Interactions of Troponin Subunits: Free Energy of Binary and Ternary Complexes†

Herbert C. Cheung,* Chien-Kao Wang, and Nouman A. Malik
Department of Biochemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294
Received February 11, 1987; Revised Manuscript Received May 5, 1987

ABSTRACT: We have determined the free energy of formation of the binary complexes formed between skeletal troponin C and troponin T (TnC·TnT) and between troponin T and troponin I (TnT·TnI). This was accomplished by using TnC fluorescently modified at Cys-98 with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine for the first complex and TnI labeled at Cys-133 with the same probe for the other complex. The free energy of the ternary complex formed between troponin C and the binary complex TnT·TnI [TnC·(TnT·TnI)] was also measured by monitoring the emission of 5-(iodoacetamido)eosin attached to Cys-133 of the troponin I in TnT·TnI. The free energies were −9.0 kcal·mol^{-1} for TnC·TnT, −9.2 kcal·mol^{-1} for TnT·TnI, and −8.7 kcal·mol^{-1} for TnC·(TnT·TnI). In the presence of Mg^{2+} the free energies of TnC·TnT and TnC·(TnT·TnI) were −10.3 and −10.9 kcal·mol^{-1}, respectively; in the presence of Ca^{2+} the corresponding free energies were −10.6 and −13.5 kcal·mol^{-1}. Mg^{2+} and Ca^{2+} had negligible effect on the free energy of TnT·TnI. From these results the free energies of the formation of troponin from the three subunits were found to be −16.8 kcal·mol^{-1}, −18.9 kcal·mol^{-1}, and −21.6 kcal·mol^{-1} in the presence of EGTA, Mg^{2+}, and Ca^{2+}, respectively. Most of the free energy decrease caused by Ca^{2+} binding to the Ca^{2+}-specific sites is derived from stabilization of the TnI–TnC linkage. The free energy coupling for the formation of troponin from the three binary complexes was positive and large, indicating that the average of the binary interactions became relatively destabilized when the binary complexes were incorporated into the three-subunit troponin structure. The destabilization of subunit–subunit interactions may facilitate Ca^{2+}-modulated interactions of the subunits with actin and tropomyosin, which occur during the contractile cycle.

It is generally recognized that signal transmission from one part of a molecule to another is essential for the regulation of many complex enzyme systems. In a simple system its mechanism may simply involve binding of a ligand to one region of the enzyme and transferring of the effect of the ligand to the catalytic site. In more complex systems such as actomyosin ATPase in skeletal and cardiac muscles, the initial molecular step is binding of Ca^{2+} as a second messenger to its receptor on the thin filament. The Ca^{2+} signal is then transmitted from its receptor, troponin C, through an assembly of three additional regulatory proteins to distant sites on the actin filament, thereby activating actomyosin ATPase and initiating the contractile cycle. The other proteins involved in signal transmission are the other two subunits of troponin, troponin I and troponin T, and tropomyosin. Ultimately, the transmission mechanism must be elucidated on the basis of altered structures and dynamic properties of the proteins that make up the entire assembly. An important foundation to the full understanding of these structural features is the general energetic aspect of the protein assembly and, more specifically, the modulating effect of Ca^{2+} on the affinities of various protein–protein interactions that exist within the assembly.

Within the three-subunit troponin the interaction between TnI[1] and TnC has been known for sometime to be substantially strengthened by the presence of Ca^{2+}. This enhanced interaction is thought to play a role in signal transmission. We (Wang & Cheung, 1985) have recently demonstrated with fluorescently modified proteins that the interaction between TnI and TnC in the isolated TnI·TnC complex is stabilized by −2.6 kcal when the two high-affinity Ca/Mg sites are saturated by either Ca^{2+} or Mg^{2+}. Subsequent saturation of the two low-affinity, Ca^{2+}-specific sites is accompanied by an additional stabilization (−2.8 kcal) of the TnI–TnC linkage. The initial stabilization appears to have little or no physiological significance since the Ca/Mg sites are likely fully occupied by Mg^{2+} in relaxed muscle. The subsequent stabilization (free energy coupling) is likely physiologically important because it is induced by Ca^{2+} binding to the regulatory sites. Its magnitude is sufficiently large (Weber, 1975) so that the TnI–TnC linkage may well play an important role in the transfer of the Ca^{2+} signal in muscle.

