ENERGETICS OF THE BINDING OF CALCIUM AND TROPONIN I TO TROPONIN C FROM RABBIT SKELETAL MUSCLE

CHIEN-KAO WANG* AND HERBERT C. CHEUNG‡
*Graduate Program in Biophysical Sciences and ‡Department of Biochemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294

ABSTRACT We determined the free energy of interaction between rabbit skeletal troponin I (TNI) and troponin C (TNC) at 10° and 20°C with fluorescently labeled proteins. The sulfhydryl probe 5-iodoacetamidoeosin (IAE) was attached to cysteine (Cys)-98 of TNC and to Cys-133 of TNI, and each of the labeled proteins was titrated with the other unlabeled protein. The association constant for formation of the complex between labeled TNC (TNC*) and TNI was 6.67×10^5 M^{-1} in 0.3 M KCl, and pH 7.5 at 20°C. In the presence of bound Mg^{2+}, the binding constant increased to 4.58×10^7 M^{-1} and in the presence of excess of Ca^{2+}, the association constant was 5.58×10^9 M^{-1}. Very similar association constants were obtained when labeled TNI was titrated with unlabeled TNC. The energetics of Ca^{2+} binding to TNC* and the complex TNI · TNC* were also determined at 20°C. The two sets of results were used to separately determine the coupling free energy for binding TNI and Mg^{2+}, or CA^{2+} to TNC. The results yielded a total coupling free energy of −5.4 kcal. This free energy appeared evenly partitioned into the two species: TNI · $TNC(Mg)_2$ or TNI · $TNC(Ca)_2$, and TNI · $TNC(Ca)_4$. The first two species were each stabilized by −2.6 kcal, with respect to the Ca^{2+} free TNI · TNC complex, and TNI · $TNC(Ca)_4$ was stabilized by −2.8 kcal, with respect to TNI · $TNC(Ca)_2$ or TNI · $TNC(Mg)_2$. The coupling free energy was shown to produce cooperatively complexes formed between TNI and TNC in which the high affinity sites were initially saturated as a function of free Ca^{2+} to yield TNI · $TNC(Ca)_4$. This saturation occurred in the free Ca^{2+} concentration range 10^{-7} to 10^{-5} M. The cooperative strengthening of the linkage between TNI and TNC induced by Ca^{2+} binding to the Ca^{2+}-specific sites of TNC may have a direct relationship to activation of actomyosin ATPase. The nature of the forces involved in the Ca^{2+}-induced strengthening of the complex is discussed.

INTRODUCTION

In vertebrate skeletal muscle, contraction is initiated by Ca^{2+} binding to troponin. The binding triggers a series of reversible molecular interactions among the proteins of the thin filament. These interactions result in structural changes that presumably play a role in the transfer of molecular information from the Ca^{2+} sites of troponin to other specific sites along the thin filament, thereby activating the actomyosin ATPase. Of the three subunits of troponin, troponin C (TNC) is the Ca^{2+} receptor with four Ca^{2+} sites. Two of the sites bind Ca^{2+} with a high affinity at which Mg^{2+} also binds competitively (Ca/Mg sites, or sites III and IV), and the other two sites, referred to as sites I and II, bind Ca^{2+} specifically with a low affinity (Potter and Gergely, 1975). When Ca^{2+} binds to the TNC in the complex formed between TNC and the subunit troponin I (TNI), or in intact troponin, the affinities are one order of magnitude higher than the affinities observed with isolated TNC. These results suggest that TNI, but not the third subunit, troponin T (TNT), plays a major role in enhancing the affinity of TNC for Ca^{2+}. The mechanism by which the initial Ca^{2+} binding signal is amplified and relayed to distant sites has been investigated by several laboratories through structural, kinetic, and biochemical studies (Levine et al., 1977; Johnson et al., 1979; Iio and Konodo, 1981; Leavis et al., 1978; Grabarek et al., 1981). The structural studies based on proton NMR generally demonstrate rearrangements of amino acid residues caused by Ca^{2+} binding to TNC. More extensive conformational changes in TNC are induced by interaction with Ca^{2+} at the high affinity sites (sites III and IV), while Ca^{2+} binding to the Ca^{2+}-specific, low affinity sites (sites I and II) causes less extensive changes. These changes are thought to provide a trigger for other molecular events that ultimately remove the inhibition of the actin-myosin interaction and activate the actomyosin ATPase. Kinetic evidence shows that the rate of Ca^{2+} dissociation from the low affinity sites of TNC, but not the high affinity sites, is more compatible with physiological events. These results have led to the suggestion that the low affinity sites have a regulatory role in contraction.

While the energetics of Ca^{2+} interaction with TNC has been determined by various methods, little is known about the energetics of the interactions among the troponin

subunits. It has been recognized that the binding of TNC with TNI is considerably enhanced in the presence of Ca^{2+}, but no detailed and quantitative studies on the energetics of the interaction have been previously reported. Here we report a study on the interaction between TNI and TNC by extrinsic fluorescence techniques. The thermodynamic properties of the interaction are discussed in terms of coupling free energy. A preliminary report of this work has been presented (Wang and Cheung, 1983).

MATERIALS AND METHODS

Materials

The fluorescent probe 5-iodoacetamidoeosin (IAE) was obtained from Molecular Probes, Inc. (Junction City, OR). Ultrapure enzyme grade urea from Schwartz/Mann was used for protein separation. A standard solution of calcium (0.1 M) was purchased from Orion Research Inc. (Cambridge, MA) and its concentration was redetermined by atomic absorption before use. All other chemicals were of reagent grade.

Protein Preparation

Troponin was prepared from rabbit skeletal muscle by a slight modification of the Ebashi method (Cheung et al., 1982). Troponin C was isolated from the troponin by DEAE-cellulose (model DE-52; Whatman Inc., Clifton, NJ) chromatography in the presence of 6 M urea (Perry and Cole, 1974). Troponin B (TNI · TNT complex) derived from troponin by the method of Wilkinson (1974) was used as the starting material for isolation of TNI. The two subunits were separated on a CM-cellulose (model CM-32; Whatman Inc.) column by using 8 M urea in 50 mM sodium formate, 15 mM β-mercaptoethanol at pH 4.0, and a linear gradient from 0 to 0.15 M NaCl. The purity of the two isolated subunits was monitored by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

The IAE derivative of TNC was prepared as previously described (Cheung et al., 1982). The probe chemically modified TNC at the single sulfhydryl site (Cys-98). Specific labeling of cysteine (Cys)-133 of TNI with IAE was carried out with troponin B before separation of the two subunits. Immediately before labeling, troponin B was first dialyzed against 0.5 M KCl, 2 mM DTT, and 25 mM Tris-HCl at pH 7.5, followed by a second dialysis against the same buffer with DTT omitted. Labeling of troponin B with IAE was carried out in the dark by using a 1.3–1.5-fold molar excess of the probe (4 mM) dissolved in 0.5 M KCl and 25 mM Tris-HCl at pH 7.5. The reaction was allowed to go on for 20–24 h at room temperature, and the resulting solution was dialyzed exhaustively to remove unreacted probe. By this procedure only Cys-133 of TNI in the TNI · TNT complex was modified, since the other two cysteine residues of TNI were protected by TNT from modification by sulfhydryl reagents (Chong et al., 1982). The labeled TNI was separated from TNT by the Wilkinson method (1974). The sulfhydryl content of the proteins was determined spectrophotometrically by using DTNB (Ellman, 1958). The concentrations of unlabeled proteins were determined by absorbance measurements using an absorption coefficient $E(1\%) = 2.3$ at 277 nm for TNC (Murry and Kay, 1972) and $E(1\%) = 3.97$ at 280 nm for TNI (Wilkinson, 1974). Where molar concentrations are reported, molecular weights of 18,000 and 23,000 were used for TNC and TNI, respectively. The extent of probe labeling in the proteins was determined spectrophotometrically by using a molar absorption coefficient of 8.3×10^4 M^{-1}-cm^{-1} at 528 nm (Cheung et al., 1982). Whenever necessary, the absorbance of TNC at 277 nm and TNI at 280 nm was corrected for IAE absorption. The correction terms were estimated from the absorption spectrum of the free probe. These values were $0.22A_p$ for IAE-TNC and $0.20A_p$ for IAE-TNI, where A_p is the peak absorbance of the attached probe in a given sample. Two fluorescent derivatives were used throughout this study: IAE-TNC and IAE-TNI, which are designated as TNC* and TNI*, respectively.

