Cooperative Effect of Calcium Binding to Adjacent Troponin Molecules on the Thin Filament-Myosin Subfragment 1 MgATPase Rate

Carol A. Butters, Jeremy B. Tobacman, and Larry S. Tobacman‡

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

The myosin subfragment 1 (S1) MgATPase rate was measured using thin filaments with known extents of Ca2+ binding controlled by varying the ratio of native cardiac troponin versus an inhibitory troponin with a mutation in the sole regulatory Ca2+ binding site of troponin C. Fractional MgATPase activation was less than the fraction of troponins that bound Ca2+, implying a cooperative effect of bound Ca2+ on cross-bridge cycling. Addition of phalloidin did not alter cooperative effects between bound Ca2+ molecules in the presence or absence of myosin S1. When the myosin S1 concentration was raised sufficiently to introduce cooperative myosin-myosin effects, lower Ca2+ concentrations were used to examine MgATPase activity. MgATPase activity was more cooperative than could be explained by cooperativeness of overall Ca2+ binding, the discrepancy between Ca2+ binding and MgATPase activity, or interactions between myosins. The results suggest the thin filament-myosin S1 MgATPase cycle requires calcium binding to adjacent troponin molecules that this binding is cooperatively promoted by a single cycling cross-bridge. This mechanism is a potential explanation for Ca2+-mediated regulation of cross-bridge kinetics in muscle fibers.

Just as isometric tension is cooperatively activated by Ca2+, so is the cardiac thin filament-myosin S1 MgATPase rate, even under conditions where there is no cooperativity in myosin S1 binding (1, 2). A possible explanation for this behavior is that ATPase activation is proportional to Ca2+ binding to the many TnCs on each thin filament and that this calcium binding is cooperative (3). We tested the idea that Ca2+ binding and MgATPase activation are proportional and found to the contrary that they are not. Instead, fractional MgATPase activation was considerably less than fractional Ca2+ binding and more closely paralleled the number of pairs of adjacent troponins with Ca2+ bound to both.

To accomplish the above experiment, we employ a constitutively inhibitory form of cardiac troponin containing an inactivating mutation of the sole regulatory site of TnC, site II (4). This troponin, designated CBMII-Tn, results in a low thin filament-myosin S1 MgATPase rate that is not increased by the addition of Ca2+, analogous to previous results in which a similar TnC mutant was exchanged into myofibrils or muscle fibers (5–7). CBMII-Tn binds to actin-tropomyosin with an affinity identical to that of normal troponin in the absence of Ca2+. This binding, which is very tight for both normal troponin and for CBMII-Tn (4, 8, 9), permits the present report in which thin filaments are assembled with defined mixtures of normal troponin and CBMII-Tn. In the presence of saturating Ca2+ concentrations, such thin filaments exhibit a fractional saturation of the TnC regulatory sites that is experimentally controllable by varying the relative concentrations of the two forms of troponin. This permits assessment of Ca2+-regulated myosin S1 MgATPase activity in a novel manner as a function of bound Ca2+ rather than as a function of the free Ca2+.

In addition to varying the ratio of the two troponins, the myosin S1 and free Ca2+ concentrations are also systematically varied in the present study. The results imply a previously unrecognized aspect of the cooperativity of muscle activation, that rapid cycling of an isolated cross-bridge depends on Ca2+ binding to adjacent troponin molecules, and also suggest that cross-bridge cycling increases Ca2+ affinity at least locally, regardless of the density of myosin on the thin filament. The relationship between the data and various models of thin filament structure and regulation are discussed.

**EXPERIMENTAL PROCEDURES**

*Protein Preparation—Cardiac troponin and tropomyosin were purified from bovine heart using an ether powder technique (10). Rabbit fast skeletal muscle F-actin was obtained by the method of Spudich and Watt (11). Because bovine cardiac myosin S1 tends to precipitate at the concentrations used in many of the experiments, most of the data were obtained using rabbit fast skeletal muscle chemotryptic myosin S1 purified by ion exchange chromatography (12). Some of the experiments (see Fig. 1) were repeated using bovine cardiac myosin S1 purified as described previously (10). CBMII-Tn was prepared by reconstitution (13) of the ternary troponin complex from bovine cardiac TnI and TnT (8, 9) and reconstituent murine TnC mutant CBMII (4).

