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# Troponin I Encompasses an Extended Troponin C in the $\text{Ca}^{2+}$ -Bound Complex: A Small-Angle X-ray and Neutron Scattering Study<sup>†</sup>

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**ABSTRACT:** We have studied the solution structure of skeletal muscle troponin C complexed with troponin I in the presence of calcium using small-angle X-ray and neutron scattering.  $4\text{Ca}^{2+}$ -troponin C in the complex has an extended dumbbell shape with a radius of gyration of  $23.9 \pm 0.5 \text{ \AA}$  and a maximum linear dimension of  $\approx 72 \text{ \AA}$ , similar to the values obtained from the crystal structure coordinates of troponin C (Herzberg & James, 1985). Troponin I is even more extended than troponin C with a radius of gyration of  $41 \pm 2 \text{ \AA}$  and a maximum linear dimension of  $\approx 118 \text{ \AA}$ . The centers-of-mass for each component of the complex are approximately coincident ( $<10\text{-\AA}$  separation) as are their long axes, and the troponin I component encompasses the  $4\text{Ca}^{2+}$ -troponin C. These data provide new insights into the nature of the conformational arrangement of this important  $\text{Ca}^{2+}$ -sensitive molecular switch.

In the sliding-filament model of muscle action, thin and thick filaments move past each other, causing contraction or relaxation. The contractile force is believed to be generated by the cyclic attachment and detachment of the myosin heads of the thick filaments to sites on the thin filaments [for reviews, see Leavis and Gergely (1984) and Zot and Potter (1987)]. The thin filaments are composed of a double-stranded helical assembly of actin monomers. Tropomyosin is polymerized head-to-tail in the grooves of the thin filament actin helix (one tropomyosin to every seven actin monomers), and each tropomyosin has one troponin complex bound to it. The troponin complex has three subunits: troponin C (TnC)<sup>1</sup> which binds  $\text{Ca}^{2+}$ ; troponin I (TnI); and troponin T (TnT). The regulation of the contraction/relaxation cycle is associated with an increase in calcium concentration and is mediated through the TnC component of the troponin complex. When TnC binds  $4\text{Ca}^{2+}$ , a signal is transmitted via TnI which releases its inhibition of the actin/myosin interaction via TnT and tropomyosin. Thus, the interactions of the thick and thin filaments are modulated to give rise to the sliding mechanism.

Low-resolution X-ray crystallographic and electron micrograph data of the troponin complex bound to tropomyosin

show it to have an elongated shape with TnC and TnI forming a globular domain while TnT forms a long ( $\approx 160 \text{ \AA}$ ) tail which interacts with tropomyosin (Flicker *et al.*, 1982; White *et al.*, 1987). The only high-resolution structural data on the components are for TnC. The crystal structures of TnC (Herzberg & James, 1985; Sundaralingam *et al.*, 1985) show this component to have an unusual dumbbell shape similar to the evolutionarily related calcium binding protein calmodulin (Babu *et al.*, 1985). These dumbbell structures consist of two globular domains connected by a solvent-exposed helix of approximately 7–8 turns. The C-terminal domain of TnC contains two high-affinity  $\text{Ca}^{2+}/\text{Mg}^{2+}$  binding sites, believed to be always occupied in muscle, while the N-terminal domain contains two lower-affinity  $\text{Ca}^{2+}$ -specific binding sites. Both the high- and low-affinity binding domains have the helix-loop-helix secondary structure known as an EF-hand [Kretsinger, 1980; review by Wylie and Vanaman (1988)]. It is  $\text{Ca}^{2+}$  binding to the N-terminal domain that regulates the contractile event via a conformational change probably involving the opening of that "cup-shaped" domain, exposing hydrophobic residues on the inner surface of the cup (Herzberg *et al.*, 1986).

We have completed small-angle X-ray and neutron scattering experiments on  $4\text{Ca}^{2+}$ -TnC complexed with TnI in order to determine structural information such as the radius of gyration ( $R_g$ ), the pairwise distribution function [ $P(r)$ ], and the maximum linear dimension ( $d_{\text{max}}$ ) for the overall complex and its components. The intensity of the scattering from a particle in solution depends upon its "contrast", i.e., the difference in scattering density between the particle and the solvent. X-rays are scattered by electrons, and the mean electron densities for most proteins are very similar. X-ray scattering thus gives information on the overall  $4\text{Ca}^{2+}$ -TnC-TnI complex. Neutrons are scattered by atomic nuclei, and hence isotopes of the same element can scatter neutrons quite differently. One of the largest differences is for hydrogen and deuterium. Thus, selective deuteration of one component in a complex, in this study TnC, provides a way of altering the neutron scattering density of that component. Further, by changing the deuterium level in the solvent, the neutron scattering contrast of each component is varied. If internal scattering density fluctuations are negligible, then "solvent

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<sup>1</sup> Abbreviations:  $A_{600}$ , absorbance at 600 nm; CaM, calmodulin;  $D$ , centers-of-mass separation;  $d_{\text{max}}$ , maximum linear dimension;  $R_g$ , radius of gyration;  $R_c$ , radius of gyration of cross section; TnC, troponin C; TnC<sub>78% d</sub>, 78% deuterated troponin C; TnI, troponin I.

matching" can be achieved by adjusting the deuteration level in the solvent so that the mean solvent and particle scattering densities are the same; i.e., there is zero contrast and hence no small-angle scattering from the particle rendering it "invisible" in the neutron experiment. "Contrast variation" techniques thus provide methods for extracting structural information on the individual components as well as their relative dispositions (Ibel *et al.*, 1975; Moore, 1981).

## MATERIALS AND METHODS

**Sample Preparation.** The neutron scattering experiment involved measuring a "contrast series" in which a complex of deuterated  $4\text{Ca}^{2+}\cdot\text{TnC}$  and nondeuterated TnI is solubilized in solvents with different  $\text{D}_2\text{O}:\text{H}_2\text{O}$  ratios. Partial deuteration of the TnC component was chosen in order to have the solvent match point for the overall complex between 65 and 72%  $\text{D}_2\text{O}$ , thus allowing neutron scattering measurements on either side of the solvent match point.

