A Kinetic Model for the Binding of Ca$^{2+}$ to the Regulatoy Site of Troponin from Cardiac Muscle

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The kinetics of the binding of Ca$^{2+}$ to the single regulatory site of cardiac muscle troponin was investigated by using troponin reconstituted from the three subunits, using a monocysteine mutant of troponin C (cTnC) labeled with the fluorescent probe 2-[(4'-iodoacetamido)anilino]naphthalene-6-sulfonic acid (IAANS) at Cys-35. The kinetic tracings of binding experiments for troponin determined at free [Ca$^{2+}$] > 1 μM were resolved into two phases. The rate of the fast phase increased with increasing [Ca$^{2+}$], reaching a maximum of about 35 s$^{-1}$ at 4 °C, and the rate of the slow phase was approximately 5 s$^{-1}$ and did not depend on [Ca$^{2+}$]. Dissociation of bound Ca$^{2+}$ occurred in two phases, with rates of about 23 and 4 s$^{-1}$. The binding and dissociation results obtained with the binary complex formed between cardiac troponin I and the IAANS-labeled cTnC mutant were very similar to those obtained from reconstituted troponin. The kinetic data are consistent with a three-step sequential model similar to the previously reported mechanism for the binding of Ca$^{2+}$ to a cTnC mutant labeled with the same probe at Cys-84 (Dong et al. (1996) J. Biol. Chem. 271, 688–694). In this model, the initial binding in the bimolecular step to form the Ca$^{2+}$-troponin complex is assumed to be a rapid equilibrium, followed by two sequential first-order transitions. The apparent bimolecular rate constant is 5.1 × 10$^{7}$ M$^{-1}$ s$^{-1}$, a factor of 3 smaller than that for cTnC. The rates of the first-order transitions are an order of magnitude smaller for troponin than for cTnC. These kinetic differences form a basis for the enhanced Ca$^{2+}$ affinity of troponin relative to the Ca$^{2+}$ affinity of isolated cTnC. Phosphorylation of the monocysteine mutant of troponin I by protein kinase A resulted in a 5-fold decrease in the bimolecular rate constant but a 2-fold increase in the two observed Ca$^{2+}$ dissociation rates. These changes in the kinetic parameters are responsible for a 5-fold reduction in Ca$^{2+}$ affinity of phosphorylated troponin for the specific site.

Muscle contraction consists of a cascade of events involving several protein structural changes and protein-protein interactions within the thick and thin filaments (1). For contraction to occur, the N-terminal domain of the myosin heavy chain in the thick filament must first bind to actin in the thin filament. The formation of this active actomyosin complex is, however, inhibited by troponin I. This inhibitory action is regulated in vertebrate skeletal and cardiac muscle through the binding of calcium to another troponin subunit, troponin C. This Ca$^{2+}$ binding releases the inhibition of formation of the actomyosin complex.

The crystal structures of a TnC$^1$ from turkey (2, 3) and chicken (4, 5) reveal a dumbbell-shaped molecule with two globular domains connected by a long central helix. Each domain contains two metal ion binding sites, designated as sites I and II in the N-domain and sites III and IV in the C-domain. Sites III and IV have a relatively high affinity for Ca$^{2+}$ ($K_{a}$ $\approx$ 10$^{5}$ M$^{-1}$) and also bind Mg$^{2+}$ competitively ($K_{a}$ $\approx$ 10$^{5}$ M$^{-1}$), and sites I and II have a lower Ca$^{2+}$ affinity and are specific for Ca$^{2+}$ ($K_{a}$ $\approx$ 10$^{6}$ M$^{-1}$). Current evidence indicates that the two Ca/Mg sites in the C-domain most likely play a structural role, and the Ca$^{2+}$-specific sites in the N-domain carry out a regulatory function.

Cardiac muscle troponin C differs from the skeletal muscle isoform in that site I cannot bind Ca$^{2+}$ due to several amino acid substitutions in critical positions within the 12-residue Ca$^{2+}$-binding loop. Although the three-dimensional structure of TnC has not been determined, it is reasonable to assume, on the basis of sequence homology and similarities in physiologic functions, that its structure is similar to that of sTnC. Reversible Ca$^{2+}$ binding to the single Ca$^{2+}$-specific site is believed to induce conformational changes in the N-domain, and these changes appear to modulate the troponin C-troponin I interaction. The precise nature of these conformational changes is still obscure. A useful biophysical approach to delineate Ca$^{2+}$-induced global structural changes is the use of extrinsic fluorescent probes that are attached to TnC. TnC contains two cysteine residues, Cys-35 located in the nonfunctional Ca$^{2+}$ binding loop I and Cys-84 located in the C-terminal end of helix D where the TnC-TnC interaction occurs. In a previous study of two monocysteine mutants of TnC (6), we showed that the probe IAANS covalently linked to Cys-84 of TnC is partially buried into troponin, the probe attached to the two cysteine residues have very different properties. The fluorescence of IAANS attached to Cys-84 of cTnC within the troponin complex is insensitive to Ca$^{2+}$ binding, but the fluorescence of the probe attached to Cys-35 within the complex decreases by a factor of 3

