Coupling of Calcium to the Interaction of Troponin I with Troponin C from Cardiac Muscle†

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ABSTRACT: The interaction of troponin I (CTnI) with troponin C (CTnC) from bovine cardiac muscle was studied using CTnC modified at Cys35 and Cys84 with the fluorescent probe 2-[(4′-iodoacetamido)-anilino]naphthalene-6-sulfonic acid ($CTnC_{IAANS}$). The association constant for complex formation between the two proteins was determined at 20 °C in 0.4 M KCl, 1 mM DTT, 1 mM EGTA, and 25 mM MOPS, pH 7.2. In the presence of EGTA, Mg^{2+}, and Ca^{2+} these constants were 1.46×10^7, 4.1×10^7, and 12.7×10^7 M^{-1}, respectively, with corresponding free energy values of −9.62, −10.23, and −10.88 kcal mol^{-1}. The $CTnI{\cdot}CTnC_{IAANS}$ complex was stabilized by −0.61 kcal when the two Ca/Mg sites of $CTnC_{IAANS}$ were saturated with Mg^{2+} and by −1.26 kcal when all three Ca^{2+} sites were occupied by Ca^{2+}. These results suggest that calcium activation in cardiac muscle may be accompanied by a coupling free energy of −0.65 kcal. This value is a factor of 4 smaller than the value previously determined, using a similar method, for the (troponin I)·(troponin C) complex from skeletal muscle [Wang, C.-K., & Cheung, H. C. (1985) *Biophys. J. 48,* 727–739]. Since CTnC has only one Ca^{2+}-specific site and troponin C from skeletal muscle has two such sites, the present result is a factor of 2 smaller than that for the skeletal complex on the basis of a single specific site. Phosphorylation of CTnI by 3′,5′-cyclic AMP-dependent protein kinase resulted in a decrease of the association constants by a factor of 2.5–3.5. This decrease is consistent with the known loss of calcium sensitivity induced by phosphorylation of CTnI in cardiac muscle. The lost affinity for CTnC was recovered upon treatment of phosphorylated CTnI with a phosphatase. There was no loss in free energy coupling by calcium in the phosphorylated complex. If the observed free energy coupling reflects the extent of coupling that occurs during activation of the myocardium, phosphorylation of CTnI does not seem to result in impaired Ca^{2+} activation.

Contraction in skeletal and cardiac muscle is regulated by the binding of Ca^{2+} to the specific sites of the troponin C subunit of troponin. Early studies with skeletal muscle proteins (Hitchcock et al., 1973; Potter & Gergely, 1974) suggested a model of regulation in which the signal of activator Ca^{2+} is transmitted along the actin filament via the other two subunits of troponin, troponin I and troponin T, and tropomyosin. The transferred Ca^{2+} signal provides a mechanism by which actomyosin ATPase and force-generating events are regulated. STnC[1] from skeletal muscle has four Ca^{2+}-binding sites, two of which are located in the N-terminal half and the other two in the C-terminal half of the molecule. Sites 1 and 2 located in the N-domain are Ca^{2+}-specific and bind Ca^{2+} with a low affinity ($\sim 2 \times 10^5$ M^{-1}), and sites 3 and 4 located in the C-domain bind both Ca^{2+} and Mg^{2+} competitively and have a high affinity for Ca^{2+} ($\sim 2 \times 10^7$ M^{-1}). Site 1 in CTnC has several amino acid substitutions which abolish its ability to bind Ca^{2+}. Thus, CTnC has only one Ca^{2+}-specific site. The affinity of both skeletal troponin C (Potter & Gergely, 1975) and cardiac troponin C (Holroyde et al., 1980) for Ca^{2+} is enhanced when each protein is incorporated into a binary complex with troponin I from the respective tissue, and this enhanced affinity of the binary complex for Ca^{2+} is about the same as the affinity of intact troponin of the same phenotype. These findings suggest a minor role of troponin T in Ca^{2+}-induced stabilization of troponin from both types of muscle.

The mechanism of Ca^{2+} regulation involves coupling of Ca^{2+}-induced structural changes of the thin filament proteins to the interactions among these proteins. On the basis of fluorescence resonance energy transfer studies, STnI has been shown to play a key role in the regulatory mechanism of skeletal muscle. These studies demonstrated Ca^{2+}-induced movements of regions of STnI and STnC in the STnI·STnC complex (Wang & Cheung, 1986; Cheung et al., 1991) and in reconstituted troponin (Tao et al., 1989, 1990). These movements are accompanied by an enhancement of the affinity of STnI for STnC. Wang and Cheung (1985) showed that the complex STnI·STnC from skeletal muscle is stabilized by −2.7 kcal when the two Ca^{2+}-specific sites are saturated. The magnitude of this coupling free energy can, in principle, provide an efficient coupling of Ca^{2+} binding to the activation of actomyosin ATPase. These and related studies have provided evidence that the STnI–STnC linkage may serve as the major transmitter of the Ca^{2+} signal (Wang & Cheung, 1986; Cheung