Partially deuterated TnC was produced using a bacterial expression system obtained from S. Hitchcock-DeGregori (University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School). The TnC gene is in a pET 3d vector in *Escherichia coli* BL21(DE53)plysS. The cells were grown in 75–80%  $\text{D}_2\text{O}$  using M9 salts with an organic supplement containing deuterated and nondeuterated components. The deuterated organic components came from Merck deuterated whole algal hydrolysate, reported by Merck to be 60–65% amino acids, 10–15% sugars, and 30–35% salts. The amino acid composition of the hydrolysate was determined by amino acid analysis. The hydrolysate was diluted with nondeuterated amino acids and glucose to give a 3:1 ratio of deuterated to nondeuterated amino acids and sugars. The final organic supplement was 10 g/L. The media also contained 20 mg/L ampicillin and 25 mg/L chloramphenicol. Cells were grown at 37 °C to an  $A_{600}$  of 0.4 in a BioFlo II fermentor (New Brunswick Scientific). TnC expression was induced by addition of IPTG to a concentration of 0.4 mM. Growth was monitored at  $A_{600}$  until it leveled off, usually at about  $A_{600} = 2.4$  after 3–4 h. Cells were harvested by centrifugation in a Beckman J21C with a J-14 rotor for 15 min at 12 000 rpm, and the pellet was frozen overnight at –80 °C.

TnC was purified according to the method of Xu and Hitchcock-DeGregori (1988) with several modifications. The ammonium sulfate fractionation they describe was always carried out, followed by the phenyl-Sepharose column and HPLC separation steps. The phenyl-Sepharose fractions were combined and dialyzed against 0.001 M  $\text{NH}_4\text{HCO}_3$  to remove salts, and the dialysate was lyophilized. The lyophilized powder was resuspended in 20 mM sodium phosphate buffer, pH 6.5, 0.1 mM  $\text{CaCl}_2$ , and applied to a Pharmacia ProRPC HR 5/10 reverse-phase HPLC column. After the column was washed with the same buffer, a linear gradient was applied: 20 mM sodium phosphate, pH 6.5, 0.1 mM  $\text{CaCl}_2$  going to 50% of 20 mM sodium phosphate with 50% acetonitrile, in 50 min and 0.3 mL/min. The TnC fractions were lyophilized to remove the acetonitrile, taken up in minimal water, dialyzed against 0.1 mM  $\text{NH}_4\text{NCO}_3$  to remove salts, and lyophilized. The purity of the final product (typically >99%) was verified via SDS gel electrophoresis.

NMR spectroscopy was used to determine the degree of deuteration of the TnC by measuring the amino acid proton signal in an ultrapure  $\text{D}_2\text{O}$  (99.996% D) phosphate buffer. The hydrogens of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$  were exchanged with deuterium by repeated dissolution in  $\text{D}_2\text{O}$  and lyophilization. A stock buffer solution was prepared by mixing

$\text{Na}_2\text{DPO}_4$  and  $\text{NaD}_2\text{PO}_4$  at a ratio to give a pH reading of 6.52 and 50 mM phosphate. Approximately 400  $\mu\text{L}$  of this stock solution was used to dissolve TnC which was added as a dry powder. The deuteration level was determined by comparing the integrated proton signal amplitudes of the partially deuterated TnC sample with that of a nondeuterated TnC sample, both of known protein concentrations ( $\approx 3.5$  mM). The deuteration level for the TnC was determined to be  $78 \pm 1\%$  for the nonexchangeable hydrogens. By comparison with a previous neutron scattering study of calmodulin (Trehwella *et al.*, 1990) and analysis of the present neutron scattering study, it was determined that approximately 45% of the exchangeable hydrogens readily exchange and it was assumed that this H/D exchange was linear with the deuterium content of the solvent.

Nondeuterated TnI was prepared by the method of Wilkinson and Grand (1975, 1978). Similar to TnC, it is assumed that 45% of the exchangeable hydrogens exchanged linearly with the deuterium content of the solvent.

$\text{TnC}_{78\text{d}}$  and TnI were completed by dissolving the appropriate quantity of each protein (in a 1:1 stoichiometry) in 0.5 mL of a 6 M urea solution. This solution was dialyzed against buffer A with 2 or 3 M urea (buffer A: 50 mM MES, 2 mM  $\text{CaCl}_2$ , 1 mM DTT, and 100 mM KCl, pH 6.1). The calcium was in  $\approx 20$  times stoichiometric excess of the TnC. Dialysis was repeated 4 times against buffer A with 0%, 40%, or 100%  $\text{D}_2\text{O}$ . The last dialysate buffer was saved and used for solvent scattering measurements. The 20%  $\text{D}_2\text{O}$  sample was made by mixing the appropriate amount of the 0% and 40%  $\text{D}_2\text{O}$  samples. Similarly, the 90%  $\text{D}_2\text{O}$  was prepared from the 0% and 100%  $\text{D}_2\text{O}$  samples. pH values measured for samples in 100%  $\text{D}_2\text{O}$  were adjusted by subtracting 0.4 pH unit. Initially, samples prepared in  $\text{D}_2\text{O}$  buffer without urea showed  $\text{D}_2\text{O}$ -induced aggregation of the complex. This aggregation was eliminated by working with low protein concentrations (1–4 mg/mL) in buffer A with 2 or 3 M urea. It is known that TnC complexed with TnI forms a stable complex under saturating  $\text{Ca}^{2+}$  conditions even in the presence of 6 M urea (Grabarek *et al.*, 1981). In the buffer composition used, the solvent match points were calculated from the chemical compositions of the components as 42%  $\text{D}_2\text{O}$  for TnI and 101%  $\text{D}_2\text{O}$  for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\text{d}}$ . The solvent match point for the overall complex was calculated as 71%  $\text{D}_2\text{O}$ .

**Scattering Data Acquisition and Reduction.** All scattering data were reduced to  $I(Q)$  versus  $Q$ , where  $I(Q)$  is the scattered neutron or X-ray intensity and  $Q$  is the amplitude of the scattering vector.  $Q$  is equal to  $4\pi \sin \theta/\lambda$ , where  $2\theta$  is the scattering angle and  $\lambda$  is the wavelength of the incident and scattered neutrons or X-rays.