Measurement of ATPase Activity

ATPase activity of actomyosin was measured in a medium containing equal parts by weight of myosin subfragment 1, actin, tropomyosin, and reconstituted troponin in 20 mM Tris (pH 7.5), 40 mM KCl, 2 mM Mg^{2+}, either 1 mM EGTA or 0.1 mM Ca^{2+}, and 1 mM ATP. The troponin was reconstituted by incubating the three subunits in equal molar concentrations before addition to the assay mixture. The activity was followed by release of inorganic phosphate (Fisk and SubbaRow, 1925). These assays were done primarily to ascertain that labeled TNC and TNI retained their ability to confer Ca^{2+} sensitivity to the actomyosin system. This was found to be the case.

Fluorescence Measurement

Steady state fluorescence measurements were made in a Perkin-Elmer (Norwalk, CT) 650:40 ratio fluorescence spectrometer. Sample temperatures were maintained at either 20 ± 0.1° or 10 ± 0.5°C. An initial volume of either 1.5 or 2.0 ml was used in most measurements. The basic sample buffer was 0.3 M KCl, 25 mM HEPES, 1 mM EGTA, and 1 mM DTT at pH 7.5. Ca^{2+} concentration was controlled by the EGTA and calculated as described in another section. The quantum yield of IAE attached to the proteins was determined by the comparative method with quinine sulfate in 0.1 N H_2SO_4 as the standard. A value of 0.70 was used as the quantum yield of the standard at 20°C (Scott et al., 1970). The apparent quantum yield was corrected for the effect of depolarization due to Brownian rotational motion by using the limiting fluorescence anisotropy of the attached probe (Shinitzky, 1972). Fluorescence anisotropy was measured in the same Perkin-Elmer instrument, equipped with a Polaroid HNP'B polarizer assembly (Polaroid Corp., Cambridge, MA) and a magnetic stirrer (Wood Manufacturing Co., Newton, PA); standard corrections were made for instrumental depolarization.

The interaction between TNC* and TNI, and between TNC and TNI*, was monitored by changes in probe fluorescence intensity. In a typical experiment, a labeled protein was titrated with an unlabeled protein under constant stirring, and the fluorescence intensity at 560 nm (with excitation at 520 nm) was recorded after each titration. Since the observed intensity in a mixture is the weighted average intensities of free and bound fluorescent species, the relationship between the observed intensity (I) and the intensities contributed from the free and bound species is

$$I = f_F I_F + f_B I_B, \tag{1}$$

where f_F and f_B represent the mole fraction of the free and bound fluorescent species, and I_F and I_B refer to the intensities of the free and bound forms. Experimentally, I_F and I_B were determined from the limits of titration. When titration is monitored by fluorescence anistropy, the observed anisotropy (A) is given by

$$A = \frac{Q_F f_F}{Q_F f_F + Q_B f_B} A_F + \frac{Q_B f_B}{Q_F f_F + Q_B f_B} A_B, \tag{2}$$

where A_F and A_B refer to the anisotropies of the free and bound fluorescent species, and Q_F and Q_B represent the quantum yields of free and bound species, respectively. Eq. 2 is rearranged to yield a useful expression to calculate the fraction of bound species f_B from experimental data:

$$f_B = \frac{A - A_F}{(A_B - A)R + A - A_F}, \tag{3}$$

where R is Q_B/Q_F. For $R = 1.0$, i.e., there is no quantum yield change upon binding, Eq. 3 reduces to

$$f_B = \frac{A - A_F}{A_B - A_F}. \tag{4}$$

The observed anisotropy is defined in the usual way by

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}, \quad (5)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescent intensities observed parallel and perpendicular to the direction of polarization of the exciting light, respectively. The initial concentration of labeled protein was usually in the range 10^{-10}–10^{-9} M when fluorescence intensity was measured, and 10^{-7}–10^{-6} M when anisotropy was determined.

Calcium Binding Measurement

In a previous study we found that the fluorescent probe IAE attached to Cys-98 of TNC responded spectrally not only to Ca^{2+} binding to the high affinity Ca/Mg sites, but also to the low affinity Ca-specific sites (Cheung et al., 1982). This change in IAE fluorescence enabled us to monitor Ca^{2+} interaction with TNC* and TNI · TNC* in the present work. The TNI · TNC* complex was prepared by incubating TNC* with an amount of unlabeled TNI, which was severalfold in excess of what was required to ensure that at least 99% of the TNC* was complexed with TNI, based on the equilibrium constant determined in the present work. The solution was exhaustively dialyzed against 0.3 M KCl, 25 mM HEPES, and 1 mM EGTA at pH 7.5 before Ca^{2+} titration. A standard calcium solution (0.1 M) was added in 0.5–2.0 μl increments from a Manostat Digipet Ultramicro pipettor (Manostat Corp., New York, NY) into 2.0 ml of protein solution contained in a cuvette thermostated in the fluorometer. Small volumes (microliters) of KOH were added, whenever necessary, to restore the pH after Ca^{2+} addition. pH measurements were made with a combined electrode on a digital pH meter (model PHM 84; Radiometer America Inc., Westlake, OH). The free Ca^{2+} concentration was calculated from the contaminating Ca^{2+} ($\leq 1.0\,\mu$M), plus the amount of calcium added, by using the computer program of Perrin and Sayce (1967). The amount of contaminant Ca^{2+} was determined by an Instrument Laboratory (model 251; Lexington, MA) atomic absorption spectrophotometer. The following standard logarithmic association constants for Ca^{2+} and H^+ to EGTA were used in the calculations: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^-, 2.0; Ca^{2+} to $EGTA^{4-}$, 11.0; Ca^{2+} to $HEGTA^{3-}$, 5.33.

Analysis of Binding Data

The binding data from the titrations of TNC* with TNI and TNI* with TNC were evaluated by fitting the observed parameter f_B to the equation

$$f_B = \sum_j \frac{n_j k_j [L]}{1 + k_j [L]}. \quad (6)$$

In this expression, n_j and k_j are the number of binding sites and the microscopic association constant, respectively, for the jth class of sites, $[L]$ is the concentration of free ligand, and f_B is the experimental parameter determined from Eq. 1. If $j = 1$, k_1 becomes identical with the macroscopic association constant denoted by K. In the case of Ca^{2+} titration of TNC* and TNI · TNC* monitored by the fluorescence of the extrinsic probe, f_B is the fractional change in fluorescence as determined from Eq. 1, n_j is the relative contribution of the jth class of binding sites to the total signal change. An iterative nonlinear least squares procedure, the Gauss-Newton method, was used to estimate n_j and k_j (Draper and Smith, 1980). Starting values for k_j and n_j were chosen and continually improved until the convergence condition was met. The goodness of fit was judged by (*a*) visual inspection of superimposed f_B (observed) and f_B (calculated) and (*b*) evaluation of the chi-square ratio which is defined by

$$\chi^2 = \sum \frac{[f_B\,(\text{observed}) - f_B\,(\text{calculated})]^2}{f_B\,(\text{observed})}. \quad (7)$$

RESULTS

Characterization of Labeled Proteins

Sulfhydryl titration of TNC labeled with IAE indicated a loss of 0.8–1.0 mol of sulfhydryl group/mol protein. This loss correlated with the degree of IAE labeling determined spectrophotometrically. Labeling specificity was also ascertained by extraction of the tryptic TR-2 fragment, as previously described (Cheung et al., 1982). The TNC* samples used in most of the experiments had a degree of labeling in the range 0.8–0.95. The −SH content of IAE-labeled TNI was in the range 2.0–2.2/TNI molecule. This suggested a degree of labeling in the range 0.8–1.0, which was confirmed by absorbance measurements of the attached probe. The labeled site was likely Cys-133, since this is the only sulfhydryl group accessible to modification in the TNI · TNT (troponin B) complex (Chong et al., 1982).

Titration of IAE-TNC with TNI

IAE-TNC exhibited an emission spectrum with the maximum at 556 nm. Addition of excess of TNI resulted in a 1.60-fold increase in fluorescence intensity, with the emission peak shifted to 560 nm. Saturation of TNC* with CA^{2+} in the presence of excess of TNI led to a further enhancement to a final level of 1.95, with the emission maximum at 562 nm. These spectral changes provided a basis for determining the energetics of the interaction between TNC and TNI and between TNI · TNC and CA^{2+}.