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‡ The abbreviations used are: s, skeletal muscle; TnC, troponin C; Tn, troponin; TnI, troponin I; TnT, troponin T; c, cardiac muscle; PKA, protein kinase A; DTT, dithiothreitol; IAANS, 2-[(4'-iodoacetamido)anilino]naphthalene-6-sulfonic acid; MOPS, 3-(N-morpholino)propane-sulfonic acid.

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in response to Ca$^{2+}$ binding to the Ca$^{2+}$-specific site. Similar fluorescence properties of IAANS attached to Cys-35 are observed with the cTnC-cTnI complex. These results provide evidence of involvement of the non-functional binding loop I of cTnC in the Ca$^{2+}$-induced interaction between cTnC and cTnI. Several previous studies (7–10) reported the dissociation and association kinetics of calcium binding to both classes of sites in isolated sTnC and in regulatory complexes containing sTnC. The binding of Ca$^{2+}$ to the two regulatory sites was suggested to be diffusion controlled (10) on the basis of binding data obtained over a narrow range of [Ca$^{2+}$]. Our recent study of a monocysteine cTnC mutant labeled at Cys-84 with IAANS showed that the Ca$^{2+}$ binding mechanism is not diffusion controlled but must be described by a three-step sequential model (11). In the present work, we have extended this kinetic study of Ca$^{2+}$ binding to the Ca$^{2+}$-specific site of cTnC in reconstituted cardiac troponin. For this work, we used the monocysteine cTnC mutant cTnC(C84S) labeled at Cys-35 with IAANS. The overall kinetic mechanism is similar to that for Ca$^{2+}$ binding to isolated cTnC, but there are significant differences in the rates of individual steps. Phosphorylation of cTnI is found to affect the rates of both Ca$^{2+}$ binding to and dissociation from the Ca$^{2+}$-specific site in troponin.

MATERIALS AND METHODS

Preparation of Cardiac Subunits—cTn was extracted from an ether powder that was prepared from the left ventricles of fresh bovine hearts (12). Troponin subunits were initially separated on a CM-Sephadex C-50–120 column in the presence of 6 M urea, 50 mM citrate, pH 6.0, 1 mM EDTA, and 1 mM DTT. Crude cTnC, cTnI, and cTnT were pooled and subsequently purified, separately, on a DEAE-Sephadex A-50 column in the presence of 6 M urea. cTnC was eluted with a gradient from 0 to 0.5 M KCl at pH 7.0, while cTnI and cTnT were eluted at pH 8.0 with a gradient from 0 to 0.5 M KCl. The purity of the proteins was monitored by SDS-polyacrylamide gel electrophoresis. The purified proteins were lyophilized in the presence of 0.1 M KCl, 0.5 mM EGTA, 0.5 mM DTT, 20 mM imidazole, pH 7.2, and stored at −20 °C.

Preparation of Labeled cTnC Mutant—Cardiac TnC mutant, cTnC(C84S), was genetically generated and characterized as previously reported (11). The mutant was labeled at Cys-35, as described previously (6), with the fluorescent probe IAANS under denatured conditions, and the degree of labeling was found to be >95%.

Troponin Reconstitution—The binary complex cTnC-cTnI and fully reconstituted cTn were prepared by incubating the IAANS-labeled cTnC mutant with a large excess of the other troponin subunits in buffer A (30 mM MOPS, pH 7.2, 1 mM DTT, and 50 mM CaCl$_2$) containing 6 M urea. After 30 min at room temperature, the solutions were dialyzed against buffer A, which also contained 3 M urea and 1 M KCl. The urea and KCl concentrations were then reduced by changing the dialysate to solutions of buffer A containing no urea and a decreasing concentration of KCl in five steps: 1, 0.7, 0.5, 0.3, and 0.1 M KCl. Uncomplexed cTnI and cTnT precipitated during the dialysis in decreasing [KCl] and the precipitate were removed by centrifugation. The samples were then dialyzed against a solution containing 30 mM MOPS, pH 7.2, 1 mM DTT, 1 mM EGTA, and 0.3 M KCl (standard buffer).