Small-angle neutron scattering (SANS) measurements were done using the low- $Q$ -diffractometer (LQD) at the Manuel Lujan Jr. Scattering Center (LANSCE) (Seeger *et al.*, 1990) and the 30 m SANS instrument (NG3) at the National Institute of Standards and Technology (NIST). The neutrons at LANSCE are produced by a spallation source which is pulsed, and hence a range of wavelengths can be used with time-of-flight methods, allowing measurement of a large  $Q$  range with a single detector position. The  $Q$  range measured using LQD was 0.005–0.35  $\text{\AA}^{-1}$ .  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\text{d}}\cdot\text{TnI}$  samples (2–4 mg/mL) in buffer A with 2 M urea in 0%, 40%, and 100%  $\text{D}_2\text{O}$  and a  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\text{d}}\cdot\text{TnI}$  (1.5–3.0 mg/mL) sample in buffer A with 3 M urea in 100%  $\text{D}_2\text{O}$  were measured. The 30 m SANS instrument at NIST uses neutrons produced by a 20 MW reactor from which we selected neutrons with a mean wavelength of 5.0  $\text{\AA}$  and a full width and half-maximum of 34%. Sample-to-detector distances of 7 and 1.5 m were

used in order to cover the appropriate  $Q$  range (0.01–0.08 and 0.04–0.3 Å<sup>-1</sup>, respectively). 4Ca<sup>2+</sup>·TnC<sub>78%<sup>d</sup></sub>·TnI samples in buffer A with 2 M urea in 0%, 20%, and 40% D<sub>2</sub>O (2–4 mg/mL) and 4Ca<sup>2+</sup>·TnC<sub>78%<sup>d</sup></sub>·TnI samples in buffer A with 3 M urea in 90% and 100% D<sub>2</sub>O (1.5–2.5 mg/mL) were measured. With 2 or 3 M urea present, the solvent density increased a few percent (2–6%), which was taken into account in the data reduction and analysis. Quartz cuvettes with 1-mm path lengths were used for the 0%, 20%, and 40% D<sub>2</sub>O samples. Since the neutron transmissions for the 90% and 100% D<sub>2</sub>O samples were higher due to their lower hydrogen content, 2- and 3-mm path-length quartz cuvettes were used for these samples, respectively. Measurements at both neutron scattering facilities were reduced by standard techniques used for buffer subtraction and detector sensitivity corrections (Chen & Bendedouch, 1986; Crawford *et al.*, 1987; Hjelm, 1988; Seeger *et al.*, 1990).

X-ray scattering data were collected using the small-angle X-ray scattering (SAXS) station at Los Alamos which is described elsewhere (Heidorn & Trehella, 1988; Olah *et al.*, 1993). The sample-to-detector distance was 64 cm. Concentration series of the complex in H<sub>2</sub>O for two different sample preparations in buffer A were performed and showed that interparticle interference is negligible over the concentration range used in the neutron scattering experiments (1.5–4 mg/mL). X-ray scattering measurements were also made on each sample used in the neutron scattering study before and after the neutron measurements to check for aggregation and structural integrity.

**Scattering Data Analysis.** The scattering from a homogeneous solution of monodisperse particles can be expressed as

$$I(Q) = \left| \int [\rho(r) - \rho_s] \exp[-i(Q \cdot r)] dr \right|^2 \quad (1)$$

where  $\rho(r)$  and  $\rho_s$  are the scattering density for the protein and solvent, respectively, and the integration is taken over the volume of the protein. If we ignore scattering density fluctuations and treat the 4Ca<sup>2+</sup>·TnC·TnI complex as a two-component system, we can write the scattering as

$$I(Q, \Delta\rho_C, \Delta\rho_I) = \Delta\rho_C^2 I_C(Q) + \Delta\rho_C \Delta\rho_I I_{CI}(Q) + \Delta\rho_I^2 I_I(Q) \quad (2)$$

where the subscripts C and I refer to TnC and TnI.  $\Delta\rho_C$  equals  $\rho_C - \rho_s$  where  $\rho_C$  is the mean scattering density for 4Ca<sup>2+</sup>·TnC. A similar definition holds for  $\Delta\rho_I$ . The three terms in eq 2 correspond to the three basic scattering functions.  $I_C(Q)$  and  $I_I(Q)$  represent the scattering of 4Ca<sup>2+</sup>·TnC and TnI in the complex, respectively, while  $I_{CI}(Q)$  is a cross-term. Each scattering measurement at a given D<sub>2</sub>O:H<sub>2</sub>O ratio of the solvent (total of nine independent measurements at five different D<sub>2</sub>O:H<sub>2</sub>O ratios) gives an equation in the form of eq 2; thus, a multiple linear regression routine was written to solve this overdefined system of equations for the three basic scattering functions. The scattering densities,  $\Delta\rho_C$  and  $\Delta\rho_I$ , are readily calculated from chemical and isotope compositions of the two components of the complex and the solvent. The inverse Fourier transform of  $I(Q)$  gives the vector distribution function,  $P(r)$ , which is the frequency of all interatomic vectors within the scattering particle and hence goes to zero at  $d_{\max}$ . The first and second moments of  $P(r)$  give the zero angle scatter,  $I_0$ , and  $R_g$  of the particle, respectively.

For a two-component system, the square of  $R_g$  can be expressed as

$$R_g^2 = f_C' R_C^2 + f_I' R_I^2 + f_C' f_I' D^2 \quad (3)$$

where

$$f_C' = \Delta\rho_C V_C / (\Delta\rho_C V_C + \Delta\rho_I V_I)$$

$f_I' = 1 - f_C'$ . Equation 3 is the “parallel axis theorem” for a two-component system (Moore, 1981).  $R_C$  and  $R_I$  are the  $R_g$ 's for each component, and  $D$  is the separation of the center-of-mass of the components. The volumes of each component,  $V_C$  and  $V_I$ , can be estimated from their molecular weights assuming a protein specific volume of 0.72 cm<sup>3</sup>/g.  $f_C'$  and  $f_I'$  are also readily calculated from the chemical and isotope composition of each component.  $R_C$ ,  $R_I$ , and  $D$  can be calculated from eq 3 using a multiple linear regression routine.

