Calcium Binds Cooperatively to the Regulatory Sites of the Cardiac Thin Filament*

Larry S. Tobacman and Dawyn Sawyer

From the Departments of Internal Medicine and Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52242

To investigate the relationship between thin filament Ca$^{2+}$ binding and activation of the MgATPase rate of myosin subfragment 1, native cardiac thin filaments were isolated and characterized. Direct measurements of $^{45}$Ca binding to the thin filament were consistent with non-cooperative binding to two high affinity sites ($K_a \approx 7.3 \pm 0.8 \times 10^5$ M$^{-1}$) and either cooperative or non-cooperative binding to one low affinity site ($K_a \approx 4 \pm 2 \times 10^5$ M$^{-1}$) per troponin at 25 °C, 30 mM ionic strength, pH 7.06. Addition of a low concentration of myosin subfragment 1 to the native thin filaments produced a Ca$^{2+}$-regulated MgATPase activity with $K_{cat}$ ($2.5 \pm 1.3 \times 10^6$ M$^{-1}$), matching the low affinity Ca$^{2+}$ site. The MgATPase rate was cooperatively activated by Ca$^{2+}$ (Hill coefficient $1.8$). To determine whether Ca$^{2+}$ binding to the low affinity sites was cooperative, native thin filament troponin was exchanged with troponin labeled on troponin C with 2-(4'-iodoacetamidinophenyl)naphtalene-6-sulfonic acid. From the Ca$^{2+}$-sensitive fluorescence of this complex, Ca$^{2+}$ binding was cooperative with a Hill coefficient of $1.7-2.0$. Using the troponin-exchanged thin filaments, myosin subfragment 1 MgATPase rate activation was also cooperative and closely proportional to Ca$^{2+}$ thin filament binding. Reconstitution of the thin filament from its components raised the Ca$^{2+}$ affinity by a factor of 2 (compared with native thin filaments) and incorporation of fluorescently modified troponin raised the Ca$^{2+}$ affinity by another factor of 2. Stoichiometrically reconstituted thin filaments produced non-cooperative MgATPase rate activation, contrasting with cooperative activation with native thin filaments, troponin-exchanged thin filaments and thin filaments reconstituted with a stoichiometric excess of troponin. The Ca$^{2+}$-induced fluorescence transition of stoichiometrically reconstituted thin filaments was non-cooperative.

These results suggest that Ca$^{2+}$ binds cooperatively to the regulatory sites of the cardiac thin filament, even in the absence of myosin, and even though cardiac troponin C has only one Ca$^{2+}$-specific binding site. A theoretical model for these observations is described and related to the experimental data. Well-known interactions between neighboring troponin-tropomyosin complexes are the proposed source of cooperativity and also influence the overall $K_a$. The data indicate that Ca$^{2+}$ is four times more likely to elongate a sequence of troponin-tropomyosin units already binding Ca$^{2+}$ than to bind to a site interior to a sequence of units without Ca$^{2+}$.

Striated muscle contraction is regulated by Ca$^{2+}$ binding to the thin filament. These results suggest that neighboring troponin-tropomyosin complexes interact along the thin filament to produce cooperative Ca$^{2+}$ binding and cooperative activation of the actomyosin MgATPase rate. A molecular model for this phenomenon is described and related to the experimental data. A distinguishing feature of the model is the treatment of cooperativity as an intrinsic property of the thin filament, rather than solely as a result of thin filament-thick filament interactions.

EXPERIMENTAL PROCEDURES

Protein Purification—Bovine ventricular actin, myosin S-1, tropomyosin, and tropinin were all isolated as previously reported (13-15), with modifications as follows. Tropinin subunits were obtained by binding crude tropinin to DEAE-cellulose, dissociating the complex with urea, and applying a 0-300 mM NaCl gradient (14). The tropinin C was further purified by Sephadex G-100 gel filtration in 1 The abbreviations used are: IAANS, 2-(4'-iodoacetamidinophenyl)naphtalene-6-sulfonic acid; S-1, myosin subfragment 1; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; dibromo-BAPTA, 1,2-bis[2-bis(o-amino-5-bromophenoxy)ethane]-N,N,N',N'-tetraacetic acid.

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the presence of 0.1 M KCl, 10 mM Tris-HCl, pH 8.0, 0.5 mM dithio-
threitol, and 0.1% NaN3. This step removed contaminating troponin T, troponin I and other impurities. DEA-DEAE-cellulose fractions containing one or the other troponin T isoform were further purified by direct application of these fractions to a hydroxyapatite column (Debu-
ning Diagnostics, fast flow) equilibrated in 6 M urea, 0.1 M KCl, 10 mM Tris/HCl, pH 8.0, 0.5 mM dithiothreitol, 0.01% NaN3. The major
impurity did not bind to the column and the troponin T was eluted by 0-200 mM KPO4 gradient. Only the larger, predominant isoform (troponin T) was used.

Troponin C was modified with IAAAS (Molecular Probes) under conditions described previously (12). The troponin C modification level ranged between 1.8 and 2.2 mol/mol. Troponin T was radiactiv-
ely labeled on Cys-39 by a 24-h incubation at 4°C with a 5-fold excess of [3H]-N-ethylmaleimide (Du Pont-New England Nuclear). Incorporation was 0.5 mol/mol. Protein concentrations were deter-
mined using molecular weights and extinction coefficients as previ-
ously compiled (15). Fluorescently modified troponin C was measured by protein assay (16) using unmodified troponin C as a standard.

