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# Effect of $Ca^{2+}$ Binding on Troponin C

## CHANGES IN SPIN LABEL MOBILITY, EXTRINSIC FLUORESCENCE, AND SULFHYDRYL REACTIVITY*

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**The $Ca^{2+}$ binding component (TnC) of troponin has been selectively labeled with either a spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, or with a fluorescent probe, *S*-mercuric-*N*-dansyl cysteine, presumably at its single cysteine residue (Cys-98) in order to probe the interactions of TnC with divalent metals and with other subunits of troponin. The modified protein has the same $Ca^{2+}$ binding properties as native TnC (Potter, J. D., and Gergely, J. (1975) *J. Biol. Chem.* 250, 4628), *viz.* two $Ca^{2+}$ binding sites at which $Mg^{2+}$ appears to compete ($Ca^{2+}$-$Mg^{2+}$ sites, $K_{Ca} = 2 \times 10^7$ M$^{-1}$) and two sites at which $Mg^{2+}$ does not compete ($Ca^{2+}$-specific sites, $K_{Ca} = 2 \times 10^5$ M$^{-1}$).**

**Either $Ca^{2+}$ or $Mg^{2+}$ alters the ESR spectrum of spin-labeled TnC in a manner that indicates a decrease in the mobility of the label, $Ca^{2+}$ having a slightly greater effect. In systems containing both $Ca^{2+}$ and $Mg^{2+}$ the mobility of the spin label is identical with that in systems containing $Ca^{2+}$ alone. The binding constants for $Ca^{2+}$ and $Mg^{2+}$ deduced from ESR spectral changes are $10^7$ and $10^3$ M$^{-1}$, respectively, and the apparent affinity for $Ca^{2+}$ decreases by about an order of magnitude on adding 2 mM $Mg^{2+}$. Thus, the ESR spectral change is associated with binding of $Ca^{2+}$ to one or both of the $Ca^{2+}$-$Mg^{2+}$ sites. Addition of $Ca^{2+}$ to the binary complexes of spin-labeled TnC with either troponin T (TnT) or troponin I (TnI) produces greater reduction in the mobility of the spin label than in the case of spin-labeled TnC alone, and in the case of the complex with TnI the affinity for $Ca^{2+}$ is increased by an order of magnitude.**

**The fluorescence of dansyl (5-dimethylaminonaphthalene-1-sulfonyl)-labeled TnC is enhanced by $Ca^{2+}$ binding to both high and low affinity sites with apparent binding constants of $2.6 \times 10^7$ M$^{-1}$ and $2.9 \times 10^5$ M$^{-1}$, respectively, calculated from the transition midpoints. The presence of 2 mM $Mg^{2+}$, which produces no effect on dansyl fluorescence itself, in contrast to its effect on the spin label, shifts the high affinity constant to $2 \times 10^6$ M$^{-1}$. Spectral changes produced by $Ca^{2+}$ binding to the TnC·TnI complex furnish evidence that the affinity of TnC for $Ca^{2+}$ is increased in the complex.**

**The reactivity of Cys-98 to the labels and to 5,5′-dithiobis(2-nitrobenzoic acid) ($Nbs_2$) is decreased by $Ca^{2+}$ or $Mg^{2+}$ both with native TnC and in 6 M urea. The reaction rate between Cys-98 and $Nbs_2$ decreases to one-half the maximal value at a $Ca^{2+}$ concentration that suggests binding to the $Ca^{2+}$-$Mg^{2+}$ sites. Formation of a binary complex between TnI and TnC reduces the rate of reaction, and there is a further reduction by $Ca^{2+}$. The effect of $Ca^{2+}$ takes place at concentrations that are 1 order of magnitude lower than in the case of TnC alone. These results suggest that the $Ca^{2+}$ binding site adjacent to Cys-98 is one of the $Ca^{2+}$-$Mg^{2+}$ binding sites.**

In vertebrate muscle the regulation of contraction by $Ca^{2+}$ involves a complex of tropomyosin and troponin located on the thin filaments. The evidence available to date (4–10) suggests that in the absence of $Ca^{2+}$, tropomyosin is in a position that overlaps the myosin binding sites of actin, stearically blocking

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the interaction. When $Ca^{2+}$ binds to TnC,[1] tropomyosin moves away from this position, allowing myosin and actin to interact and contraction to take place.

Tonomura *et al.* (11) suggested that conformational changes in troponin taking place upon combination with $Ca^{2+}$ initiate the chain of events resulting in the interaction of actin and myosin. Such changes may be reflected in the effect of $Ca^{2+}$ on circular dichroism and intrinsic and extrinsic fluorescence of TnC (12–19). TnC contains two classes of $Ca^{2+}$ binding sites (20), *viz.* two sites having a high affinity for $Ca^{2+}$, at which $Mg^{2+}$ appears to compete ($Ca^{2+}$-$Mg^{2+}$ sites), and two sites having a lower affinity for $Ca^{2+}$, which do not bind $Mg^{2+}$ ($Ca^{2+}$-specific sites).

The studies reported here used three techniques, ESR, fluorescence, and chemical reactivity, to study changes in the environment of the single cysteine residue of TnC resulting from $Ca^{2+}$ and $Mg^{2+}$ binding or formation of complexes with TnT or TnI. All three of the conformational indicators detect binding of $Ca^{2+}$ to the $Ca^{2+}$-$Mg^{2+}$ sites; only the fluorescent probe additionally detects binding to the $Ca^{2+}$-specific sites.

