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Interaction of Troponin and Tropomyosin: Spectroscopic and Calorimetric Studies†

Richard H. Ingraham and Charles A. Swenson*
Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242
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ABSTRACT: The thermodynamic parameters characterizing the interaction between rabbit fast skeletal muscle troponin and tropomyosin have been determined at 25 °C for three solution conditions: buffer containing (A) 1 mM $CaCl_2$, simulating a "turned-on" state, (B) 3 mM $MgCl_2$, simulating a "turned-off" state, and (C) 2 mM ethylenediaminetetraacetic acid, a reference state. The enthalpies were measured in two buffers with different heats of ionization to allow correction for dissociation or uptake of protons. The enthalpies corrected for proton effects are −22.1, −25.4, and −23.5 kcal/mol, respectively, in buffers A, B, and C. The interaction between troponin and tropomyosin in the presence of calcium is accompanied by release of 0.9 mol of proton per mole of complex. Proton effects in the presence of magnesium and in the absence of divalent metal ions were too small to quantitate. The association constants were measured by using tropomyosin labeled with the extrinsic fluorescent probe dansylaziridine, and binding was detected by enhancement of the probe fluorescence. The magnitudes of the association constants for unlabeled troponin are 7.5×10^5, 4.2×10^5, and 9.5×10^5 M^{-1}, respectively, for the three solution conditions corresponding to unitary free energies of −10.4, −10.1, and −10.6 kcal/mol. The unitary entropies for the interaction are −39, −51, and −43 cal/(deg·mol), respectively, for the three solution conditions. Under these conditions, the troponin–tropomyosin interaction is enthalpy driven, and a large unfavorable entropy must be overcome in the formation of the complex. The troponin–tropomyosin interaction is thought to be a crucial part of the protein interactions which regulate the actomyosin ATPase activity of skeletal muscle. These studies suggest that if changes in the troponin–tropomyosin interaction are part of the regulatory signal, they are small in terms of free energy and of its enthalpic and entropic components.

The thin-filament proteins troponin and tropomyosin mediate regulation of the actomyosin ATPase of skeletal muscle by responding to the local concentration of Ca^{2+} ions. Troponin contains three subunits: TnC,[1] the Ca^{2+}-binding subunit of troponin, TnI, the inhibitory subunit of troponin; and TnT, the tropomyosin-binding subunit of troponin (Greaser & Gergely, 1971). Tropomyosin contains two parallel α-helical chains which form a coiled-coil structure that is positioned in the grooves of the thin filament [see review by Talbot & Hodges (1982)]. Troponin binds to the thin filament through interactions between TnT and tropomyosin (Mak & Smillie, 1981), TnI and actin (Potter & Gergely, 1974), and TnI and tropomyosin (Pearlstone & Smillie, 1983). Recent studies suggest that control of the actomyosin ATPase is allosteric (Chalovich & Eisenberg, 1982; Chalovich et al., 1981). Binding of Ca^{2+} to the Ca^{2+}-specific sites of TnC alters the interactions between TnC, TnI, and TnT. This in turn alters the TnI–actin and TnI– and TnT–tropomyosin interactions in a manner that strengthens the actin–myosin interaction and results in a remarkable enhancement of the actomyosin ATPase activity. Concomitant with these changes is the well-known shift of tropomyosin in the groove of F-actin (Huxley, 1971). Although little more is known of the molecular structural details, it is likely that the Ca^{2+}-induced alterations in the interactions between troponin, tropomyosin, and actin are important in the allosteric control.

An understanding of this complex system of control requires knowledge of interactions between the components. We have studied the interaction between troponin and tropomyosin by reaction calorimetry and by fluorescence spectroscopy using an extrinsic fluorescent probe under conditions where the actomyosin ATPase would be turned on and turned off. The enthalpies are of particular interest as earlier measurements

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[1] Abbreviations: TnC, Ca^{2+}-binding subunit of troponin; TnI, inhibitory subunit of troponin; TnT, tropomyosin-binding subunit of troponin; Tn, troponin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; 1,5-IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; ANM, *N*-(1-anilinonaphth-4-yl)maleimide; DANZ-TM, tropomyosin labeled with dansylaziridine.

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(Lamkin et al., 1983; Lin & Dowben, 1982) of the free energies showed only small differences between these two states. The full set of thermodynamic parameters obtained is a direct way of quantitating this important interaction of the allosteric control system of skeletal muscle.