Bovine cardiac thin filaments were prepared by modification of a method presented in preliminary form by Ngaie et al. (17). Myofibrils were washed extensively in 10 mM KPO4, pH 7.0, 0.1 M NaCl, 5 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol. They were then extracted with 20 mM dithiothreitol and low ionic strength solutions containing the percentages of 1.5 volumes of 10 mM KH2PO4, pH 7.0, 0.1 M KCl, 5 mM ATP, 5 mM MgCl2. The pooled extracts were clarified by a 15-min centrifugation at 150,000 x g. The thin filaments were then collected by a similar centrifugation. The pellets were homogenized in 20 mM KPO4, pH 6.0, 0.1 M NaCl, 5 mM MgCl2, 1 mM ATP. After lowering the pH to 6.75, contaminating actomyosin was eliminated by a 15-
min troponin T was used. The supernatant was dialyzed overnight against 20 mM imidazole, pH 6.5, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 5 mg/ml 1-chloro-3-tosylamido-7-
amino-2-heptanone, 5 mg/liter l-chloro-3-tosylamido-7-
amino-2-heptanone, 5 mg/liter, L-1-tosylamido-2-phenylethyl chlo-
romethyl ketone. The dialyzed sample was centrifuged for 15 min at 150,000 x g, the pellets were discarded, and the purified thin filaments collected by a 2-h 150,000 x g centrifugation. The thin filaments were stored for up to 1 month as a pellet at 0 °C.

Troponin Reconstitution—Troponin subunits were mixed under denaturing conditions in 1:1:1 stoichiometry and then sequentially dialyzed to facilitate gradual subunit association (14). To eliminate uncomplexed subunits, in particular free fluorescently modified tro-
ponin C, the reconstituted troponin was concentrated with Centri-
con 30 microcentrators (Amicon) and then applied to a Sephadex G-
100 column. Fractions containing the reconstituted complex were
pooled and concentrated.

Assays—45Ca binding to thin filament was measured by a dual
label ultracentrifugation technique (18); [3H]glucose was used to correct for Ca2+ trapped in the pellet. Airfuge centrifugation was performed on native thin filaments (14 µM actin), which had been incubated for 1 h at 25 °C in the presence of 20 mM imidazole, pH 7.0, 2.5 mM MgCl2, 1 mM KCl, 5 mM ATP, varying concentrations of 45CaC1 (1.4 x 106 cpm/wmol), and 106 cpm/
ml [3H]glucose. Approximately 40% of the pellet 45Ca was truly bound to the thin filament, rather than trapped. In control experimen-
tests, 45Ca binding to actin-troponymosin was negligible (<0.2 mol/mol tro-
ponymosin). Fluorescence measurements were performed on stirred,
water-jacketed samples using an SLM-8000C spectrofluorometer.
Emission spectra were smoothed and corrected for the response of
the instrument. Calcium titrations were performed by sequential
addition of aliquots of CaCl2 to 2-ml samples. Fluorescence intensities
were corrected for progressive dilution (approximately 6% maximum).

To ensure thorough mixing of the viscous thin filament-containing samples, each aliquot of CaCl2 was mixed into the sample using a paddle-shaped micro stirrer (Precision Cell). MgATPase rates were measured as previously described. The rates were linear over the first
8 min. Free calcium concentrations were calculated using overall
CaEGTA and MgEGTA dissociation constants of 140 mM and 2.8
mM, respectively (19), under the conditions of the MgATPase and fluorescence assays: 20 mM imidazole, pH 7.06, 3.5 mM MgCl2, 1 mM
MgCl2, 0.5 mM EGTA, variable CaCl2, 25 °C, 30 mM ionic strength. Identical results were obtained using dibromo-BAPTA. The Ca2+-dibromo-BAPTA dissociation constant under these conditions is 12.7 ± 0.4 µM. After correcting for low ionic strength (20) Curve fitting was performed by nonlinear regression using the program Eznfitter (Bio-
soft).

RESULTS

FIG 1. 45Ca binding to native thin filaments. Native thin
filaments (14 µM actin) were pelleted in an Airfuge in the presence of varying free Ca2+ concentrations. The pellets were resuspended and measured for radioactivity and protein concentration. Results are expressed as moles of Ca bound/moles of troponin. (--), best fit of the experimental data to the (non-cooperative) equation: Bound Ca = 2 x 106 cpm/ml x 10 µM; 50% saturation = 2.2 µM; Kd = 0.2 ± 0.1 x 106 M; 1.0 X 106 M. The other, cooperative curve (----), is based upon the same constants and a similar equation, but with Kd/(1 + Kd) replaced by the expression in Equation 12. The value used for Y is 3.6.
Cooperative Ca\textsuperscript{2+} Thin Filament Binding

Myosin Subfragment 1 MgATPase Regulation by Native Cardiac Thin Filaments—The regulatory properties of the native thin filaments were evaluated. As shown in the right-hand curve of Fig. 2, native thin filaments activated the MgATPase rate of myosin subfragment 1 in a Ca\textsuperscript{2+}-sensitive manner. The peak MgATPase rate at saturating Ca\textsuperscript{2+} averaged 1.1 s\textsuperscript{-1} in the presence of 0.3 \mu M myosin S-1, 14 \muM thin filament (expressed as actin concentration), pH 7.06, 30 mM ionic strength, 25 °C. The Ca\textsuperscript{2+} sensitivity of the MgATPase rate was 93 ± 2%. As shown by the example in Fig. 2, the activation of the MgATPase rate by Ca\textsuperscript{2+} was cooperative, with a Hill coefficient (25) of 1.8. All of these results are in agreement with data previously reported for reconstituted cardiac thin filaments formed from a 7:2:2 actin/tropomyosin/troponin ratio (14, 15, 19).