The change of fluorescence intensity was used to determine the association constants between TNI and TNC* in the presence and absence of Ca^{2+}. Complexation between TNI and TNC*, which was already saturated with Mg^{2+} at the Ca/Mg sites, was accompanied by a small increase in IAE fluorescence. This necessitated the use of fluorescence anisotropy to monitor the interaction. Three species of TNC* were titrated with unlabeled TNI: apo-TNC*, TNC*$(MG)_2$, and TNC*$(Ca)_4$. The fully saturated TNC* species was prepared by addition of sufficient Ca^{2+} controlled by an EGTA buffer to yield the desired Ca^{2+} complex. The binding constants of TNC* for Ca^{2+} determined in the present work were used to prepare the complex. TNC*$(Mg)_2$ refers to TNC*, in which the two high affinity Ca^{2+}/Mg^{2+} sites were saturated with Mg^{2+}. It was prepared by the addition to TNC* of a sufficient amount of Mg^{2+} that was severalfold in excess of the concentration required to saturate all four Mg^{2+} sites, two of which are the Ca/Mg sites (Potter and Gergely, 1975). The results from single titration experiments that were performed with apo-TNC* and TNC*$(Ca)_4$ are shown in Fig. 1 and the corresponding results obtained with TNC*$(Mg)_2$ are shown in Fig. 2. The anisotropies of TNC*$(Mg)_2$, A_F and TNI · TNC*$(Mg)_2$, A_B, were 0.2021 and 0.2420, respectively. The ratio of the corresponding quantum yields (R) was 1.10. The association constants at

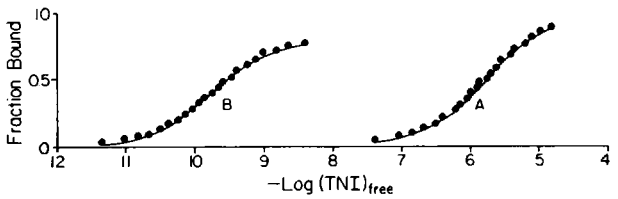


FIGURE 1 Titration of IAE-TNC (TNC*) with TNI, 0.3 M KCl, 1 mM EGTA, 1 mM DTT, and 25 mM HEPES at pH 7.5 and 20°C. The systems are TNC* + TNI (*A*), and TNC*$(Ca)_4$ + TNI (*B*). The initial TNC* concentrations were 4.60 μM in *A*, and 1.43 nM in *B*. The interactions were monitored by IAE fluorescence intensity at 560 nm with 520 nm excitation. Spectral bandwidths were 1 nm (excitation) and 2 nm (emission) for *A* and 10 nm (excitation) and 15 nm (emission) for *B*. The solid lines are the best fitted results of the binding data to Eq. 6.

20°C derived from these and additional experiments are 6.7×10^5, 4.6×10^7, and 5.6×10^9 M^{-1} for the interaction of TNI with TNC*, TNC*$(Mg)_2$, and TNC*$(Ca)_4$, respectively. These binding constants, the binding stoichiometries (n), and the corresponding free energy changes at two temperatures are listed in Table I. The observed logarithmic intervals of free TNI concentration between fractional saturations of 0.1 and 0.9 are 1.82, 2.03, and 2.00 for the titration of TNC* with TNI in the absence of cation, and in the presence of Mg^{2+} and excess of Ca^{2+}, respectively. For a macromolecule containing equal and independent binding sites, 1.908 U of the logarithmic interval in free ligand concentration are required to progress from 0.1 to 0.9 fractional saturation of the binding sites, i.e., f_B increases from 0.1 to 0.9 (Weber, 1975). A broader interval indicates either negative cooperativity or the presence of heterogeneity in the affinities of binding sites. A narrower interval indicates a positive cooperativity. The observed intervals suggest that the extent of cooperativity and affinity heterogeneity of sites for cations and TNI are relatively small.

Calcium Binding to TNC* and TNI · TNC*

When TNC* was titrated with Ca^{2+}, the probe signal increased with increasing $[Ca^{2+}]$, leveling off at ~4 mol of Ca^{2+}/mol of protein. When the fluorescence change was plotted vs. pCa, the resulting curve appeared biphasic with inflections in the pCa range 7 and 5, in agreement with the expectation of two classes of binding sites. This binding isotherm and the corresponding curve obtained with TNI · TNC* are shown in Fig. 3. The binding parameters determined from the best fit of the data to Eq. 6 are listed in Table II. Attempts to fit the data to four distinct and independent sites, each with a different intrinsic microscopic association constant, were unsuccessful. Qualitatively, when TNC* was initially complexed with TNI, the affinity of the high affinity Ca/Mg sites and the low affinity Ca^{2+}-specific sites became enhanced to about the same extent. The enhancements observed with labeled TNC are in agreement with those previously reported with unlabeled proteins (Potter and Gergely, 1975). It appears that the IAE probe located at Cys-98 of TNC does not significantly perturb the interaction between Ca^{2+} and the labeled TNC or its complex with TNI.

General Scheme for Ca^{2+} Binding to TNC and TNI · TNC

Scheme I shows the various possible interactions for the formation of the TNC$(Ca)_i$ species in the presence of

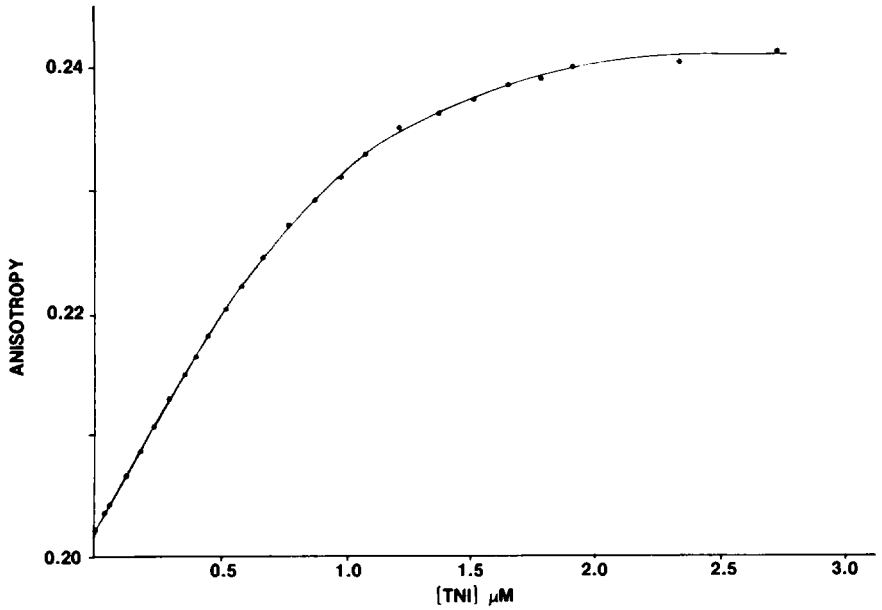
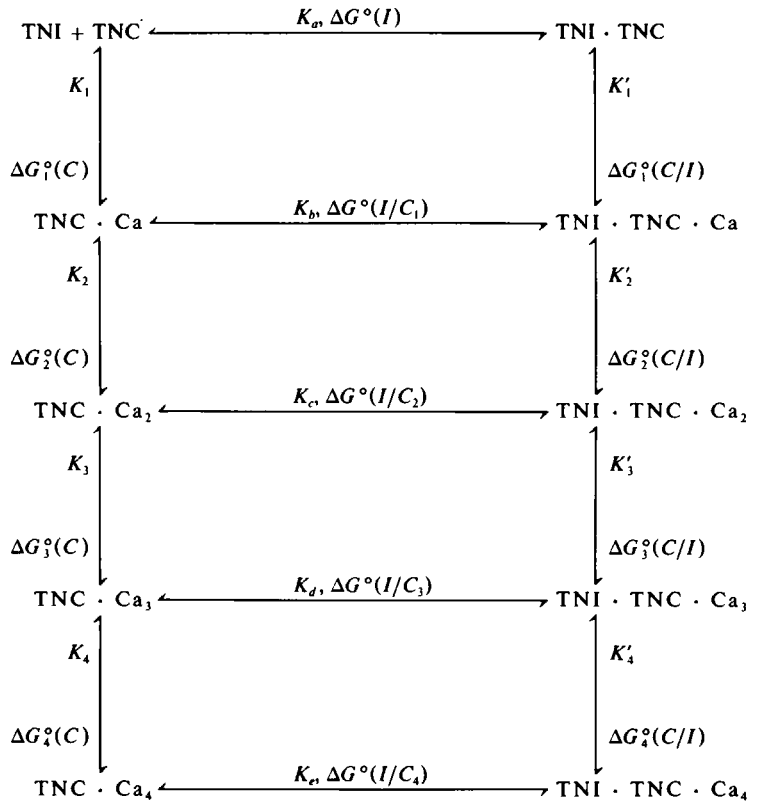


FIGURE 2 Titration of TNC*$(Mg)_2$ with TNI at 20°C, monitored by the fluorescence polarization of IAE attached to the TNC. The initial TNC* concentration was 0.87 μM. See Fig. 1 for other general conditions.



