Binary Interactions of Troponin Subunits*

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The association constants for the formation of the binary complexes of rabbit fast skeletal muscle troponin subunits have been determined for three solution conditions: (a) 1 mM CaCl₂, (b) 3 mM MgCl₂ and 1 mM EGTA, and (c) 2 mM EDTA. The subunits were labeled with extrinsic fluorescence probes, either 5-(iodoacetamid)eoosin (IAE) or dansylaziridine (DANZ), and the binding was detected by enhancement or quenching of the probe fluorescence. The association constant for TnC-TnI (where TnC is the Ca²⁺-binding subunit of troponin) interaction was measured with three probes, IAE-TnC, DANZ-TnC, and IAE-TnI. Values of 1.7 × 10⁶, 1.2 × 10⁶, and 1.0 × 10⁶ M⁻¹ were obtained, respectively, in the presence of calcium ion, in the presence of magnesium ion (no calcium), and in the absence of divalent metal ions. A mean value of 4.0 × 10⁶ M⁻¹ was obtained for the association constant of TnC-TnI using DANZ-TnC and IAE-TnC as probes in the presence of calcium or magnesium ions. A value of 4.5 × 10⁶ M⁻¹ was obtained in the absence of divalent metal ions. The results show that the presence of magnesium ion in the Ca²⁺-Mg²⁺ sites stabilizes the TnC-TnI interaction and the TnC-TnT interactions and suggest that the troponin structure would be stabilized. This likely results from the effect of magnesium ion on the Ca²⁺-Mg²⁺ domains of TnC. The presence of calcium ion in the Ca²⁺-specific sites provides an additional binding free energy for the TnC-TnI interaction which presumably reflects the changes in the subunit interactions required for the calcium regulatory switch.

The binding of calcium ions to the regulatory protein troponin initiates a series of molecular events which result in the contraction of skeletal muscle. When calcium ions bind to TnC, a conformational change is induced which alters the interactions of TnC with TnI and TnT. The latter two subunits then transmit the regulatory signal to tropomyosin and actin, thereby altering the activity of the actomyosin ATPase.

An understanding of the molecular details of regulation includes a knowledge of the thermodynamic forces involved in the interactions of the troponin subunits and of how these forces are affected in the different regulatory states available to the molecule. In our laboratory, we are involved in an ongoing effort to determine the thermodynamic parameters which characterize the troponin subunit interactions. Here we report the effect of calcium ion and magnesium ion on the association constants for the three binary interactions of troponin subunits: TnC-TnI, TnC-TnT, and TnI-TnT. The experiments were performed for three solution conditions: (a) in calcium buffer, simulating the on state, (b) in magnesium buffer, simulating the off state, and (c) in the absence of divalent cations (EDTA), a reference state. The results show that the free energy of the TnC-TnI interaction is specifically altered in the on state. Our data are consistent with a model in which magnesium ion in the Ca²⁺-Mg²⁺ sites serves as a structure stabilizer.

EXPERIMENTAL PROCEDURES

Materials.—The fluorescent labels were purchased from Sigma (dansylamine) and Molecular Probes Inc. (6-(iodoacetamidamino)eoosin). All chemicals used in the preparations were of reagent or higher grade.

Troponin was prepared from rabbit fast skeletal muscle (longissimus dorsi and psoas) using a modification of the method of Greaser and Gergely (1971) with an additional purification step on Affi-Gel Blue agarose (Bio-Rad) (Reisler et al., 1980) as described by Ingraham and Swenson (1983). The troponin subunits were separated by chromatography at 4 °C on CM-Sepharose in a buffer containing 6 M urea, 5 mM Pipes, 5 mM 2-mercaptoethanol, 2 mM EDTA, and 0.01% NaN₃ at pH 6.5. The subunits were eluted from the column using a 0.1-0.24 M KCl gradient. The TnC and TnT eluted from this column were ready for use. Purification of TnI was achieved by affinity chromatography on a TnC-agarose affinity column prepared by the reaction of TnC with Affi-Gel 15 (Bio-Rad). TnI was applied to the column in a 20 mM Tris buffer at pH 7.5 containing 1 M KCl, 1 mM CaCl₂, 5 mM 2-mercaptoethanol, and 0.01% NaN₃. Elution of TnI was monitored by UV absorption at 280 nm.

Methods.—The dansylaziridine probe was attached to TnC according to the procedure of Johnson et al. (1978). Labeling of TnC with IAE was accomplished following the method of Cheng et al. (1982) except that a Bio-Gel P-4 column was used to remove the unreacted probe rather than dialysis.

