Kinetic Studies of Calcium Binding to the Regulatory Site of Troponin C from Cardiac Muscle*  

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We have studied the kinetics of the structural transitions induced by calcium binding to the single, regulatory site of cardiac troponin C by measuring the rates of calcium-mediated fluorescence changes with a monocysteine mutant of the protein (C35S) specifically labeled at Cys-84 with the fluorescent probe 2-[4’-(iodoacetamido)anilino]naphthalene-6-sulfonic acid; IPTG, isopropyl-1-thio-

β-galactopyranoside; Mops, 3-(N-morpholino)propanesulfonic acid; PCR, polymerase chain reaction.  

Troponin C (TnC)1 is the calcium-binding subunit of the three-subunit troponin complex, which, together with tropomyosin, constitutes the regulatory system in vertebrate skeletal and cardiac muscle. TnC from skeletal muscle has two classes of calcium-binding sites. The two carboxyl-terminal sites (sites 3 and 4) bind calcium with a high affinity ($K_{Ca} \approx 2 \times 10^7$ M$^{-1}$) and also bind magnesium competitively with a lower affinity ($K_{Mg} \approx 5 \times 10^3$ M$^{-1}$) (Leavis et al., 1978). These two sites appear to have a structural role and do not contribute to calcium-dependent regulation (Potter and Gergely, 1975; Leavis et al., 1978), and are the sites that regulate contraction. TnC from cardiac muscle differs from skeletal muscle TnC most significantly in the amino-terminal half, where several critical amino acid substitutions have rendered site 1 incapable of binding calcium (Van Eerd and Takahashi, 1976; Leavis and Kraft, 1978). The calcium affinities of the two classes of binding sites in cTnC (Holroyde et al., 1980) are essentially the same as the corresponding ones for skeletal muscle TnC.

The x-ray crystallographic structures of TnC from chicken (Sundaralingam et al., 1985) and turkey skeletal muscle (Herzberg and James, 1985) show that the protein has an elongated, dumbbell-like shape in which the polypeptide is folded into two globular domains at the amino- and carboxyl-terminal ends. These domains are connected by a long $a$-helix, the middle of which is exposed to the solvent. The crystal structure provides a structural basis for understanding the potential conformational changes that may occur in the regulatory, amino-terminal domain of TnC when calcium binds to sites 1 and 2 (Herzberg et al., 1986; Strynadk and James, 1989). These putative conformational changes may involve movements of helices B and C relative to helices A and D. It has been suggested that these movements expose a segment of hydrophobic residues in the amino-terminal domain, which then become available for calcium-mediated interaction with TnI. Fluorescence (Wang and Cheung, 1986; Tao et al., 1990) and thermodynamic (Wang and Cheung, 1985; Cheung et al., 1987) studies have suggested that the interaction between TnC and TnI may serve as the Ca$^{2+}$ switch for calcium-dependent regulation of contraction.  

Maximum tension in fast skeletal muscle is observed in 10–13 ms after excitation and decays after an additional 40–50 ms (Close, 1965). During a cycle of contraction and relaxation, calcium must bind to the regulatory sites and induce structural changes in both TnC and the other thin filament regulatory proteins within 10–13 ms after excitation. Likewise, calcium dissociation from the regulatory sites and reversal of the calcium-induced conformational changes must occur within 40–50 ms after excitation. These physiologic constraints place limits within which reversible Ca$^{2+}$ binding to TnC and subsequent conformational changes must occur. Several groups (Iio and Kondo, 1982; Rosenfeld and Taylor, 1985a; Johnson et al., 1994) reported the kinetics of calcium binding to both classes of
sites in isolated skeletal TnC and in regulatory complexes containing TnC. Calcium binding to the low affinity sites of skeletal TnC has been reported to be diffusion controlled (Johnso et al., 1994). However, given the fact that most protein conformational changes occur more slowly than this, it would be expected that the kinetics of calcium binding to the regulatory sites should be saturable. Little information is available on the kinetic mechanism of calcium binding to the regulatory site of cardiac TnC, as most of the reported information is confined to the dissociation kinetics (Robertson et al., 1981, 1982).

