Properties of Troponin C Acetylated at Lysine Residues†

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ABSTRACT: We have studied the properties of rabbit skeletal troponin C (TnC) fully acetylated at its lysine residues (AcTnC). Acetylation causes a decrease in thermal stability of both domains of TnC in the absence of Ca^{2+}. At 25 °C, the acetylated C-terminal domain of TnC is almost completely unfolded and the melting temperature of the N-terminal domain monitored by far-UV circular dichroism is decreased by 16.3 °C. In the presence of 1 mM $CaCl_2$, no cooperative unfolding can be detected up to 90 °C for either TnC or AcTnC. At 25 °C, CD spectra show that AcTnC has a slightly lower α-helix content in the absence of Ca^{2+}, but higher in the presence of Ca^{2+} as compared to unmodified TnC. Acetylation causes a 3.5-fold increase in affinity for Ca^{2+} at the low-affinity sites and a 2-fold decrease at the high-affinity sites. Polyacrylamide gel electrophoresis under nondissociating conditions (no SDS, no urea, pH 8.6) indicates that acetylation has little effect on the apparent affinity of TnC for troponin I; however, the binding of the acetylated peptides corresponding to the N-terminal domain of TnC to troponin I is significantly stronger than that of the unmodified peptides. Troponin T binding to AcTnC is significantly enhanced, the altered properties of the N-terminal domain being predominantly responsible for the increase. Titration of the ATPase activity of TnC-depleted myofibrils with AcTnC or native TnC indicates that acetylation increases TnC's affinity for myofibrils in the presence of Ca^{2+} ~6 times; at saturation the ATPase activity is the same for the two forms of TnC. The Ca^{2+} dependence of the ATPase activity of myofibrils containing AcTnC is shifted to lower Ca^{2+} concentrations, consistent with the higher Ca^{2+} affinity of AcTnC at the low-affinity sites. These data indicate that positively charged lysine side chains, especially those located in the N-terminal domain, modulate TnC's structural stability and interactions with Ca^{2+} and other troponin components.

Troponin, together with tropomyosin, forms the Ca^{2+}-dependent regulatory system of the actin filament in skeletal and cardiac muscle. Each of the three troponin subunits (TnC, TnI, TnT)[1] is in contact with the other two. The TnT component also binds to tropomyosin and TnI binds to actin (Chalovich, 1992; Leavis & Gergely, 1984; Zot & Potter, 1987). The TnI−actin interaction is considered essential for the inhibition of the actomyosin ATPase in the absence of Ca^{2+}, presumably by keeping tropomyosin in the inhibitory position or state. Several inter-protein interactions and, ultimately, the state of activity of the thin filament depend on the Ca^{2+} occupancy of the Ca^{2+}-binding component—TnC.

There are four metal binding sites in TnC, each consisting of a loop flanked by two α-helical segments. Sites I and II, located in the N-terminal domain of TnC, are specific for Ca^{2+}, whereas sites III and IV, in the C-terminal domain, bind either Ca^{2+} or Mg^{2+} (Potter & Gergely, 1975). The two domains are structurally independent in the free TnC, the only connection between them being provided by a 29-residue-long α-helix, a considerable portion of which is exposed to solvent (Herzberg & James, 1985, Sundaralingam et al., 1985). Kinetic studies and, more recently, studies utilizing TnC mutants have shown that the two domains have different functions. The N-terminal domain is directly responsible for activation of contraction whereas the C-terminal domain has a structural function and provides most of the free energy of interaction with TnI [Zot and Potter (1987); for a review, see Grabarek et al. (1992)]. The key event in the activation of contraction is the conformational transition from the Ca^{2+}-free form to the Ca^{2+}-saturated form of the N-terminal domain of TnC. Herzberg et al. (1986) proposed that this regulatory transition involves a change from the antiparallel orientation of helices B and C with respect to helices A and D in the Ca^{2+}-free form to a perpendicular one in the Ca^{2+}-bound form. Such a transition exposes several hydrophobic side chains, possibly forming an interface for the interaction with TnI. Various biochemical data [reviewed in Grabarek et al. (1992)] and the atomic structure of the Ca^{2+}-filled N-terminal domain fragment of TnC recently solved by NMR (Gagne et al., 1994) and X-ray crystallography (Strynadka et al., 1995) have proved this hypothesis to be correct.

Whereas the troponin complex remains stable in solution regardless of the presence of Ca^{2+}, it is clear that its regulatory function depends on some structural rearrange-

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[1] Abbreviations: TnC, calcium binding component of troponin; TR_1C, TR_1E, TR_2C, tryptic fragments of TnC comprising residues 9−84, 1−100, and 89−159, respectively; Ac, used as a prefix indicates acetylated form of TnC, TR_1C, TR_1E, and TR_2C; TnI, TnT, inhibitory and tropomyosin binding components of troponin, respectively; Tm, tropomyosin; DANZ, dansylaziridine (5-(dimethylamino)naphthalene-1-sulfonylaziridine); DTT, dithiotreitol; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenedinitrilotetraacetic acid; NMR, nuclear magnetic resonance; CD, circular dichroism.

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ments of the components upon Ca^{2+} binding to TnC. Therefore, a significant effort has been put into characterizing the interfaces between troponin subunits. Studies on CNBr fragments of TnI have identified two segments involved in the interaction with TnC: peptide CN5 (residues 1−21) and peptide CN4 (residues 96−116) (Syska et al., 1976). The CN4 peptide and its shorter analogue comprising residues 104−116 have been found to inhibit actomyosin ATPase (Syska et al., 1976; Talbot & Hodges, 1981), and the peptide is often referred to as the inhibitory segment. Proteolytic fragments derived from both domains of TnC were found to interact with TnI (Grabarek et al., 1981a; Weeks & Perry, 1978). ^{1}H-NMR studies on the interaction of CN4 with a spin-labeled TnC (Dalgarno et al., 1982), as well as photo-cross-linking data (Leszyk et al., 1988), have indicated that the inhibitory segment of TnI interacts with the C-terminal domain of TnC. In other studies, the same segment of TnI was found to interact with the N-terminal domain of TnC (Kobayashi et al., 1991; Leszyk et al., 1990; Wang et al., 1990). Recent ^{1}H-NMR studies (Tsuda et al., 1992) show that residues from both domains of TnC are affected by, and presumably interact with, the CN4 peptide. Interaction of the inhibitory segment of TnI with both domains of TnC has also been concluded from studies on large recombinant TnI fragments (Farah et al., 1994). Thus, although only the N-terminal domain of TnC is considered critical for the regulation, both domains appear to be close to (or interacting with) the inhibitory segment of TnI. Interaction of the N-terminal segment of TnI (residues 1−40) with the C-terminal domain of TnC plays a critical role in the overall strength of interaction between the two components of troponin (Ngai & Hodges, 1992; Farah et al., 1994). Although the exact structure of the TnC−TnI complex is unknown, interdomain resonance energy transfer (Gong et al., 1994) and low- angle X-ray scattering (Olah et al., 1994) indicate that the two proteins are elongated. On the basis of the analysis of X-ray scattering data, Olah & Trewhella (1994) proposed a model of the TnC−TnI complex in which TnI wraps around the dumbbell-shaped TnC molecule.

The interaction between TnT and TnC is much less characterized. Studies on proteolytic fragments of TnT have shown that the C-terminal chymotryptic fragment T2 comprising residues 159−259 binds to TnC (Pearlstone & Smillie, 1978; Tanokura et al., 1983). However, the reactivity of some lysine residues in the N-terminal segment of TnT, which is believed to be responsible for TnT's interaction with tropomyosin, were found to decrease upon TnC binding (Hitchcock et al., 1981), suggesting more extensive interaction interface. Both the studies on proteolytic fragments of TnC (Grabarek et al., 1981a) and on lysine reactivity (Hitchcock, 1981) suggest that the N-terminal domain of TnC, possibly including the central helix, may be involved in TnT binding.

Although work to date provides significant insights into the mechanism of the Ca^{2+}-induced transitions in TnC, it is still unclear what the forces holding the troponin subunits together are, what the geometry of the complex is, and how the interactions change during the activation process. Here we address the question of the role of the overall charge on TnC in interactions with the other troponin components and in determining TnC's regulatory properties. We have increased the net negative charge of TnC by blocking all nine lysine residues with acetic anhydride. This modification causes an increase in affinity for Ca^{2+} at the N-terminal domain and a decrease at the C-terminal domain. Acetylation increases the affinity of the N-terminal domain of TnC for TnI and TnT. The Ca^{2+}-induced increase in ATPase activity of myofibrils incorporating AcTnC is shifted to lower $[Ca^{2+}]$.