In this work, we have measured the free energies of the formation of the binary complexes TnC·TnT and TnT·TnI and determined the effect of cations on these free energies. We have also determined the free energy of formation of troponin from its three subunits and the free energy coupling for the formation of troponin from its three binary complexes. This coupling is positive and large and becomes slightly more positive in the presence of cations. The three binary interactions within the three-subunit troponin are destabilized as the binary complexes are incorporated into the troponin structure, giving rise to weakened binary linkages that may be essential for optimal interactions of the subunits with actin and tropomyosin.

Materials and Methods

Troponin was isolated from rabbit skeletal muscle as previously described (Cheung et al., 1982). Its three subunits were dissociated in 6 M urea and separated by column chromatography in the presence of urea (Wang & Cheung, 1986). TnC was obtained from a Whatman DE-52 column, and TnI

† This work was supported in part by a grant from the National Institutes of Health (AM25193).

[1] Abbreviations: TnI, troponin I; TnC, troponin C; TnT, troponin T; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; IAE, 5-(iodoacetamido)eosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-5904$01.50/0

and TnT were obtained from TnB (TnI·TnT complex) with a Whatman CM-32 column (Wilkinson, 1974).

Fluorescent modifications of TnC and TnI were carried out as previously described by us (Wang & Cheung, 1985). Modification of TnC at Cys-98 with IAEDANS was performed in the presence of EGTA, and modification of TnI at Cys-133 with either IAE or IAEDANS was carried out with either intact troponin or TnB prior to separation of the subunits. These procedures allowed labeling of Cys-133 and not the other two cysteine residues of TnI (Wang & Cheung, 1985). The labeled proteins were exhaustively dialyzed against a basic medium containing 0.3 M KCl, 50 mM Tris, pH 7.5, 1 mM EGTA, and 1 mM DTT prior to fluorescence measurements. These proteins retained their biological activities as shown by their ability to confer Ca^{2+} sensitivity to actosubfragment 1 ATPase reconstituted with the labeled troponin subunits (Wang & Cheung, 1986).

Fluorescence measurements were carried out at 20 ± 0.1 °C on either a Perkin-Elmer 650/40 spectrofluorometer or a Perkin-Elmer MPF-66 fluorescence optical module interfaced to a PE 7300 computer. Proteins labeled with IAEDANS were excited at 340 nm, and its emission was measured at 470 nm. IAE-labeled TnI was excited at 520 nm, and the emission was monitored at 560 nm. For the interaction between TnC and the complex formed between IAE-labeled TnI and TnT (IAE-TnI·TnT), the fluorescent binary protein complex was formed by incubating a molar equivalent of IAE-TnI and TnT at 4 °C for several hours prior to use. Each interaction was studied in three conditions: (1) basic medium, (2) basic medium + 10 mM $MgCl_2$, and (3) basic medium + 1.5 mM $CaCl_2$. These solutions are referred to as EGTA, Mg^{2+}, and Ca^{2+} buffers, respectively. Fluorescence titrations and the nonlinear least-squares procedures used to extract binding parameters were essentially the same as those used in our previous studies (Wang & Cheung, 1985). The titrations of labeled TnC with TnT and labeled TnI with TnT and labeled TnI and TnT were carried out with initial protein concentrations in the range 1–5 μM. The titrations of labeled TnI·TnT with TnC were performed in the concentration range 1–2 μM in EGTA buffer, 100–200 nM in Mg^{2+} buffer, and 1–5 nM in Ca^{2+} buffer.

RESULTS

Spectral Properties of Labeled Proteins. Three fluorescently labeled proteins were used: IAEDANS-TnI, IAEDANS-TnC, and IAE-TnI. The emission maximum of IAEDANS-TnI was at 490 nm. Upon addition of an excess of TnT, the peak blue shifted to 485 nm with a 35% quenching of its intensity. These emission characteristics were insensitive to either Mg^{2+} or Ca^{2+}. In the presence of TnT, the emission peak of IAEDANS-TnC remained essentially unchanged at 494 nm in the presence of EGTA but shifted to 492 and 488 nm in the presence of Mg^{2+} and Ca^{2+}, respectively. The intensity of the labeled TnC was enhanced by the addition of TnT: 39% in EGTA, 20% in Mg^{2+}, and 62% in Ca^{2+}. IAE-TnI was used to form a complex with TnT, IAE-TnI·TnT, which was then titrated with TnC. Relative to IAE-TnI·TnT, the intensity of IAE in the ternary complex TnC·(IAE-TnI·TnT) was enhanced by 42% in EGTA, 22% in Mg^{2+}, and 65% in Ca^{2+}. The emission peak in the three buffers was 550, 552, and 554 nm, respectively. The choice of IAE to label TnI was dictated by its high extinction coefficient and high quantum yield. Since IAE-TnI was previously used to determine the interaction of TnC with TnI, it was appropriate to use IAE-TnI·TnT to determine the interaction between TnC and TnI·TnT.