Phosphorylation of cTnI—cTnI was phosphorylated by protein kinase A in a medium containing 50 mM KH$_2$PO$_4$, pH 7.0, 0.5 mM EGTA, 0.5 mM DTT using 125 units of the catalytic subunit/mg of cTnI. The reaction was started by adding ATP to a final concentration of 0.5 mM, followed by incubation at 30 °C for 20 min. The solution was then dialysed exhaustively at 4 °C against the standard buffer. Previous studies have shown that this protocol produces about 90% phosphorylation of PKA sites in cTnI (13).

Fluorescence Measurements—Steady-state fluorescence measurements were carried out at 20 ± 0.1 °C on an SLM 8000C spectrophotometer. The bandpass of both the excitation and emission monochromators was set at 0 nm, and the measurements were made in the ratio mode. Emission spectra were corrected for variations of the detector system with wavelengths. Fluorescence quantum yields of IAANS were measured by the comparative method as in previous work (6). A standard (15) calcium solution (Orion) was used in Ca$^{2+}$ titration experiments as described previously (11). EGTA was used to control the level of free calcium, and the free calcium concentrations were calculated by using known stability constants of the chelator for cations and proton as given in Fabiato's algorithm (14). The same procedure was used to calculate free calcium concentration for the kinetic measurements described below.

Kinetic Measurements—Transient kinetic measurements were performed at 4 °C on a Hi-Tech Scientific PQ/SF-53 stopped-flow spectrometer equipped with a 150 watt xenon lamp. The dead time of the instrument was determined to be 1.8 ms. For measurements of IAANS fluorescence, the excitation monochromator was set at 325 nm, and the emitted light was isolated at a right angle by a cut-off filter (OG 380) and detected by an EM19798QB photomultiplier. To measure Quin-2 fluorescence, the excitation wavelength was 339 nm, and the emission was measured with a 445-nm cut-off filter.

In a typical binding experiment, one syringe contained a labeled protein complex in the standard buffer containing 5 mM EGTA and 3 mM MgCl$_2$. The other syringe contained the same solution, but without protein, plus different levels of free Ca$^{2+}$ (0 to 500 μM). The concentration of the protein was about 1 μM after mixing. These conditions ensured that the two C-terminal high affinity Ca/Mg sites were saturated by Mg$^{2+}$ and that the single low affinity Ca$^{2+}$-specific site remained free prior to mixing. The free [Ca$^{2+}$] was controlled by EGTA. This control was adequate when the total Ca$^{2+}$ concentration was low, and this allowed calculation of the second-order binding rate constant from rate data obtained at low free [Ca$^{2+}$] (8, 15). Dissociation of bound Ca$^{2+}$ was measured by mixing a buffer containing EGTA with an equal volume of labeled protein saturated with Ca$^{2+}$ as described previously (11). The second-order rate constant of Ca$^{2+}$-binding to EGTA is about 10$^{-5}$ M$^{-1}$ s$^{-1}$ (8). This rate is considerably slower than the second-order rate constant of Ca$^{2+}$-binding to cTnI. The rate constant of Ca$^{2+}$-dissociation from EGTA is 0.3–0.4 s$^{-1}$ (8), much slower than the binding rate of Ca$^{2+}$-binding to cTnC. The rates involving EGTA would not contribute to the observed cTnC rates. A number of control experiments were carried out using Quin-2 to directly measure Ca$^{2+}$ dissociation (8, 15).

In these measurements, Ca$^{2+}$ dissociation was monitored by the increase of Quin-2 fluorescence. The final concentrations were 30 mM MOPS, pH 7.2, 0.3 mM KCl, 3 mM MgCl$_2$, 6 μM protein, 50 μM Ca$^{2+}$, and 150 μM Quin-2. For each experimental condition, multiple tracings (8–10) were obtained, the tracings were averaged, and the resulting tracing was fitted to a sum of exponentials by a nonlinear least-squares method (16).

RESULTS

Steady-state Fluorescence—Fig. 1 shows the fluorescence emission spectra of cTn reconstituted with the IAANS-labeled cTnC mutant. In the presence of Mg$^{2+}$, the intensity at the
emission maximum decreased by about 5% with a negligible spectral shift. Ca\textsuperscript{2+} red-shifted the spectrum by 9 nm and decreased the quantum yield by a factor of 3. Similar Mg\textsuperscript{2+} and Ca\textsuperscript{2+}-induced spectral changes were observed for the binary complex cTnC-cTnI prepared with the IAANS-labeled cTnC mutant. Table I summarizes the spectral properties of these samples and the corresponding samples prepared with cTnI phosphorylated by PKA.