MATERIALS AND METHODS

Protein Preparation. Troponin obtained from an ether-dried rabbit skeletal muscle powder was separated into subunits according to Greaser and Gergely (1971). TnC-depleted myofibrils were obtained using extraction procedure of Cox et al. (1981) except that an additional extraction step with TnI was included as described previously (Grabarek et al., 1990).

Peptides TR_1C and TR_2C were obtained by limited tryptic digestion of TnC in the presence of $CaCl_2$ (Drabikowski et al., 1977; Grabarek et al., 1981b). Lyophilized TnC (50 mg) was dissolved in 5 mL of a solution containing 6 M urea, 20 mM Hepes (pH 7.5), 20 mM DTT, and 1 mM EDTA. The solution was dialyzed overnight against 0.1 M NH_4HCO_3, and its protein concentration was determined from the absorbance at 280 nm. The concentration was then adjusted to 1 mg/mL with 0.1 M NH_4HCO_3, and $CaCl_2$ was added to 1 mM, followed by trypsin (1:100 w/w, trypsin to protein ratio). The solution was incubated at 25 °C for 2 h and the reaction terminated by addition of soybean trypsin inhibitor (2:1 w/w, inhibitor to enzyme ratio). The sample was lyophilized, dissolved in 5 mL of a solution containing 50 mM NH_4HCO_3 and 1 mM DTT, and applied to a Sephacryl S-200 column. Fractions from the column were analyzed on a urea−Tris−glycine polyacrylamide gel with 1 mM $CaCl_2$ in the running buffer. The central peak containing a mixture of TR_1C and TR_2C was collected, lyophilized, dissolved in a solution containing 0.2 M KCl, 50 mM Tris-HCl (pH 8.2), and 0.1 mM $CaCl_2$, and loaded on a DEAE-Sephadex A-25-120 (Sigma) column (40 mL of resin) equilibrated with the same solution. The column was washed with 40 mL of the loading buffer followed by a 0.2−0.5 M KCl gradient (2 × 500 mL). Under these conditions, the peptides partially separate as judged by urea−Tris−glycine PAGE. Fractions corresponding to crude TR_1C and TR_2C peptides were collected, and after dialysis using 2000 cutoff dialysis tubing against 0.2 M KCl, 50 mM Tris-HCl (pH 8.2), and 0.1 mM $CaCl_2$ run again on the same column. Fractions containing pure TR_1C (or TR_2C) were collected, dialyzed against H_2O, and lyophilized.

Peptide TR_1E was obtained by tryptic digestion of TnC in the presence of EDTA (Grabarek et al., 1981b). A TnC solution (1 mg/mL) in 0.1 M NH_4HCO_3−1 mM EDTA was digested with trypsin (1:1000 w/w, enzyme to protein ratio) for 40 min at 25 °C. The reaction was stopped by addition of soybean trypsin inhibitor (2:1 w/w, inhibitor to protein ratio). The sample was then run on a Sephacryl S-200 column equilibrated with a solution containing 50 mM NH_4HCO_3 and 1 mM DTT. Fractions containing crude TR_1E were collected and lyophilized. In the final purification step, we utilized the tendency of this peptide to form a disulfide cross-linked dimer. The lyophilized material was dissolved in a small volume of 50 mM NH_4HCO_3, dialyzed against the same solution, and oxidized by passage of a stream of oxygen through the solution for 2 h at room temperature.

The sample was then loaded onto a Sephacryl S-200 column equilibrated with 50 mM NH_4HCO_3, and the fractions containing the TR_1E dimer were collected, dialyzed against H_2O, and lyophilized. Samples of TR_1E prepared for each experiment were treated with 10 mM DTT and dialyzed against an appropriate solution. This was found sufficient to reduce the disulfide bond in the TR_1E dimer.

Protein concentration was estimated from UV absorbance measurements with $A_{280}(1\%, 1\text{ cm}) = 1.8$ for TnC; $A_{280}(1\%, 1\text{ cm}) = 3.72$ for TnI, and $A_{280}(1\%, 1\text{ cm}) = 4.58$ for TnT. Protein concentration of myofibrils was measured in the presence of 1% SDS using $A_{280}(1\%, 1\text{ cm}) = 7.0$ (Knight & Trinick, 1982). Concentrations of AcTnC's and TnC's tryptic fragments (TR_1C, TR_1E, TR_2C) in native and acetylated forms were obtained from amino acid analysis. For TnC, both UV absorbance and amino acid analysis were used and both methods gave similar results.

Acetylation of TnC and Its Tryptic Fragments. TnC and its tryptic fragments were fully acetylated with acetic anhydride (Fraenkel-Conrat, 1957). Six 1-μL aliquots of acetic anhydride were added at 10-min intervals to the protein dissolved in 50% saturated sodium acetate (2 mg/mL) at 0 °C. The samples were dialyzed overnight against 1 mM $NaHCO_3$. *O*-Acetyltyrosine formed during incubation with acetic anhydride was deacetylated by incubation with hydroxylamine (Riordan & Vallee, 1972). The progress of deacetylation was monitored spectrophotometrically at 280 nm.

Quantitative Analysis of Amino Groups. Amino groups were quantified fluorometrically upon reaction with fluorescamine, essentially as previously described (Lai, 1977; Udenfriend et al., 1972). Samples (5 μL) of protein solution were added to 1.5 mL of 0.2 M sodium borate buffer (pH 9.0). Fluorescamine (0.15 mL of 20 mg/mL solution in acetone) was added while the protein solution was vigorously mixed on a Vortex mixer. Fluorescence was measured on a Perkin-Elmer MPF4 spectrofluorometer ($\lambda_{exc} = 390$ nm, $\lambda_{em} = 475$ nm). For calibration we used *N*-α-acetyl-L-lysine. Under the conditions employed, the reaction of fluorescamine with native TnC goes to completion.

Mass Spectrometry. Molecular mass of AcTnC was determined by electrospray ionization mass spectrometry (Chait & Kent, 1992) performed on a Finnigan TSQ700 triple−quadrupole mass spectrometer at Harvard Microchem (Cambridge, MA).

Circular dichroism measurements were performed on an AVIV (Lakewood NJ) 62A DS circular dichroism spectrometer. Far-UV CD spectra were recorded at 25 °C in the 200−250-nm wavelength range in 2-mm quartz cuvettes at a protein concentration of ~0.25 mg/mL in a solution containing 0.1 M KCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mM EGTA. Spectra of the same samples were also recorded after addition of 0.5 mM $CaCl_2$ (final concentration). The same samples were then used for amino acid analysis from which the actual protein concentrations were calculated. For thermal unfolding studies, ellipticity values at 222 nm were recorded as a function of temperature in 0.5 °C steps with 25-s equilibration time and 5-s averaging time, resulting in 1 °C/min heating rate. The denaturation curves for the single-domain fragments of TnC were analyzed by assuming a two-state equilibrium between native (n) and unfolded (u) forms of the protein with the temperature-dependent equilibrium constant K_u. It is further assumed that the ellipticity of the folded and unfolded protein is a linear function of temperature T (K) over the entire temperature range. Then the observed ellipticity (θ_{obs}) can be expressed as follows:

$$\theta_{obs} = f_u(i_u + s_uT) + (1 - f_u)(i_n + s_nT)$$

$$f_u = K_u/(1 + K_u)$$

$$K_u = \exp(-\Delta G_u/RT)$$

where f_u is the fraction of unfolded protein, i and s are the intercept ellipticity and the slope, respectively, for the unfolded (subscript u) and native (subscript n) protein, and R is the universal gas constant. The free energy of unfolding ΔG_u is a function of temperature and can be expressed as

$$\Delta G_u = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)]$$

where ΔH_m and ΔC_p are the enthalpy of unfolding and heat capacity change, respectively, at the temperature T_m at which half of the molecules are unfolded (Schellman & Hawkes, 1980). The data were fitted using the nonlinear least-squares procedure (Johnson & Faunt, 1992).

Calcium Titrations. To monitor Ca^{2+} binding to the low-affinity sites I and II, we labeled TnC and AcTnC with dansylaziridine (Johnson et al., 1978) as previously described (Grabarek et al., 1983). Calcium binding to the high-affinity sites III and IV was monitored by tyrosine fluorescence. Titrations were carried out in a solution containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 1 mM NTA as previously described (Grabarek et al., 1990). The protein concentrations were 2 and 10 μM for DANZ fluorescence and tyrosine fluorescence measurements, respectively. Measurements were performed at 25 °C with a SPEX Fluorolog 2/2/2 photon counting spectrofluorometer (Edison, NJ) in 1-cm cuvette.

