# A mosaic multiple-binding model for the binding of caldesmon and myosin subfragment-1 to actin

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ABSTRACT Binding of caldesmon to actin causes a decrease in the quantity of bound myosin and results in a reduction in the rate of actin-activated adenosine triphosphate hydrolysis. It is generally assumed that the binding of caldesmon and myosin to actin is a pure competitive interaction. However, recent binding studies of enzyme digested caldesmon subfragments directed at mapping the actin binding site of caldesmon have shown that a small 8-kD fragment around the COOH-terminal can compete directly with the myosin subfragment 1 (S-1) binding to actin; at least one other fragment that binds to actin does not inhibit the actin-activated adenosine triphosphate activity of myosin. That is, only a part of the caldesmon sequence may be responsible for directly blocking the binding of S-1 to actin. This prompts us to question the actual mode of binding of intact caldesmon and myosin S-1 to actin: whether the entire intact caldesmon molecule is competing with S-1 binding (pure competitive model) or just a small part of it (mosaic multiple-binding model). To answer this question, we measured the amount of myosin S-1 and caldesmon bound per actin monomer as a function of the total concentration of S-1 added to the system at constant concentrations of actin and caldesmon. A formalism for calculating the titration data based on the pure competitive model and a mosaic multiple-binding model was then developed. When compared with theoretical calculations, it is found that the binding of caldesmon and S-1 to actin cannot be pure competitive if no cooperativity exists between S-1 and caldesmon. In contrast, the mosaic multiple-binding model can fit the binding data rather well regardless of the existence of cooperativity between S-1 and caldesmon.

## INTRODUCTION

Caldesmon is a protein present in both smooth muscle (1) and nonmuscle (2, 3) cells, which inhibits superprecipitation (4) and the actin activation of adenosine triphosphate (ATP) hydrolysis by myosin (5–10). The $NH_2$-terminal region of caldesmon binds to myosin (11–14) and possibly to calmodulin (15). The interaction of caldesmon with myosin occurs primarily through the subfragment 2 region of myosin (11, 13, 14), although there is a small degree of interaction with subfragment 1 (S-1) (11, 13). The affinity of caldesmon for myosin subfragments decreases in the following order: smooth heavy meromyosin (HMM) > skeletal HMM > smooth S-1 ≫ skeletal S-1. The COOH-terminal region of caldesmon binds to actin (16–19), calmodulin (15, 16, 17, 19–21), and tropomyosin (8, 22–24). The binding of caldesmon to actin is associated with inhibition of adenosine triphosphatase (ATPase) activity. The center region of caldesmon, which is absent in nonmuscle caldesmons (25), has a highly helical structure (24, 26, 27) and has no known activity.

The inhibition of actin-activated ATP hydrolysis by caldesmon was shown to occur with a decrease in the degree of binding of S-1 to actin (10, 11, 28, 29), and it was proposed that caldesmon is a competitive inhibitor of the binding of myosin to actin (10). Caldesmon was a less-effective inhibitor of the binding of S-1 to actin in the presence of pyrophosphate (PPi) (10), 5′-adenylyl imidodiphosphate (AMPPNP) (11), and in rigor (11). However, under these conditions, in which the binding of S-1 to actin is favored, S-1 readily displaces bound caldesmon from actin (11). Caldesmon and a 20-kD actin binding fragment of caldesmon also inhibit the binding of myosin-ATP to actin in skinned muscle fibers (30). In support of this competitive binding model, the ATPase activity of chemically cross-linked acto-S-1 was not inhibited by caldesmon (31). Furthermore, caldesmon binds to the $NH_2$-terminal region of actin (31–33) that has been implicated in the binding of myosin-ATP to actin (34, 35). Also, the steady-state kinetics of ATP hydrolysis, in the presence of caldesmon, is characterized by an increase in the apparent $K_m$, consistent with a weakening of the binding of S-1-ATP to actin (36). This latter point, though, is disputed (37).

Several recent studies directed at mapping the actin binding site of caldesmon have yielded several polypeptides that possess actin binding activity. The whole caldesmon molecule binds to ~7–10 actin monomers at full saturation (38). The 35-kD C-terminal fragment of caldesmon binds to three to four actin monomers (19), and a 20-kD COOH-terminal fragment, derived from the 35-kD fragment, binds to only two actin monomers (39). Finally, an 8-kD fragment from the 20-kD polypeptide binds with a 1:1 stoichiometry to actin (40). Intact caldesmon and the 20-kD fragment both bind to the N-terminal region of actin (32). The 20- and 8-kD fragments, as well as intact caldesmon, compete with the binding of S-1 to actin (40). Another region of caldesmon, derived from the N-terminal region of 35-kD caldesmon fragment, binds to actin but does not inhibit ATP hydrolysis (41, 42). These results indicate that only a small part of the caldesmon sequence is responsible for the inhibition of S-1 binding to actin. An interesting