Cardiac TnC has two cysteine residues that can be readily alkylated by a variety of sulfhydryl-specific reagents. Mutants containing a single cysteine at either position 35 (C84S) or 84 (C35S) have been generated (Zhang et al., 1992). The calcium-induced fluorescence change of IAANS-labeled mutant cTnC(C35S) has been shown to be coincident with calcium-activated force development (Zhang et al., 1992; Kerrick et al., 1992) and ATPase activity (Kerrick et al., 1992) in skinned cardiac muscle fibers. An advantage of using IAANS-labeled mutant cTnC(C35S) to study calcium-induced structural changes is that the fluorophore is located at a single site, which simplifies interpretation of the observed fluorescence change in terms of structural transitions.

In the present work, we have studied the kinetics of calcium binding to the single regulatory site of cTnC using a monocysteine mutant labeled with IAANS at Cys-84. The observed kinetics suggest a three-step binding model. Because the IAANS probe is strategically located, the observed kinetics of the fluorescence change also provide insight into the time course of structural changes induced by calcium binding.

**MATERIALS AND METHODS**

Preparation of Cardiac TnC Mutants—The method of Saiki et al. (1985) was used to synthesize first strand cDNA from total RNA obtained from the left ventricle of rat heart (Chomczynski et al., 1989). The clone was ligated into the polylinker sites of the expression vector pET-24a(+) (Novagen). The recombinant DNA was transformed into Escherichia coli strain BL21(DE3) lysogen (Novagen) containing a gene induced by IPTG to synthesize T7 RNA polymerase, which transcribed the inert DNA in the vector. Transformed cells were purified on a DE52 column equilibrated in 6 M urea, 25 mM Mops at pH 7.0, 0.1 M KCl, and 2 mM EGTA at 4°C to remove unreacted fluorophore and DTT. A further dialysis against the same buffer without urea was repeated three times. The concentration of the labeled protein was determined with either a turbidimetric tannin micromethod (Mebayam-Katzenelenbogen and Dobryszycia, 1959) or the Bradford method (Bradford, 1976), and the amount of label covalently attached to the protein was determined by absorbance, using a molar extinction of 24,900 M⁻¹ cm⁻¹ at 325 nm (Johnson et al., 1980). The degree of labeling was >0.9 mol of fluorophore/mol of protein. The same procedure was used to modify the sulfhydryl group of mutant cTnC(C84S).

Fluorescence Measurements—Steady-state fluorescence measurements were carried out at 200 ± 0.1°C in an SLM 8000C spectrophotometer, with both monochromators set at a 3-nm bandwidth. All measurements were made with the ratio mode. The emission spectra were corrected for variations of the response of the detector system with wavelength. The Ca²⁺ concentration was monitored by using EGTA. Free calcium concentrations were calculated by an algorithm from Dr. A. Fabiato (1988), using known stability constants of the chelator for Ca²⁺.
Kinetic Model of Calcium Binding to Cardiac Troponin C

**Fig. 1.** Fluorescence emission spectra are shown of mutant C35S labeled with IAANS at Cys-84 in the presence of Mg$^{2+}$ or different concentrations of free Ca$^{2+}$ at 20°C, 5 μM protein in 2.0 mM EGTA, 0.2 mM KCl, and 30 mM Mops at pH 7.0. Inset, Ca$^{2+}$ titration of the labeled mutant carried out in the same buffer; λ$_{em}$ = 325 nm, and λ$_{ex}$ = 450 nm. The experimental points (filled circles) were analyzed by a nonlinear least squares procedure using the following equation:

$$F - F_0 = \sum_{i=1}^{n} \frac{F_i K_i^o [Ca]}{1 + F_i K_i^o [Ca]}$$

where $F$ and $F_0$ are the intensities determined at a given [Ca$^{2+}$] and [Ca$^{2+}$] = 0, respectively; $F_i$, $K_i^o$, and $n_i$ are the intensity change, the binding constant, and the Hill coefficient of the ith site, respectively. The data were adequately fitted with one term of the equation, yielding a single binding constant corresponding to $K_i^o = 5.78$ and $n = 1.2$.

Force was 92 restored, relative to intact fibers, upon reincorporation of native skeletal TnC and 86% restored upon reincorporation of wild-type cTnC. The force was 73% restored with mutant C35S and 62% restored with mutant C84S. These results indicate that the functional properties of mutant C35S are not significantly altered.