*Assembly of Thin Filaments with Defined Fractional Saturation of TnC Regulatory Site II—F-actin, tropomyosin, and various mixtures of troponin and CBMII-Tn were combined in the indicated ratios under the ionic conditions used in the ATPase experiments. Since troponin binds to the thin filament with an affinity of 3–5 × 109 M−1 (8, 9) and the μM amounts of the troponins included in the present experiments were stoichiometric or slightly sub-stoichiometric relative to the actin concentration, it was anticipated that essentially all of both added forms of troponin would be bound to the thin filament. This was tested by a sedimentation experiment. 15.5 μM F-actin, 2 μM tropomyosin, 1 μM cardiac troponin (nonradioactive), and 1 μM reconstituent CBMII-Tn labeled with iodine131 were mixed with 100 μM CaCl2, 5 mM Tris-HCl (pH 7.5), 3.5 mM MgCl2, 8 mM KCl, 1 mM dithiotreitol. The sample was sedimented in a TLS100 centrifuge at 25 °C for 20 min at 35,000 rpm. Quantitative SDS-polyacrylamide gel electrophoresis analysis by gel scanning and standard curve comparison (14) and liquid scintillation counting of samples indicated sedimentation of 94% of both troponins combined (assessed by...
Cooperative Effect of Calcium Binding to Adjacent Troponins

SDS-polyacrylamide gel electrophoresis and 92% of the labeled troponin (assessed by radioactivity). The fraction of actin pelleting was similar, 93%. All three values agreed within experimental error.

MgATPase Assays—The ATPase rate was measured by the release of radioactive phosphate from [γ-32P]ADP (15) (NEN™ Life Science Products). For each experiment, the samples were incubated with 50% troponin-CBMII-Tn and 50% normal troponin (tropomyosin free) for 90 s. The ATPase rate was measured by the release of [γ-32P]ATP (15) (NEN™) at pH 7.5. The equilibrium constant for the interaction of thin filament regulatory sites that have bound Ca 2+ was shown to be 107 cpm/μmol. When 50% of the troponin pairs were adjacent to Ca 2+ and variable concentrations of CaCl 2 (3), the cooperative effect of bound Ca 2+ on the MgATPase rate was measured using thin filaments in which different fractions of the troponin molecules had Ca 2+ bound at TnC regulatory site II. Conditions and protein concentrations were varied as described in each figure. The free Ca 2+ concentration was varied using 0.5 mM, 1.2 mM, or 2.5 mM CaCl 2. The results for MgATPase rate activation are only 30–35% that of the maximal stimulation observed for full Ca 2+ saturation. The solid line, which does not fit the data, is the result expected if MgATPase activation were proportional to Ca 2+ binding regardless of whether Ca 2+ binding is adjacent to a troponin already with bound Ca 2+.

RESULTS

Relationship between Ca 2+ Binding to the Thin Filament Regulatory Sites and Normalized MgATPase Rate in the Presence of a Low Myosin S1:Actin Ratio—Fig. 1 shows the effect of altering the fractional Ca 2+ saturation of the thin filament by varying the relative concentrations of troponin and CBMII-Tn. The normalized results of six experiments are shown, and it is clear that the relationship between Ca 2+ binding and MgATPase rate activation is not a linear one (straight line). Rather, activation lagged behind Ca 2+ binding. When 50% of the troponin bound Ca 2+ and 50% do not, the fractional MgATPase rate activation is only 30–35% of that of the maximal stimulation observed for full Ca 2+ saturation. The solid line, which does not fit the data, is the result expected if MgATPase activation were proportional to Ca 2+ binding regardless of whether Ca 2+ binding is adjacent to a troponin already with bound Ca 2+.