TnI and TnT were labeled by reacting IAE with troponin. Purified troponin was dialyzed against 20 mM Tris buffer at pH 7.5 containing 0.1 mM KCl, 0.5 mM CaCl₂, 0.01% NaN₃, and 5 mM 2-mercaptoethanol in order to ensure reduction of the sulfhydryl residues of TnI. The troponin was next dialyzed against three 15-volume changes of the same buffer lacking 2-mercaptoethanol and centrifuged at 19,200 × g for 20 min to remove the minor turbidity which developed during dialysis. A 7-fold excess of IAE was added to the troponin solution to initiate the reaction. Following 12 h of reaction in the dark at 25 °C, the solution was dialyzed versus four changes of a 10-fold volume excess of a buffer containing 20 mM Tris buffer, pH 7.0, 0.5 M KCl, 5 mM MgCl₂, 1 mM EGTA, 0.01% NaN₃, and 5 mM 2-mercaptoethanol at pH 7.0 in the cold. The subunits were then separated and purified as described.

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† The abbreviations used are: TnC, Ca²⁺-binding subunit of troponin; TnI, inhibitory subunit of troponin; TnT, tropomyosin-binding subunit of troponin; DANZ, dansylaziridine; IAE, 5-(iodoacetamido)eoosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid; PIPES, 1,4-piperazinedionesulfonic acid.
The extents of probe incorporation into the subunits were determined as follows. Probe concentrations were measured spectrophotometrically using $e = 8.3 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 528 nm for IAE (Cherry, 1978) and $\epsilon = 3980$ M$^{-1}$ cm$^{-1}$ at 350 nm for dansylazidylase (Johnson et al., 1978). Protein concentrations were determined using the Bradford assay (Bradford, 1976) with standard curves generated using the appropriate unlabeled subunits and using ultraviolet absorption measurements employing the following extinction coefficients: TnC, $E_{280}$ = 1.59 (Leavis et al., 1978); TnI, $E_{280}$ = 3.97 (Wilkinson, 1974); TnT, $E_{280}$ = 5.04 (Margarssian and Cohen, 1973). The molecular weights used were for TnC, $M_r = 17,965$ (Collins, 1974); TnI, $M_r = 20,864$ (Wilkinson and Grand, 1975); and TnT, $M_r = 36,503$ (Pearlstone et al., 1976). Fractional incorporation was calculated as probe concentration divided by subunit concentration. For TnC, the fractional incorporation varied from 0.45 to 0.76 for the IAE probe and from 0.55 to 0.97 for the DANZ probe. Labeling of TnI with IAE yielded a fractional incorporation of 0.63. For TnT, a value of 2.23 mol of IAE/mol of subunit was determined. This value is likely to be high since two fluorescent higher molecular weight proteins are observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels which stain lightly with Coomassie Blue.

Data for the binding isotherms were obtained using a Perkin-Elmer MFP-44 spectrofluorometer. The titrations were performed at fixed excitation and emission wavelengths, respectively, of 310 and 540 nm for IAE-labeled subunits and 340 and either 510 or 520 nm for dansylazidylase-labeled TnC. Three solution conditions were used for the binding experiments: (a) 1 mM CaCl$_2$, (b) 3 mM MgCl$_2$ and 1 mM EDTA, and (c) 1 mM CaCl$_2$ and 1 mM EDTA. The solutions were buffered at pH 7.0 with 10 mM Pipes and contained 0.3 M KC1, 5 mM dithiothreitol, and 0.01% NaN$_3$. Aliquots of a cold (0 °C) concentrated solution of titrant ($5-40 \mu$l) were added to the cuvette (initial volume of 2.5 ml) with a micro burette syringe (Microinstruments Inc.) calibrated in the microliter range. Both the syringe microburette and the cuvettes were treated with a 5% solution of dimethylchlorosilane in benzene to prevent adsorption of protein.