The positions of the MgATPase rate activation curves from numerous preparations such as the one shown in Fig. 2 imply a \( K_{\text{app}} \) of 2.5 ± 1.3 \times 10\textsuperscript{-5} M\textsuperscript{-1} (\( n = 8 \)). This value is 2-3-fold weaker than results obtained from MgATPase activation of the reconstituted system (\( K_{\text{app}} \) 5-8 \times 10\textsuperscript{-9} M\textsuperscript{-1}) (14, 15, 19), suggesting that the process of dissociation, purification, and reconstitution of these proteins alters the affinity to a moderate degree. The explanation for the discrepancy is not phosphorylation on troponin I by cAMP-dependent protein kinase (26); incubation of native thin filaments with smooth muscle phosphatase I under fully dephosphorylating conditions had no effect on the thin filaments (data not shown).

More important than the discrepancy in \( K_{\text{app}} \) between native and reconstituted thin filaments is the relationship between native thin filament Ca\textsuperscript{2+} binding (4 ± 2 \times 10\textsuperscript{-5} M\textsuperscript{-1} to the lower affinity sites) and MgATPase rate activation (2.5 ± 1.3 \times 10\textsuperscript{-5} M\textsuperscript{-1}). Within experimental error, these results agree. The only discrepancy is between the cooperative MgATPase activation and the apparently non-cooperative Ca\textsuperscript{2+} binding. This discrepancy may be apparent rather than real. From the data in Fig. 1 one can conclude that overall Ca\textsuperscript{2+} binding is not cooperative and is consistent with two classes of sites. However, by these methods one cannot choose between non-cooperative and cooperative binding specifically to the weaker class of sites. The solid curve in Fig. 1 shows the expected pattern of Ca\textsuperscript{2+} binding if the low affinity sites bind with cooperativity equivalent to the cooperativity in the ATPase data. Unfortunately, the direct Ca\textsuperscript{2+}-binding data are not sufficiently sensitive to distinguish between these curves and determine if binding to the low affinity sites is cooperative.

**Effect of Calcium on Actin-Tropomyosin-Troponin IAANS Fluorescence**—A potentially sensitive method to measure Ca\textsuperscript{2+} thin filament binding is to modify bovine cardiac troponin C with IAANS. Using this modified molecule, there is an increase in fluorescence intensity on Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+}-specific site of troponin C but a decrease in fluorescence intensity when Ca\textsuperscript{2+} binds to the troponin C-troponin-I-troponin T complex (12). The fluorescent probe is localized to both cysteine residues in troponin C. The effect of calcium on the fluorescence intensity of IAANS-troponin-tropomyosin-actin has been described only in muscle fibers (not with purified proteins), where an increase in fluorescence intensity occurs (27). Similarly, the reconstituted thin filament (Fig. 3) is now shown to have a 30% increase in fluorescence intensity when saturating Ca\textsuperscript{2+} is added. It can be seen from Fig. 3 that the effect of thin filament Ca\textsuperscript{2+} binding is to change the fluorescence intensity rather than the position of the emission maximum, 450 nm.

In the presence of saturating rabbit skeletal muscle myosin subfragment 1 and no nucleotide, the emission spectrum showed minimal dependence on Ca\textsuperscript{2+} concentration and had a maximum intensity 5–10% less than the upper curve in Fig. 3 (data not shown). Consequently, IAANS troponin could not be used to study thin filament Ca\textsuperscript{2+} binding in the presence of rigor acto-myosin bonds. The fluorescence properties of free troponin were indistinguishable from the properties of troponin-tropomyosin (without actin).

**Incorporation of Labeled Troponin into Reconstituted and Native Thin Filaments**—To sensitively study Ca\textsuperscript{2+} binding to the regulatory sites, troponin labeled on troponin C by IAANS was exchanged into native thin filaments. Fig. 4A shows the identification of conditions needed for this exchange. In the

![Fig. 2. Regulation of the MgATPase rate of myosin S-1 by native thin filaments and by troponin IAANS-exchanged thin filaments. The Ca\textsuperscript{2+}-sensitive regulation of the myosin S-1 MgATPase rate by native cardiac thin filaments (○) was studied at 25 °C in the presence of 14 \muM thin filament-actin, 0.3 \muM myosin S-1, 20 mM imidazole, pH 7.06, 3.5 mM MgCl\textsubscript{2}, 1 mM ATP, 6.5 mM KCl, and varying Ca\textsuperscript{2+} concentrations. For comparison purposes, the MgATPase curve from Fig. 5A is also shown (+). 80% of the thin filament troponin has been replaced with troponin IAANS. Both curves are cooperative, with Hill coefficients of 1.8.](http://www.jbc.org/)

![Fig. 3. Effect of Ca\textsuperscript{2+} on the emission spectrum of actin-tropomyosin-troponin IAANS. Fluorescently modified bovine cardiac troponin C was reconstituted into troponin, which was purified over Sephadex G-100 and mixed with tropomyosin and actin. Conditions of fluorescence measurement are 5 \muM actin, 2 \muM tropomyosin, 0.57 \muM troponin IAANS, 20 mM imidazole, pH 7.06, 11 mM KCl, 3 mM MgCl\textsubscript{2}, 25 °C, 10\textsuperscript{-4} M Ca\textsuperscript{2+} (thin line) or 3 \times 10\textsuperscript{-5} M Ca\textsuperscript{2+} (thick line), \( \lambda_e = 332 \) nm.](http://www.jbc.org/)
consideration both added (labeled) troponin and thin filament troponin. The resuspended thin filaments after exchange had an indistinguishable specific activity (7.1 × 10^7 cpm/µmol troponin). This corresponds to a troponin/actin ratio of 0.13:1.