The dashed lines in Fig. 1 are theoretical curves for the fraction of troponin-troponin boundaries with Ca 2+ bound on both sides, which depends in part upon the degree of cooperativity in the binding of the two forms of troponin to the thin filament. The equilibrium constant Y governs the tendency of troponin and CBMII-Tn to separately cluster on the thin filament rather than bind randomly (3, 4). Y also dictates the cooperativity of Ca 2+ binding to a thin filament containing only normal troponin, with Y > 1 indicating positive cooperativity. The experimentally determined value of Y is approximately 1.5 (4), and the long dashes in Fig. 1 correspond to this value. A slightly better fit is found with Y = 4, as indicated by the theoretical curve represented with shorter dashes. This might suggest that the true value for Y is greater than the previously measured value of about 1.5. A more likely explanation is that the degree of MgATPase rate activation does not precisely correspond to the fraction of troponin-troponin pairs that have Ca 2+ on both sides. In either case, the deviation from linearity in Fig. 1 indicates that Ca 2+ binding to more than one troponin is required for full actin activation of ATP hydrolysis at any given thin filament site.

An important aspect of the cooperative process illustrated in Fig. 1 is that it is not due to interactions between myosin S1 molecules. The myosin S1 concentration was only 1% of the actin concentration, making myosin-myosin cooperativity unlikely. To confirm this experimentally, the MgATPase rate was shown to be linear with the myosin S1 concentration over a 16-fold range (0.25–4% of the actin concentration). Linearity with myosin S1 concentration was shown both at pCa 5 and at pCa 5.89 (10–15% activation) for thin filaments with...
The best fit to Equation 1 in Ref. 4, with labeled CBMII-Tn to actin $z$
factor in Fig. 1 in any case because the MgATPase rates for the
straighten a concave curve such as shown. This is not a major
din.

Main figure
troponin and no CBMII-Tn and at
CaCl$_2$, 10 mM Tris-HCl (pH 7.5), 3 mM MgCl$_2$, 300 mM KCl, 1 mM ATP,
10 $\mu$M skeletal muscle F-actin, 2 $\mu$M cardiac troponysin, 39 $\mu$M phal-
loidin. The relative amounts of troponin and CBMII-Tn were varied,
with their total concentrations maintained at 2.5 $\mu$M. The solid line
is the best fit to Equation 1 in Ref. 4, with $K_0 = 2.4 \pm 0.2$ and $Y = 1.7 \pm 0.4$. These results are indistinguishable from those found in the absence
of phallolidin (4), indicating that phallolidin has little effect on cooper-
tive interactions between troponin molecules on the thin filament. Inset,
disproportionate MgATPase rate activation and regulatory site
Ca$^{2+}$ binding. Conditions: 25 $^\circ$C, 0.1 mM CaCl$_2$, 5 mM imidazole (pH
7.1), 5 mM MgCl$_2$, 8 mM KCl, 1 mM ATP, 15.6 $\mu$M skeletal muscle
F-actin, 2.5 $\mu$M cardiac troponysin, 25 $\mu$M phallolidin, 0.3 $\mu$M cardiac
myosin S1, and troponin concentrations as in Fig. 1. The results are similar to those found in the absence of phallolidin. The curve line
corresponds to Equation 1 with $Y = 3$.

The main portion of Fig. 2 provides a measurement of Ca$^{2+}$-
dependent cooperative interactions between troponin mole-
cules on the thin filament in the absence of myosin, again in the
presence of phallolidin. This experiment differs from the
ATPase data in that the sum of the troponin and CBMII-Tn
concentrations is in constant excess relative to the sites on the
thin filament. The two forms of troponin compete for binding
sites on actin-tropomyosin, and the pattern of this competition
implies that these binding sites (for troponin) interact in a
manner sensitive to Ca$^{2+}$. This experiment measures the value
of the cooperativity parameter and equilibrium constant $Y$,
which is found to be 1.7 $\pm$ 0.4 in the presence of phallolidin. This
result implies weak Ca$^{2+}$-sensitive interactions of a strength
indistinguishable from that found previously in the absence of
phallolidin (4). Curve-fitting of the data also results in a value
for $K_R$, the fold-increase in the affinity of troponin for actin-tropomyosin that results from Ca$^{2+}$ dissociation from site
II. $K_R$ is 2.4 $\pm$ 0.2 in the presence of phallolidin, which is
indistinguishable from $K_R$ in the absence of phallolidin, 2.2 $\pm$
0.1 (4).