The binding of Ca\textsuperscript{2+} to cTn and cTnC-cTnI was monitored by the increase in the fluorescence intensity of the IAANS-labeled cTnC. The apparent Ca\textsuperscript{2+} binding constants were determined from the half-maximum increase in the fluorescence (data not shown). These equilibrium constants are listed in Table II.

**Kinetics of Ca\textsuperscript{2+} Binding to the Regulatory Site of Cardiac Troponin**—The kinetics of Ca\textsuperscript{2+} binding to the regulatory site of cardiac troponin, cTn, and cTnC was investigated. The fluorescence transients showing the time course of the change in fluorescence intensity are presented in Fig. 2. Below 1 \(\mu\)M Ca\textsuperscript{2+}, the transients could be fitted to a single exponential, above 1 \(\mu\)M, two exponential terms were required. Fig. 3 shows a tracing obtained at 1 \(\mu\)M Ca\textsuperscript{2+}. A one-exponential fit clearly was not acceptable, but an improved and acceptable fit was obtained with two exponential terms. The faster rate constant (\(\lambda_1\)) increased with [Ca\textsuperscript{2+}], whereas the slower rate constant (\(\lambda_2\)) did not depend on [Ca\textsuperscript{2+}]. The Ca\textsuperscript{2+} dependence of these two rate constants is shown in Fig. 4A. The value of \(\lambda_1\) was about 3–5 s\(^{-1}\) over the entire range of [Ca\textsuperscript{2+}]. The rate of the fast phase reached a maximum value of about 34–37 s\(^{-1}\) at large [Ca\textsuperscript{2+}], and the initial slope of this plot (Fig. 4B) was 5.1 \(\times\) 10\(^{-3}\) s\(^{-1}\). The amplitude of \(\lambda_1\) varied from 85 to 60% of the total signal above 1 \(\mu\)M Ca\textsuperscript{2+}. The ratio of the amplitude of \(\lambda_1\) to \(\lambda_2\) increased from less than 0.2 to a limiting value of about 0.55 with increasing Ca\textsuperscript{2+} concentration, and the shape of a plot of this amplitude ratio versus [Ca\textsuperscript{2+}] resembled the \(\lambda_1\) versus [Ca\textsuperscript{2+}] plot shown in Fig. 4A.

### Table I

Fluorescence properties of mutant cTnC(C84S) labelled with IAANS at Cys-35.

| Sample         | \(\lambda_{max}\) | Q  | \(\lambda_{max}\) | Q  |
|---------------|-----------------|---|-----------------|---|
| cTnC mutant   | 0.003           | 480 0.027 | 462 0.029 |
| cTnC-cTnI     | 0.098           | 451 0.083 | 459 0.033 |
| cTn           | 0.096           | 452 0.089 | 460 0.032 |
| p(cTnC-cTnI)  | 0.092           | 452 0.087 | 458 0.050 |
| p(cTnI)       | 0.089           | 451 0.086 | 458 0.053 |

### Table II

**Kinetic and equilibrium parameters for the binding of Ca\textsuperscript{2+} to troponin**

For comparison, the parameters for cTnC taken from Dong et al. (1996) are included. The equilibrium binding constants (\(K_{eq}\)) were obtained from titration curves similar to those shown in Fig. 2; the numbers in parentheses are the Hill coefficient from the least squares fits of the titration data. The kinetic measurements were made at pH 7.2 and 4 \(^\circ\)C; see legends to Figs. 3 and 4 for other conditions. \(\lambda_{a1}\) and \(\lambda_{a2}\) are the two rates observed in Ca\textsuperscript{2+} dissociation experiments. The other parameters are derived from observed rate data for Scheme I.

| Sample         | \(\lambda_{a1}\) | \(\lambda_{a2}\) | \(K_{a1}\) | \(K_{a2}\) | \(k_1\) | \(k_2\) | \(K_{eq}\) |
|---------------|-----------------|-----------------|----------|----------|--------|--------|---------|
| cTnC mutant   | 101.0           | 14 \(\times\) 10\(^7\) | 0.30 \(\times\) 10\(^6\) | 470      | 117    | 80     | 3.2 (1.2) |
| cTnC-cTnI     | 22.6            | 4.3             | 5.9 \(\times\) 10\(^7\) | 22.9 \(\times\) 10\(^6\) | 27.7    | 10\(^7\) | 2.4     | 7.0 (1.5) |
| cTn           | 20.6            | 4.7             | 5.1 \(\times\) 10\(^7\) | 2.2 \(\times\) 10\(^6\) | 23.3    | 13.3   | 2.7     | 47.3 (1.6) |
| p(cTnC-cTnI)  | 58.0            | 10.1            | 2.2 \(\times\) 10\(^7\) | 0.37 \(\times\) 10\(^6\) | 58.3    | 30.3   | 20.1    | 14.9 (1.3) |
| p(cTnI)       | 53.0            | 9.3             | 1.7 \(\times\) 10\(^7\) | 0.33 \(\times\) 10\(^6\) | 52.0    | 28.2   | 20.0    | 14.0 (1.4) |

