Time resolved fluorescence anisotropy and sedimentation velocity has been used to study the rotational and translational hydrodynamic behavior of two mutants of chicken skeletal troponin C bearing a single tryptophan residue at position 78 or 154 in the metal-free-, metal-bound-, and troponin I peptide (residues 96–116 of troponin I)-ligated states. The fluorescence anisotropy data of both mutants were adequately described by two rotational correlation times, and these are compared with the theoretically expected values based on the rotational diffusion of an idealized dumbbell. These data imply that the motion of the N- and C-terminal domains of troponin C are independent. They also suggest that in the metal-free, calcium-saturated and calcium-saturated troponin I peptide-bound states, troponin C is elongated, having an axial ratio of 4–5. Calcium or magnesium binding to the high affinity sites alone reduces the axial ratio to approximately 3. However, with calcium bound to sites III and IV and in the presence of a 1:1 molar ratio of the troponin I peptide, troponin C is approximately spherical. The metal ion and troponin I peptide-induced length changes in troponin C may play a role in the mechanism by which the regulatory function of troponin C is effected.

The three-dimensional structures of troponin C (TnC)1 (1, 2), and the homologous protein calmodulin (CAM) (3, 4) revealed molecular architectures composed of globular N- and C-terminal domains joined by an extended α-helical linker. The globular domains contain a pair of EF-hand motifs (5), which are designed to coordinate metal ions. On the basis of the crystal structure of TnC, with calcium bound to the C-terminal domain sites (1, 2), Herzberg et al. (6) propose that the major conformational change that occurs when the regulatory N-terminal domain sites bind calcium involves the exposure of a hydrophobic patch resulting from the movement of the B/C helix away from the N/A/D helices. Subsequent structural data, for example, the high resolution NMR (7) and x-ray crystal structures of calcium-saturated (8) TnC and the x-ray crystal structure of the calcium-saturated N-terminal domain of TnC (9), have confirmed the validity of this model. However, TnC is but one component of a three-component complex, and the issue of whether the structural changes that occur in “isolated” TnC directly reflect its behavior in the functioning troponin complex remains unresolved. Additionally, it has been proposed that the hydrophobic patch that is exposed in the calcium-replete N- and C-terminal domains are binding sites for troponin I (10), and the structure and dynamics that TnC displays under such conditions is not known.

We have recently completed the steady-state optical spectroscopic characterization of two mutants of chicken skeletal TnC bearing a single Trp residue at positions 78 (F78W) and 154 (F154W) in the N- and C-terminal domains, respectively.2 The introduced Trp residue in both mutants is located at position −z + 1 of the EF-hand, which is immediately after the last metal ion coordinating residue in site II (F78W) and site IV (F154W), respectively. Both mutants were iso-functional with wild-type TnC in the restoration of contractile activity to TnC-depleted-skinned muscle fibers.3 The spectroscopic properties of Trp-154 are sensitive to calcium and magnesium binding at sites III and IV (11), while Trp-78 responds to calcium binding at both the N- and C-terminal domain sites. On the basis of the spectroscopic properties of the Trp residue, we surmised that the indole moiety is in a rigid molecular environment and that this feature should render these mutants useful in studying the overall dynamic behavior of TnC. The presence of a single Trp residue in either domain may also provide information about the local dynamics of the N- and C-terminal domains of TnC.

This paper reports a study of the rotational and translational motion of F78W and F154W. We have first used minimum perturbation mapping (12, 13) to explore possible conformation of the Trp residue in both F78W and F154W starting from the x-ray crystal structure of 2-calcium TnC (2). Additionally, time-resolved anisotropy decays of the Trp fluorescence and sedimentation velocity experiments have been used to obtain information regarding the rotational motion and the shape that TnC adopts in the metal-free-, metal-bound (calcium and magnesium), and calcium-troponin I peptide (residues 96–116 of troponin I)-ligated states, respectively. Our results suggest that the dynamics of metal-free, 2-Ca2+ , and 2-Mg2+ -TnC are consistent with that of flexible dumbbell and is similar to what has been found for the metal-free state of CAM (14). However, in the calcium-saturated state (with or without TnI p) and the 2-Ca2+-TnI p state, the apparent domain motions are no longer detected, which is suggestive of a more rigid conformation. The sedimentation velocity data suggests that in the apo-, calcium-saturated- and calcium-saturated-TnI p states, TnC is elongated with an axial ratio of 4–5. Calcium or magnesium binding to
the high affinity C-terminal domain sites results in a contraction of the axial ratio to approximately 3, which is consistent with that found in the 2-calcium crystal structure of TnC (2), which has dimensions of 75 Å × 25 Å. However, in the presence of TnIp and calcium bound to the high affinity sites, TnC becomes approximately spherical.