Scheme I
THERMODYNAMIC CYCLE FOR FORMATION OF
TNI · TNC · Ca_4

TNI[1]. The equilibrium constants are the stoichiometric association constants governing the designated individual reversible reactions. A number of pathways can lead to the formation of TNI · $TNC(Ca)_4$. On the basis of free energy conservation, the following expression relating to the association constants must hold:

$$K_a K'_1 K'_2 K'_3 K'_4 = K_e K_1 K_2 K_3 K_4 \tag{8}$$

$$\frac{K_e}{K_a} = \frac{K'_1 K'_2 K'_3 K'_4}{K_1 K_2 K_3 K_4}, \tag{9}$$

with similar expressions relating K_a and K_c, and K_c and K_e: $K_c K'_1 K'_2 = K_a K_1 K_2$ and $K_c K'_3 K'_4 = K_e K_3 K_4$. K_a and K_e correspond to the constants given in Table I that were determined for the binding of TNI to TNC* in the absence and presence of a large excess of Ca^{2+}, respectively. The values K_i and K'_i are readily derived from the intrinsic association constants of TNC for Ca^{2+} from the general relationship $K_i = k(n - i + 1)/i$, where n is the number of sites and k is the intrinsic association constant. K_1 and K_2 were calculated from the intrinsic association constant k_1 given in Table II with $n = 2$ and $i = 1, 2$, and K_3 and K_4 from k_2 with $n = 2$, and $i = 1, 2$. A similar calculation yielded K'_1, K'_2, K'_3, and K'_4 for the TNI · $TNC(Ca)_i$ complexes. These stoichiometric association constants, and those for complex formation between TNI and TNC*, are summarized in Table III. K_a for the binding of TNI is of the order of 10^6 M^{-1}, and K_e of the order of 10^{10} M^{-1}. The ratio K_e/K_a is 0.84×10^4 and the ratio of the products of the Ca^{2+} binding constants (right-hand side of Eq. 9) is 1.19×10^4. This good argeement indicates that Eq. 9 is satisfied for the present system, and provides an indication of internal consistency of the various parameters that were determined in the present study.

It was difficult to obtain TNC* species that contained precisely 2 mol of bound Ca^{2+} at the Ca/Mg sites. This difficulty prevented direct determination of K_c. Since $K_c/K_a = K'_1 K'_2 / K_1 K_2$, K_c can be estimated from the other five constants listed in Tables I and III. This value (7.4×10^7) is very close to the equilibrium constant for the formation of TNI · $TNC*(Mg)_2$ (4.58×10^7, Table I). These results suggest that for the present system the

[1] K_i are the stoichiometric association constants for Ca^{2+} binding to isolated TNC, with corresponding standard free energy changes $\Delta G°_i(C)$. K'_i are the stoichiometric association constants for Ca^{2+} binding to TNC in the binary protein complex TNI · TNC. $\Delta G°_i(C/I)$ is the conditional free energy change for Ca^{2+} binding to TNC when TNI is already bound to TNC. K_a refers to the association constant of TNI for TNC, K_b, K_c, K_d, and K_e refer to the association constants of TNI for TNC, in which 1, 2, 3, and 4 mol of Ca^{2+} are already bound to the TNC. $\Delta G°(I)$ and $\Delta G°(I/C_i)$ are the standard free energy changes for the formation of the TNI · TNC complexes in the absence of bound Ca^{2+} and when i mol of Ca^{2+} are already bound to the TNC, respectively.

TABLE I
BINDING PARAMETERS FOR TNI · TNC* COMPLEX FORMATION

Temperature	TNC* species	n	K	$\Delta G°$
			M^{-1}	$kcal\text{-}mol^{-1}$
20°C	TNC*	0.95 ± 0.02	(6.67 ± 0.31) × 10^5	−7.81
	TNC*$(Mg)_2$	1.12 ± 0.02	(4.58 ± 0.29) × 10^7	−10.28
	TNC*$(Ca)_4$	0.79 ± 0.01	(5.58 ± 0.23) × 10^9	−13.07
10°C	TNC*	1.03 ± 0.09	(4.24 ± 0.67) × 10^5	−7.29
	TNC*$(Mg)_2$	1.06 ± 0.04	(2.51 ± 0.24) × 10^7	−9.59
	TNC*$(Ca)_4$	0.89 ± 0.05	(2.09 ± 0.05) × 10^9	−12.07

See Fig. 1 for experimental conditions. The errors associated with n and K are the standard errors of the best fit at the 95% confidence level.

binding constant between TNI and $TNC(Mg)_2$ and K_c were approximately equal. A value of $4.58 \times 10^7\ M^{-1}$ was used for K_c in this work.

Calculation of Coupling Free Energy

The binding data given in Table III can be analyzed according to Scheme I by the linked function theory in terms of coupling free energy (Weber, 1975). The total standard free energy change for the formation of TNI · TNC is $\Delta G°(I)$, for TNI binding to TNC that contains two bound Ca^{2+} is $\Delta G°(I/C_2)$, and for the interaction between TNI and TNC that contains four bound Ca^{2+} is $\Delta G°(I/C_4)$. These free energy changes are related to the observed association constants by the usual expressions:

$$\Delta G°(I) = -RT \ln K_a \quad (10)$$

$$\Delta G°(I/C_2) = -RT \ln K_c \quad (11)$$

$$\Delta G°(I/C_4) = -RT \ln K_e. \quad (12)$$

The principle of conservation of free energy requires that

$$\Delta G°(I/C_2) - \Delta G°(I) = \sum_{i=1}^{2} [\Delta G_i°(C/I) - \Delta G_i°(C)] \equiv 2\Delta G_{I,C}(1, 2) \quad (13)$$

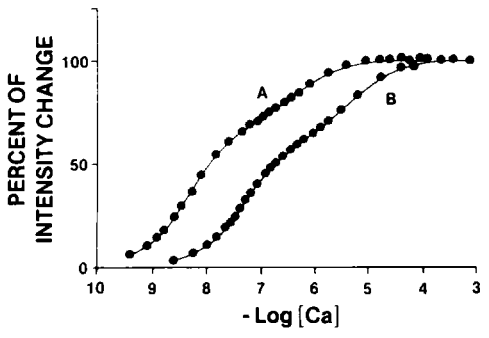


FIGURE 3 Titration of IAE-TNC (TNC*) and TNI · TNC* with Ca^{2+} at 20°C. The concentration of TNC* was 1.0 μM and the concentration of TNC* in TNI · TNC* was 0.2 μM. (*A*) TNI · TNC* + Ca^{2+}. (*B*) TNC* + Ca^{2+}. Other general conditions are given in Fig. 1.

$$\Delta G°(I/C_4) - \Delta G°(I/C_2) = \sum_{i=3}^{4} [\Delta G_i°(C/I) - \Delta G_i°(C)] \equiv 2\Delta G_{I,C}(3, 4) \quad (14)$$

$$\Delta G°(I/C_4) - \Delta G°(I) = \sum_{i=1}^{4} [\Delta G_i°(C/I) - \Delta G_i°(C)] \equiv 4\Delta G_{I,C}(1, 4). \quad (15)$$

The notation $\Delta G_{x,y}$ represents the average free energy of coupling for binding 1 mol of x and 1 mol of y simultaneously to TNC. $\Delta G_{I,C}(1, 2)$ is the average coupling free energy for binding 1 mol of TNI and 1 mol of Ca^{2+} to a high affinity site. The quantity $2\Delta G_{I,C}(1, 2)$ is the total coupling free energy for binding 1 mol of TNI and 2 mol of Ca^{2+} to both high affinity sites. $\Delta G_{I,C}(3, 4)$ is the average coupling free energy for binding 1 mol of TNI and 1 mol of Ca^{2+} to a low affinity Ca^{2+}-specific site of TNC that already contains 2 mol of bound Ca^{2+} at the high affinity sites. Finally, $\Delta G_{I,C}(1, 4)$ is the average coupling free energy for binding 1 mol of TNI and 1 mol of Ca^{2+} to TNC at any of the four sites. The quantity $4\Delta G_{I,C}(1, 4)$ represents the extent to which the TNI · TNC complex is stabilized by all four bound Ca^{2+}. Similarly, $2\Delta G_{I,C}(1, 2)$ indicates the stabilization of the protein complex by two bound Ca^{2+} at the high affinity sites, and $2\Delta G_{I,C}(3, 4)$ indicates the stabilization of TNI · $TNC(Ca)_2$ when two additional Ca^{2+} ions bind to low affinity sites of the TNC.

The free energy changes at 20°C for the formation of TNI · TNC*, TNI · TNC*$(Mg)_2$, and TNI · TNC*$(Ca)_4$ were $\Delta G°(I) = -7.81$, $\Delta G°(I/C_2) = -10.28$, and $\Delta G°(I/C_4) = -13.07$ kcal-mol^{-1}, respectively. From these values the following coupling free energy values were readily obtained: $4\Delta G_{I,C}(1, 4) = -5.26$ kcal, $2\Delta G_{I,C}(1, 2) = -2.47$ kcal, and $2\Delta G_{I,C}(3, 4) = -2.79$ kcal. The corresponding values of coupling free energy derived from the vertical pathway of Scheme I (i.e., from Ca^{2+} binding to TNC* and TNI · TNC*) were −5.47, −2.75, and −2.72 kcal. Thus, the binary complex, fully saturated with Ca^{2+}, was more stable than the apo-complex TNI · TNC by 5.4 kcal. This total free energy of coupling was equally partitioned into the two classes of sites. The relative energetic states of the TNI · TNC complexes are summarized in Fig. 4.