Fig. 4B suggests that reconstituted thin filaments (lane A), native thin filaments (lane B), and troponin-exchanged thin filaments (lane F) have very similar protein composition as expected. The only differences are in troponin C mobility. However, control experiments suggest this due to variable gel sample conditions, resulting in variable troponin C-metal binding during electrophoresis.

**Fluorescence Titration of Thin Filaments after Troponin-IAANS Exchange**—The Ca^2+*-induced fluorescence transition of troponin-exchanged thin filaments is shown in Fig. 5A. The K_s (1 × 10^6 M^−1) approximates that observed from the fluorescence of and 4Ca binding to thin filaments reconstituted using troponin IAANS (Table I), rather than that obtained from native thin filament MgATPase data before exchange (Fig. 1 and Fig. 2, right-hand curve). The dashed curve in Fig. 5A shows the shape expected for a non-cooperative binding isotherm. The data are much better described by the solid, cooperative line, which is the best fit for the model described below. The data could also be well described by the Hill equation (25), with n_H = 1.9. In five such experiments the K_H averaged 1.2 ± 0.2 × 10^6 M^−1 and the cooperativity parameter (see below) ranged between 3 and 4.2. These results suggest that Ca^2+* binding to the cardiac thin filament is cooperative and that the failure to demonstrate cooperativity by measurement of 4^Ca binding is due to the limitations of the latter technique. Significantly, Ca^2+* binding to troponin-IAANS in the absence of tropomyosin and actin is not cooperative (12, 18). Furthermore, there is only one regulatory Ca^2+*-binding site on each cardiac troponin C. Therefore, the cooperative fluorescence transition shown in Fig. 5A is most plausibly due to interactions between troponin molecules, mediated by the other components of the thin filament (actin and tropomyosin).

**Comparison of Ca^2+* Binding and MgATPase Rate Activation**—To compare Ca^2+* binding and MgATPase rate regulation the same troponin-exchanged thin filament preparation was used for both studies. A representative experiment is shown in Fig. 5B. The fluorescence transition and the MgATPase rate activation were both cooperative and were indistinguishable from each other. (The same MgATPase rate data is also shown in Fig. 2.) There is no discrepancy between Ca^2+* binding and functional activation of the thin filament. The dashed curve in Fig. 5B shows the shape of a non-cooperative binding isotherm. The solid curve, which better fits the data, is derived from the best fit of the combined data to the cooperative model described below.

In interpreting the MgATPase data it is important to note that the affinity of myosin S-1 for the thin filament is the same in the presence of EGTA and in the presence of saturating Ca^2+ (13). The interpretation of Fig. 5B also depends upon the fact that the myosin S-1/actin ratio was too low (0.3 µM S-1:1.4 µM actin) and myosin S-1 binding too weak (4% of the myosin S-1 is not actin bound (13)) to alter thin filament Ca^2+ affinity. This was confirmed by repeating the fluorescence titration in the presence of 0.3 µM myosin S-1 (data not shown). The fluorescence transition had a midpoint (K = 1.4 ± 0.2 × 10^5 M^−1) and slope (Y = 4) similar to fluorescence data obtained in the absence of myosin S-1 (K = 1.2 ± 0.2 × 10^5 M^−1, Y = 3–4). Only 25% of the ATP was hydrolyzed during the fluorescence titration.

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\textbf{THEORY}

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(28) and by Hill (11). Actin-troponin-tropomyosin undergoes a conformational change when Ca\textsuperscript{2+} binds, switching from an inhibited state to a more activated state (Fig. 6A). The activated state directly or indirectly (29) facilitates ATP turnover when myosin is present but exists independently of myosin. The ability of Ca\textsuperscript{2+} to cause a thin filament conformational change is supported by time resolved x-ray diffraction of the muscle (30). Specifically, Kress et al. reported that muscle activation produces a change in the actin second layer line which precedes cross-bridge attachment. Importantly, this thin filament alteration was neither diminished nor delayed when thick/thin filament overlap was abolished by stretching the muscle.

There is abundant experimental evidence that troponin-tropomyosin complexes interact end-to-end to form long polymers either in the absence of actin or along the actin filament (31-35). These interactions can be modeled to depend upon the conformations of each neighbor (Fig. 6B). This postulate is the proposed explanation for the observed cooperativity. As previously formulated by Hill et al. (36, 37), the interaction free energy, \(w\), between two neighbors in the same state is lower (i.e. more favorable) than the interaction free energy, \(w'\), between two neighbors in different states. Consequently, there is a tendency for several troponin-tropomyosin complexes to activate (bind Ca\textsuperscript{2+}) in a group and minimize the number of unfavorable interactions. In this initial model the interaction free energy between two activated units (\(w_{\text{on}}\)) is assumed to be the same as the interaction free energy between two inactivated units (\(w_{\text{off}}\)) and \(w_{\text{on}} = w_{\text{off}} = w\). This assumption, for which there is tentative experimental evidence (28), can be eliminated and the model still solved (see below). It is also assumed, as seems reasonable by symmetry, that \(w = w_{\text{on}} = w_{\text{off}}\).

Use of this model instead of the more commonly utilized A. V. Hill equation provides insights into the molecular interactions responsible for the observed cooperativity. The shape of the binding curve depends upon two parameters: \(K\), (or \(K\) as below), a Ca\textsuperscript{2+} affinity constant/troponin-troponin-tropomyosin unit, and \(Y\), a cooperativity parameter related to the interunit interaction free energies by:

\[
y = e^{-2w-u'/kT} (1)
\]

where \(k\) is the Boltzmann constant. \(Y\) also has a quasi-chemical significance. It is the equilibrium constant relating two different thin filament configurations (Fig. 6C). The greater the value of \(Y\), the greater the tendency of Ca\textsuperscript{2+} to cluster on adjacent troponin-tropomyosin complexes. Specifically, Ca\textsuperscript{2+} is \(Y\)-fold more likely to elongate an activated sequence than to bind to a unit interior to an inactivated sequence of units. Similarly, Ca\textsuperscript{2+} is \(Y\)-fold more likely to select an inactivated unit immediately between two activated units than to bind where it elongates the end of an activated sequence. The greater the value of \(Y\), the steeper is the slope of the Ca\textsuperscript{2+} binding curve.