Materials and Methods

Preparation of Proteins. Troponin and tropomyosin were prepared from rabbit fast skeletal muscle (longissimus dorsi and psoas) by using a modification of the method of Greaser & Gergely (1971). Additional purification steps for troponin consisted of Affi-gel blue agarose chromatography (Bio-rad) (Reisler et al., 1980) as described by Ingraham & Swenson (1983) followed by DEAE-Sepharose chromatography (van Eerd & Kawasaki, 1973). Hydroxylapatite chromatography (Eisenberg & Kielley, 1974) was used for the final purification step for tropomyosin.

Fluorescence Labeling of Tropomyosin. Sixty milligrams of tropomyosin (1.5 mg/mL) was dialyzed sequentially against three buffered solutions containing 10 mM sodium phosphate, 1 M KCl, and 1 mM EDTA at pH 8.0: first, 1 L with 16 mM 2-mercaptoethanol; second, 1 L with 2 mM dithiothreitol; third, 2 L with 0.5 mM dithiothreitol. An approximate 10-fold excess of dansylaziridine to reducing agent plus tropomyosin was then added to the dialyzed solution, and the reaction was allowed to proceed for 17 h at room temperature. Unreacted dansylaziridine was removed by extensive dialysis.

The extent of probe incorporation into tropomyosin at Cys-190 was determined as follows: The concentration of bound dansylaziridine was measured spectrophotometrically using $\epsilon = 3980\ M^{-1}\ cm^{-1}$ at 350 nm (Johnson et al., 1978). The tropomyosin concentration was determined according to the Bradford (1976) assay with the standard curve generated by using unlabeled tropomyosin and ultraviolet absorbance measurements to determine the concentrations of the standards. Fractional incorporation was calculated as the concentration ratio of bound dansylaziridine to tropomyosin.

The species present in the sample, tropomyosin molecules with zero, one, or two probes attached, were determined by cross-linking with DTNB (Lehrer, 1975) and measuring the ratio of cross-linked to un-cross-linked product on SDS slab gels after staining with Fast Green FCF. Quantitation was achieved by scanning the gels at 625 nm on a Transidyne General RFT-11 scanning densitometer.

The concentrations of unlabeled proteins were determined by employing the following extinction coefficients: tropomyosin, $E^{1\%}_{277} = 3.3$ (Woods, 1967); troponin, $E^{1\%}_{280} = 4.5$ (Hartshorne & Mueller, 1969). The molecular weights used were 65 378 for α-tropomyosin (Mak et al., 1980) and 69 332 for troponin (Pearlstone et al., 1976; Collins, 1974; Wilkinson & Grand, 1975).

Fluorescence Measurements. The fluorescence data used to construct the binding isotherms were obtained with a Perkin-Elmer MPF-44A spectrofluorometer. Titrations were performed at fixed excitation and emission wavelengths of 340 and 525 nm, respectively. Cutoff filters were used at the entrance slit of the emission monochromator to remove Rayleigh scattering.

The binding isotherms were measured for three solution conditions at 25 °C: (a) 1 mM $CaCl_2$; (b) 3 mM $MgCl_2$ and 1 mM EGTA; (c) 2 mM EDTA. In all cases, the solutions were buffered at pH 7.0 with 10 mM PIPES and contained 5 mM dithiothreitol, 0.01% NaN_3, and 0.3 M KCl.

Solutions of concentrated titrant were added to the solution in the cuvette with a syringe microburet (Micro-Metric Instrument Co.) calibrated in the microliter range. A Bascom-Turner 4120 electronic recorder was used to record and store the fluorescence data.

Analysis of Binding Curves. The data were transferred from the recorder to a computer where they were averaged and fitted to a model for binary complex formation which accounted for binding of ligand to unlabeled tropomyosin. For the Ith addition of ligand, $Y(I)$, the observed fluorescence signal is dependent on the association constants, the concentrations of labeled and unlabeled tropomyosin, the stoichiometry, and the fluorescent enhancement. For our fits, the stoichiometry was fixed at 1:1 rather than including two additional fitting parameters. The dependence of $Y(I)$ on these parameters can be written as

$$Y(I) = \theta Y_{max} \quad (1)$$

where $\theta = K_s L/(1 + K_s L)$, the fraction saturation of labeled tropomyosin with ligand, and K_s and L are respectively the association constant for binding to labeled tropomyosin and the free concentration of added ligand (troponin). For the fitting procedure, the free concentration of the ligand was calculated from the total concentration of the added ligand via the cubic equation

$$L^3 + [1/K_s + 1/K + M_s + M - X(I)]L^2 + [M/K_s + M_s/K + 1/K_sK - X(I)/K_s - X(I)/K]L - X(I) = 0 \quad (2)$$

Here, $X(I)$ is the total troponin concentration for the Ith addition, and constants K_s and K and terms M_s and M are respectively the association constants and total concentrations for labeled and unlabeled tropomyosin. The association constants K_s and K and also Y_{max} are established by the fitting procedure. The values for M_s and M were fixed by measurement.