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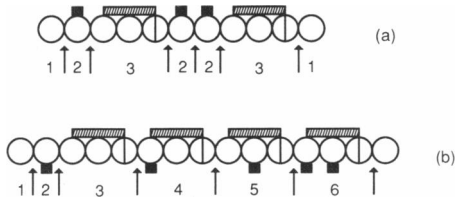


FIGURE 1 Models for simultaneous binding of two ligands to a one-dimensional lattice. Ligand 1 can cover one lattice subunit, whereas ligand 2 can cover three. Ligand 2 has a "head" part which is different from the rest of the molecule. (*a*) The pure competitive model. In this model, ligand binding sites are located at the top side of the lattice only. Thus, subunits covered by one ligand are not available for the other ligand. (*b*) A mosaic multiple-binding model. Each lattice subunit has two ligand binding sites: one located at the top for ligand 2 and the other at the bottom for ligand 1. Thus, both ligands can bind simultaneously to the same subunit of the lattice. Ligand 2 has a "head" part (indicated by the vertical line) which competes with ligand 1 for the same binding site at the bottom. Thus, when bound by the head part of ligand 2, a subunit loses its ability to bind ligand 1.

question is how a relatively small area of the caldesmon molecule (≤20 kD) that binds to actin and inhibits two molecules of S-1 binding to actin can enable the larger caldesmon molecule to inhibit the binding of additional S-1 molecules.

One possible solution is that part of a caldesmon binds relatively strongly to one (or two) actin monomers and totally inhibits the binding of S-1-ATP to these actin sites, whereas the other part may bind less tightly to additional actin sites and partially inhibit the binding of additional S-1 molecules (a mosaic multiple-binding model; see Fig. 1). In other words, the binding of intact caldesmon and S-1 to actin may not be pure competitive. This possibility is now explored by analyzing a ligand titration experiment in which the amount of bound S-1 and caldesmon molecules per actin monomer is measured as a function of the total concentration of S-1 added to the system at constant concentrations of actin and caldesmon. When the data were compared with model calculations, it was found that the binding of S-1 and caldesmon to actin cannot be purely competitive unless cooperativity between S-1 and caldesmon is assumed. On the other hand, the moaic multiple-binding model can fit the experimental data easily, regardless of the assumption of cooperativity.

Since the mosaic multiple-binding model used in this paper is new, we first describe the model and the procedure for the fitting of the model to the titration experiment.

## MODEL AND MATHEMATICAL ANALYSIS

### Competitive and multiple-binding models

We will consider a binding system involving two kinds of ligands: a short ligand 1 (S-1 in this study) that can bind to only one lattice subunit and a long ligand 2 (caldesmon) that can cover $n$ ($n$ = 7 for caldesmon; see reference 38) lattice subunits when bound to the lattice.

As shown in Fig. 1 *a*, if each lattice subunit has only one ligand binding site and both ligand 1 and ligand 2 can only bind to the same site, then the binding is purely competitive (pure competitive model). In this case, a subunit, when covered by one ligand, is not able to bind with another ligand. In contrast, if a subunit has two kinds of ligand binding sites, one for ligand 1 and the other for ligand 2, then a lattice subunit may be covered simultaneously by both ligands. That is, ligand 1 can still bind to the lattice subunits that are already bound with ligand 2 (and vice versa), although the binding strength may be altered. This mode of binding is called a multiple-binding model. If the effect of ligand 2 on the binding strength of ligand 1 is uniform for all subunits covered by the same ligand 2, the model is referred to as a "uniform" multiple-binding model. Since ligand 2 is a long ligand, it is possible that different parts of ligand 2 may exert different effects on the binding of ligand 1 to the lattice subunits that are covered by the same ligand 2. That is, the binding of ligand 1 to a subunit already bound with ligand 2 may not be uniform along the length of ligand 2. In this case, the binding model is called a "mosaic" multiple-binding model.