An alternative description of \(Y\) shows its relationship to the interaction energies \(w\) and \(w'\). Consider a section of an infinitely long thin filament with two gaps (no troponin-tropomyosin) as shown in the bottom line of Fig. 6C. On other, isolated actin filaments (not shown in the figure) are one activated (Ca\textsuperscript{2+} bound) troponin-tropomyosin and one inactivated (no Ca\textsuperscript{2+} bound) troponin-tropomyosin complexes, at equilibrium constant either \(J_1\) or \(J_2\), depending upon which unit goes to which site. The interaction energies determine \(J_1\) and \(J_2:\)

\[
J_1 = e^{-w-w'\lambda + u'/kT} (2)
\]

\[
J_2 = e^{-w-w'\lambda - u'/kT} (3)
\]

By detailed balance,

\[
y = J_2/J_1 = e^{-2w'u'/kT} (4)
\]
Cooperative Ca\textsuperscript{2+} Thin Filament Binding

**Table I**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Native Thin Filaments</th>
<th>Troponin IAANS-exchanged Thin Filaments</th>
<th>Reconstituted Actin-Tropomyosin-Troponin IAANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin S-1 MgATPase rate K\textsubscript{Mg}</td>
<td>2.5 \times 10\textsuperscript{4} M\textsuperscript{-1}</td>
<td>1.0 \times 10\textsuperscript{6} M\textsuperscript{-1}</td>
<td>1.5 \times 10\textsuperscript{6} M\textsuperscript{-1}</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} thin filament K\textsubscript{Ca} Low affinity sites</td>
<td>4.0 \times 10\textsuperscript{4} M\textsuperscript{-1}</td>
<td>ND\textsuperscript{a}</td>
<td>9.6 \times 10\textsuperscript{5} M\textsuperscript{-1}</td>
</tr>
<tr>
<td>Fluorescence K\textsubscript{Ca}</td>
<td>1.2 \times 10\textsuperscript{4} M\textsuperscript{-1}</td>
<td>ND\textsuperscript{a}</td>
<td>1.1 \times 10\textsuperscript{6} M\textsuperscript{-1}</td>
</tr>
</tbody>
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\textsuperscript{a} ND, not determined.

**Figure 6.** Theoretical model for cooperative Ca\textsuperscript{2+} binding to the thin filament. Panel A, an isolated troponin-tropomyosin unit changes its conformational state when Ca\textsuperscript{2+} binds, which it does with an affinity constant, K\textsubscript{Ca}. Panel B, each line segment represents a troponin-tropomyosin-7 actin unit. Nearest neighbor troponin-troponin complexes interact more strongly when they are in the same state (w) than when they are in different states (w'). Panel C, explanation of the cooperativity parameter, Y. The top two lines illustrate two alternatives for Ca\textsuperscript{2+} binding to one section of an infinitely long thin filament. Y is an equilibrium constant (Y \geq 1) expressing the likelihood that Ca\textsuperscript{2+} will bind to a troponin-tropomyosin complex contiguous to complexes already binding Ca\textsuperscript{2+}. The relationship of Y to w and w' is also illustrated by the dependence of Y on J\textsubscript{1} and J\textsubscript{2} (lines 2 and 3). J\textsubscript{1} and J\textsubscript{2} are equilibrium constants, each for moving two remote isolated troponin-tropomyosin complexes into the gaps, as shown. See text for details. Figure modified from Ref. 28.

The degree of thin filament activation (or Ca\textsuperscript{2+} binding) and the statistical distribution of activated units depend upon K\textsubscript{Ca}, Y, and the Ca\textsuperscript{2+} concentration. This problem can be solved using the statistical mechanical approach developed by T. L. Hill (36-39) and recently reviewed (40).

The average total binding of Ca\textsuperscript{2+}, N, is given by the fundamental relationship for equilibrium binding,

\[ N = \text{Ca} \left( \frac{\partial \ln \Xi}{\partial \text{Ca}} \right) \]  

where \text{Ca} is the free Ca\textsuperscript{2+} concentration and \Xi is the grand partition function for the system. The expression for \Xi is obtained by the matrix method, treating the thin filament as an infinitely long lattice. Since a unit (j) can exist in either of two states, and the succeeding unit (j + 1) can also be in either of these two states, there are four possible permutations for units j and j + 1. The contributions of these permutations are included as the four elements of a 2 \times 2 matrix. Each element is the product of one of the two terms (1 or K\textsubscript{Ca}) from the partition function for an isolated troponin-tropomyosin unit (\( \xi = 1 + K\text{Ca} \)) and a Boltzmann term (\( e^{-\omega kT} \) or \( e^{-\omega' kT} \)) for the interactions between neighboring units. The matrix is:

\[
\begin{pmatrix}
1 + K\text{Ca} & K\text{Ca} e^{-\omega kT} \\
K\text{Ca} e^{-\omega' kT} & 1 + K\text{Ca}
\end{pmatrix}
\]  