Effects of Ca$^{2+}$ Concentration and Myosin S1 Concentration on the Thin Filament-Myosin S1 MgATPase Rate—The experi-
mint in Fig. 1 employed a mixture of normal troponin and
CBMII-Tn. An extrapolation of these results suggests that for a
thin filament with normal troponin only, the MgATPase rate
will not increase in proportion to Ca$^{2+}$ binding as the free Ca$^{2+}$
concentration is increased. Fig. 3A shows the normalized
MgATPase rate as a function of the free Ca$^{2+}$ concentration in
the presence of either low myosin S1 concentrations as were
also present in Fig. 1 (Fig. 3, □ or in the presence of much
higher myosin S1 concentrations (Fig. 3, ×). The rightmost two
curves show the difference between Ca$^{2+}$ binding (short dashes)
and adjacent Ca$^{2+}$ pair binding (solid curve, fit to MgATPase
data (◯)) according to Fig. 1 under conditions where myosin-myosin cooperativity is precluded by low myosin S1
concentrations. The $K_{app}$ from the ATPase curve underesti-
mates the true binding constant, but this discrepancy is small,
3.7 versus $2.4 \times 10^6$ M$^{-1}$ for $K_{binding}$ versus $K_{app}$. The relation-
ship between these curves is determined by Fig. 1; if the short
dash curve in Fig. 3A is set as the independent variable and the
solid curve as the dependent variable, then a graph describing
the data in Fig. 1 is the result. However, the lines actually were
obtained by a best fit of Equation 1 to the experimental data (◯).
Assuming that the MgATPase rate is proportional to the frac-
tion of adjacent troponin pairs with Ca$^{2+}$ on both sides, then
the best fit regulatory site Ca$^{2+}$ affinity is $3.7 \pm 0.6 \times 10^6$ M$^{-1}$
and the cooperativity parameter $Y = 3.4 \pm 0.9$. The mean value
for $Y$ from 10 such experiments was $5 \pm 1$, corresponding to a
Hill coefficient of 2.2 (3) and similar to the level of cooperativity
reported previously (1, 3, 13, 26, 27).

The analysis in Fig. 3A indicates that there is little difference in
the cooperative shapes for Ca$^{2+}$ binding and for Ca$^{2+}$ pair
binding (the solid and short dash curves are equally steep).
Similarly, if $Y$ is set at a noncooperative value of 1, both curves
are less steep but they remain parallel, and the relationship
between them is still consistent with Fig. 1 (not shown). This
indicates that Fig. 1 is consistent with the data in Fig. 3A, but
only if overall Ca$^{2+}$ binding to the thin filament regulatory
sites is cooperative. Since this process is known to have little
cooperativity for reconstituted thin filaments (3, 4, 28, 29),
some other explanation will be needed to rationalize the larger
cooperativity observed for MgATPase activation versus the free
Ca$^{2+}$ concentration (Fig. 3A and Ref. 1).

Another source of cooperativity in MgATPase assays is ef-
teffects of myosin S1 on the thin filament. Careful studies of
Weber and co-workers (30) using skeletal muscle regulatory

troponin and no CBMII-Tn and at $p$Ca 5 for thin filaments with
50% troponin and 50% CBMII-Tn (data not shown).

The curvature in Fig. 1 is not attributable to hyperbolic
dependence of the MgATPase rate on the regulated actin
concentration in the presence of saturating Ca$^{2+}$ concentrations
(10, 17). Any such tendency would work in the reverse direc-
tion, producing a convex relationship or else tending to
straighten a concave curve such as shown. This is not a major
factor in Fig. 1 in any case because the MgATPase rates for the
Ca$^{2+}$-saturated thin filaments are about one-fourth to one-
third the $V_{max}$ observed with saturating thin filament concentra-
tions (data not shown). The actin concentration for the data
sets in the figure are below the actin $K_{app}$ which diminishes
the importance of this consideration.