ATPase activity of the myofibrils was determined by measuring the rate of inorganic phosphate release from ATP (Fiske & SubbaRow, 1925). Various amounts (0.05−2.0 μM) of TnC or AcTnC were added to TnC-depleted myofibrils (0.25 mL, 0.5 mg/mL) in a solution containing 90 mM KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and either 0.2 mM $CaCl_2$ or 1 mM EGTA. The reaction was initiated by addition of 1 mM ATP and terminated after 5 min by addition of 0.25 mL of 2% SDS. For measurements of ATPase activity as a function of $[Ca^{2+}]$, each sample contained 25 μL of a stock Ca^{2+}-buffering solution prepared separately for each data point. These contained 10 mM EGTA, 100 mM imidazole (pH 7.0), and various concentrations (0.5−10 mM) of $CaCl_2$ to yield free Ca^{2+} concentrations in the 10^{-8}−10^{-4} M range as computed according to Perrin and Sayce (1967) and using published stability constants (Sillen & Martel, 1964). The change in pH caused by Ca^{2+} chelation by EGTA was compensated for by addition of 0.2 M KOH.

Electrophoresis. For native gel electrophoresis we used 10% polyacrylamide slabs containing 80 mM Tris−glycine buffer (pH 8.6) (Perrie & Perry, 1970).

RESULTS

Acetylation of TnC

There are nine lysine residues in rabbit skeletal TnC. Three of them form a cluster in the central helix (Lys 84,

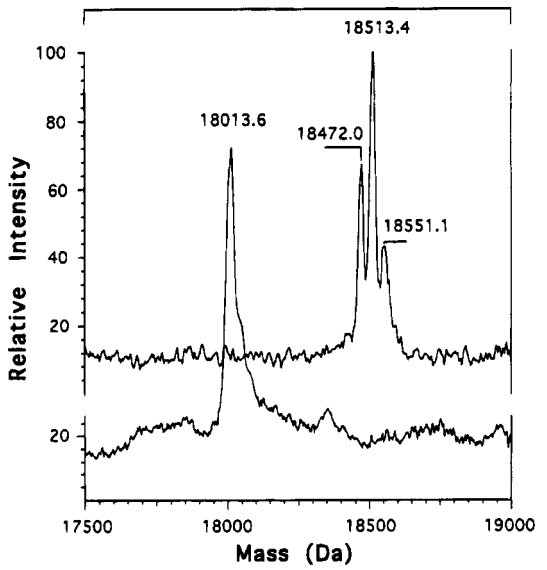

FIGURE 1: Electrospray ionization mass spectra of TnC (lower trace) and AcTnC (upper trace). Only the reconstructed spectra are shown.

88, 90) and the remaining six are equally distributed between the N-terminal (Lys 20, 37, 52) and the C-terminal (Lys 136, 140, 153) domain. Only two lysines—Lys37 and Lys140 in site I and site IV, respectively—are located in the Ca^{2+}-binding loops, both in noncoordinating positions. All Lys residues become acetylated upon treatment with acetic anhydride as judged from the lack of reaction of the modified protein with fluorescamine (data not shown). Tyr residues also become O-acetylated under these conditions, which results in disappearance of the characteristic absorption band at 280 nm. Acetylation of Tyr was readily reversed upon treatment with 0.1 M hydroxylamine (Riordan & Vallee, 1972). All acetylated proteins used in these studies were, therefore, treated with hydroxylamine and the extent of reaction was monitored spectrophotometrically. We have further analyzed AcTnC by mass spectrometry (Figure 1). The reconstructed mass spectrum of TnC obtained by the electrospray ionization method shows a single sharp peak corresponding to a mass of 18 014 Da, in good agreement with the expected value for rabbit skeletal TnC with its N-terminal α-NH_2 group acetylated (18 008 Da). The spectrum of AcTnC shows three major peaks corresponding to masses 18 472, 18 513, and 18 551 Da (Figure 1), all of which are slightly higher than the expected value for TnC fully acetylated at all its Lys residues (18 386 Da). The extra masses of 86, 127, and 165 Da correspond to two, three, and four acetyl groups (42 Da), respectively. It is not clear what the additional groups that undergo acetylation are. This additional acetylation and the resulting heterogeneity of our sample could not be detected with the other methods we used, viz. native, urea, or SDS polyacrylamide gel electrophoresis and reverse phase HPLC.

Effect of Acetylation on the Secondary Structure of TnC

The far-UV CD spectra recorded at 25 °C indicate that in the absence of Ca^{2+} AcTnC has a slightly lower α-helix content than unmodified TnC. In contrast, in the presence of Ca^{2+}, AcTnC has a higher α-helix content than TnC (Figure 2, Table 1). The latter difference corresponds to approximately two additional α-helical turns. Inspection of the amino acid sequence of TnC indicates that there would

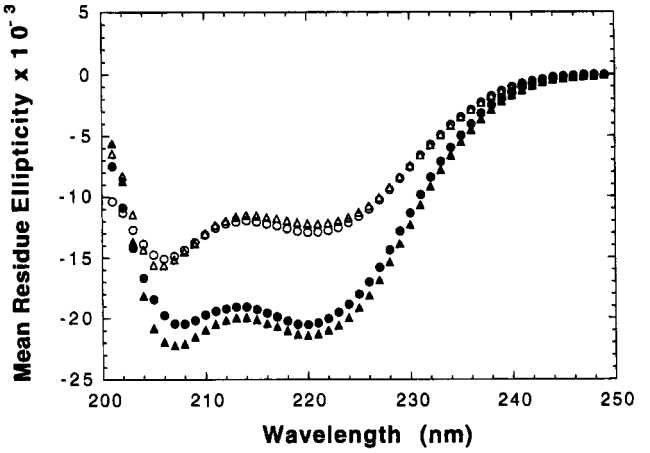

FIGURE 2: Effect of acetylation of lysine residues on the circular dichroism spectrum of TnC. CD spectra of TnC and acetylated TnC (0.25 mg/mL) in 0.1 M KCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EGTA (and 0.5 mM $CaCl_2$ when present) were recorded at 25 °C on an AVIV 62A DS circular dichroism spectrometer. Key: TnC (+EGTA) ○; TnC (+Ca^{2+}) ●; AcTnC (+EGTA) △; AcTnC (+Ca^{2+}) ▲.

Table 1: Effect of Calcium and Acetylation of Lysine Residues on the Mean Residue Ellipticity of TnC and Its Proteolytic Fragments

	$[\theta]_{222}$ (deg cm^2 dmol^{-1})		
peptide	EGTA	Ca^{2+}	$[\theta]^{Ca}/[\theta]^{EGTA}$
TnC	−12 800	−20 400	1.60
AcTnC	−12 200	−21 000	1.72
TR_1C	−17 200	−20 800	1.20
$AcTR_1C$	−16 500	−20 600	1.25
TR_1E	−16 800	−20 000	1.18
$AcTR_1E$	−14 400	−18 200	1.27
TR_2C	−6 800	−15 300	2.25
$AcTR_2C$	−5 500	−16 800	3.06

be repulsion between the side chains of Lys 84 and Lys 88 in the central helix if this segment had an α-helical conformation. Acetylation may decrease such repulsion by neutralizing the charges and thus increasing the α-helical stability of this region. It is interesting to note that the peptide bonds at Lys 84 and Lys 88 in native TnC are susceptible to proteolysis with trypsin in the presence of Ca^{2+} (Grabarek et al., 1981b), which is consistent with unfolding of this segment of the molecule. Conversely, the same lysines are inaccessible to tryptic digestion in the TnC mutant having a disulfide bond between Cys residues at positions 48 and 82, which presumably stabilizes the structure of the central helix (Gusev et al., 1991).

Measurements of ellipticity at 222 nm as a function of temperature indicate that in the absence of Ca^{2+} unfolding of TnC occurs in two transitions (Figure 3). The main transition at 71.3 °C represents a cooperative unfolding of the N-terminal domain, and a much smaller one below 30 °C represents unfolding of the C-terminal domain in agreement with microcalorimetric studies (Tsalkova & Privalov, 1980, 1985). Only one transition at 55 °C can be detected for AcTnC. Acetylation of lysine residues significantly decreases TnC's stability in the absence of Ca^{2+} as indicated by a 16.3 °C decrease in melting temperature (T_m) for the main transition (Figure 3, Table 2). In the presence of Ca^{2+} up to 90 °C, neither AcTnC nor TnC shows signs of cooperative unfolding, consistent with the previous reports (Tsalkova & Privalov, 1980, 1985; Brzeska et al., 1983).