To obtain the grand partition function, the larger of the two eigenvalues, \( \gamma \), of this matrix is needed. Dividing each term by \( e^{-\omega kT} \), which does not change the results, and substituting according to Equation 1, \( \gamma \) is the solution to:

\[
\frac{1 - \gamma}{K\text{Ca} \gamma^{\omega/\gamma}} - \frac{K\text{Ca} - \gamma}{K\text{Ca}} = 0
\]

This reduces to a quadratic equation, with the larger of two solutions,

\[
\gamma = \frac{1 + K\text{Ca}}{2} + \left( \frac{1}{2} \left( 1 - K\text{Ca} \right)^{2} + 4K\text{Ca} \gamma^{\omega/\gamma} \right)^{1/2}
\]

The usefulness of \( \gamma \) comes from its relationship to the grand partition function:

\[
\Xi = \gamma^{M}
\]

where \( M \) is the total number of units (troponin-tropomyosin complexes). Fractional saturation of the thin filament with Ca\textsuperscript{2+}, \( \theta \), is found by substituting Equation 9 into Equation 5:

\[
\theta = \frac{N}{M} = \frac{\text{Ca} \partial \ln \gamma^{\omega}}{\partial \text{Ca}}
\]

Substituting the expression for \( \gamma \) (Equation 8) into Equation 11 and simplifying gives the final expression for Ca\textsuperscript{2+} binding:

\[
\theta = \frac{2K\text{Ca}/Y}{\left[ 1 - K\text{Ca} + \left( 1 - K\text{Ca} \right)^{2} + 4K\text{Ca} \gamma^{\omega/\gamma} \right]^{1/2}}
\]

When there is no cooperativity (\( Y = 1 \)) Equation 12 appropriately reduces to the simple binding isotherm

\[
\theta = \frac{K\text{Ca}}{1 + K\text{Ca}}
\]

The qualitative relationship between Equation 12 and experimental results is straightforward. Half-saturation (\( \theta = \frac{1}{2} \)) occurs at \( K\text{Ca} = 1 \) from Equation 12, regardless of the values of \( K\text{Ca} \) and \( Y \). In other words, the midpoint of the saturation curve equals the dissociation constant of Ca\textsuperscript{2+} from a (theoretically) isolated unit. On the other hand, the slope of a typical experimental binding curve at its midpoint depends only upon \( Y \):

\[
\left( \frac{\partial \theta}{\partial \ln \text{Ca}_{\omega/\gamma}} \right)_{\omega/\gamma} = \text{Ca} \ln \frac{\partial \theta}{\partial \text{Ca}}
\]

where \( \frac{\partial \theta}{\partial \text{Ca}} \) can be obtained from Equation 12. Since \( K\text{Ca} = \ldots \)
1 when \( \theta = \frac{1}{2} \), Equation 14 reduces to

\[
\left( -\frac{\partial \theta}{\partial \log [Ca]_{\text{tot}}} \right)_{\nu_{\text{total}}} = \frac{\ln 10}{4} Y^a
\]

\[
= 0.576 Y^a
\]  

(15)

There is also a simple relationship between \( Y \) and the more commonly used A. V. Hill coefficient, \( n_H \). This can be seen from the definition of \( n_H \),

\[
n_H = \frac{\partial \ln [(1 - \theta)/\theta]}{\partial \ln [Ca]}
\]

\[
= \frac{1}{\delta(1 - \theta)} \frac{\partial \theta}{\partial \ln [Ca]}
\]

\[
= \frac{4}{\ln 10} \left( \frac{\partial \theta}{\partial \log [Ca]_{\text{tot}}} \right)_{\nu_{\text{total}}}
\]  

(16)

(17)

Substituting from Equation 15,

\[
n_H = Y^a
\]  

(19)

The above model was derived assuming that the interaction free energy (\( \omega_{\text{iso}} \)) between two activated units is the same as the interaction free energy (\( \omega_{\text{iso}} \)) between two units without bound Ca\(^{2+}\). This assumption is consistent with data obtained from the equilibrium binding of rabbit skeletal muscle troponin-tropomyosin to actin in the presence and in the absence of Ca\(^{2+}\) (33). However, these earlier data would not have detected a severalfold difference between \( e^{-\omega_{\text{act}}} \) and \( e^{-\omega_{\text{iso}}} \). In other words, the interactions between units might be “stronger,” for example, when saturating Ca\(^{2+}\) is present than when no Ca\(^{2+}\) is present. This would effectively increase the Ca\(^{2+}\) association constant. For the present model, the possibility that \( \omega_{\text{iso}} \neq \omega_{\text{act}} \) would have subtle but important implications. It can be shown (40) that Equation 12 would still hold, except \( K \) replaces \( K_0 \):

\[
K = K_0 e^{\omega_{\text{act}}/\nu_2}
\]  

(20)

where \( K_0 \) is the theoretical Ca\(^{2+}\) association constant for an isolated unit, \( \omega_{\text{act}} = e^{-\omega_{\text{act}}} \equiv e^{-\omega_{\text{iso}}} \). \( K \) represents the average Ca\(^{2+}\) association constant, per troponin-tropomyosin unit, for Ca\(^{2+}\) simultaneously binding to all units on the filament.

Experimentally \( K \) is still the reciprocal of the midpoint of the binding curve. However, in this more general model \( K \) partially depends upon the interactions between adjacent units. An important consequence is that structural changes in the “overlap” regions of tropomyosin or troponin T (such as phosphorylation, recombinantly engineered mutants (41), or isoform switching) could alter overall thin filament Ca\(^{2+}\) affinity. In this general model \( Y \) has a similar but slightly more complex value: \( Y = \gamma_{\text{iso}}/\gamma_0^2 \).