Phallloidin Does Not Alter Cooperative Interactions between
Troponin Molecules—The polymerization ability of the
troponin-tropomyosin complex (18–20) and atomic models of
actin-actin contacts in F-actin (4, 21) suggest that longitudinal
contacts along the thin filament are the most likely source
of cooperativity. However, this does not exclude the possibility
that cooperativity occurs across rather than along the actin filament.
To test this, we added phallolidin, which binds near
the thin filament axis with an orientation that is invariant with
thin filament conformation (22) and both decreases thin fila-
ment flexibility and alters strand-strand interactions (23–25).
Any cooperativity that was dependent upon such interactions
might be changed by the addition of phallolidin. The Fig. 2 inset
shows that the cooperative effect of bound Ca$^{2+}$ on MgATPase
rate activation was similar to results found in the absence
of phallolidin. The results are indistinguishable from Fig. 1.
proteins have shown increased MgATPase rates, increased Ca$^{2+}$ affinity, and apparent Ca$^{2+}$ affinity (32, 33). These effects are observed when the myosin concentration is high relative to actin or when conditions favor strong actin–myosin bond formation (34). Fig. 3B shows the potentiating effect of high myosin S1 concentrations on the thin filament-myosin S1 MgATPase rate using cardiac regulatory proteins. For an actin concentration of 5 mM, the MgATPase rate deviated from linearity when the myosin S1 concentration was $3.3 \text{mM}$. This deviation correlated with a shift in the Ca$^{2+} K_{\text{app}}$ in MgATPase versus pCa experiments. There was no shift for 3 mM myosin S1 (not shown), a small shift for 5 mM myosin S1 (not shown), and a 2.5-fold shift in $K_{\text{app}}$ in the presence of 10 mM myosin S1 (Fig. 3A, ×). The apparent Ca$^{2+}$ affinity from these and other titrations was $2.4 \pm 0.2 \times 10^5 \text{M}^{-1}$ in the presence of 0.3 mM myosin S1 and $6.0 \pm 0.9 \times 10^5 \text{M}^{-1}$ in the presence of 10 mM myosin S1.

Comparison among the three curves in Fig. 3A shows that MgATPase rate activation of 10 mM myosin S1 ($3.3 \text{mM}$) occurs at even lower Ca$^{2+}$ concentrations than the calculated Ca$^{2+}$ saturation of the regulatory sites (short dashes) when the myosin

FIG. 3. Cooperative relationships among free Ca$^{2+}$, bound Ca$^{2+}$, thin filament-myosin S1 MgATPase rate, and myosin S1 concentration. A, conditions: 25 °C, 20 mM imidazole (pH 7.3), 3.5 mM MgCl$_2$, 7 mM KCl, 1 mM ATP, 5 mM skeletal muscle F-actin, 1.5 mM cardiac troponymosin, 1 mM troponin, and either 0.3 mM (☐) or 10 mM (×) rabbit skeletal muscle myosin S1. The apparent Ca$^{2+}$ affinity is increased 2.5-fold in the presence of the higher myosin S1 concentration, from $K_{\text{app}} = 2.4 \pm 0.2 \times 10^5 \text{M}^{-1}$ to $K_{\text{app}} = 6 \pm 1 \times 10^5 \text{M}^{-1}$ (Equation 12 in Ref. 3). The transition was cooperative in both cases, with $Y = 4 \pm 1$ in the presence of 0.3 mM myosin S1 and $Y = 6 \pm 4$ in the presence of 10 mM myosin S1. The ordinate alternatively indicates (i) MgATPase rate activation (☐, ×, each separately normalized); (ii) $f_{22}$, fraction of adjacent troponin-troponin pairs with bound Ca$^{2+}$ (solid line, obtained by fitting MgATPase rate data with low myosin S1 concentrations (☐ to $f_{22}(\text{Ca}^{2+})$ (Equation 34.63 in Ref. 16); (iii) $\theta$, fractional saturation of the thin filament with Ca$^{2+}$ when the myosin S1 concentration is low (short dashes, calculated from Equation 34.83 in Ref. 16 using the parameters ($K$, $Y$) derived from the previously mentioned fit). B, MgATPase rate is cooperatively increased by myosin S1 concentrations approaching the actin concentration, the MgATPase rate was higher than the rate found with low myosin S1:actin ratios (dashed line). The pCa was 4.8, a saturating Ca$^{2+}$ concentration. C, relationship between fractional Ca$^{2+}$ binding and normalized MgATPase rate activation in the presence of a relatively high myosin S1 concentration. The data are similar to what is found with low myosin S1 concentrations (Fig. 1), implying that the shift observed in panel A involves a myosin-induced change in Ca$^{2+}$ binding. Conditions were as above, with 0.1 mM CaCl$_2$ and protein concentrations of 10 mM myosin S1, 5 mM actin, 1 mM troponymosin, and 0.7 mM troponin and CBMII–Tn.
CBMII-Tn, a gradual increase in the Ca\(^{2+}\) when only 25% of the troponins on the thin filament are treated with adjacent troponin molecules to decrease Ca\(^{2+}\) binding. This would be the opposite of the relationship in Fig. 1, a convex rather than a concave curve. This possibility is evaluated and excluded by Fig. 3C, which shows fractional MgATPase activation as a function of bound Ca\(^{2+}\). Even in the presence of high myosin S1 concentrations that "potentiate" the thin filament, Ca\(^{2+}\) binding precedes fractional activation. Therefore, the shift seen in Fig. 3A (\(\times\) versus \(\square\)) involves a myosin-induced increase in Ca\(^{2+}\) affinity. However, there may also be some change in the precise relationship between fractional Ca\(^{2+}\)-binding and fractional MgATPase rate activation; Fig. 3C appears to show less deviation from linearity than does Fig. 1. In this regard, it should be noted that strongly bound cross-bridges can activate the thin filament under appropriate conditions, even in the absence of any Ca\(^{2+}\)-binding (31).