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[1] Abbreviations: STnC, troponin C from skeletal muscle; STnI, troponin I from skeletal muscle; CTnC, troponin C from cardiac muscle; CTnI, troponin I from cardiac muscle; $CTnC_{IAANS}$, CTnC labeled with 2-[(4′-iodoacetamido)anilino]naphthalene-6-sulfonic acid (IAANS); CTnI·CTnC, binary complex formed between troponins I and C of cardiac muscle; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; cAMP, adenosine 3′,5′-cyclic monophosphate; Tris, tris(hydroxymethyl)aminomethane.

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et al., 1987) and STnI is a key element in the Ca^{2+} switch of regulation (Tao et al., 1990) in skeletal muscle.

The metabolic demand of cardiac muscle is different from that of skeletal muscle. There are important differences in the primary structures between cardiac and skeletal troponin I and troponin C. There have been several studies to quantify the binding of the Ca^{2+} to cardiac troponin and CTnC, but energetic studies comparable to those on STnI and STnC have not been reported for the cardiac proteins. In the present work, we have investigated the affinity of CTnI for CTnC which was labeled with a fluorescent probe and the coupling of Ca^{2+} to this interaction. We also report here on the effect of reversible phosphorylation of CTnI on the affinity of the protein for CTnC and on the coupling free energy for the phosphorylated system.

MATERIALS AND METHODS

Protein Preparation. Muscle from the left ventricle of fresh beef heart was used to prepare an ether powder as previously described (Potter, 1982). Troponin extracted from the ether powder was used to isolate cardiac troponin subunits on a CM-Sephadex C-50–120 column in the presence of 6 M urea. The CTnC fractions were pooled and rechromatographed on a DEAE-Sephadex A-50 column in the presence of 6 M urea and eluted with a linear gradient from 0 to 0.5 M KCl. Crude CTnI was similarly purified by a separate DEAE-Sephadex A-50 column. CTnC was eluted at 0.06 M KCl, and CTnI, at 0.16 M KCl. The purity of the two proteins was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. The purified proteins were dialyzed against 0.1 M KCl, 0.5 mM EGTA, 0.5 mM DTT, and 20 mM imidazole at pH 7.2, and the solutions were lyophilized.

Protein concentrations were determined by either the Lowry method (Lowry et al., 1951) or absorbance using the following extinction coefficients: CTnC, 0.23 g^{-1} cm^{-1} at 276 nm, and CTnI, 0.52 g^{-1} cm^{-1} at 276 nm (Byers & Kay, 1983). The molecular weights used were 18 500 and 24 000 for CTnC (Van Eerd & Takahashi, 1976) and CTnI (Leszyk et al., 1988), respectively.

Phosphorylation and Dephosphorylation of Cardiac TnI. Cardiac troponin I was phosphorylated by bovine cardiac 3′,5′-cyclic AMP-dependent protein kinase essentially as described by Perry and Cole (1974). Typically, the protein (1–2 mg/mL) was incubated with the kinase (0.01 mg/mL) in 50 mM Tris, 50 mM α-glycerophosphate, 5 mM $MgCl_2$, 25 mM NaF, 1 mM DTT, and 0.1 mM cAMP, pH 7.2, at 30 °C. The reaction was started by the addition of [γ-^{32}P]ATP to a final concentration of 1 mM and stopped after 5 min by immediate dialysis against a cold buffer containing 50 mM Tris, 0.1 M KCl, 1 mM EGTA, and 15 mM β-mercaptoethanol, pH 7.5, at 4 °C. Bound ^{32}P was quantified using a scintillation counter or by scanning autoradiograms of $NaDodSO_4$–polyacrylamide slab gels with a laser densitometer (Johnson et al., 1980). This procedure typically incorporated 1.7–1.8 mol of P/mol of CTnI. The method of Stull and Buss (1977) was used to determine the amount of incorporation of nonradioactive phosphate. Samples phosphorylated with nonradioactive ATP were used for the binding measurements described in this work.

Phosphorylated CTnI (0.5 mg/mL) was treated with *Escherichia coli* alkaline phosphatase in 0.4 M KCl and 0.05 M Tris at pH 8.0 to remove the phosphate groups. The reaction was carried out at 30 °C by incubating phosphorylated CTnI with 0.05 mg/mL enzyme for 1 h, followed by a second incubation with a another aliquot of 0.05 mg/mL enzyme for 2 h. The dephosphorylation was stopped by dialysis against 25 mM MOPS, 1 mM EGTA, 1 mM DTT, and 0.4 M KCl, pH 7.2, at 4 °C.