**DISCUSSION**

Table I summarizes binding constants and apparent binding constants determined from \(^{46}\)Ca, MgATPase, and fluorescence experiments. Within any single type of thin filament (either native, troponin IAANS-exchanged, or reconstituted), the different assays give similar results. However, whereas the regulatory Ca\(^{2+}\)-binding site of native thin filaments has an affinity of \( \sim 3 \times 10^7 \text{ M}^{-1} \), the affinity of the other two preparations is \( \sim 1.2 \times 10^6 \text{ M}^{-1} \). This discrepancy is partially attributable to reconstitution, which when performed with unmodified troponin produces a thin filament with a \( K_{\text{app}} \) of \( 5-8 \times 10^5 \text{ M}^{-1} \) (14, 15, 19). The remainder of the difference is in our hands a distinct property of thin filaments containing the fluorescently modified troponin. This may represent a local effect of the fluorescent probe on troponin C. Certainly it would be preferable to develop model systems in which these differences in \( K_0 \) did not occur. For the present it is important to note the internal consistency of the \( K_{\text{app}} \) for each preparation and the consistent level of cooperativity across preparations. The native thin filament \( K_0 \) agrees with published values obtained (in the presence of ATP) for Ca\(^{2+}\) binding (42) and tension/pCa plots (43) using canine cardiac fibers.

In considering the validity of our conclusions, it is important to mention the circumstances under which we do not observe cooperativity. The direct measurements of \(^{46}\)Ca binding to the thin filament do not demonstrate cooperativity (Fig. 1); the overall Ca\(^{2+}\) binding curve is not cooperative. However, the figure also shows that the data are not sufficiently sensitive to determine whether there is cooperative binding only to one class of sites, the Ca\(^{2+}\)-specific regulatory sites. The lack of sufficient sensitivity has two causes: 1) the similarity between the expected binding isotherms in the absence (---) and in the presence (-----) of cooperativity; 2) the need to utilize Ca\(^{2+}\)-chelator buffers, so that only 2-3% of the total \(^{46}\)Ca binds to the thin filament.

The more significant evidence against cooperativity is that both fluorescence and MgATPase rate titrations such as that shown in Fig. 5, but using stoichiometrically reconstituted thin filaments, show non-cooperative transitions. An example is shown in Fig. 7. The Hill coefficient for such experiments averages 1.2 ± 0.2. We suspect this result is spurious and reflects the imperfections introduced when reconstituting a thin filament from its five polypeptide constituents. This conclusion is partially based upon the mediocre regulatory properties of such stoichiometric thin filaments (13), which contrast with the more profound (and cooperative) MgATPase rate regulation achieved with all three of the other thin filament preparations: native thin filaments, troponin IAANS-exchanged thin filaments, and thin filaments reconstituted with a stoichiometric excess of troponin (13, 19).

![Ca\(^{2+}\) binding and MgATPase rate activation using stoichiometrically reconstituted thin filaments](http://www.jbc.org/content/159/1/937/F7.large.jpg)
However, since we cannot more precisely reconcile the stoichiometrically reconstituted thin filament results with the cooperative results from the other three preparations, some uncertainty must remain.

The newly proposed model is the simplest (but not the only) explanation for our data: a ligand-induced conformational change, a corresponding functional activation, and cooperativity attributed to well-known contacts between neighboring parts of the thin filament. We have avoided other possible sources of cooperativity, which we consider more speculative, such as longer range interactions along the thin filament or interactions between opposite sides of the thin filament.

The relationship of this biochemical model to the cooperative response of muscle fibers to Ca\(^{2+}\) is only partially clear. The degree of cooperativity described here nearly matches the levels usually (42-44) but not always (45) seen for cardiac muscle tension/pCa plots. However, it is significantly less cooperative than data sometimes obtained using fast skeletal muscle (10, 46-48). Therefore, the now described myosin-independent cooperativity may be the major explanation for the cooperative activation of cardiac muscle but only one of several factors producing skeletal muscle cooperativity. It is probably misleading, however, to attempt quantitative comparisons of cooperativity between two very different experiments. Ca\(^{2+}\) binding to a reconstituted thin filament and force generation by a muscle fiber.

The most widely held explanation for muscle cooperativity is cooperative cross-bridge binding. It is clear that myosin binds cooperatively to the thin filament (depending upon conditions) and also that by binding it increases thin filament Ca\(^{2+}\) affinity. For example, Guth and Potter (10) recently showed that under some conditions cross-bridges can alter regulatory site troponin Ca\(^{2+}\) binding not only in vitro, but also in muscle fibers. However, in the presence of ATP they found that the Ca\(^{2+}\) affinity of the regulatory site of fast skeletal muscle troponin did not depend upon the extent of thick and thin filament overlap. Furthermore, Ca\(^{2+}\) binding was highly cooperative even when thick-thin filament overlap was minimal. An extreme alternative to their own explanation of these data (that only a small overlap is sufficient for cross-bridge-induced cooperativity), is that intact thin filaments bind Ca\(^{2+}\) with high cooperativity, independently of myosin. We favor an intermediate interpretation, in which both proposed mechanisms of cooperativity are important.

Interactions between neighboring troponin-tropomyosin units, first proposed by Tawada et al. (50), have been a feature of many theoretical papers (11, 28, 36-38, 51, 52) and experimental studies of striated muscle. Muscle fibers deficient in either troponin C or tropomyosin have large shifts in the apparent Ca\(^{2+}\) affinity of the remaining troponin (49, 53-55). The role of cross-bridges in these observations is hard to define. The role of cross-bridges is well defined, however, in both alternative theoretical models proposed by Hill et al. to explain cooperative binding of myosin S-1 to the thin filament (36, 37). In the absence of both Ca\(^{2+}\) and ATP myosin S-1 binds so cooperatively to the thin filament (9) that both interunit and intraunit mechanisms have been invoked. In a theoretical model of muscle regulation based upon these data, cooperative cross-bridge binding plays a central role (11).