(data not shown). When cTn was reconstituted with phosphorylated cTnI, the kinetics was qualitatively unchanged. The fluorescence transients were also biphasic above 1 \(\mu\)M Ca\textsuperscript{2+}, with the slower rate constant of 5–8 s\(^{-1}\) that was insensitive to [Ca\textsuperscript{2+}]. The faster rate increased with increasing [Ca\textsuperscript{2+}], reaching a maximum value (about 80 s\(^{-1}\)) that is about a factor of 2 faster than that of the nonphosphorylated cTn. The initial slope of this increase of \(\lambda_1\) was reduced to 1.7 \(\times\) 10\(^7\) s\(^{-1}\). These experiments were also carried out with the binary cTnC-cTnI complex, using both nonphosphorylated and phosphorylated cTnI. The results were very similar to those obtained with the fully reconstituted cTn, indicating that cTnI in cTn had negligible effects on the rate of Ca\textsuperscript{2+} binding to the regulatory site.

**Kinetics of Calcium Dissociation from the Regulatory Domain**—The kinetics of Ca\textsuperscript{2+} dissociation from the Ca\textsuperscript{2+}-specific site was measured with the cTnC-cTnI complex and reconstituted cTn, using IAANS-labeled cTnC. Upon mixing the Ca\textsuperscript{2+}-saturated proteins in the presence of Mg\textsuperscript{2+} with an EGTA buffer, the kinetic tracings showed a large positive amplitude change. The fluorescence increase was completed in about 60 ms when the troponin was reconstituted with nonphosphorylated cTnI, and 30 ms when reconstituted with phosphorylated cTnI (Figs. 5A and 6A). The fluorescence transients from the cTn reconstituted with nonphosphorylated cTnI could be fitted with a two-exponential function with two rate constants: \(\lambda_{a1} = 20.6 \pm 1\) with a 70% of the total fluorescence change, and \(\lambda_{a2} = 4.7 \pm 1\) with a 30% of the fluorescence change. When the cTn was reconstituted with phosphorylated cTnI, the rates of both phases increased by a factor of 2: \(\lambda_{a1} = 46.9 \pm 1\) (66% fluorescence change), and \(\lambda_{a2} = 8.9 \pm 1\) (34% fluorescence change).

The one-exponential fits of these transients were not satisfactory, as shown in Figs. 5B and 6B. Very similar results were obtained with the cTnC-cTnI complexes. These observed dissociation rates are listed in Table II. The present Ca\textsuperscript{2+} dissociation results are consistent with those of a previous study (13) in that cTnI phosphorylation by PKA enhanced the rate of Ca\textsuperscript{2+} release from cTn reconstituted with native cTnC that was doubly labeled with IAANS at Cys-35 and Cys-84. However, the previous study reported a single dissociation rate, in contrast to the biphasic dissociation reported here. The difference is unlikely due to the probe located at two positions in the previous study because IAANS attached to Cys-84 is Ca\textsuperscript{2+}-insensitive in the troponin complex (6).

To establish whether Ca\textsuperscript{2+} binding to the two high affinity Ca/Mg sites in the carboxyl-terminal domain contributed to the observed dissociation rates, a dissociation experiment was performed by mixing EDTA with IAANS-labeled cTnC-cTnI and cTn saturated with either Mg\textsuperscript{2+} or Ca\textsuperscript{2+} at the two Ca/Mg sites (11). No fluorescence change was detected, indicating that IAANS attached to Cys-35 was not sensitive to Ca\textsuperscript{2+} binding to the Ca/Mg sites. These results are in agreement with the equilibrium fluorescence results.

Experiments were performed using native proteins and...
Fig. 2. Stopped-flow kinetic tracings obtained at 4 °C by mixing cTn reconstituted with cTnC mutant labeled with IAANS at Cys-35 with an equal volume of the same buffer containing an increasing concentration of free Ca²⁺. Final conditions: 0.3 M KCl, 3 mM Mg²⁺, 30 mM MOPS, pH 7.2, 5 µM EGTA, and 1 µM protein. The tracings at different [Ca²⁺] were obtained from signal averaging of eight-to-ten separate tracings over the time interval 0–1.5 s.