**Fluorescence Properties of cTnC Mutants—**Fig. 1 shows fluorescence emission spectra of mutant C35S labeled with IAANS at Cys-84 in the presence of Mg$^{2+}$ and different levels of free Ca$^{2+}$. The presence of 2 mM Mg$^{2+}$ reduced the intensity by about 10%. With increasing [Ca$^{2+}$] over the range of pCa 6.1–4.2, the intensity progressively increased by a factor of 1.6, with a small blue spectral shift of 6 nm. A calcium titration curve of the labeled mutant cTnC is shown in the inset of Fig. 1. The half-maximum increase in fluorescence occurred at pCa 5.78 with a Hill coefficient of 1.2, corresponding to an apparent dissociation constant of $6.1 \times 10^5$ M$^{-1}$. Control experiments with native cTnC IAANS yielded a Ca$^{2+}$ titration curve very similar to that shown in Fig. 1, with the half-maximum change in fluorescence at pCa 5.68, corresponding to an apparent association constant of $4.8 \times 10^5$ M$^{-1}$ and a 1.8-fold increase in maximum fluorescence (results not shown). These results indicate that the monocyteine mutant labeled at Cys-84 has similar fluorescence properties to native cTnC labeled at both Cys-35 and Cys-84. The values of the apparent affinities, as determined by fluorescence, are also similar to those determined by equilibrium dialysis. Mutant C84S labeled with IAANS at Cys-35 showed a small increase (<30%) in fluorescence over the pCa range of 8–6.7, followed by a decrease to the 10% level with further decrease in pCa. The insensitivity of labeled mutant C84S to Ca$^{2+}$ is in agreement with a previous report (Zhang et al., 1992).

**Kinetics of Ca$^{2+}$ Binding to the Regulatory Site of Mutant C35S—**The results from the Ca$^{2+}$ titration indicate that the fluorescence increase in mutant C35S labeled at Cys-84 is suitable for monitoring the kinetics of Ca$^{2+}$ binding to the regulatory site. Since our concern here was to monitor the binding kinetics of the regulatory site, it was necessary to block sites 3 and 4 by preincubation of the protein with an excess of Mg$^{2+}$ prior to mixing with Ca$^{2+}$. Two components with opposite amplitudes were observed after mixing magnesium-saturated IAANS-labeled cTnC(C35S) with saturating calcium. The first, consisting of a 1.6-fold fluorescence enhancement, was completed in <100 ms and corresponded to calcium binding to the regulatory site, while the second, consisting of a 10% fluorescence decrease, was observed over several seconds after the first component was completed and presumably was due to calcium displacement of magnesium from the high affinity sites. Thus, these two components were temporally well separated from each other. The following discussion will focus only on the first, positive amplitude phase. Fig. 2 shows kinetic tracings of this phase at increasing free Ca$^{2+}$ concentrations. At very low [Ca$^{2+}$] (<2 μM), these tracings could be fitted with a single exponential, but above 2 μM, fitting required two exponentials. Fig. 3 shows a typical tracing at [Ca$^{2+}$] >2 μM. The monoeponential fit was only marginally adequate with a χ square ratio of 1.51, but the biexponential fit was considerably improved with a χ square ratio of 1.07. A comparison of the
residual plots for the two fits also indicate an improvement of the biexponential fit over the monoexponential fit, particularly at early times.

Two observed rate constants were obtained above 2 \( \mu M \) calcium concentration. The fast rate constant, \( \lambda_f \), increased with increasing \( Ca^{2+} \) concentration, while the slow rate constant \( \lambda_s \) appeared to be insensitive to \( Ca^{2+} \) concentration. This is illustrated in Fig. 4A. The amplitude of the fast phase varied from about 60 to 30% of the total signal above 2 \( \mu M \) \( Ca^{2+} \). The ratio of the amplitude of the slow phase to that of the fast phase increased from less than 0.5 to a limiting value of about 2.5 with increasing \( Ca^{2+} \), and the shape of this plot of amplitude ratio (not shown) resembled that shown in Fig. 4A for \( \lambda_f \). The recovered limiting value of \( \lambda_s \) was close to the dead time of the instrument, and some signal of the fast phase (about 15%) was lost at higher \( Ca^{2+} \). This loss was reflected in the larger uncertainty for \( \lambda_s \) as indicated in Fig. 4A but in no way limited the analysis. The data of \( \lambda_f \) versus \( Ca^{2+} \) were fitted to Equation 2 with a maximum rate of 590 s\(^{-1}\). The initial slope of this plot (Fig. 4B) defines an apparent second-order rate constant of \( 1.4 \times 10^8 M^{-1} s^{-1} \) for calcium binding to the low affinity site.