The relationship of the present study to prior work on cooperative actin-myosin subfragment I interactions needs brief consideration, although a comprehensive model is beyond the scope of this manuscript. The thin filament model proposed by Hill et al. (36) successfully explains an impressive amount of data on the interactions of myosin S-1, Ca\(^{2+}\), and the thin filament (9, 21, 29, 56-61). According to this model, Ca\(^{2+}\) does not directly activate the interaction of myosin with the thin filament. Rather, Ca\(^{2+}\) makes the equilibrium toward another thin filament state less unfavorable. This other thin filament state is characterized by a relatively high actin-myosin affinity and a very high, or potentiated, MgATPase rate. This state can be induced by sufficient binding of cross-bridges to the thin filament or by employing high myosin S-1 concentrations.

The cooperative Ca\(^{2+}\) binding observed in the present report conflicts with this earlier model, in which Ca\(^{2+}\) alone effects little change in the thin filament. To reconcile this conflict, one could propose that what was formerly considered to be a single inactive thin filament state is really two inactive states, with and without Ca\(^{2+}\). In other words, if both of the thin filament states shown in Fig. 6 are considered inactive, then there is no conflict between our model and earlier studies of myosin S-1 binding to the thin filament. More substantial reanalysis is not required because previous experiments using the Hill et al. model were not performed at intermediate Ca\(^{2+}\) concentrations, where both of the states shown in Fig. 6 would be present.

On the other hand, we believe a new interpretation of thin filament behavior is justified. The most straightforward implication of our results (especially Fig. 5B) is that Ca\(^{2+}\) binding causes the thin filament to assume a more active conformation, facilitating rapid ATPase cycling by myosin. Significantly, it is thin filament-myosin S-1 MgATPase kinetics in the presence of Ca\(^{2+}\) and low myosin S-1 concentrations which resemble those of actin-myosin S-1 in the absence of troponin and tropomyosin. Since actin is the only protein required for myosin to produce force and movement (62-64), thin filament-myosin movement may not require an in vivo correlate of the potentiated ATPase state. Clearly, further studies are needed to definitively establish the properties of the thin filament both in solution and in muscle fibers.

The Ca\(^{2+}\)-binding model described in the present report resembles the second of the two theoretical models of actin-myosin binding proposed by Hill et al. (37). (In fact, cooperative Ca\(^{2+}\) binding is predicted by this earlier model.) The use of cardiac troponin, with only one regulatory Ca\(^{2+}\)-binding site, simplifies both the model and the experimental interpretation. For the reasons described above, we tentatively suggest that Ca\(^{2+}\) binding is sufficient for thin filament activation. We view this suggestion as the most logical conclusion from our data. However, more complicated models are also possible (29), in which neither of the states shown in Fig. 6 is active.

Previous studies of Ca\(^{2+}\) binding to the regulatory sites of the reconstituted thin filament have employed fast skeletal muscle troponin, rather than cardiac troponin. Grabarek et al. (65) reported an apparent Hill coefficient of 1.5-1.6 for the saturation of these sites in the absence of myosin. Only when the troponin was bound to the thin filament was this cooperativity observed. Since fast skeletal muscle troponin C has two Ca\(^{2+}\)-specific binding sites, intramolecular interactions between these sites were a possible explanation for this cooperativity. On the other hand, Zot and Potter (66) found no cooperativity in very similar experiments. Rosenfeld and Taylor (23) using a different fluorescent probe for Ca\(^{2+}\) binding to these same sites, also obtained steady state binding curves showing no cooperativity. However, they did report biphasic Ca\(^{2+}\)-binding kinetics when the troponin was thin filament-bound. Intramolecular interactions between sites I and II were presumed responsible, although the biphasic kinetics did not occur with free troponin or troponin C. The present work, describing cooperative Ca\(^{2+}\) binding to thin filaments containing (cardiac) troponin molecules with only one such site,
implying a mechanism of intermolecular cooperativity. Certainly, the present work does not disprove the existence of intramolecular cooperativity for skeletal muscle troponin. In fact, it is possible that the extra site on skeletal muscle troponin C substantially explains the greater cooperativity of fast skeletal muscle compared with cardiac muscle (48). This point is controversial, however (44). In any case, it seems likely that the intermolecular cooperativity now reported for the cardiac thin filament also occurs for the skeletal muscle thin filament.

Finally, it is important to note that the value of Y from Fig. 5B (Y = 3.5) is in quantitative agreement with an earlier report by Wegner and Walsh (28). These authors calculated a value of 1/4 for the reciprocal of the same cooperativity parameter used in their case to analyze a difficult and theoretical complex experiment, actin binding of mixtures of troponin-tropomyosin and troponin T-tropomyosin. The unstated implication of their experiments (performed with rabbit skeletal muscle proteins) was that Ca2+ should bind to the thin filament cooperatively, in precisely the degree shown in this present report.

In summary, the results suggest Ca2+ binds cooperatively to the cardiac thin filament in the absence of myosin. This Ca2+ binding closely parallels activation of the MgATPase rate of low concentrations of myosin subfragment 1. The cooperativity appears to result from interactions between adjacent troponin-tropomyosin complexes which make concerted binding and release of Ca2+ energetically favorable. These interactions can be described by a plausible theoretical model. It may be possible to apply this model to future studies of more intact systems, such as muscle fibers stretched beyond thick-thin filament overlap. To approach a complete understanding of the regulation of contraction, we believe it will be necessary to include the cross-bridge-independent cooperativity described in this report.

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