## METHODS

Troponin subunits were prepared by the method of Potter and Gergely (4). TnC was spin-labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (21). In initial experiments carried out to determine the optimal conditions for selective labeling, the label was incubated with TnC, unreacted label was removed by exhaustive dialysis or gel filtration on a Sephadex G-25 column, and the amount of bound label was estimated by double integration of spectra obtained after digestion of 0.1 to 0.5 mg of TnC with 3 mg of pronase for 30 min. Spectra of 1-oxyl-2,2,6,6-tetramethyl-4-acetamidopiperidine in $H_2O$ were used as standards. Digestion with pronase results in a spectrum very similar to that of free label, thereby minimizing errors inherent in comparing spectra having different shapes. Removal of $Ca^{2+}$ and $Mg^{2+}$ with EDTA was necessary for optimum reaction of TnC with the label. Of label, 0.8 to 1.2 mol were bound/mol of TnC ($M_r$ = 17,846 (22)). The amount of label bound to TnC did not change between 1 and 10 mol of added label/mol of TnC. ESR spectra were recorded with a Varian V-4502 spectrometer at room temperature.

The fact that reaction of TnC with $Nbs_2$ completely prevented the binding of the spin label supports the view that the spin label binds to Cys-98. Spin labeling of TnC was carried out routinely in 6 M urea, 0.1 mM 1,4-dithiothreitol, 0.05 M Tris, and 1 mM EDTA at pH 8.0, the solution in which TnC was eluted from the DEAE-Sephadex column used in its preparation. The spin label was added in sufficient excess over dithiothreitol to provide 1 to 2 mol/mol of TnC. After 3 h at 0° the unreacted label was removed by dialysis against 0.1 M KCl and 1 mM 3,6-*N*-[tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid (Tes) (pH 7.0) or 6 M urea, 50 mM Tris (pH 8.0), and 1 mM EDTA.

The labeling of TnC with *S*-mercuric-*N*-dansyl cysteine and fluorescence measurements with the use of a Perkin-Elmer MPF-4 spectrofluorometer equipped with thermostated cell assembly were carried out as described previously (23). The absorbance at the exciting wavelength (330 nm) was always less than 0.1.

The rate of reaction of $Nbs_2$ with the single —SH group in TnC was determined at 25° by following the time course of the absorption change at 412 nm (24). The data were plotted as a second-order reaction and the rate constant obtained from the slope of the $(1/b - a))\ \ln\ ((b - x)/(a - x))$ *versus* time plot, in which $b$ is the initial concentration of $Nbs_2$, $a$ that of the reactive —SH moiety, and $x$ is that of the colored product found.

[1] The abbreviations used are: TnC, $Ca^{2+}$ binding subunit; Tn, troponin; TnT, tropomyosin binding subunit of troponin; TnI, ATPase inhibitory subunit; SL-TnC, spin-labeled TnC; Dns, dansyl(5-dimethylaminonaphthalene-1-sulfonyl); Dns-TnC, TnC labeled with *S*-mercuric-*N*-dansyl cysteine; $Nbs_2$, 5,5′-dithiobis(2-nitrobenzoic acid); Tes, *N*-[Tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

## RESULTS

*Effects of $Ca^{2+}$ and $Mg^{2+}$ on ESR Spectra of Spin Labeled TnC (SL-TnC)* – Various parameters of the ESR spectrum of spin-labeled proteins can be used to characterize the label's mobility; among these is the height of the upfield or downfield peaks, usually normalized with respect to some other portion of the spectrum (see, *e.g.* Refs. 25 and 26). Both $Mg^{2+}$ and $Ca^{2+}$ produce changes in the ESR spectrum of SL-TnC indicative of decreased mobility of the spin label (Fig. 1), the effect of $Ca^{2+}$ being slightly larger than that of $Mg^{2+}$. The spectral changes produced by $Mg^{2+}$ in the absence of $Ca^{2+}$ are half-maximal at a concentration of $2 \times 10^{-4}$ M, which is in agreement with an apparent binding constant of $5 \times 10^3\ M^{-1}$ for $Mg^{2+}$ (Fig. 2). The

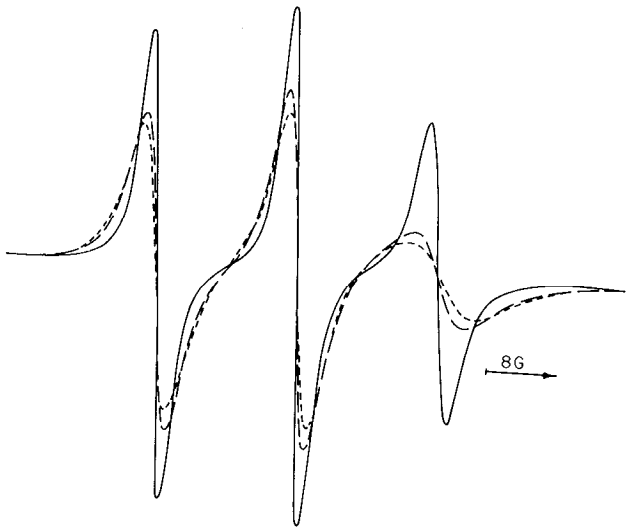


FIG. 1. The effect of $Ca^{2+}$ and $Mg^{2+}$ on the ESR spectrum of SL-TnC. SL-TnC was dialyzed against 100 mM KCl and 10 mM imidazole, pH 7.0. ESR spectra were recorded at room temperature, protein concentration, 5 mg/ml. Additions: – – –, 2 mM EDTA; - - -, 2 mM $MgCl_2$ and 2 mM EGTA; -----, 2 mM $MgCl_2$, 2 mM EGTA, and 2.4 mM $CaCl_2$ or 2 mM EDTA and 2.4 mM $CaCl_2$. In this and other figures portraying ESR spectra the *horizontal arrow* indicates the direction in which the applied magnetic field, $H_0$, increases. Scale in Gauss.