TABLE II
PARAMETERS FOR Ca^{2+} BINDING TO TNC* AND TNI · TNC*

Protein	n_1	k_1	n_2	k_2
		M^{-1}		M^{-1}
TNC*	0.63 ± 0.01	(1.99 ± 0.04) × 10^7	0.37 ± 0.01	(1.94 ± 0.11) × 10^5
TNI · TNC*	0.70 ± 0.01	(2.10 ± 0.08) × 10^8	0.29 ± 0.01	(2.01 ± 0.23) × 10^6

These parameters were obtained at 20°C. k_1 and k_2 are the intrinsic microscopic association constants for two classes of sites, with n_1 and n_2 being the relative contributions of class 1 and class 2 sites to the total fluorescence change. Experimental conditions are given in Fig. 3.

We derived from Scheme I two theoretical saturation functions of TNC. The function S_c describes the fractional saturation of TNC for Ca^{2+} as a function of free TNI concentration, and the other function S_I describes the fractional saturation of TNC for TNI as a function of free Ca^{2+} concentration. Following Weber's notation (1975), the two functions are given below:

$$S_c = \frac{\dfrac{\epsilon_1(1+\epsilon_0\beta_1)}{1+\epsilon_0} + \dfrac{\epsilon_1^2(1+\epsilon_0\beta_1^2)}{1+\epsilon_0} + \dfrac{3\epsilon_1^2\epsilon_2(1+\epsilon_0\beta_1^2\beta_2)}{1+\epsilon_0} + \dfrac{2\epsilon_1^2\epsilon_2^2(1+\epsilon_0\beta_1^2\beta_2^2)}{1+\epsilon_0}}{2 + \dfrac{4\epsilon_1(1+\epsilon_0\beta_1)}{1+\epsilon_0} + \dfrac{2\epsilon_1^2(1+\epsilon_0\beta_1^2)}{1+\epsilon_0} + \dfrac{4\epsilon_1^2\epsilon_2(1+\epsilon_0\beta_1^2\beta_2)}{1+\epsilon_0} + \dfrac{2\epsilon_1^2\epsilon_2^2(1+\epsilon_0\beta_1^2\beta_2^2)}{1+\epsilon_0}} \quad (16)$$

$$S_I = \frac{\epsilon_0}{\epsilon_0 + \left\{\dfrac{1+2\epsilon_1+\epsilon_1^2(1+\epsilon_2)^2}{1+2\epsilon_1\beta_1+\epsilon_1^2\beta_1^2(1+\epsilon_2\beta_2)^2}\right\}}, \quad (17)$$

where $\epsilon_0 = [I]/\kappa_a(I)$, $\epsilon_1 = [C]/\kappa_1(C)$, $\epsilon_2 = [C]/\kappa_2(C)$, $\beta_1 = \exp(-\Delta G_{I,C}(1,2)/RT)$, and $\beta_2 = \exp(-\Delta G_{I,C}(3,4)/RT)$. $[I]$ and $[C]$ are the respective free concentrations of TNI and Ca^{2+}, $\kappa_a(I)$ is the dissociation constant for TNI · TNC, and $\kappa_1(C)$ and $\kappa_2(C)$ are the intrinsic dissociation constants for Ca^{2+} binding to the high and low affinity sites of TNC, respectively. These dissociation constants, $\kappa_a(I)$, $\kappa_1(C)$, and $\kappa_2(C)$ are reciprocals of the association constant K_a, and the intrinsic association constants k_1 and k_2, respectively, as shown in Tables II and III. Since $\Delta G_{I,C}$ from the present system < 0, β_1 and $\beta_2 >$ 1.0. These functions indicate that saturation of TNC with Ca^{2+} should enhance the affinity of TNC for TNI and the affinity of both classes of Ca^{2+} sites of TNC is enhanced in the binary TNI · TNC complex.

TNC and TNI are complexed with troponin T in muscle with a 1:1:1 stoichiometry. The concentration of a dissociated subunit of the troponin complex under any physiological condition is irregularly high, and this concentration is unlikely to fluctuate over a wide range. On the other hand, myoplasmic-free Ca^{2+} concentration is expected to vary over a range of two to three orders of magnitude during a relaxation-contraction cycle. This fluctuation must modulate the saturation of TNC by the other two subunits. The function S_I is of immediate interest, as it depicts the extent of saturation of the various TNC$(Ca)_i$ species for TNI. At a given free Ca^{2+} level, S_I increases with increasing [TNI]. The saturation curve is expected to shift toward higher free $[Ca^{2+}]$ with decreasing [TNI]. In the region of very low free $[Ca^{2+}]$, i.e., $[Ca^{2+}] \ll \kappa_1(C)$ and $\kappa_2(C)$, S_I reduces to a simple saturation function, $S_I = [TNI]/\{[TNI] + \kappa_a(I)\}$, in which the dissociation of TNI · TNC is governed by $\kappa_a(I)$ as would be expected. In the region of large saturation, i.e., $[Ca^{2+}] \gg \kappa_1(C)$, and $\kappa_2(C)$, S_I also reduces to a simple saturation function, in which the dissociation of the complex is described by a dissociation constant equal to $\kappa_a(I)\beta_1^{-2}\beta_2^{-2}$. Since β_1 and $\beta_2 > 1.0$, the equivalent dissociation constant is considerably smaller than $\kappa_a(I)$, the magnitude of which is determined by coupling free energies. The extent of cooperativity can be shown (Weber, 1975) to increase with decreasing ϵ_0, hence with decreasing [TNI]. These characteristics are shown in Fig. 5 *A* and several of the parameters are

TABLE III
STOICHIOMETRIC BINDING CONSTANTS FOR Ca^{2+} BINDING TO TNC* AND TNI · TNC* AND FOR INTERACTION BETWEEN TNI AND TNC*

TNC* + 4Ca → TNC*$(Ca)_4$	TNI · TNC* + 4Ca → TNI · TNC*$(Ca)_4$	TNI + TNC*$(Ca)_i$ → TNI · TNC$(Ca)_i$
M^{-1}	M^{-1}	M^{-1}
K_1 3.98 × 10^7	K_1' 4.20 × 10^8	$K_a(i=0)$ 6.67 × 10^5
K_2 0.99 × 10^7	K_2' 1.05 × 10^8	$K_c(i=2)$ 4.58 × 10^7
K_3 3.88 × 10^5	K_3' 4.02 × 10^6	$K_e(i=4)$ 5.58 × 10^9
K_4 0.97 × 10^5	K_4' 1.01 × 10^6	

K_i and K_i' were calculated from the observed intrinsic association constants shown in Table II as described in the text. K_a, K_c, and K_e are taken from Table I and listed here to provide a summary of all association constants of Scheme 1. The constant for formation of TNI · TNC*$(Mg)_2$ is assumed to be equivalent to that for formation of TNI · TNC*$(Ca)_2$, K_c (see text).

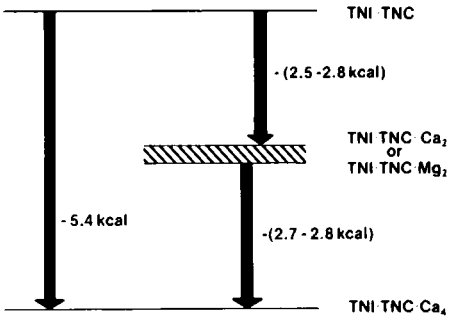


FIGURE 4 Summary of coupling free energy for binding of TNI and Ca^{2+} (or Mg^{2+}) to TNC. The stabilization of the binary protein complex by occupation of the high affinity Ca/Mg sites was derived from the first and third horizontal pathways in Scheme I for Mg binding, and from the first two vertical pathways for Ca binding. The coupling free energy for transition for TNI · TNC to TNI · $TNC(Ca)_4$ was derived from both sets of horizontal and vertical pathways.

listed in Table IV. The saturation curves were calculated with various observed parameters for two different values of [TNI]. At 10^{-7} M, the extent of cooperativity is small (logarithmic interval of free Ca^{2+} is Ca 2.17), and it becomes appreciably higher (logarithmic interval 1.83) at 10^{-8} M with the free Ca^{2+} concentration for half-maximum saturation shifted from 7.5 to 6.5. It is of interest to examine the curves predicted for a system in which the initial TNC species is $TNC(Mg)_2$. This system mimics in vivo relaxed muscle in which the Ca/Mg sites of TNC are saturated by Mg^{2+}. Eq. 17 is easily reduced to describe such a system. The curves depicted in Fig. 5 *B* describe the saturation of TNI · TNC for TNI as a function of free Ca^{2+}, where the TNC initially contains two bound cations at the Ca/Mg sites. This saturation shows considerable positive cooperativity, particularly at [TNI] = 10^{-8} M (Table IV). The concentration of TNI at which maximum cooperativity is expected for transition from TNI · $TNC(Ca)_2$ (or TNI · $TNC(Mg)_2$) to TNI · $TNC(Ca)_4$ is obtained by maximizing the difference between $K_3(C)$ and $K_4(C)$ (Weber, 1975). This condition is $\epsilon_0'^2 = \beta_2^{-3}$, where $\epsilon_0' = [TNI]K_c(I)$. From the observed values β_2 and $K_c(I)$, [TNI] ≃ 0.5 nM. Under the present experimental conditions, maximum cooperativity of TNI and Ca^{2+} binding to the low affinity sites occurs in the free Ca^{2+} range 10^{-7}–10^{-5} M, and when [TNI] is just below 1 nM.