In this paper, we consider a special mosaic multiple-binding model for the simultaneous binding of S-1 and caldesmon to actin in which the effect of bound caldesmon on S-1 binding is uniform for all actin monomers along the length of a bound caldesmon, except one monomer that does not bind S-1 at all (the binding of S-1 to this monomer is prohibited). That is, among the seven actin monomers covered by a caldesmon molecule, one monomer completely loses its ability to bind S-1, whereas the rest bind S-1 with a common binding constant. This model is based on the finding mentioned above that only a small part of caldesmon is actually competing with S-1 for the same binding site on actin. Since the detailed structure of a bound caldesmon molecule on actin is not known, it is hard to determine where the S-1 competing part of a caldesmon molecule is located. However, if interaction (cooperativity) between two neighboring S-1 molecules on actin does not exist, then the binding formalism to be developed below will not depend on the exact position on a caldesmon molecule of this S-1 competing part. It is known that S-1 binds noncooperatively to bare actin (see below). It is reasonable to assume that S-1 binds similarly to caldesmon-bound actin. Thus, we arbitrarily assume in this model that the S-1 competing part of a caldesmon molecule is located at its "head" (see Fig. 1 *b*).

It is known that actin has polarity. It is reasonable to assume that the two ends of a caldesmon molecule are also structurally different. Thus, it is possible that the binding of caldesmon to actin may depend on the relative orientation of their polarities. For simplicity, we as-

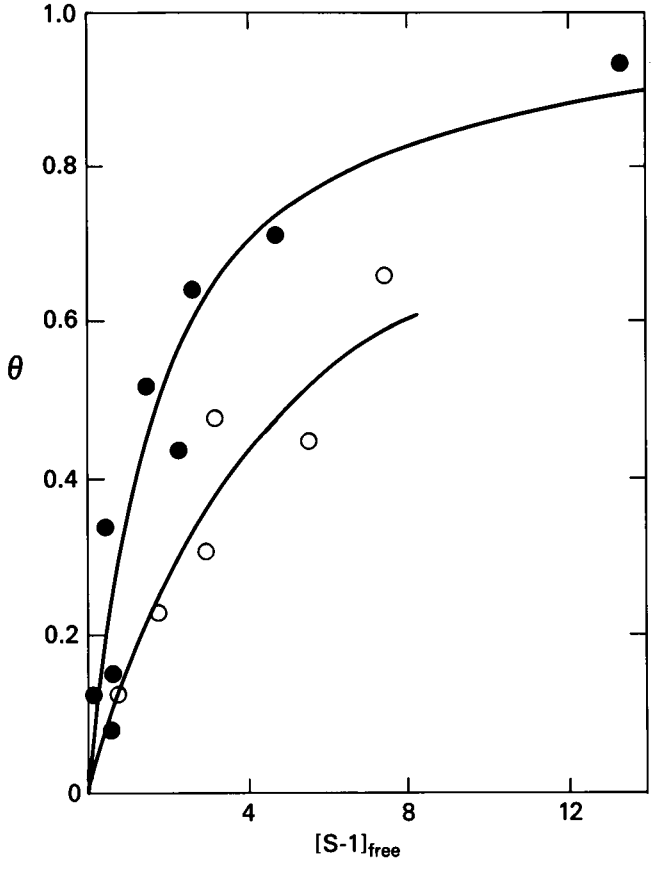


FIGURE 2 Binding of S-1 to actin in the presence (●) and absence (○) of smooth muscle tropomyosin. Binding was measured using either [$^{14}$C]-labeled S-1 (●) or using unmodified S-1 (○) as described in Materials and Methods. Binding was measured at 25°C in solutions containing 4 mM AMPPNP, 4.8 mM $MgCl_2$, 42 mM NaCl, 9.6 mM imidazole-HCl, pH 7.0, 0.25 mM EGTA, 0.6 mM dithiothreitol, 10 $\mu$M actin, 2.8 $\mu$M smooth muscle tropomyosin, where applicable, and variable concentrations of skeletal S-1. The data were fit by theoretical curves giving association constants of 6.25 and $1.95 \times 10^5$ $M^{-1}$ in the presence and absence of tropomyosin, respectively.

sume that caldesmon binds with only one direction (see Fig. 1 *b*). In this case, there is only one binding constant and one cooperativity parameter for the binding of caldesmon to actin. One must note that this assumption is made only to simplify the mathematical analyses to be presented below and that this assumption has been used implicitly in the analyses of binding of caldesmon to actin (38).

## Binding constants and cooperativity parameters of the models

An analysis of the competition between caldesmon and S-1 requires that the binding of each ligand to actin be characterized first in the absence of the other ligand. Caldesmon has been shown to bind to about seven actin monomers with a slight degree of cooperativity (38). This interaction is strengthened somewhat by the presence of smooth muscle tropomyosin. The binding of skeletal S-1-AMP PNP to actin and actin-tropomyosin was measured under identical conditions and is shown in Fig. 2. S-1 binds to actin without any apparent cooperativity and is facilitated by smooth muscle tropomyosin. The binding isotherm for S-1-AMP PNP is reasonable in regard to data obtained at different experimental conditions (43, 44). The parameters characterizing the binding of caldesmon or S-1 to actin are given in Table 1.