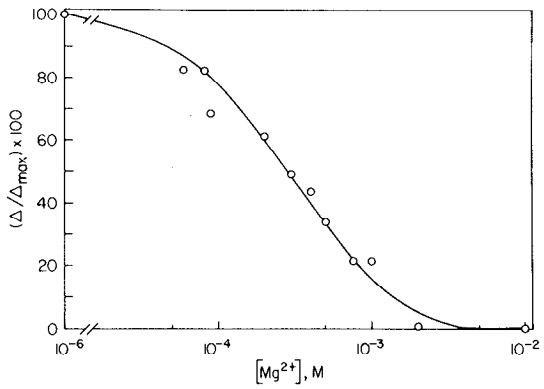


FIG. 2. Changes in the ESR spectrum of SL-TnC as a function of $[Mg^{2+}]$. Conditions: TnC was exhaustively dialyzed against 0.1 M KCl, 10 mM imidazole, pH 7.0, and 0.1 mM EGTA; protein concentration 1 mg/ml. Enough $MgCl_2$ was added to the above sample to produce the desired $[Mg^{2+}]$ shown on the *abscissa*. *Ordinate:* Δ and $\Delta_{max}$ are defined as follows: $\Delta = r - r_{min}$, where $r$ is the ratio of the height of the upfield peak and the height of the midfield peak of the spectrum at the concentration indicated on the abscissa; $r_{min}$ is the value of $r$ at a saturating concentration; $\Delta_{max}$ is the value of Δ without adding $Mg^{2+}$ (or in other figures $Ca^{2+}$).

full effect of $Ca^{2+}$ cannot be produced by $Mg^{2+}$ even at concentrations as high as 10 mM.

The dependence of the spectral change on $Ca^{2+}$ concentration is shown in Fig. 3, both in the presence and absence of $Mg^{2+}$. In the presence of 2 mM $Mg^{2+}$, the spectral change is half-maximal at 0.16 μM $Ca^{2+}$; in the absence of $Mg^{2+}$ the midpoint of the spectral change is shifted to 0.035 μM. Direct measurements of $Ca^{2+}$ binding to SL-TnC revealed two classes of sites, each containing two binding sites per molecule with binding constants of $2 \times 10^7$ $M^{-1}$ and $2 \times 10^5$ $M^{-1}$ (Table I). In the presence of 2 mM $MgCl_2$ the higher binding constant is reduced to $3 \times 10^6$ $M^{-1}$ with no effect on the lower one. Thus, $Ca^{2+}$ binding to SL-TnC is the same as to unlabeled TnC with regard to the number of sites in each class and the affinity constants (20). The range of $Ca^{2+}$ concentration, in which the ESR spectrum of SL-TnC changes, corresponds to that in which binding to the $Ca^{2+}$-$Mg^{2+}$ sites takes place.

*Effect of $Ca^{2+}$ and $Mg^{2+}$ on Fluorescence of Dansyl-labeled TnC*—The fluorescence of Dns-Hg-TnC is enhanced biphasically upon $Ca^{2+}$ binding; a 40% increase upon saturation of the $Ca^{2+}$-$Mg^{2+}$ sites followed by another 35% for the $Ca^{2+}$-specific sites (Fig. 4). Binding to the latter sites also produces a shift in the peak of the spectrum from 522 to 512 nm (Table II), indicative of a decrease in the polarity of the environment of the fluorophor (27, 28). These changes represent the only spectral demonstration to date of molecular changes associated with $Ca^{2+}$ binding to both classes of sites. The binding constants estimated from the midpoints of the spectral transitions are $2.6 \times 10^7$ $M^{-1}$ and $2.9 \times 10^5$ $M^{-1}$, in good agreement with those obtained from direct binding studies (20). $Mg^{2+}$ alone has no effect on the fluorescence of Dns-Hg-TnC in contrast to the results obtained with the spin label; however, it does shift the concentration dependence of the fluorescence change associated with $Ca^{2+}$ binding to the $Ca^{2+}$-$Mg^{2+}$ sites by about an order of magnitude toward higher $Ca^{2+}$ concentrations. The absence of energy transfer from the tyrosine residues of TnC to the Dns probe is apparent from the observations that (*a*) tyrosine fluorescence is unaffected by the presence of the probe, and (*b*) $Ca^{2+}$ binding produces the same relative enhancement of the Dns fluorescence whether the probe is excited at its own excitation maximum of 330 nm or at 275 nm.