**MATERIALS AND METHODS**

**Minimum Perturbation Mapping**—The initial structures used to obtain minimum perturbation maps were generated from the 2-calcium x-ray crystallographic coordinates of TnC (2), with bad contacts removed by 5 steps of conjugate gradient minimization. The side chains of Trp-78 and Trp-154 were subsequently built from the topology and parameter internal coordinates. The following residues were allowed to be free during the simulations: 20, 24, 35, 40, 43, 44, 47, 71, 73, and 80 (F78W; 98, 101, 102, 105, 113, 149, 151, 155, 157, and 158 (F154W). All nonpolar hydrogens were treated as extended heavy atoms, and the dielectric constant was equal to the distance in angstroms between interacting atoms. The charge on ionized side chains was reduced by 80%, and nonbonded interactions were switched off over the range 7–11 Å. Calculations were performed on a Silicon Graphics, Inc. Indigo-2 computer using executable code derived from CHARMm (15) version 22 with topology and parameter files taken from CHARMm version 19.

Minimum perturbation mappings were computed on a 5° grid of Trp-78 and Trp-154 x1 × x2 torsion space (13). During minimization, x1 and x2 were constrained with a harmonic constraint energy constant of 400 kcal mol⁻¹ rad². At each of the 72 × 72 grid points, the free atoms were minimized using 40 steps of the steepest descent method followed by 240 steps with the Powell (16) method. SHAKE (17) was applied to all bonds involving hydrogen, and the system was minimized for an additional 40 steps by the Powell method. Grid points for which the final minimized energy exceeded 50 kcal mol⁻¹ were considered outliers, and the map was interpolated at these points using bivariate interpolation (18).

**Protein Preparation**—The expression and purification of the TnC mutants has been described previously.² Metal-free protein samples were prepared by dialysis (twice) against a medium composed of 120 mM MOPS, 90 mM KCl, 2 mM EGTA at pH 7.0. After dialysis, the volume was adjusted with dialysate to yield a protein concentration of 7–10 mg ml⁻¹. The high concentration of MOPS used ensured that the free calcium ion concentration was negligible.

**Time-resolved Fluorescence**—The time-resolved anisotropy decay of the Trp fluorescence was measured using time-correlated single photon counting (20). Protein samples of approximately 7 mg ml⁻¹ concentration were excited at 300 nm using the frequency-doubled output of a cavity-dumped synchronously pumped Coherent 700 nm dye laser (Coherent), which was itself pumped by the frequency-doubled output of a Coherent Antares YAG laser. The emission was isolated using an interference filter (351 nm; 4 nm bandpass) and detected with a Coherent Antares YAG laser. The emission was isolated using an interference filter (351 nm; 4 nm bandpass) and detected with a Coherent Antares YAG laser. The emission was isolated using an interference filter (351 nm; 4 nm bandpass) and detected with a Coherent Antares YAG laser.