Ibel and Stuhmann (1975) showed that the  $R_g^2$  dependence on the scattering contrast can be written in another form as

$$R_g^2 = R_m^2 + \alpha / \Delta\bar{\rho} - \beta / \Delta\bar{\rho}^2 \quad (4)$$

where  $R_m$  is the  $R_g$  at infinite contrast and  $\Delta\bar{\rho} \equiv \bar{\rho} - \rho_s$ , where  $\bar{\rho}$  is the mean scattering density for the total particle and  $\rho_s$  is the scattering density of the solvent. The coefficient  $\alpha$  is related to the second moment of the scattering density fluctuations about the mean value for the scattering particle, while  $\beta$  is related to the square of the first moment of the density fluctuations about the mean. For a two-component system,  $R_m$ ,  $\alpha$ , and  $\beta$  can be expressed as

$$R_m^2 = f_C R_C^2 + f_I R_I^2 + f_C f_I D^2 \quad (5a)$$

$$\alpha = (\rho_C - \rho_I) f_C f_I [R_C^2 - R_I^2 + (f_I^2 - f_C^2) D^2] \quad (5b)$$

$$\beta = (\rho_C - \rho_I)^2 f_C^2 f_I^2 D^2 \quad (5c)$$

where

$$f_C = V_C / (V_C + V_I)$$

$$f_I = 1 - f_C$$

$R_m$ ,  $\alpha$ , and  $\beta$  can be determined from eq 4 using a polynomial least-squares fit of  $R_g^2$  versus  $1/\Delta\bar{\rho}$ . If the sign of  $\alpha$  is positive, then the lower scattering density component (in this case the TnI) is located more toward the inside of the complex than the higher scattering density component (the partially deuterated TnC). A negative  $\alpha$  indicates the reverse case.  $\beta$  is proportional to the square of the separation of the two components. If  $\beta$  is zero, then the centers-of-mass are coincident. Once  $R_m$ ,  $\alpha$ , and  $\beta$  are determined, the three linear eqs 5 can be solved for  $R_C$ ,  $R_I$ , and  $D$ .

The measured scattering data are “smeared” as a result of the finite wavelength distribution and beam shape of the incident radiation. To correct for these effects, all intensity curves were individually analyzed using the indirect Fourier transform algorithm described by Moore (1980). This algorithm models the intensity curves,  $I(Q)$ , by calculating the pair-distance, or vector distribution function, for a homogeneous scattering particle,  $P(r)$ . The  $P(r)$  function is determined by setting  $P(r)/r$  to zero at  $r = 0$  and at some maximum dimension,  $d_{\max}$ , and representing it as a sine series. The number of terms in the sine series is limited by  $d_{\max} Q_{\max} / \pi$ , where  $Q_{\max}$  is the maximum  $Q$  of the experiment. The smearing effects due to the wavelength distribution and beam shape can be incorporated into the coefficients of the sine series from which the corresponding unsmeared  $I(Q)$ , used in the analyses described by eqs 1–5, can be calculated using a Fourier transform:

$$I(Q) = 4\pi \int P(r) \sin(Q \cdot r) / (Q \cdot r) dr \quad (6)$$

## RESULTS

X-ray scattering data for 4Ca<sup>2+</sup>·TnC<sub>78%<sup>d</sup></sub>·TnI are shown in Figure 1, with the corresponding  $P(r)$  function in Figure 4(top).

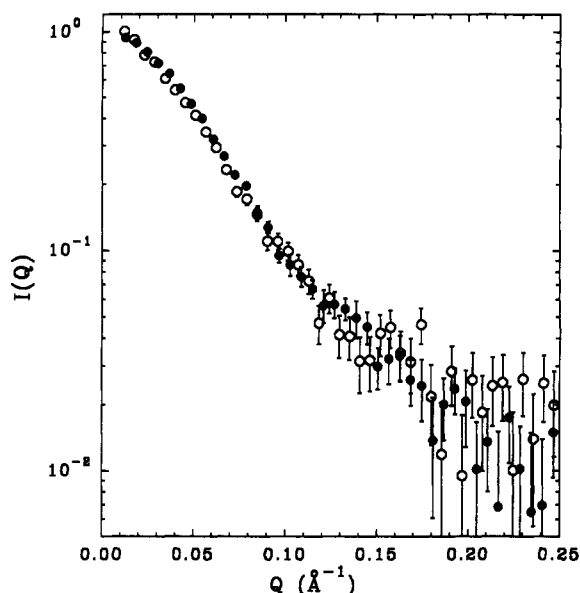


FIGURE 1: Comparison of X-ray scattering data from the  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}\cdot\text{TnI}$  complex,  $\approx 3$  mg/mL protein, in buffer A (O) with  $\text{H}_2\text{O}$  and 0 M urea and (●) in buffer A with  $\text{D}_2\text{O}$  and 3 M urea.

Since TnC and TnI have approximately equal X-ray scattering densities, the scattering curve and  $P(r)$  profile correspond to those for the overall complex. The  $P(r)$  function is asymmetric, showing a broad peak at  $\approx 38$  Å with a tail extending to  $\approx 115$  Å, indicating the complex itself is asymmetric. Indeed, Guinier analysis for “rod”-shaped particles [reviewed in Glatter (1982)] shows the complex to be elongated with an average radius of cross section,  $R_c$ , of  $16.2 \pm 1.5$  Å (Figure 3, bottom, and Table 2).

