectra A, B, and F). The difference spectra are obtained by subtracting the troponin C spectrum (A) from each of the spectra B and F. [Troponin C] $\simeq 4.1 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.1). Negative peaks in the difference spectra are the result of conformational and/or environmental changes occurring in troponin C on the successive addition of peptide. Positive peaks are attributed to the added peptide. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 15 000.

*Preparation of Troponin C Titrant.* Troponin C was dissolved to a final concentration of 52 mg/mL in either the calcium or calcium-free buffer at pH 8.0, and small aliquots (1–2 $\mu$L) of these solutions were taken to titrate the peptide NMR samples. Standard troponin C spectra were recorded by adding aliquots of these samples to 350 $\mu$L of the same buffer system in the absence of peptide. The pH was adjusted during these titrations with 5 M HCl or 5 M KOH solutions in $H_2O$.

## Results and Discussion

The inhibitory peptide chosen for this study represents the minimum sequence [troponin I-(104–115)] necessary for the

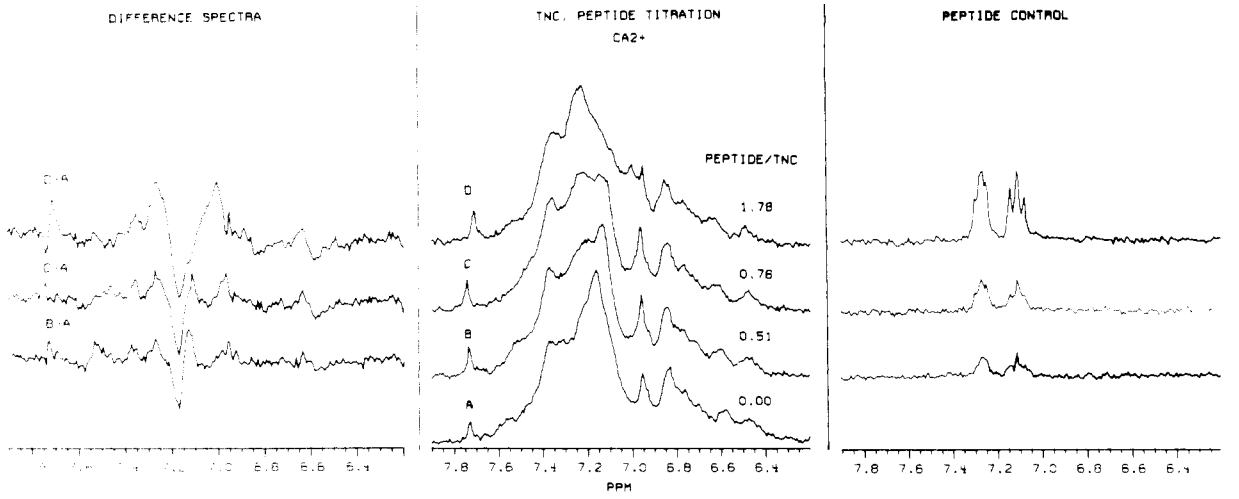


FIGURE 3: $^1$H NMR spectra of the aromatic region (6.2–7.9 ppm) of troponin C plus $Ca^{2+}$ (3 mM) at various stages of addition of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide (i.e., spectra B–D inclusive). The difference spectra are obtained by subtracting the troponin C spectrum (A) from each of the succeeding spectra B–D. [Troponin C] $\simeq 4.1 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.1). Negative peaks in the difference spectra are the result of conformational and/or environmental changes occurring in troponin C on successive addition of increasing amounts of peptide. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 3170.

inhibition of actomyosin ATPase in the presence of tropomyosin (Talbot & Hodges, 1981). The objectives of this study are 3-fold: first, to demonstrate that this minimum inhibitory region binds to troponin C; second, to locate the site of interaction on troponin C; and last, to show the relevance of these findings to the regulation of muscle contraction.