Fluorescence data at each addition (500 points over 25 s) were recorded and stored on a Bascom-Turner Model 4120 electronic recorder. The standard deviations for the measured 500 points ranged from less than 0.01% at the highest concentrations to 0.4% at the lowest concentrations. The means, which are used in the calculations, have standard errors which are about 4-fold smaller. There were no detectable time-dependent fluorescence changes over this time interval. The data were sent to a computer where they were averaged and fit to Equation 1 for the complexation of two components using the criterion of minimization of the sum of deviations squared.

$$nE_0 + X(I) + 1/K_1 = Y(I) = \frac{(nE_0 + X(I)) + 1/K_1}{nE_0X(I)} - 4nE_0X(I)^n \cdot Y_{max}$$

$$2nE_0$$

In this equation, $E_0$ is the molar concentration of the labeled protein component which is held constant (corrected for dilution), $X(I)$ is the molar concentration of the unlabeled protein component which is varied, and $Y(I)$ is the fluorescence signal after the nth addition of the varied component. The three parameters which are obtained from the fit are $n$, the number of binding sites/ labeled molecule, $K$, the association constant, and $Y_{max}$, the maximum fluorescence signal. In some cases, the fitting was done with the number of binding sites fixed at 1 with only negligible effects on the fitted association constants. Error in the fitted parameters was less than 10%. The limiting error is the variation from run to run. The values of the association constants reported are the geometric means with the 70% confidence limits. The values of $n$ reported are the arithmetic means with the standard deviations.

RESULTS AND DISCUSSION

Troponin C from fast skeletal muscle contains four Ca$^{2+}$-binding sites. Two of these, the Ca$^{2+}$-Mg$^{2+}$ sites, will bind either Ca$^{2+}$ ($K = 2 \times 10^3$ M$^{-1}$) or Mg$^{2+}$ ($K = 5 \times 10^5$ M$^{-1}$) and are located in the C-terminal half of TnC (Potter and Gergely, 1975; Leavis et al., 1978). The two Ca$^{2+}$-specific sites ($K = 2 \times 10^3$ M$^{-1}$) are in the N-terminal half of the molecule (Potter and Gergely, 1975; Leavis et al., 1978). Considerable experimental evidence indicates that the Ca$^{2+}$-specific sites are the actual regulatory sites which "switch on" the actomyosin ATPase in muscle (Potter and Gergely, 1975; Johnson et al., 1979). The Ca$^{2+}$-Mg$^{2+}$ sites of TnC in resting muscle are occupied by magnesium ion and are thought to stabilize the TnC and troponin structures (Zot and Potter, 1982). Troponin C presumably functions by presenting different interfaces for interaction with the other subunits depending on the presence or absence of calcium ion in the Ca$^{2+}$-specific sites. In these experiments, we have examined the effects of calcium and magnesium ions on the strength of the binding interactions.

The concentration of calcium ion used in the calcium buffer (1 mM) is sufficient to ensure saturation of all four binding sites. This was done in order to minimize any coupling between the interactions involving the subunits and those involving TnC and calcium ion. The concentration of magnesium ion used in the magnesium buffer for these experiments (3 mM) is approximately the physiological concentration. Included in the magnesium buffer is 1 mM EGTA which assures chelation of any contaminating calcium ion present. For experiments in which neither divalent metal ion is desired, we included 2 mM EDTA in the buffer solution. In all cases, 0.3 M KC1 was present in order to prevent aggregation of the relatively insoluble TnI and TnT subunits (Greaser and Gergely, 1973).

Four different fluorescent troponin subunit probes were prepared for these experiments: IAE-TnC, DANZ-TnC, IAE-TnI, and IAE-TnT. The probable locations of the probes in the individual subunits are described below.

Labeling of TnC with DANZ was performed in the presence of 0.5 mM calcium ion. Under these conditions, the apparent accessibility of the single cysteinyl residue of TnC, Cys-98, is low (Potter et al., 1976). As a consequence, it is likely that the probe reacts with 1 or more of the 10 methioyd residues of TnC. Using tryptic digests of TnC labeled in this manner, Johnson et al. (1978) have demonstrated that this is indeed the case. Moreover, they discovered that approximately 60% or more of the labeling occurred at a single residue, Met-25. In addition, they examined the effect of the probe on the circular dichroic spectrum of TnC as a function of calcium ion concentration. Comparison of these data with data from their previous study of unlabeled TnC suggests that DANZ labeling of TnC does not significantly affect either the secondary structure of the protein or the conformational changes which occur as a result of calcium ion binding. Thus, probe effects on the binary interactions are expected to be small. Labeling of TnC with IAE was done in the presence of 2 mM EDTA, i.e. in the absence of calcium and magnesium ions. Under these conditions, Cys-98 is quite reactive (Potter et al., 1976), and Cheung et al. (1982) have demonstrated convincingly that it is the labeling site for IAE. The possibility that labeling of this site has an effect on the ability of troponin to confer calcium ion sensitivity on the actomyosin ATPase has been examined by Sutoh (1980). He found that troponin reconstituted using TnC labeled with N-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide exhibited the same calcium ion sensitivity as did native troponin.