Kinetics of Calcium Dissociation from the Amino-terminal and Carboxyl-terminal Domains—The kinetics of calcium dissociation from the low affinity site of cTnC was measured by mixing EGTA with cTnC saturated with \( Ca^{2+} \) in the presence of \( Mg^{2+} \). Upon mixing, there was a fast phase with a large negative amplitude (decreasing fluorescence), which was completed in about 20 ms at 20 \(^\circ\)C and 60 ms at 4 \(^\circ\)C (Fig. 5), followed by a slow phase with a small positive amplitude (increasing fluorescence), which was observed over several seconds (data not shown). Similar results were obtained in the absence of \( Mg^{2+} \). Both fast and slow phases each could be fitted adequately with a single exponential function. The rate constant for the fast phase was 102 s\(^{-1}\) at 4 \(^\circ\)C and 296 s\(^{-1}\) at 20 \(^\circ\)C, and the rate constant of the slow phase was 0.06 ± 0.06 s\(^{-1}\) (data not shown). To establish whether this slow phase arose from dissociation of bound \( Ca^{2+} \) at the two high affinity sites in the carboxyl-terminal domain, a dissociation experiment was performed by mixing EDTA with IAA-N-labeled mutant C35S, which was saturated with \( Mg^{2+} \) or \( Ca^{2+} \) at sites 3 and 4. The resulting kinetic tracings are shown in Fig. 6. The tracing for \( Mg^{2+} \) dissociation was adequately fitted to a single exponential function, with a rate constant of 0.83 s\(^{-1}\). This experiment was also carried out under identical conditions over a shorter time interval. There was no evidence of a fast phase with a negative amplitude, indicating that the slow fluorescence transient in Fig. 6 was due to dissociation of bound \( Mg^{2+} \) from the two carboxyl-terminal domain, high affinity sites. Also shown in Fig. 6 is a tracing obtained by mixing EDTA with labeled mutant C35S at pH 7.0. At this pH, sites 3 and 4 would be expected to be close to fully saturated with \( Ca^{2+} \), while site 2 would be \( Ca^{2+} \) free. The increase in fluorescence also followed a single exponential function with a rate constant of 0.73 s\(^{-1}\). A lag phase was apparent in both dissociation tracings. This could be due to different rates of \( Ca^{2+}/Mg^{2+} \) dissociation from the two high affinity sites (Rosenfeld and...
Ca\(^{2+}\) in the presence of Mg\(^{2+}\). Thus, Ca\(^{2+}\) and Mg\(^{2+}\) dissociated from the carboxyl-terminal domain sites with rates that were at least two orders of magnitude slower than dissociation of Ca\(^{2+}\) from the regulatory site.

Effect of Temperature on the Kinetics of Calcium Binding to the Regulatory Site—The experiment described for the binding of Ca\(^{2+}\) to the regulatory site was repeated at several temperatures and at a saturating level of Ca\(^{2+}\). The observed rate constants of the fast and slow phases are plotted versus reciprocal temperature, as shown in Fig. 7. The activation energy for the fast phase was 7.2 ± 0.4 kJ/mol and for the slow phase was 26 ± 3 kJ/mol.

**DISCUSSION**

In this paper, we have studied the kinetics of the binding of Ca\(^{2+}\) to the single regulatory site of cardiac TnC. Native cTnC labeled with IAA NS at both Cys-35 and Cys-84 has been extensively used to investigate the equilibrium properties of Ca\(^{2+}\) binding to the single regulatory site of the isolated protein and of the protein reconstituted into myofibrils and skinned muscle fibers. Because the label is located at two different sites that are far separated in the x-ray structure of TnC, the question arises as to whether the labels at both sites sense the same events. Using bacterially produced mutants containing a single cysteine residue, recent studies have shown that the fluorescence of IAA NS attached to Cys-84 in mutant C35S tracks Ca\(^{2+}\) binding to the regulatory sites with rates that were at least two orders of magnitude slower than dissociation of Ca\(^{2+}\) from the regulatory site.