*Troponin C: Titration with Peptide in the Presence and Absence of Calcium.* In these $^1$H NMR studies, the changes that occur in troponin C in the absence of calcium on addition of peptide (Figures 1 and 2) involve redistribution of the Phe envelope ($\delta \simeq 7.35$), broadening of Tyr resonances [Tyr-10 and -109; 2 and 6 protons, $\delta$ 7.10, d, $J$ = 10.8 Hz; 3 and 5 protons, $\delta$ 6.85, br d, $J$ = 10.8 Hz; Levine et al. (1977)], and doubling of the C-2 proton resonance of His-125 at $\delta$ 7.75 (cf. Figure 1, spectra C–F exhibiting two peaks at $\delta$ 7.74 and 7.77). Broadening is also observed in regions associated with Asp and Glu $\beta$-$CH_2$ and $\gamma$-$CH_2$ protons (centered at $\delta$ 2.77 and 2.30, respectively), the $\beta$-$CH_3$ protons of Ala, the $\gamma$-$CH_2$ protons of Lys and Ile, and $\beta$-$CH_2$ and $\gamma$-CH protons of Leu ($\delta$ 1.50), and the Leu, Ile, and Val $CH_3$ protons (at $\delta$ 0.90). Singlet resonances associated with Met $CH_3$ protons and the $N^{\alpha}$-acetyl $CH_3$ protons of troponin C are found at $\delta$ 2.1 (Levine et al., 1976), and changes in this region due to addition of peptide result in sharp peaks in the difference spectra. In the region from $\delta$ 3.90 to 4.40, changes associated with the $\alpha$-$CH_2$ protons of Gly and other $\alpha$-CH backbone rearrangements are also indicated. The titration with peptide was conducted over the range of peptide/troponin C mole ratios of 0–2.0.

Levine et al. (1977) have titrated calcium-free troponin C with calcium and have observed the doubling effect on both the C-2 and C-4 protons of His-125, effects on the 3 and 5 protons of Tyr, and phenylalanine signal intensity redistribution. They have noted that, at calcium concentrations known to saturate the calcium high-affinity sites only, the resonances associated with Tyr-109 undergo broadening associated with restriction of molecular motion. This restriction results from an ordering of the region about Tyr-109, which lies in the calcium-binding loop associated with high-affinity site III of troponin C. Phenylalanine signal intensity redistribution has also been shown by Levine et al. (1977) to arise during calcium saturation of the high-affinity sites. Phenylalanine signal intensity redistribution was also reported by Seamon et al. (1977) in their study of the calcium titration of calcium-free troponin C. By analogy with work by Murray & Kay (1972), who suggest that calcium binding results in a conformational change in troponin C providing a more compact structure, Seamon et al. (1977) suggest that the upfield shift observed in the phenylalanine signal intensity on addition of calcium could represent further structuring of a hydrophobic core. A large portion of this redistribution from $\delta$ 7.35 to 7.19 occurs when only the high-affinity sites are occupied (sites III and IV), although changes in the Phe envelope are observed to occur over the entire range of calcium concentration and are associated with progressive binding at all sites. Calcium titration of the 34-residue synthetic analogue of site III of troponin C (residues 90–123) (Gariépy et al., 1982) has also demonstrated that in the presence of calcium an upfield shift of the signal intensity is observed (Phe-99, -102, and -119).

Our results demonstrate that an upfield shift of Phe signal intensity takes place in response to the accumulation of peptide over the range of the titration shown. The comparison with the $Ca^{2+}$ titration results suggests that similar residues may be affected by the addition of calcium or the peptide to calcium-free troponin C and thus peptide binding may also promote structuring in troponin C. The observed changes occurring in the aromatic region of calcium-free troponin C on addition of peptide have been used to calculate binding constants in the absence and presence of calcium: calcium-free $K_B = (7.1 \pm 2.9) \times 10^3$ M$^{-1}$; calcium-saturated $K_B = (6.7 \pm 3.4) \times 10^4$ M$^{-1}$. These binding constants are characteristic of weak binding between the peptide and troponin C and also suggest that complex formation is calcium-dependent since binding is enhanced by a factor of 9 in the presence of calcium. Though this interaction may be weak, it must be remembered that native troponin C has been shown to have three binding sites for troponin I (Grabarek et al., 1981; Weaks & Perry, 1978) and two binding sites on troponin I for troponin C have been located in the regions of residues 1–21 and 96–116 (Syska et al., 1976).