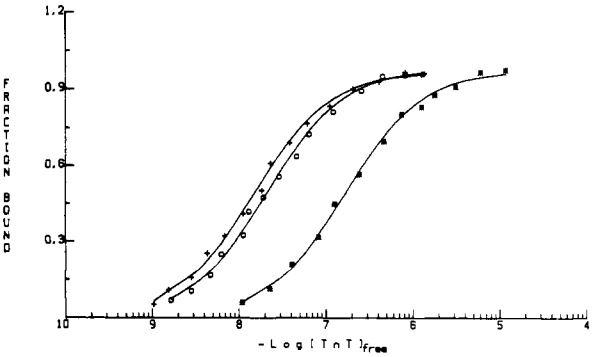

FIGURE 1: Binding isotherms for the formation of TnC·TnT. The right curve was obtained in the presence of EGTA, the middle curve in the presence of Mg^{2+}, and the left curve in the presence of Ca^{2+} sufficient to saturate all four sites. The curves were the best-fit isotherms.

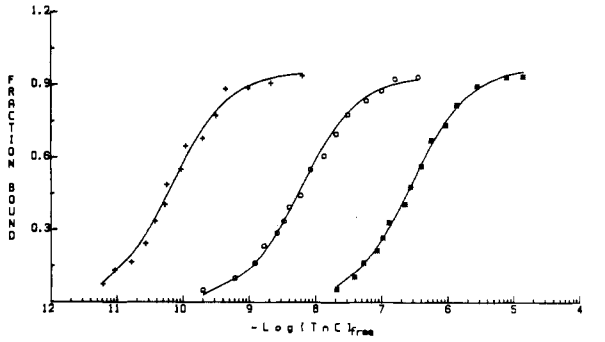

FIGURE 2: Binding isotherms for the formation of the ternary complex formed between TnC and TnT·TnI, TnC·(TnT·TnI). The right curve was obtained in EGTA buffer, the middle curve in Mg^{2+} buffer, and the left curve in Ca^{2+} buffer. The curves were the best-fit isotherms for each set of data.

Table I: Binding Parameters for Formation of TnC·TnT: C + T → CT[a]

buffer	K (M^{-1})	n
EGTA	(5.50 ± 0.66) × 10^6	0.95 ± 0.02
Mg^{2+}	(4.49 ± 0.37) × 10^7	1.01 ± 0.03
Ca^{2+}	(7.91 ± 1.01) × 10^7	0.97 ± 0.02

[a] Experiments were performed at 20 ± 0.1 °C by titrating IAEDANS-TnC with TnT. The errors associated with the stoichiometric constant (K) and binding stoichiometry (n) are the standard errors of the best fit at the 95% confidence level.

Binding Studies. The changes in emission intensity resulting from titration of labeled proteins with unlabeled proteins were in the range 20–65%. These changes were very adequate for monitoring their interactions. Unlike in the previous study of the interaction of IAE-TnC with TnI, which in the presence of Mg^{2+} was accompanied by less than 10% change in intensity, it was not necessary to use changes in polarization to evaluate binding parameters in this work.

Figure 1 shows the binding isotherms that were obtained for the formation of the binary complex TnT·TnC with labeled TnC. There was a 10-fold increase in the association constant for TnT·TnC when Mg^{2+} was present. The effect of Ca^{2+} was very comparable to that of Mg^{2+}. Figure 2 displays the isotherms for the formation of the ternary complex TnC·TnI·TnT by addition of TnC to IAE-TnI·TnT. The formation of troponin from TnC and TnI·TnT was considerably more favorable in the presence of Ca^{2+} than in the absence of cations by almost 4 orders of magnitude. The binding parameters derived from these data are summarized in Tables I and II.