The binding parameters in Table 1 are used for both models without change. Thus, the only unknown parameters in the pure competitive model are the two cooperativity parameters between S-1 and the two ends of a caldesmon molecule. On the other hand, additional binding constants are needed in the mosaic multiple-binding model: $K'_1$, the binding constant of S-1 to an isolated actin monomer already bound with a caldesmon, and $K_{2s}$, s = 1, 2, . . . , the binding constants of caldesmon to an aggregate of seven actin monomers already bound with s S-1 molecules (see Fig. 3). Since the binding of S-1 to caldesmon-covered actin is assumed to be noncooperative (see before), the value of $K_{2s}$ can be shown easily (e.g., see Fig. 3) to be related to $K'_1$ as

$$K_{2s} = K_{20}(K'_1/K_1)^s \quad s = 1, 2, \ldots, n-1, \tag{1}$$

where $K_1$ and $K_{20}$ are the binding constants of S-1 and caldesmon, respectively, to bare actin (Table 1). Thus, all binding constants of the system can be obtained when the value of $K'_1$ is known. In principle, $K'_1$ can be obtained experimentally by measuring the binding isotherm of S-1 to caldesmon-saturated actin. In this paper, we will treat it as an adjustable parameter. It is important to note that when $K'_1 = 0$, the mosaic multiple-binding model becomes equivalent to a pure competitive binding model.

## $\theta_I$–$\theta_{II}$ curve

The titration experiment carried out in this study involves the measurement of fractions of bound S-1 and caldesmon per actin monomer ($\theta_I$ and $\theta_{II}$) at various concentrations of the total S-1 present in the system ($C_I^0$), with the total concentrations of actin ($C_A^0$) and caldesmon ($C_{II}^0$) in the system being kept constant. The mea-

TABLE 1 **Basic binding parameters of S-1 and caldesmon**

| | | With tropomyosin | Without tropomyosin |
|---|---|---|---|
| Binding constant | S-1, $K_1$ | $0.625 \times 10^6$ $M^{-1}$ | $0.2 \times 10^6$ $M^{-1}$ |
| | Caldesmon, $K_{20}$ | $1.9 \times 10^6$ | $0.55 \times 10^6$ |
| Cooperativity | S-1, $y'$ | 1 | 1 |
| | Caldesmon, $y$ | 4 | 3 |

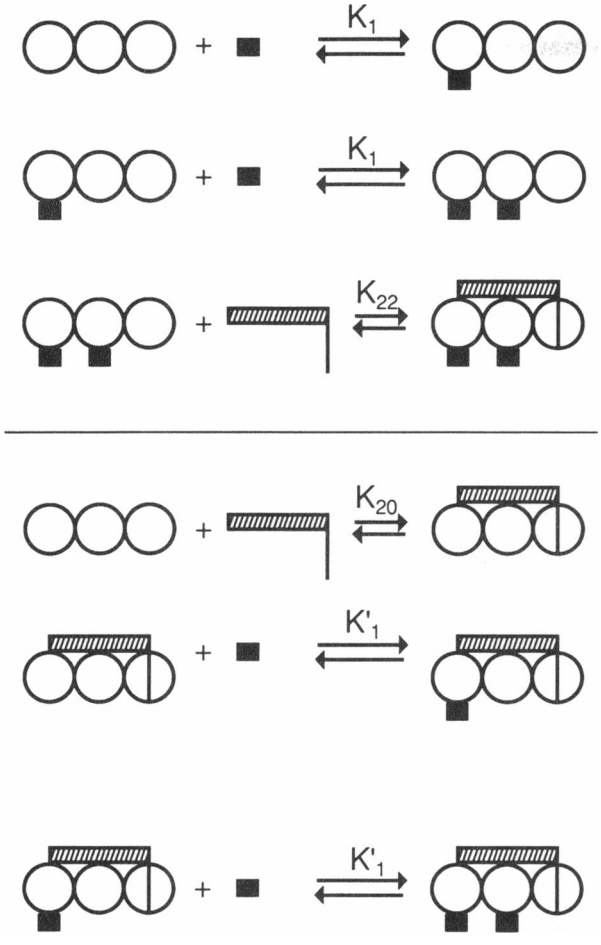


FIGURE 3 The two possible reaction routes for the formation of unit six in Fig. 1 *b*. The *K*'s are the equilibrium binding constants of the reactions. From these reactions, the weighting factor of unit six, $x_6$, can be written down easily.

sured $\theta_I$ at a number of $C_I^0$ values are then plotted against the corresponding $\theta_{II}$, as those shown in Fig. 4. In this section, we show how to calculate the $\theta_I$–$\theta_{II}$ curves for the pure competitive and the mosaic binding models.

