The Calcium and Magnesium Binding Sites on Cardiac Troponin and Their Role in the Regulation of Myofibrillar Adenosine Triphosphatase*

(Received for publication, February 14, 1980, and in revised form, July 16, 1980)

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The cardiac troponin (Tn) complex, consisting of a Ca\(^{2+}\)-binding subunit (TnC), an inhibitory subunit (TnI), and a tropomyosin-binding subunit (TnT), has been reconstituted from purified troponin subunits isolated from bovine heart muscle. The Ca\(^{2+}\)-binding properties of cardiac Tn were determined by equilibrium dialysis using either EGTA or EDTA to regulate the free Ca\(^{2+}\) concentration. Cardiac Tn binds 3 mol Ca\(^{2+}\)/mol and contains two Ca\(^{2+}\)-binding sites with a binding constant of 3 \(\times 10^8\) M\(^{-1}\) and one binding site with a binding constant of 2 \(\times 10^9\) M\(^{-1}\). In the presence of 4 mM MgCl\(_2\), the binding constant of the sites of higher affinity is reduced to 2 \(\times 10^8\) M\(^{-1}\), while Ca\(^{2+}\) binding to the site at the lower affinity is unaffected. The two high affinity Ca\(^{2+}\)-binding sites of cardiac Tn are analogous to the two Ca\(^{2+}\)-Mg\(^{2+}\) sites of skeletal Tn, while the single low affinity site is similar to the two Ca\(^{2+}\)-specific sites of skeletal Tn (Potter, J. D., and Gergely, J. (1978) J. Biol. Chem. 250, 4825-5633). The Ca\(^{2+}\)-binding properties of TnC and TnI (1:1 molar ratio) are similar to those of Tn. Cardiac TnC also binds 3 mol of Ca\(^{2+}\)/mol and contains two sites with a binding constant of 1 \(\times 10^9\) M\(^{-1}\) and a single site with a binding constant of 2 \(\times 10^8\) M\(^{-1}\). Assuming competition between Mg\(^{2+}\) and Ca\(^{2+}\) for the high affinity sites of TnC and Tn, the binding constants for Mg\(^{2+}\) were 0.7 and 3.0 \(\times 10^6\) M\(^{-1}\), respectively. The Ca\(^{2+}\) dependence of cardiac myofibrillar ATPase activity was similar to that of an actomyosin preparation regulated by the reconstituted troponin complex. Comparison of the Ca\(^{2+}\)-binding properties of cardiac Tn and the cardiac myofibrillar ATPase activity as a function of [Ca\(^{2+}\)] and at millimolar [Mg\(^{2+}\)] suggests that activation of the ATPase occurs over the same range of [Ca\(^{2+}\)] where the Ca\(^{2+}\) specific site of cardiac Tn binds Ca\(^{2+}\).

Regulation of cardiac and skeletal myofibrillar ATPase activity is similar in both that the presence of the tropomyosin-troponin complex on the thin filament to confer Ca\(^{2+}\) sensitivity to the actin-myosin interaction (1). Yet the exact nature of the Ca\(^{2+}\) regulation is probably different in these two muscle types since the corresponding proteins in the regulatory complex display characteristically different properties. For example, rabbit cardiac tropomyosin consists of \(\beta\) subunits, while skeletal tropomyosin contains both \(\alpha\) and \(\beta\) subunits (2). Moreover, the cardiac troponin (Tn)\(^1\) subunits (TnT, a tropomyosin-binding subunit, TnI, a subunit inhibiting actomyosin ATPase activity, and TnC, a subunit binding Ca\(^{2+}\)) differ from the corresponding subunits of skeletal Tn in molecular weight, amino acid composition (3), and immunological properties (4). Cardiac TnI and TnT are higher molecular weight proteins than their counterparts in skeletal Tn, and in the case of cardiac TnI, the extra 26 amino acids at the NH\(_2\) terminus may be involved in a regulatory function specific for control of myofibrillar activity by a cyclic AMP-dependent phosphorylation. Solaro et al. (5) showed that this region of TnI contains a serine residue, absent in skeletal TnI, which is phosphorylated in vivo following perfusion of rabbit hearts with catecholamines (5).