Fluorescent Labeling of CTnC. CTnC (~50 μM) was labeled with the probe 2-[(4′-iodoacetamido)anilino]naphthalene-6-sulfonic acid in 90 mM KCl, 2 mM EGTA, 2.6 mM $CaCl_2$, and 10 mM MOPS, pH 7.0, at 4 °C in the dark for 24 h. Prior to labeling the protein was first dialyzed against a buffer containing 6 M urea, 20 mM imidazole (pH 6.0), 1 mM EGTA, and 1 mM DTT, followed by a second dialysis in which urea and DTT were omitted. The labeling reaction was started by first adding $CaCl_2$ to the protein to 2.6 mM and then adding a 5-fold molar excess of IAANS dissolved in a 10 mM phosphate buffer at pH 7.0. When necessary, the pH was restored to 7.0 by adding KOH. Unreacted probe was removed by exhaustive dialysis against 25 mM MOPS, 0.1 M KCl, and 1 mM DTT at pH 7.2. The sulfhydryl content of CTnC was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) using an extinction coefficient of 12 400 M^{-1} cm^{-1} (Ellman, 1958). The labeled protein was found to contain about 0.1–0.2 mol of free sulfhydryl group/mol of $CTnC_{IAANS}$. The degree of labeling by IAANS was determined by absorbance using an extinction coefficient of 24 900 M^{-1} cm^{-1} at 325 nm for IAANS (Johnson et al., 1980), and the labeled protein was found to contain about 1.75–1.85 mol of IAANS/mol of CTnC. Thus, IAANS was likely attached to the two cysteine residues of CTnC, as previously reported (Johnson et al., 1980). The concentration of the labeled protein was determined at 276 nm after correction for the contribution of the probe on the basis of the absorbance of the probe in its adduct with β-mercaptoethanol.

Fluorescence Measurement. Fluorescence measurements were carried out on a Perkin-Elmer MPF-66 spectrofluorometer equipped with an electronic temperature-controlled cell compartment. Quantum yields were determined as previously described (Wang & Cheung, 1986). In a typical binding experiment, labeled CTnC [$\sim(0.38–8) \times 10^{-8}$ M] was titrated at 20 °C with CTnI in 0.4 M KCl, 1 mM DTT, 1 mM EGTA, and 25 mM MOPS at pH 7.2. When Ca^{2+} or Mg^{2+} was present, $CaCl_2$ was added to 1.5 mM and $MgCl_2$ to 5 mM. The fluorescence intensity (F) of $CTnC_{IAANS}$ increased upon addition of CTnI, and the increase in intensity observed at 450 nm with excitation at 335 nm was used to determine the fraction of CTnC that was bound to CTnI using the equation

$$y = \frac{F - F_0}{F_\infty - F_0} \tag{1}$$

where F_0 and F_∞ are the initial intensity of the labeled protein in the absence of CTnI and the limiting intensity determined in the presence of an excess of CTnI, respectively. F_∞ was obtained from extrapolation of a plot of $1/(F - F_0)$ vs $1/[\text{CTnI}]$. The values of y were then fitted to the following equation to obtain the stoichiometric binding constant K and the number of binding site n:

$$y = \frac{nK[\text{x}]}{1 + K[\text{x}]} \tag{2}$$

where [x] is the concentration of free CTnI. An iterative nonlinear least-squares procedure (Gauss–Newton algorithm) was used to estimate K and n.

Chemicals and Reagents. Bovine 3′,5′-cyclic AMP-dependent protein kinase, *E. coli* alkaline phosphatase, and cAMP were purchased from Sigma Chemical Co. (St. Louis,

Table 1: Relative Quantum Yield of CTnC Labeled with IAANS

CTnC*[a]	CTnC* + Ca^{2+}	CTnC* + Mg^{2+}	CTnI.CTnC*	CTnI.CTnC* + Mg^{2+}	CTnI.CTnC* + Ca^{2+}
1.00	2.00	0.80	1.79	1.95	3.44

[a] CTnC* is CTnC labeled with IAANS. The quantum yield of unliganded CTnC* was 0.105, and the relative quantum yields of different species are shown relative to the quantum yield of unliganded CTnC*.