Fig. 3. A typical kinetic tracing fitted to a one-exponential and a two-exponential function. The tracing was obtained as described in Fig. 2, and the free [Ca²⁺] after mixing was 1 µM. Panel A shows the two-exponential fit with rate constants \( \lambda_1 = 25.3 \text{ s}^{-1} \) (amplitude = 6.6) and \( \lambda_2 = 3.1 \text{ s}^{-1} \) (amplitude = 1.9), \( \chi^2_1 = 1.01 \). Panel B is the residual plot of the two-exponential fit. Panel C shows the residual plot for the best one-exponential fit with a rate constant of 15.2 s⁻¹ (the best fitted curve is not shown), \( \chi^2_2 = 1.99 \).

Fig. 4. Ca²⁺ dependence of the two observed first-order rate constants determined with reconstituted cTn in binding experiments. The two rate constants were determined at 4 °C from the fluorescence transients illustrated in Fig. 2 and plotted as a function of free [Ca²⁺]. Closed circles, the faster rate constant (λ₂); open circles, the slower rate constant (λ₁). Top panel, the data are shown for the entire range of [Ca²⁺] studied, and the solid curve was obtained by fitting the data of \( \lambda_2 \) to Equation 1. Bottom panel, \( \lambda_1 \) is shown over a narrow range of [Ca²⁺]. The slope is 5.1 × 10⁻¹ M⁻¹ s⁻¹.

Quin-2 as the fluorescent Ca²⁺ chelator to establish whether the IAANS fluorescence transients accurately reflected removal of bound Ca²⁺ from the Ca²⁺-specific site rather than conformational changes. The fluorescence of Quin-2 is expected to increase upon chelation of Ca²⁺ (8). When Quin-2 was mixed with cTnC previously incubated with an excess of Ca²⁺ sufficient to saturate all three sites, biphasic dissociation kinetic tracings with a positive amplitude were observed (data not shown). The two rate constants were 133 s⁻¹ (35% amplitude) and 7 s⁻¹ (65% amplitude). These amplitudes suggested that the fast phase reflected removal of bound Ca²⁺ from the single Ca²⁺-specific site, and the slow phase was associated with Ca²⁺ removal from the two Ca/Mg sites. These results were in agreement with previous Ca²⁺ dissociation rate data obtained from cTnC with Quin-2 as the chelator (15, 17). The assignment of the fast phase to Ca²⁺ removal from the specific site supports our previous conclusion that the single-exponential IAANS transient with cTnC-cTnI complex and reconstituted cTn, the Quin-2 transients were also biphasic: the fast rate constant (λ₁) being in the range of 33–38 s⁻¹ (amplitude A₁ = 26%), and the slow rate constant (λ₂) in the range of 2–4 s⁻¹ (amplitude A₂ = 74%). If Ca²⁺ dissociation from the specific site was in fact biphasic as the IAANS transients suggested, the Quin-2 amplitude change associated with this dissociation would be partitioned into two phases, with amplitudes A₁ and A₂, for the fast and slow phases, respectively. The sum of these amplitudes (A₁ + A₂) should be about one-third of the total amplitude. Thus, the amplitude A₂ should be less than 33%, approximately 20–25% on the basis of the biphasic IAANS amplitudes. The observed amplitude of the fast Quin-2 phase (A₁) was in agreement with this expectation. The rate of the slow phase associated with Ca²⁺ removal from the specific site and the rate of removal of Ca²⁺ from the Ca/Mg sites would be expected to be similar and not easily resolvable, and a single composite slow rate would be expected. The observed slow Quin-2 rate reflected this composite rate, and the associated amplitude would be larger than two-thirds because it contained contributions from the slow phase associated with the specific site and removal of Ca²⁺ from the other two sites. The observed amplitude...
A′<sub>ds</sub> is consistent with this prediction. Thus, the biphasic IAANS kinetics observed with cTn and the cTnC-cTnI complex was not limited by slow conformational changes and reflected closely Ca<sup>2+</sup> removal from the specific site.

Effect of Temperature on the Ca<sup>2+</sup> Binding Kinetics—The effect of temperature on the two Ca<sup>2+</sup> binding rates was studied with phosphorylated and nonphosphorylated cTn. From the Arrhenius plots of these data (data not shown), the following activation energies were obtained for the fast and slow phase: ΔE<sub>f</sub> = 55.3 ± 3.2 kJ/mol and ΔE<sub>s</sub> = 46.4 ± 2.7 kJ/mol for nonphosphorylated cTn, and ΔE<sub>f</sub> = 33.2 ± 2.5 kJ/mol and ΔE<sub>s</sub> = 31.9 kJ/mol for phosphorylated cTn.