*Effect of TnI and TnT on Labeled TnC*—TnT and TnI, which are insoluble at KCl concentrations less than 0.3 M, are insoluble in the presence of equimolar TnC (29). In the absence

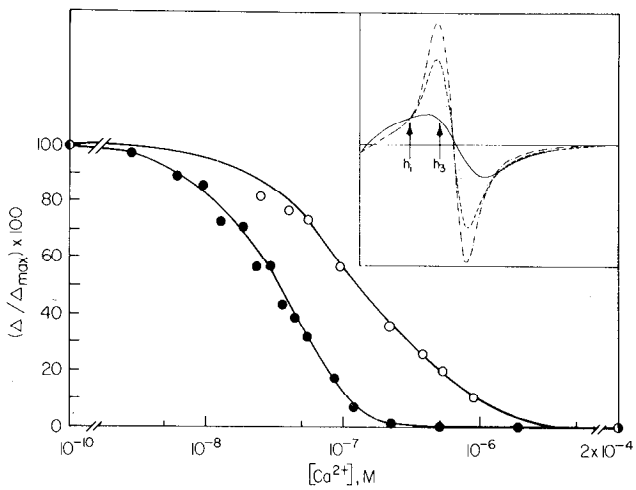


FIG. 3. Changes in the ESR spectrum of SL-TnC as a function of $[Ca^{2+}]$ in the presence and absence of $Mg^{2+}$. ○, 2 mM $MgCl_2$, 0.3 M imidazole, pH 7.0, 20 mM EGTA, and 6.4 mg of protein/ml; ●, no $Mg^{2+}$, 0.15 M imidazole, pH 7.0, 10 mM EDTA, and 2 mg of protein/ml. Enough $CaCl_2$ was added to produce the $[Ca^{2+}]$ shown on the abscissa (see "Methods"). Ordinate: Δ and $\Delta_{max}$ are defined as follows: $\Delta = r - r_{min}$, where $r$ is the ratio height of the upfield peak ($h_3$) and the height at the isosbestic point ($h_1$) shown in the *inset* at the $[Ca^{2+}]$ indicated on the abscissa $r_{min}$ is the value of $r$ at saturating $[Ca^{2+}]$; $\Delta_{max}$ is the value of Δ without added $Ca^{2+}$.

TABLE I

*$Ca^{2+}$ binding to spin-labeled TnC*

Measurements were carried out as described previously (20). $K_1$, and $K_2$ are the binding constants and $n_1$ and $n_2$ the number of binding sites in each class.

| Addition of $MgCl_2$ | $n_1$ | $K_1$ | $n_2$ | $K_2$ |
|---|---|---|---|---|
| *nm* | *mol/mol* | $M^{-1}$ | *mol/mol* | $M^{-1}$ |
| None | 1.9 | $2 \times 10^7$ | 2.1 | $2 \times 10^5$ |
| 2 mM | 1.9 | $3 \times 10^6$ | 2.1 | $1 \times 10^5$ |

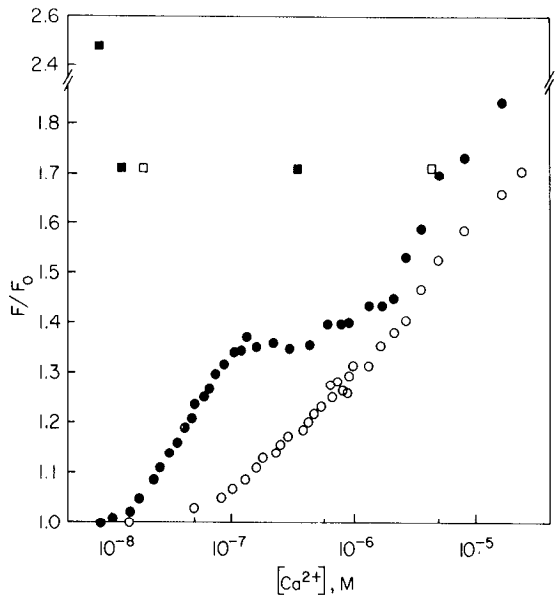


FIG. 4. Effect of $[Ca^{2+}]$ on the fluorescence of TnC labeled with Dns-Cys-(Hg). Measurements were carried out at room temperature on a solution containing 0.05 mg of protein/ml, 60 mM KCl, 10 mM imidazole, pH 7.0, 2 mM EGTA, and, when added, 2 mM $MgCl_2$. $CaCl_2$ was added to achieve the $[Ca^{2+}]$ indicated on the *abscissa*. Excitation and emission wavelengths were 330 and 530 nm, respectively. ●, TnC, no $Mg^{2+}$; ○, TnC and $Mg^{2+}$; ■, TnC and TnI, no $Mg^{2+}$; □, TnC, TnI, and $Mg^{2+}$.