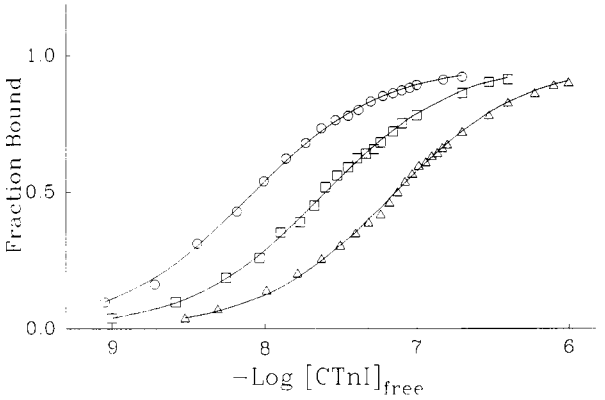


FIGURE 1: Binding isotherms for the formation of the binary complex CTnI·$CTnC_{IAANS}$ in 0.4 M KCl, 1 mM DTT, 1 mM EGTA, and 25 mM MOPS, pH 7.2, at 20 °C in the presence of EGTA (△), Mg^{2+} (□), and Ca^{2+} (○). The initial concentrations of $CTnC_{IAANS}$ were 3.0×10^{-7} M (EGTA), 1.0×10^{-7} M (Mg^{2+}), and 5.0×10^{-8} M (Ca^{2+}). See text for other conditions. The solid curves were the best fitted results obtained from eq 2.

MO). Radioactive ATP was obtained from Amersham (Arlington, IL), and IAANS was from Molecular Probes, Inc. (Eugene, OR) and was used without further purification. Ultrapure enzyme-grade urea and ammonium sulfate were purchased from Schwartz/Mann (Cleveland, OH). Chemicals used for gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA), and other chemicals were of reagent grade.

RESULTS

Interaction of $CTnC_{IAANS}$ with CTnI. Cardiac troponin C was previously shown to react with IAANS at the two –SH groups of Cys35 and Cys84 (Johnson et al., 1980). The quantum yield of IAANS in $CTnC_{IAANS}$ increased by a factor of 2.0, and the emission peak shifted from 452 to 450 nm in the presence of a large excess of Ca^{2+} (results not shown), in agreement with the previously reported fluorescence properties of the labeled protein. The fluorescence of $CTnC_{IAANS}$ was also sensitive to the addition of Mg^{2+} and CTnI, and in the presence of CTnI the fluorescence also responded to both Mg^{2+} and Ca^{2+}. These spectral changes (Table 1) were used to monitor the interaction of the labeled CTnC with CTnI in the absence and presence of the two cations. Three sets of binding curves are shown in Figure 1. It is clear that the midpoint of the curves shifted to lower free CTnI concentration in the following order: EGTA, Mg^{2+}, and Ca^{2+}. The association constants recovered from these data are given in Table 2. A 3-fold increase in the binding constant was observed in the presence of a large excess of Mg^{2+} when compared with the absence of any divalent cation. Under this condition, $CTnC_{IAANS}$ was fully saturated with Mg^{2+} at the two Ca/Mg sites. Replacement of Mg^{2+} by Ca^{2+} in the medium resulted in another 3-fold increase in the binding constant. Under the conditions of the experiments, the free Ca^{2+} concentration was in the range of 0.1 mM and all three sites were saturated with Ca^{2+}. The observed logarithmic intervals of free CTnI

Table 2: Binding Parameters for Complexation between CTnI and CTnC Labeled with IAANS[a]

CTnI species	CTnC species	$K \times 10^{-6}$ (M^{-1})	n	$\Delta G°$ (kcal/mol)
nonphosphorylated CTnI	CTnC	(14.6 ± 0.6)	0.96 ± 0.02	−9.62
	$CTnC(Mg)_2$	(41.7 ± 0.1)	0.97 ± 0.01	−10.23
	$CTnC(Ca)_3$	(127.0 ± 5.6)	0.96 ± 0.01	−10.88
phosphorylated CTnI	CTnC	(5.3 ± 0.3)	0.99 ± 0.03	−9.02
	$CTnC(Mg)_2$	(12.2 ± 0.4)	0.95 ± 0.01	−9.51
	$CTnC(Ca)_5$	(51.0 ± 2.7)	0.96 ± 0.02	−10.35

[a] The binding parameters were obtained from data similar to those shown in Figures 1 and 2. The parameters are given as the mean ± standard deviation of the best fitted values obtained from several preparations. The free energy was calculated from the standard relationship $\Delta G° = -RT \ln K$.

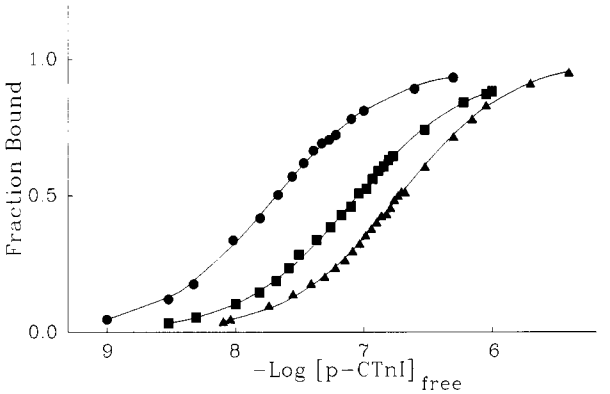


FIGURE 2: Binding isotherms for the formation of the binary complex between phosphorylated CTnI (p-CTnI) and $CTnC_{IAANS}$ in the presence of EGTA (▲), Mg^{2+} (■), and Ca^{2+} (●). Conditions are the same as in Figure 1.