The Ca\(^{2+}\)-binding properties of cardiac TnC also differ from that of skeletal TnC. Skeletal TnC contains two Ca\(^{2+}\)-binding sites at which Mg\(^{2+}\) competes, termed Ca\(^{2+}\)-Mg\(^{2+}\) sites, and two low affinity sites unaffected by physiological concentration of free Mg\(^{2+}\), termed Ca\(^{2+}\)-specific sites (6). At maximum steady state activation of skeletal myofibrillar ATPase or tension, skeletal TnC most likely contains 4 mol of Ca\(^{2+}\)/mol. By comparison of the Ca\(^{2+}\)-binding properties of skeletal TnC, carp parvalbumin and rat testis calmodulin with their amino acid sequences, it is possible to predict from the sequence data, the stoichiometry and nature of Ca\(^{2+}\) binding to TnC (7). Recently, Van Eerd and Takahashi (8) reported the amino acid sequence of bovine cardiac TnC and predicted that cardiac TnC should bind only 3 mol of Ca\(^{2+}\)/mol since one of the Ca\(^{2+}\)-binding sites was thought to be defective due to the amino acid replacement of 2 aspartic acid residues by a leucine and alanine residue. Potter et al. (7) and subsequently Leavis et al. (9) have shown that Regions I and II of skeletal TnC are the location of the two Ca\(^{2+}\)-specific sites and that Regions III and IV are the two Ca\(^{2+}\)-Mg\(^{2+}\) sites. Since Region I is defective in cardiac TnC (7, 8, 10), this protein contains only one low affinity Ca\(^{2+}\)-specific site (Region II) and two high affinity Ca\(^{2+}\)-Mg\(^{2+}\) sites (Regions III and IV) (7).

While some studies (7, 10) indicated that the Ca\(^{2+}\)-binding properties of cardiac TnC agrees with the prediction from the primary structure, conflicting reports exist concerning the

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*This work was supported by grants from The Muscular Dystrophy Association, The American Heart Association AM 78-1167, AM 79-1001, The Southwestern Ohio Heart Association, The National Institutes of Health, NIH (HL 22619-3A,3B, and HL 22231), and postdoctoral research Training Grants HL 07382-03 and AM 05998-02. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of Research Career Development Award HL 00464.

The abbreviations used are: Tn, troponin; TnI, tropomyosin subunit of troponin; TnT, ATPase inhibitory subunit; TnC, Ca\(^{2+}\) binding subunit; MOPS, morpholinoopanesulfonic acid; pCa, \(-\log [\text{Ca}^{2+}]); pMg, \(-\log [\text{Mg}^{2+}]); EGTA, ethylene glycol bis(\beta-amoenoethyl ether) N,N,N',N' tetraacetic acid.
exact nature of the Ca\textsuperscript{2+} - and Mg\textsuperscript{2+}-binding sites of both cardiac TnC and Tn. Burtnick and Kay (11) recently described the Ca\textsuperscript{2+-}binding properties of cardiac TnC, using a gel filtration technique and report a stoichiometry of 3 mol of Ca\textsuperscript{2+}/mol of TnC in apparent agreement with the predictions detailed above. However, they conclude from their Ca\textsuperscript{2+-}binding studies that all three Ca\textsuperscript{2+}-binding sites possess the same apparent affinity. Stull and Buss (12) have reported Ca\textsuperscript{2+}-binding data for bovine cardiac Tn as has Kohama (13) for chicken heart Tn. Despite the observation by both investigators that cardiac Tn binds 1.5 to 2.0 mol of Ca\textsuperscript{2+}/mol of Tn, a discrepancy exists concerning the presence of high and low affinity Ca\textsuperscript{2+}-binding sites. Stull and Buss (12) describe a single class of high affinity Ca\textsuperscript{2+}-binding sites for cardiac Tn while Kohama (13) reports the presence of both high and low affinity sites. Johnson et al. (14) have shown that in addition to the two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites, C-TnC has a Ca\textsuperscript{2+}-specific site with the same Ca\textsuperscript{2+} affinity as the two Ca\textsuperscript{2+}-specific regulatory sites of skeletal TnC as judged by fluorescence measurements. Clearly, the exact nature of the Ca\textsuperscript{2+}-binding properties of cardiac TnC and Tn remains unclear. In this paper, we report the results of a detailed study of the Ca\textsuperscript{2+}-binding properties of reconstituted and native cardiac troponin together with studies on TnC in its isolated state and as a complex with TnI.

