Equilibrium-binding studies at 4°C show that, in the instance of crayfish, troponin C contains only one Ca-binding site with an affinity in the range of physiological free [Ca²⁺] (K = 2 × 10⁸ M⁻¹). At physiological levels of Mg²⁺, this site does not bind Mg²⁺. In the complexes of troponin C-troponin I, troponin and troponin-tropomyosin, the regulatory site-specific site exhibits a 10- to 20-fold higher affinity (K = 2–4 × 10⁸ M⁻¹). The latter affinity is reduced to that of troponin C upon incorporation of the troponin-tropomyosin complex into the actin filament (regulated actin), as determined at 4°C by the double isotope technique. The Ca-binding constant is again shifted to a higher value (7 × 10⁸ M⁻¹) when regulated actin is associated with nucleotide-free myosin. Both crayfish myofibril and rabbit actomyosin regulated by crayfish troponin-tropomyosin display a steep rise in ATPase activity with [Ca²⁺]. Comparison of the pCa/ATPase relationship and the Ca-binding properties at 25°C for the crayfish troponin-regulated actomyosin indicates that while the threshold [Ca²⁺] for activation corresponds to the range of [Ca²⁺] where the regulatory site in its low affinity state (K = 1 × 10⁶ M⁻¹) starts to bind Ca²⁺ significantly, full activation is reached at [Ca²⁺] for which the Ca-specific site in its high affinity state (K = 3 × 10⁸ M⁻¹) approaches saturation. These results suggest that, in the actomyosin ATPase cycle, there are at least two calcium-activated states of regulated actin (one low and one high), the high affinity state being induced by interactions of myosin with actin in the cycle.

The activity of vertebrate-striated muscle is primarily controlled by the reversible binding of Ca²⁺ to TnC, one of the three subunits of Tn in the thin filament which is composed of Tn, Tm, and F-actin in a 1:1:7 molar ratio (2). The steric blocking model has been proposed as a mechanism for the calcium-regulated control (3, 4). This all-or-none model suggests that, when TnC is Ca²⁺-free, Tm sterically prevents the binding of the myosin heads to actin, thus relaxing the muscle. In the presence of Ca²⁺, Tm does not block the binding of myosin cross-bridges. Recent data suggest that the Tn-linked regulation may be better viewed as an allosteric system (5, 6).

In many invertebrate muscles, the Tn-linked regulatory system coexists with the myosin-linked system (7). Yet, in fast striated muscle of arthropods such as horseshoe crab (8) and crayfish (9), Ca²⁺ binding to TnC seems to be the primary trigger for contraction. Similarly to its vertebrate counterpart, Tn isolated from arthropods appears to consist of three components (10-12). The latter differ, however, from the corresponding subunits of vertebrate Tn (13) in molecular weight and amino acid composition. The smallest (M₆ = 16,000 to 18,000) and medium (M₆ = 23,000 to 29,000) subunits are analogous in function to vertebrate TnC and TnI, respectively (11, 12). As for the TnT-like protein (M₆ = 50,000 to 60,000), its involvement in troponin function has not yet been studied in detail (10-12).

Rabbit skeletal TnC contains four potential binding sites for Ca²⁺ (14, 15). Only two sites that bind Ca²⁺ specifically are thought to be functional (15). The two other sites that bind both Ca²⁺ and Mg²⁺ seem to play a structural role (16). Bovine cardiac TnC is analogous to the skeletal muscle protein, with two Ca²⁺-Mg²⁺ sites but only one Ca²⁺-specific site (17). There is still some doubt about the regulatory sites in vertebrate systems, since magnesium ions affect the calcium activation of myofibrillar ATPase (18) and of tension development (19). Invertebrate TnC binds less Ca²⁺ than does vertebrate TnC (11, 20–22); arthropod TnC seems to have no more than one Ca²⁺-binding site (20, 21).

The aim of this work was to study the nature of the Ca²⁺- and Mg²⁺-binding sites on crayfish TnC and their role in the regulation of actomyosin ATPase. We measured Ca²⁺ and Mg²⁺ binding to crayfish TnC in a vast range of [Ca²⁺] or [Mg²⁺] and found that in the limited range of physiological [Ca²⁺] only a single site binds Ca²⁺. This site is analogous to the Ca²⁺-specific sites of vertebrate TnC. Hence, the Tn-M complex with the simplest physiologically significant Ca²⁺-binding properties constitutes a convenient system to study Ca²⁺ regulation. For instance, conflicting reports exist concerning the mechanism responsible for the sharp transition in Ca²⁺ dependence of myofibrillar ATPase and of tension development (15, 23–25). We compared the Ca²⁺-binding properties of crayfish Tm-Tn-containing actin (alone or associated with myosin) with the pCa/ATPase relationship in the instance of both regulated actomyosin and myofibrils. This system has the advantage over the vertebrate one that...
the results are blurred neither by Ca$^{2+}$ binding to the nonrelevant sites on TnC nor by the requirement for activation of multiple bound Ca$^{2+}$ on the TnC molecule.