The binding constant for the formation of TnI and TnT was determined with IAE-TnI. The best estimate of the stoi-

Table II: Binding Parameters for Formation of TnC·(TnI·TnT) from TnC and TnI·TnT: C + TI → CIT[a]

buffer	K (M^{-1})	n
EGTA	(3.14 ± 0.52) × 10^6	0.96 ± 0.02
Mg^{2+}	(1.28 ± 0.42) × 10^8	0.94 ± 0.01
Ca^{2+}	(1.17 ± 0.35) × 10^{10}	0.94 ± 0.02

[a] IAE-TnI·TnT was titrated with TnC at 20 ± 0.1 °C.

chiometric constant was 7.2 × 10^6 M^{-1} when determined in EGTA. The values determined in Mg^{2+} and Ca^{2+} were within a factor of 3 from the EGTA value. Thus, this interaction was not sensitive to cations.

Discussion

The observed stabilization of TnC·TnT by Mg^{2+} binding to the high-affinity Ca/Mg sites of TnC is similar to the stabilization of TnI·TnC by Mg^{2+}, but the magnitude of stabilization (−1.2 kcal) for TnC·TnT is only about 50% of that for the TnI–TnC linkage (Wang & Cheung, 1985). Unlike TnI·TnC, the linkage between TnC and TnT is not further stabilized when the two Ca^{2+}-specific sites are filled. This conclusion is based on the finding that the binding constants obtained in the presence of Mg^{2+} and Ca^{2+} differed by a factor of only 1.6 (Table I). There is no coupling between TnT and the two Ca^{2+} bound to the Ca^{2+}-specific sites within the complex TnT·TnC$(Mg)_2(Ca)_2$ or TnT·TnC$(Ca)_2(Ca)_2$. This finding is in agreement with a previous study (Ingraham & Swenson, 1984) in which the interaction of TnC with TnT was studied with two different species of fluorescently labeled TnC: one species (dansylaziridine-TnC) for the interaction in EGTA and in Mg^{2+} and the other (IAE-TnC) for that in the presence of Ca^{2+}. Our study, which was carried out with a single species of labeled TnC in all three buffers, avoided the possibility that different probes at different locations might sense the interaction in different ways. The absence of stabilization of TnC·TnT by specific Ca^{2+} binding is in qualitative accord with the early observation that troponin and the complex TnI·TnC both bind Ca^{2+} with about the same affinity. Insofar as binary linkages of troponin subunits are concerned, the regulatory effect of Ca^{2+} on the contractile cycle is likely expressed via the TnI-TnC linkage.

The free energies of the three binary complexes are known. The sum of these free energies determines the equilibrium between individual subunits and the three complexes:

$$2I + 2C + 2T \rightleftharpoons IC + CT + TI \quad (1)$$

$\Delta G_1^\circ = \Delta G^\circ(I + C) + \Delta G^\circ(C + T) + \Delta G^\circ(T + I)$, where $\Delta G^\circ(I + C)$ is the standard free energy of the formation of TnI·TnC from TnI and TnC, $\Delta G^\circ(C + T)$ is the free energy of the formation of TnC·TnT, and $\Delta G^\circ(T + I)$ is free energy of formation of TnT·TnI. The standard free energy of the formation of troponin (CIT) from the three subunits can be determined from the experimentally measured standard free energies of any one binary complex and a ternary complex that is formed from the binary complex and the third subunit. For example

$$T + I \rightleftharpoons TI$$
$$C + TI \rightleftharpoons CIT$$

These two reactions yield

$$C + I + T \rightleftharpoons CIT \quad (2)$$

$\Delta G_2^\circ = \Delta G^\circ(T + I) + \Delta G^\circ(C + TI)$. Combination of (1) and (2) leads to

$$IC + CT + TI \rightleftharpoons CIT + C + I + T \quad (3)$$

In terms of the linked-function concept (Weber, 1975), the free energy of (3) is defined as the free energy coupling (ΔG_{coup}) for the formation of troponin from the three binary complexes of its subunits. This free energy coupling is given by $\Delta G^\circ_{coup} = \Delta G_3^\circ = \Delta G_2^\circ - \Delta G_1^\circ$. It represents an average of the coupling values for all three binary complexes of troponin. By convention, a negative ΔG°_{coup} corresponds to stabilizing binary linkages or positive interaction between any two subunits that are complexed to the third. These relationships are presented in Figure 3. In the case of positive interaction, the binary interactions within troponin are more stable than they are as isolated species.