The rotational diffusion coefficient for rotation about an axis perpendicular to the main symmetry axis, \( D_j \), is

\[
\frac{8 \pi \eta r_j^3}{k_B T} D_j = 0.2675 \exp \left[ \sum_{i=1}^{3} \frac{1}{\alpha_i} \left( \frac{\sigma_i}{\sigma_j} \right)^{3/2} \right]
\] ²

where \( \eta \) is the viscosity at temperature \( T \), and \( k_B \) is the Boltzmann constant. The coefficients \( c_i \) are given by Table I of Garcia de la Torre and Bloomfield (28–30). This procedure considers a dumbbell to be composed of two large spheres of diameter \( \sigma_j \) connected by smaller spheres of diameter \( \sigma_i \). The equation describing the rotational diffusion coefficient for rotation about an axis perpendicular to the main symmetry axis, \( D_j \), is

\[
\frac{8 \pi \eta r_j^3}{k_B T} D_j = 0.2675 \exp \left[ \sum_{i=1}^{3} \frac{1}{\alpha_i} \left( \frac{\sigma_i}{\sigma_j} \right)^{3/2} \right]
\]

with \( \gamma(t) \) being modeled by a multieponential function (20). Molecules lacking spherical symmetry are usually modeled as prolate or oblate ellipsoids, and under such circumstances, the anisotropy is expected to decay as a sum of exponentials given by

\[
I(t) = \frac{1}{3} I(1 + 2\gamma(t))
\]

(1)
The diffusion coefficient for rotation about the main symmetry axis, $D_1$, given by

$$D_1 = \frac{k_BT}{4\pi\eta} 14\sigma^2 + \rho_0^2$$  \hspace{1cm} (Eq. 9)

is also required (31). The functional form for the decay of the fluorescence anisotropy in terms of the diffusion coefficients for rotation about $D_1$ and $D_2$, for a system with three rotational correlation times is similar to Eq. 3 with $\phi_1 = (4D_1 + 2D_2)^{-1}$, $\phi_2 = (D_1 + 5D_2)^{-1}$, and $\phi_3 = (6D_2)^{-1}$ (31).

The expected rotational correlation times for the TnC mutants were calculated using hydrated volumes of 27,973 Å$^3$ and 34,045 Å$^3$, corresponding to a commonly used value for the degree of hydration for globular proteins of 0.2 g of H$_2$O/g of protein (27) and the value of 0.4 g of H$_2$O/g of protein suggested by Hubbard et al. (32). In all calculations, the value of $\sigma$ was 3.5 Å.

**Ultracentrifugation**—Equilibrium sedimentation experiments were performed at 23.3 °C using short column (0.7 mm) cells (33) and Rayleigh interference optics on a Beckman Model E analytical ultracentrifuge equipped with a electronic speed control, RTIC temperature controller, and a pulsed laser diode light source (670 nm). Data were acquired at speeds of 10,000, 20,000, 30,000, and 40,000 revolutions/minute using a television camera-based online data acquisition and analysis system (34). Identical experiments were also conducted in a Beckman XL-I ultracentrifuge at 20 °C. Sample concentrations were approximately 16, 8, and 5 μM, and data was collected at intervals after the estimated time to equilibrium and tested for equilibrium by subtracting successive scans (35). Data within the optical window were selected and analyzed to estimate the molecular weight of the various species using the program NONLIN (36). Sedimentation velocity experiments were performed on a Beckman XL-I instrument equipped with absorption or Rayleigh interference optics (37), a 4-hole titanium rotor, 6-channel, 12-mm-thick charcoal-filled epon centerpieces, and appropriate windows. Experiments were conducted at 60,000 revolutions/minute at 20 °C. Data were acquired at 1-s intervals to produce sedimentation coefficient distributions according to the method described by Stafford (38). Estimates of the shape of the TnC mutants were obtained by calculating Perrin shape factors and the corresponding axial ratio of an ellipsoid. The Perrin shape factor, $F$, is defined as

$$F = \frac{s_0}{S_{obs}}$$  \hspace{1cm} (Eq. 10)

where $s_{obs}$ is the measured sedimentation coefficient, and $s_0$, that of an equivalent spherical molecule having a radius $R_C$, is calculable from

$$s_0 = \frac{M(1 - \pi\rho_0)}{6\pi\eta\nu_0 R_C^2}$$  \hspace{1cm} (Eq. 11)

where $M$ is the mass of the particle, $\nu$, the calculated partial specific volume (0.72 g cm$^{-3}$) based on the amino acid sequence, $\eta$ is the solvent viscosity, $N_A$ is Avogadro’s number, $R_C = [3M(1 + \delta)4\pi N_A^2]^\frac{1}{3}$, $\rho_0$ is the density and $\delta$ is the degree of hydration.