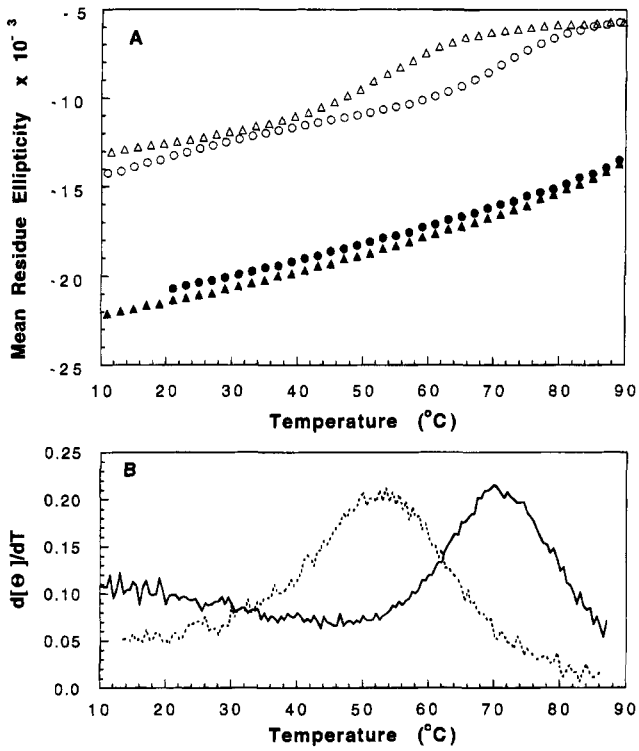

FIGURE 3: Thermal unfolding of TnC and AcTnC monitored by circular dichroism. Ellipticity at 222 nm for TnC (○, ●) and AcTnC (△, ▲) in the absence (empty symbols) and the presence (solid symbols) of Ca^{2+} is shown in (A). The first derivative of the unfolding profiles in the absence of Ca^{2+} for TnC (solid line) and AcTnC (dotted line) is shown in (B). Ellipticity at 222 nm was recorded as a function of temperature in 0.5 °C steps with 25-s equilibration time and 5-s averaging time, resulting in 1 °C/min heating rate. The samples contained 0.25 mg/mL protein in a solution containing 0.1 M KCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mM EGTA.

Table 2: Thermodynamic Parameters of the Thermal Unfolding of TnC and Its Fragments, Monitored by Circular Dichroism

protein/ peptide	T_m (°C)	ΔH (kcal/mol)	ΔC_p (cal/mol·deg)
TnC	71.3[a]		
AcTnC	55.0 (−0.7, +0.8)	32.7 (−1.2, +1.2)	140 (−280, +260)
Tr_1C	66.9 (−0.3, +0.3)	38.9 (−0.9, +0.9)	870 (−90, +90)
$AcTR_1C$	53.1 (−0.3, +0.4)	35.3 (−0.7, +0.7)	440 (−180, +160)
TR_1E	72.1 (−0.9, +0.5)	40.0 (−1.0, +0.9)	880 (−66, +62)
$AcTR_1E$	46.2 (−0.4, +0.4)	25.4 (−0.8, +0.9)	550 (−68, +65)
Tr_2C	39.2[a]		

[a] Only the second transition for TnC was analyzed (data were truncated at 40 °C). ΔH and ΔC_p were considered unreliable and are not listed; however, they had little effect on T_m. ΔH and ΔC_p for TR_2C were also unreliable because no adequate baseline before the transition could be recorded (cf. Figure 7). Numbers in parentheses indicate 67% confidence limit.

Effect of Acetylation on the Ca^{2+}-Binding Properties of TnC

We have used the Ca^{2+}-induced increase in tyrosine fluorescence to measure calcium affinity of TnC and AcTnC at the C-terminal sites III and IV (Figure 4). We found that acetylation causes a 2-fold decrease in Ca^{2+} affinity at these sites as indicated by a shift in the transition midpoint of the fluorescence titration curve toward higher $[Ca^{2+}]$; $K_a = 2.1 \times 10^7\ M^{-1}$ and $K_a = 1.0 \times 10^7\ M^{-1}$ for TnC and AcTnC, respectively. Calcium binding to AcTnC appears to be more cooperative (Hill coefficient $n = 1.5$) as compared to

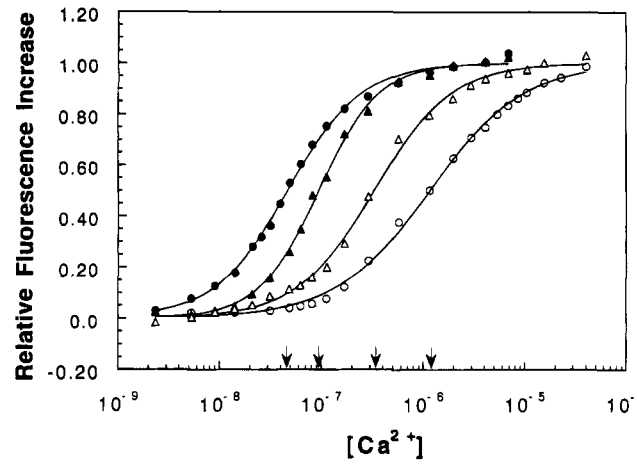

FIGURE 4: Calcium titrations of TnC and AcTnC. Calcium binding to TnC (○, ●) and to AcTnC (△, ▲) was monitored by tyrosine fluorescence (solid symbols) and by dansylaziridine fluorescence (empty symbols). Free Ca^{2+} concentration was controlled by an EGTA−NTA Ca^{2+}-buffering system. Each data point is an average of two titrations. Data points were fitted with the Hill equation: $F = F_o + \Delta F(K_a[Ca^{2+}])^n/(1 + (K_a[Ca^{2+}])^n)$ using a least-squares method. F_o and ΔF is the initial fluorescence and maximal change in fluorescence, respectively, K_a is the apparent binding constant, and n is the Hill coefficient. The solid line represents the best fit with parameters given below. Arrows indicate $[Ca^{2+}]$ at the transition midpoints. The data were fitted and plotted using a Macintosh computer and the program Kaleidagraph (Synergy Software, Reading, PA).

	sites III and IV (Tyr)		sites I and II (DANZ)	
	$10^{-6}K_a$ (M^{-1})	n	$10^{-6}K_a$ (M^{-1})	n
TnC	21.3 (±0.9)	1.21 (±0.06)	0.86 (±0.05)	0.94 (±0.04)
AcTnC	10.5 (±0.3)	1.48 (±0.07)	3.04 (±0.16)	1.15 (±0.06)

unmodified TnC ($n = 1.2$). Also, the fluorescence increase is larger in AcTnC ($F_{max}/F_o = 1.66$) than in TnC ($F_{max}/F_o = 1.55$), suggesting a larger Ca^{2+}-induced change in the environment of Tyr 108.

For monitoring Ca^{2+} binding to the low-affinity sites I and II, the proteins were labeled with dansylaziridine (DANZ). Ca^{2+}-induced increase in fluorescence intensity of AcTnC-DANZ is much smaller than that of TnC-DANZ; 1.4- and 2.1-fold increases, respectively. Ca^{2+} titrations of TnC and AcTnC monitored with DANZ fluorescence show that acetylation of lysine residues causes a 3.5-fold increase in Ca^{2+} affinity at sites I and II; $K_a = 8.6 \times 10^5\ M^{-1}$ and $K_a = 3.0 \times 10^6\ M^{-1}$ for TnC and AcTnC, respectively. Thus, acetylation has opposite effects on Ca^{2+} affinity at the two classes of sites in TnC.

Effect of Acetylation on the Interaction of TnC with TnI and TnT

We have used polyacrylamide gel electrophoresis at pH 8.6 under nondenaturing conditions (no SDS, no urea) to study the effect of acetylation on the ability of TnC to interact with the other troponin subunits TnI and TnT. Under these conditions, neither TnI nor TnT enters the gel unless it is complexed with TnC. Complex formation is indicated by a decrease of the intensity of the TnC band and by formation of a slower migrating band corresponding to the complex (Figure 5). An increase in the net negative charge in TnC upon acetylation is reflected by a small increase in its mobility on the gel. Similarly, the complexes of AcTnC with TnI and TnT have higher mobility than the corresponding