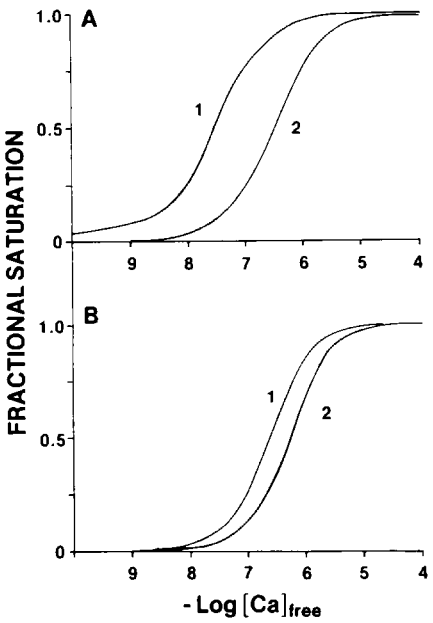


FIGURE 5 Calculated fractional saturation of TNC by TNI as a function of free Ca^{2+} concentration at 20°C, S_f (Eq. 17). (*A*) Saturation of apo-TNC for TNI at [TNI] = 10^{-7} M (curve 1) and 10^{-8} M (curve 2). The curves were calculated with $\Delta G_{I,C}(1, 2) = -1.24$ kcal-mol^{-1}, and $\Delta G_{I,C}(3, 4) = 1.40$ kcal-mol^{-1}; $\kappa_a(I) = 1.50 \times 10^{-6}$ M, $\kappa_1(C) = 5.02 \times 10^{-8}$ M, and $\kappa_2(C) = 5.05 \times 10^{-6}$ M. (*B*) Saturation of TNI · $TNC(Mg)_2$ for TNI. Eq. 17 was reduced to $S_f = \epsilon_0'/\{\epsilon_0' + [(1 + \epsilon_2)/(1 + \epsilon_2\beta_2)]^2\}$ for this case, where $\epsilon_0' = [TNI] \cdot K_c(I)$, since $K_c = 4.58 \times 10^7$ M^{-1} was the relevant stoichiometric association constant for the initial complexation of TNI with $TNC(MG)_2$. Curve 1, [TNI] = 10^{-7} M, curve 2 [TNI] = 10^{-8} M. $\beta_2 = \exp[-\Delta G_{I,C}(3, 4)/RT]$, where $\Delta G_{I,C}(3, 4) = -1.40$ kcal-mol^{-1}; $\kappa_2(C) = 5.15 \times 10^{-6}$ M.

Titration of IAE-TNI with TNC

Since Cys-98 of TNC is not generally accessible to chemical modification when TNC is complexed with TNI, the residue is assumed to be located at the interface between the two proteins in their complex. The binding affinity between the TNC* and TNI may not truly reflect their interaction that occurs with unlabeled proteins. To examine this possibility, IAE was used to label Cys-133 of TNI. Since this residue is readily modified in either intact troponin or troponin B, it is unlikely to be at the interface between TNC and TNI (Chong and Hodges, 1982). The labeled TNI emitted maximally at 557 nm upon excitation with 520 nm radiation. The fluorescence was insensitive to either Mg^{2+} or Ca^{2+}. When a large excess of TNC was added to the TNI*, the probe intensity increased by ~40%, with a 2-nm red shift of the emission peak. Addition of an excess of Ca^{2+} to the protein mixture resulted in a further fluorescence enhancement to a final level of ~80% and a final red spectral shift of 3 nm. The interaction between TNI* and TNC was monitored by fluorescence intensity changes when titrated with apo-TNC and TNC containing four bound CA^{2+}. The corresponding titration with TNC saturated with Mg^{2+} at the Ca/Mg sites was monitored by fluorescence polarization. Fig. 6 shows typical results from titration of TNI* with TNC. The anisotropy values derived from the TNI* · $TNC(Mg)_2$ system were $A_F = 0.2100$ and $A_B = 0.2563$ at 20°C, and $A_F = 0.2173$ and $A_B = 0.2668$ at 10°C. The binding parameters for this system are summarized in Table V. The results compared favorably with those derived from TNC*. The logarithmic intervals between 0.1 and 0.9 fractional saturation of TNI* by apo-TNC, $TNC(Mg)_2$, and $TNC(Ca)_4$ were 1.93, 2.00, and 1.93, respectively.

TABLE IV
PARAMETERS DERIVED FROM SATURATION OF TNC BY TNI AND CALCIUM

Transition	[TNI]	Total coupling free energy	$\Delta G_{I,C}(1,2)$	$\Delta G_{I,C}(3,4)$	Logarithmic interval	pCa(½)
	M	*kcal*	*kcal-mol*$^{-1}$	*kcal-mol*$^{-1}$		
TNI · TNC to TNI · TNC(Ca)$_4$	10^{-7}	−5.26	−1.24	−1.40	2.17	7.5
	10^{-8}	−5.26	−1.24	−1.40	1.83	6.5
TNI · TNC(Mg)$_2$ to TNI · TNC(Ca)$_4$	10^{-7}	−2.79	—	−1.40	1.67	6.6
	10^{-8}	−2.79	—	−1.40	1.50	6.3

The saturation function S_f was calculated from Eq. 17 with the parameters listed in columns 2–5 and other appropriate parameters taken from Tables I and III. The last two columns were obtained from the calculated S_f curves shown in Fig. 5. The logarithmic interval refers to the interval in free Ca^{2+} concentration for fractional saturation to progress from 0.1 to 0.9.

Temperature Effect on the TNI-TNC Interaction

From the association constants determined at 10° and 20°C the changes in enthalpy and entropy associated with complex formation between TNI and TNC were calculated in the usual way from the expression $\Delta G = \Delta H - T\Delta S$. These values are listed in Table VI. The complex formation was endothermic, and the reaction was driven by a large increase in entropy. The stabilization of the protein complex by cations was accompanied by a large increase in both enthalpy and entropy.

DISCUSSION

Calcium binding to troponin C is the first step in Ca^{2+} activation of actomyosin ATPase and force generation in skeletal muscle. This binding is not a cooperative process, while the other two processes are highly cooperative. The initial Ca^{2+} binding event must be translated into molecular signals that are propagated in a cooperative manner to ultimately activate the actin-myosin interaction. These signals can be expressed through different processes, including protein-protein interactions. As a first step toward elucidating the effects of Ca^{2+} on the interactions among the proteins involved in Ca^{2+} regulation, we have investigated the nature of the interaction between the two troponin subunits, TNI and TNC. The initial choice of proteins was dictated by the fact that Ca^{2+} binds to troponin and the complex formed between TNI and TNC with essentially identical affinities that are significantly higher than the affinities observed with isolated TNC. Insofar as Ca^{2+} binding is concerned, the presence of TNT in troponin appears to have little effect on the Ca^{2+} binding properties. The TNI · TNC complex served as an initial model to investigate protein-protein interactions that may be modulated by Ca^{2+}. The free energy of interaction between these two subunits was quantitatively evaluated in the present work with the fluorescent sulfhydryl probe IAE attached to one or the other protein. The interaction was monitored by following either the increases of IAE fluorescence intensity or its fluorescence anisotropy. Since the probe has a large absorption coefficient and high quantum yield, it was possible to monitor the reaction with the same labeled protein in the nanomolar to micromolar range. To optimize the conditions for experiments to determine binding constants, it was necessary to perform titrations in the presence of excess of Ca^{2+} with proteins in the nanomolar and subnanomolar concentrations, since the dissociation constant for TNI · TNC fully saturated with Ca^{2+} is of this magnitude. A single probe thus enabled us to determine the binding affinities between the two subunits in the range 10^5 to 10^9 M^{-1} in the absence and presence of bound Ca^{2+}. From these results we have determined the coupling energetics for binding TNI and Ca^{2+} to TNC. IAE was well suited for this purpose, since when attached to TNC it also detected two classes of Ca^{2+} binding sites. The attached probe also allowed evaluation of Ca^{2+} binding constants to yield complementary information on the stabilization of the TNI · TNC complex by bound Ca^{2+}.

