Calcium-Induced Dimerization of Troponin C: Mode of Interaction and Use of Trifluoroethanol as a Denaturant of Quaternary Structure

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ABSTRACT: Protein aggregation can be a problem, especially as a large number of proteins become available for structural studies at fairly high concentrations using solution techniques such as NMR spectroscopy. The muscle regulatory protein troponin C (TnC) undergoes a calcium-induced dimerization at neutral pH with a dissociation constant for the dimerization of 0.4 mM at 20 °C. The present study indicates that the mode of dimerization involves the N-domain of one monomer interacting with the N-domain of another monomer. Addition of the solvent trifluoroethanol (TFE) to a concentration of 15%, v/v, results in a 10-fold increase in the dimer dissociation constant of calcium-saturated TnC (4 mM at 20 °C), making TnC predominantly a monomer for spectroscopic studies. Further, TFE, at the concentrations used herein, acts to perturb the quaternary structure of TnC without adversely affecting the secondary or tertiary structure as evidenced by minimal changes to its CD spectra and ^{1}H, ^{13}C, and ^{15}N NMR chemical shifts.

Troponin C (TnC)[1] is the calcium binding protein of the thin filament of muscle involved in the regulation of muscle contraction. Chicken skeletal TnC binds four metal ions in four separate metal ion binding sites numbered consecutively from the N-terminus of the protein. The structure of half-saturated avian skeletal TnC (with calcium binding sites III and IV filled) has been solved using X-ray crystallographic techniques (Satyshur et al., 1988; 1994; Herzberg & James, 1988) and reveals two domains (the N-terminal and C-terminal domains), each with two EF-hand helix−loop−helix calcium binding sites. The N-terminal domain contains the lower affinity regulatory calcium-specific sites, whereas the C-terminal domain contains the high-affinity calcium/magnesium binding sites. Knowledge of the structure of TnC in its half and fully calcium saturated forms is essential to understanding the molecular mechanism of muscle contraction.

Nuclear magnetic resonance (NMR) spectroscopy has developed into a technique capable of determining three-dimensional structures of proteins in solution. Developments in molecular biology not only have allowed large amounts of proteins to be expressed but have facilitated the incorporation of ^{15}N and ^{13}C isotope labels into proteins. Spectral editing and internuclear correlations based on the attached heteronuclei coupled with greater dimensionality result in better resolution than is possible by two-dimensional methods. Our goal has been to use these new techniques to solve the 3D solution structure of calcium-saturated TnC. TnC, however, undergoes a reversible calcium-induced dimerization. This dimerization/aggregation is detrimental to the NMR structural characterization of TnC since the molecular weight of the dimer is beyond the limits of present NMR techniques.

The first characterization of the calcium-induced dimerization of TnC involved hydrodynamic studies using ultracentrifugation techniques (Murray & Kay, 1972). These authors showed that TnC aggregated at saturating calcium concentrations and neutral pH. It was later shown that the calcium-induced dimer had a K_d of 0.08 mM (at pH 7.0 and 5 °C in 10 mM imidazole, 5 mM potassium phosphate, 1 mM $MgCl_2$, and 1 mM DTT), and a monomer apparent molecular weight of 18 700 was estimated from a two-species plot of the data (Margossian & Stafford, 1982). TnC aggregation has also been studied using small angle X-ray scattering techniques (Fujisawa et al., 1990; Blechner et al., 1992).

In addition to the calcium-induced dimerization, TnC also undergoes a reversible acid-induced aggregation (Wang et al., 1989). As the pH of TnC is lowered from 7 to 5, the affinity of the N-domain sites for calcium is reduced (McCubbin et al., 1986). The crystal structure of TnC was determined at a pH near 5, where the N-domain sites are in the apo form. The acid-induced aggregation is most likely similar to the molecular packing of TnC seen in the crystal, which involves helix A of the N-domain interacting with the C-domain hydrophobic pocket (Satyshur et al., 1988; Herzberg & James, 1988). The interaction is hydrophobic in nature and allows for polymerization of TnC. This paper will show that the mode of the calcium-induced dimerization

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[1] Abbreviations: TnC, recombinant chicken skeletal troponin C (and unless stated, TnC will refer to the calcium-saturated state of TnC); TFE, 2,2,2-trifluoroethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS 4-morpholinepropanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); OD, optical density; MW, molecular weight; UV, ultraviolet; NMR, nuclear magnetic resonance; HNCO, three-dimensional $^{1}H-^{15}N-^{13}C'_{(i-1)}$ correlation; HNCA, three-dimensional $^{1}H-^{15}N-^{13}C\alpha$ correlation; HN(CO)CA, three-dimensional $^{1}H-^{15}N-^{13}C\alpha_{(i-1)}$ correlation; HCACO, three-dimensional $^{1}H\alpha-^{13}C\alpha-^{13}C'$ correlation; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser effect; HCCH−COSY, three-dimensional $^{1}H-^{13}C-^{13}C-^{1}H$ correlation; HSQC, heteronuclear single quantum correlation; 2D and 3D, two- and three-dimensional; CSI, chemical shift index.

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is different from that of the acid-induced aggregation.

Protein aggregation can be a general problem especially as a large number of proteins become available for structural studies at fairly high concentrations using solution techniques such as NMR spectroscopy. Some proteins are completely insoluble in water, such as membrane proteins (Barsukov et al., 1990; Orekhov et al., 1994; Johansson et al., 1994; Girvin & Fillingame, 1993; Lycksell et al., 1992), whereas other are "sticky" and thus aggregate at higher concentrations. It has recently been shown that one way to prevent protein aggregation during NMR studies has been to use the detergent CHAPS (Anglister et al., 1993; Grzesiek & Bax, 1993; Davies & Riechmann, 1994). Other methods used to break up protein aggregation have included the use of organic cosolvents. One of the more studied protein aggregates is insulin. A variety of cosolvents have been used to overcome its self-association, such as 35% acetonitrile (Kline & Justice, 1990), 20% acetic acid (Hua & Weiss, 1991a), 10% DMSO (Hua & Weiss, 1991b), or 33% trifluoroethanol (Higgens et al., 1990; Craik & Higgens, 1991). We have chosen to use the solvent trifluoroethanol (TFE) to prevent TnC aggregation.

TFE has been characterized as a structure-enhancing cosolvent. TFE has been shown to stabilize a variety of structures in peptides including β-sheet (Goodman et al., 1971; Mutter & Altmann, 1985; Maser et al., 1984), β-turn (Cann et al., 1987; Greff et al., 1976; Siligardi et al., 1987; Blanco et al., 1994), and α-helix (Nelson & Kallenbach, 1989; Lu et al., 1984; Lehrman et al., 1990; Segawa et al., 1991; Marion et al., 1988; Jiméniz et al., 1987; Reutimann et al., 1981). The dielectric constant of TFE is approximately one-third that of water, and it was proposed that the net effect of TFE would be to strengthen the interactions between charged groups (Llinás & Klein, 1975; Nelson & Kallenbach, 1986). It was shown, however, that secondary structure is stabilized due to the decreased hydrogen bonding of amide protons to the solvent resulting in increased intramolecular hydrogen bonds (Nelson & Kallenbach, 1986; Sönnichsen et al., 1992). TFE has also been found to disrupt hydrophobic protein interactions without substantial structural change of the individual monomeric units involved, thereby acting as a denaturant of quaternary structure (Lau et al., 1984a,b). The fully folded native structure of hen egg white lysozyme was studied with 15%, v/v, TFE, and it was shown that the structure of this protein was relatively unchanged at this low concentration of TFE (Buck et al., 1993). This paper also illustrates that, for this concentration of TFE, no substantial structural changes occur for TnC.