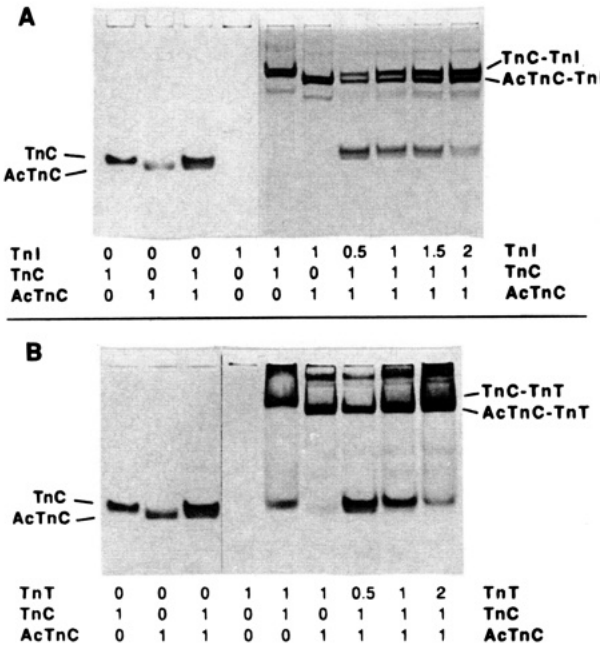

FIGURE 5: Effect of acetylation of TnC on its interaction with TnI and TnT. Acetylated TnC (5 μg) was mixed with unmodified TnC (5 μg), and various amounts of TnI (2.9−11.7μg) (A) or TnT (4.2−17.0 μg) (B) were added. Relative molar amounts are shown for each lane. Samples were run on 10% polyacrylamide gels in Tris−glycine buffer (pH 8.6), containing 1 mM $CaCl_2$. Note that under these conditions TnI and TnT do not enter the gel unless they are bound to TnC or AcTnC.

complexes of unmodified TnC. We have utilized this difference in electrophoretic mobilities to compare the apparent affinities of TnC and AcTnC for TnI and TnT. Figure 5 shows titrations of a 1:1 mixture of AcTnC and TnC with TnI (A) and with TnT (B). Although the extent of complex formation can be estimated both from the decrease in free proteins (TnC, AcTnC) and from the appearance of the complexes, the former proved less reliable owing to weak staining with Coomassie Blue of the AcTnC bands. From the essentially identical intensities of the bands corresponding to the TnC−TnI and AcTnC−TnI complexes, it appears that acetylation does not change significantly the affinity of TnC for TnI. On the other hand, the binding of TnT to AcTnC is stronger than to the native TnC since under the conditions where substoichiometric amounts of TnT are present the AcTnC−TnT complex is predominant.

Identification of the Regions of TnC Most Affected by Acetylation. TnC Tryptic Fragments

In our attempt to identify regions of the TnC molecule whose properties are most affected by acetylation, we have used three proteolytic fragments of TnC and their acetylated forms. These were TR_1C (residues 9−84), TR_1E (residues 1−100), and TR_2C (residues 89−159). Peptide TR_1C consists of the N-terminal Ca^{2+}-binding sites I and II, whereas the TR_1E peptide also contains the N-terminal helix as well as helix E, which is a part of Ca^{2+}-binding site III. The TR_2C peptide contains intact high-affinity sites III and IV. There are four Lys residues in TR_1C and in TR_2C, whereas TR_1E contains six out of the nine Lys of TnC. We have shown previously that these peptides preserve their secondary structure, Ca^{2+}-binding properties, and the ability to bind TnI (Evans et al., 1980; Grabarek et al., 1981a; Leavis et al., 1978).

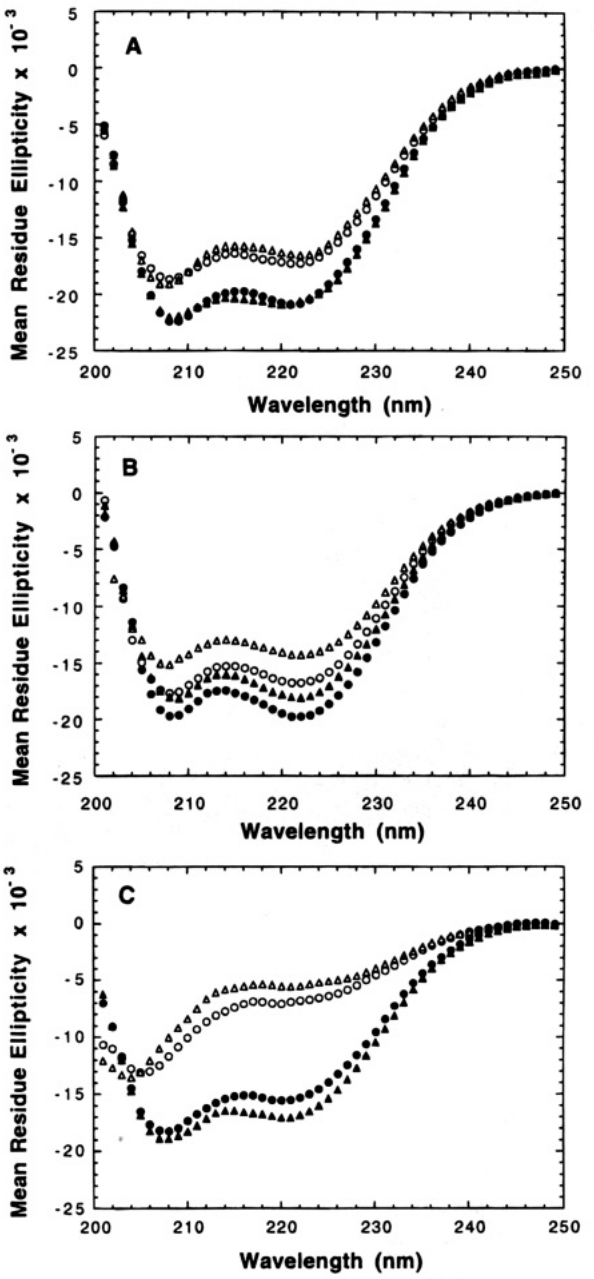

FIGURE 6: Circular dichroism spectra of tryptic fragments of TnC and their acetylated forms. Spectra of TR_1C (A), TR_1E (B), and TR_2C (C) in their native and acetylated forms were recorded in the presence of 0.1 mM EGTA and then again after addition of 0.5 mM $CaCl_2$ (final concentration). For experimental details, see legend to Figure 2. Key: native peptide (+EGTA) ○; native peptide (+Ca^{2+}) ●; acetylated peptide (+EGTA) △; acetylated peptide (+Ca^{2+}) ▲.

CD Spectra. There is virtually no effect of acetylation on the mean residue ellipticity of TR_1C whether or not Ca^{2+} is present (Figure 6, Table 1). In the case of the TR_1E peptide, the acetylated form has a lower α-helix content than the native form in both the absence and presence of Ca^{2+}. In the case of TR_2C, which in the absence of Ca^{2+} has very little α-helical structure, acetylation causes a further decrease in α-helix content. However, in the presence of Ca^{2+}, the α-helix content of $AcTR_2C$ is significantly higher than that of TR_2C (Figure 6, Table 1).

The dependence of CD on temperature for the three TnC peptides and their acetylated forms shows that in the absence of Ca^{2+} acetylation causes a large decrease in the stability of both N- and C-terminal domains of TnC (Figure 7). At

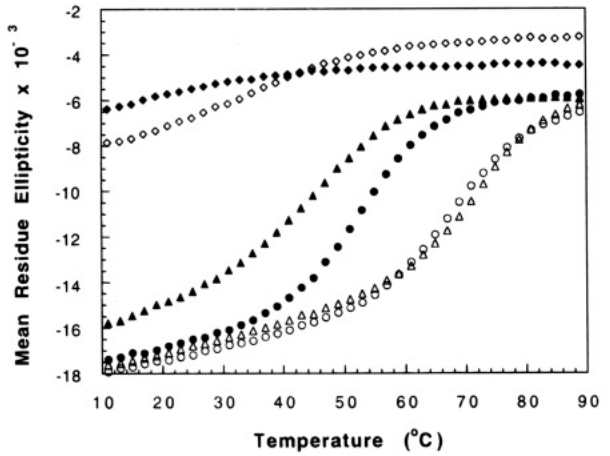

FIGURE 7: Thermal unfolding of TnC tryptic peptides and their acetylated forms monitored by circular dichroism. Key: TR_1C ○; $AcTR_1C$ ●; TR_1E △; $AcTR_1E$ ▲; TR_2C ◇; $AcTR_2C$ ◆. For experimental details see the legend to Figure 3. The data were fitted as described in Materials and Methods. Parameters of the fit are given in Table 2.

25 °C, the C-terminal domain (peptide TR_2C) is partially unfolded in the native form and almost completely unfolded in the acetylated form. In view of the fact that even at 10 °C $AcTR_2C$ is mostly unfolded, we were unable to estimate the melting temperature and ΔH for this peptide. The melting temperatures for TR_1C and TR_2C obtained here (Table 2) are in good agreement with those obtained from microcalorimetric studies by Tsalkova and Privalov (1985); ΔH_m values are, however, slightly lower. The melting temperatures of acetylated forms of TR_1C and TR_1E are 13.8 and 25.9 °C lower, respectively, compared to the native forms of these peptides (Table 2). In the presence of Ca^{2+}, none of the peptides, including their acetylated forms, shows signs of cooperative unfolding up to 90 °C (data not shown). Comparison of these results with those in Figure 3 confirms our conclusion that the transition in AcTnC and the main transition in TnC represent unfolding of the N-terminal domains of these proteins. The lack of "pretransition" in AcTnC is apparently related to the extremely low stability of the C-terminal domain in AcTnC. It is also clear that only the CD spectra recorded at 25 °C in the absence of Ca^{2+} (Figures 2 and 6) are affected by differences in stability between acetylated and unmodified proteins (peptides). Differences observed in the presence of Ca^{2+} can be attributed solely to changes in secondary structure induced by acetylation of lysine residues.