**EXPERIMENTAL PROCEDURES**

**Materials**—DEAE-Sephadex A-25 and SE (sulfoethyl)-Sephadex C-50 were obtained from Pharmacia (Uppsala, Sweden). Polyacrylamide gel electrophoresis reagents were purchased from Serva (Heidelberg, Germany). $^{45}$Ca (30 mCi/mg) and $^{3}H$-labeled [3H]glucose (13 Ci/mmol) were from Amersham International plc (Amersham, England). All other chemicals were reagent grade and were utilized without further purification, except for urea (Merck, Darmstadt, Germany) solutions which were deionized by means of a mixed bed solution from an all-quartz apparatus and contained inhibitors of bacterial growth and proteolysis: 0.5 mM NaN$_{3}$ (Merck), 20 bidistilled water from an all-quartz apparatus and contained inhibi-

FIG. 1. 
**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the crayfish preparations.** a, 10 µg of Tm-Tn complex; b, 5 µg of Tn; c, 5 µg of Tn complex; d, 3 µg of TnT; e, 2 µg of TnC; f, 6 µg of TnC. The apparent $M_{r}$ of polypeptide chains was: Tm, 37,000; TnT, 45,000; TnI, 26,000; TnC, 16,000.

**Preparation of Myofibrils—**Myofibrils were prepared by the method of Lehman (26) from crayfish (Astacus leptodactylus) tail muscle and from rabbit hind leg and back muscle. The myofibrils were stored at 0–4 °C.

**Preparation of Rabbit Myosin and Actin—**Myosin was prepared according to Watson and Schaub (27) and then stored at −20 °C in 10 mM potassium phosphate, pH 6.5, 0.6 M KCl, 5 mM dithiothreitol, and 50% glycerol. Actin was purified by the procedure of SPUDICH and WATTENBERG (28) and stored on ice as F-actin.

**Preparation of Tm-Tn Complex from Rabbit and Crayfish—**Both complexes were extracted from fresh myofibrils in 15 mM 2-mercaptoethanol, 2 mM Tris/HCl buffer, pH 7.0, using a procedure similar to that described by Murray (29). The complexes were stored in the above buffer at −20 °C.

**Preparation of Crayfish Tn and Tn Subunits—**Crayfish Tn was obtained either from the Tm-Tn complex by the isoelectric precipitation of Tm (30) or from myofibrils by a procedure similar to that of EBASHI et al. (31). The Tn complex was dialyzed against 0.2 M NaCl, 5 mM EDTA, 15 mM 2-mercaptoethanol, 20 mM Tris/HCl buffer, pH 7.8. When applied to a column of DEAE-Sephadex A-25, only TnC was retained. The latter protein was eluted at 0.4 M NaCl. The proteins not absorbed were dialyzed against 6 M urea, 5 mM 2-mercaptoethanol, 50 mM sodium barbital, pH 8.0, and loaded on a column of SE-Sephadex C-50. TnT and TnC were eluted by a linear gradient of 0 to 0.3 M NaCl (at 0.08 and 0.20 M NaCl, respectively). Appraisal of purity of the preparations (Fig. 1) and determination of the apparent $M_{r}$ of the polypeptide chains (32) were carried out by means of an sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedure (33) in 12.5% acrylamide gels. The TnT-TnC complex was obtained by mixing purified subunits using a procedure similar to that described by Holroyde et al. (17).

**Preparation of Reconstituted Regulated Actin and Actomyosin—**Regulated actin was prepared by polymerizing rabbit actin in the presence of an excess of the Tm-Tn complex from crayfish or rabbit, as described by Murray (29). The thin film preparations and rabbit myosin were dialyzed against 80 mM KCl, 1 mM dithiothreitol, 0.1 mM EGTA, pH 7.0, 77 mM imidazole, 40 mM imidazole, pH 7.0, and myosin were then mixed (4:1 molar ratio) to produce a concentration of reconstituted regulated actomyosin of approximately 6 mg/ml.