**EXPERIMENTAL PROCEDURES**

**Materials**—DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-150 were obtained from Pharmacia. \textit{\textsuperscript{45}CaCl\textsubscript{2}} (25 mCi/mg) from New England Nuclear was diluted to the required specific activity with analytical grade CaCl\textsubscript{2}.

**Preparation of Native Bovine Cardiac Tn**—Cardiac troponin was prepared by a modification of the procedure of Stull and Buss (15). All preparative procedures were performed at 4°C. The troponin-tropomyosin complex that had been purified by gel filtration chromatography on Bio-Gel A-0.5M was further purified by a 30 to 42.5% ammonium sulfate fractionation to remove tropomyosin, followed by chromatography on a DEAE-A-50 Sephadex column (2.5 x 30 cm) equilibrated in 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.1 mM dithiothreitol. The troponin was eluted by a linear KCl gradient (0.1 to 0.6 M KCl; 2 x 500 ml). The procedure described here results in the isolation of approximately 10 to 20 mg of purified troponin from 30 g of ether powder. The stoichiometry of the troponin subunits was measured by densitometry of the Coomassie blue stain of the sodium dodecyl sulfate polyacrylamide gels. Several aspects of the purification procedure for cardiac troponin, described by Stull and Buss (15) deserve comment. Ammonium sulfate fractionation (40 to 60%) of the extract of myofibrillar ether powder results in substantial loss of troponin in the 0 to 40% ammonium sulfate fraction. Furthermore, removal of the 24,000-dalton contaminant from the troponin-tropomyosin complex by gel filtration chromatography on Bio-Gel A-0.5M leads to a further loss of troponin due to poor resolution of the contaminant from the troponin-tropomyosin complex.

**Preparation of Cardiac Tn Subunits**—The cardiac troponin subunits (TnC, TnI, and TnT) were prepared by procedures similar to those described by Stull and Buss (15) and Brekke and Greaser (16). A manuscript detailing our procedures for the isolation of the cardiac subunits is presently in preparation.

**Preparation of Reconstituted Cardiac Tn and TnC-TnI Complex from Isolated Tn Subunits**—Cardiac Tn and TnC-TnI complex were prepared by mixing equimolar amounts of purified subunits together in 6 M urea, 2.5 x 10\textsuperscript{-3} M CaCl\textsubscript{2}, 0.5 mM dithiothreitol, and 10 mM MOPS/KOH, pH 7.0 to a final concentration of 1 x 10\textsuperscript{-5} M complex. After 1 h of incubation at room temperature, this solution was dialyzed for at least 8 h at 4°C against each of the following buffers: (a) 1.0 M KCl, 10 mM MOPS/KOH, 0.1 mM dithiothreitol, pH 7.0 (4 liters); (b) 90 mM KCl, 10 mM MOPS/KOH, 0.1 mM dithiothreitol, pH 7.0 (4 liters, repeat dialysis), and (c) 90 mM KCl, 100 mM MOPS/KOH, 2 mM EGTA, pH 7.0 (4 liters, repeat dialysis). The complex was then spun at full speed on an IEC clinical table top centrifuge to remove any precipitate and the supernatant concentrated by Amicon ultrafiltration with a UM 10 membrane followed by chromatography at 4°C on a Sephadex G-150 column (2.0 x 90 cm) equilibrated in 10 mM MOPS/KOH, pH 7.0, 90 mM KCl, and 2 mM EGTA. Flow rate was 15 ml/h and fraction size 2 ml/A\textsubscript{225}. Protein fractions were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Reconstituted Tn composed of TnT, TnI, and TnC in a molar ratio of 1:1:1 was obtained in Fractions 17 to 25. Both TnI and TnC were detected in Fractions 29 to 32 while TnC was found in Fractions 34 to 44.