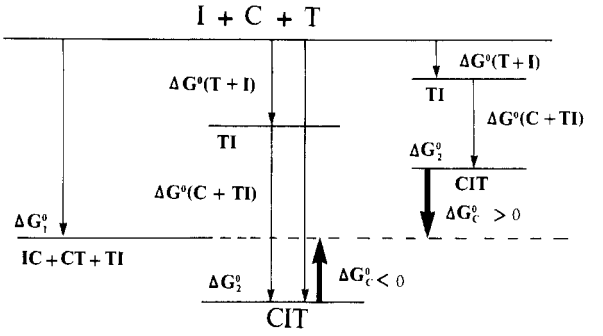

FIGURE 3: Representation of changes in standard free energy (ΔG_1°) associated with the formation of the three binary complexes of troponin from the individual subunits (left panel). The middle and right panels, which represent free energy changes (ΔG_2°) associated with the formation of troponin (CIT) from one binary and one ternary complex, illustrate free energy coupling ($\Delta G_2^\circ - \Delta G_1^\circ$) for the formation of troponin from the three binary complexes. Negative free energy coupling (ΔG_c°) indicates positive interaction, and positive free energy coupling indicates negative interaction.

Table III: Free Energy Coupling for Formation of Troponin from Binary Complexes[a]

	EGTA	Mg^{2+}	Ca^{2+}
ΔG_1° (kcal·mol^{-1})	−26.1	−29.8	−32.9
ΔG_2° (kcal·mol^{-1})	−17.7	−19.9	−22.5
ΔG_c° (kcal·mol^{-1})	+8.4	+9.9	+10.4

[a] ΔG_1° is the sum of the free energies of the three isolated binary complexes. The free energy of TnC·InI was taken from previous work (Wang & Cheung, 1985). A single association constant (7 × 10^6 M^{-1}) was used for the formation of TnT·TnI in all three buffers. ΔG_2° is the free energy of troponin (TnC·TnI·TnT), which was determined from (2). ΔG_c° is the free energy coupling for the formation of troponin from the three binary complexes as defined by (3).

We have previously reported that $\Delta G^\circ(I + C)$ is −7.9 kcal in EGTA, −10.4 kcal in Mg^{2+}, and −13.2 kcal in Ca^{2+} (Wang & Cheung, 1985). From these free energies and the data given in Tables I and II, ΔG_1°, ΔG_2°, and the free energy coupling as defined by (3) are readily calculated. The results are listed in Table III. The free energy of troponin (ΔG_2°) becomes progressively more negative as the high-affinity and the low-affinity sites of TnC are sequentially filled. Apotroponin is stabilized by −2.2 kcal when the Ca/Mg sites are saturated by Mg^{2+}. The protein is further stabilized by −2.6 kcal when the Ca^{2+}-specific sites are filled. The stabilization by Mg^{2+} is likely to provide a more stable structure for troponin, a structure corresponding to that in relaxed muscle. This is the case because TnC in relaxed muscle is likely saturated with Mg^{2+} due to the relatively high intracellular Mg^{2+} concentration. The second stabilization that occurs under conditions that mimic in vivo transition from relaxed to active muscle has physiological relevance since this specific Ca^{2+} binding activates actomyosin ATPase. The extent of this stabilization is very close to that previously determined for the isolated TnI·TnC complex. These results suggest that approximately equal free energy coupling is obtained with either TnI·TnC

or troponin when muscle is turned on by Ca^{2+} interaction with the regulatory sites in TnC. We have previously discussed the possible significance of negative free energy coupling (Wang & Cheung, 1985). If the stabilization is to be correlated with a biological function, a coupling of −2.76 kcal at 300 K should correspond to a 90% positive correlation (Weber, 1975). This is the level of correlation that is obtained with either the binary complex TnI·TnC or troponin when Ca^{2+} binds to the specific sites.