For comparison, some of the data were fit to the equation for binary complex formation where the association constant was the same for binding to labeled and unlabeled tropomyosin. The equation (eq 3) is given below and uses the same definitions as eq 1 except that M is the total concentration of tropomyosin. Minimization of the summation of deviations squared was used as the criterion for all the fits.

$$Y(I) = \{[M + X(I) + 1/K - \sqrt{[M + X(I) + 1/K]^2 - 4MX(I)}]Y_{max}\}/2M \quad (3)$$

Calorimetry. The enthalpies were measured on a calorimeter constructed in our laboratory which operates on the heat-leak principle. The reaction was initiated in a gold cell (total volume ~3.0 mL) by rotation of the calorimetric unit. The heat effect was measured by two thermopiles interposed between the sides of the cylindrical cell and the heat sink. Output from the thermopiles was sent to a Keithley Model 147 nanovolt null detector where it was amplified and then sent to a Bascom–Turner Model 4120 electronic recorder for storage. For each measured heat effect, which takes ~8 min, 2000 points were stored. The traces were smoothed and integrated by using the hard-wired functions of the electronic recorder.

In a typical experiment, 1.0 mL of tropomyosin (5–8 mg/mL) was mixed with 0.5 mL of troponin (4–6 mg/mL) after an equilibration time of 2 h. The buffers were the same as those used in the fluorescent measurements. For each run, three viscous heat effects were measured. Calibration was achieved in two ways: electrically and chemically by neutralization of Tris with hydrochloric acid. The two methods agreed to better than 5%. Heats of dilution for tropomyosin and troponin were measured in separate experiments and subtracted from the heat of mixing the reactants. Samples of troponin and tropomyosin from these calorimetric runs were

Table I: Association Constants for Troponin–Tropomyosin[a]

buffer	% enhancement	K_s (M^{-1})	K (M^{-1})
Ca^{2+}	69	2.0×10^6 (1.8×10^6, 2.2×10^6)[a]	7.5×10^5 (4.9×10^5, 3.2×10^6)
Mg^{2+}	45	1.3×10^6 (1.2×10^6, 1.5×10^6)	4.2×10^5 (1.4×10^5, 1.3×10^6)
EDTA	68	2.8×10^6 (2.8×10^6, 2.9×10^6)	9.5×10^5 (6.8×10^5, 1.3×10^6)

[a] The values of the association constants presented are the geometric means with the 70% confidence intervals of the logarithmically transformed (base ten) variates in parentheses.

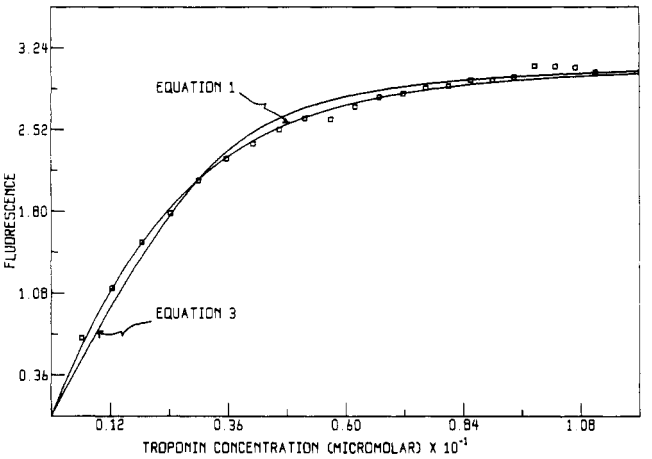

FIGURE 1: Binding isotherm obtained when DANZ-TM (5.0 μM, 28% labeled) was titrated with Tn (53.2 μM) in a standard buffer with EDTA containing 0.3 M KCl, 5 m M dithiothreitol, 10 mM PIPES buffer at pH 7.0, and 0.01% NaN_3 at 25 °C. The solid lines are best fits of the data using (a) eq 1 with the association constants for labeled and unlabeled tropomyosin and the maximum value of the fluorescence as parameters and the stoichiometry fixed at 1:1 and (b) eq 3 with a single association constant for labeled and unlabeled tropomyosin, the maximum value of the fluorescence, and the stoichiometry as parameters.