**RESULTS**

**Minimum Perturbation Mapping**—The minimum perturbation approach is based on the assumption that the overall structure of a stable mutant differs from the wild-type protein only in the positions of the backbone and side chain atoms that are neighbors of the mutant side chain. Consequently, the method provides simple estimates of the possible conformation of the mutant side chain (12). Minimum perturbation maps for TnC mutants to obtain information about the rotational dynamics of TnC. Fig. 2 shows typical time-resolved intensity decay data, which upon analysis, yielded the parameters given.
The time-resolved fluorescence anisotropy decays of F78W were dominated by $\phi_1$, which accounted for 72–86% of the total anisotropy decay. In the metal-free state, the recovered $\phi_1$ value of 7.04 ns for this mutant was approximately 2 × greater than the corresponding value for F154W. This suggests that the motional components of TnC that are sensed by Trp-78 and Trp-154 are different. Magnesium binding to the high affinity sites results in a reduction of the $\phi_1$ value relative to that of the metal-free state. This implies that magnesium binding to sites III and IV alters the conformation of the C-terminal domain such that the motional component detected by Trp-78 is different than that of the apo state. When calcium replaces magnesium at the high affinity sites, the recovered $\phi_1$ value is the same as that obtained for the metal-free state. The $r_0$ and $r_{ss}$ values for the metal-free, magnesium-saturated, and 2-Ca$^{2+}$-bound states are almost identical. TnI$p$ binding to 2-Ca$^{2+}$-F78W increases the recovered $\phi_1$ value by approximately 3 ns. The $r_0$ and $r_{ss}$ values are similar to those of the metal-free, 2-Ca$^{2+}$, and 2-Mg$^{2+}$ states. The $\phi_1$ and $\phi_2$ values for the calcium-saturated state of F78W are 11.47 ns and 0.09 ns, respectively. The recovered $r_0$ value is identical to that obtained by steady-state emission anisotropy measurements in 67% glycerol at $-46 ^\circ C$, and the $r_{ss}$ value was 0.16. The parameters recovered for 4-Ca$^{2+}$ state of F78W are remarkably similar to those obtained for the analogous state of F154W as well as the 4-Ca$^{2+}$-TnI$p$ state of F154W and suggest that Trp-78 and Trp-154 are detecting the same components of TnC motion.

The available high resolution structures have established that TnC adopts a dumbbell shape in the 2-Ca$^{2+}$- and 4-Ca$^{2+}$ states (1, 7, 8). While high resolution structural data of the metal-free state of TnC is currently unavailable, such data exists for the homologous protein CAM (39). Metal-free CAM exists as a dumbbell, and it is plausible that metal-free TnC is similarly shaped. To compare the recovered rotational correlation times to those expected for TnC, we have calculated the rotational diffusion coefficients for the dumbbell shape depicted in the x-ray and NMR high resolution structures of TnC using the analytical procedures developed by Garcia de la Torre and Bloomfield (28–30). Fig. 3 shows the predicted rotational correlation times of a dumbbell as a function of its length, assuming hydration values of 0.2 and 0.4 g of H$_2$O/g of protein, and Fig. 4 depicts some of the possible motions of TnC. As expected, the values of the rotational correlation time depend on the presumed degree of hydration. Assuming that the length of TnC in solution is 75Å, which is the value observed in the 2-Ca$^{2+}$ crystal structure (2), the predicted rotational correlation times at 20 °C would be 17, 14, and 10 ns for a hydration of 0.4 g of H$_2$O/g of protein and 14, 12, and 8 ns for a hydration of 0.2 g of H$_2$O/g of protein. For 2-Ca$^{2+}$-F154W, the recovered $\phi_1$ value of approximately 4 ns is substantially less than the shortest expected rotational correlation time (6D$^{-1}$). This also holds for the metal-free and 2-Mg$^{2+}$-bound states, if the shape of TnCs under these conditions is similar to that of the 2-Ca$^{2+}$ state. For the 4-Ca$^{2+}$- and 4-Ca$^{2+}$-TnI$p$ states of F154W and the 4-Ca$^{2+}$ state of F78W, the $\phi_1$ values recovered from the r(t) data are consistent with those calculated from (D$_1$ + 5D$^{-1}$)$^{-1}$, assuming that the degree of hydration is 0.2 g of H$_2$O/g of protein. The $\phi_1$ values recovered from the r(t) data for the metal-free, 2-Ca$^{2+}$, and 2-Mg$^{2+}$ states of F78W are shorter than 1/6D$^{-1}$, whereas for the 2-Ca$^{2+}$-TnI$p$ state of F154W is close to this value for a degree of hydration of 0.2 g of H$_2$O/g of protein.