**Measurement of Ca\textsuperscript{2+} Binding**—For measurements of Ca\textsuperscript{2+} binding (6) in the presence of Mg\textsuperscript{2+}, TnC and the reconstituted Tn and TnC and TnC complexes were dialyzed exhaustively against a solution of 20 mM MOPS/KOH, pH 7.0, 150 mM KCl (see legends), and 0.1 mM EGTA at 4°C. To study the Ca\textsuperscript{2+} binding in the absence of Mg\textsuperscript{2+}, the proteins were dialyzed exhaustively against the above buffer except that EDTA was substituted for EGTA. The dialyzed proteins were then used for equilibrium dialysis as follows: a 0.5-ml portion of protein was dialyzed with gentle shaking for 24 h at 4°C against 100 ml of a solution containing 20 mM MOPS/KOH, pH 7.0, 150 mM KCl, 25 \textmu M of \textit{\textsuperscript{45}CaCl\textsubscript{2}}. In experiments with Mg\textsuperscript{2+}, the solution also contained 4 mM MgCl\textsubscript{2} (see legend) and 0.1 mM EGTA while those without Mg\textsuperscript{2+} contained either 0.1 mM EDTA or EGTA (see legends). In all cases, a calculated amount of CaCl\textsubscript{2} was added to achieve the desired free Ca\textsuperscript{2+} concentration. The radioactivity of portions of the outside and inside solutions was determined by scintillation counting. The protein concentration in each bag was determined by the Lowry method (18) utilizing a bovine serum albumin protein solution standardized by nitrogen determination (19). The free Ca\textsuperscript{2+} concentration was calculated from the contaminant Ca\textsuperscript{2+} (~10\textsuperscript{-5} M determined by atomic absorption) plus the amount of added Ca\textsuperscript{2+} using the computer program of Perrin and Sayce (20) and the association constants for metals and H\textsuperscript{+} to EGTA and EDTA used by Potter and Gergely (6).

**Measurements of Mg\textsuperscript{2+} Binding**—For measurement of Mg\textsuperscript{2+} binding to TnC, the protein (3 mg/ml) was dialyzed against 20 mM MOPS/KOH, pH 7.0, 150 mM KCl, and 2 mM EDTA to remove bound Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. This was followed by exhaustive dialysis against the same solution except that EDTA was replaced by 2 mM EGTA to ensure removal of trace Ca\textsuperscript{2+}. Equilibrium dialysis of 0.6-ml portions of TnC for 24 h at 4°C was carried out against 100 ml of the above solution containing the desired amount of Mg\textsuperscript{2+}. Internal and external Mg\textsuperscript{2+} were determined by atomic absorption after the addition of sufficient

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**Fig. 1.** Gel filtration chromatography of a reconstituted cardiac Tn preparation on a Sephadex G-150 column (2.0 x 90 cm) equilibrated in 10 mM MOPS/KOH, pH 7.0, 90 mM KCl, and 2 mM EGTA. Flow rate was 15 ml/h and fraction size 2 ml/A\textsubscript{225}. Protein fractions were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Reconstituted Tn composed of TnT, TnI, and TnC in a molar ratio of 1:1:1 was obtained in Fractions 17 to 25. Both TnI and TnC were detected in Fractions 29 to 32 while TnC was found in Fractions 34 to 44.
trichloroacetic acid and LaCl₃ to achieve concentrations of 6% and 1%, respectively. These reagents were added to precipitate the protein and release bound Mg²⁺.