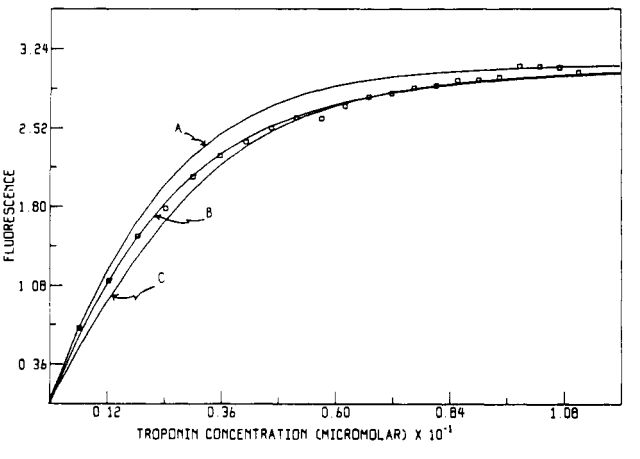

FIGURE 2: Binding isotherm obtained when DANZ-TM (5.0 μM, 28% labeled) was titrated with Tn (53.2 μM) in a standard buffer with EDTA containing 0.3 M KCl, 5 mM dithiothreitol, 10 mM PIPES buffer at pH 7.0 and 0.01% NaN_3 at 25 °C. The solid lines are (A) fit of data when $K_s = 6 \times 10^6$ M^{-1} and $K = 2 \times 10^6$ M^{-1}, (B) best fit when $K_s = 3 \times 10^6$ M^{-1} and $K = 1 \times 10^6$ M^{-1}, and (C) fit of data when $K_s = 3 \times 10^6$ M^{-1} and $K = 1 \times 10^{6-1}$. For curves A and B, $M_s = 1.4 \times 10^{-6}$ M and $M = 3.6 \times 10^{-6}$ M (28% labeled). For curve C, $M_s = 2.8 \times 10^{-6}$ M and $M = 2.2 \times 10^{-6}$ M (56% labeled). For all curves, the maximum value of the fluorescence was 3.15.

analyzed on SDS–PAGE slab gels to determine if significant breakdown had occurred during the time of the calorimetric experiment.

Results

The free energies of the interactions were determined from fluorescence measurements using α-tropomyosin labeled with dansylaziridine at Cys-190. Fluorescent enhancements were 69%, 45%, and 68%, respectively, in the buffers containing Ca^{2+}, Mg^{2+}, and EDTA. Analysis showed that 28% of the cysteine sites of the tropomyosin molecules were labeled. A typical binding curve for this interaction of troponin and tropomyosin is shown in Figure 1. The shape of the curve suggests small but significant preferential binding of troponin to labeled tropomyosin. This is particularly apparent if the data are fit to eq 3 for simple binary complex formation. The theoretical curve rises too slowly initially when troponin binds preferentially to labeled tropomyosin and too rapidly in the mid-portion of the graph where binding to unlabeled tropomyosin is occurring and saturation of labeled tropomyosin is slowed. These data are well fitted by eq 1, and values of the association constants for binding to labeled and unlabeled tropomyosin are obtained. In Figure 2, we show in addition to a typical best fit two other fits which give an indication of the sensitivity to changes in the parameters. The values of the association constants for binding to labeled and unlabeled tropomyosin with 70% confidence intervals are given in Table I. For the three cases presented, the association constants are 2–3 times greater for binding to labeled tropomyosin. These values for the association constants to labeled tropomyosins compare well with values determined by other workers. A binding constant of 2.7×10^6 M^{-1} was obtained by Lamkin et al. (1983) using 1,5-IAEDANS-labeled α-tropomyosin in the presence of Ca^{2+} or Mg^{2+}. Lin & Dowbin (1982) measured values of 1.9×10^6 and 1.7×10^6 m^{-1} in the presence and absence of Ca^{2+}, respectively. Morris & Lehrer (1984) investigated the interaction of ANM–tropomyosin and troponin and obtained an association constant range of $(1.2 \times 10^6) - (3 \times 10^7)$ M^{-1}. The small differences reflect in part the slightly different solution conditions used for the studies.