FIG. 7. Arrhenius plots of the observed rate constants obtained from experiments of Ca\(^{2+}\) binding to the regulatory sites of IAA NS-labeled mutant C35S as described in Fig. 2. The rate constants were obtained at [Ca\(^{2+}\)] = 300 μM. The activation energy for the fast phase was 7.2 ± 0.4 kJ/mol and for the slow phase was 26 ± 3 kJ/mol. The coefficient of correlation was 0.99 for both lines.

Taylor, 1985a). The slower dissociation process might dominate the observed overall fluorescence change. Taken together, these results indicate that the dissociation of Mg\(^{2+}\) or Ca\(^{2+}\) from the carboxyl-terminal domain sites occurs with slow but very comparable rates. These rates were comparable to the rate (0.65 s\(^{-1}\)) of the slow phase with positive amplitude observed upon mixing EGTA with labeled mutant fully saturated with Ca\(^{2+}\) (Fig. 5). The final levels of fluorescence were not the same because the observed overall fluorescence change. Taken together, these events. Using bacterially produced mutants containing a single cysteine residue, recent studies have shown that the fluorescence of IAA NS attached to Cys-84 in mutant C35S tracks Ca\(^{2+}\) binding to the regulatory site and produces Ca\(^{2+}\) titration curves that superimpose on those for the Ca\(^{2+}\) activation of ATPase and force development in skinned muscle fibers (Zhang et al., 1992). As will be further elaborated below, Cys-84 is strategically located in the amino-terminal domain, and a probe linked to this position might sense Ca\(^{2+}\)-activated conformational changes involving specific helices.

To focus on the kinetics of Ca\(^{2+}\) binding to the regulatory site, the binding kinetics were studied in the presence of a large excess of Mg\(^{2+}\) so that the two carboxyl-terminal sites were occupied throughout the binding reaction. Since the dissociation of bound Mg\(^{2+}\) is at least two orders of magnitude slower than the binding of Ca\(^{2+}\) to the amino-terminal domain, this protocol enables us to follow the time course of the binding reaction directly. The kinetic transients for calcium binding to the low affinity site were biexponential at high Ca\(^{2+}\) concentration. The rate constant of the fast phase (λ\(_{f}\)) varies with increasing [Ca\(^{2+}\)] in a hyperbolic manner, whereas λ\(_{s}\) is insensitive to [Ca\(^{2+}\)]. We propose a three-step mechanism to account...
for these kinetic data:

\[
K_0 = k_0, \quad k_1 = k_1, \quad k_2 = k_2
\]

\[
Ca + TnC \rightarrow CaTnC \rightarrow (CaTnC)^+ \rightarrow (CaTnC)^{**}
\]

\[
\text{Scheme I.}
\]

The initial binding in the bimolecular step is assumed to be a rapid equilibrium, which is characterized by a rate constant \(k_0\). The normalized fluorescence transient can be described by two exponential terms (Bernasconi, 1976). The rate constants from the fluorescence transient are identified with \(\lambda_1\) and \(\lambda_2\). The relationships of the observed rate constants to the kinetic parameters in Scheme I are as follows:

\[
\lambda_1 = \left( \frac{k_1}{1 + k_2} \right) k_1 k_1 \]  \hspace{1cm} (Eq. 2)

\[
\lambda_2 = k_2 \]  \hspace{1cm} (Eq. 3)

The initial slope of a plot of \(\lambda_1\) versus \([Ca^{2+}]\) (Fig. 4) yields an apparent second order association rate constant \(K_{J_1}\) of \(1.4 \times 10^{7} \text{M}^{-1} \text{s}^{-1}\). Extrapolation to \([Ca^{2+}] = 0\) yields \(k_{-1} = 117 \text{ s}^{-1}\). At large \([Ca^{2+}]\), \(\lambda_1 = k_1 + k_{-1} = 587 \text{ s}^{-1}\). Thus, \(k_1 = 470 \text{ s}^{-1}\), \(k_{-1} = 117 \text{ s}^{-1}\), and \(K_0 = 2.95 \times 10^{8} \text{M}^{-1}\) at \(4°C\). The sum of \(k_2\) and \(k_{-2}\) is approximately 100 \text{ s}^{-1}. While their individual values cannot be directly extracted from the present kinetic data, they can be estimated from the experimental value of the equilibrium constant \(K_{eq} = 3.2 \times 10^{9} \text{M}^{-1}\) obtained from the Ca²⁺ titration at the same temperature: \(K_{f1,2} = \frac{k_2}{k_{-2}}(k_1/k_{-1})\). This yields \(k_2 = 20 \text{ s}^{-1}\) and \(k_{-2} = 80 \text{ s}^{-1}\). These parameters are listed in Table I. Alternatively, Scheme I can be treated as described previously (Benson, 1960), and similar conclusions are obtained. The present model is the first kinetic mechanism for cardiac TnC using any preparation of the protein.