The low $\phi_1$ value obtained for the apo state of F154W prompted us to examine the effect of viscosity on the rotational motion of Trp-154. Fig. 5 shows the effect of varying the external viscosity by additions of sucrose on the long and short rotational components of the apo state of F154W. The $\phi_1$ value

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5 M. C. Moncrieffe and F. G. Prendergast, unpublished data.
Parameters recovered from the analysis of the time-resolved fluorescence anisotropy decay data of the TnC mutants at 20 °C

The two rotational correlation times \( \phi_1 \) and \( \phi_2 \) and their corresponding amplitudes, \( \beta_1 \) and \( \beta_2 \) as well as the steady-state anisotropy \( r_0 \) and the anisotropy at zero time \( r_z \) are shown. The uncertainties in the recovered parameters were obtained from 100 Monte-Carlo simulations. Protein concentrations were approximately 7 μM, and the solution composition was 100 mM MOPS, 90 mM KCl, 2 mM EGTA, pH 7.0, at 20 °C.

| Sample     | \( \phi_1 \) | \( \phi_2 \) | \( \beta_1 \) | \( \beta_2 \) | \( r_0 \) | \( r_z \) | \( \chi^2 \) |
|------------|-------------|-------------|-------------|-------------|--------|--------|--------|
| F154W      | 3.07 ± 0.07 | 0.57 ± 0.02 | 0.11 ± 0.01 | 0.12 ± 0.01 | 0.23 ± 0.01 | 0.07 ± 0.12 | 1.2     |
| 2-Mg       | 3.44 ± 0.03 | 0.25 ± 0.01 | 0.17 ± 0.01 | 0.04 ± 0.01 | 0.21 ± 0.01 | 0.09 ± 1.2  | 1.2     |
| 2-Ca       | 4.15 ± 0.04 | 0.32 ± 0.02 | 0.16 ± 0.01 | 0.05 ± 0.01 | 0.21 ± 0.01 | 0.10 ± 1.1  | 1.2     |
| 2-Ca-Mg    | 8.09 ± 0.07 | 0.70 ± 0.03 | 0.16 ± 0.01 | 0.06 ± 0.01 | 0.22 ± 0.01 | 0.12 ± 1.2  | 1.2     |
| 4-Ca       | 11.75 ± 0.10| 0.09 ± 0.01 | 0.21 ± 0.01 | 0.08 ± 0.01 | 0.29 ± 0.01 | 0.16 ± 1.3  | 1.3     |
| 4-Ca-Mg    | 11.46 ± 0.08| 0.08 ± 0.01 | 0.21 ± 0.01 | 0.06 ± 0.01 | 0.27 ± 0.01 | 0.16 ± 1.2  | 1.2     |
| F78W       | 7.04 ± 0.05 | 0.45 ± 0.04 | 0.18 ± 0.01 | 0.03 ± 0.01 | 0.21 ± 0.01 | 0.12 ± 1.2  | 1.2     |
| 2-Mg       | 6.46 ± 0.07 | 0.28 ± 0.03 | 0.18 ± 0.01 | 0.03 ± 0.01 | 0.21 ± 0.01 | 0.12 ± 1.2  | 1.2     |
| 2-Ca       | 7.03 ± 0.07 | 0.18 ± 0.03 | 0.15 ± 0.01 | 0.03 ± 0.01 | 0.18 ± 0.01 | 0.10 ± 1.2  | 1.2     |
| 2-Ca-Mg    | 9.89 ± 0.15 | 0.59 ± 0.04 | 0.17 ± 0.01 | 0.04 ± 0.01 | 0.21 ± 0.01 | 0.13 ± 1.2  | 1.2     |
| 4-Ca       | 11.47 ± 0.03| 0.09 ± 0.01 | 0.21 ± 0.01 | 0.08 ± 0.01 | 0.29 ± 0.01 | 0.16 ± 1.3  | 1.3     |