The present investigation indicates a calcium-induced dimerization of TnC, using ultracentrifugation and NMR studies, and shows that this dimerization involves the respective N-domains of the two monomeric units. Further, this interaction is hydrophobic in nature and may be disturbed upon addition of a small amount of TFE. TFE does not significantly alter the characteristics of TnC as a calcium binding protein, nor does it alter the tertiary structure of monomer. The added TFE thereby permits NMR structural studies to be accomplished on this protein. Implications involving the use of TFE as a general perturbant of quaternary structure are also discussed.

MATERIALS AND METHODS

Preparation of Protein. Chicken skeletal muscle TnC was expressed in *Escherichia coli* using either of two vectors, both based on the pET3a system of Studier et al. (1990). The construction of the first of these (pET3a-TnC) has been described by Quaggio et al. (1993). The second (pET3a-TnC*), a smaller version of pET3a-TnC, was constructed from pLcII-Fx-TnC (Reinach & Karlsson, 1988) as a template using the polymerase chain reaction and two oligonucleotides. One of these was designed to include codons for amino acid residues 1−6 (underlined) of the sequence of TnC flanked on the 5′ side by nucleotides of the pET3a vector including the *Nde*I restriction enzyme site (GAG ATA TAC ATA TGG CGT CAA TGA CGG ACC). The second corresponded to codons for amino acid residues 159−162 of the noncoding strand of TnC (underlined) flanked on the 5′ side by a stop codon and the *Bam*HI restriction enzyme site and nucleotides of the pET3a vector (GGA ATG TCT GGA TCC TTA CTG CAC ACC CTC). Following the polymerase chain reaction, the amplified DNA fragment was digested with the restriction enzymes *Nde*I and *Bam*HI and ligated into the *Nde*I−*Bam*HI sites of expression vector pET3a plasmid DNA (Studier et al., 1990). Expression of TnC protein was in *E. coli* strin BL21(DE3) pLysS.

Unlabeled TnC was prepared by growing this strain of *E. coli* in LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, and 10 g/L NaCl, pH 7.5, containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol). For preparation of ^{15}N-labeled or ^{13}C/^{15}N-labeled protein, 50 mL of ZB medium (1% N-Z-amine A casein enzymatic hydrolysate + 0.5% NaCl) (Studier et al., 1990) containing 100 μg/mL ampicillin + 25 μg/mL chloramphenicol was inoculated with a fresh colony and allowed to grow until an OD_{600} of 0.6−0.9 was reached. This culture was then used to inoculate 1 L of minimal medium which consisted of M9 salts without NH_4Cl (as described by Maniatis et al. (1986)), 5 g of D-glucose or 3 g of D-[$^{13}C_6$]glucose (for ^{13}C-labeled protein), and 1 g of $(^{15}NH_4)_2SO_4$, each of which was dissolved in water and filter sterilized for use in the medium. To enhance the expression level, a mixture of vitamins and minerals was added to the minimum medium: 1 mL/L of a filter-sterilized mixture of vitamins (at pH 7.5) (0.1 g/100 mL of biotin, choline chloride, folic acid, niacinamide, D-pantothenate, and pyridoxal; 0.5 g/100 mL of thiamine; and 0.01 g/100 mL of riboflavin in H_2O) (Venters et al., 1991; Hoffman & Spicer, 1991); 1 mL/L of a filter-sterilized mixture of minerals (2 M $MgSO_4$ (Hoffman & Spicer, 1991), 1 mM $FeCl_3$, and 25 mM $ZnSO_4$ (Venters et al., 1991)); 1 mL/L of a filter-sterilized solution of 100 mM $CaCl_2$ (Hoffman & Spicer, 1991); 100 μg/mL ampicillin; and 25 μg/mL chloramphenicol. The cells were then allowed to grow until an OD_{600} between 0.5 and 1.0 was reached, and filter-sterilized IPTG was added to a final concentration of 1% to induce TnC production. The cells were allowed to grow for a further 3.5 h, after which time they were centrifuged and French pressed. Pure TnC was recovered using either DEAE-Sephacel containing urea as outlined by Golosinka et al. (1991) and the same column without urea or a phenyl-Sepharose column (elution of TnC with EDTA). To remove calcium and other possible impurities, TnC was dissolved in 0.5 M EDTA at pH 8.0 and purified on a Sephadex G25 size-exclusion column equilibrated with 25 mM NH_4HCO_3. The purity of TnC was determined to be >98% using UV spectroscopy and reverse-phase HPLC. The overall yield of TnC was approximately 50 mg/L of culture.

The single cysteine in TnC was reacted with iodoacetamide to prevent oxidation of TnC. The carboxamidomethylation

was performed by dissolving TnC in 0.5 M EDTA and 100 mM DTT at pH 7.5. Iodoacetamide was added to a final concentration of 100 mM (by first dissolving the powder in H_2O and NaOH), and the reaction was allowed to proceed in the dark for 10 min. TnC was then recovered on a Sephadex G25 size-exclusion column equilibrated with 25 mM NH_4HCO_3. The reaction proceeded easily to greater than 95% as determined by DTNB assay, reverse-phase HPLC, and mass spectroscopy (MW(determined) = 18 315).

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

Ultracentrifugation. Ultracentrifugation analysis was performed either in 30 mM MOPS, 150 mM KCl, 20 mM $CaCl_2$, and 15%, v/v, TFE at pH 7.0 or in 30 mM MOPS, 150 mM KCl, and 20 mM $CaCl_2$ at pH 7.0. Both buffers were filtered after preparation. The concentrations of TnC were 0.390 mM in the TFE buffer and 0.392 mM in the buffer containing no TFE. Both runs were done on a Beckman Model E ultracentrifuge at 16 000 rpm and 20 °C employing a photoelectric scanner optical system, where the absorbance was measured at 267 nm over a 1.2-cm path length. All absorbance values were corrected to a 1-cm path length. Molecular weights and dissociation constants were determined by fitting the following equation (Burrows et al., 1994):

$$A_{267,r} = c_m\epsilon_{267}\exp[HM(r^2 - r_0^2)] + (c_m c_m/K_d)2\epsilon_{267}\exp[2HM(r^2 - r_0^2)]$$

where r is the radial distance from the axis of rotation, r_0 is the radial distance at the meniscus, M is the monomer molecular weight, K_d is the dissociation constant for the dimer (K_d = [monomer]^2/[dimer]), $A_{267,r}$ is the absorbance at radius r, c_m is the concentration of the monomer at radius r, and ϵ_{267} is the molar extinction coefficient for TnC at 267 nm, which was experimentally determined (after correcting for scatter) to be 0.632 mM^{-1} cm^{-1} for TnC in the absence of TFE and 0.845 mM^{-1} cm^{-1} for TnC in the presence of TFE. The operational constants H and ω are defined by the equations

$$H = \frac{(1 - \bar{v}\varrho)\omega^2}{2RT} \quad \text{and} \quad \omega = \frac{2\pi(\text{rpm})}{60}$$

where R is the universal gas constant (8.314 J/(mol K); T is the temperature in degrees K; ω is the angular velocity; ϱ is the density of the solution, which was 1.0087 g/mL for TnC and 1.0811 g/mL for TnC in 15%, v/v, TFE; and $\bar{v}$ is the partial specific volume of the solute, which is 0.71 mL/g (Byers & Kay, 1982). The partial specific volume may be calculated to give a value of 0.742 mL/g (McCubbin et al., 1985). The value of 0.71 mL/g was used because for highly charged proteins, such as calmodulin, troponin C, and S-100, electrostriction of H_2O leads to a lower experimentally determined value for the partial specific volume (Crouch & Klee, 1980; Byers & Kay, 1982; Mani & Kay, 1984). The equation was fit using an iterative nonlinear least squares analysis on an in-house-written program, where c_m, M, and K_d were allowed to vary to best fit the data points.