Interaction with TnI and TnT. As shown in Figure 8, acetylation increases the electrophoretic mobility of all three peptides as well as that of their complexes with TnI. Acetylation has no apparent effect on the affinity of TR_2C for TnI (Figure 8C) or TnT (Figure 9). In contrast, acetylation of both TR_1C and TR_1E significantly increases their affinity for TnI (Figure 8A,B). Even more striking is the enhancing effect of acetylation on the interaction of TR_1C and TR_1E with TnT. Both $AcTR_1C$ and $AcTR_1E$ form complexes with TnT, unlike their unmodified forms which show no complex formation with TnT on the native polyacrylamide gel (Figure 9). Thus, the N-terminal domain of TnC is primarily responsible for the acetylation-induced increase in TnC's affinity for TnT. These results are consistent with the previous findings that the N-terminal domain and possibly the central helix of TnC are involved

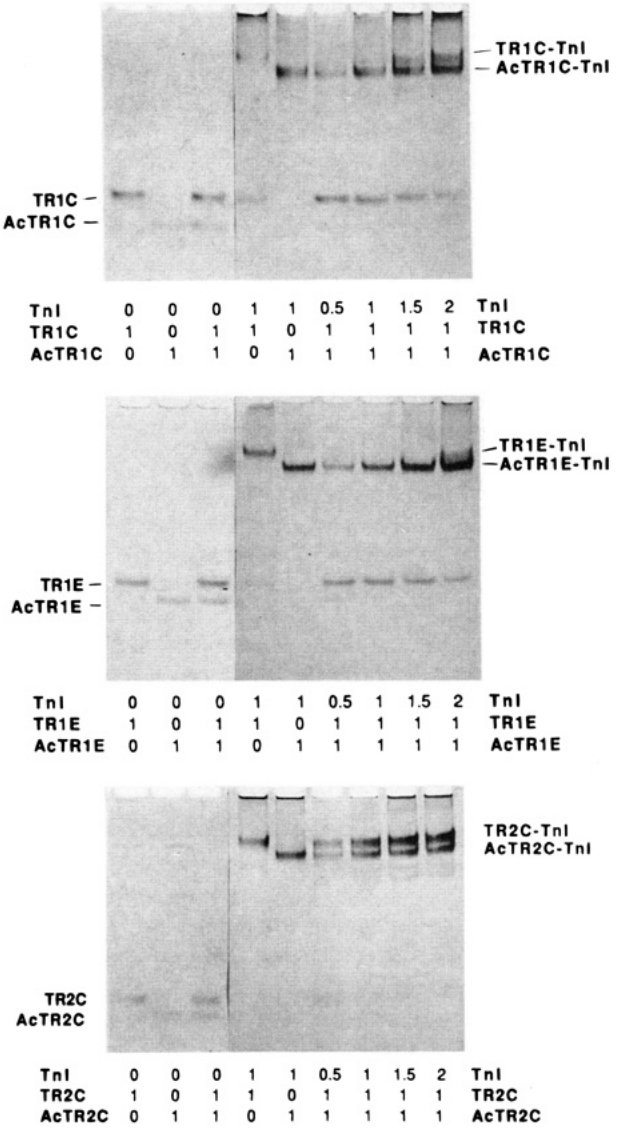

FIGURE 8: Interaction of the TnC tryptic fragments with TnI. Native and acetylated tryptic peptides of TnC (3 μg) were run separately and in a 1:1 mixture in the absence and presence of TnI, as indicated, on native polyacrylamide gels. To obtain the indicated molar ratios between TnI and the peptides, the following molecular weights were used: M_r(TnI) = 21000; M_r(TR_1C) = 8500; M_r(TR_1E) = 11200; M_r(TR_2C) = 8100. Note that staining of the acetylated peptides with Coomassie Blue is weaker (A−C, top to bottom, respectively).

in TnT binding (Grabarek et al., 1981a; Hitchcock, 1981). These experiments also indicate that acetylation enhances the interaction between the N-terminal domain of TnC and TnI—an effect that was less apparent in the experiments on the intact TnC (Figure 5).

Regulatory Activity of AcTnC

We have tested the ability of AcTnC to regulate the ATPase activity of myofibrils. Figure 10 shows the ATPase activity of TnC-depleted myofibrils as a function of increasing concentrations of TnC or AcTnC in the presence and absence of Ca^{2+}. Neither protein can relieve inhibition in the absence of Ca^{2+}, although in the presence of Ca^{2+}, both the AcTnC and TnC can activate the myofibrillar ATPase. The rise of activity on titration with AcTnC is sharper than with TnC, suggesting a higher affinity. This also results in

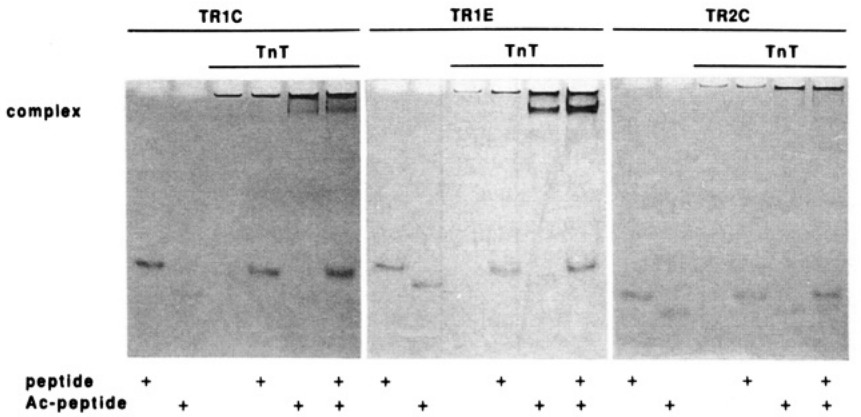

FIGURE 9: Interaction of TnC tryptic fragments with TnT. Native and acetylated TnC tryptic peptides (3 μg of each) were run separately and in a mixture with TnT on a native polyacrylamide gel electrophoresis. The proteins are mixed in a 1:1 molar ratio. The molecular weight M_r = 30 500 was used for TnT.

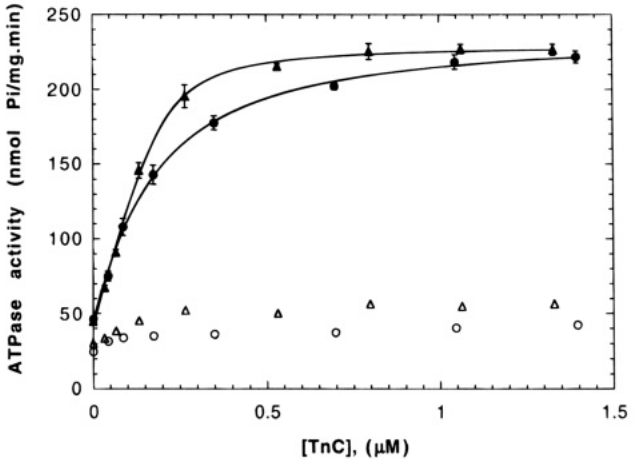

FIGURE 10: Effect of acetylation on the regulatory properties of TnC. ATPase activity of the TnC-depleted myofibrils as a function of increasing concentrations of TnC (○, ●) or AcTnC (△, ▲). The reaction mixture contained 0.5 mg/mL myofibrils, 30 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM imidazole (pH 7), 0.5 mM DTT, and 1 mM EGTA (empty symbols) or 0.1 mM $CaCl_2$ (solid symbols). The reaction was initiated by addition of ATP and terminated with 1% solution of SDS. The ATPase activity was calculated from the amount of phosphate released during 10-min incubation at 25 °C. Each data point is an average of six measurements (three separate titrations in duplicate). Error bars indicate the standard error of the mean. The solid line is a least-squares fit. The ATPase activity (V) is assumed to be proportional to the fraction of TnC-binding sites occupied (ν): $V = V_o + \Delta V\nu$, where V_o and ΔV are the initial ATPase activity and the TnC-induced maximal increase in ATPase activity, respectively. The fraction of sites occupied, ν, is one of the roots of the quadratic equation $nA\nu^2 - (K^{-1} + nA + B)\nu + B = 0$, where K is the apparent equilibrium binding constant, A is the total concentration of the TnC-binding sites, B is the total concentration of TnC or AcTnC, and n is the apparent stoichiometry (Morris & Lehrer, 1984). The data were fitted and plotted using a Macintosh computer and the Kaleidagraph program (Synergy Software, Reading, PA).