Peptide addition to calcium-saturated troponin C (Figures 3 and 4) results in perturbations observed in both the aromatic and aliphatic regions of the spectrum. In the aromatic region (Figure 3), the most significant effects are again observed to occur in the Phe envelope centered at $\delta$ 7.16. Addition of

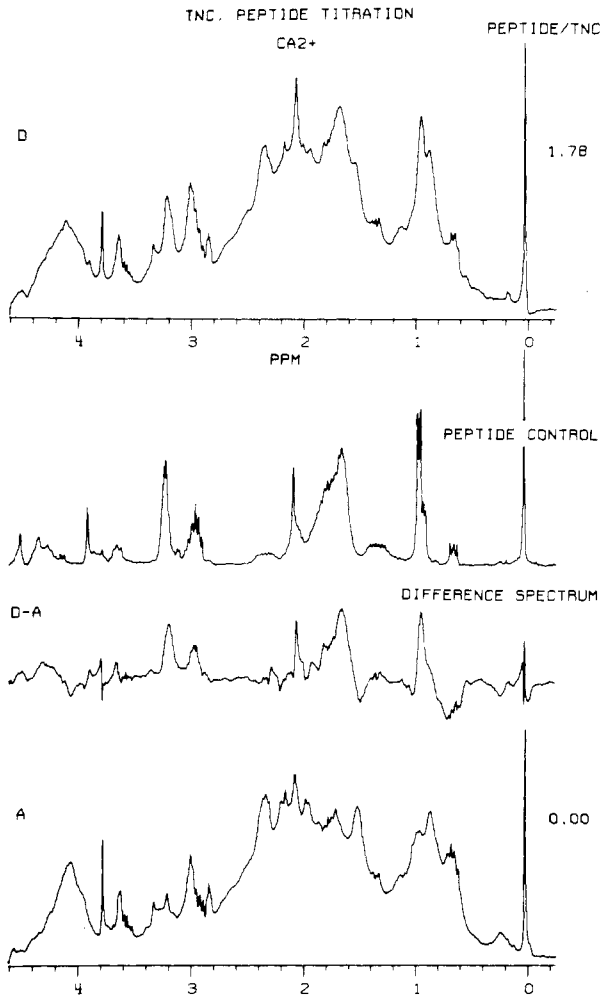


FIGURE 4: $^1$H NMR spectrum of the aliphatic region (0.0–4.6 ppm) of troponin C plus $Ca^{2+}$ (3 mM) in the presence of synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide at a peptide to troponin C ratio of 1.78. The difference spectrum (D – A) was obtained by subtracting the troponin C spectrum (A) from spectrum D. [Troponin C] $\simeq 4.1 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.1). Negative peaks in the difference spectrum are the result of conformational and/or environmental changes occurring in troponin C as a result of the addition of peptide to this sample. Positive peaks are attributed to the added peptide. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 15 000.