Fig. 3. Predicted rotational correlation times \( \phi_{1,2} = 1–3 \) of a rigid dumbbell as a function of total length and the degree of hydration. —, \( \phi = 0.4 \) g of H₂O/g of protein; -----, \( \phi = 0.2 \) g of H₂O/g of protein.

Fig. 4. Possible rotational motions of TnC. A, rotational diffusion about an axis parallel to the main symmetry axis \( (D_1^*) \) and perpendicular to the main symmetry axis \( (D_2^*) \); B, motion about a hinge in the central helix.

scales linearly with the external viscosity, whereas the short rotational component, \( \phi_2 \), does not. Similar results were also observed when the external viscosity was altered by changes in temperature. Thus, although the magnitude of the \( \phi_3 \) value for the apo state of F154W is perhaps not indicative of the overall rotational motion of TnC, its dependence on the external viscosity suggests that is probably represents “hinge bending” motions of the two domains of TnC.

Analytical Ultracentrifugation—It is difficult to ascribe physical meaning to the rotational correlation times recovered from the \( r(t) \) data and make inferences regarding the shape that TnC adopts in solution from the fluorescence anisotropy data only (41). To obtain information about TnC shapes, especially under solution conditions for which high resolution structural data is unavailable, we have measured sedimentation coefficients by velocity sedimentation. Fig. 6 shows typical results for F154W in the metal-free, 2Ca²⁺, 2Mg²⁺-states and also in the presence of saturating concentrations of calcium (in the absence of trifluoroethanol). In general, the \( g(s) \) distributions had a single Gaussian component, except in those experiments conducted in trifluoroethanol that usually had a small component at 0.95–1.0 S. The experimentally obtained sedimentation coefficients, the calculated Perrin shape factors, and the resulting axial ratios are presented in Table II.

In the metal-free and calcium-saturated states, the sedimentation coefficient is 1.6 S. From this value, an axial ratio of 4–5 can be calculated that is at the higher limit of axial ratios normally associated with globular proteins (42), suggesting that the molecule is in an extended conformation. The addition of either calcium or magnesium to the high affinity sites results in a contraction of the axial ratio to a value of 3. An axial ratio of 3 for the 2-Ca²⁺ state of TnC is consistent with that observed in the x-ray crystal structure (2) and suggests that the overall shape of molecule when the high affinity sites are saturated...
some conditions (the calcium saturates states, for example) are metal-free (consistent with previous reports in the literature (43–45). Experimentally, the inability to recover more than two rotational correlation times expected of an ideal dumbbell having the dimensions of a natively prolate ellipsoid. We have calculated the rotational correlation times expected of an ideal dumbbell having the dimensions of the NMR solution structure of TnC at pH 7.2 (8) as well as the NMR solution structure of calcium-saturated TnC at pH 5.1 (2) and of calcium-saturated TnC at pH 7.0, at 20 °C.

The Perrin shape factor (F) and the corresponding axial ratio as well as the predicted hydrodynamic shapes are also given. F was calculated assuming a hydration of 0.4 g of H₂O/g of protein (32) and a calculated i of 0.7213 cm²g⁻¹ based on amino acid sequences. The error in the calculated sedimentation coefficients is approximately 10%. Protein concentrations were 16, 8, and 5 μM, and the solution composition was 100 mM MOPS, 90 mM KCl, 2 mM EGTA, pH 7.0.