**DISCUSSION**

Native cTnC has two cysteine residues at positions 35 and 84. These two residues are readily alkylated by the fluorescent probe IAANS. The doubly labeled cTnC has been extensively used to study the equilibrium properties of Ca<sup>2+</sup> binding to the regulatory site of both isolated cTnC and cTnC reconstituted into myofibrils and skinned muscle fibers. In a previous study (6), we reported that the fluorescence of IAANS attached to Cys-84 of the monocysteine mutant cTnC(C35S) tracked Ca<sup>2+</sup>-binding to the regulatory site in isolated cTnC. The probe attached to Cys-84 became insensitive to Ca<sup>2+</sup> upon complex formation with either cTnI or cTnI plus cTnT. The fluorescence of the probe attached to Cys-35 in the mutant cTnC(C84S), however, was found to be sensitive to Ca<sup>2+</sup> binding to the regulatory site only upon reconstitution with cTnI or with both cTnI and cTnT. We report here the kinetics of Ca<sup>2+</sup> binding to and dissociation from the single Ca<sup>2+</sup>-specific site of cTnC reconstituted into the cTnI-cTnC and the troponin complex, using the mutant cTnC(C84S) labeled at Cys-35 with IAANS.

Cys-35 is located at the −y coordinate of the 12-residue inactive Ca<sup>2+</sup> binding loop of site I. The low fluorescence quantum yield of IAANS attached to this residue in the cTnC mutant clearly indicates a highly exposed environment. This property is little affected by Ca<sup>2+</sup> binding to the regulatory site in the isolated cTnC, but is significantly changed when the labeled mutant is reconstituted with the other troponin subunits. This change reflects the interaction between cTnI and the inactive binding loop I, which alters the local conformation of Cys-35. The presence of Mg<sup>2+</sup> has little effect on this new conformation, but Ca<sup>2+</sup> binding to the regulatory site of cTnC in the nonphosphorylated reconstituted complexes causes a significant conformational change which leads to a large reduction of fluorescence quantum yield. In phosphorylated troponin, this Ca<sup>2+</sup> induces a 2-fold reduction in quantum yield, indicating that the phosphorylation of cTnI at Ser-23 and Ser-24 affects Ca<sup>2+</sup>-induced conformational changes in the N-domain of cTnC in the complexes. These large Ca<sup>2+</sup>-induced changes in IAANS fluorescence provide a convenient signal to study the kinetics of Ca<sup>2+</sup> binding to troponin.

The Ca<sup>2+</sup> dependence of the two observed Ca<sup>2+</sup> binding rates is similar to that previously observed with uncomplexed cTnC.
(11) and suggests a similar kinetic scheme for the binding of Ca$^{2+}$ to both isolated cTnC and cardiac troponin (cTn).

\[
\begin{align*}
\text{Ca} + \text{cTn} & \quad \xrightarrow{K_1} \quad \text{Ca-cTn} \\
& \quad \xrightarrow{k_1} \quad \text{(Ca-cTn)}^* \\
& \quad \xrightarrow{k_2} \quad \text{(Ca-cTn)**}
\end{align*}
\]

Scheme I

The initial binding step is assumed to be a rapid equilibrium, which is characterized by $K_1$, followed by two sequential first-order transitions in which the probe fluorescence changes. If $k_1 k_{-1} \gg k_2 + k_{-2}$, the normalized fluorescence transient can be described by two exponential terms (11). The rate constants of the fluorescence transient are identified with $\lambda_1$ and $\lambda_2$. The relationships between the observed rate constants and the kinetic parameters in Scheme I are as follows.

\[
\lambda_1 = \left( \frac{K_1[\text{Ca}]}{1 + K_1[\text{Ca}]} \right) k_1 + k_{-1} \quad (\text{Eq. 1})
\]

and

\[
\lambda_2 = k_2 + k_{-2} \quad (\text{Eq. 2})
\]