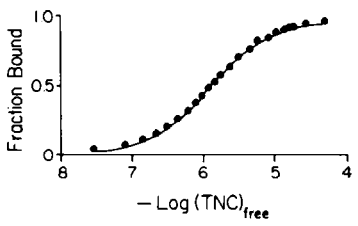


FIGURE 6 Titration of IAE-TNI (TNI*) with TNC at 20°C. The initial TNI* concentration was 4.0 μM, and the reaction was monitored by changes in IAE fluorescence intensity. See Fig. 1 for other conditions.

Cations bound to the Ca/Mg sites of TNC are not needed for TNI binding to TNC. The present results yielded for the first time a set of thermodynamic parameters characterizing the interaction between two troponin subunits, and a quantitative measure of the coupling free energy for binding TNI and a substoichiometric/stoichiometric number of Ca^{2+} simultaneously to TNC. The immediate effect of Ca^{2+} binding to TNC is expressed in the enhanced stability of the TNI-TNC linkage. Since

TABLE V
BINDING PARAMETERS FOR COMPLEXATION BETWEEN TNI* AND TNC

Temperature	TNC species	n	K	$\Delta G°$
			M^{-1}	$kcal\text{-}mol^{-1}$
20°C	TNC	0.96 ± 0.01	$(8.37 \pm 0.34) \times 10^5$	−7.94
	$TNC(Mg)_2$	1.06 ± 0.04	$(5.77 \pm 0.47) \times 10^7$	−10.41
	$TNC(Ca)_4$	0.80 ± 0.01	$(7.02 \pm 0.21) \times 10^9$	−13.20
10°C	TNC	1.01 ± 0.02	$(7.51 \pm 0.43) \times 10^5$	−7.61
	$TNC(Mg)_2$	0.97 ± 0.01	$(2.74 \pm 0.10) \times 10^7$	−9.64
	$TNC(Ca)_4$	0.79 ± 0.01	$(2.68 \pm 0.09) \times 10^9$	−12.21

Experimental conditions are given in Fig. 6.

Ca^{2+} binding to the same labeled TNC* and its complex with TNI was also determined, the additional results provided a second set of data to evaluate the coupling free energy values. This is possible as is shown in Scheme I and Eqs. 13–15. The increases in the stoichiometric association constants of the complex TNI · TNC* for Ca^{2+} over that of TNC* (Table III) require that $4\Delta G_{I,C}(1, 4) = -5.47$ kcal. This compares favorably with the value (−5.26 kcal) derived from $\Delta G°(I/C_4) - \Delta G°(I)$ that was determined directly from titration of TNC* with TNI. The qualitative nature of this large stabilization of the complex by Ca^{2+} was previously recognized from several lines of evidence. The present results provide a quantitative measure of the stabilization.

The intrinsic binding constants of unlabeled TNC and TNI · TNC for Ca^{2+} were previously determined (Potter and Gergely, 1975). From the reported intrinsic binding constants, stoichiometric constants corresponding to the K_i and K_i' listed in Table III are readily calculated. The ratio $K_1K_2K_3K_4/K_1'K_2'K_3'K_4'$ from unlabeled proteins is 10,000 in excellent agreement with our value derived from labeled TNC*. The previous K_i and K_i' from unlabeled TNC correspond to $4\Delta G_{I,C}(1, 4) = -5.34$ kcal in agreement with the present value obtained from Ca^{2+} binding data. This comparison should be taken with caution, since the results with unlabeled proteins were obtained at 4°C. The coupling free energy is determined not by the absolute values of free energy of complex formation, but by the ratio of the binding constants of the two species TNI · $TNC(Ca)_i$ and $TNC(Ca)_i$. If both species have a similar temperature dependence, then the coupling free energy values would be similar at two temperatures.

The approximately equal partition of the total free energy of coupling into the two classes of binding sites was not previously recognized. The present finding is compatible with Ca^{2+} binding data previously obtained from unlabeled proteins. Subsequent to the initial report of our preliminary results on the binding of TNC* with TNI (Wang and Cheung, 1983), two preliminary reports (Ingraham and Swenson, 1984; Grabarek et al., 1984) appeared in which the interaction between TNI and TNC was similarly studied. The former group investigated the interaction with dansylaziridine attached to TNC and IAE to TNI. Their K_a was higher than that reported here by a factor of 2, the K_c for TNI* · $TNC(Mg)_2$ was ~3.5 times lower than ours, and the K_e for TNI* · $TNC(Ca)_4$ was ~threefold smaller than the present result. These association constants yielded the following coupling free energies: $\Delta G_{I,C}(1, 4) = -4.16$ kcal, $2\Delta G_{I,M}(1, 2) = -2.67$ kcal, and $2\Delta G_{I,C}(3, 4) = -1.49$ kcal. In contrast to Fig. 4, these results indicated that Ca^{2+} binding to the Ca^{2+}-specific sites stabilized TNI · TNC to a considerably lesser extent than the binding at the Ca/Mg sites. The total stabilization by four bound Ca^{2+} was smaller than the present result by >1 kcal. Grabarek et al. (1984) studied the same interaction using TNC labeled at Cys-98 with 1,5I-AEDANS (*N*-[iodoacetyl]-*N'*-[1-sulfo-5-napthyl]ethylene diamine. Their K_a was 5.3×10^5 M^{-1} and K_e was 2.2×10^7 M^{-1}. They attributed the small K_e to interference of the probe located at the interface between TNI and TNC in

TABLE VI
THERMODYNAMIC PARAMETER FOR THE INTERACTION BETWEEN TNI AND TNC

System	TNC species	$\Delta H°$	$\Delta S°$	$\Delta G°$
		$kcal\text{-}mol^{-1}$	$cal\text{-}K^{-1}\text{-}mol^{-1}$	$kcal\text{-}mol^{-1}$
TNI + TNC*	TNC*	7.47	52.1	−7.81
	$TNC*(Mg)_2$	9.92	68.9	−10.28
	$TNC*(Ca)_4$	16.19	99.8	−13.07
TNI* + TNC	TNC	1.79	33.2	−7.94
	$TNC(Mg)_2$	12.28	77.4	−10.41
	$TNC(Ca)_4$	15.88	99.2	−13.20

the complex. The effect of this interference was evaluated from kinetic measurements and the observed effect was used to adjust the association constants. The adjusted values were $K_a = 3 \times 10^6\ M^{-1}$ and $K_e = 1.5 \times 10^9\ M^{-1}$. These association constants yielded a total free energy coupling $4\Delta G_{I,C}(1, 4) = -3.61$ kcal at 20°C, considerably smaller than the values obtained by Ingraham and Swenson and by us. It is not immediately apparent why the unadjusted K_e determined by this group was so much smaller than our value, since both probes were located at Cys-98 of TNC. The difference might be due to different perturbations of the Cys-98 region induced by different probes. The possibility of probe interference will be further discussed in another section.

If the coupling free energy has a relationship in the ultimate activation of actomyosin ATPase, its magnitude is expected to be sufficient to provide an efficient correlation with function. There is reasonable consensus that Ca^{2+} binding to the Ca/Mg sites has little physiological significance, insofar as activation of muscle is concerned. It is of interest to consider the process(es) that are modulated by Ca^{2+} binding to the Ca^{2+}-specific, physiologically relevant sites. The present study has demonstrated that one such process is strengthening of the linkage between TNI and TNC. TNC has a preference to exist as a doubly liganded species. On theoretical ground Weber (1975) has discussed that the extent of correlation between the binding of multiple ligands to a macromolecule may be directly related to the efficiency of the biological function that is modulated by the binding. This correlation is expressed by the magnitude of the coupling free energy. At 300°K, a 90% positive correlation requires a coupling free energy of -2.76 kcal and a 99% correlation requires -5.52 kcal. The coupling free energy determined in this work (~ -2.8 kcal) for transition from TNI · $TNC(Mg)_2$ to TNI · $TNC(Ca)_4$ represents a 90% correlation of multiple ligand binding to TNC. To achieve this level of correlation, both Ca^{2+}-specific sites would be required. From a purely energetic point of view, this would explain why two apparently identical and independent Ca^{2+}-specific binding sites are present in skeletal TNC. The efficiency provided by the coupling free energies derived from the other two recent reports would be considerably less for the same transition. The function of the two high affinity Ca/Mg sites is thought to provide a favorable conformation of TNC for interaction with other proteins and with Ca^{2+} at the specific sites. This favorable conformation is brought about in relaxed muscle by saturation of the high affinity sites with either Ca^{2+} or Mg^{2+}. Since the total coupling free energy for this saturation is also sufficiently large to yield a 90% correlation of multiple ligand binding to TNC, it appears that the conformation of troponin subunits in relaxed muscle is energetically close to optimal for Ca^{2+} activation. The magnitude of the coupling free energy would also require two high affinity sites to provide the structural requirement.