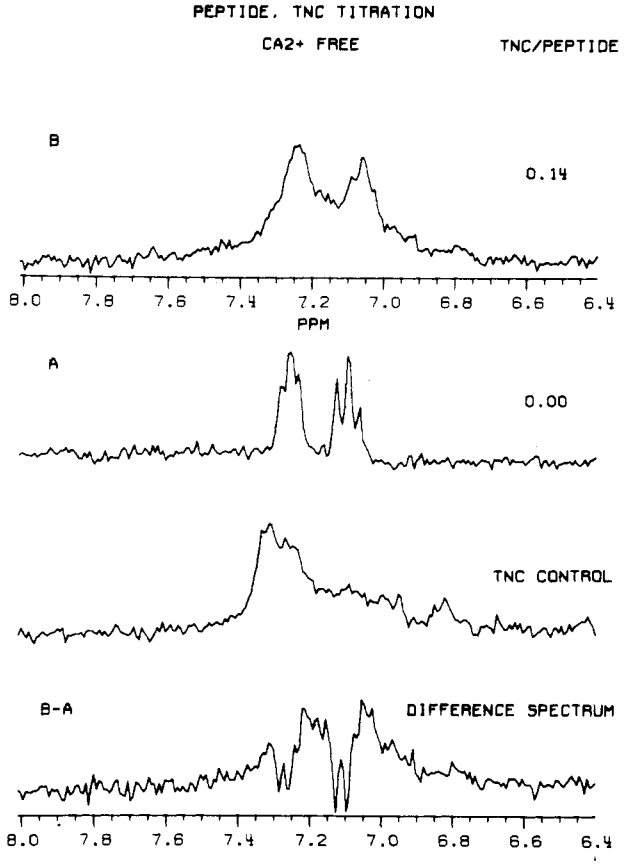


FIGURE 5: $^1$H NMR spectrum of the aromatic region (6.4–8.0 ppm) of a $Ca^{2+}$-free solution of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide at a troponin C to peptide ratio of 0.14. The difference spectrum (B – A) is obtained by subtracting spectrum A from spectrum B. [Peptide] $\simeq 3.6 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.10). Negative peaks in the difference spectrum are the result of conformational and/or environmental changes occurring in the peptide on addition of troponin C. The titration with troponin C was carried out to a final troponin C to peptide ratio of 0.68. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 642.

peptide results in a downfield signal intensity redistribution with subsequent development of a new peak at $\delta$ 7.34. (cf. difference spectra of Figure 3). Tyrosine meta 2 and 6 protons, found under the C-4 His-125 resonance at $\delta$ 6.96, are gradually shifted downfield by the addition of peptide to their new position at $\delta$ 7.00, while the ortho 3 and 5 protons at $\delta$ 6.84, although unshifted, are broadened by the addition of peptide. In the presence of calcium, the C-2 and C-4 protons of His-125 remain unaffected. In the aliphatic region (Figure 4), negative peaks centered at $\delta$ 0.25 and 0.70 in the difference spectra result from shifts experienced by ring-shifted methyl-group resonances (probably due to Leu, Ile, and Val residues). These methyl resonances merge with the main methyl-group envelope centered at $\delta$ 0.90 probably as a result of the restructuring of Phe groups as indicated by the changes in the aromatic region (Figure 3). Small negative peaks are also observed at $\delta$ 1.50, 3.78, and 4.07 and are associated with perturbed $\beta$-$CH_2$ and $\gamma$-CH protons of Leu, $\gamma$-$CH_2$ protons of Lys and Ile, and $\beta$-$CH_3$ protons of Ala ($\delta$ 1.50), $\alpha$-$CH_2$ protons of Gly ($\delta$ 3.78), and backbone $\alpha$-$CH_2$ protons ($\delta$ 4.07).

These results demonstrate that the peptide binds to troponin C and affects similar residues in both the presence and absence of calcium. However, one should keep in mind that the conformation of the peptide binding site on troponin C may be different in the presence and absence of calcium, since the conformation of the protein is unique in each case.

*Peptide: Titration with Troponin C in the Presence and Absence of Calcium.* In the spectra referred to in this section (Figures 5–8), the maximum mole ratios of troponin C to peptide achieved were 0.68 (calcium-free troponin C) and 1.04 (calcium-saturated troponin C). As troponin C concentrations were increased above 0.14 (calcium free) or 0.39 (calcium saturated), perturbation effects on the peptide resonance frequencies were complicated by increasing contributions from troponin C.