TABLE II

*Maxima of fluorescence emission peaks and relative enhancements*

Number of bound calcium was estimated from titration and binding data.

| Moiety | pCa | Calcium bound | $F/F_0$ | | $\lambda_{max}$ | |
|---|---|---|---|---|---|---|
| | | | No $Mg^{2+}$ | 2 mM $Mg^{2+}$ | No $Mg^{2+}$ | 2 mM $Mg^{2+}$ |
| | | *mol/mol* | *nm* | | *nm* | |
| Dns-Hg-TnC | 9.0 | 0 | 1.00 | 1.00 | 522 | 522 |
| Dns-Hg-TnC | 6–8 | 2 | 1.40 | 1.40 | 522 | 522 |
| Dns-Hg-TnC | 4.0 | 4 | 1.72 | 1.72 | 512 | 512 |
| Dns-Hg-TnC-TnI | 9.0 | 0 | 2.48 | 1.72 | 502 | 512 |
| Dns-Hg-TnC-TnI | 7.8 | 2 | 1.72 | 1.72 | 512 | 512 |
| Dns-Hg-TnC-TnI | 4.0 | 4 | 1.72 | 1.72 | 512 | 512 |

of divalent cations the ESR spectrum of SL-TnC is unchanged by TnI or TnT. However, if TnI is added in the presence of $Ca^{2+}$ or $Mg^{2+}$ the metal-induced immobilization of the spin label is further increased (Fig. 5). As with TnC alone, the immobilization by $Ca^{2+}$ and $Mg^{2+}$ is greater than by $Mg^{2+}$ alone. The effect of TnT on the immobilization by $Ca^{2+}$ is the same as that of TnI. The spectral change associated with the combination of TnI or TnT with SL-TnC increases linearly up to 1 mol of TnI or TnT/mol of TnC; further additions have no effect (Fig. 6).

TnI increases the affinity of all four $Ca^{2+}$ binding sites on TnC by a factor of 10 (20). This is also reflected in the lower concentration of $Ca^{2+}$ required to produce the half-maximal change in the ESR spectrum of the complex of SL-TnC and TnI (Fig. 7). The value of $5 \times 10^{-9}$ M at the midpoint of the curve in Fig. 7 agrees well with the $Ca^{2+}$ binding constant of the $Ca^{2+}$-$Mg^{2+}$ sites in the unlabeled complex.

In contrast to the results with the spin label, the Dns label on TnC responds to TnI in the absence of divalent cations (Fig. 4); there is a 2.5-fold enhancement of the fluorescence accompanied by a 20 nm blue shift to 502 nm. The fluorescence drops to a relative value of 1.72 upon adding 10 nM $Ca^{2+}$ and does not change to millimolar $Ca^{2+}$. In 2 mM $Mg^{2+}$ the fluorescence is the same as with $Ca^{2+}$. Both $Ca^{2+}$ and $Mg^{2+}$ ions cause a red shift of the fluorescence maximum of the Dns·Hg·TnC·TnI complex to 512 nm (Table II).

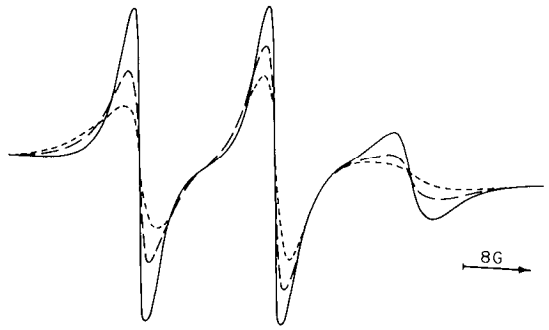


FIG. 5. The effect of $Ca^{2+}$ on the ESR spectrum of SL-TnC complexed with TnI. SL-TnC and TnI in 6 M urea, 50 mM Tris, pH 8.0, and 1 mM EDTA were mixed in an equimolar ratio. Urea was removed by dialysis against 0.1 M KCl, 2 mM EGTA, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7.0. ——, SL-TnC (2.0 mg/ml), no addition; - - -, SL-TnC-TnI (TnI 2.4 mg/ml, TnC 2.0 mg/ml), no additions; -----, same plus 2.4 mM $CaCl_2$.

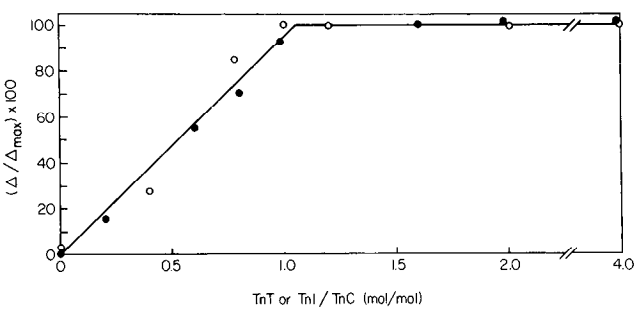


FIG. 6. The effect of TnI and TnT on the ESR spectrum of SL-TnC. SL-TnC, TnI, and TnT were dialyzed against 1 M KCl, 1 mM Tes, pH 7.0, and $10^{-5}$ M $CaCl_2$. SL-TnC concentration, 1.3 mg/ml. SL-TnC-TnI and SL-TnC-TnT were mixed at various ratios in the above solvent and then dialyzed against 1 mM Tes, pH 7.0. *Ordinate:* Δ and $\Delta_{max}$ are defined as follows: $\Delta = r_0 - r$, where $r$ is the ratio of the height of the downfield peak and the midfield peak of the spectrum at the protein ratio indicated on the abscissa and $r_0$ is the value of $r$ for spin labeled TnC alone; $\Delta_{max}$ is the value of Δ at saturating ratios of TnT or TnI to TnC.