### MgATPase Activation as a Function of the Free Ca\(^{2+}\) Concentration for Thin Filaments Containing Mostly CBMII-Tn

When only 25% of the troponins on the thin filament are capable of binding Ca\(^{2+}\), i.e. 75% of the troponin is of the form CBMII-Tn, a gradual increase in the Ca\(^{2+}\)-concentration produces a small level of activation that is shown in Fig. 4. The figure is a normalized composite of four experiments, and in all of them the noise precluded any assessment of Y. The data is noisy because a 25:75 ratio of tropinin:CBMII-Tn produces only a low MgATPase rate (Fig. 1); the average Ca\(^{2+}\)-saturated rate is twice the EGTA rate for these data sets. The solid curve is a noncooperative binding isotherm. Comparison of the data points to this theoretical curve suggests that cooperativity may actually be present (the data deviates from the curve), but this may be an artifact of the normalization of each data set.

The \(K_{app}\) could be measured with enough precision, \(4.3 \pm 1.2 \times 10^{-5}\) M\(^{-1}\), to permit comparison to the value found for thin filaments with fully normal troponin, \(2.4 \pm 0.2 \times 10^{-5}\) M\(^{-1}\) (n = 10, with representative data shown in Fig. 3A, \(\square\)). This was unexpected, since the CBMII-Tn might have cooperatively interacted with adjacent troponin molecules to decrease Ca\(^{2+}\) affinity. It is unclear why a modest increase in apparent affinity occurred instead, but the effect is small in any case.

---

**DISCUSSION**

The thin filament has at least three conformations: an inhibited state in the presence of EGTA, a Ca\(^{2+}\)-induced state, and an active state observed in the presence of strongly binding myosin cross-bridges (35–37). These structures have been compared with three-dimensional reconstructions of myosin S1-decorated thin filaments (38, 39), leading to the conclusion that troponyosin interferes with the binding site for myosin S1 in the inhibited state and (to a lesser extent) in the Ca\(^{2+}\) state but not in the active state. Solution studies of cross-bridge thin filament binding support this conclusion (40) even though the initial stage of myosin S1-ATP binding to the thin filament is Ca\(^{2+}\)-insensitive (10, 41). The structural data strongly suggest that completion of the MgATPase cycle requires a local conformational change in the thin filament from the Ca\(^{2+}\)-induced state to a myosin-induced state. Otherwise, troponyosin would prevent tight actin-troponin binding that is part of the cycle. To explain the deviation from linearity in Fig. 1, we now suggest that this single cross-bridge-induced conformational change requires Ca\(^{2+}\) binding to more than one troponin positioned on adjacent tropomyosin molecules along the thin filament. This interpretation parallels implications drawn from the very cooperative equilibrium binding of myosin S1 to the thin filament (42–44). Such binding is so cooperative that theoretical models (45, 46) explaining it invoked a myosin-promoted conformational change for the tropomyosin-troponin-7 actin unit that strongly depended upon the same myosin-induced conformational change in adjacent units. The kinetics of tight thin filament-myosin S1 binding suggest a similar conclusion (47). Longitudinal cooperativity of this type is also implied by studies of muscle fibers subjected to partial extraction of TnC (48–51). In fact, the nonlinear relationship in Fig. 1 is very similar to tension versus TnC data in skeletal muscle fibers (50). We suggest that this type of cooperative interaction between adjacent regulatory units also occurs during the MgATPase cycle, even for single, isolated myosin heads along the thin filament.