$4\text{Ca}^{2+}\cdot\text{TnC}\cdot\text{TnI}$  in  $\text{H}_2\text{O}$  is monodisperse for concentrations less than 5 mg/mL. This was determined by measuring the forward scatter,  $I_0$ , which is directly proportional to the molecular weight of the scattering species and comparing with a standard protein (lysozyme) known to be monodisperse (Kringbaum & Kugler, 1970). Initial attempts to measure scattering data from samples in  $\text{D}_2\text{O}$ , however, showed the complex to have a tendency to aggregate in solutions with  $>20\%$   $\text{D}_2\text{O}$ .  $\text{D}_2\text{O}$ -induced aggregation often arises from hydrophobic interactions which can sometimes be disrupted by adding mild denaturants. We therefore added urea or guanidine thiocyanate with concentrations between 0.5 and 3 M. We also tried using 10–40% (v/v) glycerol, as well as varying the complex concentration from 1.5 to 7 mg/mL. X-ray scattering data measured for samples under these various conditions were compared with the scattering data for monodisperse samples in  $\text{H}_2\text{O}$  in order to evaluate aggregation. 2–3 M urea proved the most effective agent for eliminating aggregation without affecting the structure of the complex as determined from the scattering data. A comparison of the X-ray scattering profiles for the complex in buffer A using  $\text{H}_2\text{O}$  with no urea and using  $\text{D}_2\text{O}$  with 3 M urea is given in Figure 1. The profiles are identical in the  $Q$  range used for analysis ( $0.015$ – $0.25$  Å $^{-1}$ ). Samples in buffer A with 2 M urea also gave the same profile over this  $Q$  range. The difference between the mean scattering solvent density for samples in buffer A with 2 or 3 M urea was negligible. Therefore, X-ray scattering data taken for samples with 2 and 3 M urea, a total of nine separate measurements, were combined to give a final X-ray scattering profile with improved statistics for the structural analysis.

The neutron scattering profiles for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}\cdot\text{TnI}$  samples measured at NIST are given in Figure 2. The data are not normalized with respect to concentration or path length

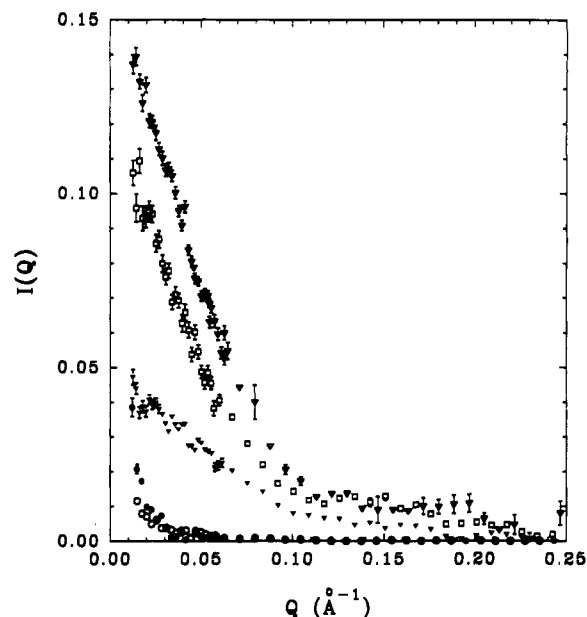


FIGURE 2: Neutron scattering data for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}\cdot\text{TnI}$  collected on beamline NG3 at NIST with (▽) 0%, (□) 20%, (▼) 40%, (●) 90%, and (○) 100%  $\text{D}_2\text{O}$ . The 0%, 20%, and 40%  $\text{D}_2\text{O}$  samples contained 2 M urea, and the 90% and 100%  $\text{D}_2\text{O}$  samples contained 3 M urea. Data are on the same arbitrary normalization scale, but not corrected for concentration or path-length differences.

Table 1:  $R_g$  Values from  $P(r)$  Analysis of Neutron and X-ray Scattering Data

	% $\text{D}_2\text{O}$	[urea] (M)	$R_g$ (Å)
neutron (NIST)			
JAN93	0	2	$27.7 \pm 0.5$
JAN93	20	2	$27.1 \pm 0.5$
JAN93	40	2	$23.3 \pm 0.5$
SEP93	90	3	$48.0 \pm 4.0$
SEP93	100	3	$41.8 \pm 2.0$
neutron (LANSCE)			
SEP92	0	2	$28.5 \pm 2.0$
SEP92	40	2	$23.1 \pm 1.2$
SEP92 <sup>a</sup>	100	2	$44.0 \pm 4.0$
AUG93	100	3	$42.5 \pm 4.0$
X-ray <sup>b</sup>			$33.3 \pm 0.8$

<sup>a</sup> This data set showed no evidence of aggregation for  $q > 0.027$  Å $^{-1}$ , and, therefore, it was used in the analyses. <sup>b</sup> From the X-ray scattering profile calculated by combining nine separate measurements as described under Results.

in order to show the differences in intensity of the raw data between samples. The results from the measurements of LANSCE were consistent with the NIST data. Table 1 gives  $R_g$  values for the data collected at both facilities.

The basic scattering functions determined from eq 2 using nine neutron scattering data sets for five different  $\text{D}_2\text{O}:\text{H}_2\text{O}$  ratios plus the combined X-ray scattering data set are shown in Figure 3. The basic scattering functions were quite robust; that is, they did not change in shape with the number of data sets included in the analysis down to the minimum required of three. Inclusion of more data sets simply improved statistics. The basic scattering function corresponding to the  $4\text{Ca}^{2+}\cdot\text{TnC}$  component extends to larger scattering angle than that for the TnI component, indicating TnC is less extended than TnI. The scattering curve calculated from the crystal structure of TnC is also given in Figure 3, and it is in good agreement with the basic scattering function of  $4\text{Ca}^{2+}\cdot\text{TnC}$  in scattering space. The inverse Fourier transform of the scattering curves for  $4\text{Ca}^{2+}\cdot\text{TnC}$  and TnI in Figure 3 gives the  $P(r)$  functions shown in the middle and bottom panels, respectively, of Figure 4. The  $P(r)$  function of  $4\text{Ca}^{2+}\cdot\text{TnC}$  is very similar to that expected