The ternary complex of TnT, TnI, and SL-TnC was reconstituted by mixing the three proteins in 6 M urea and then removing the urea by dialysis against successively lower concentrations of KCl to a final level of 0.15 M (see legend to Fig. 8). The ternary complex thus formed is completely soluble at low ionic strengths in the presence or absence of $Ca^{2+}$. The ESR spectra of either TnI-SL-TnC or TnT-SL-TnC and the effect of $Ca^{2+}$ on the two complexes are essentially the same (Fig. 7) as with the ternary complex (Fig. 8).

*Effect of $Ca^{2+}$, $Mg^{2+}$, Urea, and TnI on Reactivity of Cys-98 in TnC* – Since the initial studies on the labeling of TnC with the spin label indicated that removal of divalent cations from TnC was necessary for the reaction to occur, it was of interest to study the $Ca^{2+}$ dependence of the reactivity of the –SH group. The reaction of $Nbs_2$ with TnC, the stoichiometry being 1 mol/mol, at different $Ca^{2+}$ concentrations in the presence and absence of $Mg^{2+}$ followed second order kinetics at all concentrations of $Ca^{2+}$; $Ca^{2+}$ inhibits the reaction and the dependence of the rate on $Ca^{2+}$ concentration (Fig. 9) is nearly identical with that of the change in mobility of the spin label (*cf.* Fig. 3). In the presence of 2 mM $Mg^{2+}$ the rate is maximally reduced and there is no further effect on the rate by adding $Ca^{2+}$. The fact that even in urea spin labeling took place only if divalent

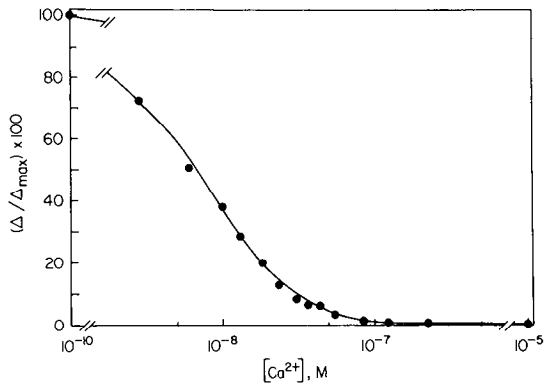


FIG. 7. The effect of $[Ca^{2+}]$ on the ESR spectrum of a complex of TnI and SL-TnC. TnI and SL-TnC were mixed in a 1:1 molar ratio as described in the legend to Fig. 5. (TnI 2.45 mg/ml, SL-TnC 1.82 mg/ml). Urea was then removed by dialysis against 0.15 M imidazole, pH 7.0, and 10 mM EDTA. Sufficient $CaCl_2$ was added to achieve the desired free $Ca^{2+}$ concentration (see "Methods"). *Ordinate:* for definition of Δ and $\Delta_{max}$ see the legend to Fig. 1, except that $r$ is the ratio of the height of the downfield peak and the height of the midfield peak.

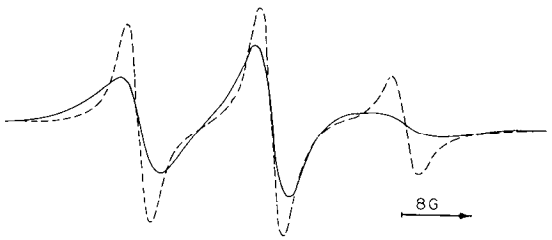


FIG. 8. The effect of $[Ca^{2+}]$ on the ESR spectrum of SL-TnC when complexed with TnT and TnI. SL-TnC, TnT, and TnI in 6 M urea, 50 mM Tris, pH 8.0, and 1 mM EDTA were mixed in an equimolar ratio and then dialyzed free of urea against 1.0 M KCl and 25 mM imidazole, pH 7.0. The KCl concentration was then slowly lowered over a period of 48 h by stepwise changes of the dialysis solution of 0.8 M, 0.6 M, 0.5 M, 0.4 M, 0.3 M, 0.2 M, and finally 0.15 M; all solutions contained 50 mM imidazole, pH 7.0 (TnI, 0.72 mg/ml, TnT, 1.08 mg/ml, and SL-TnC, 0.53 mg/ml). Solvent conditions: 0.15 M KCl and 50 mM imidazole, pH 7.0. Additions: - - -, 2 mM EDTA; ——, 0.01 mM $CaCl_2$.

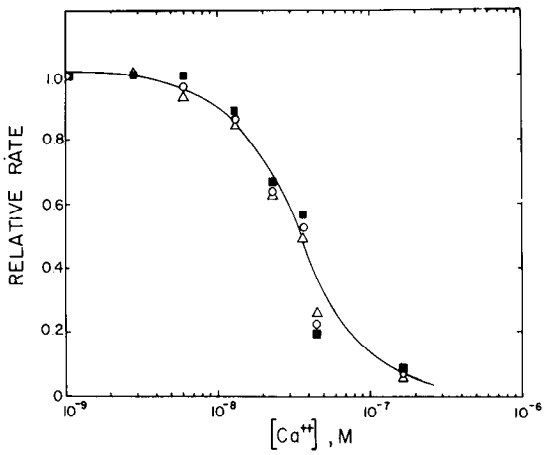


FIG. 9. The effect of $[Ca^{2+}]$ on the reactivity of Cys-98 in TnC. Conditions: 25°, 0.1 M KCl, 150 mM imidazole, pH 7.0, and 2 mM EDTA (TnC: 1.12 mg/ml). Enough $CaCl_2$ was added to produce the $[Ca^{2+}]$ shown on the *abscissa*. The rate of reaction was followed by monitoring the change in absorbance at 412 nm with time. The reaction was started by adding 0.01 M $Nbs_2$ (final concentration, 0.1 mM). Second-order rate constants, $k$, were evaluated as described under "Methods." *Ordinate:* $(k - k_{[Ca^{2+}]=0.4\ mM})/k_{[Ca^{2+}]=0}$. The sample with no added $Ca^{2+}$ was taken as $[Ca^{2+}] = 0$.