**Metal and Protein Analyses—**Calcium, magnesium, and $^{45}$Ca were determined as described previously (34). Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard. Protein concentrations were standardized by either amino acid analysis or determination of dry weight. The concentrations of rabbit proteins were measured spectrophotometrically using the following absorption coefficients: 630 cm$^{-1}$/g at 280 mg for G-actin, 540 cm$^{-1}$/g at 280 mg for myosin, and 280 cm$^{-1}$/g at 278 nm for Tm-Tn. The molecular weights used for rabbit actin, myosin, and Tm-Tn complex were 42,000, 460,000, and 150,000, respectively. Those for crayfish proteins were: Tm, 45,000; Tn complex, 161,000; TnC, 87,000; TnC-Tn complex, 42,000; TnT, 16,000.

**Quantification of TnC in crayfish preparations was achieved by densitometry of the Coomasie Blue-stained gels (36), using increasing amounts of pure TnC as internal standards. Extrapolation to zero concentration of added TnC gave the amount of TnC in the original solution.

**Calcium- and Magnesium-binding Measurements—**The binding of calcium to crayfish TnC (3 mg/ml), TnC-Tn complex (6 mg/ml), Tn (8 mg/ml), and Tm-Tn (16 mg/ml) was measured by equilibrium dialysis at 27 °C. For Mg$^{2+}$ binding to TnC in the absence of Ca$^{2+}$, EGTA was contained 40 mM imidazole, pH 7.3, 80 mM KCl, 0.1 mM EGTA, 0.1 µCi/ml of $^{45}$CaCl$_{2}$, and the appropriate amount of MgCl$_{2}$ to achieve the desired free Ca$^{2+}$ concentration. In experiments without added Mg$^{2+}$, the contaminating Mg$^{2+}$ concentration was about 0.1 µM. In experiments with 1 mM MgCl$_{2}$, KCl concentration was diminished to 77 mM. For Mg$^{2+}$ binding to TnC in the absence of Ca$^{2+}$, EGTA concentration was increased to 1 mM, and the desired concentrations of MgCl$_{2}$ were used. The free Ca$^{2+}$ and Mg$^{2+}$ concentrations were calculated by means of the computer program of Perrin and Sayce (37). The association constants for metals and H$^{+}$ to EGTA were also performed without EGTA, and the free [Ca$^{2+}$] was regulated by appropriate amounts of Chelex (Bio-Rad) in the dialysis fluid, as described by Crouch and Klee (39).

The binding of calcium to the reconstituted regulated actin and actomyosin was measured at 4 and 25 °C by means of a double-isotope technique. Regulated actin (2 mg/ml) and regulated actomyosin (6 mg/ml) were incubated for 30 min at the chosen temperature in 1 ml of the solution described above containing also 0.3 µCi of [3H]glucose and 5 mM glucose. For measurements of Ca$^{2+}$ binding to regulated actomyosin in the presence of 1 mM MgATP, 20 mM creatine phosphate and 0.2 mg of creatine phosphokinase were included. MgCl$_{2}$ concentration was increased to 2 mM, and KCl concentration was diminished to keep ionic strength constant; ATP was brought to 1.3 mM (4 °C) or 1.2 mM (25 °C) just before centrifugation. The suspensions (1.5-ml fractions) were centrifuged in a Beckman Airfuge at 165,000 $g$ for 30 min. The pellets and supernatants were carried out at 4 °C in a boiling water bath for 5 min, and then neutralized by addition of 0.1 ml of 1 M HCl and 5 µl of 0.5 M sodium phosphate, pH 7.0. Bound calcium was calculated from the $^{45}$Ca/$^{3}H$ ratio of the dissolved pellets relative to that of the supernatant solutions and from the total concentration of calcium in the supernatant. Corrected values of bound calcium binding to pure actin (in the case of regulated actin) and myosin combined with pure actin (for regulated actomyosin) in the same assay conditions.

**ATPase Assays—**One-ml reaction mixture, equilibrated at 25 °C, contained 0.2 to 0.4 mg of regulated actomyosin or 0.1 to 0.5 mg of myofibrils, 40 mM imidazole, pH 7.0, 70 mM KCl, 0.1 mM EGTA, 2
mm MgCl₂, 4 mm phospho(enol)pyruvate, 0.02 mg of pyruvate kinase, and the desired concentrations of CaCl₂. Reactions were initiated by adding ATP to 1.2 mm and stopped at various times with 0.25 ml of ice-cold 25% trichloroacetic acid. The supernatant obtained following a low speed centrifugation of the precipitate was assayed for inorganic phosphate (40) and calcium. The pCa values were calculated as shown above.