The probable location of the IAE probe on the TnI subunit is Cys-133. Chong and Hodges (1982a) have shown that of the 3 cysteinyl residues of TnI, Cys-48, Cys-64 and Cys-133, only Cys-133 is exposed for reaction with iodoaceticamide when the reaction is performed on native troponin. Our reaction conditions should have yielded a similar result. Work performed by Sutoh and Matsuizaki (1980) suggests that labeling at this site does not affect the physiological function of troponin. When troponin was reconstituted using TnI labeled at Cys-133 with N-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide, they found no significant difference between...
the reconstituted and native troponin in their ability to regulate the actomyosin ATPase.

The locations of the IAE probes in the structure of TnT are unknown. Since TnT has no cysteinyl residues, it is probable that the probe is attached to 2 or more of the 5 methionyl residues present (Means and Feeney, 1971). Proteolytic evidence indicates that at least 2 residues in different regions of the molecule are involved. This evidence stems from the action of trace amounts of protease in our TnT samples, which slowly cleave TnT into two fragments, M, ~ 24,000 and ~ 10,000. They are similar to the TnT1 and TnT2 fragments obtained by chymotryptic digestion (Ohtsuki, 1979). When a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel was run on an old IAE-TnT sample, both proteolytic fragment bands were observed to fluoresce.

Table I summarizes the changes in fluorescence intensity which occur upon complex formation. The largest changes observed involved binding of TnI to IAE-TnT. Under all three buffer conditions, fluorescence enhancements of approximately 47% were noted. Two methionyl residues, Met-70 and Met-151, are located either in, or immediately adjacent to, regions of TnT which are thought to be involved in the TnI-TnT interaction (Pearlstone and Smillie, 1980; Chong and Hodges, 1982b). In light of this, the relatively large enhancements which we observed for this interaction suggest the possibility that the probe may be in the immediate vicinity of the site of interaction for the two subunits. If such is indeed the case, it is possible that the presence of the probe on IAE-TnT alters the strength of the interaction between the two subunits. Fortunately, binding of TnT to IAE-TnI yields reasonable fluorescence enhancements for the three buffer systems (9-11%). Consequently, complex formation can also be examined using IAE-TnI.

Three different approaches were used in investigating the TnC-TnI interaction. The approach which proved most satisfactory utilized IAE-TnI. Binding of TnC to IAE-TnI causes a 6% decrease in fluorescence intensity in the calcium buffer and 11% quenching in the magnesium buffer, but does not affect the fluorescence intensity in the EDTA buffer. Since experiments utilizing labeled TnC indicate complex formation under identical conditions, we presume that the binding interaction in the absence of calcium and magnesium ions does not significantly affect the environment of the probe on the TnI subunit.

The interaction between TnI and IAE-TnC results in enhancements which are small for all the three buffer conditions, varying between approximately 5 and 6%. Although reasonable binding isotherms can be obtained using this approach, it appears that the presence of the probe may adversely affect the TnC-TnI interaction. When we studied the TnC-TnI interaction with the IAE-TnC probe, a small lag was noted which roughly corresponded to the fraction of unlabeled TnC molecules. We do not report association constants from studies with this probe as this effect might be expected. A similar probe effect has been observed and quantitated by Grabarek et al. (1983) using TnC labeled with N-(idoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid. Several laboratories have noted that the inhibitory region of TnI binds to the III domain of TnC which Cys-98 is a part (Grabarek et al., 1981; Cachia et al., 1983). It is then reasonable that the TnC-TnI interaction would be affected by the covalent attachment of IAE probe to Cys-98 of TnC.

The extent of change in the fluorescence intensity of DANZ-TnC upon complex formation with TnI varied significantly with the buffer system being used. For the magnesium and EDTA buffer systems, values of 27 and 20% enhancement were observed, respectively, when TnI bound. When the Ca++-specific sites are filled, TnI binding causes 9% quenching of the DANZ probe. Since enhancement is observed when calcium ions bind to the Ca++-specific sites (Johnson et al., 1978), the observation that TnI binding causes enhancement in the presence of either magnesium ion or EDTA suggests that the same conformational states of TnC could be stabilized by both TnI and calcium ion binding.