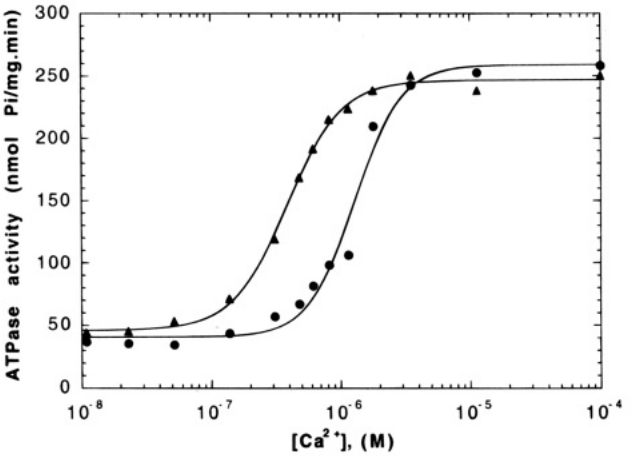

FIGURE 11: Calcium dependence of the ATPase activity of myofibrils reconstituted with TnC and AcTnC. ATPase activity of myofibrils as a function of [Ca^{2+}] was measured under conditions similar to those in Figure 10. The reaction mixture contained 0.5 mg/mL myofibrils, 1 μM TnC (●) or 1 μM AcTnC (▲), 30 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 20 mM imidazole (pH 7.0), 0.5 mM DTT, 1 mM EGTA, and various concentrations (0−1 mM) of $CaCl_2$. Each point is an average of data from two experiments. The solid line is a least-squares fit with the Hill equation (see legend to Figure 4).

a slightly higher ATPase activity of AcTnC containing myofibrils at lower, presumably substoichiometric, concentrations of the regulatory component (Figure 10). There is no difference in the maximal ATPase activities. We have attempted to fit the titration data with an equilibrium binding isotherm. Since the concentration of the binding sites is not known exactly, we used an estimation based on the composition of myofibrils (Yates & Greaser, 1983). Assuming that troponin accounts for 5% of the weight of myofibrils and using M_r = 80 000 for troponin, the 0.5 mg/mL concentration of myofibrils used in our studies would correspond to a concentration of 0.3 μM for TnC-binding sites. If we further assume that increase in the ATPase activity is proportional to TnC binding, we obtain the apparent equilibrium binding constants $K_a = 7.5 \times 10^6$ M^{-1} and $K_a = 4.4 \times 10^7$ M^{-1} for TnC and AcTnC, respectively.

In another set of experiments we have evaluated the Ca^{2+} dependence of ATPase activity of myofibrils reconstituted with AcTnC and with TnC (Figure 11). In these experiments, the total concentration of the regulatory component was kept constant at 1 μM, which based on the titration data (Figure 10) was sufficient to saturate the myofibrils. Free Ca^{2+} concentration was varied in the 10^{-8}−10^{-4} M range with an EGTA−$CaCl_2$ buffer. The AcTnC-containing myofibrils show a 3.3-fold higher affinity for Ca^{2+} as compared with those containing unmodified TnC [$K_a = 0.77$ (±0.06) $\times 10^6$ M^{-1} and $K_a = 2.58$ (±0.09) $\times 10^6$ M^{-1} for TnC and AcTnC, respectively]. The shift in Ca^{2+} sensitivity is consistent with the fluorescence data showing a higher affinity for Ca^{2+} at the N-terminal domain in acetylated TnC (Figure 4). Unlike the DANZ fluorescence titration curves the ATPase activity curves, show significantcooperativity,

Hill coefficients $n = 2.06$ and $n = 2.4$ for the AcTnC- and TnC-containing myofibrils, respectively.

DISCUSSION

In this work we found that acetylation of lysine residues in TnC causes a dramatic decrease in overall stability of the protein. This may suggest that intramolecular electrostatic interactions provide a considerable stabilization of the native protein. This, however, would be rather surprising since all Lys residues are located on the surface of TnC; therefore, their interactions with carboxyls may be expected to be weak due to the interference from the solvent. Another explanation for the lower stability of the acetylated form may be that the increase in the overall negative charge of the molecule causes intramolecular repulsive forces.

It is also surprising that lysine acetylation has opposite effects on the Ca^{2+} affinity of the two classes of sites in TnC. Although Lys 37 and Lys 140 are located in the Ca^{2+}-binding loops I and IV, respectively, neither residue is directly involved in Ca^{2+} binding. It is possible that the removal of the positive charge upon acetylation of these Lys residues has some subtle specific structural effects on the immediate environment of Ca^{2+} ions in sites I and IV resulting in the observed change in Ca^{2+} affinities. It is likely, however, that other structural effects of acetylation, for example, the decrease in the structural stability, may be more important.

Another interesting finding of this work is that the interactions of TnC with the other troponin components are enhanced upon acetylation of its lysine ϵ-NH_2 groups. Altered properties of the N-terminal domain are predominantly responsible for these effects. Consideration of the significance of electrostatic interactions in the troponin complex may throw some light on these results. There are 45 acidic (Asp, Glu) and 16 basic amino acids (Lys, Arg) in TnC. Thus, at neutral pH the molecule has a net charge of -29 and -21 in the apo- and Ca^{2+}-bound forms, respectively. Since both TnI and TnT are positively charged at neutral pH (4.5 and 4.0 for TnI and TnT, respectively), there must be some electrostatic component in the interaction between TnC and these proteins. Upon acetylation of Lys residues, the net negative charge of TnC increases to -38 and -30 for the Ca^{2+}-free and Ca^{2+}-bound form, respectively. This may increase the electrostatic attraction between TnC and the other troponin components, resulting in an increase in affinity. This would happen even if Lys residues were not directly involved in the interaction. Alternatively, one or more Lys side chains of TnC may be located at the interface with the other troponin subunits and may be directly involved in the interaction. This, however, would have to be a repulsive interaction with another positively charged group on TnI or TnT, since the neutralization of the charge upon acetylation enhances the affinity. Although the accessibility of Lys residues at positions 52, 84, 88, and 90 was indeed found to decrease upon complex formation with TnI or TnT, the effect was small (Hitchcock, 1981).

Acetylation of Lys residues may have further indirect effects on intersubunit interactions by changing some features of the secondary structure of TnC. Our CD studies indicate that in the presence of Ca^{2+} both AcTnC and its C-terminal peptide $AcTR_2C$ have a slightly higher α-helix content as compared to their native forms. In contrast, there is either no effect of acetylation or a decrease in α-helix content in the TR_1C and TR_1E peptides, respectively. Although it is difficult to interpret these results in terms of their possible effects on the interactions with the other troponin components, they indicate that the structure or stability of some specific segments of TnC is altered upon acetylation. It is, perhaps, significant that one third of all Lys residues in TnC are located within a stretch of only seven residues (84–90) in the central helix. Recent data of Hitchcock-DeGregori and her colleagues (Dobrowolski et al., 1991a,b) show that significant changes in the regulatory properties of TnC mutants are associated with deletions in this region. These observations and our present data point to the significance of charge distribution or charge–charge interactions for the intersubunit interactions in troponin.

These results raise important questions concerning the mechanism of intersubunit interactions in troponin and, in general, the Ca^{2+}-regulatory mechanism. In our early attempts to map TnC–TnI interaction using proteolytic fragments of TnC, we did show that both N- and C-terminal domains of TnC are involved in the interaction (Grabarek et al., 1981a). Although this conclusion has been supported by several lines of experimentation involving ^{1}H-NMR (Dalgarno et al., 1982; Grand et al., 1982), spectroscopic probes (Grabarek et al., 1986, Potter et al., 1976) and chemical cross-linking (Leszyk et al., 1987, 1988), our more specific prediction of the three helical segments being involved in the binding (Grabarek et al., 1981a) appears to be an oversimplification. An important insight into this problem is provided by the atomic structure of the complex of calmodulin (CaM) with a 26-residue peptide (M13)–the CaM-binding fragment of myosin light chain kinase (Ikura et al., 1992; Meador et al., 1992). The complex has a compact structure owing to a disruption of the long central helix of CaM that enables the two domains of CaM to clamp the bound peptide, which adopts a helical conformation. In addition to numerous contacts between the hydrophobic side chains of the peptide and the hydrophobic pockets formed by the four α-helical segments in each of the two globular domains of CaM, there are also several ionic interactions between carboxyls on CaM and positively charged side chains of M13. The question arises as to the extent one may extrapolate the model based on the CaM–M13 complex to the TnC–TnI complex. Since both domains of TnC interact with TnI, and in view of the sequence homology and similar 3D structures of TnC and CaM, it seems that the basic features of the complexes formed by these proteins may be similar. ^{1}H-NMR (Campbell et al., 1991; Slupsky et al., 1992) and molecular modeling studies (Strynadka & James, 1990) suggest that this indeed is the case in so far as the hydrophobic pocket in each domain of TnC is the docking site for the segments of TnI. However, there are some important differences. Trewhella and her colleagues (Olah et al., 1994) have shown using a low-angle X-ray scattering method that TnC has an extended structure in the complex with TnI. A similar conclusion was reached based upon energy transfer studies on TnC mutants (Gong et al., 1994). It appears that although each domain of TnC has a specific

function, a contribution from all parts of TnC is required for regulation.