In the formation of the ternary complex (troponin) from the three binary complexes, the average binary interaction becomes less stable than the isolated complexes. This decreased stability is reflected in the positive and large free energy coupling (Table III). In the presence of Mg^{2+} bound to TnC, the coupling becomes still more positive by about 1.5 kcal. Subsequent addition of Ca^{2+} to fill the Ca^{2+}-specific sites has only a marginal effect on the binary interactions within the three-subunit troponin structure. These data are interpreted in terms of less rigid binary linkages which exist in troponin. The positive free energy coupling describes destabilization of the interaction of subunit A with subunit B caused by the presence of subunit C bound to either A or B. This effect can arise from factors such as (1) electrostatic or van der Waals repulsion, (2) conformational changes, and (3) loss of entropy resulting from binding of one subunit when another is already present. It is not possible to infer from the present data as to which binary interaction is more likely affected when troponin is formed. Regardless of the origin of this antagonistic coupling, the weakened binary interactions offer advantages for Ca^{2+} regulation because certain interactions of the subunits with tropomyosin and actin are modulated by Ca^{2+} binding to TnC. It has been shown with oligopeptides that the segment of residues 96–116 of TnI (I_{96-116}) binds to either actin–tropomyosin (Talbot & Hodges, 1981) or TnC (Cachia et al., 1983). These studies are compatible with other proton NMR studies of cyanogen bromide fragments of TnI (Grand et al., 1982), which showed perturbations of certain residues in the CN4 fragment (I_{96-116}) in the presence of TnC and of other residues in CN4 upon interaction with actin. Some of these perturbations observed with TnC are Ca^{2+}-dependent. The interaction of TnT with tropomyosin in the presence of TnI and TnC is likely to occur at two sites: one between the TnI-1 segment (residues 1–158) and the C-terminal region of tropomyosin and the other between the amino TnT-2 segment and a region of tropomyosin near Cys-190. The latter interaction is either broken or weakened when Ca^{2+} binds to TnC (Perlstone & Smillie, 1983). A picture has emerged from these and other studies that I_{96-116} may interact alternately with actin and a segment of TnC (residues 89–100) during a relaxation–excitation cycle. This alternate interaction regulated by Ca^{2+} may constitute the main Ca^{2+} switch by which the effect of bound Ca^{2+} is relayed. If Ca^{2+} binding to TnC indeed weakens one of the two TnT–tropomyosin interactions, this effect may have a bearing on the lateral movement of tropomyosin on the actin helix that was suggested by early structural studies (Huxley, 1972). These multiple interactions, which must be responsible for the transmission of the Ca^{2+} signal from its sites on TnC to distant sites along the actin filament, can be facilitated if the subunits in troponin are not rigidly held through multiple binary linkages. The observed destabilization of binary linkages may be a necessary, but not a sufficient, requirement for transmission of the Ca^{2+} signal because the extent to which the binary linkages are destabilized is little affected (free energy coupling 0.5 kcal) by specific Ca^{2+} binding. It is the decrease in the standard free energy of troponin ($\Delta G_2°$) caused by specific Ca^{2+} binding that plays a pivotal role. As demonstrated by the two reactions studied in this work to determine the free energy, this decrease (−2.6 kcal) comes entirely from stabilization of the complex formed between TnC and TnT·TnI with no contribution from the binary interaction of TnT with TnI. The free energy of troponin can also be determined from the free energies of either of the following two pairs of complexes: (1) TnC·TnT and TnI·(TnC·TnT) and (2) TnI·TnC and TnT·(TnI·TnC). Since specific Ca^{2+} binding has little effect on the binding constant for TnC·TnT, the decrease in $\Delta G_2°$ caused by such Ca^{2+} binding must be from the stabilization of the interaction between TnI and TnC·TnT, in which a linkage between TnI and TnC is established. For the other pair, the decrease in free energy is expected from the stabilization of TnI·TnC rather than the other interaction. Thus most, if not all, of the decrease in the free energy of troponin caused by specific Ca^{2+} binding is partitioned into the TnI–TnC linkage, thereby turning on the Ca^{2+} switch. This consideration, on the basis of binding energetics, lends further support to the notion that the TnI–TnC linkage within troponin serves as the main Ca^{2+} signal transmitter. Work with fully reconstituted thin filament is in progress to further examine this idea.

In summary, we have determined the free energies of the formation of the three binary complexes that are formed between troponin subunits and the formation of troponin from the three subunits. From these free energies, the free energy coupling for the formation of troponin from the three binary complexes was calculated. The coupling is large and positive, indicating that the binary interactions within troponin are destabilized relative to the isolated interactions. These destabilized binary linkages may facilitate multiple interactions between the subunits and actin and tropomyosin that are necessary in the transfer of the Ca^{2+} signal to activate actomyosin ATPase.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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