Troponin-I-Troponin T Interaction—The ability of TnI and TnT to form a binary complex has been investigated using a number of techniques: gel filtration (Horwitz et al., 1979; Pearlstone and Smillie, 1980), circular dichroism (Horwitz et al., 1979), chemical cross-linking (Hitchcock, 1975; Chong and Hodges, 1982b), and lysine protection from acetic anhydride labeling (Hitchcock et al., 1981; Hitchcock-DeGregori, 1982). It is apparent from these studies that these two subunits interact in a specific manner within the troponin complex.

A binding isotherm for the formation of the TnI-TnT complex in the presence of EDTA using IAE-TnT as the probe is presented in Fig. 1. The fitted curve, shown by the continuous line, corresponds to an association constant of 7.9 \times 10^6 M^{-1} and a stoichiometry of 1:1. The association constants for this complex are given in Table II for the three

**Table I**

| Buffer   | TnC % | TnI % | TnT % |
|----------|-------|-------|-------|
| DANZ-TnC | Ca++  | 9     | 19    |
|          | Mg++  | 27    | 24    |
|          | EDTA  | 20    | 12    |
| IAE-TnC  | Ca++  | 5     | 12    |
|          | Mg++  | 6     | 16    |
|          | EDTA  | 6     | 5     |
| IAE-TnI  | Ca++  | 6     | 9     |
|          | Mg++  | 11    | 9     |
|          | EDTA NC | 11 | |
| IAE-TnT  | Ca++  | ND    | 49    |
|          | Mg++  | NC    | 47    |
|          | EDTA ND | 46 | |

* Indicates quenching.

* NC, no change; ND, not determined.

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**Fig. 1.** Binding isotherm obtained when TnI was added to IAE-TnT (0.25 μM) in EDTA buffer. ΔF is the observed fluorescence increase. The open squares are the experimental points. The solid line is the calculated best fit; this yields a stoichiometry of 1 mol of TnI/mol of IAE-TnT and an association constant of 7.9 \times 10^6 M^{-1}.
buffers used. It is clear from these results that magnesium and calcium ions do not affect formation of this binary complex. Measurement of the association constants utilizing IAE-TnI as the probe gives the same results. When the overall geometric means for the association constants are calculated from the data for each probe under all three buffer conditions, the following values are obtained: for IAE-TnI, 8 x 10⁶ M⁻¹; and for IAE-TnT, 9 x 10⁹ M⁻¹. The good agreement for the association constants using the same probe in two different subunits strongly suggests that the presence of the probe does not affect formation of the TnI-TnT complex.

**Troponin C-Troponin I Interaction**—Fig. 2 displays a typical binding isotherm for the formation of the TnC-TnI complex in the presence of 1 mM calcium ion using IAE-TnI as the probe. Using this probe and DANZ-TnC, the association constants presented in Table III were obtained. These values (1.7 x 10⁶ to 1.1 x 10⁹ M⁻¹) agree well with each other and are comparable to the value of 5.6 x 10⁶ M⁻¹ obtained by Wang and Cheung (1983) using IAE-TnC as the probe and with the value of 1.5 x 10⁹ M⁻¹ measured by Grabarek et al. (1984) using TnC labeled with N-(iodoacetylamoethyl)-5-naphthalene-1-sulfonic acid. When 3 mM magnesium ion is present rather than calcium ion, the association constant is about an order of magnitude lower (1.2 x 10⁶ M⁻¹). The measured association constant in the absence of divalent cations is 1.0 x 10⁶ M⁻¹. This is quite comparable to the value of 3.0 x 10⁶ M⁻¹ determined by Grabarek et al. (1984) using the TnC labeled with N-(iodoacetylminoethyl)-5-naphthalene-1-sulfonic acid. Using DANZ-TnC as the probe in calcium buffer. In studying a related interaction (DANZ-TnI and brain calmodulin), Olwin et al. (1982) obtained a value of -8.4 kcal/mol. The equilibrium dialysis data of Potter and Gergely (1975) for calcium ion binding to TnC and TnC-TnI for magnesium ion. Calculation of the interaction free energy using their results yields a value of -2.7 kcal/mol. Hence, these two independent measurements show remarkable agreement. The interaction free energy which corresponds to the free energy difference for the binding of TnI to TnC in the presence and absence of calcium ion is -4.4 kcal/mol. The equilibrium dialysis data of Potter and Gergely (1975) for calcium ion binding to TnC and TnC-TnI in qualitative agreement that they yield a negative interaction free energy. However, their data indicate a significantly larger value (-7.6 kcal/mol). The reason for this discrepancy is unknown, but at least a portion of it may be due to the fact that the equivalent of eight association constants rather than two are used in the determination of their value. A smaller discrepancy is noted when our data are compared to the interaction free energy of -5.2 kcal/mol obtained by Cheung et al. (1983) using IAE-TnC as probe in calcium buffer. In studying a related interaction (DANZ-TnI and brain calmodulin), Olwin et al. (1982) obtained a value of -8.4 kcal/mol. We have reached two conclusions based on our data. First, the presence of magnesium ion in the Ca²⁺-Mg²⁺ sites of TnC helps significantly to maintain the structural integrity of the TnC-TnI complex.
This provides support for the concept that the primary role for magnesium ion binding to TnC in resting muscle is to maintain the integrity of troponin on the thin filament (Zot and Potter, 1982). Second, when calcium ion is bound to TnC, the TnC-TnI interaction is strengthened. Presumably this is achieved by altering one or more of the interfaces which TnI and TnC share.