Fluorescence Measurements—The tyrosyl fluorescence of crayfish TnC was measured in 10 mm HEPES, pH 7.0, and 80 mm KCl, at 4 and 25 °C, in a Baird Atomic FC 100 spectrofluorimeter equipped with a thermostatted cuvette holder. The free Ca²⁺ concentrations were controlled using an EGTA buffer system as described above. The TnC concentration was 20 μg/ml. Upon excitation at 280 nm, emission was monitored at 310 nm. In a first approximation, the intensity of the fluorescence was considered as directly proportional to the absolute quantum yield.

RESULTS

Ca- and Mg-binding Studies on Crayfish TnC—Fig. 2 depicts typical Ca-binding curves, with and without added Mg²⁺, for crayfish TnC. In order to estimate the binding parameters, a curve of the form

\[ r = \frac{c}{\sum n_i K_i [Me^{i+}]} \]

was fitted to the experimental binding data by means of a computerized nonlinear least squares regression procedure (34). In this formula, \( r \) is the amount of metal bound per mol of TnC, \( K_i \) and \( n_i \) are the affinity 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 \( K_i, n_i, \) and \( c \) were obtained from the Scatchard plot (Fig. 3). Two classes of Ca-binding sites were obtained, one (\( n_1 = 1 \)) with higher affinity for Ca²⁺ (\( K_1 = 2.6 \times 10^9 \) M⁻¹, Table I) and the other (\( n_2 = 5 \)) with lower affinity for Ca²⁺ (\( K_2 = 2.0 \times 10^7 \) M⁻¹). Mg²⁺ (1 mM) reduces the binding constants of the sites of lower affinity to

\[ K_2 = 9 \times 10^6 \text{M}^{-1} \]

1.0 × 10⁻⁵ M⁻¹ without affecting significantly the site of higher affinity. Fig. 2 shows that in 1 mM Mg²⁺, when the protein is calcium-free, it binds about 2.5 mol of Mg²⁺/mol; saturation of the first site by Ca²⁺ occurs without significant decrease in the amount of magnesium bound to crayfish TnC, whereas Ca²⁺ binding to the other sites is accompanied by a release of Mg²⁺ from the protein. Furthermore, the best fit of the inverted magnesium release curve as a function of free Ca²⁺ concentration (where \( r_{Ca} = 2.5 - r_{Mg} \) represents calcium bound to sites that may also accommodate Mg²⁺) is obtained with a single binding constant for Ca²⁺ (1.3 × 10⁻² M⁻¹). This value agrees with that obtained from Ca²⁺-binding measurements for the lower affinity calcium sites, suggesting that Mg²⁺ competes with Ca²⁺ for these sites. Our data and the competition equation

\[ K_{Ca} = K_{Ca(Mg)} = K_{Ca(Mg)} - K_{Ca}[Mg^{2+}] \]

where \( K_{Ca} \) and \( K_{Ca(Mg)} \) are the binding constants of Ca²⁺ in the absence of Mg²⁺ and at given [Mg²⁺], respectively, yield a value of 1.0 × 10⁻² M⁻¹ for \( K_{Mg} \), the constant of Mg²⁺ in the absence of Ca²⁺. Similar binding parameters were found irrespective of whether or not EGTA was used to regulate the free [Ca²⁺].

The Mg-binding properties of crayfish TnC were investigated to ascertain that the effect of Mg²⁺ on Ca-binding sites with lower affinity was the result of direct competition between Mg²⁺ and Ca²⁺ for these sites. The results are shown in Fig. 4. The best fit of the data was obtained with about 5 mol of Mg²⁺ per mol and a binding constant of 9 × 10⁻⁵ M⁻¹. This value is in good agreement with the constant calculated from the Mg²⁺ effect on Ca²⁺ binding. Thus, crayfish TnC contains a single site that displays an affinity for Ca²⁺ within the physiological range of [Ca²⁺] and that does not bind Mg²⁺ at millimolar Mg²⁺ levels (regulatory Ca-specific site). In addition, there are five low affinity Ca²⁺-binding sites that also bind Mg²⁺ competitively (Ca-Mg sites).