concentrations between 0.1 and 0.9 fractional saturation were 2.11, 2.05, and 2.11 for formation of the CTnI·$CTnI_{IAANS}$ complex in the presence of EGTA, Mg^{2+}, and Ca^{2+}, respectively. The logarithmic interval between the two levels of saturation for ligand binding to a single class of noninteracting site is 1.908. An interval much larger than 1.908 would suggest binding heterogeneity and smaller interval would suggest cooperative binding (Weber, 1975). The observed intervals suggested that the extent of binding heterogeneity in each case was very minor.

Addition of a large excess of phosphorylated CTnI to $CTnC_{IAANS}$ in the absence of divalent cation resulted in a 1.78-fold increase in the IAANS fluorescence intensity. The intensity enhancement was 1.93- and 1.42-fold in the presence of Mg^{2+} and Ca^{2+}, respectively. These intensity changes were used to generate the binding curves shown in Figure 2 for the interaction of phosphorylated CTnI with $CTnC_{IAANS}$. The recovered binding constants are also listed in Table 2. These binding constants are a factor of 3 smaller than those for nonphosphorylated CTnI. As is the case with nonphosphorylated CTnI, the binary protein complex was stabilized by the presence of Mg^{2+} and Ca^{2+}. The extents of stabilization were comparable to those observed with nonphosphorylated CTnI. The logarithmic intervals of free CTnI concentrations between 0.1 and 0.9 saturation were 1.95, 2.18, and 2.11 for the binding curves obtained in the presence of EGTA, Mg^{2+}, and Ca^{2+}, respectively. As for the binding with nonphosphorylated CTnI, the binding heterogeneity was also very minor with phosphorylated CTnI.

Interaction of $CTnC_{IAANS}$ with Dephosphorylated CTnI. To investigate the extent to which the phosphorylation of CTnI could be reversed, binding experiments were carried out with phosphorylated CTnI that had been treated with an alkaline

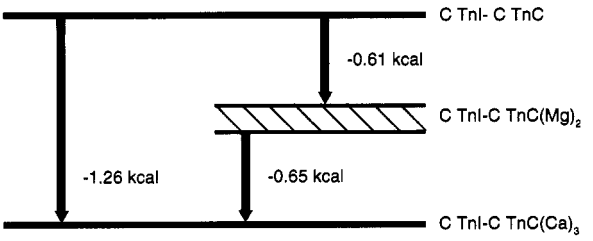


FIGURE 3: Summary of coupling free energy for formation of complexes between CTnI and CTnC and between CTnI and CTnC containing bound cations. The complex in which CTnC contains two bound cations (Mg^{2+} or Ca^{2+}) is more stable than the apo complex by –0.61 kcal, and the complex in which all three CTnC sites are occupied by Ca^{2+} is more stable than the apo complex by –1.26 kcal. The difference between these two values is the coupling free energy for formation of CTnI·CTnC$(Ca)_3$ from CTnI and CTnC·$(Ca)_2$.

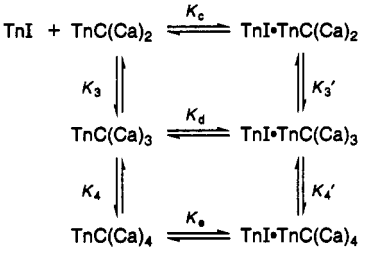
phosphatase. The association constants obtained for the interaction of dephosphorylated CTnI with $CTnC_{IAANS}$ were 1.31×10^7 M^{-1} (EGTA), 3.99×10^7 M^{-1} (Mg^{2+}), and 13.8×10^7 M^{-1} (Ca^{2+}). These values were essentially the same as those obtained with CTnI without phosphorylation. There were no evidence for any increased binding heterogeneity with this preparation of CTnI because the logarithmic intervals of free CTnI concentrations between 0.1 and 0.9 saturation were essentially the same as those shown in Figure 1. These results suggest that, after dephosphorylation, CTnI interacted with $CTnC_{IAANS}$ identically as nonphosphorylated CTnI.

DISCUSSION

We have determined the affinity of troponin I for troponin C from bovine cardiac muscle by using a fluorescently labeled CTnC. The labeled protein was previously shown to respond to both Mg^{2+} and Ca^{2+} and bind Ca^{2+} at the single Ca^{2+}-specific site with an affinity very similar to that determined with unmodified CTnC. The affinity of $CTnC_{IAANS}$ in the complex with CTnI for Ca^{2+} was also shown to be very similar to that determined with CTnI·CTnC. The labeled CTnC should retain full Ca^{2+}-binding activity, and the binding studies of this labeled CTnC with CTnI in different ionic conditions should reflect the effects of cation binding to CTnC on the stability of the protein complex.