TABLE III

*Effect of urea and TnI on reactivity of Cys-98 in TnC*

Reactivities are expressed as second-order rate constants; for details see "Methods." Conditions: TnC concentration in terms of titratable −SH, $6 \times 10^{-5}$ M; 0.1 M KCl, 150 mM imidazole, pH 7.0, and 0.1 mM $Nbs_2$, 25°. $+Ca^{2+}$: 2 mM EDTA + 2.4 mM $CaCl_2$. $-Ca^{2+}$: 2 mM EDTA. Additions: 6 M urea as indicated. The complex of TnI and TnC was made as described in the legend to Fig. 5. Weight ratio of TnI and TnC, 2:3.

| Additions | $-Ca^{2+}$ | $+Ca^{2+}$ |
|---|---|---|
| | $M^{-1}\ s^{-1}$ | |
| | 712 | 112 |
| 6 M urea | 1200 | 79 |
| TnI | 300 | 5 |

cations were removed suggested that urea treatment does not abolish metal binding to TnC. Urea has little effect on the rate of reaction (Table III); identical reduction by $Ca^{2+}$ of the rate of reaction with $Nbs_2$ occurs with or without 6 M urea.

Since TnI decreases the mobility of the spin label attached to TnC, we tested the effect of TnI on the reactivity of Cys-98 with $Nbs_2$ and found that TnI reduces the reactivity in the presence and absence of $Ca^{2+}$ (Table III). The concentration of $Ca^{2+}$ required to inhibit the reaction in the binary complex TnC·TnI is the same as that required to reduce the mobility (Fig. 7) of SL-TnC-TnI ($K_{app} = 2 \times 10^8\ M^{-1}$).

## DISCUSSION

The fact that 1 mol of spin label or fluorescent label (23) can be incorporated/mol of TnC and that the reaction with both labels is blocked by $Nbs_2$, which also reacts in a 1:1 molar ratio, indicates that the labels bind selectively to Cys-98, the single cysteine residue in TnC (22). The labeling of TnC does not seem to affect its physicochemical properties as shown by: (*a*) the lack of change in $Ca^{2+}$ binding properties produced by spin labeling; (*b*) the identical behavior of native and Dns-TnC with respect to $Ca^{2+}$-induced changes in CD or tyrosine fluorescence; and (*c*) the fact that labeled TnC forms binary and ternary complexes with the other Tn subunits. $Ca^{2+}$ or $Mg^{2+}$ inhibits the reaction of TnC with thiol reagents, which shows that the thiol group of Cys-98 becomes less accessible as a consequence of conformational changes. Fuchs' observation (30) that $Ca^{2+}$ reduces the reactivity of whole troponin with sulfhydryl modifiers may be interpreted in the light of the present results as indicating that one of the —SH groups of troponin, the reactivity of which is altered by $Ca^{2+}$, is that found in TnC.

Responses to $Ca^{2+}$ or $Mg^{2+}$ of the spin (see also Ref. 31) and fluorescent labels and of the reactivity of the —SH group in Cys-98 are probably consequences of the overall changes in secondary and tertiary structure that are indicated by the large changes in circular dichroism (17). While the response of the spin label clearly parallels the effect of $Ca^{2+}$ or $Mg^{2+}$ on circular dichroism, *viz.* the effect of $Ca^{2+}$ is somewhat larger than that of $Mg^{2+}$, the Dns fluorescence is enhanced by $Ca^{2+}$ but not by $Mg^{2+}$. This would suggest that the response of the fluorescent label depends more strongly on finer cation-specific differences in the immediate environment of the label than does the mobility of the spin label. It should be pointed out that the intrinsic tyrosine fluorescence of TnC is also much more strongly affected by $Ca^{2+}$ than by $Mg^{2+}$ (17).

The $Ca^{2+}$-induced changes in ESR spectra and in the reactivity of Cys-98 can be attributed to binding to the $Ca^{2+}$-$Mg^{2+}$ sites. The apparent affinity constant for $Ca^{2+}$ determined from the midpoints of the titration curves is approximately $2 \times 10^7$ $M^{-1}$, and the $Ca^{2+}$ concentration necessary for changing the ESR spectra shifts to higher values in the presence of $Mg^{2+}$. The effect of $Mg^{2+}$ on the $Ca^{2+}$ dependence of the reactivity could not be studied since the lower reactivity produced by $Mg^{2+}$ was not affected by $Ca^{2+}$.

The ESR titration curves are somewhat steeper and shifted to lower free $Ca^{2+}$ concentrations than the $Ca^{2+}$ binding curves, suggesting that binding of $Ca^{2+}$ to either of the two $Ca^{2+}$-$Mg^{2+}$ sites may produce the spectral change; however, further work is needed to substantiate this point.