Ca²⁺ titration of crayfish TnC results in an enhancement of tyrosine fluorescence (Fig. 5). The best fit of the titration curve was obtained with a single binding constant \( K = 3.9 \times 10^6 \text{M}^{-1} \) (4°C). The binding constant is similar to that obtained from direct Ca²⁺-binding measurements for the Ca-specific site. Mg²⁺ (2 mM) in itself has no effect on the fluorescence nor does it affect the Ca²⁺ titration curve (not shown). These results suggest that solely the Ca-specific site participates in the structural change in crayfish TnC upon Ca²⁺ binding. Moreover, Fig. 5 shows that the affinity of Ca²⁺ for the regulatory site varies significantly between 4 and 25 °C. Assuming that the enthalpy change, \( \Delta H \), for Ca²⁺ binding to
A Single Regulatory Ca-binding Site on Crayfish Troponin C

Effect of protein-protein interactions on the Ca-binding properties of the regulatory site on crayfish TnC

The Ca-binding parameters were measured by equilibrium dialysis for crayfish TnC and its TnI-TnC, Tn, Tm-Tn complexes, and by double-isotope technique for reconstituted regulated actin and actomyosin. Calcium bound per mol of the TnC and N are averages (± S.E.) for several (N) experiments carried out in both the absence and presence (1 mM) of MgATP except for regulated actomyosin which was studied only at 1 mM [MgATP].

| Ca bound | K | N | Temperature |
|----------|---|---|-------------|
| mol/mol TnC | M⁻¹ | | °C |
| TnC | 0.9 ± 0.1 | 2.6 ± 0.9 ¥ 10⁻⁵ | 12 | 4 |
| TnI-TnC | 0.9 ± 0.1 | 2.6 ± 0.9 ¥ 10⁻⁵ | 2 | 25 |
| Tn | 0.8 ± 0.1 | 2.5 ± 0.9 ¥ 10⁻⁵ | 4 | 4 |
| Tm-Tn | 1.0 ± 0.1 | 2.4 ± 0.9 ¥ 10⁻⁵ | 6 | 4 |
| Regulated actin | 1.0 ± 0.1 | 2.3 ± 0.5 ¥ 10⁻⁵ | 6 | 4 |
| Regulated actomyosin | 1.1 ± 0.2 | 2.9 ± 0.8 ¥ 10⁻⁵ | 5 | 4 |
| (1 mM MgATP) | 1.1 ± 0.2 | 6.8 ± 2.0 ¥ 10⁻⁵ | 4 | 4 |
| (-ATP) | 1.0 | 3.1 ¥ 10⁻⁶ | 1 | 25 |

*Estimated by tyrosyl fluorescence.

Calcium Dependence of Myofibrillar and Actomyosin ATPase—The ATPase activity of rabbit actomyosin, regulated by crayfish Tm-Tn complex, shows the same Ca²⁺ dependence as that of the crayfish myofibrillar ATPase (Fig. 8f). Hence, the replacement of crayfish actin and myosin by the corre-
The curves represent the least squares fitting of the data. In the absence of ATP, the best fit was obtained with $n = 1.0$ and $K = 5.8 \times 10^6 \text{ M}^{-1}$. In the presence of $1 \text{ mM MgATP}$, the best fit was $n = 1.1$ and $K = 3.5 \times 10^6 \text{ M}^{-1}$.

**Fig. 7.** Calcium binding at $4^\circ \text{C}$ to regulated actomyosin in the presence of $1 \text{ mM free Mg}^{2+}$ by double-isotope technique. $\bullet$, binding of Ca$^{2+}$ in the absence of ATP; $\bigcirc$, binding of Ca$^{2+}$ in the presence of $1 \text{ mM MgATP}$. The curves represent the least squares fitting of the data.

**Fig. 8.** Calcium dependence of ATPase activity at $25^\circ \text{C}$ for crayfish and rabbit Tm-Tn complex. The ATPase rates were determined in the presence of $1 \text{ mM MgATP}$ and $1 \text{ mM free Mg}^{2+}$. Activities (A) are expressed as per cent of maximum activity. Basal ATPase at $\text{pCa} 7.5$ is subtracted from each experimental point before normalization. $I$, activation curve for crayfish myofibrils ($\Delta$, $0.45 \text{ mg/ml}$, $A_{\text{max}} = 0.18 \mu\text{mol of P$_i$/mg/min}$) and for the crayfish Tm-Tn-regulated actomyosin ($\Delta$, $0.36 \text{ mg/ml}$, $A_{\text{max}} = 0.13 \mu\text{mol of P$_i$/mg of myosin/min}$) compared to the Ca-binding curves at $25^\circ \text{C}$ for the crayfish Tm-Tn-regulated actomyosin. For binding of Ca$^{2+}$ in the presence of $1 \text{ mM MgATP}$ ($\bigcirc$), the best fit was obtained with $n = 1.0$ and $K = 1.3 \times 10^4 \text{ M}^{-1}$; for that in the absence of ATP ($\bullet$), the best fit was obtained with $n = 1.0$ and $K = 3.1 \times 10^6 \text{ M}^{-1}$. $II$, activation data for rabbit myofibrils ($\Delta$, $0.35 \text{ mg/ml}$, $A_{\text{max}} = 0.22 \mu\text{mol of P$_i$/mg/min}$) and for the rabbit Tm-Tn-regulated actomyosin ($\bigcirc$, $0.40 \text{ mg/ml}$, $A_{\text{max}} = 0.15 \mu\text{mol of P$_i$/mg of myosin/min}$).