**Analysis of Ca²⁺ and Mg²⁺ Binding Data**—To estimate the Ca²⁺- and Mg²⁺-binding parameters of cardiac TnC and its complexes, we fitted the data by nonlinear least squares regression analysis with the following equation:

\[
\frac{\text{mol Ca}^2+ \text{bound}}{\text{mol protein}} = \sum_{i=1}^{n_1} \frac{n_i}{1 + 10^{pK_i - pK}} + \sum_{j=1}^{c} 10^{pK_j - pK} \]

where \( K_i \) and \( n_i \) are the affinities and number of binding sites for the \( i \)th class of sites and \( c \) is the number of different classes of binding sites. Initial values for \( pK_i, n_i, \) and \( c \) were obtained from Scatchard analysis. The positive cooperativity present when Ca²⁺ binds to the high affinity Ca²⁺-Mg²⁺ sites on TnC was fit with the Hill equation:

\[
n = \frac{s}{1 + 10^{pK - pK}} \]

where \( s \) is the slope of the curve at 50% saturation (i.e. when \( pC_a = pK) \) and \( n \) is the number of these sites per molecule. The equation used to analyze the binding data for TnC therefore was:

\[
\frac{\text{mol Ca}^2+ \text{bound}}{\text{mol TnC}} = \frac{n_1}{1 + 10^{pK_1 - pK}} + \frac{n_2}{1 + 10^{pK_2 - pK}} + \cdots + \frac{n_c}{1 + 10^{pK_c - pK}} \]

**Preparation of Reconstituted Regulated Actomyosin**—Cardiac actin, troponycin, and reconstituted tropolin were dialyzed against 40 mM KCl, 10 mM MOPS/KOH, 0.1 mM CaCl₂, and 0.1 mM dithiorthreitol, pH 7.0 at 4°C and then a thin filament preparation was made by mixing together these proteins in a ratio of 7:1:1.5, respectively. The thin filament preparation was centrifuged for 10 min at 105,000 × g and the supernatant discarded. Cardiac myosin and the thin filament preparation were suspended and dialyzed against 120 mM MOPS/KOH, pH 7.0, 90 mM KCl, 2 mM EGTA, and 0.1 mM dithiorthreitol to yield protein concentrations of 14 and 4 mg/ml, respectively. The dylized proteins were then mixed together with myosin and seven actins to produce a concentration of reconstituted regulated actomyosin of approximately 1 mg/ml. The Ca²⁺ dependence of the reconstituted regulated actomyosin complex was determined as described in the legend of Fig. 6.

**Measurement of Myofibrillar ATPase**—Bovine cardiac myofibrils free of membrane contaminants were prepared using the procedure of Solaro et al. (21). Myofibrillar ATPase was determined as previously described by measuring inorganic phosphate liberated in incubation mixtures (6). The reaction was stopped at various times by adding samples of the incubation mixture, to an equal volume of ice-cold 10% trichloroacetic acid, filtering the mixture, and determining the phosphate in the filtrate. Incubation conditions for myofibrillar ATPase determinations are given in the legend of Fig. 6.

**RESULTS**

**Ca²⁺ and Mg²⁺ Binding Studies on Cardiac TnC**—Cardiac TnC exhibits Ca²⁺-binding properties similar to those described by Potter and Gergely (6) for skeletal TnC with the exception that one of the low affinity Ca²⁺-specific sites is absent leaving two high affinity Ca²⁺-Mg²⁺ sites (\( K_i = 1.0 \times 10^9 \) M⁻¹) and one low affinity Ca²⁺-specific site (\( K_i = 2.5 \times 10^5 \) M⁻¹). Results of measurements of Ca²⁺ binding to cardiac TnC with and without added Mg²⁺ are shown in Fig. 2. The Ca²⁺-binding data provide evidence for several very low affinity (\( K_i = 9 \times 10^6 \) M⁻¹) Ca²⁺ binding sites on TnC. These sites are clearly not physiologically significant because of their very low affinity for Ca²⁺. The Ca²⁺-binding parameters derived from the titration curves are summarized in Table I. In order to obtain an objective estimate of the number of binding sites and of the magnitude of the affinity constants, the Ca²⁺-binding data were analyzed by nonlinear least squares regression analysis (see “Experimental Procedures”). The least squares fit of the binding data had to account for positive cooperativity in Ca²⁺ binding to the two high affinity Ca²⁺-Mg²⁺ sites on cardiac TnC. This positive cooperativity was evident when we plotted the binding data as shown in Fig. 3A, where the downward concavity indicative of positive cooperativity is evident. Mg²⁺ depressed the extent of positive cooperativity of Ca²⁺ binding to the Ca²⁺-Mg²⁺ sites. The curve best fitting the Ca²⁺-binding data in the presence and absence of 4 mM Mg²⁺ was obtained with Hill coefficients of 1.2 and 1.5, respectively. The extent of cooperativity, the number of binding sites, and the magnitude of the binding constants were, however, unaffected by the type of divalent metal chelator (EGTA or EDTA) that we used to regulate the free Ca²⁺. Fig. 3B is a Scatchard plot of the low affinity Ca²⁺-binding data revealing the two classes of Ca²⁺-binding sites: one site is Ca²⁺-specific with an affinity in the range of physiological free Ca²⁺ (\( K_i = 2.5 \times 10^5 \) M⁻¹) and the other class of sites (\( n = 5 \)) binds Ca²⁺ with lower affinity (\( K_i = 9 \times 10^6 \) M⁻¹).