Let $C_I$ and $C_{II}$ be the concentrations of free S-1 and free caldesmon in solution when the total concentrations of actin, S-1, and caldesmon in the system are $C_A^0$, $C_I^0$, and $C_{II}^0$, respectively. Then, the conservation of material requires that

$$C_I^0 = C_A^0 \cdot \theta_I + C_I \tag{2}$$

$$C_{II}^0 = C_A^0 \cdot \theta_{II} + C_{II}. \tag{3}$$

At constant $C_A^0$ and $C_{II}^0$, the values of $\theta_I$ and $\theta_{II}$ at any $C_I^0$ can be evaluated from Eqs. 2 and 3 if we have the binding isotherms of the system, which describe the relation between the amount of ligands that are bound to actin ($\theta_I$, $\theta_{II}$) and the concentrations of free ligands in solution ($C_I$, $C_{II}$).

Many statistical mechanical techniques can be used to derive the binding isotherms of one-dimensional ligand binding models. Recently, Chen (45) has developed a very simple procedure in deriving the secular equation (and therefore the binding isotherms) of the system that is applicable to many binding models, including the mosaic model discussed in this paper. In the Appendix, the application of the procedure to the present model is briefly discussed. The final expressions for the binding isotherms are obtained as

$$\theta_I = \left(\frac{x_I}{D}\right) \cdot \left\{\gamma_1^n - x_{II}(1 + x_I\delta)^{n-2} \cdot \left[\delta(n-1)(y - 1 - \gamma_1 y) + (1 + nx_I\delta)\left(y - y_{1a}y_{1b} + \frac{(1 - y_{1a})(1 - y_{1b})}{\gamma_1}\right)\right]\right\} \tag{4}$$

$$\theta_{II} = \left(\frac{x_{II}}{D}\right) \cdot \left\{1 - y + \gamma_1 y - x_I \times \left[y - y_{1a}y_{1b} + \frac{(1 - y_{1a})(1 - y_{1b})}{\gamma_1}\right]\right\} \tag{5}$$

where

$$D = \gamma_1^{n+1} - n\gamma_1^n(1 + x_I - \gamma_1) - x_{II}(1 + x_I\delta)^{n-1} \cdot \left[\gamma_1 y + \frac{(1 - y_{1a})(1 - y_{1b})}{\gamma_1}\right] \tag{6}$$

$$\delta = \frac{K_1'}{K_1} \tag{7}$$

$$x_I = K_1 C_I \tag{8}$$

$$x_{II} = K_2 C_{II}. \tag{9}$$

$y$ is the cooperativity parameter between two caldesmon molecules, $y_{1a}$ and $y_{1b}$ are those between S-1 and the two ends of a caldesmon molecule, and $\gamma_1$ is the largest root of the secular equation:

$$\gamma^{n+1}(1 + x_I - \delta) - x_{II}(1 + x_I\delta)^{n-1} \cdot \{\gamma(y - 1) - \gamma^2 y + x_I \cdot [\gamma(y - y_{1a}y_{1b}) + (1 - y_{1a})(1 - y_{1b})]\} = 0. \tag{10}$$

Two points are emphasized here. First, the binding isotherms in Eqs. 4 and 5 are applicable to both the pure competitive and the mosaic multiple-binding models discussed in this paper. The pure competitive case is obtained by setting the value of $\delta$ to zero. Second, the only unknown or adjustable binding parameters in Eqs. 4–10 are $\delta$ (or $K_1'$), $y_{1a}$, and $y_{1b}$. The others are known and listed in Table 1.

The values of $\theta_I$ and $\theta_{II}$ at different $C_I^0$ (at constant $C_A^0$ and $C_{II}^0$) can be evaluated numerically from Eqs. 2 to 5 with the use of Eqs. 6–10. For simplicity, $y_{1a}$ is set equal to $y_{1b}$ in all calculations. That is, no difference in interaction is assumed between the head and the end of a caldesmon and a S-1 molecule.

## MATERIALS AND METHODS

Rabbit back and leg muscle was the source of actin (46, 47), myosin (48), and myosin S-1 (49). Tropomyosin (50) and caldesmon (38) were isolated from turkey gizzards. The concentrations of actin and S-1 were

determined by absorbance measurements at 280 nm, whereas the Lowry assay (with a bovine serum albumin standard) was used for other proteins. We have shown earlier that the Lowry assay gives the true protein concentration for caldesmon (38); this is essential for binding studies. The molecular weights were assumed to be 120,000 for S-1, 42,000 for actin, 68,000 for tropomyosin, and 86,974 for caldesmon (26). (The estimate of the molecular weight of caldesmon has continued to change from 140,000 (1) to 97,000 (51) and recently to 86,974 (26). Previous estimates of binding constant and cooperativity parameter for the binding of caldesmon to actin were based on the molecular weight of 97,000 (38). The corrected values based on the new molecular weight are listed in Table 1.)