**DISCUSSION**

Our Ca$^{2+}$- and Mg$^{2+}$-binding studies on crayfish TnC show that this protein has one Ca$^{2+}$-binding site with an affinity in the range of physiological free [Ca$^{2+}$] and a high selectivity for this metal as compared to Mg$^{2+}$ (regulatory Ca$^{2+}$-specific site). TnC is thought to have evolved from a four-domain ancestor which was common to many intracellular calcium-binding proteins; each domain contained a calcium-binding domain. As many as three of the four putative domains of skeletal TnC, there are four Ca$^{2+}$-binding sites, and two of these are thought to regulate hydrolysis of MgATP (15). Because there is only one Ca$^{2+}$-binding site of physiological significance on crayfish TnC, such a cooperative-like behavior in calcium activation cannot be explained as a consequence of multiple sites on the TnC molecule. On rabbit skeletal TnC, there are four Ca$^{2+}$-binding sites, and two of these are thought to regulate hydrolysis of MgATP (15).

Therefore, it was of interest to compare, in the same experimental conditions, rabbit and crayfish myofibrils with respect to their pCa/ATPase relationship. The rabbit myofibrillar ATPase activity as a function of [Ca$^{2+}$] is shown in Fig. 8II. Both the position and the slope of the activation curve are not significantly different from those for crayfish myofibrils. Thus, in rabbit myofibrils also, the sharp response of ATPase to Ca$^{2+}$ is controlled by factor(s) other than the requirement of more than one bound Ca$^{2+}$ on TnC for activation.

The pCa/ATPase curve for crayfish myofibrils and for the crayfish Tm-Tn-regulated actomyosin was compared with the Ca-binding curves determined at the same temperature ($25^\circ \text{C}$) for crayfish Tm-Tn-containing actomyosin (Fig. 8I). The relation between free [Ca$^{2+}$] and ATPase activity fits with neither “weak” (regulated actomyosin during steady-state ATP hydrolysis) nor “strong” (regulated actomyosin, no ATP) binding of Ca$^{2+}$ to TnC. The threshold [Ca$^{2+}$] for activation corresponds to the range of [Ca$^{2+}$] where the Ca-specific site in its low affinity state starts to bind Ca$^{2+}$ significantly. On the other hand, comparison of the activation curve and the “strong” binding of Ca$^{2+}$ to TnC on regulated actomyosin (no ATP) shows that full activation is reached at [Ca$^{2+}$] where the regulatory site in its high affinity state approaches saturation. This suggests that during calcium activation of the actomyosin ATPase, the Ca-specific site on TnC displays at least two affinity states (one low and one high), the high affinity state being induced by interaction of myosin with regulated actin in the ATPase cycle.
predicted before amino acid sequence data for crayfish TnC become available.

The Ca\(^{2+}\)-binding properties of crayfish TnC again raise the question of the role played by domains III and IV on TnC. A structural role for the Ca\(^{2+}\)-Mg\(^{2+}\) sites on skeletal TnC has been postulated, since these sites would always contain either Ca\(^{2+}\) or Mg\(^{2+}\) in vivo, and their occupancy by either metal is required for attachment of TnC to TnI in intact myofibrils (16). Crayfish TnC possesses about five sites with the same low affinity ($K = 1 - 2 \times 10^4 \text{ M}^{-1}$) for Ca\(^{2+}\) and Mg\(^{2+}\). If some of these Ca\(^{2+}\)-Mg\(^{2+}\) sites were the mutated sites in domains III and IV, then one could postulate that their occupancy by Mg\(^{2+}\) in vivo is required for maintaining the integrity of the Tn complex; however, this turns out not to be the case. Unpublished results of ours indicate that removal of metal ions from whole crayfish myofibrils by extractions with metal chelators does not cause a dissociation of TnC from the myofibrils. This suggests that in crayfish TnC, there is a region that binds TnI irrespective of Ca\(^{2+}\) or Mg\(^{2+}\). The four-domain ancestor of calcium-binding proteins probably possessed only sites specific for Ca\(^{2+}\) as in calmodulin (43). Therefore, it is likely that evolution of domains III and IV in TnC proceeded in two ways, toward either the loss of ion-binding capacity (invertebrates) or the acquisition of high affinity Ca\(^{2+}\)-Mg\(^{2+}\) sites (vertebrates). In both cases, these two domains maintain the structure of the Tn complex intact independently of the cytosolic levels of Ca\(^{2+}\), thus allowing the Ca\(^{2+}\)-specific site(s) to function in the regulation of Ca\(^{2+}\) binding to TnC proceeds in two ways, toward either the loss of ion-binding capacity (invertebrates) or the acquisition of high affinity Ca\(^{2+}\)-Mg\(^{2+}\) sites (vertebrates). In both cases, these two domains maintain the structure of the Tn complex intact independently of the cytosolic levels of Ca\(^{2+}\), thus allowing the Ca\(^{2+}\)-specific site(s) to function in the regulation of Ca\(^{2+}\) activation.