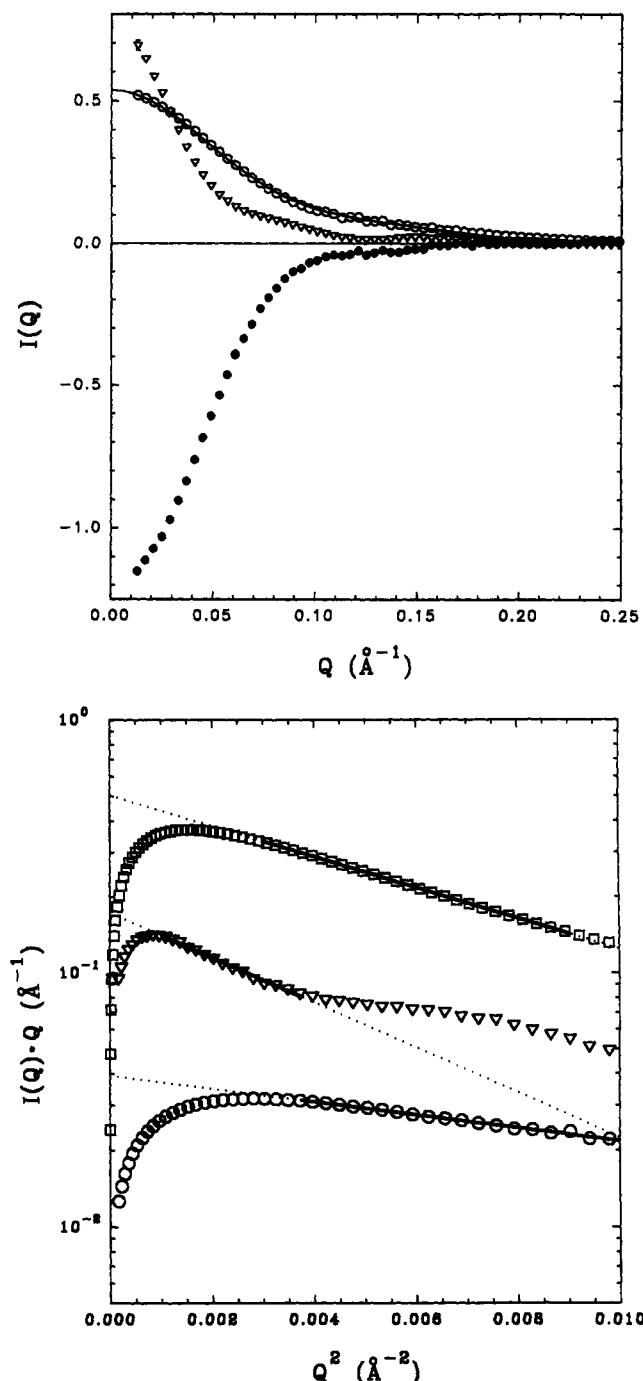


FIGURE 3: (Top) Basic scattering functions calculated using eq 2 for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}$  (O), TnI (▽), and the cross-term (●) between the two proteins in the complex. The solid line is the calculated scattering curve from the known crystal structure of  $2\text{Ca}^{2+}\cdot\text{TnC}$  (Herzberg & James, 1985). (Bottom) Guinier showed that for a "rod-shaped" particle, a plot of  $\ln[I(Q)\cdot Q]$  vs  $Q^2$  is linear at certain small  $Q$  values, with a slope proportional to the mean  $R_g$  value of the scattering particle. Shown here are the Guinier plots for  $4\text{Ca}^{2+}\cdot\text{TnC}$  (O) and TnI (▽) (using the basic scattering functions) and for  $4\text{Ca}^{2+}\cdot\text{TnC}\cdot\text{TnI}$  (□) (using the X-ray scattering data). The solid lines show the regions of the Guinier plots fitted for each profile, and the dotted lines are extrapolations of those fits. The roll-over observed at the lowest  $Q$  values is expected because of the finite lengths of the rod-shaped particles. The linearity is also expected to break down at high  $Q$  values, and the  $Q$  value at which this breakdown occurs is indirectly proportional to the  $R_g$  value (as we observe for these data sets).

for the crystal structure of  $2\text{Ca}^{2+}\cdot\text{TnC}$  (Herzberg & James, 1985). It shows a peak at  $\approx 18$  Å, corresponding to the approximate radius of each of the globular lobes, and a secondary peak at  $\approx 45$  Å resulting from the separation of the two lobes. The  $P(r)$  function calculated from the crystal

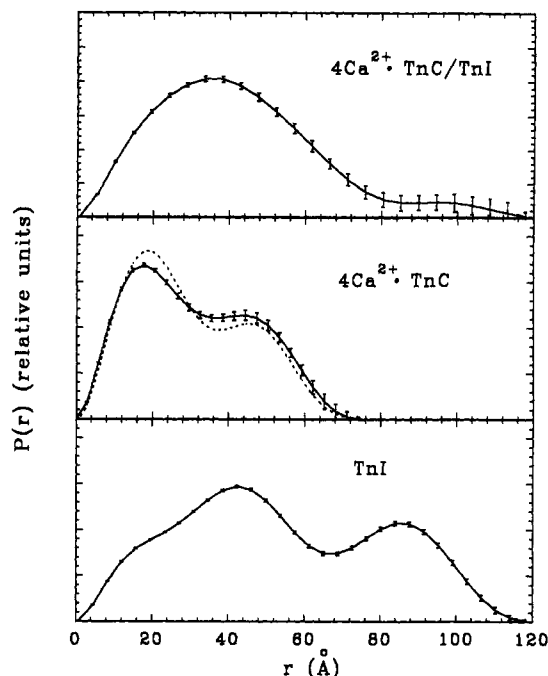


FIGURE 4: (Top)  $P(r)$  function for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}\cdot\text{TnI}$  determined from the X-ray scattering data. Middle and bottom panels are the  $P(r)$  functions for  $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}$  and TnI, respectively, as determined from the basic scattering functions given in Figure 3. The dotted line in the middle graph is the calculated  $P(r)$  function from the crystal structure of TnC (Herzberg & James, 1985).

Table 2

	$4\text{Ca}^{2+}\cdot\text{TnC}^a$	TnI	$4\text{Ca}^{2+}\cdot\text{TnC}\cdot\text{TnI}$
$R_g$ (Å) [and $d_{\text{max}}$ (Å)] Values Derived from Three Methods of Analysis			
basic scattering functions	$23.9 \pm 0.5$ ( $72 \pm 3$ ) <sup>b</sup>	$41.2 \pm 2.0$ ( $118 \pm 4$ )	$33.0 \pm 0.5$ ( $115 \pm 4$ )
parallel axis theorem <sup>c</sup>	$23.9 \pm 0.4$	$41.6 \pm 1.2$	$34.5 \pm 1.0$
Stuhrmann analysis <sup>c</sup>	$23.5 \pm 1.0$	$41.5 \pm 2.0$	$32.7 \pm 1.0$
$R_c$ (Å) Values Derived from Guinier Analysis of the Basic Scattering Functions			
	$10.7 \pm 1.0$	$20.5 \pm 2.0$	$16.2 \pm 1.5$