TABLE II
Calculated sedimentation coefficients of F154W obtained from sedimentation velocity measurements at 20 °C

| Sample            | s (Svedberg) | F  | Axial ratio | Predicted shape |
|-------------------|--------------|----|-------------|-----------------|
| apo-TnC           | 1.60         | 1.2| 4–5         | Prolate ellipsoid |
| 2-Mg²⁺-TnC        | 1.70         | 1.1| 3           | Prolate ellipsoid |
| 2-Ca²⁺-TnC        | 1.69         | 1.1| 3           | Prolate ellipsoid |
| 2-Ca²⁺·TnC·TnIp   | 1.92         | 1.0| 1           | Spherical       |
| 4-Ca²⁺-TnC        | 1.60         | 1.2| 4–5         | Prolate ellipsoid |
| 4-Ca²⁺·TnC·TnIp   | 1.56         | 1.2| 4–5         | Prolate ellipsoid |

At 20 °C, the recovered φ₁ value for metal-free F78W is approximately two times greater than the corresponding value for F154W, implying that the motion “sensed” by the Trp moiety in these two mutants are different. Although not supported by available data (47), the glycine residue in the central helix of TnC has been suggested to be a possible “hinge region” (1) that would allow the N- and C-terminal domains to move relative to each other. In an NMR study of the backbone and methyl dynamics of the regulatory domain of chicken skeletal TnC (residues 1–90), Gagné et al. (48) report an overall rotational correlation time for the metal-free state of 4.86 ± 0.15 ns at 29.6 °C. From our measurements, the φ₁ value of Trp-78 in the metal-free state at 30 °C is 5.12 ± 0.05 ns. This lends support to the notion that Trp-78 senses the motion of the N-terminal domain. Also, the 3 ns φ₁ value recovered for the apo state of F154W likely represents the rotational motion of the C-terminal domain. This value is somewhat smaller than the 4 ns that would be expected of a spherical C-terminal domain based on molecular weight and most likely reflects the less ordered nature of this domain relative to the N-terminal domain in the apo state (49, 50). This interpretation is supported by the fact that the recovered φ₁ values for the 2-Ca²⁺ state of F154W, where presumably, the C-terminal domain is more ordered, is approximately 4 ns.

Additional support for the idea that the values of the long correlation times of F154W reflect domain motion, particularly in the apo, 2-Mg²⁺, and 2-Ca²⁺ states of these mutants is obtained when the effect of viscosity on the recovered φ₁ values is examined. Because the frictional coefficient is proportional to solvent viscosity, protein motions consisting of large scale fluctuations (the hinge-bending motions of two domains, for example) will be influenced by the solvent viscosity (40). Thus, the component representing the overall motion is expected to scale with the external solvent viscosity, whereas those representing local motions should be relatively insensitive to the external viscosity. As is evident from Fig. 5, the φ₁ values of Trp-154 in the apo state of F154W scale with external viscosity in contrast to the φ₂ values. The recovered φ₂ values are significantly greater than the approximately 40 ps expected for

with calcium or magnesium is similar. The addition of TnIp to 2-Ca²⁺-F154W results in a further reduction of the axial ratio to 1.0, indicative of a spherically symmetric molecule.

DISCUSSION

The primary objectives of this work are interpretation of the parameters recovered from an analysis of time-resolved fluorescence anisotropy decay data of the single Trp mutants of TnC and to make inferences regarding the effect of ligand binding on the dynamic behavior of TnC. The x-ray crystal structures of 2-Ca²⁺·TnC at pH 5.1 (2) and of calcium-saturated TnC at pH 7.2 (8) as well as the NMR solution structure of calcium-saturated TnC at pH 7 (7) reveal that TnC adopts a dumbbell shape. We have calculated the rotational correlation times expected of an ideal dumbbell having the dimensions of TnC using the analytical procedure developed by Garcia de la Torre and Bloomfield (29–30) and Small and Anderson (31). Ideally, a comparison of the theoretically predicted and experimentally obtained rotational correlation times should allow one to ascribe each measured correlation time to rotational diffusion about a particular molecular axis. A comparison of the predicted rotational correlation times with those obtained from an analysis of the time-resolved anisotropy decays reveals that, although the model predicts three rotational correlation times (φ₁, φ₂, φ₃) for the dumbbell-shaped TnC, only two are recovered experimentally. The inability to recover more than two rotational components from time-resolved anisotropy decays is consistent with previous reports in the literature (43–45).