The CTnI·$CTnC_{IAANS}$ complex is stabilized by the presence of Mg^{2+} by –0.61 kcal. The concentration of Mg^{2+} used in the measurements was sufficiently high to ensure that the two high-affinity Ca/Mg sites were saturated by Mg^{2+}. Thus, the coupling free energy for simultaneous binding of Mg^{2+} and CTnI to $CTnC_{IAANS}$ is –0.61 kcal. This ionic environment mimics that of relaxed muscle in which troponin C is expected to be saturated by Mg^{2+} because of the millimolar concentration of the cation in myocyte. When the ionic condition of the solution is changed from Mg^{2+} to Ca^{2+} in the range of pCa ~4, all three sites are expected to be saturated by Ca^{2+}. Under this condition, the binary protein complex is stabilized by –1.26 kcal. This total coupling is partitioned between saturation of the two Ca/Mg sites and saturation of the single regulatory site. These free energy values are summarized in Figure 3. The coupling free energy due to the binding of activator Ca^{2+} to the regulatory site is –0.65 kcal. In myocyte an elevation of Ca^{2+} concentration to pCa ~4 is expected to result in saturation of the single Ca^{2+}-specific site and displacement of bound Mg^{2+} at the two high-affinity sites by Ca^{2+}. The binding of Ca^{2+} to CTnC under this condition activates actomyosin ATPase and initiates force generation. The change of solution condition from Mg^{2+} to Ca^{2+} simulates the activation process insofar as Ca^{2+} binding is concerned. The present results suggest that Ca^{2+} activation in cardiac muscle may be accompanied by a coupling free energy on the order of –0.65 kcal.

The magnitude of the coupling free energy between the Mg^{2+} and the Ca^{2+} states of the CTnI·$CTnC_{IAANS}$ complex is very different from the value –2.7 kcal which we previously reported for skeletal muscle (Wang & Cheung, 1985). The previous value is expected to provide a 90% correlation of Ca^{2+} binding to the activation process. In the present system, the coupling free energy corresponds to only a 50% correlation. This large difference may be related, at least in part, to the fact that CTnC has only one active Ca^{2+} specific site and STnC has two such sites. If the affinity of the dormant site 1 in CTnC is restored, the following simple scheme describes the sequential binding of Ca^{2+} to the two active Ca^{2+}-specific sites when the two high-affinity Ca/Mg sites are occupied by Ca^{2+} (or Mg^{2+}):



where K_3 and K_4 are the stoichiometric association constants for the binding of Ca^{2+} to the two specific sites in isolated CTnC and K_3' and K_4' are the corresponding constants for the CTnI·CTnC complex. From detailed balance, $K_d/K_c = K_3'/K_3$ and $K_e/K_c = K_3'K_4'/K_3K_4$. Since $K_d = 1.3 \times 10^8$ M^{-1} and if K_c is assumed to be 4.2×10^7 M^{-1} (the same as that determined in the presence of Mg^{2+}), $K_3'/K_3 = 3$. If site 2 and activated site 1 are equivalent and bind Ca^{2+} independently of each other in both isolated CTnC and its complex with CTnI, $K_3 = 4K_4$ and $K_3' = 4K_4'$. Hence, $K_4'/K_4 = 3$, and the value of K_e is predicted to be 4×10^8 M^{-1} with a free energy of –11.49 kcal mol^{-1}. The hypothetical free energy coupling with two Ca^{2+}-specific sites would then be –1.26 kcal [–11.49 – (–10.23)], corresponding to a 75% correlation of calcium binding with activation. This coupling still is less than that observed with skeletal proteins.

When site 2 of CTnC was rendered inactive in binding calcium by site-directed mutagenesis, the mutant also lost the ability to trigger contraction in skinned fibers (Putkey et al., 1989). When the calcium-binding ability of the dormant site 1 was restored by mutation, the resulting mutant which also contained unaltered site 2 was found to increase the cooperativity of calcium-dependent contraction. Sweeney et al. (1990) showed that a mutant CTnC in which site 1 was restored to bind Ca^{2+} and site 2 was inactivated was unable to trigger Ca^{2+}-dependent contraction in TnC-depleted skinned fibers. These findings demonstrate that sites 1 and 2 have different functions and only site 2 of CTnC is essential for triggering contraction in slow muscle fibers. Since the binding of Ca^{2+} to both sites 1 and 2 of CTnC mutant in troponin (or in CTnI·CTnC complex) is cooperative, the stoichiometric association constant K_4' in the binding scheme would be larger than those for independent and equivalent binding. A consequence of this is that $K_4'/K_4 > 3$ and $K_e > 4 \times 10^8$ M^{-1}, a value predicted on the basis of noncooperative Ca^{2+} binding. Such a system would have a larger correlation than predicted from the present data.