CD Spectroscopy. TnC was prepared in 1 mL of a buffer containing 50 mM MOPS, 100 mM KCl, and 1 mM EGTA with or without 1 mM DTT at pH 7.1. To this was added 100 μL of 8 M guanidine HCl in 50 mM MOPS buffer, and the resultant TnC solution was dialyzed for 48 h with two 1-L changes of buffer (50 mM MOPS, 100 mM KCl, and 1 mM EGTA). The final concentration of TnC was 0.044 mM. Calcium was then added to a final concentration of 4 mM to obtain the calcium-saturated sample. TFE was added to this sample to obtain the spectrum with 15%, v/v, TFE. Two runs were averaged to obtain the final far-UV CD spectra, while only one run of the near-UV CD spectrum was taken.

NMR Spectroscopy. For NMR spectroscopy, TnC was dissolved to a final concentration of approximately 1.4 mM in 15%, v/v, TFE in 150 mM KCl, and 15 mM $CaCl_2$ at a pH of 7.0 in either 75% H_2O, 10% D_2O, and 15% TFE or 85% D_2O and 15%, v/v, TFE. Assignments of TnC were done using a combination of triple-resonance, three-dimensional NMR experiments including HNCO, HNCA, HN(CO)CA, HCACO, ^{15}N-edited-TOCSY, ^{15}N-edited-NOESY, HCCH-COSY, and ^{13}C-edited-NOESY and will be presented elsewhere (Slupsky et al., 1995).

All 2D ^{1}H^{15}N HSQC NMR experiments were carried out on a Varian Unity-600 NMR spectrometer or a Varian VXR-500 NMR spectrometer using the pulse scheme of Bodenhausen and Reuben (1980) or using the modified form (water saturation using spin lock) as outlined by Messerle et al. (1989). For these experiments, the ^{1}H dimension was centered at the water frequency. Typical acquisition parameters for these spectra were 128 × 512 complex points in F_1 (^{15}N) and F_2 (^{1}H), respectively. The proton dimension had a spectral width of 6667 Hz (for experiments on the VXR500 spectrometer) or 8000 Hz (for experiments on the Unity 600 spectrometer) and the ^{15}N dimension had a spectral width of 1500 or 1824 Hz on the VXR500 or the Unity 600 NMR spectrometer, respectively. The spectra were normally processed using cosine-squared weighting, and the ^{15}N dimension was zero-filled to 2K data points. For the TFE titration, the intensities were scaled for dilution effects. For the TFE titration, ^{1}H linewidths were measured from the width of the line at half-height. The digital resolution was 6.5 Hz in the ^{1}H dimension.

2D ^{15}N T_2 relaxation experiments were carried out at 40 °C on a Varian Unity spectrometer operating at 600 MHz ^{1}H frequency, using the pulse sequence as described by Barbato et al. (1992), except that quadrature detection in F_1 was obtained by the method of States et al. (1982). One hundred twenty-eight transients were acquired for each t_1 increment. The ^{1}H carrier was centered at 4.70 ppm on the water frequency, and the ^{15}N carrier was positioned at 119 ppm. The spectral widths used were 8000 Hz in F_2 (^{1}H) and 1824 Hz in F_1 (^{15}N). For seven different durations of the T_2 relaxation delay, T = 0, 14.14, 28.29, 42.43, 56.58, 84.86, and 141.44 ms, 128 × 1024 complex points were acquired. A 1.8-s delay was used between scans. The spectra were processed with a Lorentzian-to-Gaussian filtering function and zero-filling to 2048 data points in F_1 and a cosine squared weighting function in F_2 with zero-filling to 2048 points. Resonance intensities were used to determine the relaxation rates. ^{15}N T_2 data were analyzed using an iterative nonlinear least squares determination where T_2 and M_0 were allowed to vary using the equation $y = M_0\exp[-t/T_2]$, where y is the intensity at the relaxation time t, M_0 is

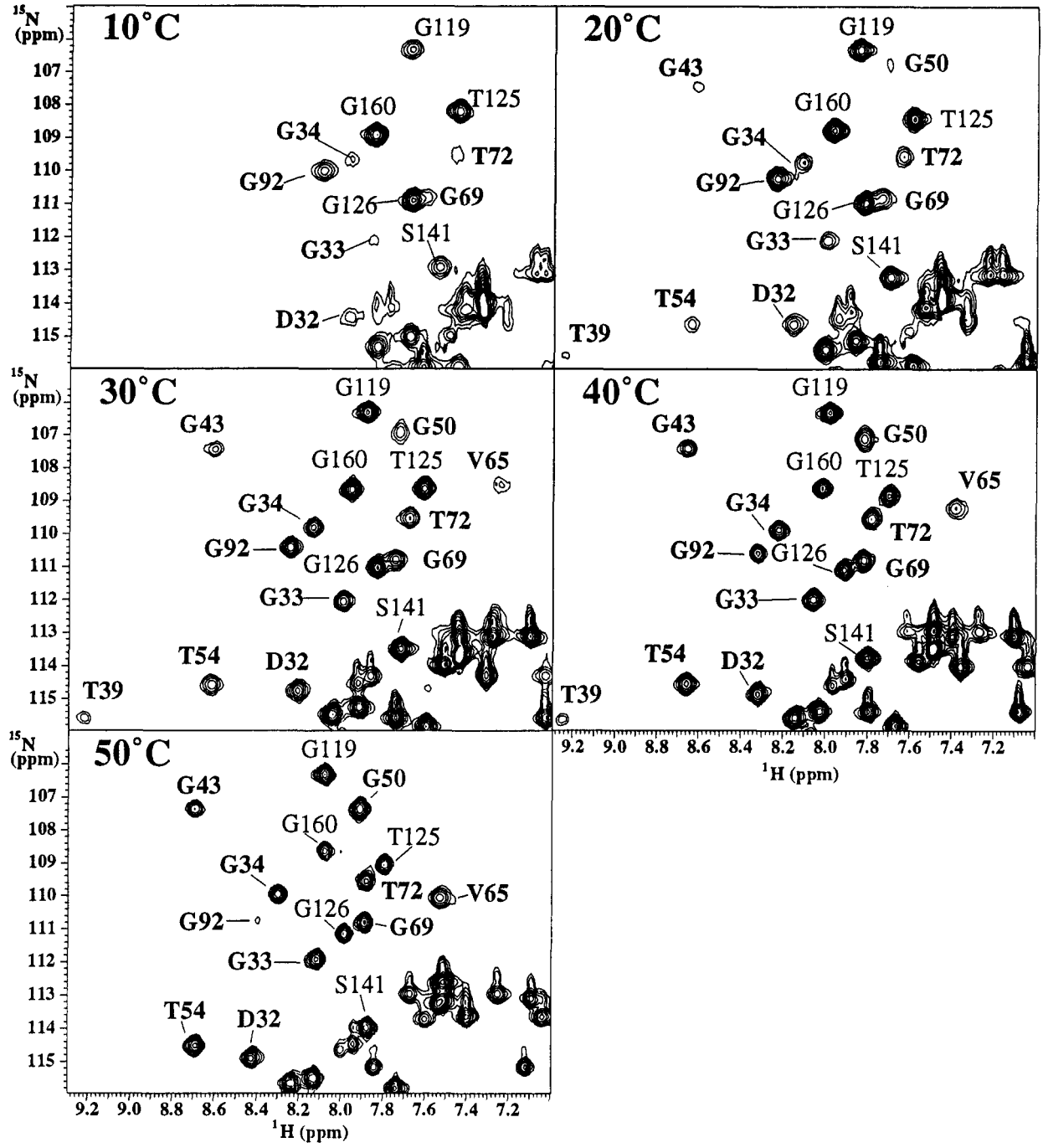


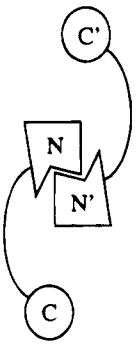
FIGURE 1: Effect of temperature on intensity of cross-peaks in a series of 600-MHz 2D $^{1}H-^{15}N$ HSQC NMR spectra recorded with spin-lock water suppression (Messerle et al., 1989) of TnC which has been uniformly labeled with ^{15}N (>95%). Shown is the portion of the HSQC spectrum corresponding to glycine residues. Cross peaks labeled in boldface type correspond to resonances from the N-domain, while those in light face type correspond to resonances from the C-domain [for assignments, see Slupsky et al. (1995)].

the intensity when $t = 0$, and T_2 is the ^{15}N T_2 relaxation time.