The binding of S-1 or caldesmon to actin was measured by a cosedimentation assay. Myosin S-1 was used in the competition experiments since there is little, if any, interaction between caldesmon and skeletal S-1. Myosin S-1 and/or caldesmon were mixed with skeletal F-actin at 0°C in a solution containing 4 mM AMP-PNP, 4.8 mM $MgCl_2$, 42 mM NaCl, 9.6 mM imidazole-HCl, pH 7.0, 0.25 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, and 0.6 mM dithiothreitol. After incubation at 25°C for 20 min, the mixture was centrifuged at 160,000 *g* for 25 min at 25°C to sediment the actin and bound ligand. The supernatant was carefully removed, and the free ligand protein concentration was determined. The concentration of bound ligand was determined from the free and total ligand concentrations. In the case of skeletal S-1, the free ligand concentration was determined either by measuring the $NH_4^+$-ethylenediaminetetraacetate ATPase rate of the S-1 in the supernatant (52) or by preparing [$^{14}$C]-labeled S-1 and determining the loss of radioactivity in the supernatant after centrifugation. The labeled S-1 was prepared by reaction with [$^{14}$C]-iodoacetamide and was purified before use by ATP-agarose affinity chromatography (10). Free caldesmon was determined by using a radioactive probe. Caldesmon was modified with either [$^{14}$C]iodoacetamide or $^{125}$I and purified using cellulose phosphate chromatography (38). Binding values were corrected for the amount of S-1 and caldesmon that sedimented even in the absence of actin (5–10%), as well as for that fraction of ligand that did not bind even in the presence of a large excess of actin (negligible for S-1 but 10% for [$^{14}$C]caldesmon and 25% for [$^{125}$I]caldesmon).

## RESULTS AND DISCUSSION

The purpose of this paper was to study whether the simultaneous binding of caldesmon and myosin S-1 molecules to actin can be described by a mosaic multiple-binding model, instead of the usual competitive model. In this model, some of the actin monomers when bound with caldesmon can still bind S-1. The motivation comes from the recent findings that not all of the small subfragments of an enzyme-digested caldesmon are directly competing with S-1 in binding to actin (16, 18–20, 28). To this end, we first carried out a ligand titration experiment in which S-1 was added to a system containing fixed amount of actin and caldesmon, and values of $\theta_I$ and $\theta_{II}$ (bound S-1 and caldesmon per actin monomer) were measured at different S-1 concentrations. The data were then compared with those calculated theoretically from the model. To test the effect of tropomyosin on the binding, two types of actins were used in the study: actins with and without tropomyosin. In each case, the titration experiment was carried out at two caldesmon concentrations. The experimental and calculated results are shown in Fig. 4.

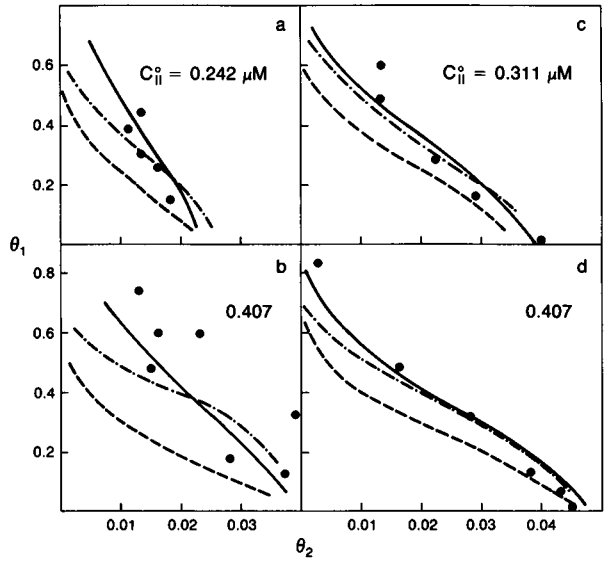


FIGURE 4 The $\theta_I$–$\theta_{II}$ curves in the absence (*a* and *b* on the left) and presence (*c* and *d*) of tropomyosin. Each panel represents a S-1 titration experiment with constant actin and caldesmon concentrations. In all four cases, the concentration of actin is fixed at 7.14 μM. The concentration of caldesmon (in units of μM) is shown in each figure. The measured data are shown as filled circles. The solid curves are those calculated for the mosaic multiple-binding model described in the text with $y_{1a} = y_{1b} = 1$ (no cooperativity between S-1 and caldesmon). The δ value for the two panels on the right (actin with tropomyosin) is 0.3 and 0.6 for those on the left. The two broken curves in each figure are those calculated from the pure competitive model. The case without cooperativity between S-1 and caldesmon ($y_{1a} = y_{1b} = 1$) is represented by the dashed curve. The dashed curve with dots is for the case that $y_{1a} = y_{1b} = 5$.