The Mg²⁺-binding properties of TnC were investigated to determine whether the effect of Mg²⁺ on the two high affinity Ca²⁺-binding sites was the result of direct competition of Mg²⁺.
for Ca$^{2+}$ at these sites. Assuming competition between Ca$^{2+}$ and Mg$^{2+}$ for the high affinity sites, our data and the relation

$$K'_c = K_{Ca}/(1 + K_{Mg}[Mg^{2+}])$$

where $K'_c$ is the binding constant of Ca$^{2+}$ in the presence of Mg$^{2+}$, yield $K_{Mg} = 0.7 \times 10^5$ M$^{-1}$. Fig. 4 shows the Mg$^{2+}$-binding properties of cardiac TnC. No positive cooperativity was observed in Mg$^{2+}$ binding to TnC. TnC binds approximately 4 mol of Mg$^{2+}$/mol at pH 2.7. When sufficient Ca$^{2+}$ (pCa 4) was added to saturate all three Ca$^{2+}$-binding sites, approximately 2 to 3 mol of Mg$^{2+}$ remained bound/mol of TnC in the presence of 2 mM MgCl$_2$ (data not shown). Thus, under the conditions used in these experiments, TnC contains the following divalent metal binding sites, two high affinity Ca$^{2+}$-binding sites, and two Mg$^{2+}$-binding sites, one site with lower affinity for Ca$^{2+}$ that does not bind Mg$^{2+}$ in the presence of physiological Mg$^{2+}$ concentrations (Ca$^{2+}$-specific site), and several sites that bind Mg$^{2+}$ independent of the [Ca$^{2+}$] used in this experiment.

**Ca$^{2+}$-binding Studies on Native Tn, Reconstituted Tn, and TnI-TnC Complex**—The preparation of native cardiac Tn described in this paper results in the isolation of relatively small amounts of Tn from bovine heart muscle. Other procedures used to prepare larger quantities of cardiac troponin resulted in the isolation of troponin that proved to be unstable during equilirium dialysis experiments and thus were unsuitable for these measurements. This instability of the native Tn may be due to endogenous proteases. Since large quantities of protein were required for the measurements and since the Ca$^{2+}$-binding properties of native and reconstituted Tn measured in the absence of added Mg$^{2+}$ were similar (see Table 1), we decided to characterize the Ca$^{2+}$-binding properties of the reconstituted Tn preparation due to the ready availability of larger quantities of the Tn subunits and to the stability of the reconstituted Tn. The reason for the greater stability of the reconstituted Tn is unknown, however, it may be due to the removal of contaminating proteases either by the urea treatment required for chromatography of the subunits or during one of the additional chromatographic steps required for subunit purification.