Figures 5 and 6 contain the spectra obtained from the synthetic peptide, employing calcium-free troponin C as titrant. Figure 5 represents the aromatic portion of the $^1$H NMR spectrum of $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide, vis spectrum A. This region displays resonance frequencies belonging to the 2 and 6 and 3 and 5 aromatic ring protons of the *p*-fluorophenylalanine moiety. The 3 and 5 protons occur as an apparent triplet centered at $\delta$ 7.09 ($J$ = 10.8 Hz), while the 2 and 6 protons appear as a broad triplet centered at $\delta$ 7.26 ($J$ = 5.8 Hz). The difference spectrum (B – A) contains large negative contributions at $\delta$ 7.10 and 7.26, indicating that the peak intensities associated with the 2 and 6 and 3 and 5 protons

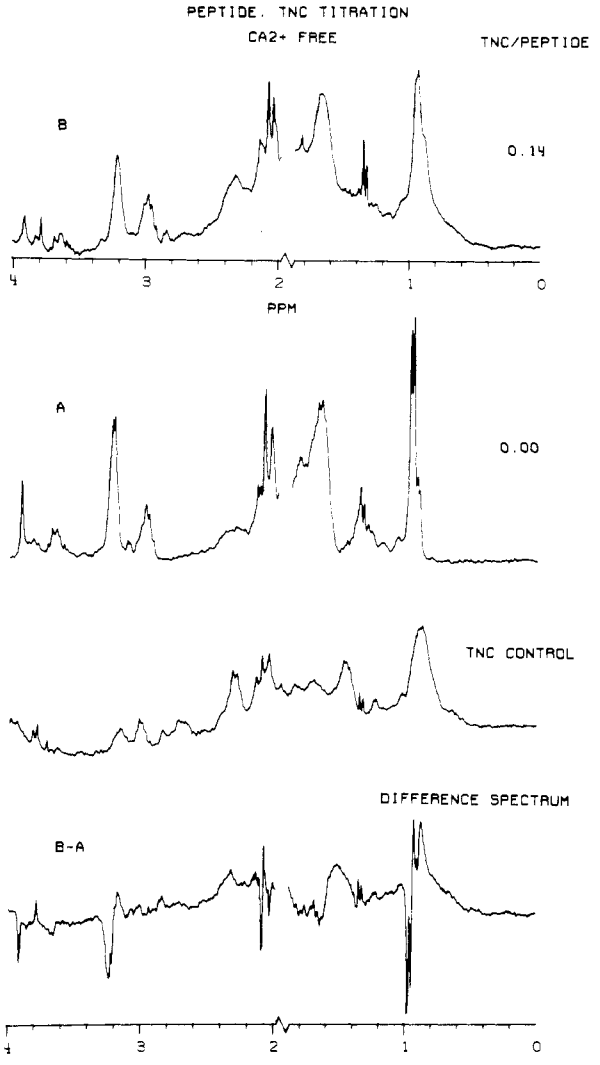


FIGURE 6: $^1$H NMR spectrum of the aliphatic region (0.0–4.0 ppm) of a $Ca^{2+}$-free solution of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]TnI-(104–115) amide at a troponin C to peptide ratio of 0.14. A resonance, due to an acetate salt contaminant present in the peptide, was removed from these spectra by omitting the region between 1.90 and 1.98 ppm. The difference spectrum (B – A) is obtained by subtracting spectrum A from spectrum B. [Peptide] $\simeq$ $3.6 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.10). Negative peaks in the difference spectrum are the result of conformational and/or environmental changes occurring in the peptide on addition of troponin C. Positive peaks are attributed to the added troponin C. The titration with troponin C was carried out to a final troponin C to peptide ratio of 0.68. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 2446.