We have taken advantage of the simplicity of Ca\(^{2+}\) binding in crayfish TnC to study those features that are more difficult to discern in the vertebrate Tn-linked regulatory system because of the presence of multiple and diverse sites which bind Ca\(^{2+}\) in the range of its physiological concentrations. Previous studies on the TnI-TnC and whole Tn complexes from skeletal (15) and cardiac (17) muscle indicated that the interaction of TnI and TnC results in a 10- to 20-fold increase in the affinities of both the Ca-Mg sites and the Ca-specific sites. This is also the case for TnC from crayfish with respect to its single Ca\(^{2+}\)-specific site. Moreover, our Ca-binding studies reveal that while the regulatory site displays essentially the same affinity ($K = 4 \times 10^5 \text{ M}^{-1}$) in the crayfish Tm-Tn complex as in the Tn complex, in regulated actin the Ca-specific site exhibits a markedly lower affinity ($K = 2 \times 10^5 \text{ M}^{-1}$). As reported in our earlier studies (1), these data show that the actin filament affects the Ca-binding properties of TnC. Similar results have been recently reported by Zot et al. (45) for the rabbit system, where an effect of similar magnitude was found exclusively for the Ca-specific sites. Interestingly, the reduced affinity approaches the level of that of Ca\(^{2+}\) in its isolated state; this suggests that the effect of actin, mediated through TnI and (or) transmitted via Tm to TnT and to TnI, depresses those interactions between TnI and TnC which increase the affinity of the Ca-specific site on TnC in the absence of actin. This is in agreement with studies showing that the binding of Tm-Tn to F-actin appears to be weaker in the presence of Ca\(^{2+}\) than in its absence (46). Indeed, it follows from thermodynamic reasoning that, if the binding of Ca\(^{2+}\) to TnC decreases the binding constant of Tm-Tn to F-actin, the binding of the Tm-Tn complex to actin filament decreases the binding constant of Ca\(^{2+}\) to TnC.

Bremel and Weber (14) first reported a slight increase in affinity of skeletal TnC for Ca\(^{2+}\) when regulated actin and myosin form complexes in the absence of ATP hydrolysis, i.e., under equilibrium conditions. Our studies on actomyosin, regulated by crayfish Tm-Tn, indicate that in the absence of ATP the interaction of myosin and regulated actin results in a 20- to 30-fold increase in the affinity of the Ca\(^{2+}\)-specific sites on TnC. The affinity of regulated actomyosin for Ca\(^{2+}\) ($K = 7 \times 10^6 \text{ M}^{-1}$) is similar to that of the Tm-Tn complex. Hence, in the rigor state, where the Tm-Tn complex is “pushed” into the groove of the actin helix by myosin heads, the effect of actin on the Ca-specific site is suppressed. The rigor state can be assumed to correspond only to the final step of an actomyosin ATPase cycle. The affinity of actin to myosin-nucleotide intermediates of the cycle is lower than in the rigor state and varies over four orders of magnitude depending on the state of the nucleotide bound to myosin. Therefore, the effect of myosin intermediates on binding of Ca\(^{2+}\) to regulated actin is difficult to measure directly.