For all these earlier studies, the labeling of the Cys-190 sites was complete with two probe molecules per tropomyosin molecule, or at least it was assumed that the association constants were the same for labeled and unlabeled tropomyosin. In our experiments with 28% of the sites labeled, different molecular species can exist, namely, zero, one, or two molecules of probe per tropomyosin molecule. We found that the data could be fit satisfactorily only when we assume that each labeled tropomyosin molecule has two molecules of probe bound; i.e., 28% of the molecules are doubly labeled. This is in agreement with analysis of our sample on SDS slab gels after cross-linking with DTNB. Quantitation of the peaks after labeling with Fast Green FCF gave a ratio of cross-linked to un-cross-linked tropomyosin species of 3.0 ± 0.5. For 28% labeled sample, this ratio is consistent only with the presence of species containing two probes or zero probe per tropomyosin molecule (theoretical ratio 2.6). The presence of only this species may be a consequence of the large excess of negative charge in the vicinity of Cys-190 which would slow the reaction with the probe; however, when the probe has reacted with one of the cysteines, an opening of the coiled-coil structure occurs, and reaction with a second molecule of probe is facilitated.

The association constants for the interaction of unlabeled tropomyosin and troponin as determined by this analysis are 2–3 times lower than those reported for labeled tropomyosins, and they were used in the calculation of thermodynamic pa-

Table II: Thermodynamic Parameters for Association of Troponin and Tropomyosin

buffer	no. of trials	ΔH_{obsd} (PIPES)[b,d]	no. of trials	ΔH_{obsd} (Tris)[b,d]	$\Delta G°$ [b]	$\Delta H°$ [b]	$\Delta S°$ [c]
Ca^{2+}	12	−24.4 ± 0.3[e]	6	−31.9 ± 1.6[e]	−8.0 (−10.4)[a]	−22.1	−47 (−39)[a]
Mg^{2+}	18	−25.3 ± 1.0	6	−25.1 ± 1.5	−7.7 (−10.1)	−25.4	−59 (−51)
EDTA	3	−23.5 ± 1.5			−8.2 (−10.6)	−23.5	−51 (−43)

[a] The unitary free energies and entropies are given in the parentheses. [b] In kilocalories per mole. [c] In calories per degree per mole. [d] For the measurements in PIPES buffer, four, six, and one different sample preparations were used respectively in calcium, magnesium, and EDTA buffers. In Tris buffer, two different preparations were used. [e] The errors are standard errors.

rameters. They show little dependence on the presence or absence of the divalent metal ions Ca^{2+} or Mg^{2+} in the buffer.

Mixing of tropomyosin and troponin at pH 7.0 and 25 °C in the calorimeter resulted in the evolution of heat in all cases. The magnitude of the heat effect ranged from ~0.1 Mcal to 0.9 Mcal. The endothermic heats of dilution of tropomyosin and troponin were respectively ~0.04 and ~0.12 Mcal. Several binding isotherms were measured by the addition of variable amounts of tropomyosin to fixed quantities of troponin. In all cases, the binding isotherms were comparable to those determined by fluorescence. When they were fit to eq 3, the association constants obtained were in good agreement with those for unlabeled tropomyosin determined by fluorescence except with larger errors. Once it was determined that titration gave a saturable binding curve, measurements of the enthalpies were done at a fixed TM:Tn mole ratio of ~3:1. On the basis of the magnitudes of the association constants determined for these complexes, greater than 94% saturation was achieved at the concentrations used in calorimetry. The enthalpies measured in PIPES and Tris buffers were corrected for the degree of saturation and are reported in columns 3 and 5 of Table II as ΔH_{obsd}. From the observed values of the enthalpies in the two buffers, the extent of proton dissociation or uptake which occurs simultaneously with the binding interactions can be determined, and enthalpies of binding which are free of proton effects, ΔH_b, can be calculated (column 7). In the Ca^{2+}-containing buffer, 0.9 mol of protons was dissociated on binding, and in the Mg^{2+}-containing buffer, 0.02 mol of protons was taken up on binding.

The enthalpy and free-energy values can be used to calculate the entropy values from the Gibbs equation by assuming a standard state of 1 mol/L. These values are presented in columns 6–8 of Table II.

Discussion

The thermodynamic parameters presented in Tables I and II are interesting in view of our understanding of the troponin and tropomyosin structures and interactions.