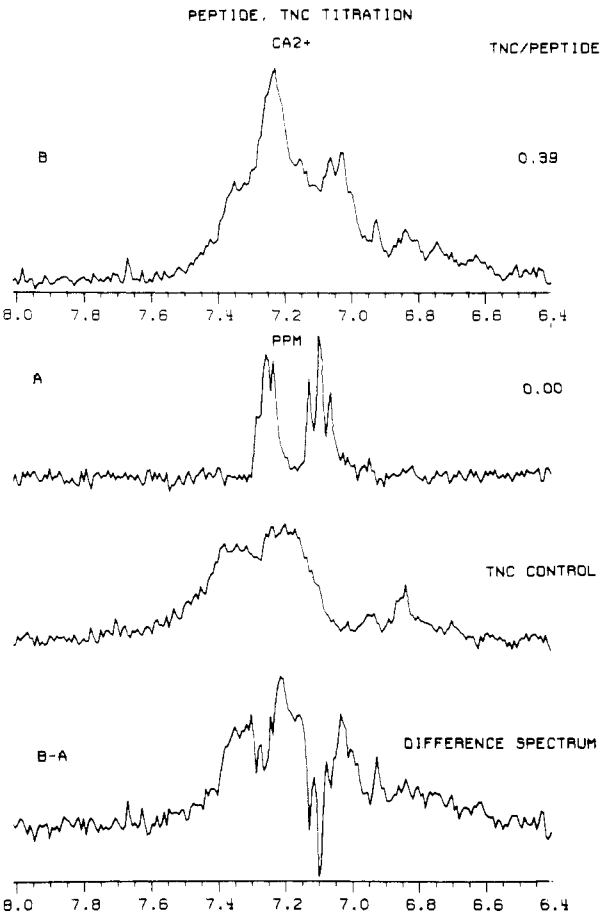


FIGURE 7: $^1$H NMR spectrum of the aromatic region (6.4–8.0 ppm) of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]-TnI-(104–115) amide plus $Ca^{2+}$ (3 mM) at a troponin C to peptide ratio of 0.39. The difference spectrum (B – A) is obtained by subtracting spectrum A from spectrum B. [Peptide] $\simeq$ $3.5 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.10). Negative peaks in the difference spectrum are the result of conformational and/or environmental changes occurring in the peptide on addition of troponin C. The titration with troponin C was carried out to a final troponin C to peptide ratio of 1.04. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 281.

of the aromatic system have broadened or shifted in the presence of troponin C. Similarly, Figure 6 demonstrates that the resonance frequencies of several groups in the aliphatic region also undergo chemical shift changes, vis difference spectrum B – A, which results in discrete negative peaks associated with the $\delta$-$CH_3$ protons of Leu and the $\gamma$-$CH_3$ protons of Val at $\delta$ 0.90–0.95, $\beta$-$CH_2$ and $\gamma$-CH protons of Leu and $\gamma$-$CH_2$ protons of Arg at $\delta$ 1.68–1.90, the $N^{\alpha}$-acetyl group of Gly at $\delta$ 2.09, a broad negative peak due to $\delta$-$CH_2$ groups of Arg centered at $\delta$ 3.24, and a sharp singlet resonance for the $\alpha$-$CH_2$ protons of Gly at $\delta$ 3.92. There are six charged residues present in this peptide at pH 8.0: four Arg residues and two Lys residues, each carrying one positive charge. Of these, however, only the Arg residues are affected by interaction with the protein as indicated by the broad negative peak at $\delta$ 3.24 and the lack of any peak associated with the Lys $\epsilon$-$CH_2$ groups ($\delta$ 2.99).

Evans & Levine (1980) provide evidence to show that Arg residues are involved in stabilization of the complex formed between the proteins troponin C and troponin I. However, Grand et al. (1982), working with troponin I inhibitory peptide (residues 96–116), have indicated that Lys residues are involved in the interaction between this peptide and troponin C, while little perturbation of Arg residues is observed. These results were obtained on cyanogen bromide fragment 4, which contains three Lys residues (98, 105, and 107), one more than is present in our synthetic fragment (Lys-105 and -107).

The results of the titration of the peptide with calcium-saturated troponin C are shown in Figures 7 and 8. The difference spectra (B – A) in both Figures 7 and 8 are very similar to the results obtained during the titration of peptide with calcium-free troponin C (Figures 5 and 6). This result suggests that the same perturbations in the peptide are observed under both sets of conditions and that the same residues are involved in this interaction although troponin C is in different states, i.e., calcium free and calcium saturated.