RESULTS

Troponin C has been shown in the crystal structure to be a dumbbell-shaped molecule with separate N- and C-terminal domains attached by a helical linker (Herzberg & James, 1988; Satyshur et al., 1988, 1994). The first indication that the calcium-induced dimerization of TnC in solution involves the interaction of the N-terminal domain from two monomeric TnC units was obtained from the observation of differential linewidths of NMR resonances from the N- and C-terminal domains and the effect of temperature on this phenomenon. In 1D NMR spectroscopy, the peak height increases as the linewidth decreases; in 2D NMR, the cross-peak intensity reflects the linewidths of the two correlated resonances because of relaxation in the indirectly detected dimension and line broadening in the observed dimension. Since the linewidths in NMR spectra increase with increasing molecular weight, the cross-peak intensity in *n*D spectra are therefore increasingly sensitive to larger molecular weights (Weiss et al., 1984). Figure 1 presents a study of the effect of temperature on a cross-peak intensity in 2D NMR spectra of calcium-saturated TnC. Shown are 2D $^{1}H-^{15}N$ HSQC NMR spectra correlating the proton and nitrogen chemical shifts of the amide residues of TnC. At 10 °C, the most obvious feature of this spectrum is the differential intensities of cross-peaks from the N- and C-domains, with some

Chart 1



N-domain cross-peaks not even visible. The residues T39, G43, G50, and T54 are broadened to the point where they are not visible. The other N-domain peaks (D32, G33, G34, G69, and T72) are also very broad and are barely visible. The C-domain cross-peaks, however, are all present, and all have strong intensities in comparison to the N-domain peaks. As the temperature is increased, the intensities of the N-domain cross-peaks (D32, G33, G34, G43, G50, T54, V65, G69, and T72) gradually increase, whereas the intensities of the C-domain cross-peaks (G119, T125, G126, S141, and G160) remain fairly constant. At much higher temperatures, however, some cross-peaks in the $^{1}H-^{15}N$ HSQC NMR spectrum are lost due to increased amide exchange with the solvent; see for example the residues threonine 39 and glycine 92 at 50 °C.

There is a difference in the molecular weights of the N-terminal (90 residues) and C-terminal (72 residues) domains. This difference, however, is not sufficient to explain the difference in intensities nor the change in intensities as the temperature is increased, as this change should be the same for both domains regardless of their size. Further, any difference between the two domains could only be seen if there was a flexible linker between them, or if the internuclear vectors dominating the relaxation of the observed nuclei all had a different orientation relative to the principal axis system of an anisotropic diffusion tensor for a rigid dumbbell molecule (Barbato et al., 1992). A simple model consistent with these data is therefore proposed wherein the dimerization involves the association of two N-terminal domains to which the C-terminal domains are attached via a flexible linker as shown in Chart 1. As the temperature is raised, not only are the linewidths of resonances from both the N- and C-terminal domains decreased slightly (or the intensities are increased slightly) by viscosity and temperature effects, but the monomer−dimer equilibrium is shifted toward the monomer, resulting in an additional strong decrease in linewidth for resonances from the N−terminal domain.

It was previously shown for the homologous and also dumbbell-shaped protein calmodulin that the two domains have different average ^{15}N T_2 relaxation rates and thus different average correlation times, and that the correlation times derived for the individual amides depend only weakly on the orientation of the N−H bond vector (Barbato et al., 1992). Thus, it was hypothesized that the central helix of calmodulin serves as a flexible tether (Barbato et al., 1992). There is no evidence, however, for a calcium-induced dimerization of calmodulin. The difference in linewidths observed for the two domains of calmodulin is much less than observed here and is similar to that observed for TnC in TFE (see below).

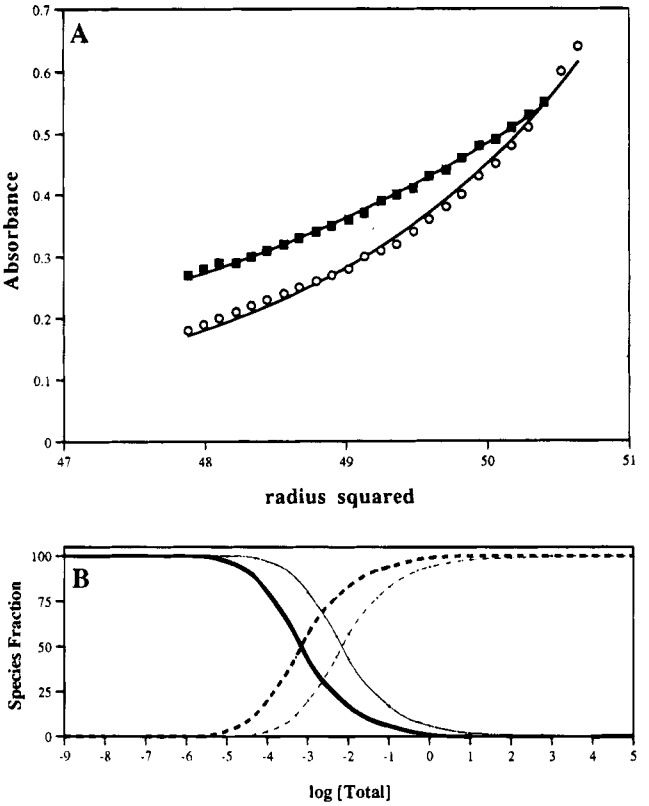


FIGURE 2: (A) Sedimentation equilibrium data for TnC in the presence (■) and absence (○) of TFE. Shown is the absorbance (1-cm path length) versus the radial distance (to the center of the rotor) squared. The data was fit as outlined in Materials and Methods, and molecular weights and dissociation constants were obtained. (B) Percent of monomer or dimer versus the logarithm of the total concentration of TnC at 40 °C. The solid lines represent monomer, the dashed lines represent dimer, the black lines represent TnC in the absence of TFE, and the gray lines represent TnC in the presence of TFE.

Figure 2A represents low-speed sedimentation equilibrium studies on TnC in the presence of calcium and either in the presence or in the absence of TFE.[2] These data were fit to a monomer and dimer equilibrium (see Materials and Methods). The molecular weight that best fits the ultracentrifugation data at 20 °C is 18 415 for both TnC and TnC in TFE, which is close to the calculated molecular mass of 18 315 Da. The K_d for TnC at 20 ° was determined to be 0.43 mM in the absence of TFE and 4.2 mM in the presence of TFE. If one assumes that the K_d changes by a factor of approximately 2 for every 10 °C, then our results would predict a K_d of 0.14 mM at 5 °C, which is within experimental error of that obtained by Margossian and Stafford (1982). It is thus possible to estimate a K_d of 1.6 mM for TnC and 16 mM for TnC in TFE at 40 °C (assuming that there is a 10-fold increase in K_d in the presence of TFE at all temperatures). Figure 2B is a plot of the percent of monomer or dimer versus the log of the total concentration of TnC at 40 °C. This plot indicates that, in the absence of TFE, the concentration of TnC where half is monomer and half is dimer is 1.5 mM, whereas in the presence of TFE, the concentration of TnC where half is monomer is approximately 15 mM. For 1.4 mM TnC in the presence of TFE (the concentration used in our studies), the amount of monomer present is at least 87%.