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REFERENCES

Brzeska, H., Venyaminov, S. Vu., Grabarek, Z., & Drabikowski, W. (1983) *FEBS Lett. 153*, 169−73.
Campbell, A. P., Cachia, P. J., & Sykes, B. D. (1991) *Biochem. Cell Biol. 69*, 674−81.
Chait, B. T., & Kent, S. H. (1992) *Science 257*, 1885−94.
Chalovich, J. M. (1992) *Pharmacol. Ther. 55*, 95−148.
Cox, J. A., Comte, M., & Stein, E. A. (1981) *Biochem. J. 195*, 205−11.
Dalgarno, D. C., Grand, R. J. A., Levine, B. A., Moir, A. J. G., Scott, G. M. M., & Perry, S. V. (1982) *FEBS Lett. 150*, 54−8.
Dobrowolski, Z., Xu, G. Q., Chen, W., & Hitchcock-DeGregori, S. E. (1991a) *Biochemistry 30*, 7089−96.
Dobrowolski, Z., Xu, G. Q., & Hitchcock-DeGregori, S. E. (1991b) *J. Biol. Chem. 266*, 5703−10.
Drabikowski, W., Grabarek, Z., & Barylko, B. (1977) *Biochim. Biophys. Acta 490*, 216−24.
Evans, J. S., Levine, B. A., Leavis, P. C., Gergely, J., Grabarek, Z., & Drabikowski, W. (1980) *Biochim. Biophys. Acta 623*, 10−20.
Farah, C. S., Miyamoto, C. A., Ramos, C., Dasilva, A., Quaggio, R. B., Fujimori, K., Smillie, L. B., & Reinach, F. C. (1994) *J. Biol. Chem. 269*, 5230−40.
Fiske, C. H., & SubbaRow, Y. (1925) *J. Biol. Chem. 66*, 375−400.
Fraenkel-Conrat, H. (1957) *Methods Enzymol. 4*, 247−69.
Fujimori, K., Sorenson, M., Herzberg, O., Moult, J., & Reinach, F. C. (1990) *Nature 345*, 182−4.
Gagne, S. M., Tsuda, S., Li, M. X., Chandra, M., Smillie, L. B., & Sykes, B. D. (1994) *Protein Sci. 3*, 1961−74.
Gong, B.-J., Wang, Z., Tao, T., & Gergely, J. (1994) *Biophys. J. 66*, A346.
Grabarek, Z., Drabikowski, W., Leavis, P. C., Rosenfeld, S. S., & Gergely, J. (1981a) *J. Biol. Chem. 256*, 13121−7.
Grabarek, Z., Drabikowski, W., Vinokurov, L., & Lu, R. C. (1981b) *Biochim. Biophys. Acta 671*, 227−33.
Grabarek, Z., Grabarek, J., Leavis, P. C., & Gergely, J. (1983) *J. Biol. Chem. 258*, 14098−102.
Grabarek, Z., Leavis, P. C., & Gergely, J. (1986) *J. Biol. Chem. 261*, 608−13.
Grabarek, Z., Tan, R. Y., Wang, J., Tao, T., & Gergely, J. (1990) *Nature 345*, 132−5.
Grabarek, Z., Tao, T., & Gergely, J. (1992) *J. Muscle Res. Cell Motil. 13*, 383−93.
Grand, R. J., Levine, B. A., & Perry, S. V. (1982) *Biochem. J. 203*, 61−8.
Greaser, M. L., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226−33.
Gusev, N. B., Grabarek, Z., & Gergely, J. (1991) *J. Biol. Chem. 266*, 16622−6.
Herzberg, O., & James, M. N. (1985) *Nature 313*, 653−9.
Herzberg, O., Moult, J., & James, M. N. (1986) *J. Biol. Chem. 261*, 2638−44.
Hitchcock, S. E. (1981) *J. Mol. Biol. 147*, 153−73.
Hitchcock, S. E., Zimmerman, C. J., & Smalley, C. (1981) *J. Mol. Biol. 147*, 125−51.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632−8.
Johnson, J. D., Collins, J. H., & Potter, J. D. (1978) *J. Biol. Chem. 253*, 6451−8.
Johnson, M. L., & Faunt, L. M. (1992) *Methods Enzymol. 210*, 1−37.
Knight, P. J., & Trinick, J. A. (1982) *Methods Enzymol. 85*, 9−12.
Kobayashi, T., Tao, T., Grabarek, Z., Gergely, J., & Collins, J. H. (1991) *J. Biol. Chem. 266*, 13746−51.
Lai, C. Y. (1977) *Methods Enzymol. 47*, 236−43.
Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235−305.
Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452−9.
Leszyk, J., Collins, J. H., Leavis, P. C., & Tao, T. (1987) *Biochemistry 26*, 7042−7.
Leszyk, J., Collins, J. H., Leavis, P. C., & Tao, T. (1988) *Biochemistry 27*, 6983−7.
Leszyk, J., Grabarek, Z., Gergely, J., & Collins, J. H. (1990) *Biochemistry 29*, 299−304.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251−5.
Morris, E. P., & Lehrer, S. S. (1984) *Biochemistry 23*, 2214−20.
Ngai, S. M., & Hodges, R. S. (1992) *J. Biol. Chem. 267*, 15715−20.
Olah, G. A., & Trewhella, J. (1994) *Biochemistry 33*, 12800−6.
Olah, G. A., Rokop, S. E., Wang, C.-L. A., Blechner, S. L., & Trewhella, J. (1994) *Biochemistry 33*, 8233−9.
Pearlstone, J. R., & Smillie, L. B. (1978) *Can. J. Biochem. 56*, 521−7.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31−8.
Perrin, D. D., & Sayce, J. G. (1967) *Talanta 14*, 833−42.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628−33.
Potter, J. D., Seidel, J. C., Leavis, P., Lehrer, S. S., & Gergely, J. (1976) *J. Biol. Chem. 251*, 7551−6.
Riordan, J. F., & Vallee, B. L. (1972) *Methods Enzymol. 25*, 500−6.
Schellman, J. A., & Hawkes, R. B. (1980) in *Protein folding* (Jaenicke, R., Ed.) pp 331−341, Elsevier/North-Holland Biomedical Press, New York.
Sillen, L. C., & Martel, A. E. (1964) *Stability constants of metal ion complexes*, 2nd ed., special publication 17 ed., The Chemical Society, Burlington House, London.
Slupsky, C. M., Shaw, G. S., Campbell, A. P., & Sykes, B. D. (1992) *Protein Sci. 1*, 1595−603.
Strynadka, N. C., & James, M. N. (1990) *Proteins 7*, 234−48.
Strynadka, N. C. J., Chernaia, M., Li, M., Smillie, L. B., & James, M. N. G. (1995) *Biophys. J. 68*, A359.
Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science 227*, 945−8.
Syska, H., Wilkinson, J. M., Grand, R. J., & Perry, S. V. (1976) *Biochem. J. 153*, 375−87.
Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798−802.
Tanokura, M., Tawada, Y., Ono, A., & Ohtsuki, I. (1983) *J. Biochem. 93*, 331−7.
Tsalkova, T. N., & Privalov, P. L. (1980) *Biochim. Biophys. Acta 624*, 196−204.
Tsalkova, T. N., & Privalov, P. L. (1985) *J. Mol. Biol. 181*, 533−44.
Tsuda, S., Aimoto, S., & Hikichi, K. (1992) *J. Biochem. 112*, 665−70.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science 178*, 871−2.
Wang, Z. Y., Sarkar, S., Gergely, J., & Tao, T. (1990) *J. Biol. Chem. 265*, 4953−7.
Weeks, R. A., & Perry, S. V. (1978) *Biochem. J. 173*, 449−57.
Yates, L. D., & Greaser, M. L. (1983) *J. Biol. Chem. 258*, 5770−4.
Zot, A. S., & Potter, J. D. (1987) *Ann. Rev. Biophys. Biophys. Chem. 16*, 535−59.

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