Our results, employing inhibitory peptide (residues 104–115), suggest that of the basic residues only the Arg residues are affected during the interaction with troponin C in both the presence and absence of calcium. Although we do not observe perturbation of Lys residues (105 and 107) on

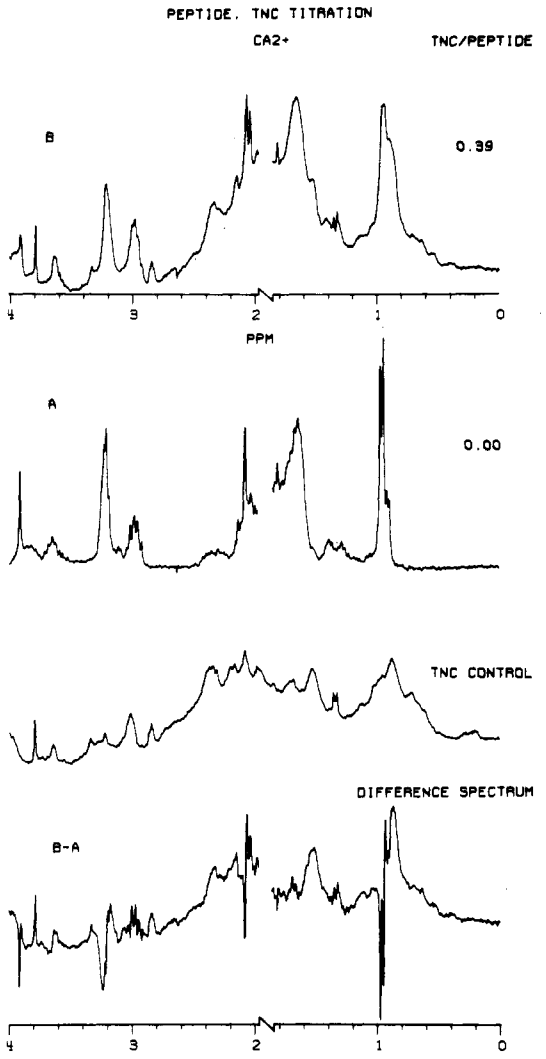


FIGURE 8: $^1$H NMR spectrum of the aliphatic region (0.0–4.0 ppm) of the synthetic troponin I inhibitory peptide $N^{\alpha}$-acetyl[FPhe$^{106}$]-TnI-(104–115) amide plus $Ca^{2+}$ (3 mM) at a troponin C to peptide ratio of 0.39. The difference spectrum (B – A) is obtained by subtracting spectrum A from spectrum B. [Peptide] $\simeq 3.5 \times 10^{-4}$ M in 50 mM KCl and 20 mM $NH_4HCO_3$ (pH 8.10). A resonance, due to an acetate salt contaminant present in the peptide, was removed from the spectrum by omitting the region between 1.90 and 1.98 ppm. Negative peaks in the difference spectrum are the result of conformational and/or environmental changes occurring in the peptide on addition of troponin C. The titration with troponin C was carried out to a final troponin C to peptide ratio of 1.04. FPhe denotes *p*-fluorophenylalanine. The vertical scaling factor = 1372.

interaction with troponin C, actomyosin ATPase inhibition studies employing troponin I inhibitory region (104–115) (Talbot & Hodges, 1981) have demonstrated that Lys-105 is essential to the inhibitory activity brought about by its interaction with actin–tropomyosin.