[2] Unless otherwise stated, TFE will refer to 15%, v/v, TFE.

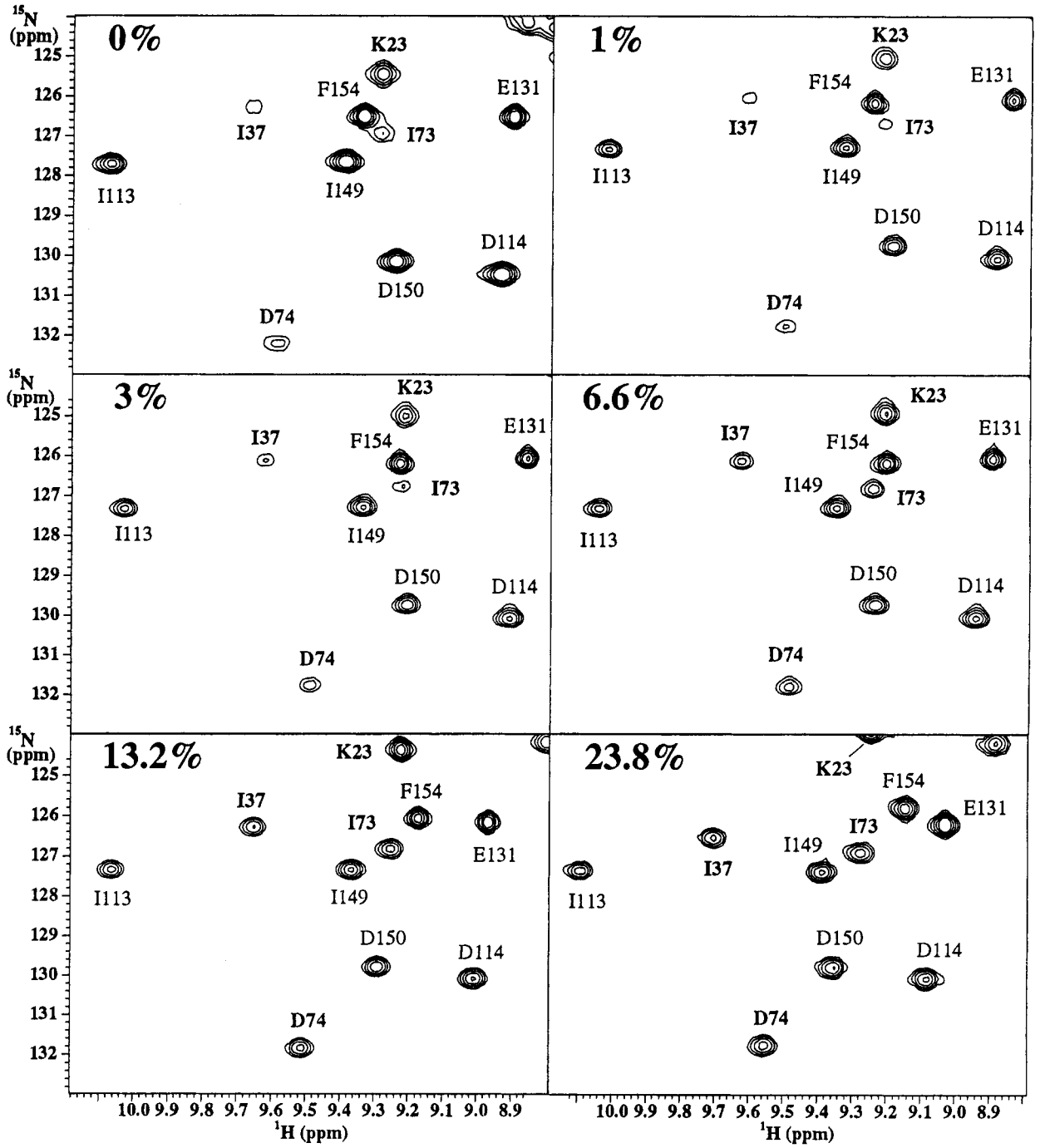


FIGURE 3: Effect of TFE on the intensity of cross-peaks in a series of 600-MHz 2D ^{1}H−^{15}N HSQC NMR spectra of uniformly ^{15}N-labeled TnC at a temperature of 40 °C. Shown is the portion of the HSQC spectrum corresponding to downfield-shifted residues of TnC. Cross peaks labeled in boldface type represent resonances from the N-domain, while those in lightface type correspond to resonances from the C-domain [for assignments, see Slupsky et al. (1995)].

Figure 3 represents an NMR-monitored titration of TnC with TFE. Shown are amide correlations in the downfield region of 2D ^{1}H−^{15}N HSQC NMR spectra. Again, one can see in the panel for 0% TFE the differential intensities for the cross-peaks for the residues from the N- and C-terminal domains. I37, I73, and D74 are barely visible, and K23 is weaker in intensity at 0% TFE. As the amount of TFE is increased, the intensities of cross-peaks arising from the N-domain (K23, I37, D74 and I73) increase, whereas the intensities of cross-peaks arising from the C-domain (I113, D114, E131, I149, D150, and F154) remain virtually constant. These differences may be seen more quantitatively in Figure 4, where residues in homologous positions in the N- and C-domains are compared: I37 in the β-sheet portion of calcium binding site I and I113 in the β-sheet portion of calcium binding site III. The intensity and ^{1}H linewidth of I113 remains constant throughout the TFE titration, whereas the intensity and ^{1}H linewidth of I37 increase or decrease, respectively to levels comparable to those of I113. This plot shows that the minimum concentration of TFE that could be used, so that both N- and C-domain cross-peaks were similar in intensity and linewidth, was 15%, v/v, at 40 °C. The maximum attainable concentration of TnC at 15%, v/v, TFE was 1.4 mM.

Measurement of ^{15}N or ^{13}C relaxation rates is useful for obtaining dynamic information since the relaxation of these nuclei is governed predominantly by the dipolar interaction with directly bound protons and to a much smaller extent