<sup>a</sup> For comparison,  $2\text{Ca}^{2+}\cdot\text{TnC}$  has an  $R_g$  of 23.9 Å and a  $d_{\text{max}}$  of 72 Å (Herzberg & James, 1985; Trewella & Heidorn, 1988). <sup>b</sup> Values in parentheses are  $d_{\text{max}}$ . <sup>c</sup> The best fit gave values for the center-of-mass separation ( $D$ ) are  $0 \pm 10$  Å.

structure differs from that shown in Figure 4(middle) only in the relative amplitudes of these two peaks. The ratio of the peak at  $\approx 18$  Å to that at  $\approx 45$  Å is 20% greater for the crystal structure  $P(r)$ . The  $R_g$  and  $d_{\text{max}}$  values for  $4\text{Ca}^{2+}\cdot\text{TnC}$  in the complex (Table 2) agree well with those calculated from the crystal structure coordinates. These data indicate that the interconnecting helix of TnC is extended when it is complexed with TnI, more like the crystal structure as compared with the solution structure in which the globular domains are on average closer together (Heidorn & Trewella, 1988). The average  $R_c$  value for  $4\text{Ca}^{2+}\cdot\text{TnC}$  is  $10.7 \pm 1.0$  Å (Figure 3, bottom, Table 2).

The  $P(r)$  function for TnI is even more extended than that for  $4\text{Ca}^{2+}\cdot\text{TnC}$ . It shows two peaks, at  $\approx 43$  and 86 Å, and a shoulder at  $\approx 10$ –20 Å. The shape and amplitudes of the two peaks cannot arise from a simple dumbbell shape like TnC. The  $R_c$  value for the TnI component is 20.5 Å (Figure 3, bottom, Table 2). A detailed modeling procedure based on a Monte Carlo method (Heidorn *et al.*, 1988) has been developed to model and test possible TnI structures (Olah and Trewella, unpublished results).

A Stuhrmann plot ( $R_g^2$  versus  $1/\Delta\rho$ ) is shown in Figure 5. The lines were calculated using eq 5 with  $R_g$  values determined

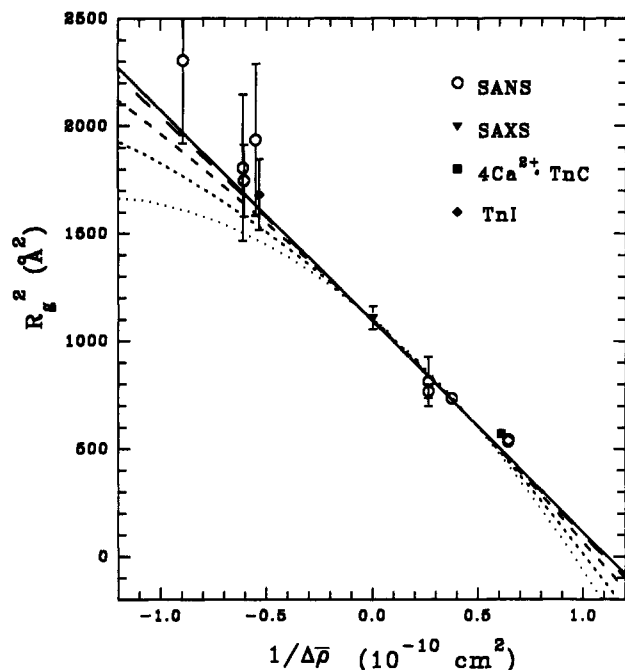


FIGURE 5: Stuhrmann plot ( $R_g^2$  versus  $1/\Delta\rho$ ). The line fits were determined from eqs 4 and 5 by assuming fixed  $R_g$  values of 23.9 and 41 Å for  $4\text{Ca}^{2+}\cdot\text{TnC}$  and TnI, respectively, and varying the separation,  $D$ , of the two components. Separations are (—) 0, (---) 5, (- - -) 10, (- - -) 15, and (····) 20 Å.

from the basic scattering functions and assuming center-of-mass separations ranging from 0 to 20 Å. The scattering data exclude solutions for which the center-of-mass separation of the two components is  $>10$  Å. The negative slope ( $\alpha$  in eq 4) indicates that the component with the larger neutron scattering power ( $4\text{Ca}^{2+}\cdot\text{TnC}_{78\%}$ ) is located more toward the center of the total complex. The  $R_g$  value calculated from the X-ray scattering data is approximately equivalent to scattering at infinite contrast and is also included in the Stuhrmann plot.  $R_c$ ,  $R_l$ , and  $D$  resulting from solution of the Stuhrmann analysis (eqs 4 and 5) are given in Table 2. Also, in Table 2 are the structural parameters determined from the "parallel axis theorem" (eq 3) along with those determined from the basic scattering functions showing that the different methods of analysis are in excellent agreement.

The  $R_c$  values for the two components and the complex give information on the relative orientations of their long axes. In the limiting case in which  $4\text{Ca}^{2+}\cdot\text{TnC}$  is approximated as a simple rod shape surrounded by TnI approximated as a simple annular cylinder, with each component constrained to have their centers-of-mass coincident and long axes parallel, the  $R_c$  value for the total complex ( $R_{cT}$ ) can be calculated using  $R_{cT} = f_c R_{cC}^2 + f_l R_{cI}^2$  where  $f_{l,c}$  are the mass fractions of each component and  $R_{cC,I}$  are the respective  $R_c$  values. Substitution of  $R_c$  values for  $4\text{Ca}^{2+}\cdot\text{TnC}$  and TnI (Table 2) into this equation gives an  $R_{cT}$  value of 16.6 Å, in agreement with the measured value for the complex. Nonparallel axes would give increasingly larger  $R_{cT}$  values (up to 21 Å for the limit of perpendicular axes). Therefore, we conclude the long axes of  $4\text{Ca}^{2+}\cdot\text{TnC}$  and TnI (as well as that for the overall complex) are approximately coincident.