The steep pCa/ATPase curve of rabbit skeletal myofibrils has been attributed to the requirement that all four sites (23) or both Ca-specific sites (15) on TnC must be filled by Ca\(^{2+}\) for activation to occur. However, experimental data have been recently obtained that do not support such an explanation. First, as monitored by tension development of rabbit psoas fibers (28) and by ATPase of rabbit skeletal myofibrils (16, 24), the responses to Ca\(^{2+}\) are much too steep to be explained solely by a requirement for 2 or even 4 calcium ions bound on TnC. Second, the pCa/ATPase curve of cardiac myofibrils is sloping as sharply as that of skeletal myofibrils despite the suggestion that among three Ca-binding sites on cardiac TnC, only the site which is specific for Ca\(^{2+}\) is regulatory (17). Similarly, our studies show that crayfish myofibrils and rabbit actomyosin, regulated by crayfish Tm-Tn complex, display a steep rise of ATPase activity with [Ca\(^{2+}\)]. Moreover, we observe a close similarity in the Ca\(^{2+}\) dependence of ATPase activity with rabbit and crayfish myofibrils as well as with actomyosin regulated by rabbit and crayfish Tm-Tn complex. Thus, in Tn-linked regulation of actomyosin ATPase, the requirement of more than one site occupied by Ca\(^{2+}\) on TnC does not play any role in the steep responses to Ca\(^{2+}\).

The Ca-binding measurements carried out on the crayfish Tm-Tn-containing actomyosin during steady state ATP hydrolysis reflect essentially the noncooperative Ca-binding properties of regulated actin which is dissociated from myosin. With the simplest kinetic models, the increase in ATPase rate would be expected to be proportional to the fractional occupancy of the Ca-specific site in its low affinity state ($K = 1 - 2 \times 10^5 \text{ M}^{-1}$) over the entire range of [Ca\(^{2+}\)]. However, this is not the case. When comparing the overall "weak" binding of Ca\(^{2+}\) with the ATPase activity as a function of [Ca\(^{2+}\)], it appears that the only common range of [Ca\(^{2+}\)] for both curves is the one where the Ca-specific site begins to bind Ca\(^{2+}\) significantly and activation starts (Fig. 8).

In order to explain the sensitive activation of isometric muscle contraction by Ca\(^{2+}\), Hill (47) has developed a model with two major ingredients in the regulation of contraction: (a) Ca\(^{2+}\) binds much more strongly on TnC if myosin is already attached to actin; (b) there is positive cooperativity in the system because of nearest-neighbor Tm-Tm interactions which are responsible for the steep response to Ca\(^{2+}\). The increased calcium affinity of myofilaments as a result of cross-bridge interaction has recently been shown for muscle fibers in the isometric state (48). In considering the application of the model of Hill (47) to the regulation of actomyosin ATPase, we note that under the conditions used in our experiments (where the actomyosin or myofibrillar preparations are under no tension) the fraction of myosin bridges attached to actin at any instant is too small to alter the overall Ca\(^{2+}\) affinity of TnC in regulated actin (Fig. 7, Table 1). This, of course, does not mean that the cross-bridge-induced increase in the cal-

\[ \text{Ca}^{2+} \]
cium affinity of the Ca-specific site on TnC is not instrumental in the kinetic cycle of ATP hydrolysis. One wonders, however, whether under these conditions the interactions between adjacent Tm molecules along the thin filament can sharpen the response to Ca$^{2+}$.

It is also of interest to consider the situation when more than one step in the actomyosin ATPase cycle are affected by the Tm-Tn complex. Chalovich and Eisenberg (49) found that Ca$^{2+}$ has little effect on the binding of S-1 ATP and S-1 ADP-Pi to regulated actin, although this cation largely increases the regulated actin-activated ATPase activity of S-1. They postulated that a kinetic step, perhaps $P$, release, is the Ca-sensitive part of the cycle. On the other hand, Wagner and Stone (50) observed that single- and two-headed meromyosin bind to regulated actin (in the presence of ATP) with higher affinity in the presence of Ca$^{2+}$ than in its absence. They also showed that the Ca-sensitive binding of myosin to regulated actin requires that both the head-tail junction and the light chain 2 be intact. It is, therefore, likely that at least two steps in the cycle are controlled by Tm-Tn, the attachment of myosin to actin and a kinetic step which occurs after binding. Such a regulation would explain the discrepancy between ATPase activation and Ca$^{2+}$ binding to the Ca-specific site in TnC shown in Fig. 8. While the "weak" binding of Ca$^{2+}$ may relieve the inhibition of binding of myosin to regulated actin and the attachment may then augment the binding constant of Ca$^{2+}$ to the regulatory site, a rapid equilibrium between Ca$^{2+}$ and the site in its high affinity state may become (with increasing $[Ca^{2+}]$) more and more important in suppressing the inhibition by Tm-Tn of the rate-limiting step in the ATPase cycle. This may explain why the ATPase activity rises steeply with $[Ca^{2+}]$ and reaches its maximum value in the range of $[Ca^{2+}]$ which is close to that where the Ca-specific site in its high affinity state ($K = 3 \times 10^6 M^{-1}$) approaches saturation. Further work will be required to determine if this hypothesis is correct.

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