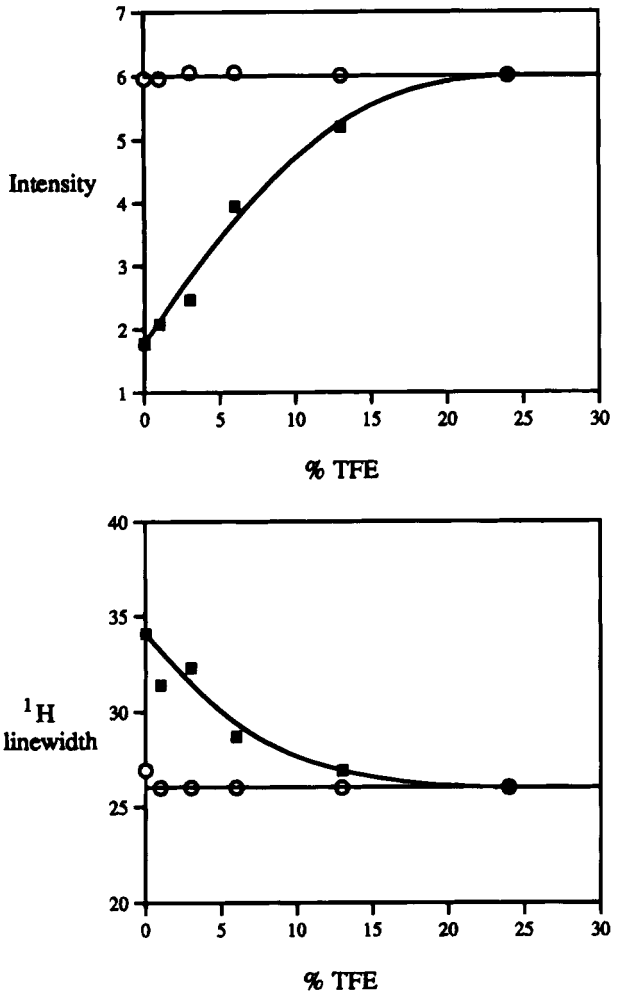


FIGURE 4: (a) Effect of TFE on the intensity of ^{1}H−^{15}N HSQC cross-peaks from the N-terminal and C-terminal domains. Shown are the cross-peaks from I37 (■), representative of the N-domain, and I113 (○), representative of the C-domain. Residue I37 is the N-domain calcium binding site I and is part of the β-sheet structure of the N-domain, and I113 has the same position in the C-domain in calcium binding site III. (b) Effect of TFE on the ^{1}H linewidth of peaks from residues in the N-terminal and C-terminal domains. Shown are the cross-peaks from I37 (■), representative of the N-domain, and I113 (○), which is representative of the C-domain.

by the chemical shift anisotropy mechanism (Allerhand et al., 1971), and thus these measurements provide a direct measure of mobility. Figure 5 shows ^{15}N T_2 relaxation studies on TnC in the presence of TFE. The average T_2 value for the N-domain is 83 ± 13 ms (residues 5−85), whereas the average T_2 value for the C-domain is 103 ± 18 ms (residues 95−158). The residues with the highest mobility are located at the C-terminus. The N-terminus exchanges too rapidly to observe, but most likely has mobility similar to the C-terminus. Residues K107 and K143 (residues in calcium binding sites III and IV of the C-domain) also appear quite flexible. Of particular importance are the residues in the linker region between the two domains (86−88) which are highly flexible and allow the two domains to rotate freely about these residues. These data account for the differences in linewidths between the two domains and explain the differential broadening of residues in the N-domain upon dimerization.

To determine the effects of TFE on the structure of TnC, two spectroscopic methods were employed: NMR and CD. One way to determine the secondary structure of a molecule is by the use of chemical shifts (Wishart et al., 1991a,b; Wishart & Sykes, 1994a). In particular, the consensus of ^{13}Cα, CαH, and ^{13}CO chemical shift indices predicts to greater than 90% efficiency the secondary structure of a protein, which is at least as good as having four interresidue NOEs per residue in the protein (Wishart & Sykes, 1994a). Figure 6 illustrates the chemical shift differences of ^{15}N, NH, CαH, ^{13}Cα, and ^{13}CO resonances between TnC in the presence and absence of TFE. In general, the chemical shift changes are small. As far as changes to secondary structure (in terms of the CαH, ^{13}Cα, and ^{13}CO resonances), V65, E76, E88, D89, and A90 incur the biggest changes. These changes, however, do not alter the secondary structure of these residues as determined by the chemical shift index method (Wishart & Sykes, 1994a). Of all of the chemical shifts, the greatest changes occur with residues valine 45 and methionine 48 in helix B; leucine 49 and glycine 50 in the B−C linker; glutamic acid 64 and valine 65 in helix C; glutamic acid 76 in helix D; and glutamine 85, glutamic acid 88, aspartic acid 89, alanine 90, lysine 91, and glycine 92 in the linker between the N- and C-domains). Smaller changes occur for residues threonine 4, alanine 8, and phenylalanine 13, all in the N-helix; alanine 25 in helix A; glutamic acid 57 in helix C; phenylalanine 105 in helix E; aspartic acid

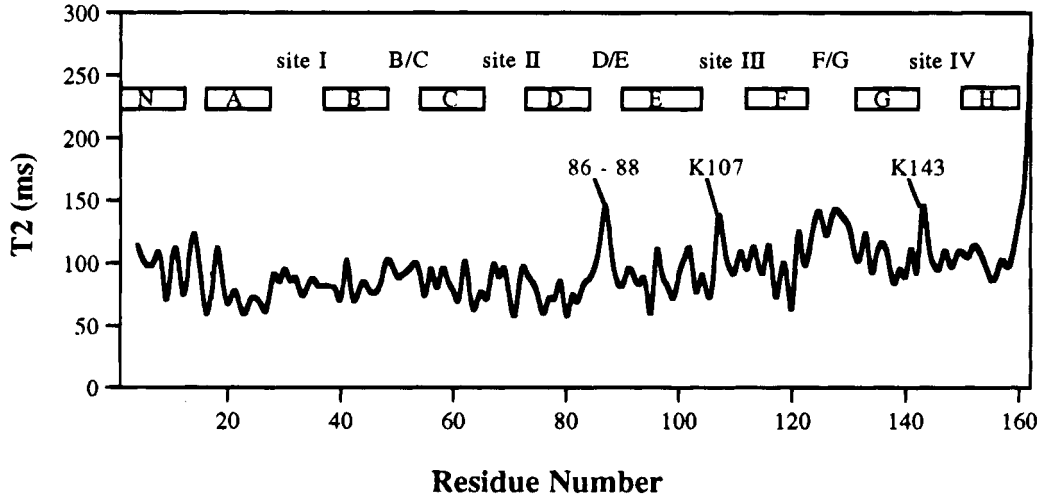


FIGURE 5: ^{15}N T_2 relaxation time for TnC in the presence of 15% TFE versus residue number. Relaxation times not shown are for residues A1 to M3, D5, Q6, L49, P53, and H128. For overlapping resonances, it was assumed that the ^{15}N T_2 relaxation time was the same for all. The helices of TnC are indicated by boxes. The calcium binding sites and linker regions are also indicated.