**Fig. 5.** Effect of [Mg$^{2+}$] on Ca$^{2+}$ binding to cardiac TnC. Calcium binding to cardiac TnC was measured by equilibrium dialysis and the data analyzed as described under “Experimental Procedures.” Scatchard plots of the Ca$^{2+}$ binding data for the two high affinity sites and the low affinity site are presented in A and B, respectively.
Ca\textsuperscript{2+} Binding to Cardiac Troponin

Ca\textsuperscript{2+} and Mg\textsuperscript{2+} for the high affinity sites, a $K_M$ of $3 \times 10^5 \text{ M}^{-1}$ can be calculated for TnC when complexed with either TnI or TnI and TnT. The data indicate that formation of these complexes may increase the affinity of the Mg\textsuperscript{2+}-binding sites of TnC approximately 5-fold.

Measurement of Myofibrillar ATPase—The ATPase activity of reconstituted regulated actomyosin showed the same Ca\textsuperscript{2+} dependence as that of the myofibrillar ATPase. The relation between free [Ca\textsuperscript{2+}] and cardiac myofibrillar ATPase activity measured in the presence of 4 mM free Mg\textsuperscript{2+} is shown in Fig. 6 together with a calcium binding curve for cardiac Tn. It is clear that the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites of Tn are titrating before the onset of activation of the myofibrillar ATPase while the Ca\textsuperscript{2+}-specific site is titrating over the same range of free Ca\textsuperscript{2+} that is responsible for activation of the myofibrillar ATPase. The data indicate that with these conditions Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+}-specific site is more clearly related to activation of the myofibrillar ATPase than Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites of cardiac Tn.

**DISCUSSION**

Ca\textsuperscript{2+} binding data on cardiac TnC obtained by equilibrium dialysis have been presented in this paper as evidence that bovine cardiac TnC has three Ca\textsuperscript{2+}-binding sites, two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites ($K_2 = 1.0 \times 10^4 \text{ M}^{-1}$) and only one low affinity Ca\textsuperscript{2+}-specific site ($K_2 = 2.5 \times 10^4 \text{ M}^{-1}$). These results are similar to those reported by Potter and Gergely (6) for rabbit skeletal TnC except that cardiac TnC contains only one low affinity Ca\textsuperscript{2+}-specific site. While there is general agreement that cardiac TnC contains three Ca\textsuperscript{2+}-binding sites, conflicting reports exist concerning the precise nature of these three binding sites. In agreement with the studies reported here, Potter et al. (7) and Leavis and Kraft (10) described two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites and one low affinity Ca\textsuperscript{2+}-binding site for cardiac TnC while Burtnick and Kay (11) describe three high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites. The inability of Burtnick and Kay to separate the contributions of the two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites and the one low affinity Ca\textsuperscript{2+}-binding site reported here may be attributed to the lack of sufficient Ca\textsuperscript{2+} binding measurements made in the pCa range 6 to 4 over which the one low affinity Ca\textsuperscript{2+}-binding site is binding Ca\textsuperscript{2+}.

Potter et al. (7) and Leavis and Kraft (10) report affinities for the two Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites of cardiac TnC in the range 1 to $3 \times 10^4 \text{ M}^{-1}$ in good agreement with our data. Leavis and Kraft (10) could not determine precisely the affinity of the low affinity site for Ca\textsuperscript{2+} but estimate the affinity to be in the range $10^4$ to $10^5 \text{ M}^{-1}$. A Ca\textsuperscript{2+}-specific electrode was used in their studies. Potter et al. (7) reported an affinity of $2 \times 10^4 \text{ M}^{-1}$ for the low affinity Ca\textsuperscript{2+}-specific site on cardiac TnC, lower than that reported here ($K_2 = 2.5 \times 10^4 \text{ M}^{-1}$) and is probably the result of the difficulty involved in separating the Ca\textsuperscript{2+}-specific site of TnC ($2.5 \times 10^4 \text{ M}^{-1}$) from the several very low affinity sites ($K_2 = 9 \times 10^4 \text{ M}^{-1}$). By collecting Ca\textsuperscript{2+}-binding data for TnC between pCa 4 and 2 and accounting for the positive cooperativity in Ca\textsuperscript{2+}-binding to the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites in our data analysis, we were able to obtain a more objective estimate of the affinity of the Ca\textsuperscript{2+}-specific site of cardiac TnC than that reported previously (7, 10). The Ca\textsuperscript{2+}-binding constant described here for the low affinity Ca\textsuperscript{2+}-specific site of cardiac TnC is in good agreement with fluorescent probe studies of Ca\textsuperscript{2+} binding to cardiac Tn reported by Johnson et al. (14).