Troponin C-Troponin T Interaction.—The ability of TnC and TnT to form a complex has been established by affinity chromatography (Pearlstone and Smillie, 1978), nonadenaturating polyacrylamide gel electrophoresis (van Eerd and Kawasaki, 1973; Burtnick et al., 1976; Jackson et al., 1975), fluorescence spectroscopy (van Eerd and Kawasaki, 1973; Mani et al., 1974), and hydrodynamic studies (Mani et al., 1974). The strength of this binding interaction, however, has never been determined, neither have the effects of calcium and magnesium ions on the interaction been quantitated. We have utilized IAE-TnC and DANZ-TnC to investigate this interaction as TnT labeled with IAE gave no significant fluorescence change upon complex formation. Difficulty was encountered in measuring the association constants for this interaction, mainly because the factor responsible for the usual trend of TnT to form aggregates in solution (Greaser and Gergely, 1973). In fact, hydrodynamic studies of bovine cardiac TnT suggest that monomeric TnT may be in equilibrium with dimers, trimers, and tetramers (Byers and Kay, 1983). This factor occasionally manifested itself in either high values of n or isotherms which did not display saturation over the course of the titration. Despite these difficulties, we have obtained association constants for this interaction which we think are correct but with substantially larger error. We are investigating this interaction further using fluorescence depolarization.

The association constants for the TnC-TnT interaction in the calcium, magnesium, and EDTA buffers are, respectively, \(4.2 \times 10^6 \text{ M}^{-1}\), \(3.8 \times 10^6 \text{ M}^{-1}\), and \(4.5 \times 10^5 \text{ M}^{-1}\). DANZ-TnC was the probe utilized for the experiments in magnesium and EDTA buffers, and IAE-TnC was used for the experiments in calcium buffer. These results indicated that the presence of \(\text{Mg}^{2+}\) strengthens the interaction between these two subunits. As with the TnC-TnI interaction, this may play a role in maintaining the structural integrity of troponin in the tropomyosin complex in resting muscle. The presence of calcium ion does not strengthen the interaction further as was observed for the TnC-TnI complex.

In summary, our data clearly show a stabilization of the TnC-TnI and TnC-TnT complexes by magnesium ion bound to TnC as compared to the absence of divalent ions in the Ca\(^{4+}\)-Mg\(^{2+}\) sites. This supports the view that the function of magnesium ion bound to the Ca\(^{4+}\)-Mg\(^{2+}\) sites is to enhance the structural integrity of TnC and troponin (Zot and Potter, 1982). The results show that the binding of calcium ions to the Ca\(^{4+}\)-Mg\(^{2+}\) sites and the Ca\(^{4+}\)-specific sites provides an additional strengthening of the TnC-TnI interaction. The strength of the TnI-TnT interaction was unaffected by metal ions. In muscle, the Ca\(^{4+}\)-Mg\(^{2+}\) sites of TnC are occupied by either calcium or magnesium ion. Our results suggest that when contraction is initiated by the binding of calcium ion to the Ca\(^{4+}\)-specific sites, the only binary interaction which has an altered free energy is TnC-TnI. Some caution needs to be exercised when interpreting these results. Absence of an effect of metal ions on the free energy of interaction does not require the interfaces between the subunits to be identical; there could be compensating enthalpy and entropy changes. Furthermore, it is possible that calcium and magnesium ion-dependent changes occur only in native troponin (ternary complex) (Hitchcock-DeGregori, 1982). We are at present investigating both of these possibilities by measuring the enthalpies of binary complex formation and the free energies and enthalpies for ternary complex formation.

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