Strongly binding cross-bridges increase the affinity of Ca\(^{2+}\) for the thin filament (28, 31, 52–55). This process has been invoked to explain the leftward shift in MgATPase versus pCa curves that occurs with high myosin S1 concentrations or low ATP concentrations (32, 33) using skeletal muscle proteins. The most direct explanation for the shift would be a true change in Ca\(^{2+}\) affinity at the regulatory site(s) of TnC. The present data show that this shift also occurs when cardiac troponin-troponyosin is used and, more importantly, confirms the previous interpretation. By studying the relationship between MgATPase rate and bound Ca\(^{2+}\) using CBMII-Tn, Fig. 3 demonstrates that this shift is caused primarily by a myosin-induced increase in affinity and not primarily by a change in the relationship between Ca\(^{2+}\) binding and activation.

Perhaps the greatest significance of the above conclusion is that it suggests a mechanism for the problematic cooperativity of MgATPase activation in the presence of low myosin S1 concentrations. This cooperativity is difficult to explain because Ca\(^{2+}\) binding per se is much less cooperative (4, 56) and because the newly described cooperative effects of bound Ca\(^{2+}\) (Fig. 1) fail to provide an explanation. This is demonstrated in Fig. 3A, which shows the relationship between cooperative activation by Ca\(^{2+}\) of the MgATPase rate under low myosin S1 conditions (\(\square\), solid line) and implied fractional Ca\(^{2+}\) binding under the same conditions (short dashes). The theoretical curves in Fig. 3A are based upon the nearest neighbor analysis described under "Experimental Procedures," but the general shape of the curves is dictated by the data, not the equations.

The steepness of the solid curve is chosen to match the empirical, model-independent MgATPase observations (\(\square\)), and the
A long-standing issue in muscle regulation is whether and to what extent the Ca$^{2+}$ concentration alters cross-bridge kinetics (58) as opposed to controlling the recruitment of a variable number of cross-bridges, all with the same kinetics (59). More recent analyses of cross-bridge function have established that several processes have a graded response to the Ca$^{2+}$ concentration (49, 60–67). The present data pertain to this problem. For example, force development occurs in several steps, including at least one transition before phosphate release (49, 63, 65, 68). Most studies indicate that the rate of force development is very sensitive to the Ca$^{2+}$ concentration (49, 60, 61, 65, 66). These observations can be explained if the MgATPase model in Fig. 5 is also applicable in muscle fibers. An early kinetic step producing strong myosin binding can be expected to alter the position of the tropomyosin strand and raise the Ca$^{2+}$ affinity of adjacent troponin(s) (36, 56). The Ca$^{2+}$ dependence of force generation kinetics can be explained if the concentration-dependent binding of additional Ca$^{2+}$ to adjacent troponin(s) alters the rate constants for completion of the power stroke and/or reversal of the early transition. This suggested mechanism for graded activation is an additional aspect of regulation, compatible with an additional control point dependent upon the density of bound cross-bridges (sites 2 and 3 in Fig. 5 have different properties) and with either steric or allosteric effects on recruitment (61, 69–71). Mechanical studies will be needed to explore this proposal. Investigation of the transient and steady state properties of muscle fibers in which native TnC has been partially replaced by CDBII may prove a useful approach.

Acknowledgments—We thank Earl Homsher for many informative discussions of Ca$^{2+}$-mediated regulation and for helpful critique of an earlier version of this manuscript. We also thank Jay Chyung for valuable assistance during development of the theoretical model.

REFERENCES
1. Tobacman, L. S. (1987) Biochemistry 26, 492–497
2. Walsh, T. P., Trueblood, C. E., Evans, R., and Weber, A. (1984) J. Mol. Biol. 182, 265–269
3. Tobacman, L. S., and Sawyer, D. (1990) J. Biol. Chem. 265, 931–939