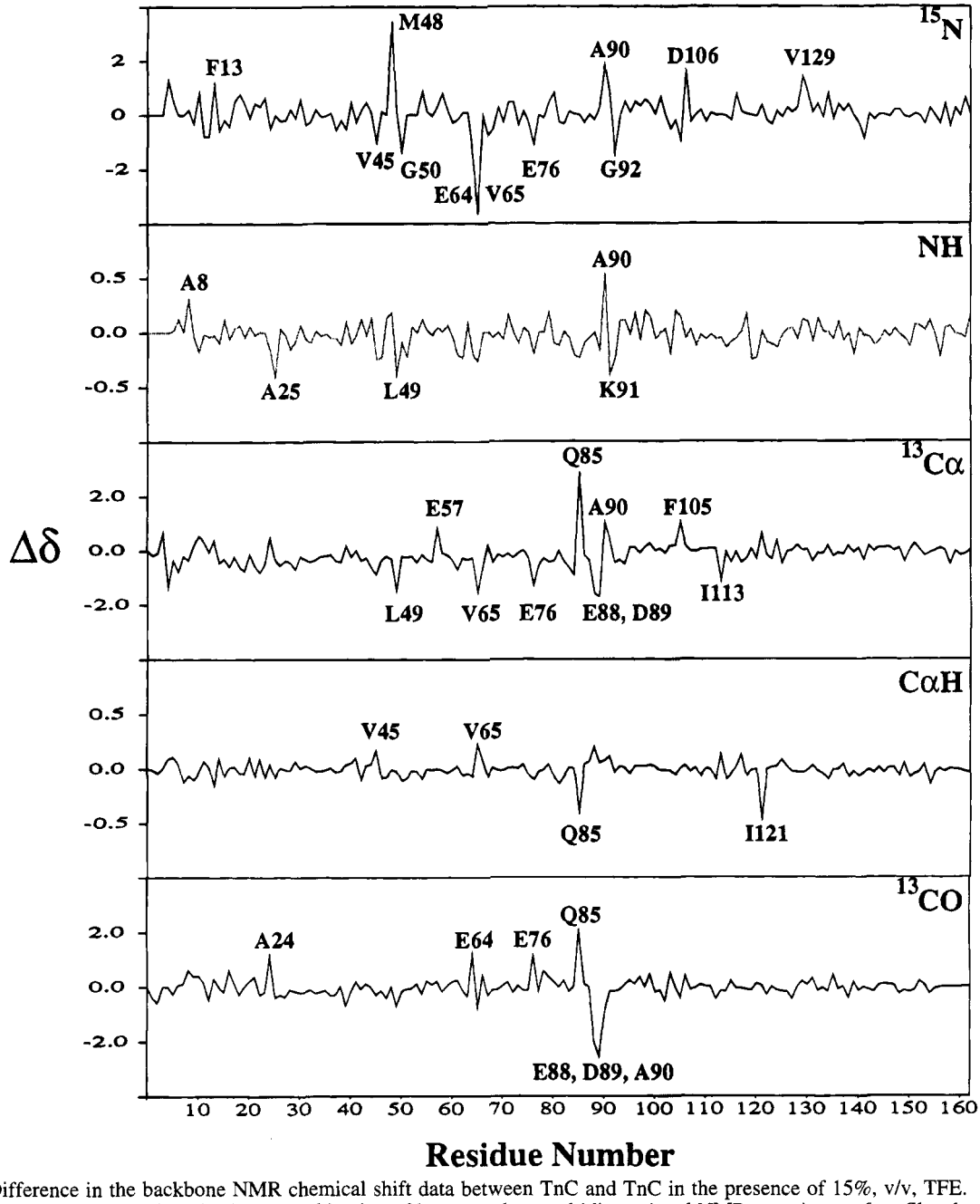


FIGURE 6: Difference in the backbone NMR chemical shift data between TnC and TnC in the presence of 15%, v/v, TFE. The chemical shifts for TnC in TFE were assigned using a combination of heteronuclear multidimensional NMR experiments [see Slupsky et al. (1995)]. Chemical shifts for the TnC dimer were obtained in the same manner as for TnC in TFE, except where discrepancies in the shift data were obtained (such as where the resonances were too broad or had too short a T_2 relaxation time), shifts were obtained from the isolated N-domain (Gagné et al., 1995) or the isolated C-domain (Larry Calhoun, personal communication).

106 and isoleucine 113 in calcium binding site III; isoleucine 121 in helix F; and valine 129 in the linker between the F and G helices. These smaller changes, which generally affect only one chemical shift value, are most likely due to small changes in the hydrogen-bonding character of the residue induced by TFE, or very small changes in the hydrophobic pocket due to the presence of TFE. The largest changes occur in the region at the end of helix B and the end of helix C, as well as in the linker between the two domains. The changes in helices B and C are probably due to the breakup of the dimer (since they are very, very broad resonances in the absence of TFE, especially residues around valine 65). The chemical shift change of residues in the linker region between the two domains of TnC could be due to some stabilization of the secondary structure in this region induced by TFE, but may also be due to the breakup of the dimer.

Figure 7A represents the far-UV CD spectrum of TnC apo, calcium-saturated, and calcium-saturated in the presence of TFE. The apo spectrum has a molar ellipticity of approximately $-11\,300 \pm 500$ deg cm^2 dmol^{-1} at 220 nm. This value is similar to that obtained for recombinant TnC (Golosinska et al., 1991). When calcium is added to apo TnC, the negative molar ellipticity increases to approximately $-16\,400 \pm 500$ deg cm^2 dmol^{-1} at 220 nm. This value is slightly lower by approximately 1000° than previously reported (Golosinska et al., 1991). The addition of TFE to calcium-saturated TnC produces a further increase in negative molar ellipticity of approximately 1400° at 220 nm. This change could be due to a small increase in secondary

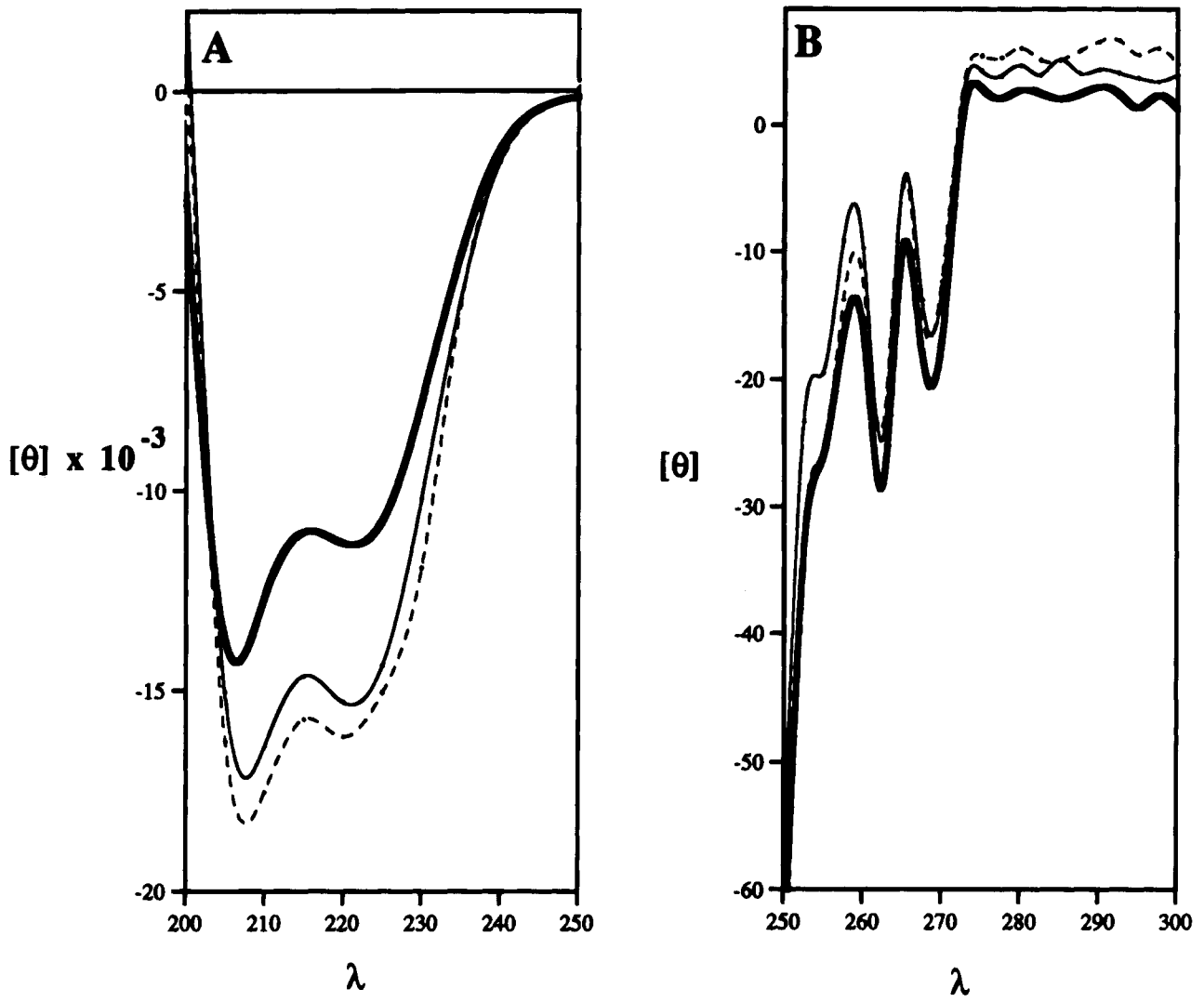


FIGURE 7: (A) Far-UV CD spectra of apo TnC (dark solid line), calcium-saturated TnC (light solid line), and calcium-saturated TnC in the presence of 15% TFE (dashed line). Experimental conditions were as described in Materials and Methods. (B) Near-UV CD spectra of apo TnC (dark solid line), calcium saturated TnC (light solid line), and calcium saturated TnC in the presence of 15% TFE (dashed line). Experimental conditions were as described in Materials and Methods.

structure, or due to the effects of tertiary or quaternary structural changes such as a slight rearrangement of helices (or breakup of the dimer) (Manning, 1989).