The kinetic data indicate that step 1 is thermodynamically favorable with an equilibrium constant of about 4, but step 2 is unfavorable. This is consistent with a 3.5-fold higher activation for slow phase. Equilibrium studies have shown that the \(K_{eq}\) for calcium binding to the regulatory site of a TnC-TnI complex is considerably larger than that for isolated TnC. If the same kinetic scheme holds for the complex, this enhanced affinity must arise from increases in the equilibrium constants of one or all steps of the model. Since step 2 is unfavorable with isolated TnC, this step may become more favorable in the binary protein complex. This possibility can be tested with either the cTnC-TnI complex or reconstituted troponin.

The dissociation kinetics of Ca²⁺ from the regulatory site are monoeponential at both 4 and 20 °C. Step 1 is thermodynamically favorable, but step 2 is unfavorable with an equilibrium constant of 0.25. The observed initial fluorescence change in dissociation kinetics is expected to arise from the dominant species, (CaTnC)⁰, with only a small contribution from (CaTnC)⁰. Thus, the observed dissociation rate constant of 102 s⁻¹ largely reflects \(k_{-1}\). Furthermore, the dissociation rate constant for the (CaTnC)⁰ state is predicted to be \(k_{-1}k_{-2}/(k_{-1} + k_{-2})\), which is approximately 50-60 s⁻¹ (Trybus and Taylor, 1982). It would be unlikely that a small amplitude phase in the dissociation transient occurring at 50-60 s⁻¹ could be resolved from the main phase, occurring at 117 s⁻¹ determined from the intercept in Fig. 4B.

The x-ray structure of skeletal TnC shows that in the amino-terminal domain Cys-84 is located on helix D and is surrounded by helices B and C. The calcium-saturated structural model of the amino-terminal domain suggests that Ca²⁺ binding to the two amino-terminal domain sites induces movements of helices B and C relative to helices A and D, such that two EF hands are formed. These movements result in a more “open” conformation of the amino-terminal domain (Strynadk and J ames, 1989) and expose a hydrophobic patch on helix B. This exposed patch may be a site for calcium-mediated interactions with troponin I. In this model, helix D in the 4Ca state is stationary relative to the 2Ca state. A recent study of cTnC suggests similar helix movements in the amino-terminal domain when the single regulatory site is occupied (Ovsak and Taskinen, 1991). These models suggest that the observed Ca²⁺-mediated fluorescence enhancement of IAANS attached to Cys-84 on helix D may result from movements of helices B and C away from helix D. A recent study of cTnC with fluorescent probes, showed that the Ca²⁺-mediated enhancement of probe fluorescence was related to strong internal quenching by interactions of a dipolar nature in the apo and 2Mg states. This quenching is reduced or eliminated upon reorientation of helices B and C from helix D. This lends further support to the interpretation that the two first-order transitions that lead to fluorescence enhancement of the IAANS probe may reflect these helix movements. It is not known whether the movements actually occur in two steps, but it is unlikely that they move independently of each other because only the first fluorescence transition is Ca²⁺dependent. At a saturating level of Ca²⁺, these transitions would take about 5 ms to reach a 95% completion. This rate and the observed dissociation rate are likely overestimates of the rates that would occur in muscle. It is well known that both the time-to-peak-tension after excitation and the relaxation time can vary widely with temperature, and they are species-dependent. These time windows are generally longer in cardiac muscle than skeletal muscle, but structural transitions occurring in 5 ms in isolated cTnC may be too fast to be compatible with physiologic events. However, the Ca²⁺ on- and off-rates in reconstituted troponin are expected to be slower, as the Ca²⁺ dissociation rate from the regulatory site in the TnCIAANS-cTnI complex is only 21 s⁻¹ at 20 °C (Robertson et al., 1982), a factor of 10 slower than with cTnC determined in the present work. The on-rate in reconstituted troponin or in the reconstituted thin filament can also be expected to be significantly slower (Robertson et al., 1981). If tension transients follow Ca²⁺ transients, this anticipated reduction in the rates of reversible Ca²⁺ binding should still be fast enough to support contraction.