Because of the difficulty of simultaneously measuring the binding of two ligands, the measured data are rather scattered. However, because of the large difference in the calculated curves for different binding models, the following interesting points can be readily observed from Fig. 4. (*a*) For the pure competitive model, if no cooperativity exists between bound S-1 and caldesmon, then the fitting of the model with experiment is rather poor (see the dashed curves in the figure). (*b*) However, the fitting of the model improves slightly when a small cooperativity ($y_{1a} = y_{1b} = 5$) is invoked between S-1 and caldesmon (the broken curves with dots). (*c*) On the other hand, the mosaic multiple-binding model discussed in this paper can fit the experimental data reasonably well, even without cooperativity between S-1 and caldesmon (the solid curves). (*d*) If the binding is of the mosaic model, then the effect of bound caldesmon on actin monomer is to reduce the S-1 binding affinity (δ is always <1 or $K_1'$ is always $<K_1$), and the effect is stronger when the actin is saturated with tropomyosin ($\delta = 0.3$) than without tropomyosin ($\delta = 0.6$). From these findings, it is reasonable to conclude that the mosaic multiple-binding model discussed in this paper works well in describing the simultaneous binding of caldesmon and S-1 to actin. In contrast, the binding cannot be of the pure competitive type if there is no cooperativity between S-1 and caldesmon.

However, the pure competitive model is not completely ruled out if cooperativity exists between S-1 and caldesmon.

One must note that in this paper we have discussed only the mosaic model that just one of the seven actin monomers covered by the same caldesmon is assumed to be excluded from S-1 binding. In fact, it has been found that this mosaic model can still fit the experimental data even if two or three of the seven actin monomers lose the S-1 binding ability (data not shown). In these cases, the value of $\delta$, the ratio of the binding constants of S-1 to bare and to caldesmon-covered actin monomers, becomes larger.

The mosaic multiple-binding model discussed in this paper implies that only a small part of a caldesmon molecule is directly competing with S-1 for the same binding site on actin. This agrees rather well with recent structural studies in which the actin binding sites on a caldesmon are mapped by measuring the binding of various enzyme digested subfragments of a caldesmon (19, 39). The finding that reduction in the binding of S-1 to caldesmon-covered actin monomers is larger when the actin is saturated with tropomyosin is in line with the greater inhibition of actin-activated S-1 ATPase activity by caldesmon in the presence of tropomyosin (29, 38, 53). This is consistent with the suggestion that the inhibition of S-1 ATPase activity by caldesmon is primarily due to a reduction in Kapp (30, 36).

We would like to emphasize that the binding titration experiment presented in this paper was carried out by changing the total concentration of S-1 ($C_I^0$) at a constant caldesmon concentration. A similar experiment would be to titrate the binding system with caldesmon instead of S-1. The same formalism presented in this paper can be applied directly to this case.

Finally, we would like to point out that the present formalism is also useful for analyzing the binding of caldesmon fragments and S-1 to actin. In fact, the simultaneous binding of S-1 and the 20-kD fragment to actin has been studied recently (L. Velaz and J. M. Chalovich, personal communication). The preliminary results show that each fragment covers two actin monomers and that the binding is pure competitive. A possible implication of this finding is that the number of actin monomers excluded from S-1 binding by a bound caldesmon molecule may be two instead of one.

In conclusion, we propose in this paper a mosaic multiple-binding model for the simultaneous binding of S-1 and caldesmon to actin. We also present a formalism for analyzing the binding of a two-ligand titration experiment based on the model. The formalism is new and is useful in analyzing other two-ligand multiple-binding systems, such as in tropomyosin-myosin-actin binding system. In this system, tropomyosin molecules bind to the helix groove of actin (actin is a double-stranded helix) and myosin molecules bind to the sites on the outer surface of each actin monomer, forming a double-binding structure (54, 55). In general, the possibility of having a multiple-binding mechanism exists when the lattice for ligand binding is a double- or multiple-stranded polymer (such as actin, DNA, or microtubule).