The regulatory systems in skeletal and cardiac muscle have evolved for different physiologic demands. Gulati et al. (1992)

recently reported that the N-terminal 41 residues of CTnC (the N-helix plus the dormant helix–loop–helix site 1) dominated the performance of the calcium switch in cardiac muscle. The cardiac phenotype is expressed by this segment of the cardiac type and cannot be expressed by chimeric TnC in which the cardiac-type segment is replaced by the skeletal-type segment. Apparently, a strong interaction between the N-helix and the dormant site 1 is essential for full responses of the myocardium to Sr^{2+} and Ca^{2+}, including a nearly 2-fold smaller Hill coefficient in the Ca^{2+}–force relationship when compared with the skeletal phenotype. Other differences between the two phenotypes are found in the interaction between troponin C and troponin I. The inhibitory regions of cardiac and skeletal troponin I have identical amino acid sequences, except for a single substitution (threonine in the cardiac sequence and proline in the skeletal sequence). The inhibitory peptides of both isoforms inhibit tension development and ATPase in cardiac and skeletal muscle. The cardiac peptide, however, is a less effective inhibitor than the skeletal peptide (Talbot & Hodges, 1981; Ruegg et al., 1989). This difference may be related to the presence of a threonine rather than a proline in the cardiac peptide in which an optimal "turn conformation" thought to be needed for binding to TnC might be statistically less favorable (Campbell et al., 1992). Thus, there are substantial differences in the coupling of the calcium switch to the troponin I–troponin C linkage in the two different phenotypes. These differences may account for the observed difference in Ca^{2+} coupling free energies of the linkage between cardiac and skeletal muscle.

Two intrinsic binding constants were previously reported for the interaction of CTnC with Ca^{2+} (Holroyde et al., 1980). One constant (1×10^7 M^{-1}) was assigned to the two high-affinity Ca/Mg sites, and the other constant (2×10^5 M^{-1}) was assigned to the single Ca^{2+}-specific site. Each constant was increased by a factor of about 10 when CTnC was incorporated into the binary complex CTnI·CTnC or in intact cardiac troponin. Similar binding constants were obtained with $CTnC_{IAANS}$ (Johnson et al., 1980). These results would predict a 10-fold increase in the binding constant of CTnC for CTnI in the presence of Mg^{2+} when compared with the presence of EGTA and another 10-fold increase when Mg^{2+} is replaced by Ca^{2+} (i.e., $K_3'/K_3 = 10$). What we have found, however, is a 3-fold increase in each case. The difference is not likely due to impaired cation binding by the labeled protein or insufficient cations to saturate the respective sites because the spectral properties of the labeled CTnC in the presence of these cations are essentially the same as previously reported. The previous studies of Ca^{2+} binding to cardiac troponin and CTnC were carried out in low KCl concentrations (0.05–0.15 M), whereas the present study of the interaction between CTnI and $CTnC_{IAANS}$ was carried out in 0.4 M KCl to ensure solubility of unbound CTnI. One of us (Liao & Gwathmey, 1994) has recently shown that the affinity of CTnI·$CTnC_{IAANS}$ for calcium at the specific site determined in 0.4 M KCl is only 2.4-fold larger than that of $CTnC_{IAANS}$. This result is consistent with the small calcium-enhanced affinity between the two proteins reported here.

The physiological relevance of CTnI phosphorylation is in the ability of the myocardium to respond to hormonal fluctuations. Troponin I can be phosphorylated in response to adrenaline as demonstrated with perfused hearts (England, 1975; Solaro et al., 1976). The present work shows that CTnI phosphorylation by cAMP-dependent kinase decreases the affinity of CTnI for $CTnC_{IAANS}$ by a factor of 2.8. In spite of this decrease, the total coupling free energy (–1.33 kcal) for the binding of Ca^{2+} and phosphorylated CTnI to the labeled CTnC is not altered. This is because the binding constants for complex formation are enhanced by similar factors in the presence of divalent cations as with nonphosphorylated CTnI, and the coupling is partitioned between saturation of the Ca/Mg sites (–0.49 kcal) and the Ca^{2+}-specific site (–0.84 kcal). The loss in affinities resulting from phosphorylation is recovered upon dephosphorylation, indicating reversibility of the phosphorylation as expected. The loss of Ca^{2+} sensitivity in cardiac myofibrillar ATPase (Holroyde et al., 1979) and contractility in skinned fibers (Mope et al., 1980) resulting from phosphorylation of CTnI was related to a decreased affinity of CTnC for Ca^{2+} at the specific site. A 1.9-fold decrease of this affinity was shown with cardiac troponin reconstituted from $CTnC_{IAANS}$, and the decrease was shown to be related to a small enhancement in the rate of Ca^{2+} dissociation from the specific site (Robertson et al., 1982). The reported loss of Ca^{2+} affinity with phosphorylated CTnI is compatible with the present finding of decreases in the binding affinity of $CTnC_{IAANS}$ for phosphorylated CTnI, both in the absence and in the presence of divalent cations.