Figure 7B illustrates the near-UV CD spectrum of TnC apo, calcium-saturated, and calcium-saturated in the presence of TFE. The spectra in the presence and absence of calcium or TFE show minima in ellipticity in the region of 250–270 nm associated with the major absorption bands of phenylalanine. The addition of calcium to TnC results in minor changes to the spectrum due to burying of the phenylalanine residues to the C-terminal domain hydrophobic pocket, as well as in the hydrophobic dimer interface. Addition of TFE to calcium-saturated TnC produced minor changes most likely attributable to the breakup of the dimer.

DISCUSSION

$^{1}H-^{15}N$ HSQC NMR spectra of calcium-saturated TnC at various temperatures gave the first clue that the mode of dimerization was via the N-domain as evidenced by differential line broadening of resonances from the N-domain at lower temperatures. To determine the structure of calcium-saturated TnC therefore required that the monomeric form be obtained either by raising the temperature or by other intervention. The results shown here illustrate that, at temperatures of 50 °C and higher, some of the TnC structure was disrupted as some of the amide residues lost intensity at higher temperatures. Simply increasing the temperature was therefore not an option, and it became necessary to find a solvent which would break apart the dimer without adversely affecting the tertiary structure of the monomer. We chose TFE for its known characteristic as a denaturant of quaternary structure (Lau et al., 1984a,b) but keeping in mind that it could adversely affect the structure of the monomer.

A titration of TnC with TFE showed an increase in intensity with a concomitant decrease in linewidth of the N-domain NMR resonances, while the C-domain NMR resonances were largely unaffected by the addition of TFE. Line widths in NMR spectra are directly proportional to the rotational correlation time, which in turn is directly proportional to the molecular weight. It may therefore be concluded that the molecular weight of the N-domain increased to a much greater extent than the molecular weight of the C-domain upon binding of calcium (and therefore dimerization of TnC as demonstrated by ultracentrifugation studies). This also suggests that there must be some sort of flexibility between the domains since the linewidths and

intensities are so different for both domains. The ^{15}N T_2 relaxation data supports the idea of a flexible linker between the two domains.

TnC is homologous in sequence and structure to another calcium binding protein, calmodulin. The solution structure of calmodulin illustrates the central region to be like a flexible tether between the two domains (Ikura et al., 1992). The ^{15}N NMR T_2 values for calmodulin were determined, and it was found that there was a difference in correlation times for both domains and there was an increase in T_2 for six residues in the linker region between the two domains (Barbato et al., 1992). Our results, however, show an increase in the T_2 relaxation time for only three residues (M86-K87-E88), and these residues do not show the same increase in T_2 relaxation time as the residues in calmodulin. This implies that the linker is less flexible in TnC than in calmodulin. This result is also supported by the work of Wang et al. (1993), who used fluorescence anisotropy decay measurements, and the work of Gulati et al. (1993), who studied mutants of the central helix.

To study the effects of TFE on secondary and tertiary structure, two types of spectroscopy (CD and NMR) were utilized. Far-UV CD spectroscopy revealed only a small change in negative ellipticity when TFE was added to calcium-saturated TnC. This change is most likely attributable to a breakup of the dimer interface since this region of the CD spectrum is sensitive not only to secondary structure but to interactions amongst secondary structure elements (Manning, 1989). Near-UV CD also revealed very little change in the hydrophobic phenylalanine region when TFE was added to calcium-saturated TnC. The change observed could be attributed to an exposure of the hydrophobic pocket of the N-domain which contains the phenylalanine residues.

The chemical shifts of the backbone resonances are largely unaffected by the addition of TFE, as was shown in Figure 6. The ^{15}N and NH chemical shifts experience some of the greatest changes; however, chemical shift changes of these nuclei are difficult to rationalize in terms of secondary structure due to helix dipole effects and amide chemical shift periodicities (Wishart & Sykes, 1994b). The chemical shift changes of the $^{13}C\alpha$, CαH, and ^{13}CO resonances are, however, very good indicators of secondary structure. The shift downfield from random coil chemical shifts (positive deviations) of CαH resonances and the shift upfield from random coil chemical shifts (negative deviations) of $^{13}C\alpha$ and ^{13}CO signify the production of helical structure. The upfield shift of CαH and the downfield shift of $^{13}C\alpha$ and ^{13}CO resonances signify the production of β-sheet structure. Although there are chemical shift deviations when TFE is added to TnC, these deviations do not affect the secondary structure, as the chemical shift index indicates the same secondary structure in the presence and absence of TFE (except for glutamine 85). A glance at Figure 6 reveals that changes in chemical shift for all nuclei studied seem to be localized in the same regions of the molecule: Helix B, helix C, and helix D/E which links the N and C domains together. The region of TnC linking the two domains together is largely exposed to solvent according to the crystal structure of half-saturated TnC, and thus when TFE is added, this region may have strengthened hydrogen bonds. ^{15}N T_2 relaxation data indicate that residues 86−88 are mobile in the presence of TFE, and there is a difference in rotational correlation times of the two domains signifying that the effect of TFE in this region does not provide an uncharacteristically stable structure. The chemical shift changes in this region could therefore be due to the breakup of the dimer as has been suggested for the changes in chemical shift of residues in helices B and C. The side-chain chemical shifts (data not shown) also experience minimal change with the addition of TFE indicating minimal, if any, changes to the tertiary structure of TnC.

A model for the calcium-induced dimer based upon low-angle X-ray scattering data has been presented (Blechner et al., 1992) which suggests that the N- and C-domains both interact in a way similar to that seen in the crystal packing of TnC. The authors used Gly-92 in the interconnecting helix as a point of flexibility, allowing the N- and C-domains to rotate about this residue, and tested random structures which would approximate the X-ray scattering data. The final model generated is not consistent with the results reported herein, as the C-terminal domain of each monomer is not flexible. Further, glycine 92 has not been shown to be flexible according to the T_2 data. The present study indicates that dimerization involves the N-domains from two monomers, and that the C-domains have minimal, if any, interaction with each other or the N-domain. Also, the interface for the dimer may involve helices B, C, and possibly D/E, and residues M86 to E88 are flexible enough to cause the two domains to have different rotational correlation times.

The use of TFE, in this case, does not appear to alter the secondary or tertiary structure of TnC to any significant extent, due most likely to the fact that TnC has already formed its structure in the absence of TFE. This is a similar result to the one obtained for hen egg white lysozyme (Buck et al., 1993). Indeed, TFE has been shown to induce significant structure in peptides, or to alter the structure of peptides when compared to the aqueous solvent. We show here that the primary effect of TFE in small concentrations on proteins which have stable secondary and tertiary structures is to break up to the quaternary structure, as was also shown for coiled coil peptides (Lau et al., 1984a,b). Structure calculations of TnC in the presence of TFE are thus relevant, as the change in secondary or tertiary structure of TnC is minimal at the low concentration of TFE used in this study. Any denaturing agent, however, should be used with caution. A complete study of the differences in secondary and tertiary structure should be done (in terms of CD and chemical shift information). The structure of TnC in TFE should provide an insight into the hydrophobic faces of TnC presented to other components of the thin filament as calcium is added, and hopefully give an indication as to how the domains relate to one another.

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