In contrast to cardiac TnC, several studies on the kinetics of Ca²⁺ binding to skeletal TnC have been reported. The early studies of Iio and Kondo (1980a, 1980b, 1982), based on tyro-

### Table I

| Parameter           | Unit | Value     |
|---------------------|------|-----------|
| \(K_{eq}\)         | M⁻¹  | 3.2 \times 10⁶ |
| \(k_{-1}\)         | s⁻¹  | 117       |
| \(k_{-2}\)         | s⁻¹  | 80        |
| \(k_0\)            | M⁻¹  | 2.95 \times 10⁸ |
| \(k_1\)            | s⁻¹  | 470       |
| \(k_2\)            | s⁻¹  | 20        |

The parameters were derived from kinetic measurements made at 4 °C with cTnC mutant C3S labeled at Cys-84 with IAANS. The overall equilibrium constant \(K_{eq}\) was obtained from Ca²⁺ titration experiments under identical conditions.
sine fluorescence, proposed a rapid diffusion-controlled binding step followed by conformational changes. Rosenfeld and Taylor (1985a, 1985b) investigated the binding of Ca\(^{2+}\) to both classes of sites with isolated skeletal TnC and reconstituted troponin, using a fluorescently labeled preparation. A two-step binding mechanism with a second-order rate constant of \(1.3 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\) was proposed for Ca\(^{2+}\) binding to the high affinity sites. This value is about 100 times lower than that for Ca\(^{2+}\) binding to the low affinity site in cTnC determined in the present work. Rosenfeld and Taylor (1985a, 1985b) also reported very fast Ca\(^{2+}\) binding rates to the regulatory sites at low calcium concentration, and the rates at high [Ca\(^{2+}\)] became too large to be measured. It could not be determined whether the rate reached a maximum, and the binding data did not establish whether the binding kinetics could be described by a two-step or a three-step model. However, the rates increased linearly with increasing [Ca\(^{2+}\)] at low concentration, and an apparent second-order binding rate constant of \(5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) was obtained. This is at least one order of magnitude larger than the corresponding rate at the high affinity sites determined using the same protein preparation. More recently, Johnson et al. (1994) reported the kinetics of Ca\(^{2+}\) binding to the two regulatory sites with a tryptophan mutant of skeletal TnC(F29W). They observed monoexponential kinetic traces over a narrow range of [Ca\(^{2+}\)] \((0–6 \mu\text{M})\) and assumed a simple one-step, diffusion-controlled mechanism for the binding of Ca\(^{2+}\) to the two regulatory sites. Since no data were reported at higher Ca\(^{2+}\) concentrations, it is not known whether the observed rate would have saturated at high [Ca\(^{2+}\)]. Their apparent second-order binding rate constant is in the range of \(1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\) to \(2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\), essentially the same as that reported here for cardiac TnC (1.4 \(\times 10^8 \text{ M}^{-1} \text{s}^{-1}\)). It appears that the Ca\(^{2+}\) binding rate at the regulatory sites is one to two orders of magnitude faster than that at the high affinity sites, regardless of isoform. The previous interpretation of calcium binding to the regulatory sites as a simple diffusion-controlled process (Johnson et al., 1989; 1994), however, is not consistent with the present evidence. The reporter group of the skeletal TnC mutant is Trp-29 located on helix A immediately adjacent to the amino-terminal end of the Ca\(^{2+}\)-binding loop 1, whereas in the present work the reporter group is located on helix D. The different locations of the two probes may be a reason why first-order transitions arising from activator Ca\(^{2+}\)-mediated movements of helices B and C were not sensed with mutant F29W.

In summary, we have used the fluorescence of an extrinsic probe linked to the single cysteine residue on helix D of a monocysteine mutant to investigate the kinetics of Ca\(^{2+}\) binding to cardiac TnC. The dissociation of Ca\(^{2+}\) from the regulatory site is two orders of magnitude faster than the dissociation of Ca\(^{2+}\) or Mg\(^{2+}\) from the two carboxyl-terminal domain sites. The kinetics of Ca\(^{2+}\) binding to the regulatory sites is consistent with a three-step mechanism in which the bimolecular binding step is in rapid equilibrium and is followed by two sequential first-order transitions.

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