Cardiac tropinin I from different species contains an extension of amino acid residues at the N-terminus not present in skeletal troponin I. This extension has 33 residues in bovine CTnI and contains two serine residues in positions 23 and 24. Ser23 is conserved in CTnI from different species and was implicated in early studies as the site of phosphorylation by cAMP-dependent protein kinase. A second site of phosphorylation was thought to be Ser151, which is an invariant in all known sequences of TnI from both cardiac and skeletal muscle (Leszyk et al., 1988). Swiderek et al. (1988) showed that both Ser23 and Ser24 of bovine CTnI were readily phosphorylated by cAMP-dependent protein kinase and reported no phosphorylation at Ser151. It is unclear what sites were phosphorylated in our samples, but it is reasonable that at least one of the two serines alluded to in the N-terminal segment was phosphorylated. The secondary structure of CTnI predicted by the methods of Chou and Fasman (1978) and Garnier et al. (1978) indicates that there is little secondary structure in the N-terminal extension (Liao et al., 1992). Both Ser23 and Ser24 are hydrophilic with large surface probabilities (10.3 and 6.0, respectively) (Emini et al., 1985). Ser151, on the other hand, is considerably less hydrophilic with a much smaller surface probability (1.7) and is located at the N-terminal end of a long α-helix. Four arginines are located within a 11-residue segment that includes Ser23 and Ser24: ^{19}VRRRSSANYRA^{29}. If these two serines are phosphorylated, the two phosphate groups could be involved in interactions with adjacent arginyl side chains, and these interactions could result in a different local conformation of the N-terminal extension and possibly a very different ovrall conformation of the protein. The rotational correlation time of CTnI determined from the anisotropy decay of the single tryptophanyl residue in position 192 is 24 ns, and this correlation time is reduced to 15 ns upon phosphorylation under conditions identical to those used in the present work (Liao et al., 1992). These correlation times suggest a change of the axial ratio from a value of about 4–5 for nonphosphorylated CTnI to a value of about 2 for phosphorylated CTnI. The asymmetry of the protein appears to collapse into a more symmetric shape as a result of phosphorylation. This change in molecular shape is also observed in the complex formed between phosphorylated CTnI and CTnC. The reduced affinity between phosphorylated CTnI and CTnC must be related to this large change in the global conformation

of CTnI. The known decrease in Ca^{2+} sensitivity induced by CTnI phosphorylation may also be related to this more compact conformation of phosphorylated CTnI.

Ample evidence exists pointing to multiple contact regions for the interaction of STnI with STnC (Syska et al., 1976; Grabarek et al., 1981; Leszyk et al., 1990). The N-terminal segment of STnC (helix A and part of site 1) does not appear to be involved in the interaction. Similar interaction sites in CTnI and CTnC can be deduced from sequence homology between the skeletal and cardiac proteins. STnI does not have the N-terminal extension segment found in CTnI, and to what extent this segment might be involved in the interaction cannot be inferred. Since phosphorylation of CTnI decreases the affinity between CTnI and CTnC by only a small factor, it seems unlikely that the main contact regions between the two proteins are significantly perturbed. This raises the question as to whether there may be an additional contact region in each cardiac protein whose interaction is more specifically affected by phosphorylation. The N-terminal segment of CTnI has five basic residues (positions 3, 20, 21, 22, and 28) with a patch of three such residues located immediately adjacent to Ser23. The N-terminal segment of CTnC (^{1}MDDIYKAAVEQLTEEQ^{16}) contains five acidic residues at positions 2, 3, 10, 14, and 15. If the N-terminal segments of the two proteins are also contact regions in their complex, this interaction can be interrupted when two phosphate groups are introduced next to the patch of positive charges in CTnI. Although no crystallographic information is available on cardiac TnC, modeling results on STnC suggest that the binding of activator Ca^{2+} should result in movements of some helical segments in the N-domain to bring about a strong interaction between troponin C and troponin I (Herzberg et al., 1986). The N-terminal segment of STnC does not seem to be involved in this Ca^{2+}-dependent movement. If similar structural rearrangements also occur in CTnC, the interaction between the N-terminal segments of the two proteins is unlikely to be involved in signal transduction and, therefore, would not be expected to modulate the Ca^{2+} activation process. These ideas are consistent with the findings that phosphorylation of CTnI does not result in suppression of the coupling of Ca^{2+} to formation of the CTnI·CTnC complex.

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