Our Ca\textsuperscript{2+}-binding studies on the cardiac TnI-TnC complex and the native and reconstituted whole troponins provide evidence that in the absence of Mg\textsuperscript{2+} the interaction of TnI and TnC results in an approximately 10-fold increase in the affinities of both the two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites ($K_2 = 3 \times 10^4 \text{ M}^{-1}$) and the one low affinity Ca\textsuperscript{2+}-specific site ($K_2 = 2 \times 10^4 \text{ M}^{-1}$). Recent studies on the Ca\textsuperscript{2+}-binding properties of native cardiac Tn preparations by Stull and Buss (12) report that Tn contains two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites with affinities of $4 \times 10^4 \text{ M}^{-1}$ and no Ca\textsuperscript{2+}-specific site. Clearly, a discrepancy exists concerning the studies of Stull and Buss (12) and those reported here. Our studies show that both native and reconstituted troponin binds 3 mol of Ca\textsuperscript{2+} and exhibits two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites and one low affinity Ca\textsuperscript{2+}-specific site. We are unable to explain the inability of Stull and Buss to demonstrate the existence of the low affinity Ca\textsuperscript{2+}-specific site reported here in both native and reconstituted troponin and by Johnson et al. (14) with fluorescent probe studies of Ca\textsuperscript{2+} binding to cardiac Tn. Despite the apparent agreement on the existence of the two high affinity Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites on cardiac Tn, Stull and Buss report a 10-fold lower affinity of the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites for Ca\textsuperscript{2+}. The affinities that they observe for the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites of whole Tn are similar to these described here for uncomplexed TnC.

Regulation of actomyosin by a reconstituted troponin-troponyosin complex is reported as evidence of the functional integrity of the reconstituted troponin complex used to characterize the Ca\textsuperscript{2+}-binding properties of cardiac Tn (see Fig. 7). Additional evidence is provided by the close similarity between the Ca\textsuperscript{2+} dependence of the myofibrillar and reconstituted regulated actomyosin ATPase activities.

The Ca\textsuperscript{2+}-specific site of cardiac Tn binds Ca\textsuperscript{2+} over the same range of free [Ca\textsuperscript{2+}] that activates the cardiac myofibrillar ATPase (Fig. 6). In the experiments described in this paper the two Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites of cardiac Tn are filled before the onset of activation of the myofibrillar ATPase, indicating that the Ca\textsuperscript{2+}-specific site is more important in the regulation of ATPase than the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} sites. A similar conclusion has been reached in studies by Potter and Gergely (6) for the role of the Ca\textsuperscript{2+}-specific sites in skeletal Tn.

Cardiac Tn is a phosphoprotein, which undergoes rapid phosphorylation of the Tn subunits in response to epinephrine in a manner that suggests an important role in regulation of contractile activity. Several investigators (22, 23) including ourselves have demonstrated that phosphorylation of TnI results in a decreased sensitivity of the myofibrillar ATPase to Ca\textsuperscript{2+}. Furthermore, Holroyde et al. (23) have shown that decreased myofibrillar Ca\textsuperscript{2+} binding accompanies the decreased Ca\textsuperscript{2+} sensitivity of the ATPase following phosphorylation of the myofibrillar TnI. In this paper, we report the
results of studies on the Ca\textsuperscript{2+}-binding properties of cardiac Tn reconstituted using TnI containing a constant 0.1 mol of P/mol of TnI. We are presently determining what effect TnI phosphorylation may have on the Ca\textsuperscript{2+}-binding properties of cardiac Tn.

Acknowledgments—We would like to acknowledge Patricia Walker, Denise Robinson, Elizabeth Howe, and Henry Zot for their excellent technical assistance during the course of this work.

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