At the temperature of these studies, 25 °C, the binding of troponin to tropomyosin is enthalpy driven and has a substantial unfavorable entropy. The large negative unitary entropy is surprising as hydrophobic interactions involving large positive entropy contributions are generally expected to play a major role in protein–protein interactions (Chothia & Janin, 1975). This is thought to arise from the release of ordered water from the binding interfaces of the two proteins. The observed negative entropy must arise from substantial contributions from nonhydrophobic effects. The most likely contribution is from a decrease in soft vibrational modes upon binding—a tightening of the structure (Sturtevant, 1977). This explanation is reasonable considering the dominance of the TnT–tropomyosin interaction to the formation of the troponin–tropomyosin complex. The TnT subunit is thought to have a relatively loose ternary structure, on the basis of its primary sequence (Pearlstone et al., 1976) and thermal denaturation studies (Mani et al., 1974; Ingram, 1981). Hence, the binding interaction could be expected to result in a considerable tightening of the structure of this subunit even beyond that which occurs in the formation of troponin. It is also likely that this interaction involves burial of fewer hydrophobic surface residues than is typical with formation of most protein–protein complexes. The coiled-coil model of tropomyosin structure predicts that few hydrophobic residues will be at the surface (Hodges et al., 1981). Similarly, Pearlstone et al. (1976) have pointed out that the primary sequence of TnT indicates that this troponin subunit is very polar; it contains only one significant stretch of nonpolar residues. These observations suggest that the positive hydrophobic contribution to the entropy of formation of the troponin–tropomyosin complex is smaller than if the molecules had been less polar. To further partition the entropic contributions to the binding process, measurements of the binding would need to be performed at several other temperatures.

Qualitative studies using TnT and TnT fragments in complex with the other subunits have suggested that there are two sites of interaction between TnT and tropomyosin: a Ca^{2+}-independent site near the C-terminal end of tropomyosin and a Ca^{2+}-dependent site near the disulfide link at Cys-190 (Morris & Lehrer, 1982; Ohtsuki, 1979; Chong & Hodges, 1982; Pearlstone & Smillie, 1983). The importance of the tropomyosin–TnT interaction is clear in view of data that show this complex can inhibit the acto-S1 ATPase to 30% of its control value (Chong et al., 1983). Partial release of the inhibition can be achieved by addition of TnC and Ca^{2+}. Evidence from the studies of Pearlstone & Smillie (1983) suggests that troponin also interacts with tropomyosin via the TnI subunit. Although the role of this interaction in regulation is unknown, it is reasonable to anticipate that it would be affected by Ca^{2+} as the binary interaction of TnC and TnI is strongly affected (Ingraham & Swenson, 1984).

In view of the Ca^{2+} dependencies of the troponin subunit interactions, it is surprising that the thermodynamic parameters for the interaction of troponin and tropomyosin are not significantly affected by the presence or absence of divalent metal ions. Compensating enthalpy and entropy effects leading to the same free energy often occur in biological reactions; however, in this case the enthalpies and entropies are also similar. The difference we note is that when Ca^{2+} is bound to TnC the interaction with tropomyosin is accompanied by dissociation of protons, suggesting a pH dependence whereas when Mg^{2+} is bound no proton dissociation occurs.

Thus, it appears that for our experimental conditions either the effect of calcium ion on the interactions between tropomyosin and troponin involves small changes in the thermodynamic parameters or compensating changes in the parameters related to both TnI–tropomyosin and TnT–tropomyosin interactions make the net change small.

The magnitude of the difference in thermodynamic binding parameters required to achieve allosteric control in this system is of course unknown. The small changes observed for the simulated turned-on and turned-off states could form part of the regulatory signal. Since considerable evidence exists which suggests that the interaction between the TnI subunit of troponin and actin is important to the performance of the control

system (Talbot & Hodges, 1981; Eaton et al., 1975; Greaser et al., 1972; Sutoh & Matsuzaki, 1980), an alternate explanation is that actin is required for full expression of the changes in the interactions involved in contractile regulation. The results of this study are a first step toward obtaining thermodynamic parameters for the whole system. Reaction calorimetric studies involving actin are difficult due to the viscosity of the actin solutions at the concentrations required for calorimetry. As yet, calorimetric studies on the whole regulatory complex, troponin, tropomyosin, and actin, have not proved feasible, and analysis of all the changes in the protein interactions which constitute the complete regulatory signal in thermodynamic terms is precluded.

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