Although the magnitude of the recovered φ₁ values, under some conditions (the calcium saturates states, for example) are consistent with those expected for the shortest predicted times (1/60²), the φ₂ values are much too short to represent overall rotational motions of TnC. The simplest and perhaps most intuitive explanation for these findings is that in solution, TnC is not the rigid dumbbell depicted by the crystal structures. Indeed, the finding that there is detectable fluorescence energy transfer between Tyr-10 and Tyr-109 of rabbit skeletal TnC (46), a condition which necessitates that the separation between these residues be less than or equal to 10 Å, supports this idea, or at the very least, that there is flexibility, presumably, about the central helix of the molecule. Alternatively, it may be argued that motions leading to the predicted long correlation times exist but are undetected by the fluorescence anisotropy decay measurements or that current methods of data analysis are incapable of resolving more than two rotational components. An inspection of Eq. 3 reveals that the magnitude of pre-exponential factor, γ₃, will dramatically influence the ability to recover a particular rotational correlation time. Specifically, if γ₃ → 0, that rotational component, φ₃, would not be recovered. Given the angle (θ) between the absorption and emission dipoles, the dependence of the anisotropy decay amplitudes on the angle between a chosen symmetry axis and the dipoles can be determined. Such an analysis reveals, as was the case for the dityrosine derivative of CAM (31), that there are only a few possible orientations of the dipoles where one might recover more than one correlation time describing the rotational motion of the molecule.
the local motion of the Trp residue. Given our interpretation that the mutants are ostensibly sensing the independent motions of the N- and C-terminal domains, we believe that the \( \phi \) values actually represent the superposition of the domain motion of TnC on the localized motion of the Trp residue.

We have used sedimentation velocity measurements to obtain estimates regarding the shape TnC adopts when various ligands are bound, as this is not readily obtained from the \( r(t) \) data alone. Although a distinction between an axial ratio of 4 and 5 cannot be made, the sedimentation data (see Table II) clearly suggests that in the metal-free, 4-Ca\(^{2+}\), and 4-Ca\(^{2+}\)-TnI\(_n\) states, TnC is in an extended conformation. The increased steady-state anisotropy values for the Ca\(^{2+}\) and Ca\(^{2+}\)-TnI\(_p\) states relative to the metal-free state implies a decrease in the "flexibility" of the Trp residue, and this is perhaps responsible for the longer \( \phi \) values obtained under these conditions. For the 2-Ca\(^{2+}\)-TnI\(_p\) state, however, TnC has approximately spherical symmetry, and consequently, the \( \phi \) value should truly represent the rotational motion of the whole molecule. The compact globular shape that TnC apparently adopts in the presence of TnI\(_n\) and calcium bound to the high affinity sites as suggested by the sedimentation data disagrees with interpretations of the x-ray scattering data of Blechner et al. (51), who infer that the molecule was extended in the 2-Ca\(^{2+}\)-TnI\(_p\) state. The recently solved crystal structure of TnC bound to a TnI peptide (residues 1–47 of TnI) (52) revealed a complex having a compact globular shape in contrast to the dumbbell structure of the 2-Ca\(^{2+}\) and 4-Ca\(^{2+}\) states and suggests that peptide binding to TnC can result in structures similar to that seen with CAM (53).

The data presented here may have important implications regarding the function of the troponin complex, assuming that the behavior of the TnC-TnI\(_n\) complex mirrors that of TnC-TnI. These (i) within the troponin complex, under conditions where the high affinity sites of TnC are occupied by calcium ions, TnC has a compact, essentially spherical shape; ii) calcium binding to the regulatory sites, which provides the signal for muscle contraction, results in TnC becoming elongated. This latter observation is consistent with previous reports (7) and the results of Stone et al. (54), who found that in a complex consisting of calcium-saturated TnC and TnI, TnC was elongated. Thus, the conformational switch that signals muscle contraction may involve not only the exposure of the hydrophobic patch in the N-terminal domain but alterations in the shape of TnC, which either allow or prevent its interactions with other members of the troponin complex.

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