The plot of $\lambda_1$ versus $[\text{Ca}^{2+}]$ shown in Fig. 4A is analyzed by Equation 1. The limiting value of $\lambda_1$ at large $[\text{Ca}^{2+}]$ is $36.5 \, s^{-1}$, and extrapolation to $[\text{Ca}^{2+}] = 0$ yields $k_{-1} = 13.3 \, s^{-1}$. Since $\lambda_1 = k_1 + k_{-1}$ at large $[\text{Ca}^{2+}]$, $k_1 = 23.2 \, s^{-1}$. The initial slope of Fig. 4A defines the second-order binding rate constant, $K_1$. Thus, $K_1 k_1 = 5.1 \times 10^7 \, M^{-1} \, s^{-1}$ and $K_1 = 2.2 \times 10^6 \, M^{-1}$. The sum of $k_2 + k_{-2}$ is approximately 5 s$^{-1}$ and their individual values can be estimated from $K_{1}\text{eq} = K_1(k_1/k_{-1})(k_2/k_{-2})$, where $K_{1}\text{eq}$ is the experimental equilibrium constant of Ca$^{2+}$ binding to the Ca$^{2+}$-specific site. These kinetic and equilibrium parameters are given in Table II for the cTnC-cTnI complex and reconstituted cTn. The corresponding parameters for isolated cTnC are also included in Table II for easy comparison.

The overall kinetic mechanism for the binding of Ca$^{2+}$ to the regulatory site of cTnC is similar for isolated cTnC and reconstituted cTn, but there are significant quantitative differences. With cTn, both the limiting value of the observed fast binding rate and the slow Ca$^{2+}$-insensitive binding rate are reduced by more than 1 order of magnitude. These lower rates may be related to the higher activation energies of the two phases when compared with the activation energies previously observed for isolated cTnC (11). These differences lead to a second-order binding rate constant ($K_1 k_1$) that is about 3-fold smaller. The rates of the first-order transitions, however, are more than 10-fold reduced. Our previous spectroscopic study suggested that these movements may be less extensive in cTn than as suggested by the 4Ca$^{2+}$ model of isolated troponin C (6). At a saturating level of Ca$^{2+}$ and 4°C, the binding transitions in cTn would take about 40 ms to reach a 95% completion, and the dissociation would take about 50 ms. At elevated temperatures, these events could be faster. These time constants are likely more compatible with physiologic events than those previously determined with cTnC.

Having elucidated the kinetic mechanism of Ca$^{2+}$ binding to cTn, we then examined the effect of phosphorylation of cTnI by PKA on the various steps of the mechanism and the contributions of the kinetic steps to the equilibrium Ca$^{2+}$ binding constant. The phosphorylation results in a 2–3-fold increase of the bimolecular binding rate constant and the two first-order rate constants associated with step 1. The rate constants of step 2 are increased by a factor of 7–8. The net effect of these changes is a substantial decrease in $\lambda_1$ and reduction of the overall equilibrium binding constant. The latter result is consistent with a similar decrease in the equilibrium constant for formation of the cTnC-cTnI complex resulting from phosphorylation of cTnI (21). It is also in agreement with previous reports of decreased Ca$^{2+}$ affinity of cTn upon complex formation with phosphorylated cTnI (13). The present results also corroborate the previous report that cTn phosphorylation results in an increase in the observed rate of Ca$^{2+}$ removal from cTn. This phosphorylation effect, however, is not limited to the rate of Ca$^{2+}$ dissociation; it also reduces the bimolecular Ca$^{2+}$ binding rate. These kinetic effects may be related to a new conformation of cTn. The hydrodynamic shape of cTn is less asymmetric in the phosphorylated state than in the nonphosphorylated state, as suggested by a smaller rotational correlation time of the phosphorylated protein (22). Consistent with this finding, phosphorylation of a mutant cTnI by PKA has recently been shown to shorten the mean distance between Trp-192 and Cys-5 by 8–9 Å, which is accompanied by a considerable nar-
rowing of the distribution of the distances between the two sites (23). Similar phosphorylation-induced decreases in the distance parameters of cTnI also occur in the cTnC-cTnI complex in the presence of Mg$^{2+}$ and Ca$^{2+}$. This substantial decrease in distance likely arises from a folding of the N-terminal extension of cTnI that contains the two unique PKA target sites (Ser-23 and Ser-24). The functional role of PKA phosphorylation of cTnI has been amply demonstrated (24). Since this phosphorylation is thought to mediate the dynamics of Ca$^{2+}$ binding to the regulatory site, it is important to delineate the kinetic mechanism by which the dynamics of Ca$^{2+}$ binding is modulated. The present study provides this information. What is unresolved at this time is whether the folded N-terminal segment is sufficient to bring about the observed changes in both the binding and dissociation kinetics.

In conclusion, we have used the fluorescence of an extrinsic probe linked to Cys-35 of a monocysteine TnC mutant to study the kinetics of Ca$^{2+}$ binding to cardiac muscle troponin complexes. The kinetics is consistent with a three-step sequential mechanism. Phosphorylation of cTnI by PKA alters both the binding and dissociation rates but not the overall kinetic mechanism.

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