## DISCUSSION

The neutron and X-ray scattering data presented here give the first view of the nature of the conformational interaction between  $4\text{Ca}^{2+}\cdot\text{TnC}$  and its regulatory target TnI. The data show  $4\text{Ca}^{2+}\cdot\text{TnC}$  in the complex is extended, similar to the crystal structure for  $2\text{Ca}^{2+}\cdot\text{TnC}$ , as determined from the  $R_g$

and  $d_{\max}$  values and the  $P(r)$  function. The small discrepancy in the relative amplitudes of the peaks in the  $P(r)$  function determined from the basic scattering functions and that determined from the  $2\text{Ca}^{2+}\cdot\text{TnC}$  crystal structure coordinates could be attributed to  $\text{Ca}^{2+}$ -induced conformational changes such as the opening of the "cup"-shaped N-domain of  $4\text{Ca}^{2+}\cdot\text{TnC}$  (Herzberg et al., 1986). The basic conclusion that the TnC component in the complex is dumbbell-shaped and the connecting region between the two globular lobes is extended similar to the crystal structure remains firm. The TnI component is even more extended than  $4\text{Ca}^{2+}\cdot\text{TnC}$ , and the maximum linear dimensions of the TnI component and the overall complex ( $\approx 118$  Å) are the same. The long axes of the two components are approximately coincident, as are their centers-of-mass, and the TnI component is more toward the outside of the complex encompassing the  $4\text{Ca}^{2+}\cdot\text{TnC}$ .

An extended  $4\text{Ca}^{2+}\cdot\text{TnC}$  complexed with TnI is consistent with an earlier observation using energy transfer methods (Wang et al., 1987). However, this is quite different from the current view of the interactions between the evolutionarily related calmodulin (CaM) and a number of its regulatory targets. Structural studies of  $4\text{Ca}^{2+}\cdot\text{CaM}$  complexed with synthetic peptides based upon binding domains from smooth muscle and skeletal muscle myosin light chain kinase (MLCK) (Heidorn et al., 1989; Ikura et al., 1992; Meador et al., 1992, 1993) show CaM contracts around the short ( $\approx 20$  residue) target sequence, which forms a bifurcated basic amphipathic  $\alpha$ -helix, via flexibility in the "helical" sequence connecting the two globular lobes of CaM. Hydrophobic residues along the helical target sequence form tight interactions with the hydrophobic pockets on each of CaM's globular lobes. This mode of interaction has also been observed for a variety of peptide sequences, including a number that appear to have no physiological relevance but do form basic, amphipathic helices (Kataoka et al., 1989, 1991; Yoshino et al., 1989). Sequence analysis of a variety of  $\text{Ca}^{2+}\cdot\text{CaM}$ -regulated enzymes indicates this may be a general feature of a number of targets (O'Neil & DeGrado, 1989). The target enzymes that share these CaM binding domain features generally only bind CaM in the presence of  $\text{Ca}^{2+}$ . In contrast, phosphorylase kinase is a multisubunit enzyme made up of a tetramer of tetramers,  $(\alpha\beta\gamma\delta)_4$ , and the  $\delta$ -subunit is CaM which modulates the activity of the catalytic or  $\gamma$ -subunit in a  $\text{Ca}^{2+}$ -dependent manner [for a review, see Pickett-Giess and Walsh (1986)]. Like TnC in the troponin complex, CaM remains an integral part of phosphorylase kinase in the presence and absence of  $\text{Ca}^{2+}$ . The CaM binding region in the  $\gamma$ -subunit is distinct from other CaM binding domains in that it is made up of two noncontiguous subdomains that bind to CaM simultaneously (Dasgupta et al., 1989). Further, Dasgupta et al. (1989) identified sequence similarities between the CaM binding subdomains and TnI. The regions of sequence similarity include a four-residue region of sequence identity that overlaps with the TnI inhibitory peptide sequence [TnI(104-115)] which is the shortest peptide segment needed to inhibit actin-myosin interaction and ATPase activity (Syska et al., 1976; Talbot & Hodges, 1981; Van Eyk & Hodges, 1988). Small-angle X-ray and neutron scattering data on CaM complexed with the two CaM binding subdomains identified in the  $\gamma$ -subunit of phosphorylase kinase showed CaM to be extended (Trehwella et al., 1990). Small-angle X-ray scattering experiments (Blechner et al., 1992) on TnC complexed with a number of peptides, including the TnI inhibitory peptide sequence, showed TnC to be extended in its interaction with this peptide sequence, as well as with another TnC binding sequence, TnI(1-30), which has been suggested to act as a negative regulator of the



TnI inhibitory region (Ngai & Hodges, 1992). These observations led us to speculate (Trewella *et al.*, 1990; Blechner *et al.*, 1992) that the interaction of TnC with TnI may be similar to the interaction between CaM and the  $\gamma$ -subunit of phosphorylase kinase. The data presented here support this speculation, and give further support to the idea that the extended TnC and CaM structures may be important in maintaining  $\text{Ca}^{2+}$ -independent associations in some systems.

Several studies have concluded that the TnI inhibitory peptide interacts with regions in both the C- and N-terminal domains of TnC [Farah *et al.*, 1994; for a review, see Ngai and Hodges (1992), Swenson and Frederickson (1992), and Kobayashi *et al.* (1991)]. Nuclear magnetic resonance (NMR) studies of the TnI inhibitory peptide sequence complexed with TnC in the presence of  $\text{Ca}^{2+}$  show it to form a one-turn amphiphilic  $\alpha$ -helix which is distorted around two central prolines (Campbell & Sykes, 1991; Campbell *et al.*, 1992). This peptide structure would not be long enough to span the distance between the N- and C-terminal domains determined from the data presented here, indicating that the peptide could not simultaneously interact with both domains. Alternatively, one could consider the possibility that the inhibitory peptide has a propensity to interact independently with each domain since there is structural similarity between the two domains. This possibility has been suggested elsewhere (Ngai & Hodges, 1992; Swenson & Frederickson, 1992). Further, Ngai and Hodges (1992) showed that a subtle difference exists between the action of the TnI(1–40) regulatory peptide and the regulatory region of TnI when each binds to TnC. These observations suggest caution must be taken when interpreting results from studies utilizing peptides to model the interaction between TnC and TnI, and there may also be differences between the interaction with TnC of the TnI inhibitory peptide and the corresponding region in intact TnI.

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