## APPENDIX

## Derivation of binding isotherms of a mosaic multiple-binding model

In this appendix, the binding isotherms of S-1-caldesmon-actin binding system will be derived for the mosaic multiple-binding model described in the text, using the procedure developed recently by Chen (45). For simplicity, we will start with the $n = 3$ case (a caldesmon molecule is assumed to cover only three actin monomers when bound; see Fig. 1 *b*) and extend the results to an arbitrary $n$ at the end. As mentioned before, the mosaic model reduces to a pure competitive model when $K_1'$ (or $\delta$) is set to zero. Thus, the binding isotherms derived here are also applicable to the pure competitive case.

As shown in Fig. 1 *b*, there are six elementary units in this binding system. Let the cooperativity parameters between S-1 and the two ends of a caldesmon molecule be denoted as $y_{1a}$ and $y_{1b}$ and between two caldesmon molecules as $y$. Then, as shown by Chen (45), the secular equation of this binding system can be obtained as

$$\begin{vmatrix} 1-\gamma & 1 & 1 & 1 & 1 & 1 \\ x_2 & x_2-\gamma & x_2y_{1a} & x_2y_{1a} & x_2y_{1a} & x_2y_{1a} \\ x_3 & x_3y_{1b} & x_3y-\gamma^3 & x_3y & x_3y & x_3y \\ x_4 & x_4y_{1b} & x_4y & x_4y-\gamma^3 & x_4y & x_4y \\ x_5 & x_5y_{1b} & x_5y & x_5y & x_5y-\gamma^3 & x_5y \\ x_6 & x_6y_{1b} & x_6y & x_6y & x_6y & x_6y-\gamma^3 \end{vmatrix} = 0, \quad \text{(A1)}$$

where $x_i$, $i = 1, 2, \ldots, 6$, is the "weighting factor" of the elementary unit $i$. The value of $x_i$ can be expressed as the product of binding potentials of all binding reactions leading to the formation of unit $i$ (45). For example, as shown in Fig. 3, $x_6$ can be expressed as

$$x_6 = K_{22}C_{II}\cdot(K_1C_I)^2, \quad \text{(A2)}$$

or

$$x_6 = K_{20}C_{II}\cdot(K_1'C_I)^2. \quad \text{(A3)}$$

From these two equations, the relation in Eq. 1 can be easily verified. Using Eqs. 7–9, the weighting factors of the six elementary units in Fig. 1 *b* can be easily identified as

$$\begin{aligned} x_1 &= 1 \\ x_2 &= x_I \\ x_3 &= x_{II} \\ x_4 &= \delta x_I x_{II} \\ x_5 &= \delta x_I x_{II} \\ x_6 &= (\delta x_I)^2 x_{II}. \end{aligned} \quad \text{(A4)}$$

After carrying out the following two sets of operations—(*a*) successively substracting column 1 from column 2, column 4 from column 3, column 5 from column 4, et cetera, followed by (*b*) adding row 2 to row 1, row 3 to row 4, row 4 to row 5, et cetera—the matrix in Eq. A1 can be transformed into a partial diagonal form and reduced to

$$\begin{vmatrix} 1 + x_2 - \gamma & 0 & 1 + x_2 y_{1a} \\ x_2 & -\gamma & x_2 y_{1a} \\ \Sigma & \Sigma \cdot (y_{1b} - 1) & \Sigma \cdot y - \gamma^3 \end{vmatrix} = 0, \quad \text{(A5)}$$

where

$$\Sigma = x_3 + x_4 + x_5 + x_6 = x_{II}(1 + x_I\delta)^2. \quad \text{(A6)}$$

It is easy to show that the secular equation in Eq. A5 is applicable to any $n$ value, if the $\Sigma$ in Eq. A6 is replaced by

$$\Sigma = x_{II}(1 + x_I\delta)^{n-1}. \quad \text{(A7)}$$

After expanding the determinant in Eq. A5, the polynomial form of the secular equation in Eq. 10 for an arbitrary $n$ is obtained.

The analytical expressions of $\theta_I$ and $\theta_{II}$ can be obtained from this secular equation using the relations:

$$\theta_I = \frac{\partial \ln \gamma_1}{\partial \ln x_I}, \quad \text{(A8)}$$

$$\theta_{II} = \frac{\partial \ln \gamma_1}{\partial \ln x_{II}}, \quad \text{(A9)}$$

where $\gamma_1$ is the largest root of the secular equation of the system (Eqs. A5 or 10). Thus, after differentiating Eq. 10 with respect to $x_I$ and $x_{II}$, respectively, and substituting the $\gamma$ in the resulting equations with $\gamma_1$, the binding isotherms in Eqs. 4 and 5 are obtained.

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