In contrast to the response of the spin label, the fluorescence of the Dns probe reflects binding of $Ca^{2+}$ to the $Ca^{2+}$-$Mg^{2+}$ sites and to the $Ca^{2+}$-specific sites. The change in Dns fluorescence is the first reported spectral change which can be attributed to the binding of $Ca^{2+}$ to $Ca^{2+}$-specific sites.

While $Ca^{2+}$ binding to the $Ca^{2+}$-$Mg^{2+}$ sites produces an enhancement of Dns fluorescence, the binding of $Mg^{2+}$ does not. $Mg^{2+}$ only indirectly affects fluorescence by shifting the $Ca^{2+}$ concentration required for the enhancement to higher values.

Formation of complexes of SL-TnC with TnI or TnT in the absence of divalent cations does not lead to a change in the mobility of the spin label. However, the response to $Ca^{2+}$, as manifested in reduced mobility, is greater in the complexes (31). This greater $Ca^{2+}$-induced change in the ESR spectra of the binary complexes may be related to the stabilization of the TnI·TnC and TnT·TnC complexes by $Ca^{2+}$ (32, 33). The $Ca^{2+}$ binding constant of the TnI·SL-TnC complex estimated from the ESR spectral change, $2 \times 10^8$ $M^{-1}$, is consistent with the fact that the interaction of TnI with TnC increases the affinity for $Ca^{2+}$ of the $Ca^{2+}$-$Mg^{2+}$ sites by an order of magnitude (*cf.* Ref. 20).

The behavior of the ESR spectrum of spin-labeled TnC is in contrast to that of the fluorescence of Dns-TnC, where the complex formation with TnI in the absence of $Ca^{2+}$ leads to a

considerable enhancement of the fluorescence of the label. $Ca^{2+}$ or $Mg^{2+}$ slightly reduces the enhanced fluorescence to the maximum level for Dns-TnC alone. It should be noted that in the Dns-labeled complex there is no indication of a response to $Ca^{2+}$ binding to the $Ca^{2+}$-specific sites. The enhanced fluorescence of the Dns label in the TnC·TnI complex suggests a more hydrophobic environment for the label. The difference between the spin-labeled and Dns-labeled TnC with respect to complex formation with TnI again brings to light the difference in response between the two labels. It may well be that the Dns label is chiefly an indicator of the hydrophobic character of the environment of the label (27, 28), while the mobility of the spin label may depend chiefly on the geometry or restrictiveness of the immediate neighborhood.

TnC contains four regions (I to IV) similar in sequence to each other and to the two $Ca^{2+}$ binding regions of parvalbumin, and it has been suggested that these sequences contain the four $Ca^{2+}$ binding sites of TnC (22). Each region in parvalbumin or in the proposed model for TnC (34) consists of two $\alpha$ helical segments roughly at right angles to each other, joined by a $Ca^{2+}$ binding loop. Kretsinger and Barry (35) suggested that the $Ca^{2+}$ binding sites in regions III and IV of TnC correspond to the $Ca^{2+}$-$Mg^{2+}$ sites, while, according to Weeds and McLachlan (36), it is the sites in region I and II that correspond to the $Ca^{2+}$-$Mg^{2+}$ sites. The present work shows that the reactivity of Cys-98, located in one of the putative $\alpha$ helices in region III, and the mobility of a spin label bound to this residue are influenced by metal binding to the $Ca^{2+}$-$Mg^{2+}$ sites. This, together with the proximity of Cys-98 and the $Ca^{2+}$ binding site of region III in the amino acid sequence and in the proposed three-dimensional structure, as well as the location of one of the tyrosines (Tyr-109) in the calcium binding loop of region III and the reported changes in Tyr fluorescence on $Ca^{2+}$ and $Mg^{2+}$ binding (17, 37), suggests that the site in region III is one of the $Ca^{2+}$-$Mg^{2+}$ binding sites of TnC. This conclusion is supported by the fact that in the presence of 6 M urea $Ca^{2+}$ changes the reactivity of Cys-98 and induces formation of the same amount of $\alpha$ helical structure as in the absence of urea (38). In addition, $Ca^{2+}$ alters the reactivity of Cys-98 and tyrosine fluorescence in the cyanogen bromide peptide, CB-9, consisting of amino acid residues 94 through 135 (39, 40).

The relationship of the $Ca^{2+}$-induced conformational changes discussed in this and other papers (12–19) to the regulatory function of troponin is still not clear. It seems that most of the changes in TnC observable *in vitro* are due to $Ca^{2+}$ binding to the $Ca^{2+}$-$Mg^{2+}$ sites and the effects of $Mg^{2+}$ are not much smaller than those produced by $Ca^{2+}$. Recent data on the effect of $Ca^{2+}$ and $Mg^{2+}$ on myofibrillar ATPase activity suggest that the regulatory sites are the $Ca^{2+}$-specific sites (20), while reports on tension development by muscle fiber preparations (41, 42) suggest the participation of the $Ca^{2+}$-$Mg^{2+}$ sites as well.

In either case, the $Ca^{2+}$-$Mg^{2+}$ sites of troponin in relaxed muscle would be occupied by either $Ca^{2+}$ or $Mg^{2+}$; the concentration of the latter in living muscle is of the order of 1 mM or higher (43) so that whatever the changes are in TnC that underlie the transition from the relaxed to the active state, they must be of a more subtle nature than those observed so far.

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