Two of the saturation curves in Fig. 5 show large positive cooperativity. Fig. 5 *B* refers to a cooperative process that occurs when Ca^{2+} binds to the regulatory, Ca^{2+}-specific sites. As this binding occurs, the TNC species that already contain two bound Ca^{2+} interact with TNI cooperatively. Within the constraints imposed by the observed association constants and the coupling free energies, the saturation of $TNC(Ca)_i$ ($i \geq 2$) for TNI occurs with a degree of cooperativity and over free Ca^{2+} concentrations that are qualitatively similar to Ca^{2+} activation of actomyosin ATPase (Grabarek et al., 1981). In their recent paper, Grabarek et al. (1983) reported Ca^{2+} binding properties to TNC in reconstituted thin filament that were monitored by extrinsic fluorescence. They found no cooperativity in Ca^{2+} binding to the Ca/Mg sites, and a small cooperativity in Ca^{2+} binding to the Ca^{2+}-specific sites. The latter cooperativity was enhanced in the presence of myosin, but was still considerably less than the cooperativity displayed in plots of the Ca^{2+} dependence of actomyosin ATPase activity. These authors concluded that there was no apparent direct correlation between Ca^{2+} binding to the Ca^{2+}-specific sites of TNC and Ca^{2+} activation of actomyosin ATPase. The present results show that the strengthening of the linkage between TNC and TNI could be induced by Ca^{2+} with a high degree of cooperativity that is dependent upon coupling free energy and free TNI concentration, and that Ca^{2+} binding per se is only one of several determinants of the observed cooperativity. It is possible that, in fully reconstituted thin filament and in the presence of myosin, the linkage between TNI and TNC could be additionally stabilized, and the shape of the saturation curves such as those depicted in Fig. 5 *B* could approach that of actomyosin ATPase activation. It seems that a correlation is more likely to exist between actomyosin ATPase and processes that are induced by Ca^{2+}, rather than Ca^{2+} binding itself. Ultimately, energetic information on the various protein-protein interactions is required to elucidate the mechanism of thin filament regulation.

It has been suggested that Cys-98 of TNC is in the region of the protein that is involved in Ca^{2+}-independent interaction with TNI (Leavis et al., 1978; Chong and Hodges, 1982). Insofar as the binding affinity is concerned, the association constant reported by IAE attached to Cys-98 of TNC is very similar to that sensed by the same probe placed at Cys-133 of TNI. A close examination of the other thermodynamic parameters reveals several interesting features. Both $\Delta H°$ and $\Delta S°$ for formation of TNI · TNC are positive and large. These values would suggest that the interaction is entropy driven and could be predominantly hydrophobic, as it is well known that for hydrophobic interaction $\Delta G° < 0$, $\Delta S° > 0$, and $\Delta H° > 0$ (Kauzmann, 1959). This relationship alone cannot always account for the experimental energetic parameters observed in many protein-association reactions. The initial complexation between two proteins most likely involves nonpolar interactions in which hydrophobic amino acid

side chains that were previously accessible to solvent became buried in the complex. Chothia and collaborators (Chothia and Janin, 1975; Janin and Chothia, 1978) have considered this problem in detail, based on the idea that the surface area of a protein accessible to water decreases upon association with a second protein. This decrease, in effect, results in a net gain in entropy. After the initial interactions, secondary interactions may occur that involve different types of residues. Interactions between ionic species in aqueous environment are characterized by $\Delta G° < 0$, $\Delta S° > 0$, and $\Delta H° \simeq 0$. H-bonding and van der Waals interactions are accompanied by $\Delta S° < 0$ and $\Delta H° < 0$. The secondary interactions in TNI · TNC* could involve electrostatic interactions that contribute negligibly to the enthalpy term, but positively to the entropy term. This mechanism is compatible with the suggestion that the interaction between the two proteins is largely electrostatic, involving positively charged side chains of TNI and negatively charged side chains of TNC (Perry, 1979). When compared with TNI · TNC*, formation of TNI* · TNC was accompanied by a much less positive enthalpy change and a smaller increase in entropy, although the free energy change was virtually the same. The difference in $\Delta H°$ and $\Delta S°$ between the two species suggests that the mode of interaction between the two proteins is in some way influenced by IAE attached to Cys-98 of TNC. It is difficult to interpret whether the overall interaction is more entropy driven in TNI · TNC* or in TNI* · TNC, since conformational changes in proteins can give rise to unpredictable changes in thermodynamic parameters. While the specific interactions appear energetically different in the two species, the compensating changes in $\Delta H°$ and $T\Delta S°$ give rise to an equally stable complex regardless of the probe location.

Apart from the nature of the interaction between TNI and TNC, which occurs in the absence of bound cations, the energetics of the Ca^{2+}-dependent reaction that gives rise to the regulatory phenomenon is of more interest. The increase in $\Delta H°$ for transition from TNI* · TNC to TNI* · $TNC(Mg)_2$ was very substantial from 1.8 to 8.5 kcal, and that for transition to TNI* · $TNC(Ca)_4$ was from 1.8 to 16.2 kcal. The corresponding increase observed with TNI · TNC* was also large, and the change in $\Delta S°$ was positive and large in every case. For transition from TNI · $TNC(Mg)_2$ to TNI · $TNC(Ca)_4$ the coupling enthalpy obtained with either labeled protein is very similar, and the corresponding coupling free energy is identical. It would seem that these coupling energetics are a reasonable reflection of the coupling process. As already indicated, it is difficult to decide, from the changes in $\Delta H°$ and $\Delta S°$, the type of force that contributes predominantly to the stabilization of the complex by Ca^{2+} binding to the TNC. If the effect of conformational changes resulting from Ca^{2+} binding on enthalpy and entropy were negligible, the observed increase in $\Delta H°$ and $\Delta S°$ would reflect an entropy-driven stabilization, with hydrophobic interaction being an important determinant. This would be the case, since it seems unlikely that electrostatic interactions could contribute to the substantial increases in $\Delta H°$ that were observed between Ca^{2+}-free and Ca^{2+}-bound species. The overall energetics of protein associations necessarily reflects a delicate balance of several opposing forces. While the effect of Ca^{2+}-induced structural changes on thermodynamic parameters is unknown, the Ca^{2+} stabilization is likely a result of both electrostatic and hydrophobic interactions.

Several lines of evidence suggest the importance of nonpolar residues in the interaction between TNI and TNC. A recent report (Brzeska et al., 1983) demonstrated from Phenyl-Sepharose chromatography that a large proportion of hydrophobic residues of TNC are exposed upon Ca^{2+} binding. Almost all of this exposure is confined to the TR-1 fragment at the NH_2-terminal half of the molecule. Exposure of nonpolar residues was also demonstrated by CD and NMR studies (Nagy et al., 1978; Reid et al., 1981; Gariepy et al., 1982). These results are consistent with the notion that in the presence of Ca^{2+}, TNC may interact with other proteins via hydrophobic interactions. A small peptide corresponding to the sequence 104–115 of TNI is the smallest that possesses inhibitory activity of actomyosin ATPase and binds TNC in a Ca^{2+}-dependent manner (Talbot and Hodges, 1981; Cachia et al., 1983). NMR results indicated that both basic and nonpolar residues of this peptide interact with TNC. In addition, several of the hydrophobic residues in the sequence 1–21 of TNI were also shown by NMR to be involved in interaction with TNC in the presence of Ca^{2+} (Grand et al., 1982). When considered in conjunction with spectroscopic information, the present energetic results point to the role of hydrophobic interactions in Ca^{2+}-induced stabilization of the TNI · TNC complex.

In summary, we have presented energetic evidence that Ca^{2+} binding to the Ca^{2+}-specific sites of TNC is correlated with stabilization of the TNI-TNC linkage. This linkage may have a special functional role in the overall Ca^{2+} regulation in contraction. The dominant forces responsible for the TNI-TNC interaction that occurs in the absence of bound cations may have a considerable ionic character, but the principal forces responsible for Ca^{2+} stabilization of the complex are likely to have a considerably nonpolar character. The present results provide a basis for additional studies to investigate the coupling energetics for binding Ca^{2+} and other proteins to troponin C.

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