Grabarek et al. (1981) have demonstrated that three regions of troponin C are involved in interaction with troponin I. Two of these regions are near calcium-binding sites II and III and require calcium for the interaction, while a third region, near calcium-binding site IV, is involved in a calcium-independent interaction with troponin I. Weeks & Perry (1978) also concluded that cyanogen bromide fragment 9 (residues 83–134) of troponin C forms a calcium-dependent complex with troponin I and that this fragment mimics the properties of the intact protein in its ability to release troponin I mediated actomyosin ATPase inhibition in the presence of calcium. This same fragment also inhibits the in in vitro phosphorylation of rabbit skeletal troponin I at Ser-117. Talbot & Hodges (1979, 1981) have shown that synthetic analogues of the inhibitory region of troponin I (residues 104–115) adjacent to Ser-117 mimic the activity of the troponin I molecule in its ability to inhibit actomyosin ATPase activity. This inhibition is released by troponin C in the presence of calcium in a manner similar to that exhibited by the action of troponin C on troponin I (Chong et al., 1983). The N-terminal region of site III is known to undergo coil to helix transformation on addition of calcium (Leavis et al., 1978; Reid et al., 1981). If binding between troponin C and our synthetic fragment occurs at this site, then it too may induce coil to helix transformation. Circular dichroism studies have demonstrated that calcium-free troponin C undergoes $\alpha$-helix induction in the presence of the inhibitory peptide.[2] Photochemical cross-linking studies using troponin C (photoreactive azido moiety on Cys-98) and troponin I (Chong & Hodges, 1981) resulted in cross-linking to the troponin I inhibitory region (residues 96–116).[3] Perry, in his review (Perry, 1979), has suggested that calcium-induced conformational changes that occur on binding calcium bring negatively charged residues in site III, not involved in calcium binding, into favorable position for interaction with the appropriate residues on troponin I. Furthermore, circular dichroism and NMR studies (Nagy et al., 1978; Reid et al., 1981; Gariépy et al., 1982) suggest that coil to helix transformation in site III arranges hydrophobic residues of the N-terminal region such that Leu-95, Ala-96, Phe-99, and Phe-102 form a hydrophobic surface available for interaction with nonpolar residues in troponin I. All these results support our conclusion that there is a weak but specific interaction between the synthetic troponin I inhibitory peptide and troponin C at the N-terminal region of site III (Figure 9).

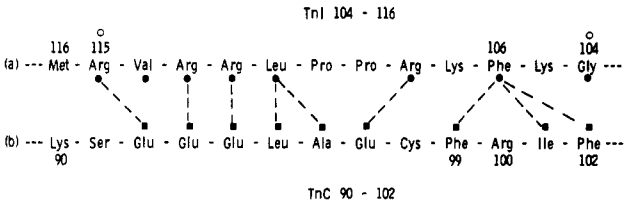


FIGURE 9: Amino acid residues that may be perturbed on interaction of the inhibitory region of troponin I with troponin C. (a) Sequence of the minimum region for inhibition of the protein troponin I (Talbot & Hodges, 1981). (b) Sequence of the N-terminal region of site III of troponin C. FPhe was substituted for Phe in the synthetic peptide. (●) All the residues that may be perturbed in the presence of troponin C. (○) Residues found to be nonessential for inhibition (Talbot & Hodges, 1981). (■) All the residues that may be perturbed in the presence of inhibitory peptide in the N-terminal region of site III of troponin C. Broken lines (– –) between residues on the two proteins indicate possible interacting residues.

## Conclusions

Since previous studies have demonstrated that this synthetic inhibitory troponin I peptide mimics the whole protein by binding to actin–tropomyosin, inhibiting actomyosin ATPase activity (Talbot & Hodges, 1979, 1981), we conclude that the troponin I sequence (residues 104–115) respresents an essential component of the calcium-sensitive chemical switch that regulates actomyosin ATPase activity, the driving force of muscle contraction. In the absence of calcium, this region of troponin I is bound to the surface of actin–tropomyosin, inhibiting the actomyosin ATPase activity and contraction. In the presence of calcium, binding occurs between the N-ter-

[2] P. J. Cachia and R. S. Hodges, unpublished results.
[3] P. C. S. Chong and R. S. Hodges, unpublished results.

minal region of site III in troponin C and residues 104–115 of troponin I and aids in the release of actomyosin ATPase inhibition.

**Registry No.** $N^{\alpha}$-Acetyl[FPhe$^{106}$]TnI-(104–115) amide, 86129-34-2; DL-*p*-fluorophenylalanine, 51-65-0; Boc-ON, 58632-95-4; Boc-L-*p*-fluorophenylalanine, 41153-30-4; Boc-DL-*p*-fluorophenylalanine methyl ester, 86129-35-3.

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